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Consequences of photosystem I damage and repair on photosynthesis and carbon utilisation in *Arabidopsis thaliana*

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Running title

PSI photoinhibiton and recovery

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Summary

Natural growth environments commonly include fluctuating conditions that can disrupt photosynthetic energy balance and induce photoinhibition through inactivation of the photosynthetic apparatus. Photosystem II (PSII) photoinhibition is efficiently reversed by the PSII repair cycle, while photoinhibited photosystem I (PSI) recovers much more slowly. In the current study, treatment of the *Arabidopsis thaliana* mutant *proton gradient regulation 5*

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(*pgr5*) with excess light was used to compromise PSI functionality in order to investigate the impact of photoinhibition and subsequent recovery on photosynthesis- and carbon metabolism. The negative impact of PSI photoinhibition on CO_2 fixation was especially deleterious under low irradiance. Impaired starch accumulation after PSI photoinhibition was reflected in reduced dark respiration, but this was not attributed to impaired sugar synthesis. Normal chloroplast and mitochondrial metabolism were shown to recover despite the persistence of substantial PSI photoinhibition for several days. The results of this study indicate that recovery of PSI function involves reorganisation of light-harvesting antenna, and suggest a pool of surplus PSI that can be recruited to support photosynthesis under demanding conditions.

Introduction

In photosynthesis, plants convert light energy into chemical energy for fixing atmospheric CO_2 to build complex carbon-based molecules that eventually comprise plant yield. As sessile organisms, plants are subjected to various environmental changes that modulate photosynthetic activity. Among them, light intensity is especially important since it is directly related to photon incidence on leaves. High light intensities, and particularly the fluctuating light conditions that are normal under natural environments, induce damage to the photosynthetic apparatus, leading to a condition of reduced photosynthetic capacity called photoinhibition (for reviews, see Powles, 1984; Aro *et al*., 1993; Gururani *et al*., 2015). Photosystem II **(**PSII) is especially susceptible to damage in high light and the mechanisms of PSII photoinhibition have been extensively studied (Aro *et al*., 1993; Gururani *et al*., 2015), while photosystem I (PSI) photoinhibition has received less attention. Under optimal conditions, electrons discharged from the PSI reaction centre P700 are ejected to the PSI electron transfer chain comprising A_0, A_1, and the FeS centres F_X, F_A, and F_B (Amunts *et al*., 2007; Kozuleva and Ivanov, 2016) and finally to ferredoxin (Fd), while the electron hole $P700^+$ in the reaction centre is filled by donation of an electron from plastocyanin (PC). PSI photoinhibition occurs when the capacity of stromal electron acceptors is saturated, but the flow of electrons from PC to $P700^+$ remains functional. It has been postulated that in PSI photoinhibition conditions, molecular oxygen functions as an electron acceptor from PSI, leading to the generation of reactive oxygen species (ROS), which can react with FeS centres at PSI and lead to inhibition of electron transport activity (reviewed in Sonoike, 2011). PSI photoinhibition can occur at low temperatures (Havaux and Davaud 1994; Terashima *et al*.,

1994; Tjus *et al*., 1998), but also under fluctuating light conditions (Suorsa *et al*., 2012; Tikkanen and Grebe, 2018) and under high light (Tiwari *et al*., 2016; Gollan *et al*., 2017).

Recent studies have shown that PSI photoinhibition severely affects net carbon assimilation, photoprotection, starch accumulation, plant growth and retrograde signalling (Brestic *et al*., 2015; Zivcak *et al*., 2015; Yamori and Shikanai, 2016; Gollan *et al*., 2017). In comparison to PSII, PSI is more resistant to photoinhibition (Barth *et al*., 2001; Huang *et al*., 2010), and recovery of inhibited PSI, which is thought to involve degradation of the protein complex and replacement of damaged redox cofactors, is much slower (Li *et al*., 2004; Zhang and Scheller, 2004; Sonoike, 2011; Zhang *et al.*, 2011; Tikkanen and Grebe, 2018). For this reason, PSI photoinhibition is believed to have more severe consequences on plant metabolism than PSII photoinhibition under environmental stresses (Takagi *et al*., 2016a).

PSI photoinhibition is mitigated by both upstream and downstream regulation mechanisms; the latter include increases in chloroplast electron sink strength (Sonoike, 1995; Takagi *et al*., 2016b), water-water cycle activity (Driever and Baker, 2011; Cai *et al*., 2017) and antioxidant capacity (Takagi *et al*., 2016a), and the former involve regulating the flow of electrons from PSII to PSI (reviewed in Tikkanen and Aro, 2014). Among photosynthetic regulation mechanisms, accumulation of protons in the thylakoid lumen and subsequent downregulation of electron transport by the cytochrome b_6f complex is especially important for PSI protection. The PROTON GRADIENT REGULATION 5 (PGR5) protein is necessary for lumen acidification, and has been shown to be a key player in protecting PSI functionality (Munekage *et al*., 2002; Tiwari *et al*., 2016). Due to the absence of pH-dependent protection of PSI in *pgr5* mutant plants (Munekage *et al*., 2002; Suorsa *et al*., 2012; Kono *et al*., 2014; Kono and Terashima, 2016; Tiwari *et al*., 2016; Yamori *et al*., 2016), exposure of *pgr5* to increases in light intensity is a convenient system for induction of PSI photoinhibition (Tiwari *et al.*, 2016; Gollan *et al*., 2017). Using this system, we recently found that PSI damage severely inhibits carbon fixation and starch accumulation, and affects chloroplast regulation of nuclear gene expression (Gollan *et al*., 2017). However, the cumulative impact of this condition over the course of slow PSI recovery was not clarified. In the current study, Arabidopsis *pgr5* mutants were used to investigate the consequences of PSI inhibition and subsequent recovery of PSI function over several days, on gas exchange and carbon assimilation processes. These results reveal important details about the depletion and restoration of photosynthesis and primary metabolism after severe PSI photoinhibition, and

indicate that a substantial proportion of PSI may to be surplus to the plant's metabolic requirements under normal growth conditions, as previously proposed (Zhang and Scheller 2004).

Results

High light induces photosystem II photoinhibition in both WT and *pgr5* mutant

WT and *pgr5* mutant *Arabidopsis* plants were treated with high light (HL) for 4 h, followed by 5 days of recovery in growth light (GL) conditions. Control sets of WT and *pgr5* mutant plants were treated similarly, but exposed only to GL instead of the 4 h HL treatment. Maximum chl *a* fluorescence (Fm) was used to assess PSII function in preference to the calculated Fv/Fm parameter, in order to avoid the confounding effect of PSI photoinhibition on fluorescence, especially on Fo values (Tikkanen *et al.*, 2017; see Figure S1b). Fm measurements revealed that 4 h HL treatment induced PSII photoinhibition in both WT and *pgr5* mutants (35% and 55% decreases, respectively) compared to the GL levels (Figure 1a). After 24 h recovery in GL, Fm values in WT plants were equivalent to pre-treatment levels, while Fm in *pgr5* plants 24 h after HL treatment was slightly lower than in GL-treated plants, and was fully restored to pre-treatment levels on recovery Day 2 (Figure 1a).

High light inhibits P700 oxidation and ferredoxin reduction

In order to evaluate PSI photoinhibition and recovery, the maximum oxidation capacity of P700 at the PSI reaction centre (Pm) was monitored as an indicator of PSI functionality (Figure 1b). The results showed that *pgr5* mutants have lower content of oxidisable PSI in normal growth light conditions, in comparison to WT plants, as has been observed previously (Tiwari *et al*., 2016; Gollan *et al*., 2017). HL treatment induced an 80% decrease in the Pm value in *pgr5* plants compared with the Pm value of GL-treated control plants. Recovery of Pm in HL-treated *pgr5* plants occurred over four days, after which the Pm value of *pgr5* plants was restored to a similar level to that of untreated plants. A minor decrease in the Pm value was observed in the WT after 4 h HL treatment, although there were no significant differences in Pm between GL- and HL-treated WT plants.

To further characterise the consequences of PSI photoinhibition on the photosynthetic electron transport chain, the redox state of the electron donor (plastocyanin; PC) and acceptor (ferredoxin; Fdx) pools of PSI were assessed (Figure 1c, d). HL-treated WT plants showed a

slight decrease in the maximum reduced state of the ferredoxin pool (Fdm), in comparison to GL-treated WT plants (Figure 1c), while HL-treated *pgr5* mutants showed a 60% decrease in Fdm in comparison to GL-treated *pgr5* mutants. The Fdm level in HL-treated *pgr5* was restored to the control levels on recovery Day 3. The maximum oxidised state of the plastocyanin pool (PCm) in HL-treated *pgr5* plants was marginally, although significantly, higher in *pgr5* plants directly after treatment than in GL-treated *pgr5* plants, while PCm also remaining slightly higher in HL-treated *pgr5* during the recovery phase (Figure 1d). No differences in PCm values between GL and HL-treated WT plants were observed. A strong and positive correlation was observed between Pm and Fdm (Figure 1e), but not between Pm and PCm (Figure 1f).

PSI photoinhibition correlates with depletion of the PsaB subunit

The abundance of PSI core subunit PsaB and the extrinsic stromal subunits PsaC and PsaD were assessed by immunoblots of thylakoid membranes isolated from WT and *pgr5* plants treated with GL, and during the course of PSI photoinhibition and recovery (Figure 2a–c). Total thylakoids were loaded on SDS-PAGE according to chlorophyll equivalence, which corresponded with protein equivalence between samples as shown by Coomassie Brilliant Blue-stained membranes (Figure 2e) and protein:chlorophyll ratio calculations (not shown). No differences in PSI subunits were apparent in thylakoids isolated from WT plants, between GL and HL treatments, while the abundance of PsaB was noticeably lower in *pgr5* thylakoids isolated from leaves immediately after HL treatment, in comparison to pre-treatment controls. Cross-reaction between anti-PsaB antibody and product of approximately 18 kDa was observed in *pgr5* thylakoids isolated from HL-treated plants directly after HL-treatment (Figure S2), but other known PsaB degradation fragments (Sonoike, 1996b; Sonoike *et al*., 1997, Kudoh and Sonoike 2002) were not detected. After 4 days of recovery in GL, PsaB protein in *pgr5* thylakoids remained less abundant than in untreated controls (Figure 2, Figure S2). PsaC and PsaD protein levels were not noticeably different in *pgr5* thylakoids isolated from HL treated leaves, in comparison to GL and WT controls (Figures 2b and c).

Changes in thylakoid protein phosphorylation were detected by Western blotting with an anti-phosphothreonine antibody. The results showed a strong decrease in phosphorylated LHCII and a slight increase in phosphorylated PSII core proteins CP43, D1 and D2 directly after 4 h HL treatment (Day 0) of WT leaves, in comparison to samples harvested prior to treatment (Figure 2d). Moderate phosphorylation of both LHCII and PSII core proteins was observed in

WT samples during recovery days 1–4. In contrast to WT, the LHCII phosphorylation state was not diminished by HL treatment of *pgr5* plants, while phosphorylation of LHCII and PSII core proteins was substantially greater in the *pgr5* mutant during recovery, compared to untreated plants.

Capacity for CO_2 assimilation after HL treatment is light intensity-dependent in both genotypes

To better understand the consequences of PSI photoinhibition and subsequent slow recovery of PSI function on primary metabolism, the CO_2 assimilation rates of WT and *pgr5* mutants were measured under 50 µmol m^{-2} s^{-1}, 125 µmol m^{-2} s^{-1} and 1000 µmol m^{-2} s^{-1} light intensities during the course of our experimental protocol of pre-treatment (Pre), after HL treatment (Day 0) and during subsequent recovery (Days 1-5). In each case, *pgr5* leaves showed distinct inhibition of CO_2 assimilation rates (*A*) immediately after the HL treatment; however, the magnitude of decrease was dependent on the intensity of the measuring light (Figure 3). When measured under low light, *pgr5* showed almost no CO_2 assimilation immediately after HL treatment, while HL-treated WT plants showed only around 50% reduction in *A* (Figure 3a). CO_2 assimilation under low light was restored to the pre-treatment level in HL-treated *pgr5* after three days of recovery, while for WT plants a full recovery was observed already after 24 h.

When *A* was measured under 125 µmol m^{-2} s^{-1} (GL), HL-treated *pgr5* mutants displayed an 80% decrease in CO_2 assimilation, but after 24 h recovery in GL (Day 1) there was no significant difference between HL and GL-treated *pgr5*. Small increases in *A* were observed in HL-treated WT plants on Days 2 and 3 of recovery, compared to GL-treated WT (Figure 3b). Under 1000 µmol m^{-2} s^{-1} measuring light, CO_2 assimilation in HL-treated *pgr5* immediately after treatment was 45% lower than in untreated *pgr5* leaves, while, inversely, WT showed a 45% increase compared to untreated leaves (Figure 3c). In both genotypes, CO_2 assimilation measured under 1000 µmol m^{-2} s^{-1} was slightly higher in the HL-treated plants during the recovery phase (i.e., Days 1–3) in comparison to their respective GL-treated control plants. Plotting these data as light response curves for each recovery day clearly illustrated depression of the initial part of the curve in HL-treated *pgr5* leaves on Day 0, indicating increased light limitation of photosynthesis, which was not seen after 24 h recovery (Day 1–5; Figure S3).

High light stress altered carbohydrate metabolism

To further characterise the effects of PSI photoinhibition and recovery on plant primary metabolism, we evaluated the concentration of key molecules of carbohydrate metabolism in WT and *pgr5* leaves from plants exposed to HL treatment and recovery, as previously described. Starch content was 85% higher in WT directly after HL treatment in comparison to the concentration found in plants grown under GL, while there was no significant change in starch in *pgr5* directly after HL treatment (Figure 4a). After 24 h of recovery, starch concentrations were substantially lower in both WT and *pgr5* leaves (50% and 65%, respectively), compared to leaves immediately after HL treatment. In *pgr5* plants, starch levels steadily increased during recovery Days 2–4, reaching values similar to GL-treated *pgr5* plants by Day 4. HL-treated WT leaves also contained less starch than GL-treated WT controls until Day 4 (Figure 4a).

Glucose and fructose contents were evaluated as an index of sugar metabolism status. The HL treatment induced substantial increases in glucose and fructose concentrations in both WT and *pgr5* leaves, with 85% and 70% increases in glucose, respectively, and 210% and 85% increases in fructose, respectively, compared to the respective GL-treated controls (Figure 4b and c). During the initial 2 days of recovery in GL, glucose concentrations in WT and *pgr5* were slightly, but significantly lower than in GL-treated controls. After 3 days of recovery, glucose concentrations in HL-treated leaves of both genotypes were equivalent to those found in GL-exposed leaves. Fructose concentrations measured in GL- and HL-treated WT and *pgr5* plants during the recovery phase were very similar (Figure 4c).

WT and *pgr5* display distinct changes in mitochondrial respiration

Mitochondrial metabolism is directly related to photosynthetic energy production, and therefore mitochondrial respiration rates were evaluated in leaves of GL- and HL-treated WT and *pgr5* plants (Figure 5). Day-time respiration measured by changes in CO_2 flux after 15 min dark adaptation (Brooks and Farquhar, 1985) was around 50% higher in WT plants directly after HL-treatment compared to GL levels, while a slight increase was also observed in HL-treated *pgr5* in comparison to GL-control (Figure 5a). However, there were no significant differences between GL and HL-treated plants for both WT and *pgr5* plants during the recovery phase. The effect of PSI photoinhibition on dark respiration rates was evaluated by measuring O_2 uptake in WT and *pgr5* leaves throughout 4 h of dark incubation directly following 4 h HL or GL treatment (Figure 5b). In all cases, the rate of O_2 uptake

showed a consistent and linear decline over 4 h in the dark (Figure 5b). The rates of decrease in both GL-treated genotypes, and in HL-treated *pgr5* leaves were closely correlated, and were about 3 times faster than the rate of decrease in HL-treated WT leaves (Figure 5b). Analysis of O_2 uptake during night-time respiration (measured each night during the recovery period, 6 h after sunset) showed no significant effects of HL-treatment in WT plants (Figure 5c), while in the *pgr5* mutants night-time respiration decreased significantly in the second night after HL treatment and recovered to the level of GL-treated plants by the following night (Figure 5d).

Discussion

When photosynthetic electron transport exceeds the capacity of electron acceptors, both PSI and PSII are susceptible to photoinhibition through the activity of reactive oxygen species (ROS), although the mechanisms of inhibition and repair differ vastly between the two photosystems. PSII photoinhibition is quickly reversed by the efficient PSII repair cycle (reviewed in Aro *et al*., 1993), while recovery of photoinhibited PSI takes place very slowly (Li *et al*., 2004; Zhang and Scheller, 2004; Sonoike, 2011; Zhang *et al*., 2011). Therefore, PSI is robustly protected from over-reduction, especially by regulation of electron transport through the cyt b_6f complex that is sensitive to lumenal pH (reviewed in Tikhonov, 2014). In the current study, we used HL treatment of *Arabidopsis* WT and the *pgr5* mutant to study the interactions between PSII and PSI photoinhibition and their effects on photosynthesis and sugar metabolism during light-stress and recovery in normal growth conditions.

PSI photoinhibition recovery impacts photosynthetic electron transport

Substantial decreases in maximal chl *a* fluorescence (Fm) in both WT and *pgr5* plants after 4 h HL exposure is attributed to PSII photoinhibition, including both LHCII excitation quenching and PSII damage through over-reduction of the electron transport chain and subsequent excitation back-pressure on PSII. Fm was fully restored within 24 h in HL-treated WT plants, but slightly less quickly in *pgr5* (discussed below). In contrast, the maximum level of P700 oxidation (Pm) was severely diminished in the *pgr5* mutant after HL treatment and was slowly restored to the level of untreated controls over 4 days of recovery in GL, while only a minor Pm decrease was apparent in HL-treated WT leaves. Although PSII photoinhibition may have made a small contribution to low Pm measured in *pgr5*, by decreasing linear electron transport (Baker *et al*., 2007), the Pm decrease in HL-treated WT was minor and not statistically significant, despite substantially lower Fm in the same plants.

Instead, the severe PSI photoinhibition in *pgr5* is attributed to inactivation of FeS clusters F_A, F_B and F_X, inhibiting electron transport from reduced P700 (Sonoike, 2011; Tiwari *et al.*, 2016). Despite the protective effect of PSII photoinhibition on PSI over-reduction (Tjus *et al.* 1998; Tikkanen *et al*., 2014), PSII inactivation in *pgr5* during the HL treatment did not effectively protect PSI. A larger Fm decrease and slower restoration of Fm levels in *pgr5* compared to WT after HL treatments (Figure 1) indicate strong excitation pressure on PSII, which is induced by PSI photoinhibition (Kudoh and Sonoike 2002; Zhang and Scheller 2004). However, full recovery from PSII photoinhibition in HL-treated *pgr5*, as shown by restoration of Fm (Figure 1) and Fv/Fm (Figure S1a), was achieved after 48 h in GL, despite the persistence of severe PSI photoinhibition and abnormally high PSII excitation pressure at this time-point (see Figures S1b and S1d). PSII recovery may be partly attributed to the increase in light-harvesting complex (LHCII) protein phosphorylation observed in HL-treated *pgr5* (Figure 2d), which would increase the channelling of excitation from phospho-LHCII towards PSI centres. In fact, damaged PSI is an efficient quencher of excited LHCII, with the effect of relaxing the excitation pressure within the electron transport chain (Tiwari *et al.*, 2016). Increased levels of both LHCII and PSII core protein phosphorylation in *pgr5* plants is similar to the effects induced by "State 2 light", and likely facilitated the recovery of both PSI and PSII after PSI photoinhibition (Tikkanen *et al.*, 2008; Mekala *et al.*, 2015). High LHCII phosphorylation in *pgr5* directly after HL treatment showed an absence of STN7 kinase inactivation, reflecting a relatively oxidised state of the stromal redox system due to impaired electron transport through the partially-inactivated PSI pool (Rintamäki *et al*., 2000). This effect of stromal "under-reduction" was also evident in the severely diminished Fdm during PSI photoinhibition and recovery, showing that Fd was more oxidised in HL-treated *pgr5* plants (Figure 1) and that normal Fd reduction recovered in strong correlation with Pm recovery. The decline in Fdm was not due to any changes in thylakoid-associated Fd abundance (Figure S2), but instead reflects the inability of inhibited PSI to transport enough electrons to the stroma to fully reduce its primary electron acceptor Fd.

The metabolic penalty of PSI photoinhibition varies according to light intensity

CO_2 assimilation and starch synthesis can be compromised by both PSI and PSII photoinhibition (Munekage *et al*., 2008; Nishikawa *et al*., 2012; Belgio *et al*., 2015; Gollan *et al*., 2017); however, PSI photoinhibition is likely to have more persistent consequences for plant fitness due to relatively slow recovery compared to PSII. In the current work, inactivation of PSI caused almost complete loss of CO_2 assimilation when measured under

low light (50 µmol m^{-2} s^{-1} PPFD; Figure 3a), and the recovery of CO_2 assimilation capacity over 3 days in GL followed the trend of Pm and Fdm recovery that reflects slow restoration of PSI activity. The contribution of PSII photoinhibition to low CO_2 assimilation in HL-treated *pgr5* cannot be excluded, as a decrease in CO_2 fixation was also detected in HL-treated WT leaves with intact PSI, under low light. This may be due to diminished yield from the partially damaged PSII pool, as well as increased consumption of NADPH and ATP for PSII repair at the expense of the Calvin-Benson-Bassham (CBB) cycle reactions (Murata and Nishiyama, 2018). Nonetheless, the effect of severe PSI photoinhibition on limiting the stromal content of NADPH and ATP is the primary reason for decreased CO_2 fixation observed here. The diminished abundance of stromal reductants (discussed above) may lead to relative oxidation of ferredoxin-thioredoxin reductase (FTR) and the stromal thioredoxin network, resulting in impaired redox-activation of CBB cycle enzymes under non-saturating light conditions (Haldrup *et al*., 2003; Buchanan, 2016; Nikkanen *et al*., 2016).

HL treatment of *pgr5* has a smaller negative impact on CO_2 assimilation when measured under 125 µmol photons m^{-2} s^{-1} irradiance, compared to 50 µmol m^{-2} s^{-1}, and smaller again under 1000 µmol m^{-2} s^{-1}. This result is in line with our previous study, which showed PSI photoinhibition to be especially deleterious to primary metabolism under low light intensities (Gollan *et al.*, 2017). These results clearly demonstrate that PSI photoinhibition does not necessarily limit primary metabolism, as higher light intensities require fewer functional PSI centres to transport electrons required for primary metabolism. This can be explained in the context of PSI quantum yield (Φ_{PSI}), which is high under light limitation and decreases over increasing light intensities as the formation of $P700^+$ increases (Baker *et al*., 2007). PSI photoinhibition would therefore intensify high Φ_{PSI}, especially under low light, while improved PSI efficiency in high irradiance would increase electron transport, thus enabling higher rates of metabolism. This light intensity-dependent effect on CO_2 fixation is similar to the observation that higher intensities of far-red light were required to oxidise P700 directly after chilling-induced PSI photoinhibition, compared to untreated plants (Kudoh and Sonoike 2002; Zhang and Scheller 2004). In addition to the improved CO_2 fixation under high irradiance, the current results also show that CO_2 fixation under lower irradiances in HL-treated *pgr5* recovered to the level of GL-treated controls after 24 h recovery (Fig. 3b, Fig. S3b), despite PSI operating at only around 35% of its full capacity (according to Pm values) at that point. The capability of a partially inactivated PSI population to support normal CO_2 assimilation rates during recovery, even at low light intensity, shows an improvement in PSI

efficiency after photoinhibition. This may be attributed to the increase in PSI antenna size under strong LHCII phosphorylation (Figure 2d), or may suggest the recruitment of a pool of photo-oxidisable PSI centres that is not involved in electron transport under normal conditions (Zhang and Scheller 2004).

The natural outcome of CO_2 assimilation is the synthesis of triose phosphate, which can be exported from the chloroplast and converted to sucrose in the cytosol, or retained in the chloroplast for starch synthesis to support dark metabolism (Stitt *et al*., 2010). Concentrations of simple sugars glucose and fructose were found to be higher in both genotypes after HL treatment, compared to GL-treated controls, as a result of high photosynthetic activity during 4 h HL exposure. However, rapid onset of PSI photoinhibition in *pgr5*, and subsequent limitation of CO_2 assimilation, is likely responsible for approximately 50% less glucose and fructose in *pgr5* compared to WT after HL treatment (Day 0). Nonetheless, the levels of these sugars were equivalent in *pgr5* and WT plants throughout recovery (Days 1 – 5), which shows that normal sugar synthesis can be sustained in the presence of considerable PSI inhibition. In contrast, starch accumulation was substantially lower in *pgr5* than WT, upon recovery, for several days after HL treatment. Even directly after the treatment, starch content in *pgr5* leaves was only marginally higher than pre-treatment levels, despite the 50-75% increase in sugars in those plants, and the doubling of starch levels in the WT. These results suggest that impairment of starch synthesis by PSI inhibition may be independent of CO_2 fixation and accumulation of sugars. Instead, a shortfall of reducing power caused by the combined effect of PSI damage and low irradiance (growth light) may leave little energy available for starch synthesis, as the limited reductants are used to support more immediate metabolic demands, including sugar synthesis. It is also possible that PSI damage and subsequent under-reduction of stromal acceptors (discussed above) has a negative impact on redox activation of chloroplast stromal enzymes, such as the starch-branching ADP-Glc pyrophosphorylase (AGPase), which is redox-regulated by NADPH-thioredoxin reductase C (NTRC; Michalska *et al*., 2009). It should be noted that WT plants also had less starch on Days 1–2 after HL treatment, compared to untreated controls, suggesting that the HL treatment affected starch accumulation independently of PSI photoinhibition; however, the starch deficiency in *pgr5* plants was much greater than in WT.

PSI damage in HL-treated *pgr5* mutants was shown to impair mitochondrial respiration relative to that in WT during both the day and the night. The rates of day respiration increased in both genotypes directly after HL treatment, although to a smaller degree in *pgr5*, and were largely unaffected by PSI inhibition during the recovery phase (Figure 5a). These results correlated with the accumulation of simple sugars (Figure 4b and c) and therefore it is reasonable to assume that the lower day respiration rate in *pgr5* in relation to WT after the 4 h HL treatment was a consequence of a low synthesis/accumulation of simple sugars. In comparison, night respiration (Figure 5c and d) followed the trend of starch accumulation (Figure 4a), which was negatively affected by PSI inhibition during recovery. This is in accordance with the role of starch in supporting night-time metabolism (Graf *et al*., 2010) and suggests that growth and development processes occurring during the night may be particularly susceptible to PSI photoinhibition.

Assessing PSI photoinhibition through PSI damage, PSI function and primary metabolism during recovery from PSI photoinhibition

PSI photoinhibition was accompanied by rapid depletion of PsaB content from thylakoids of HL-treated *pgr5* plants, with around 50% of WT PsaB levels remaining after 4 h of HL treatment (Figure 2). This was in contrast to studies of light/chilling stress-induced PSI photoinhibition that show PsaB degradation occurring only after several hours in recovery at normal temperature (Kudoh and Sonoike 2002; Zhang and Scheller 2004). This difference may relate the inhibitory effect of chilling temperatures on proteolytic enzyme activity (Kudoh and Sonoike, 2002), which was not a factor in our experiment. However, proteolytic removal and turnover of PSI complexes was not apparent at all in the current study, as there was no detectable decrease in the abundance of PsaC and PsaD subunits (Figure 2). Furthermore, most of the proteolytic PsaB fragments reported in chilling experiments (e.g., Sonoike *et al*. 1997) were not detected and no significant changes were observed in chlorophyll abundance or chl *a*:*b* ratio (not shown) in photoinhibited thylakoids. These disparities may indicate mechanistic differences in PSI photoinhibition between chilling stress and HL treatment of *pgr5*. In the former case, downregulation of stromal metabolism in the light leads to over-reduction of PSI and ROS-induced photoinhibition (Soinoike 2006), but at the same time, lower ATP consumption induces pH-dependent regulation of electron transport that likely affords some protection to PSI (Kanazawa and Kramer 2002). No such protection occurs in *pgr5* mutants (Munekage *et al*. 2002; Suorsa *et al*. 2012). Instead, the PSI reaction centre of HL-exposed *pgr5* is assaulted by a relentless current of reducing

power, which quickly overwhelms stromal acceptors and results in rapid ROS-induced destruction of PSI cofactors and core proteins (Tiwari *et al.*, 2016), perhaps similar to the effects reported in Inoue *et al.* (1989). PSI photoinhibition by chilling stress is likely less severe, especially in cold-tolerant Arabidopsis, instead triggering controlled proteolytic degradation of PsaB and other PSI subunits to prevent further ROS formation (Tjus et al 1999; Kudoh and Sonoike 2002; Zhang and Scheller 2004).

When observations above are brought together with the recovery of normal P700 oxidation within 4 days under GL, it appears that severely damaged PSI complexes may not be repaired within the time-frame of the current study. Increased LCHII phosphorylation likely compensates, at least partially, for decreased PSI oxidation capacity at limiting light conditions (GL), while restoration of PSI function may also take place by substituting damaged reaction centre proteins/cofactors and recycling other subunits. Indeed, turnover of PSI core proteins PsaA and PsaB was recently found to be faster than turnover of peripheral subunits (Li et al. 2018), suggesting that further investigation into PSI damage and repair after different types of injury, using quantitative protein mass spectrometry is warranted. The PSI assembly complex known as "PSI*" lacking peripheral subunits and LHCI antennae (Ozawa *et al.*, 2010; Wittenberg *et al.*, 2017) may be a reserve of immature PSI that is visible in Pm measurements, although inactive in electron transport, which can be brought online to support electron transport and metabolism under demanding conditions such as low light intensity or after PSI photoinhibition.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana L. Heynh. *pgr5* mutant (Munekage *et al*., 2002) and its reference ecotype Columbia *glabrous 1* wild type (WT) were used for all experiments. Plants were grown in a growth chamber at 23 °C constant temperature, 60% relative humidity and 8 h photoperiod of white growth light (GL) of photosynthetic photon flux density (PPFD) of 125 µmol photons m^{-2} s^{-1}, for six weeks after sowing. Plants were either kept under GL for the reference treatment or shifted to a high light (HL) treatment of 1000 µmol photons m^{-2} s^{-1} in a controlled growth chamber set at 23 °C/60% relative humidity for 4 h. The shift to HL treatment occurred one hour after the beginning of the photoperiod. After 4 h treatment, HL-treated plants were used for the first measurements (Day 0) and returned to GL until the end

of the experimental period (Day 5). All *in vivo* measurements and plant harvesting were performed at the same time every day (i.e., 5 h after beginning of the photoperiod), except for the O_2 uptake measurements, which were performed as described below. The experiments were repeated at least twice and at least three biological replicates were used in every experiment.

Photochemistry measurements

Photosystems II and I photochemical parameters were simultaneously measured using a Dual-PAM-100 system (Walz, Germany) based on chlorophyll *a* fluorescence (Schreiber *et al*., 1995) and the P700 oxidation signal (Klughammer and Schreiber, 1998) methods, respectively. Fo and Fm measurements were taken from detached leaves after 30 min of dark acclimation. F' measurements were taken after 5 min exposure to actinic light intensities of 125 µmol photons m^{-2} s^{-1} for GL and 1000 µmol photons m^{-2} s^{-1} for HL. Maximal reduction values of ferredoxin (Fdm), and maximum oxidation values of P700 (Pm) and plastocyanin (PCm) were measured with a Kinetic LED-Array Spectrophotometer (Walz, Germany), through the deconvolution of their redox changes in intact leaves (Klughammer and Schreiber, 2016). Measurements were performed as previously described (Schreiber and Klughammer, 2016; Schreiber, 2017).

Gas exchange measurements

Leaf CO_2 exchange was measured at 400 ppm CO_2 and 23 °C using the LI-6400XL Portable Infrared Gas Analyzer system (LI-COR Biosciences, USA). Leaves were acclimated in the dark for 15 min and CO_2 assimilation values of each leaf were assessed with a PPFD gradient of 0, 50, 125, and 1000 µmol photons m^{-2} s^{-1}. Data were taken after the infrared gas analyser parameters reached a steady-state value following the onset of the respective PPFD (usually around 120 s). The results from 0 PPFD measurements were used as to estimate day respiration, which is the rate of CO_2 evolution from processes other than photorespiration (Brooks and Farquhar, 1985). O_2 uptake was measured for 5 min in darkness at 23 °C using an "OX-NP" oxygen microsensor (Unisense A/S, Denmark) from three detached leaves submerged in 50 mM sodium phosphate buffer (pH 7.2) in a gas-tight vial fitted with a rubber septum. Leaves were dark acclimated for at least 15 min prior to each O_2 consumption rate measurement.

Carbohydrates quantification

Leaves were collected, frozen until the last day of the experiment and oven-dried at 60 °C for 72 h for determination of starch, D-glucose, and D-fructose contents. Total starch concentration was determined using the K-TSTA assay kit (Megazyme, Ireland). D-glucose and D-fructose concentrations were determined using the K-SUFRG assay kit (Megazyme, Ireland) after ethanolic extraction (80% v/v) at 99 °C for 15 min. Assays were performed according to the manufacturer protocols.

Immunoblotting

Thylakoids were isolated from mature leaves as previously described (Järvi *et al*., 2011). Total thylakoid proteins equivalent to 0.5 μg chlorophyll were separated by SDS PAGE, transferred to PVDF membranes and blotted with polyclonal antibodies against PsaB (Agrisera; AS10 695), PsaC (Agrisera; AS10 939), and PsaD (a kind gift from Prof. Poul Erik Jensen).

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Conflict of interest

The co-authors of this manuscript declare no conflict of interest.

Supporting information

Figure S1: The impact of PSI photoinhibition on chlorophyll *a* fluorescence parameters.

Figure S2: The impact of PSI photoinhibition on the PsaB subunit degradation of PSI and abundance of thylakoid membrane-associated ferredoxin (Fdx).

Figure S3: The impact of PSI photoinhibition and recovery on CO_2 assimilation.

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Figure Legends

Figure 1. Parameters associated with PSII and PSI integrity in WT and *pgr5* mutants with and without a 4 h HL treatment, and during the subsequent recovery phase of 5 days.
(a) Maximum chlorophyll *a* fluorescence (Fm); (b) maximum oxidizable P700 (Pm); (c) maximal reduction state of ferredoxin (Fdm); (d) maximum oxidation state of plastocyanin (PCm) measured with a Dual/KLAS-NIR spectrophotometer in detached leaves of WT and *pgr5* plants exposed to growth light (GL, 125 µmol m^{-2} s^{-1}) or to 4 h high light (HL, 1000 µmol m^{-2} s^{-1}) on Day 0, and subsequently returned to GL conditions for recovery (Days 1 – 5). Correlations (e) between Fdm and Pm, and (f) between PCm and Pm were plotted using the data shown in plots A, C and D. Error bars show standard deviation among replicates (n = 4). Significant differences between treatments and between genotypes are indicated by non-overlapping error bars (Student's *t* test, $p < 0.05$). The vertical yellow bar represents application of the 4 h HL treatment.

Figure 2. The impact of PSI photoinhibition on the abundance of PSI protein subunits and thylakoid protein phosphorylation.
Immunoblots against PsaB (a), PsaC (b), PsaD (c) and phosphorylated threonine residues (d) performed on thylakoid membranes isolated from wild type (WT) and *pgr5* plants before treatment (Pre-tr), directly after 4 h treatment with 1000 µmol m^{-2} s^{-1} (Day 0) and during the following 4 days of recovery in 125 µmol m^{-2} s^{-1} (recovery Day 2 omitted due to spatial constraints). Dilution series (200% - 50%) of the WT Pre-tr. sample were included for each antibody. All lanes (except dilution series) contain 0.5 µg chlorophyll, Coomassie brilliant blue staining of a representative PVDF membrane (e) is included to show protein equivalence between lanes. Arrow in (b) indicates the specific PsaC cross-reaction. Phosphorylated photosynthetic proteins (d) are indicated.

Figure 3. The impact of PSI photoinhibition on CO_2 assimilation under different light intensities.
Changes in net CO_2 assimilation (*A*) in leaves of WT and *pgr5* plants before treatment (Pre), after exposure to either growth light (GL, 125 µmol m^{-2} s^{-1}) or to 4 h high light (HL, 1000 µmol m^{-2} s^{-1}) (Day 0), and during the subsequent 5 days recovery under growth light (Day 1 – 5). Gas exchange measurements were performed under (a) 50 µmol photons m^{-2} s^{-1}; (b) 125 µmol photons m^{-2} s^{-1}; (c) 1000 photons µmol m^{-2} s^{-1}. Error bars show standard deviation

among replicates (n = 4). Significant differences between treatments and genotypes are indicated by non-overlapping error bars (Student's *t* test, $p < 0.05$). The vertical yellow bar represents application of the HL treatment.

Figure 4. The effects of PSI photoinhibition on accumulation of carbohydrates.
Abundances of (a) starch; (b) D-glucose; (c) D-fructose in leaves of WT and *pgr5* plants before treatment (Pre), after exposure to either growth light (GL, 125 µmol m^{-2} s^{-1}) or to 4 h high light (HL, 1000 µmol m^{-2} s^{-1}) (Day 0), and during the subsequent 5 days under growth light (Day 1 – 5). Error bars show standard deviation among replicates (n = 4). Significant differences between treatments and genotypes are indicated by non-overlapping error bars (Student's *t* test, $p < 0.05$). The vertical yellow bar represents application of the 4 h HL treatment.

Figure 5. The effects of PSI damage on mitochondrial respiration.
(a) Day-time respiration (measured after 15 min incubation in darkness) after 4 h HL (1000 µmol m^{-2} s^{-1}) treatment of WT and *pgr5* plants and during subsequent recovery of 5 days, shown relative to growth light (GL, 125 µmol m^{-2} s^{-1}) treated controls; (b) decline in the rate of O_2 uptake in leaves of GL- and HL-treated WT and *pgr5* plants throughout 4 h dark incubation directly after treatment with HL or GL, r^2 values represent correlation of trend lines that were fitted to data points from HL-treated samples (dotted lines); (c and d) night-time O_2 uptake in leaves of WT (c) and *pgr5* (d) plants treated with 4 h HL, measured 6 h after "sunset". HL data are shown relative to GL-treated controls. Error bars show standard deviation among replicates (n = 4). Significant differences between treatments and genotypes are indicated by non-overlapping bars (Student's *t* test, $p < 0.05$). The vertical yellow bar represents application of the 4 h HL treatment.