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Steady-state phosphorylation of light-harvesting complex II proteins preserves Photosystem I under fluctuating white light

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Abstract

According to the ‘state transitions’ theory, the light-harvesting complex II (LHCII) phosphorylation in plant chloroplasts is essential to adjust the relative absorption cross section of photosystem (PS) II and PSI upon changes in light quality. The role of LHCII phosphorylation upon changes in light intensity is less thoroughly investigated, particularly when changes in light intensity are too fast to allow the phosphorylation/dephosphorylation processes to occur. Here, we demonstrate that the stn7 Arabidopsis mutant, devoid of the STN7 kinase and LHCII phosphorylation, shows a growth penalty only under fluctuating white light due to a low amount of PSI. Under constant growth light conditions stn7 acquires chloroplast redox homeostasis by increasing the relative amount of PSI centers. Thus, in plant chloroplasts, the steady-state LHCII phosphorylation plays a major role in preserving PSI upon rapid fluctuations in white light intensity. Such protection of PSI results from LHCII phosphorylation-dependent equal distribution of excitation energy to both PSII and PSI from the shared LHCII antenna and occurs in co-operation with non-photochemical quenching (NPQ) and the PGR5-dependent control of electron flow, which are likewise strictly regulated by white light intensity. LHCII phosphorylation is concluded to function both as a stabilizer (in time scales of seconds to minutes) and dynamic regulator (time scales from tens of minutes to hours and days) of redox homeostasis in chloroplasts, subject to modifications by both environmental and metabolic cues. Exceeding the capacity of LHCII phosphorylation/dephosphorylation to balance the distribution of excitation energy between PSII and PSI results in readjustment of photosystems stoichiometry.

Key words: STN7 kinase, Photosystem I, Photosystem II, LHCII phosphorylation, state transitions, LHC complex, non-photochemical quenching, PGR5, PsbS, fluctuating light, light acclimation, redox signaling, retrograde signaling
Introduction

Plant acclimation to different quantity and quality of light has been extensively investigated. The light quality experiments have usually concerned the red/blue and far red light acclimation strategies, which have been closely related to the state transitions and the phosphorylation of the light-harvesting II (LCHII) proteins, Lhcb1 and Lhcb2, by the STN7 kinase (Allen 2003; Bellafiore et al., 2005; Bonardi et al., 2005; Tikkanen et al., 2006; Rochaix 2007). Such studies on acclimation to different qualities of light have uncovered key mechanisms required for maintenance of photosynthetic efficiency in dense populations and canopies (Dietzel et al., 2008). However, the role of LHCII phosphorylation under fluctuations in the quantity of white light has been scarcely investigated. Light conditions in natural environments may be very complex with respect to the quantity of white light, which constantly fluctuates both in short- and long-term durations (Smith, 1982; Kulheim et al., 2002). Thus the acclimation strategies to natural environments must concomitantly meet the challenges of both the high and low light acclimation. Changing cloudiness, for example, would initiate both the high light and low light acclimation signals in the time scale of minutes and hours, whereas the movements of leaves in the wind or the rapid movement of clouds would initiate even more frequent light acclimation signals. The kinetics of reversible LHCII phosphorylation is far too slow to cope with rapid environmental changes.

The phosphorylation level of LHCII proteins in the thylakoid membrane is regulated by both the STN7 kinase and the counteracting PPH1/TAP38 phosphatase (Pribil et al., 2010; Shapiguzov, et al., 2010). No definite results are available about regulation of the PPH1/TAP38 phosphatase but the STN7 kinase is strongly under redox regulation (Lemeille et al., 2009) and controls the phosphorylation level of LHCII proteins under varying white light intensity as well as according to chloroplast metabolic cues, as described already decades ago (Fernyhough et al., 1983; Rintamaki et al., 2000; Hou et al., 2003). So far, the research on the role of the STN7 kinase and LHCII phosphorylation in light acclimation of higher plants has heavily focused on reversible LHCII phosphorylation and concomitant state transitions. The state 1 to state 2 transition, by definition, means the phosphorylation of LHCII proteins, their detachment from PSII in grana membranes and migration to the stroma membranes to serve in collection of excitation energy to PSI (Fork and Satoh, 1986; Williams and Allen, 1987; Wollman, 2001; Rochaix, 2007; Kargul and Barber, 2008; Murata, 2009; Lemeille et al., 2010; Minagawa, 2011). Concomitantly, the absorption cross section of PSII
decreases and that of PSI increases (Canaani and Malkin, 1984; Malkin et al., 1986; Ruban and Johnson, 2009). Indeed, state transitions have been well documented when different quality (blue/red and far red) of light, preferentially exciting either PSII or PSI, has been applied.

Different from state transitions, the white light intensity-dependent reversible LHCII phosphorylation does not result in differential excitation of the two photosystems (Tikkanen et al., 2010). Instead, both photosystems remain nearly equally excited independently whether the LHCII proteins are heavily phosphorylated or strongly dephosphorylated. Moreover, it is worth noting that the different qualities of light generally used to induce reversible LHCII phosphorylation and state transitions (blue/red and far red lights) have usually been of very low intensity (See for a review (Haldrup et al., 2001)) and apparently minimal protonation of the lumen takes place under such illumination conditions. Yet another difference between induction of LHCII protein phosphorylation by different quality of light or different quantity of white light concerns concomitant induction of PSII core protein phosphorylation. In the former case, the level of PSII core protein phosphorylation follows the phosphorylation pattern of LHCII proteins whereas under different quantities of white light the phosphorylation behavior of PSII core and LHCII proteins is the opposite (Tikkanen et al., 2008b).

To gain a more comprehensive understanding of the physiological role of white light-induced changes in LHCII protein phosphorylation, we have here integrated LHCII phosphorylation with other light-dependent regulatory modifications of light harvesting and electron transfer in the thylakoid membrane, which include the non-photochemical quenching of excitation energy (See for recent reviews Niyogi, 1999; Horton and Ruban, 2005; Barros and Kuhlbrandt, 2009; de Bianchi et al., 2010; Jahns and Holzwarth, 2012; Ruban et al., 2012) and the photosynthetic control of electron transfer by the Cytb6f complex (Rumberg and Siggel, 1969; Witt, 1979; Tikhonov et al., 1981; Bendall, 1982; Nishio and Whitmarsh, 1993; Joliot and Johnson, 2011; Suorsa et al., 2012; for review see Foyer et al., 1990; Foyer et al., 2012), both strongly dependent on lumenal protonation.

It is demonstrated that the steady-state LHCII phosphorylation is particularly important under rapidly fluctuating light conditions. It ensures equal energy distribution to both photosystems, prevents accumulation of electrons in the intersystem electron transfer chain, eliminates perturbations in chloroplast redox balance and maintains PSI functionality upon rapid fluctuations in white light intensity.
Results

Phenotype of Arabidopsis WT and *stn7* mutants grown under different light conditions

In order to investigate the mechanisms that enable the acclimation of Arabidopsis to rapidly changing light intensities, we set up an illumination device in which the intensity of white light fluctuates (FL), i.e. low light (60 µmol photons m$^{-2}$ s$^{-1}$) is interrupted every five minutes by a one minute-lasting high light (600 µmol photons m$^{-2}$ s$^{-1}$) peak. Plants, developed in this condition for their entire life, apparently make use of the full flexibility of the photosynthetic machinery in order to acclimate in the best possible way to such a dynamic growth light condition. As controls, we used plants grown under three different constant light intensities: low, moderate and high light (LL, ML and HL, respectively). ML light plants received roughly an equal number of photons as the FL plants during the 8-h photoperiod.

Different Arabidopsis mutant lines grown under FL and the three different constant light intensities are depicted in Table I. In addition to the *stn7* single mutant, we tested also other thylakoid mutants for growth under FL, including *stn8*, *stn7 stn8*, *npq4*, *npq4 stn7* and *npq1* with known defects in regulation of the LHCII-PSII function (Niyogi et al., 1998; Bonardi et al., 2005; Niyogi et al., 2005; Vainonen et al., 2005; Frenkel et al., 2007; Tikkanen et al., 2008a). Under FL, all mutant plants lacking the STN7 kinase grew significantly slower compared to WT or the *stn8* and *npq* mutants (Fig 1A, Supplemental Fig. S1). On the contrary, growth under constant LL, ML and HL illumination did not result in the visual phenotype of the mutants different from that of WT.

Chlorophyll *a/b* ratio (chl *a/b*) was first used as a parameter to monitor the acclimation processes. Under constant growth light conditions only the *stn7* mutants showed slightly lower value of chl *a/b* compared to WT and the other mutants (Fig. 1B). Intriguingly, however, in plants grown under FL the chl *a/b* ratio was distinctly lower in all the *stn7* mutants than in WT or the *stn8* and *npq* mutants. These differences in the chl *a/b* ratio in plants grown under fluctuating light suggested modification(s) in the stoichiometry of the main photosynthetic pigment protein complexes.
Importantly, the FL light conditions were designed such that the one-minute high light pulse was not long enough to induce changes in the level LHCII protein phosphorylation of WT plants (Fig. 1C).

**Stoichiometry of thylakoid protein complexes and extent of protein phosphorylation during light acclimation**

To gain deeper insights into structural modifications of the thylakoid membrane in the absence of STN7, we next focused on the WT and stn7 mutant plants grown under constant LL, ML and HL conditions as well as under FL. Thylakoid pigment protein complexes were solubilized with DM and separated by lpBN-PAGE (Järvi et al., 2011) in the first dimension, and then the individual proteins comprising the protein complexes were separated by SDS-PAGE. The gels were first stained with ProQ® Diamond, a quantitative dye for phosphoproteins, and then with SYPRO® Ruby, a quantitative dye for total proteins. Figure 2A shows the representative 1D and 2D gels from thylakoid membranes of WT and stn7 grown under FL. The nomenclature of the protein complexes separated in lpBN-PAGE is based on Järvi et al., 2011, and the identification of the individual proteins is based on (Aro et al., 2005). The relative amounts of PSI and PSII, and their respective antennae LHCI and LHCII, were analysed by spot densitometry from the 2D gels using representative proteins of the chlorophyll-binding complexes: CP47 and CP43 for PSII, PsaA and PsaB for PSI, Lhcb1 and Lhcb2 for LHCII, Lhca2 and Lhca3 for LHCI. These data allowed the comparison of the ratios of different pigment protein complexes between WT and stn7 on one hand, and on the other hand they made possible to analyse the acclimation strategy at the level of pigment protein complexes of both WT and stn7 to different growth light conditions (Fig. 2B).

The stn7 mutant plants grown under constant LL, ML and HL showed a lower PSII/PSI ratio as compared to WT. On the contrary, for plants grown under FL an opposite result was obtained, the PSII/PSI ratio being clearly higher in the stn7 mutant than in WT (Fig. 2B). In plants developed under constant light the LHCII/(PSII+PSI) ratio was similar between WT and the stn7 mutant but the FL growth conditions again resulted in a difference, this ratio being higher in the stn7 plants compared to WT. This result was partially due to higher LHCII/PSII ratio in the stn7 mutant plants grown under FL compared to WT but was also contributed by remarkably higher LHCII/PSI ratio in the stn7 mutant plant (Fig. 2B).
Analysis of thylakoid protein phosphorylation in WT and the stn7 plants from different growth conditions was performed by normalizing the phospho-signal to the amount of the respective protein signal determined from the same gel after staining with ProQ and SYPRO, one after another (Fig. 2C). It was first tested whether the phosphorylation level of thylakoid proteins changed in the course of light intensity fluctuations under FL growth conditions. As shown in Fig. 1C, fluctuations in light intensity in minutes time scale did not significantly affect the phosphorylation level of thylakoid proteins. Comparisons of steady-state thylakoid protein phosphorylation between different growth conditions and between the WT and stn7 thylakoids were always made between samples run side by side in the same gel. As can be seen from Fig. 2C, the relative phosphorylation of the PSII core proteins in WT was several times higher than that of the LHCII proteins, and the highest phosphorylation level of LHCII proteins occurred under FL growth conditions. As expected, the LHCII phosphorylation in the stn7 mutant was only negligible. Nevertheless, the steady-state phosphorylation level of the PSII core proteins CP43, D2 and D1 under the light period was higher in the stn7 mutant than in WT under all different growth conditions. This could suggest that the stn7 mutant, in the absence of LHCII protein phosphorylation, generally keeps the PQ pool more reduced compared to WT. The phosphorylation level of neither the PSII core nor the LHCII proteins was drastically affected whether the plants were grown under LL, ML or HL conditions. Since the phosphorylation of the PSII core proteins by the STN8 kinase is regulated by the redox state of the PQ pool (Tikkanen et al., 2008a), these results suggest that plants achieve a redox balance upon acclimation to different light intensities.

Immunoblot analyses of WT and stn7 grown under ML and FL were performed as a second approach to estimate the relative amounts of representative proteins of the main photosynthetic protein complexes (Fig. 3): PSII (D1), PSI (PsaB), LHCII (Lhcb2), LHCI (Lhca1) and Cyt b6/f (Cyt f) for linear electron flow; NAD(P)H-dehydrogenase (NdhL), PTOX, PGRL1 and PGR5 for alternative pathways and regulation of photosynthetic electron flow; ATP synthase (ATPβ), and Rubisco (RbcL) for the stromal carbon fixation. WT and stn7 plants revealed similar changes in photosystems stoichiometry as observed by the 2D lpBN-PAGE analysis (Fig. 2). The PSII level, revealed by the D1 protein, was only slightly lower in stn7 compared to WT in both ML and FL, whereas no distinct differences were found in the relative amounts of ATP synthase, Cyt b6/f or NAD(P)H-dehydrogenase (NdhL), PTOX, PGRL1 and PGR5. RuBisco showed some decline in FL growth conditions but this occurred both in WT and stn7. Interestingly, a clear difference between the stn7 mutant and
WT thylakoids from constant ML growth condition was a higher relative amount in stn7 of the PsAaB protein, indicative of the content of the PSI centers, as well as of the LHCI antenna (Fig. 3, A and B). In sharp contrast to ML growth conditions, under FL growth conditions the amounts of PSI as well as LHCI were substantially lower in the stn7 mutant than in WT, thus corroborating the results obtained from total Sypro staining experiments (Figure 2).

To reveal the origin of a substantially lower chl a/b ratio of the stn7 thylakoids from FL grown plants compared to WT, and a minor difference between stn7 and WT under ML conditions, the major chlorophyll protein bands were excised from the 1D lpBN-PAGE, chlorophyll was extracted and the chl a/b ratios of the pigment protein complexes were measured (see Supplemental Fig. S2). A minor decrease in chl a/b in stn7 grown under ML as compared to WT seemed to be evenly distributed among all antenna and photosystem complexes. The chl a/b ratios in FL grown-stn7 plants also showed a similar pattern as in WT except for the PSII dimer + PSI monomer band, which revealed a lower chl a/b in stn7 than the corresponding band of WT or in the ML grown stn7 plants. This result is consistent with a low content of PSI centers in stn7 grown in FL. The fraction of total chlorophyll associated with each pigment protein complex in WT and stn7 under FL and ML is depicted in Supplemental Fig. S2.

To further investigate the relative differences in the pigment protein complexes, as described above, the distribution of excitation energy between PSII and PSI was examined by 77 K chlorophyll a fluorescence emission spectra, recorded from thylakoids isolated from WT and the stn7 mutant plants grown in all the four different light conditions (Supplemental Fig. S3A). The PSII/PSI fluorescence ratios calculated from the spectra are shown in Supplemental Fig. S3B. In all growth conditions the stn7 mutant showed a higher value compared to WT. As expected, the lack of LHCI phosphorylation favored the excitation of PSII. However, the difference in PSII/PSI fluorescence ratio between WT and stn7 plants was remarkably higher in fluctuating light compared to the differences observed in constant growth light conditions (P<0.01). This observation confirmed that the PSII/PSI ratio increased in stn7 grown in FL and, moreover, indicated that such stoichiometric change greatly favors the excitation of PSII.

**Functional properties of WT and stn7 thylakoids upon acclimation to fluctuating light**
Functional consequences of the modifications in the amounts of thylakoid protein complexes, which on one hand led to the acclimation of the \textit{stn7} mutant to constant growth light conditions but, on the other hand, resulted in impaired growth of \textit{stn7} under FL, were addressed next. The first functional parameter to be addressed was the Q\textsubscript{A} redox state that is an indicator of the redox balance between PSII and PSI and hence describes the flow of electrons in ETC. The Q\textsubscript{A} redox state is commonly measured by the 1-q\textsubscript{P} and 1-q\textsubscript{L} chlorophyll fluorescence parameters that estimate the fraction of closed PSII reaction centers, based on the puddle and the lake models, respectively, in steady state conditions (Dietz et al., 1985; Weis and Berry, 1987; Huner et al., 1996; Gray et al., 1996; Kramer et al., 2004). However, during the high light phases of fluctuating light and in the first half of low light phases, the steady state condition was not reached. Actually, the aim of using HL pulses was to disturb the steady state in order to challenge the dynamic responses of photosynthetic mechanisms. Therefore, as an indicator of the Q\textsubscript{A} redox state we used the chlorophyll \textit{a} fluorescence yield values under actinic light normalized to the maximal fluorescence F\textsubscript{m} (the F'/F\textsubscript{m} parameter). Although F'/F\textsubscript{m} is not linearly correlated to [Q\textsubscript{A}−] due to the antenna connectivity (Joliot and Joliot, 1964; Lavergne and Trissl, 1995), we used this parameter as a semiquantitative estimation of the reduction level of QA (denoted as ‘Relative Q\textsubscript{A} redox state’), relevant to indicate relative changes under non-steady state conditions.

Fluorescence was first monitored from the WT and the \textit{stn7}, \textit{stn8}, \textit{stn7 stn8}, \textit{npq4}, \textit{npq4 stn7} and \textit{npq1} mutants grown both in ML and FL conditions, by mimicking the same illumination program as during the growth under FL (actinic fluctuating light (AFL) = 5 min low green light and 1 min high green light repeating the entire measuring period) using JTS-10 spectrofluorometer (BioLogic, France) (Fig. 4). The first low intensity period of the three fluctuating light cycles was different from the other cycles and gave information on the activation of photosynthesis after the dark incubation while all the following ones showed a cyclic repetition during the AFL experiment (only two such cycles are shown in Fig. 4). The Q\textsubscript{A} reduction showed a similar low steady-state value upon low illumination phases of AFL in all \textit{stn7} mutants grown under ML and in WT grown in both ML and FL. However, in FL grown \textit{stn7} plants the Q\textsubscript{A} reduction level reached a much higher value during the low illumination phase of AFL, indicating an imbalance in the excitation of PSII and PSI, in favor of PSII, and resulting in accumulation of electrons in the intersystem electron transfer chain (ETC). Since the F'/F\textsubscript{m} parameter in the second half of the low light illumination phases of
AFL reached a steady-state value, it can be used also as an indicator of the PQ pool redox state.

These results raised the question whether the observed redox unbalance was related to the lack of STN7 kinase itself or rather to the lack of LHCII docking to PSI. To this end, we analyzed the psal mutant plants, which revealed similar visual phenotype as the stn7 plants when grown under FL conditions (Supplemental Fig. S4A), and have previously been shown to be impaired in P-LHCII binding to PSI and consequently also in state transitions (Lunde et al., 2000). The same measurements as shown in Fig. 4 were carried out on psal mutant plants, grown in constant moderate (ML) and in fluctuating light conditions (FL) (Supplemental Fig. S4B). Functional analysis of the PQ pool redox state under AFL revealed that the lack of the PsaL protein results in a significant increase in the PQ pool reduction state during the low light phases of AFL in plants grown under FL (P<0.01) In ML growth condition, on the contrary, the psal mutant showed a full compensation of mutation. These results follow the same trends observed in the stn7 plants, even though in a less drastic way. This is apparently due to the fact that the psal mutant is still able to perform state transitions in an extent equal to about 30% of WT (Lunde et al., 2000). The similarity of psal and stn7 mutant phenotypes under FL suggested that the STN7 kinase-induced LHCII phosphorylation is a key factor for redox balancing under fluctuating light, not the STN7 kinase per se. It was concluded that the capacity to maintain the dynamics of the light harvesting apparatus and balanced excitation of both PSII and PSI, despite fluctuations in the light intensity, determines the success of growth under strongly fluctuating light conditions.

Fluctuating growth light-mimicking fluorescence experiments were repeated with FL-grown WT and the mutant plants using a Dual-PAM spectrofluorometer, which enables simultaneous measurements of both PSII and PSI functionality. Four photosynthetic parameters were measured: (i) the reduction level of QA (F'/Fm), (ii) the reduction level of P700 reaction centers, (iii) the non-photochemical quenching of excitation energy (NPQ) and (iv) the PSI acceptor side limitation (Y(NA)) (Fig. 5).

The F'/Fm of WT leaves slightly increased in the beginning of the low intensity light period of AFL and then stabilized to a low steady-state level. In the stn7 and stn7 stn8 mutants, the F'/Fm in the beginning of the low intensity light period had almost the same value as WT but then increased greatly and stayed high during the entire low light illumination phase of AFL. In the npq4 stn7 mutant, F'/Fm was high as well and remained at
this high level for the entire duration of AFL. Indeed, all plants lacking the STN7 kinase showed nearly similar steady-state of \( F'/F_m \), which reached the level much higher than that in WT and the other mutants (Fig. 5A). This result thus confirmed the high reduction level of the PQ pool in all \( stn7 \) mutants when grown under FL conditions.

The behavior of PSI and NPQ under actinic FL (AFL) were recorded next. During the LL illumination period of AFL, the P700 reaction centers were fully reduced (Fig. 5B) and NPQ was very low both in WT and the \( stn7 \) mutant (Fig. 5C). Upon switch on the 1 min HL pulse of AFL, NPQ was rapidly induced in WT. The PSI reaction centers were rapidly oxidized in WT upon the HL pulse (Fig. 5B). Similar trends were monitored in the \( stn7 \) mutant during the HL period of AFL. Distinctively, however, in \( stn7 \) mutants PSI was oxidized more slowly and to a lesser extent than in WT. NPQ was similarly low during the LL phase in all plants (Fig. 5C). Upon the HL phase, WT as well as the \( stn8 \) and \( stn7 \) mutants showed very similar development of NPQ, while it was low in \( npq1 \) and nearly non-existent in \( npq4 \) and \( npq4 \) \( stn7 \).

The Y(NA) parameter was measured to estimate the fraction of P700 reaction centers which are closed due to acceptor side limitation, i.e. due to the reduction of the first electron acceptors of PSI (Klughammer and Schreiber, 1994). A striking feature of all plants lacking the STN7 kinase was a clearly higher PSI acceptor limitation values during the HL phases of AFL compared to the other plants (Fig. 5D).

Comparison in the \( stn7 \) mutant plants of the behavior of \( F'/F_m \) and NPQ (Fig. 5, A and C) revealed that the strongly reduced PQ pool during low light periods was gradually oxidized to the WT level when NPQ became induced upon subsequent high light pulse. This indicates that the induction of NPQ helps in restoring the redox balance of the PQ pool altered by the lack of LHCII phosphorylation. This compensation is, however, not operational under low quenching states.

**PSI modifications in the acclimation process of the \( stn7 \) mutants**

Based on results above, it is conceivable that upon growth under constant light intensities the lack of STN7 is compensated by increasing the amount of PSI complexes (Fig. 2 and 3). Increase in PSI, in turn, maintained a relatively low reduction level of the PQ pool observed during the measurements under AFL. Such adjustment of PSI centers, however, did
not occur in \textit{stn7} grown under FL. On the contrary, the abundance of PSI centers in \textit{stn7} diminished under FL growth conditions. This prompted us to take a closer look at the role of PSI in the light acclimation process of the \textit{stn7} mutants.

A method was set up to correlate the amount of PSI with the structural and functional alterations of \textit{stn7} plants described above, by measuring the oxidation level of the P700 reaction centers. A saturating pulse (SP) was given to a leaf disc during far-red light illumination, allowing the calculation of the maximal oxidizable P700 centers, called $P_m$ (Klughammer and Schreiber, 1994), being proportional to the total amount of PSI (see Supplemental Fig. 5). The $P_m$ values were normalized to the leaf disc chlorophyll content ($P_m/\mu g$ chl). In these same measurements, prior to flashing the SP, the P700 oxidation level reached a plateau during the far-red light illumination (720 nm wavelength), which however did not result in full oxidization of PSI centers. Consequently, the redox steady state during the far red light illumination implied the presence of an electron flow that partially reduced intersystem ETC, and also PSI. The difference between the value of the plateau and $P_m$ was normalized against the $P_m$ value, giving the parameter $\Delta P/P_m$, proportional to the reduction level of the ETC. The possible sources of electrons maintaining the plateau level of P700 oxidation upon far red illumination include the excitation of PSII at 720 nm light and the cyclic electron flow (CEF) around PSI. Since no significant differences were observed in the amount of CEF complexes (Fig. 3) or in the extent of CEF (Supplemental Fig. S6) between WT and the \textit{stn7} mutant, we attributed the source of this electron flow to PSII. Therefore, in this specific case of comparison of WT and \textit{stn7}, the $\Delta P/P_m$ parameter can be used to estimate the redox balance between PSII and PSI (intersystem redox balance). (For details on the method see Materials and methods and Supplemental Fig. S5)

The two parameters, $P_m/\mu$ g chl and $\Delta P/P_m$, were plotted against the chl $a/b$ ratio, the simplest parameter used above to monitor the acclimation processes (Fig 1B). The results from five different experiments with WT and \textit{stn7} sets of plants (6 plants in each experiment) grown under ML and FL are shown in Figure 6. The \textit{stn7} mutants grown under constant ML conditions showed a similar pattern as WT (Fig. 6, A and C). Both the amount of PSI ($P_m/\mu g$ chl) and the $\Delta P/P_m$ parameter were stable despite clear differences in the chl $a/b$ ratio of individual plants from different experiments. WT plants grown under FL conditions behaved similarly as the WT plants grown under continuous ML conditions (Fig. 6). The \textit{stn7} mutant grown under FL light behaved very differently from WT plants and from the \textit{stn7} mutant grown under constant ML conditions. First, the amount of PSI ($P_m/\mu g$ chl) appeared to be
directly proportional to the chl \( a/b \) ratio in FL grown \( stn7 \) mutants (Fig. 6B). Indeed, the lower the chl \( a/b \) ratio the smaller was the PSI content in the \( stn7 \) mutant leaves upon growth under FL conditions. Second, the \( \Delta P/P_m \) parameter, which describes the reduction level of the ETC, was in the \( stn7 \) mutant grown under FL conditions inversely proportional to the chl \( a/b \) ratio (Fig. 6D) and thus also to the amount of PSI. It is worth noticing that every single set of plants grown under FL (grown at differed dates) showed a very characteristic and reproducible pattern - WT individuals clustered together showing very similar values of chl \( a/b \), \( P_m/\mu g \) chl and \( \Delta P/P_m \) whereas in the \( stn7 \) plants the chl \( a/b \) ratio was linearly related to variable amount of PSI (\( R^2 = 0.87\pm0.09 \)) and the \( \Delta P/P_m \) parameter values (\( R^2 = 0.88\pm0.08 \)). In \( stn7 \) plants grown in FL, \( \Delta P/P_m \) was linearly correlated to \( P_m/\mu g \) chl (\( R^2 = 0.90\pm0.03 \)), indicating that the lower the amount of PSI the higher is the intersystem redox unbalance (for details see Supplemental Fig. S5). WT and \( stn7 \) plants from the four different growth light conditions (constant LL, ML and HL as well as FL), except for the FL grown \( stn7 \) mutants, possessed a rather similar and low value of \( \Delta P/P_m \), ranging from 0.2 to 0.3, whereas in the FL grown \( stn7 \) mutants the \( \Delta P/P_m \) parameter was generally higher and greatly variable (Fig. 6C and D; results from LL and HL grown plants are shown in Supplemental Fig. S7). This provides further evidence that the STN7 kinase ensures functional flexibility of the thylakoid membrane, allowing the maintenance of optimal redox balance between PSII and PSI despite of changing light intensities and noticeable structural variations in the photosynthetic apparatus. These data together with the structural analyses above provide strong evidence that the low amount of PSI in the \( stn7 \) mutant results in impaired growth under FL as compared to WT.
Discussion

Light acclimation of the photosynthetic apparatus is likely to follow the signals originating in the photosynthetic machinery itself. It is clear both from our experiments here (Figures 4 and 5A) and from reports published earlier (Bailey et al., 2004; Miyake et al., 2009) that the successful acclimation of plants to changing light environments relates to the capacity of chloroplasts to keep the PQ pool largely oxidized also in the new light environment of both short- and long-term durations. A minimum of three different mechanisms controlling the linear electron transfer and chloroplast redox harmony have been identified so far. (i) The PsbS protein and xanthophyll-dependent non-photochemical quenching of excitation energy (NPQ) (for reviews, see (Niyogi, 1999; Horton and Ruban, 2005; Demmig-Adams and Adams, 2006)) is the mechanism that senses the energetic state of the photosynthetic machinery from the extent of lumenal protonation and quenches the excess excitation energy accordingly, affecting not only the redox state of the PQ-pool but also the stromal side of PSI. (ii) The STN7 kinase also senses the chloroplast redox state both in ETC and on the reducing side of PSI (Rintamäki et al., 2000), and via phosphorylated LHCII proteins balances the effects of NPQ to concern both photosystems, thus enabling the maintenance of excitation and redox balance upon changes in the efficiency of light harvesting (Bellafiore et al., 2005; Tikkanen et al., 2008b; Tikkanen et al., 2010). Indeed, these two mechanisms are not independent but function in close cooperation (Tikkanen et al., 2011). (iii) The dynamic “photosynthetic control” of linear electron transfer via the Cyt b6/f complex between PSII and PSI, according to lumenal protonation, is yet another mechanism that plays a vital role in preventing strong redox fluctuations in ETC (Hope, 1993; Joliot and Johnson, 2011; Suorsa et al., 2012).

Using the mutants of both the LHCII protein phosphorylation (stn7 mutants) and NPQ (npq1 and npq4) as well as various double mutants, we addressed the strategies of both the short- and long-term acclimation of Arabidopsis to constant and fluctuating white light conditions. The stunted growth phenotype (Fig. 1A, Supplemental Fig. S1) and low chl a/b ratio (Fig. 1B) were properties of all stn7 mutants (stn7, npq4 stn7, stn7 stn8) but only when grown under fluctuating light whilst WT or the npq mutants did not reveal distinct differences in the visual phenotype or chl a/b ratio upon growth under any of the light condition applied here (Fig. 1, Supplemental Fig. S1). Success in acclimation to natural fluctuating growth light
is dependent both on the steady-state LHCII phosphorylation and its dynamic regulation via the STN7 kinase and PPH1/TAP38 phosphatase activities.

**Steady-state LHCII phosphorylation preserves PSI upon rapid fluctuations of white light intensity**

Molecular background behind the distinct visual phenotype of *stn7* upon growth under fluctuating light (Fig. 1) (Bellafiore et al., 2005; Tikkanen et al., 2010) has remained elusive. Actually, depending on the strength of light gradients under fluctuating growth light conditions, a repertoire of *stn7* growth phenotypes, from invisible to very slow growth with chlorotic lesions, can be induced. To reveal the primary factors that lead to *stn7* phenotype, we first attempted to distinguish whether they result from the lack of STN7-induced LHCII phosphorylation and subsequent perturbations in regulation of redox homeostasis in chloroplasts or from the lack of the STN7 kinase as a putative retrograde signaling component. To this end, the *psal* mutant of Arabidopsis, which has an active STN7 kinase and LHCII phosphorylation but lacks the energy transfer from P-LHCII to PSI (Lunde, et al., 2000), was analysed. It revealed a similar phenotype and redox unbalance as the *stn7* mutant under FL growth conditions (Supplemental Fig. S4). These facts provide evidence that the *stn7* phenotype is primarily caused by the excitation and redox unbalance, not by a lack of any unknown phosphorylation-dependent signaling cascade mediated by the STN7 kinase.

As to the mechanism causing the stunted phenotype of *stn7*, it was striking to find out that the FL grown *stn7* mutant has a high PSII to PSI ratio (Figures 2, 3, 6 and Supplemental Fig. S3). Indeed, this is just opposite to the strategy of the *stn7* mutant to increase PSI centers for successful growth under constant light. Thus, either the high light pulse under FL growth conditions disturbs the redox-based signaling strategy for enhanced synthesis of PSI or a specific damage of PSI under fluctuating light causes the *stn7* growth phenotype. Dissecting between these two possibilities required a more detailed analysis of the redox behavior in chloroplasts under fluctuating light.

Under low light periods, the efficiency of excitation energy transfer to photosystems is at the maximum and under high light periods it is at the minimum, as regulated by NPQ (Fig. 7). NPQ thus buffers the effects of changing light intensity on the redox state of the thylakoid membrane. Nonetheless, this occurs successfully only when both photosystems are equally excited, and thus under low quenching state requires LHCII phosphorylation that
enables redox balance between the PQ-pool and the stromal side of PSI (Rintamäki et al., 2000) (Figures 4, 5 and 6). On the contrary, in the absence of LHCII phosphorylation (the stn7 mutants) (Fig. 7), the high efficiency of LHCII at low light intensity preferentially excites PSII as compared to PSI, leading to concomitant strong reduction of the PQ pool but a low reduction state of the PSI stromal side (Figures 4, 5, 6 and 7). Shift of stn7 from low to high light induces NPQ (Fig. 7), which leads to rapid oxidation of the “over”-reduced PQ pool and concomitant reduction of PSI stromal side electron acceptors. Indeed, the fluctuations between the non-quenched and quenched states in the absence of the STN7 kinase lead to strong short term changes in the redox state of the PQ-pool (Figures 4B and 5A).

In theory, the unbalanced redox state of the PQ pool could disturb the signaling cascades for enhanced PSI synthesis potentially initiated from the PQ-pool (Pfannschmidt et al., 1999; Fernandez and Strand, 2008) and thereby lead to a stunted phenotype of stn7. Nonetheless, the stn7 npq4 double mutant shows a high reduction level of ETC both in low and high light phases of FL (Fig. 5A), yet it has the stn7 FL growth phenotype. Accordingly, the most likely reason behind the scarcity of PSI centers in the stn7 mutants grown under FL conditions is their enhanced rate of damage and degradation due to unbalanced excitation energy distribution and electron transfer in the absence of LHCII phosphorylation.

To further investigate this option, we searched for common features for the stn7 single and stn7 npq4 double mutants that could lead to a damage of PSI. A striking feature of both mutants is the deficiency in oxidation of P700 due to an acceptor side limitation of PSI upon the high light phase of fluctuating growth light, as indicated by the measurements under AFL (Fig. 5, B and D). In general, under high light, the protonation of the lumen not only induces NPQ but also regulates electron transfer via the Cyt b₆f complex ((Rumberg and Siggel, 1969; Witt, 1979; Tikhonov et al., 1981; Bendall, 1982; Nishio and Whitmarsh, 1993); for review see (Foyer, et al., 1990; Foyer, et al., 2012)). Control via the Cyt b₆f complex particularly slows down the reduction of the plastocyanin (PC) pool (Eberhard et al., 2008), and it was recently shown that missing of this central mechanism in the pgr5 mutant has hazardous consequences on PSI (Joliot and Johnson, 2011; Suorsa et al., 2012). Do the stn7 mutants fail in control of electron flow via the Cytb₆f complex and thereby induce damage of PSI like in pgr5 (Suorsa et al., 2012)? Under the low light phases of FL growth conditions the lumen is only poorly protonated and consequently there is no control of electron flow by NPQ or the Cyt b₆f complex in WT or the stn7 mutants. LHCII collects photons in its full
efficiency and in WT the intersystem ETC remains mostly oxidized. This balance in WT keeps LHCII phosphorylated and both photosystems equally excited. Moreover, the reducing stromal side of PSI in WT can easily cope with the slow incoming electron flow (Fig. 5D). The situation with the stn7 mutants, however, is different and PSII becomes overexcited as compared to PSI due to the lack of LHCII phosphorylation (Figures 4 and 5). PSI simply cannot oxidize the intersystem electron transfer chain in the lack of enough excitation energy at low illumination phases. Indeed, a high reduction state of the ETC is a common and unique feature for both the stn7 and stn7 npq4 mutants under low illumination phases of FL.

In general, the over reduction of the intersystem ETC (PQ pool and PC pool) is not dangerous for PSI. Nevertheless, when the low light phase is followed by a high light phase (Fig. 7), a rapid oxidation of the highly reduced intersystem ETC (mainly the PC pool) in the stn7 mutants exceeds the capacity of immediate PSI electron acceptors, as indicated by the PSI acceptor side limitation in these mutants (Fig. 5D). Such excess of electrons has a potential to damage PSI. Indeed, it seems that the mechanism behind PSI phenotype of the stn7 mutants results from a sudden burst of electrons accumulated in ETC towards the acceptor side of PSI upon a shift from low to high light phase of the fluctuating growth light. It is well known that excess electrons are hazardous to the iron-sulfur clusters of PSI (Sonoike et al., 1995). We have recently shown that the protection of PSI from photodamage upon a shift from low to high light needs a PGR5-dependent mechanism that controls the speed of intersystem electron transfer via the Cyt b6f complex under the high light phase (Suorsa et al., 2012). Here we provide evidence that the excitation balance between PSII and PSI, provided by the STN7 kinase and steady state LHCII protein phosphorylation, is required to prevent an accumulation of excess electrons in the PC pool under the low light illumination phase. As a consequence of electron accumulation in the PC pool, a switch on of the HL phase suddenly supplies PSI with enough excitation energy to transfer the excess of electrons from the PC pool to PSI acceptors, causing an electron burst that damages PSI, possibly the Fe-S clusters.

The stn7 mutant acclimates to different constant light intensities by decreasing the PSII to PSI ratio

When changes in white light intensity occur in the time scale of hours, it is likely that the first attempt to balance the redox state of ETC originates from reversible phosphorylation
of LHCII. Under constant white light conditions the extent of LHCII phosphorylation is not at the maximum level. By the interplay between the STN7 kinase and the PPH1/TAP38 phosphatase, upon decrease in light intensity enhanced phosphorylation occurs whereas strong dephosphorylation takes place upon increase in light intensity as well as in strongly reduced light intensity (Rintamäki et al., 1997). Thus, the redox unbalances are sensed and transiently corrected by modulations of the level of LHCII protein phosphorylation. Nevertheless, the WT plants grown under LL, ML or HL conditions serve as an example of the fact that the phosphorylation of LHCII proteins eventually reaches a steady-state level that is rather similar irrespective of the light intensity (Fig. 2C). If the changes in light intensity are long-lasting and drastic enough, signals for readjustment of the photosynthetic machinery are initiated in order to meet the challenges of the new environment. The fact that the absence of the STN7 kinase causes light intensity dependent redox unbalance between the intersystem ETC and the PSI stromal side makes the \textit{stn7} mutant an excellent tool to elucidate the roles of the PQ-pool and the PSI acceptor side in regulation of the stoichiometry of various components of the photosynthetic machinery.

As shown above, the absence of the STN7 kinase leads to a strong reduction of the PQ-pool upon short-term fluctuations in white light intensity. In long term, the \textit{stn7} mutant compensates this unbalance and shows oxidized PQ pool at all different constant light conditions. Intriguingly, such compensation does not occur by decreasing the amount of LHCII. Instead, the \textit{stn7} mutant decreases the PSII to PSI ratio, which helps in keeping the PQ pool more oxidised. Such regulation strongly suggests, in accordance with previous studies on acclimation to changes in light quality (Pfannschmidt et al., 1999; Tullberg et al., 2000), that the signal(s) for adjusting the relative amounts of the PSII and PSI complexes are triggered by the redox state of the PQ-pool (see Supplemental Fig. S8).

Nevertheless, it has been unambiguously documented that the size of the LHCII antenna increases upon long term decrease in the light intensity (Leong and Anderson, 1984; Yang et al., 1998; Bailey et al., 2001). Both WT and the \textit{stn7} kinase mutants show rather similar long term regulation of the LHCII antenna size. Yet, the \textit{stn7} mutant increases the LHCII antenna size under limiting growth light even more than WT (Fig. 2), and this occurs despite much higher reduction level of the PQ pool as compared to WT (Figures 4B, 5A and 6D). Thus, our results with the \textit{stn7} mutant provide evidence that the actual signal for regulation of the LHCII antenna size originates from the redox state of components on the acceptor side of PSI (Supplemental Fig. S8). Manipulation of the redox state of these two
compartments, the ETC and the stroma, in WT Arabidopsis has also earlier led us to conclude that the redox compounds (or metabolites) in the stroma are crucial in regulation of the LHCII antenna size (Walters, 2005; Piippo et al., 2006).

In summary, comparison of the acclimation strategy of WT and \textit{stn7} to different constant light intensities lead to two important conclusions regarding the initiation or triggering of the retrograde signaling cascades for adjustment of thylakoid composition. First, the PQ pool redox state provides signals, by a yet unknown mechanism(s), to the chloroplast and nuclear genomes to initiate long term acclimation to modulate the relative amounts of the PSII and PSI core complexes according to environmental and metabolic cues. This conclusion is in line with experiments applying different quality of growth light on mustard (Pfannschmidt et al., 1999). Second, the increase in the size of the LHCII antenna is regulated by signals originating from the acceptor side of PSI, when the chloroplast redox compounds in the stroma are highly oxidized (see Supplemental Fig. S8).

\textit{Concluding remarks}

The STN7 kinase-dependent phosphorylation of LHCII is essential to maintain redox stability in chloroplast under fluctuating intensities of white light. Steady-state LHCII phosphorylation, in cooperation with NPQ and PGR5-dependent control of electron flow, maintains the functionality of PSI and is crucial for plants to cope with rapid changes in light intensity, for example with the sun flecks in natural environments. Long-lasting redox changes in ETC are dynamically reflected at the level of LHCII phosphorylation, which then transiently helps in acclimation to a new light environment. Thus, in very short-run, the excitation and redox balance provided by steady-state phosphorylation of LHCII is essential to preserve PSI. In longer-run, reversible LHCII phosphorylation allows a smooth transition phase before establishment of a new stoichiometry between PSII and PSI and the re-establishment of the steady-state LHCII phosphorylation level. Redox signals for the adjustment of photosystems stoichiometry initiate from the ETC whereas the signals for LHCII accumulation result from oxidation of redox components on the reducing side of PSI in the chloroplast stroma.
Materials and Methods

Growth of plants

Wild-type Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia and the *stn7* (Bellafiore et al., 2005), *stn8* (Bonardi et al., 2005), *stn7 stn8* (Bonardi et al., 2005), *npq4* (Niyogi et al., 2005), *npq4 stn7* (Frenkel et al., 2007) and *npq1* (Niyogi et al., 1998) mutant plants were grown in phytotron at 23 ºC, in relative humidity of 60 %, 8-h photoperiod, under three constant white light intensities: low light (LL, 50 µmol photons m$^{-2}$ s$^{-1}$), moderate light (ML, 130 µmol photons m$^{-2}$ s$^{-1}$) and high light (HL, 500 µmol photons m$^{-2}$ s$^{-1}$). The fluctuating growth light condition (FL) consisted of alternations of five minutes of LL (60 µmol photons m$^{-2}$ s$^{-1}$) and 1 min of HL (600 µmol photons m$^{-2}$ s$^{-1}$) provided by an automatic shading system, during the 8-h photoperiod. The total number of photons at FL growth condition corresponded to that of the constant ML growth condition. OSRAM PowerStar HQIT 400/D Metal Halide Lamps served as a light source. Mature rosette leaves from 3 (HL), 4 (ML) and 8 weeks (LL and FL) old plants were used for experiments. From fluctuating light, leaves were collected at the end of the low light period. The one minute high light period upon FL illumination was not long enough to change the phosphorylation of thylakoid proteins.

Chlorophyll content measurements

Chlorophyll amount and *a/b* ratio in leaves were determined in N,N-dimethylformamide (DMF) (Inskeep and Bloom, 1985) using fresh leaf discs (9 mm diameter).

Relative chlorophyll amount and *a/b* ratio values were measured from BN-gel bands after overnight methanol extraction, based on absorbance at 646.6 nm and 665.2 nm (for chl *b* and chl *a*, respectively).

2D large pore blue native (lpBN) electrophoresis

2D lpBN gel electrophoresis of n-Dodecyl β-D-maltoside (DM)–solubilized thylakoid membranes was carried out according to Järvi et al., 2011. SYPRO® Ruby and ProQ® Diamond staining was performed according to Invitrogen instructions. Gels images were acquired by Géliance 1000 Imaging System (Perkin Elmer). Spots densitometry was
performed by ProFinder 2D, version 2005 (Nonlinear Dynamics Ltd.). The intensity value of every spot in ProQ and Sypro stained gels was normalized based on the total intensity of all spots in the sample.

**SDS-PAGE and immunoblotting**

For isolation of total foliar extracts, leaves were immediately frozen in liquid nitrogen, homogenized in ice cold buffer (50 mM Hepes-KOH, pH 7.5, 100 mM sorbitol, 10 mM MgCl₂, 20 mM NaF) and filtrated through Miracloth. Chlorophyll content was determined as described in (Porra et al., 1989) and samples were solubilised and separated by SDS-PAGE according to (Laemmli, 1970) using 15% polyacrylamide and 6 M urea in the separation gel, and finally transferred to an Immobilon-P membrane (Millipore; http://www.millipore.com). After blocking with 5% milk (www.bio-rad.com), proteins were immunodetected with antibodies raised against D1, CP43, PsA, Lhcb2, Lhca1, Cyt f, NdhL, PGRL1, PGR5, PTOX, ATPβ or RbcL. Anti-Cyt f antibodies were kindly provided by L. Zhang, anti-NdhL and anti-PGR5 by T. Shikanai, anti-PGRL1 by D. Leister, anti-PTOX by M. Kuntz. All the other antibodies were purchased by Agrisera. Horseradish peroxidase–linked secondary antibody and enhanced chemiluminescence reagents (GE Healthcare; http://www.gehealthcare.com) were used for detection.

**In vivo measurements of PSII and PSI photosynthetic parameters**

Dual-PAM-100 (Heinz Walz GmbH, Effeltrich, Germany) was used for the simultaneous measurement of PSII and PSI photosynthetic parameters, based on chlorophyll a fluorescence and P700 oxidation signal (depending on the absorbance at 830 nm, see Klughammer and Schreiber 2008), respectively. The measuring light (460 nm wavelength) intensity for fluorescence measurements was 5 μmol photons m⁻² s⁻¹. Red actinic light (635 nm wavelength) was used at the intensities of 56 and 632 μmol photons m⁻² s⁻¹ to mimic the low and high light phases of fluctuating growth light. The relative QA redox state was measured as F’/Fm, where F’ is the fluorescence yield under actinic light and Fm is the maximal fluorescence from a dark adapted leaf during saturating pulse (SP, 6000 μmol photons m⁻² s⁻¹, 300 ms). NPQ was measured as (Fm-Fm‘)/Fm‘ (Bilger and Bjorkman, 1990), where Fm‘ is the maximal fluorescence yield from illuminated leaf during SP (a SP was given every 30 seconds). The PSI redox state and the PSI acceptor side limitation [Y(NA)] were determined according to (Klughammer and Schreiber, 1994 and 2008).
Chlorophyll fluorescence parameter $F'/F_m$ from intact leaves was determined also by
JTS-10 spectrometer (Bio-Logic SAS), using a green actinic light (520 nm wavelength) of 67
and 590 $\mu$mol photons m$^{-2}$ s$^{-1}$ intensities and a 250 ms saturating pulse (SP, 7900 $\mu$mol
photons m$^{-2}$ s$^{-1}$) provided by green light-emitting diodes.

The measurements of $Y(I)/Y(II)$ were carried out by using Dual-PAM-100. Detached
leaves were incubated in darkness for 15 min and subsequently illuminated by red actinic
light (635 nm wavelength).

The $F_0$ rise was detected in intact leaves after 5 min illumination under white actinic
light (100 $\mu$mol photons m$^{-2}$ s$^{-1}$). The fluorescence signal was normalized to the $F_m$ value.

For all the chlorophyll $a$ fluorescence experiments leaves were incubated in darkness
for 15 min and a SP was applied before turning on the actinic light. Leaves from minimum of
three different plants of each mutant and the wild-type were analysed.

The $\Delta P/P_m$ and $P_m$ parameters were measured based on changes of P700 redox state
measured according to Dual-PAM-100 instructions (Klughammer and Schreiber, 1994 and
2008). Leaf discs (9 mm diameter), collected from light adapted plants, were incubated in
darkness for five minutes and illuminated by far red light (720 nm wavelength, 191 $\mu$mol
photons m$^{-2}$ s$^{-1}$) for 9 seconds. On the top of far red light, a SP (10000 $\mu$mol photons m$^{-2}$ s$^{-1}$,
300 ms,) was applied and then both lights were turned off simultaneously. The difference
between the signal upon SP and darkness, and the signal upon SP and the far red light steady
state level were denoted as $P_m$ and $\Delta P$, respectively (see Supplemental Fig. S5). $P_m$ values
were normalized based on the leaf discs chlorophyll content ($P_m/\mu g$ chl), determined after
solubilization in DMF. It is worth noticing that the $P_m/\mu g$ chl parameter underestimates the
differences in PSI amount between samples, mainly because of the scattering of light passing
through the leaf. Since WT and $stn7$ plants showed similar leaf morphology and chlorophyll
concentration per area, we retain that this technical limitation constituted a systematic error
that cannot determine the observed linear correlations.

77 K chlorophyll $a$ fluorescence emission spectra were recorded (Murata et al., 1966).
Thylakoids were isolated and diluted in buffer containing 50 mM HEPES/KOH, pH 7.5, 100
mM sorbitol, 10 mM MgCl2, and 10 mM NaF to a chl concentration of 10 $\mu$g mL$^{-1}$ and
subjected to measurements of 77 K chl $a$ fluorescence emission spectra using a diode array
spectrophotometer (S2000; Ocean Optics) equipped with a reflectance probe. To record the
77 K fluorescence emission curves, the samples were excited with blue light (wavelengths
below 500 nm, derived from white light filtered by LS500S and LS700S filters (Corion)).
The emission between 600 and 800 nm was recorded and normalized according to the value at 685 nm.
Supplemental material

Supplemental Figure S1. Rosette diameter (relative values) of Arabidopsis wild-type (WT) and mutant plants grown under constant and fluctuating light.

Supplemental Figure S2. Chlorophyll a/b ratio and distribution of chlorophyll between pigment-protein complexes in WT and stn7 mutant plants grown under moderate and fluctuating light conditions.

Supplemental Figure S3. A, 77K chlorophyll a fluorescence spectra of WT and stn7 mutant plants grown in constant low (LL), moderate (ML) and high (HL) light growth conditions and under fluctuating light (FL). B, WT and stn7 PSII/PSI emission peak ratios.

Supplemental Figure S4. Visual phenotype (A) and relative QA reduction (chlorophyll a fluorescence parameter F'Fm) (B) in WT and psal mutant plants grown under fluctuating light.

Supplemental Figure S5. Supplemental information on data shown in Figure 6.

Supplemental Figure S6. Cyclic electron flow around PSI (CEF) in WT and stn7 plants grown under moderate (ML) and fluctuating light (FL) conditions.

Supplemental Figure S7. Amount of photosystem I (PSI) and intersystem electron transfer chain (ETC) reduction, plotted against chlorophyll a/b ratio (Chl a/b), in WT and stn7 mutant plants grown under low (LL) and high light (HL).

Supplemental Figure S8. Schematic model of the redox state of the electron transfer chain (PQ and PC) and the reducing side of PSI (stroma) in WT and stn7 under low (LL) and high (HL) light.
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Author Contributions
EMA, MG, MT and SK designed the experiments; MG and VP conducted most of research and analyzed the data together with MT and EMA; all authors contributed to the writing of the manuscript.
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Figure Legends

Figure 1. Phenotype of Arabidopsis wild-type (WT) and mutant plants grown under constant and fluctuating light. A, Visual phenotypes of plants grown under constant low (LL), moderate (ML) and high (HL) white light intensity (50, 130 and 500 μmol photons m⁻² s⁻¹, respectively) and under fluctuating light (FL) i.e. alternation of 5 min of low white light (60 μmol photons m⁻² s⁻¹) and 1 min of high white light (600 μmol photons m⁻² s⁻¹) during the 8-h photoperiod. Plants were photographed 8 weeks (LL), 4 weeks (ML), 3 weeks (HL) and 6 weeks (FL) after sowing the seeds. B, Chlorophyll a/b ratio (Chl a/b). Asterisks (*) indicate statistically significant differences between WT and stn7 plants at a P value < 0.001. Values are means (±SD) from three independent growth experiments, five plants from each. C, Phosphorylation of thylakoid proteins in WT plants during the cyclic phases of fluctuating growth light. Wild type plants were grown under fluctuating light (FL) for 8 weeks. Leaf samples were collected at the end of two different cycles of low (L) and high (H) light phases of FL after 4 and 8 hours from the beginning of 8-h photoperiod, and at the end of dark phase (D). Phosphorylation of thylakoid proteins was determined by immunoblotting with P-Thr antibody. P-CP43, P-D2, P-D1, and P-LHCII indicate the phosphorylated PSII core proteins CP43, D2, and D1 and the LHCII proteins Lhcb1 and Lhcb2. The shown immunoblot is representative of three independent experiments.

Figure 2. Relative amounts and phosphorylation pattern of photosystems and antennae from WT and stn7 plants grown under low (LL), moderate (ML), high (HL) and fluctuating (FL) light conditions. A, Two-dimensional large pore blue native gel electrophoresis (2D lpBN-PAGE) of thylakoid proteins isolated from WT and stn7 mutant plants grown under fluctuating light (FL WT and FL stn7, respectively). The thylakoid membranes were entirely solubilized by dodecyl-maltoside (DM). Every gel was stained both with SYPRO® Ruby, a quantitative dye for total proteins, and with ProQ® Diamond, a quantitative dye for phosphoproteins. The protein spots used for the densitometry are labeled. Proteins were identified based on Aro et al., 2005. MC = megacomplex; SC = supercomplex. B, Relative amount of antennae and photosystems in plants grown under LL, ML, HL and FL. PSII/PSI = Photosystem II/photosystem I ratio. LHCII/PSII+PSI = Light harvesting complex II/photosystems ratio. Data were obtained by spot densitometry of 2D Large Pore-Blue Native gels stained by SYPRO® Ruby dye. Asterisks (*) indicate statistically significant
differences between WT and stn7 plants at a P value < 0.05. Values are means of three independent experiments (±SD). C, Phosphorylation pattern of photosystem II (P-CP43, P-D2, P-D1) and LHCII (P-Lhcb1+2) in plants grown under LL, ML, HL and FL. The protein phosphorylation level was calculated as a ProQ/Sypro densitometric ratio of 2D lpBN-PAGE spots. Gels were loaded based on chlorophyll content (8 µg chl per sample). SC = supercomplex; dim = PSII dimer; mon = PSII monomer; TRIM = LHCII trimer; MON = LHCII monomer. Values are means of three independent experiments (±SD).

Figure 3. Photosynthetic proteins in WT and stn7 mutant plants grown under moderate (ML) and fluctuating light (FL). A, Immunoblotting. Gels were loaded based on chlorophyll content (chlorophyll amount varied depending on the primary antibody). B, Densitometry of D1 and PsAβ proteins. Asterisks (*) indicate statistically significant differences between WT and stn7 plants at a P value < 0.05. Gels are representative of three independent experiments.

Figure 4. Relative QA reduction in WT and the stn7, stn8, stn7 stn8, npq4, npq4 stn7 and npq1 mutant plants during the low light phases of fluctuating light.

The relative QA reduction was measured by chlorophyll fluorescence F/Fm parameter, in plants grown in (A) moderate (ML) and (B) fluctuating (FL) light conditions, under actinic light that mimicked the FL growth condition (AFL). Detached leaves were incubated in darkness for 15 minutes before measurements. L = low light phase of AFL; H = high light phase of AFL. No measuring points were detected during H.

Figure 5. Photosynthetic parameters of WT and the stn7, stn8, stn7 stn8, npq4, npq4 stn7 and npq1 mutant plants grown in fluctuating light condition (FL). Measurements were performed under actinic light that mimicked the FL growth condition (AFL). A, Relative QA reduction measured by chlorophyll fluorescence parameter F/Fm. B, PSI reduction monitored as a percentage of reduced P700 reaction centers. C, Non-photochemical quenching of excitation energy (NPQ). D, PSI acceptor side limitation [Y(NA)]. Detached leaves were incubated in darkness for 15 minutes before measurements. Values are the means from 3 independent experiments. L = low light phase of AFL; H = high light phase of AFL.

Figure 6. Amount of photosystem I (PSI) and the reduction state of intersystem electron transfer chain (ETC), plotted against chlorophyll a/b ratio (Chl a/b), in WT and stn7 mutant plants grown under moderate (ML) and fluctuating light (FL).
The amount of PSI (measured as the P_m signal and normalized to chlorophyll content) is plotted against chl a/b in leaf disks from plants in ML (A) and FL (B) growth conditions. The state of intersystem ETC reduction (measured as ΔP/P_m, see Supplementary Fig. S5A) is plotted against chl a/b in ML (C) and FL (D) growth conditions from the same leaf disks used in A and B, respectively.

**Figure 7.** Model of the interplay between LHCII phosphorylation, the photosynthetic control of electron transfer by Cyt b_6f and NPQ in WT and stn7 plants in constant growth light condition and in low (L) and high (H) light phases of fluctuating growth light condition (FL).

**A, Steady-state constant growth light conditions.** In WT, LHCII is phosphorylated, protonation of lumen is low, NPQ and the photosynthetic control of electron transfer by Cyt b_6f are not induced, ensuring fluent electron flow and redox harmony. In stn7, the redox harmony is achieved by increasing the amount of PSI centers.

**B, Low light phase of fluctuating growth light (FL).** In WT, redox harmony is achieved as under steady state condition. In stn7, when NPQ and the photosynthetic control of Cyt b_6f are not induced, excess electrons are accumulated in the entire ETC.

**C, High light phase of fluctuating growth light (FL).** In WT, the activity of PSII is slowed down by NPQ preventing the reduction of the PQ pool and the control of Cyt b_6f complex has a similar effect on the PC pool. In stn7, a shift to H phase provides energy to PSI to oxidize the over reduced ETC. Photosynthetic control of Cyt b_6f hinders the electron transfer from PQ but does not affect the rapid oxidation of the PC pool. A burst of electrons from over reduced PC damages PSI. Since PSI repair is almost non-existing, this results in a loss of PSI centers. Opposite to constant growth light conditions where stn7 increases the amount of PSI centers.

Red P denotes protein phosphorylation. Arrows indicate energy transfer. Wavy lines indicate LHCII heat dissipation. The size of circles represents the reduction states of the PQ and PC pools. Acc = capacity of stromal electron acceptors.

**Supplemental Figure S1.** Rosette diameter (relative values) of Arabidopsis wild-type (WT) and mutant plants grown under constant and fluctuating light.

Plants were grown under constant low (LL), moderate (ML) and high (HL) white light intensity and under fluctuating light (FL). The rosette diameter of every mutant was normalized according to the value of WT grown in the same condition. Measurements were carried out 8 weeks (LL), 4 weeks (ML), 3 weeks (HL) and 6 weeks (FL) after sowing the
seeds. Asterisks (*) indicate statistically significant differences between WT and stn7 plants at a P value < 0.01 (n=5).

**Supplemental Figure S2.** Chlorophyll a/b ratio and distribution of chlorophyll between pigment-protein complexes in WT and stn7 mutant plants grown under moderate and fluctuating light conditions. Values are means of three independent experiments (±SD).

**Supplemental Figure S3.** A, 77K chlorophyll a fluorescence spectra of WT and stn7 mutant plants grown in constant low (LL), moderate (ML) and high (HL) light growth conditions and under fluctuating light (FL). B, WT and stn7 PSII/PSI emission peak ratios. Exciting light was blue (wavelengths < 500 nm). Values are means of three different experiments (±SD).

**Supplemental Figure S4.** Visual phenotype (A) and relative QA reduction (chlorophyll a fluorescence parameter F’/Fm) (B) in WT and psal mutant plants grown under fluctuating light.

**Supplemental Figure S5.** Supplemental information on data shown in Figure 6. A, Method to determine Pm and ΔP/Pm parameters. P700 oxidation level was measured in intact leaves as the intensity of transmitted light at 875 nm minus the intensity of transmitted light at 830 nm (expressed in volts, V) by Dual-PAM-100 (Klughammer and Schreiber, 2008). After 5 min of dark incubation, far red light (FR) was switched on. Subsequently, a saturating pulse (SP) was applied and both FR and SP were switched off. The plateau reached during FR illumination reveals a steady-state that results from the intersystem electron transfer chain (ETC) reduction (reducing P700) and FR illumination (oxidizing P700). Part of the picture was obtained as a screenshot from Dual PAM v1.11 software. B-F, Data from a single set of WT and stn7 plants grown under fluctuating light (FL) (representative of five independent experiments, whose overall data are shown in Fig. 6, B and D). B-C, The values of the relative amount of PSI (Pm/µg chl) are shown as (B) average ±SD and (C) plotted against the chlorophyll a/b ratio (chl a/b). Asterisk (*) indicates statistically significant differences between WT and stn7 at a P value < 0.001 (n= 6). D-E, The values of the intersystem ETC reduction level (ΔP/Pm) are shown as (D) average ±SD and (E) plotted against chl a/b. Asterisk (*) indicates statistically significant differences between WT and stn7 at a P value < 0.05 (n= 6).
The decrease in chl \( a/b \) ratio observed in \textit{stn7} grown under FL is shown to correlate with decreased amount of PSI centers and with increase in the reduction level of intersystem ETC. F, The values of intersystem ETC reduction (\( \Delta P/P_m \)) are plotted against the amount of PSI (\( P_m/\mu g \) chl). In \textit{stn7}, the low amount of PSI causes redox unbalance in intersystem ETC. The coefficients of determination \( R^2 \) related to the linear regression of \textit{stn7} plants data are shown on the right.

**Supplemental Figure S6.** Cyclic electron flow around PSI (CEF) in WT and \textit{stn7} plants grown under moderate (ML) and fluctuating light (FL) conditions.

A, Photosystem I / photosystem II yields ratio [Y(I)/Y(II)], plotted against the photosynthetically active radiation (PAR) intensity. The increase of Y(I)/Y(II) is indicative of the capacity of CEF (Harbinson and Foyer, 1991).

B, NDH-dependent change in chlorophyll a fluorescence in darkness (\( F_0 \) rise). \( F_0 \) rise was detected in intact leaves after 5 min illumination under white actinic light (100 \( \mu \)mol photons \( m^{-2} \) \( s^{-1} \)). The fluorescence signal was normalized to the \( F_m \) value (\( F/F_m \)) and its relative increase in darkness is reported in the plot.

**Supplemental Figure S7.** Amount of photosystem I (PSI) and intersystem electron transfer chain (ETC) reduction, plotted against chlorophyll \( a/b \) ratio (Chl \( a/b \)), in WT and \textit{stn7} mutant plants grown under low (LL) and high light (HL).

The amount of PSI (measured as \( P_m \) signal and normalized to chlorophyll content) is plotted against chl \( a/b \) in LL (A) and HL (B) growth conditions. The intersystem ETC reduction level (measured as \( \Delta P/P_m \), see Supplemental Figure S5) is plotted against chl \( a/b \) in LL (C) and HL (D) growth conditions.

**Supplemental Figure S8.** Schematic model of the redox state of the electron transfer chain (PQ and PC) and the reducing side of PSI (stroma) in WT and \textit{stn7} under low (LL) and high (HL) light.

Left panel: Conditions that trigger the acclimation upon shift of plants to low and high light. ↑ upregulation, ↓ downregulation. Right panel: Acclimated state. Red P denotes protein phosphorylation. White arrows indicate energy transfer. The size of circles represents the reduction states of the PQ and PC pools and stroma.
Tables

Table I. Thylakoid regulatory mutants used in the experiments

| Mutant     | Defective protein product | Function                              | References                                      |
|------------|---------------------------|---------------------------------------|------------------------------------------------|
| stn7       | STN7 kinase               | LHCII phosphorylation                 | Bellafiore et al. 2005; Bonardi et al. 2005    |
|            |                           |                                       | Bonardi et al. 2005; Tikkanen et al. 2006      |
| stn8       | STN8 kinase               | PSII core phosphorylation             | Bonardi et al. 2005; Vainonen et al. 2005      |
| stn7 stn8  | STN7 and STN8 kinases     | LHCII and PSII core phosphorylation   | Bonardi et al., 2005                           |
| npq4       | PsbS                      | Feedback de-excitation (NPQ)           | Niyogi et al., 2005                            |
|            |                           | LHCII phosphorylation and feedback    |                                                |
| npq4 stn7  | STN7 kinase and PsbS      | de-excitation (NPQ)                    | Frenkel et al., 2007                           |
| npq1       | violaxanthin de-epoxidase (VDE) | Zeaxanthin-dependent NPQ | Niyogi et al., 1998                           |
**Figure 1.** Phenotype of Arabidopsis wild-type (WT) and mutant plants grown under constant and fluctuating light. A, Visual phenotypes of plants grown under constant low (LL), moderate (ML) and high (HL) white light intensity (50, 130 and 500 μmol photons m\(^{-2}\) s\(^{-1}\), respectively) and under fluctuating light (FL) i.e. alternation of 5 min of low white light (60 μmol photons m\(^{-2}\) s\(^{-1}\)) and 1 min of high white light (600 μmol photons m\(^{-2}\) s\(^{-1}\)) during the 8-h photoperiod. Plants were photographed 8 weeks (LL), 4 weeks (ML), 3 weeks (HL) and 6 weeks (FL) after sowing the seeds. B, Chlorophyll a/b ratio (Chl a/b). Asterisks (*) indicate statistically significant differences between WT and sin7 plants at a P value < 0.001. Values are means (±SD) from three independent growth experiments, five plants from each. C, Phosphorylation of thylakoid proteins in WT plants during the cyclic phases of fluctuating growth light. Wild type plants were grown under fluctuating light (FL) for 8 weeks. Leaf samples were collected at the end of two different cycles of low (L) and high (H) light phases of FL after 4 and 8 hours from the beginning of 8-h photoperiod, and at the end of dark phase (D). Phosphorylation of thylakoid proteins was determined by immunoblotting with P-Thr antibody. P-CP43, P-D2, P-D1, and P-LHClI indicate the phosphorylated PSII core proteins CP43, D2, and D1 and the LHClI subunit proteins, respectively. The immunoblotting results are representative of three independent experiments.
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Red P denotes protein phosphorylation. Arrows indicate energy transfer. Wavy lines indicate LHCII heat dissipation. The size of circles represents the concentration of a particular electron acceptor in the stromal electron acceptor pool.