Running head: Active avoidance of chloroplast state transitions

Corresponding authors:
Eva-Mari Aro:
Address: Molecular Plant Biology, Dept. Biochemistry, University of Turku, 20014, Turku, Finland
Email: evaaro@utu.fi
Phone: +35823338075

Mikko Tikkanen:
Address: Molecular Plant Biology, Dept. Biochemistry, University of Turku, 20014, Turku, Finland
Email: misati@utu.fi
Phone: +35823338255

Research area:
Photosynthesis research
Plants actively avoid state-transitions upon changes in light intensity - role of light-harvesting complex II protein dephosphorylation in high light

Nageswara Rao Mekala, Marjaana Suorsa, Marjaana Rantala, Eva-Mari Aro* and Mikko Tikkanen*

*Shared correspondence between Mikko Tikkanen and Eva-Mari Aro

Molecular Plant Biology, Dept. Biochemistry, University of Turku, 20014, Turku, Finland.

Summary:

PSII core and LHCII protein phosphorylation and dephosphorylation upon changes in light intensity are shown to maintain the excitation balance between photosystems by preventing state transitions.
Footnotes:

Research was supported by the Academy of Finland (projects 271832, 273870 and 260094).

Corresponding authors:
Eva-Mari Aro:
Email: evaaro@utu.fi

Mikko Tikkanen:
Email: misati@utu.fi
Abstract

Photosystem II (PSII) core and light-harvesting complex II (LHCII) proteins in plant chloroplasts undergo reversible phosphorylation upon changes in light intensity, being under control of redox regulated STN7 and STN8 kinases and TAP38/PPH1 and PBCP phosphatases. Shift of plants from growth light to high light results in increase of PSII core phosphorylation while LHCII phosphorylation concomitantly decreases. Exactly the opposite takes place when plants are shifted to lower light intensity. Despite distinct changes occurring in thylakoid protein phosphorylation upon light intensity changes, the excitation balance between PSII and PSI remains unchanged. This differs drastically from canonical state transition model induced by artificial “state 1” and “state 2” lights that concomitantly either dephosphorylate or phosphorylate, respectively, both the PSII core and LHCII phosphoproteins. Analysis of the kinase and phosphatase mutants revealed that TAP38/PPH1 phosphatase is crucial in preventing state transition upon increase in light intensity. Indeed, \textit{tap38/pph1} mutant revealed strong concomitant phosphorylation of both the PSII core and LHCII proteins upon transfer to HL, thus resembling WT under “state 2” light. Coordinated function of thylakoid protein kinases and phosphatases is shown to secure balanced excitation energy for both photosystems by preventing state transitions upon changes in light intensity. Moreover, PROTON GRADIENT REGULATION5 (PGR5) is required for proper regulation of thylakoid protein kinases and phosphatases, and the \textit{pgr5} mutant mimics phenotypes of \textit{tap38/pph1}. This demonstrates that there is a close co-operation between the redox- and proton gradient-dependent regulatory mechanisms for proper function of the photosynthetic machinery.
Introduction

Photosynthetic light reactions take place in the chloroplast thylakoid membrane. Primary energy conversion reactions are performed by synchronized function of the two light energy-driven enzymes, photosystem II (PSII) and photosystem I (PSI). PSII uses excitation energy to split water into electrons and protons. PSII feeds electrons to the intersystem electron transfer chain (ETC) consisting of plastoquinone, cytochrome b6f and plastocyanin. PSI oxidizes the ETC in a light-driven reduction of NADP to NADPH. Light energy is collected by the light harvesting antenna systems in the thylakoid membrane, composed of specific pigment-protein complexes (LHCI and LHCII). Majority of the light absorbing pigments are bound to LHCII trimers that can serve the light harvesting of both photosystems (Galka, et al., 2012; Kouril, et al., 2013; Wientjes et al. 2013b). Energy distribution from LHCII is regulated by proteins phosphorylation (Bennett, 1979; Bennett, et al., 1980; Allen, et al., 1981) under control of the STN7 and STN8 kinases (Depege, et al., 2003; Bonardi, et al., 2005b; Bellafiore, et al., 2005; Vainonen, et al., 2005) and the TAP38/PPH1 and PBCP phosphatases (Pribil, et al., 2010; Shapiguzov, et al., 2010; Samol, et al., 2012). LHCII trimers are composed of LHCB1, LHCB2 and LHCB3 proteins and, in addition to reversible phosphorylation of LHCB1 and LHCB2, also the protein composition of the LHCII trimers affect the energy distribution from the light harvesting system to photosystems (Damkjaer, et al., 2009; Pietrzykowska, et al., 2014). Most of the LHCII trimers are located in the PSII-rich grana membranes and PSI- and PSII-rich grana margins of the thylakoid membrane and only a minor fraction resides in PSI- and ATP synthase-rich stroma lamellae (Tikkanen, et al., 2008b; Suorsa, et al., 2014). Both photosystems bind a small amount of LHCII trimers in biochemically isolatable PSII-LHCII and PSI-LHCII complexes (Caffarri, et al., 2014; Pesaresi, et al., 2009; Jarvi, et al., 2011). Large portion of the LHCII, however, does not form isolatable complexes with PSII or PSI and therefore it separates as free LHCII trimers upon biochemical fractionation of the thylakoid membrane by sucrose gradient centrifugation or in native gel analyses (Jarvi, et al., 2011; Caffarri, et al., 2009), the amount being dependent on the thylakoid isolation method. Nonetheless, in vivo this major LHCII antenna fraction serves the light harvesting function. This is based on the fact that fluorescence from free LHCII, peaking at 680 nm in 77K fluorescence emission spectra, can only be detected when the energy transfer properties of the thylakoid membrane are disturbed by detergents (Grieco, et al., 2015).
Regulation of excitation energy distribution from LHCII to PSII and PSI has, for decades, been linked to LHCII phosphorylation and state transitions (Bennett, 1979; Bennett, et al., 1980; Allen, et al., 1981). It has been explained that a fraction of LHCII gets phosphorylated and migrates from PSII to PSI, which can be evidenced as increase in PSI cross section and was assigned as transition to State 2 (See for reviews: ((Allen, 2003; Rochaix, et al., 2012)). The LHCII proteins are, however, phosphorylated all over the thylakoid membrane i.e. in the PSII and LHCII rich grana core, in grana margins containing PSII, LHCII and PSI as well as in PSI-rich stroma lamellae also harboring PSII-LHCII, LHCII and PSI-LHCII complexes in minor amounts (Tikkanen, et al., 2008b; Grieco, et al., 2012; Wientjes, et al., 2013a; Leoni, et al., 2013) – making the canonical state transition theory inadequate to explain the physiological role of reversible LHCII phosphorylation (Tikkanen and Aro, 2014). Moreover, the traditional state transition model is based on lateral segregation of PSII-LHCII and PSI-LHCl to different thylakoid domains. It however seems likely that PSII and PSI are energetically connected via shared light harvesting system composed of LHCII trimers (Grieco, et al., 2015) and there is efficient excitation energy transfer between the two photosystems (Yokono, et al., 2015). Nevertheless, it is clear that LHCII phosphorylation is a prerequisite to form an isolatable PSI-LHCII complex called “state transition” complex (Pesaresi, et al., 2009; Jarvi, et al., 2011). Existence of a minor state transition complex, however, does not explain why LHCII is phosphorylated all over the thylakoid membrane and how the energy transfer is regulated from the majority of LHCII antenna that is shared between PSII and PSI, but does not form isolatable complexes with them (Grieco, et al., 2015).

Plants grown under any steady state white light condition demonstrate the following characteristics of the thylakoid membrane: PSII core and LHCII phosphoproteins are moderately phosphorylated, phosphorylation takes place all over the thylakoid membrane and the PSI-LHCII state transition complex is present (Jarvi, et al., 2011; Grieco, et al., 2012; Wientjes, et al., 2013b). Upon changes in the light intensity, the relative phosphorylation level between PSII core and LHCII phosphoproteins drastically changes (Rintamaki, et al., 1997; Rintamaki, et al., 2000) in the time scale of 5 to 30 min. When light intensity increases, the PSII core protein phosphorylation increases, while the level of LHCII phosphorylation decreases. On the contrary, a decrease in light intensity decreases the phosphorylation level of PSII core proteins, but strongly increases the phosphorylation of the LHCII proteins (Rintamaki, et al., 1997; Rintamaki, et al., 2000). The presence and absence of the PSI-LHCII
“state transition” complex correlate with LHCII phosphorylation (similarly to the state transitions) (Pesaresi, et al., 2009; Wientjes, et al., 2013b). Despite all these changes in thylakoid protein phosphorylation, the relative excitation of PSII and PSI (i.e. the absorption cross section of PSII and PSI measured by 77 K fluorescence) remains nearly unchanged upon changes in white light intensity i.e. no state transitions can be observed despite massive differences in LHCII protein phosphorylation (Tikkanen, et al., 2010).

The existence of the opposing behavior of PSII core and LHCII protein phosphorylation, as described above, has been known for more than 15 years (Rintamaki, et al., 1997; Rintamaki, et al., 2000), yet the physiological significance of this phenomenon has remained elusive. It is known that PSII core protein phosphorylation in high light (HL) facilitates the unpacking of PSII-LHCII complexes required for proper processing of the damaged PSII centers and thus prevents oxidative damage of the photosynthetic machinery (Tikkanen, et al., 2008a; Fristedt, et al., 2009; Goral, et al., 2010; Kirchhoff, et al., 2011). It is also known that the damaged D1 needs to be dephosphorylated before its proteolytic degradation upon PSII turnover (Koivuniemi, et al., 1995). There is, however, no coherent understanding available to explain why LHCII proteins are dephosphorylated upon exposure of plants to HL and PSII core proteins, respectively, upon exposure to low light (LL).

The above-described light quantity-dependent control of thylakoid protein phosphorylation drastically differs from the light quality-dependent protein phosphorylation (Tikkanen, et al., 2010). State transitions are generally investigated by using different light qualities, preferentially exciting either PSI or PSII. “State 1” light favors PSI excitation, leading to oxidation of the ETC and dephosphorylation of both the PSII core and LHCII proteins. “State 2” light, in turn, preferentially excites PSII leading to reduction of ETC and strong concomitant phosphorylation of both the PSII core and LHCII proteins (Haldrup, et al., 2001). Shifts between the “state 1” and “state 2” lights induce state transitions, a mechanism that changes the excitation between PSII and PSI (Murata and Sugahara, 1969; Murata, 2009). Similar to shifts between state lights, the shifts between LL and HL intensity also change the phosphorylation of the PSII core and LHCII proteins (Rintamaki, et al., 1997; Rintamaki, et al., 2000). Importantly, the white-light-intensity-induced changes in thylakoid protein phosphorylation do not change the excitation energy distribution between the two photosystems (Tikkanen, et al., 2010). Despite this fundamental difference between the light-quantity and light quality-induced thylakoid protein phosphorylations, a common feature for both mechanisms is a strict requirement of LHCII phosphorylation for formation of the PSI-
LHCII complex. Yet, it is worth noting that LHCII phosphorylation under “state 2” light is not enough to induce the state 2 transition but also the P-LHCII docking proteins in the PSI complex are required (Lunde, et al., 2000; Jensen, et al., 2004; Zhang and Scheller, 2004; Leoni et al 2013).

Thylakoid protein phosphorylation is a dynamic redox-regulated process dependent on the interplay between two kinases (STN7 and STN8) (Depege, et al., 2003; Bonardi, et al., 2005b; Bellaﬁore, et al., 2005; Vainonen, et al., 2005) and two phosphatases (TAP38/PPH1 and PBCP) (Pribil, et al., 2010; Shapiguzov, et al., 2010; Samol, et al., 2012). Concerning the redox regulation mechanisms in vivo, only the LHCII kinase (STN7) has so far been thoroughly studied (Rintamaki, et al., 2000; Vener, et al., 1997; Lemeille, et al., 2009). The STN7 kinase is considered as the LHCII kinase and indeed it phosphorylates the LHCB1 and LHCB2 proteins (Bellaﬁore, et al., 2005; Bonardi, et al., 2005a; Tikkanen, et al., 2006). In addition to this, STN7 takes part in the phosphorylation of PSII core proteins (Vainonen et al. 2006), especially in LL (Tikkanen, et al., 2008b; Tikkanen, et al., 2010). The STN8 kinase is required for phosphorylation of PSII core proteins in HL but does not signiﬁcantly participate in phosphorylation of LHCII (Bellaﬁore, et al 2005; Bonardi et al, 2005; Vainonen, et al., 2006; Tikkanen, et al., 2010). It has been shown that in traditional “state 1” condition, which oxidizes the ETC, the dephosphorylation of LHCII is dependent on TAP38/PPH1 phosphatase (Pribil, et al., 2010; Shapiguzov, et al., 2010) whereas the PSII core protein dephosphorylation is dependent on the PBCP phosphatase (Samol, et al., 2012). However, it remains unresolved whether and how the TAP38/PPH1 and PBCP phosphatases are involved in the light intensity-dependent regulation of thylakoid protein phosphorylation, typical for natural environments.

Here we have used the two kinase (stn7 and stn8) and two phosphatase (tap38/pph1and pbc p) mutants of Arabidopsis to elucidate the individual roles of these enzymes in reversible thylakoid protein phosphorylation and in distribution of excitation energy between PSII and PSI upon changes in light intensity. It is demonstrated that the TAP38/PPH1-dependent redox regulated LHCII dephosphorylation is the key component to maintain excitation balance between PSII and PSI upon increase in light intensity, which at the same time induces strong phosphorylation of the PSII core proteins. Collectively, reversible but opposite phosphorylation and dephosphorylation of the PSII core and LHCII proteins upon increase or decrease in light intensity are shown to be crucial for maintenance of even distribution of excitation energy to both photosystems, thus preventing state transitions. Moreover, evidence
is provided indicating that the pH gradient across the thylakoid membrane is yet another important component in regulation of the distribution of excitation energy to PSII and PSI, possibly by affecting the regulation of thylakoid kinases and phosphatases.

Results

Thylakoid protein phosphorylation and excitation energy distribution between PSII and PSI in growth light as compared to the “state 1” and “state 2” lights

Traditionally the role of LHCII phosphorylation has been studied by using light qualities preferentially exciting either PSI (“State 1”) or PSII (“State 2”). Here phosphor threonine (P-Thr) immunoblot in Fig 1 A is used to demonstrate the phosphorylation levels of LHCII phosphoproteins LHCB1 and LHCB2 (migrate together and designed as P-LHCII) and the PSII core phosphoproteins P-D1, P-D2 and P-CP43 in growth light (GL) and in state lights. LHCII and PSII core proteins are moderately phosphorylated when plants are acclimated to the GL. “State 1” light leads to dephosphorylation of both PSII core and LHCII proteins and “State 2” slightly increases both the PSII core and LHCII phosphorylation as compared to GL (Fig 1 A). Despite strong change in thylakoid protein phosphorylation upon transfer of plants from GL to “State 1” light (Fig 1 A), only slight decrease occurred in the relative PSII to PSI absorption cross section, deduced from the 77K fluorescence emission spectrum revealing the PSII peaks at 685 nm and 695 nm and the PSI peak 733 nm (Fig 1 B). Transfer of plants from GL to “State 2” light, in turn, strongly increased the relative excitation of PSI (Fig 1 B) despite the fact that the changes in PSII core and LHCII protein phosphorylation were rather minor (Fig 1 A).

Phosphorylation of LHCII and PSII core proteins in WT and the stn7, stn8, tap38/pph1, pbcp and pgr5 mutant plants

With the purpose of exploring the individual physiological roles of the thylakoid protein kinases and phosphatases, the thylakoid phosphorylation pattern of wild type (WT) and that of the kinase mutants stn7 and stn8 as well as the phosphatase mutants tap38/pph1 and pbcp was investigated from three different illumination conditions: (1) from steady state GL acclimated plants, (2) from plants subjected to 20 min shifts between LL and HL and (3) from plants shifted from GL to HL for 1 and 2 hours. Previously, by screening the light intensity-dependent phosphorylation of thylakoid proteins in different mutant plants, we had
found that the pgr5 mutant, unable to generate trans-thylakoid proton gradient (ΔpH) upon increase in light intensity (Munekage, et al. 2002), is also impaired in regulation of thylakoid protein phosphorylation. Thus, the pgr5 mutant was likewise included in the study, in order to elucidate the role of ΔpH on protein phosphorylation and energy distribution in the thylakoid membrane. The most relevant and already published facts of the mutants used in this study are collected to Table 1. Table 1 also contains the chlorophyll a/b ratios of the WT and mutant plants in GL conditions.

First the phosphorylation levels of LHCII and the PSII core phosphoproteins in WT and mutant plants acclimated to steady state GL conditions were explored. In the stn7 mutant, the phosphorylation of LHCII proteins was below the detection level, whilst the phosphorylation levels of D1, D2 and CP43 were clearly higher as compared to WT. The stn8 mutant had strongly decreased phosphorylation of D1 and D2, yet the phosphorylation level of CP43 was not significantly different from that of WT. In tap38/pph1, the phosphorylation levels of all thylakoid phosphoproteins were very similar to those in WT. In turn, the lack of the PBCP phosphatase caused slightly higher phosphorylation level of PSII core proteins than observed in WT. All the results on kinase and phosphatase mutants described above are line with several earlier studies (Bonardi, et al., 2005; Bellafiore, et al., 2005; Vainonen, et al., 2005; Pribil, et al., 2010; Shapiguzov, et al., 2010; Samol, et al., 2012). The pgr5 mutant (Munekage, et al., 2002) demonstrated similar phosphorylation levels of LHCII and PSII core proteins at steady state growth light as WT (Fig 2 A), indicating no drastic difference in the kinase and phosphatase system or the redox status of ETC in constant growth light.

Next, the plants from GL (with thylakoid phosphorylation as shown in Fig. 2) were exposed to repetitive 20 min LL and HL periods. As demonstrated in Fig. 3, in WT the LHCII proteins were highly phosphorylated upon shift of plants to LL and, respectively, the PSII core proteins became more phosphorylated upon shift of plants from LL to HL (see also Tikkanen, et al., 2010). On the contrary, the stn7 mutant lost its capacity to keep PSII core proteins phosphorylated upon shift to HL whilst the minor PSII core protein phosphorylation present in the stn8 mutant was further enhanced upon the second HL shift. This suggests substrate overlap and delicate interactions between the STN7 and STN8 kinases in regulation of PSII core protein phosphorylation upon changes in light intensity. Results with kinase mutants demonstrated that STN7 phosphorylates not only the LHCII proteins but in LL also PSII core proteins, especially CP43 (Vainonen, et al. 2006), whereas STN8 is rather specific only for the PSII core proteins and most active in HL (Rintamäki et al., 1997; Bonardi, et al., 2005b;
Vainonen, et al., 2006; Tikkanen, et al., 2010; Tikkanen, et al., 2008a). The apparent paradox that the phosphorylation of the PSII core proteins in stn7 increased upon shift to LL and decreased subsequently in HL, which is opposite to the situation in WT (Fig 3 and Tikkanen, et al., 2008a; Tikkanen, et al., 2010) results from the fact that the reduction state of ETC increases in stn7 upon shift of plants from HL to LL (Bellaire, et al., 2005; Tikkanen, et al., 2006; Tikkanen, et al., 2010) whereas upon a similar shift of WT plants the ECT becomes oxidized (Tikkanen, et al., 2010; Grieco, et al., 2012). Importantly, the tap38/pph1 mutant demonstrated no LHCII dephosphorylation upon a shift of plants from LL to HL (Fig 2). This indicated that the TAP38/PPH1 phosphatase is solely responsible for dephosphorylation of LHCII in HL, similar to that in “state 1” light and in darkness (Pribil, et al., 2010; Shapiguzov, et al., 2010). Interestingly, the pbcp mutant behaved in light intensity shifts like WT (Fig 3), despite the fact that the PBCP phosphatase has been shown to be responsible for PSII core protein dephosphorylation in “state 1” light (Samol, et al., 2012) and the mutant also showed slightly higher PSII core protein phosphorylation than WT in steady state GL. (Samol, et al., 2012 and Fig 2).

Relationship of phosphorylation of PSII core and LHCII proteins with excitation energy distribution between PSII and PSI

After having gained understanding about regulation of the PSII and LHCII protein phosphorylation in WT and the two kinase (stn7 and stn8) and two phosphatase (tap38/pph1 and pbcp) mutants, we next focused on comparisons between thylakoid protein phosphorylation and the 77 K fluorescence emission spectra, which reflect the distribution of excitation energy between PSII and PSI. Earlier experiments had revealed no difference in excitation energy distribution in light shifts of WT and kinase mutant plants between LL, GL and HL (Tikkanen, et al. 2010). As the major aim of the present study was to understand the role TAP38/PPH1-dependent dephosphorylation of LHCII upon increase in light intensity, the focus was put on shifts from GL to HL with two (1 h and 2 h) HL exposure times thus allowing the thylakoids to stabilize the excitation energy distribution with changing protein phosphorylation status (Fig 4). As expected, in WT the HL treatment led to a high phosphorylation of the PSII core proteins and to a distinct decrease in the phosphorylation of the LHCII proteins (Fig 4). In the stn7 mutant, PSII phosphorylation increased and LHCII remained dephosphorylated in both GL and HL intensities (Fig 4). In the stn8 mutant, the
LHCII and the PSII core proteins, both being phosphorylated in GL, became largely dephosphorylated upon transfer to HL (Fig 4). In the tap38/pph1 mutant, the PSII and LHCII proteins were moderately phosphorylated in GL and the shift to HL increased the PSII core protein phosphorylation but failed to dephosphorylate the LHCII proteins (Fig 4). As in GL (Fig. 2), the pbcP mutant did not show any distinct phosphorylation phenotype as compared to WT upon shift to HL (Fig 4).

Despite clear differences in the behavior of thylakoid protein phosphorylation upon shift of WT, the kinase mutants (stn7 and stn8) and the pbcP phosphatase mutant from GL to HL, the concomitant 77 K fluorescence emission measurements did not reveal distinct changes in the relative excitation energy distribution between PSII and PSI i.e. no state transitions were evident (Fig 4), in line with earlier results (Tikkanen, et al., 2010). Intriguingly, the tap38/pph1 mutant, incapable to dephosphorylate LHCII and therefore keeping both the PSII core and LHCII proteins strongly phosphorylated in HL, behaved completely differently from the WT, the kinase mutants and the pbcP phosphatase mutant. Indeed, the 77K fluorescence spectrum of the tap38/pph1 mutant demonstrated strong increase in relative excitation of PSI already after 1h shift to HL (Fig 4), resembling the artificial “state 2” condition both with respect to protein phosphorylation pattern and excitation energy distribution (Fig 1). Thus, the HL exposure induced the state 2 transition in the tap38/pph1 mutant due to the fact that it cannot regulate reversible LHCII phosphorylation in the same way as WT upon changes in light intensity. It is worth noting that these changes in the tap38/pph1 mutant were more distinct after 1h HL exposure than after 2h HL exposure (Fig. 4).

ΔpH across the thylakoid membrane has a strong influence on thylakoid protein phosphorylation and distribution of excitation energy

The entire light intensity dependent regulation system of LHCII protein phosphorylation was lost in the pgr5 mutant (Fig 5 A). The pgr5 mutant cannot protonate the thylakoid lumen upon increase in light intensity and thus suffers from impaired “photosynthetic control”, i.e. control of electron transfer from PSII to PSI via the cytochrome b6f complex, leading to damage of PSI upon shift to HL (Suorsa, et al., 2012). This most likely lead to an incapability to properly reduce the electron acceptors of PSI that, in turn, are required for inhibition of the LHCII kinase in high light (Rintamäki, et al., 2000). The amounts of the TAP38/PPH1 phosphatase and the STN7 kinase were, however, also estimated by immunoblotting from
WT and the pgr5 mutant (Fig. 5 B). Although minor differences were observed in the amounts of the STN7 kinase and the TAP38/PPH1 phosphatase between WT and the pgr5 mutant, they were not regarded big enough to explain the missing regulation of LHCII phosphorylation in the pgr5 mutant upon changes in light intensity.

As demonstrated in Fig 6 A, the pgr5 mutant behaved similar to the tap38/pph1 mutant (Fig 4) also with respect to the failure in dephosphorylation of the LHCII proteins during 1 h and 2 h HL illumination. This, in turn, led to strong concomitant phosphorylation of both the PSII and LHCII proteins in the pgr5 mutant, similar to that in the tap38/pph1 mutant, upon exposure of plants to HL. Importantly, as in case of the tap38/pph1 mutant, strong phosphorylation of both the PSII and LHCII proteins in the pgr5 mutant was accompanied by strongly increased relative excitation of PSI (Fig 6 B), observed in the 77K fluorescence spectrum, thus mimicking the transition to state 2 (Fig 1).

In case of both the tap38/pph1 (Fig 4) and pgr5 (Fig 6 A) mutants, prolonged exposure of leaves to HL (2 hours) already started diminishing the superior PSI fluorescence emission at 77K. This is an indication of initiation of a general acclimation strategy of plants towards equal distribution of excitation energy from the light-harvesting antenna to both PSII and PSI. Imbalanced excitation energy distribution in these mutants, observed most strongly after 1 hour HL illumination, either directly or indirectly initiated a signaling cascade to restore the excitation balance in the thylakoid membrane.

**The effect of missing LHCII dephosphorylation on organisation of thylakoid protein complexes**

With the aim of elucidating the molecular mechanism that leads to increased excitation of PSI (state 2) in tap38/pph1, the thylakoid membranes were isolated from the GL acclimated and 1 h HL shifted WT and tap38/pph1 plants, then subjected to solubilisation with two different detergents and separation of the pigment-protein complexes by blue native gel electrophoresis. Dodecyl maltocide (DM) was used to solubilise the entire thylakoid membrane in order to explore the packing of PSII-LHCII super complexes (Tikkanen, et al., 2008a). In this treatment, no clear difference in pigment-protein complexes between GL and HL and between WT and tap38/pph1 were observed, indicating no differences in the PSII-LHCII complexes in grana cores (Tikkanen, et al. 2008a; Goral, et al. 2010). Digitonin is a
much milder detergent than DM and does not solubilise the highly packed PSII-LHCII complexes of grana core, but can be used to investigate the less packed grana margin and stroma lamellae domains of the thylakoid membrane (Järvi, et al., 2011). Digitonin maintains the weak interactions between pigment-protein complexes and allows the analysis of different large PSII-LHCII-PSI-LHCII complexes, large PSI-LHCI complexes, the PSI-LHCII “State transition” complex, in addition to the PSII and PSI monomer and free LHCII trimer and monomer complexes. The digitonin-solubilisation experiment demonstrated that the LHCII-PSI-LHCI complex (“state transition” complex) was similarly present in WT and tap38/pph1 under GL, but disappeared only from WT upon shift to HL. Moreover, the content of high molecular mass megacomplexes containing both PSI-LHCI and PSII-LHCII (Järvi, et al., 2011) was more abundant in HL in the tap38/pph1 mutant than in WT. The molecular structure and the energy transfer properties of these large complexes are not yet fully understood despite extensive investigation in different laboratories. However, the appearance of these large complexes in tap38/pph1 in HL is a clear sign of major re-arrangements of pigment protein complexes in grana margin regions as compared to WT.

Discussion

Evidence has accumulated during the past ten years indicating that reversible thylakoid protein phosphorylation is one important component in multifaceted and highly integrated regulatory network for optimal harnessing of light energy and its transduction into chemical energy in the thylakoid membrane (for a review see Tikkanen and Aro, 2014). Reversible but opposite phosphorylation of thylakoid proteins is particularly important in naturally changing environmental conditions (Tikkanen, et al., 2010; Grieco, et al., 2012). Nonetheless, the mechanisms, consequences and mutual interactions of such opposite PSII and LHCII protein phosphorylation in regulation of thylakoid function needed re-investigation. Instead of traditional views assigning reversible LHCII phosphorylation a unique role in state transitions (See for reviews: (Haldrup, et al., 2001; Allen and Forsberg, 2001; Rochaix, 2007)) and the PSII core protein phosphorylation in fluent PSII turnover in high light (See for reviews: (Aro, et al., 1993; Tikkanen and Aro, 2011)), we provide here compelling evidence emphasizing that instead of such individual roles, the co-operation of LHCII and PSII core protein phosphorylation is a key factor in adjusting the photosynthetic apparatus to changing light intensities. Moreover, the primary physiological significance of thylakoid protein
phosphorylation is shown to guarantee the excitation balance between PSII and PSI, despite changes in light intensity that strongly modify both the PSII core and LHCII protein phosphorylation (Fig 4 and Fig 6).

The crucial early observation was the fact that high light illumination does not only lead to increased phosphorylation of PSII core proteins, but also to dephosphorylation of the LHCII proteins in WT plants (Rintamäki, et al., 1997). These facts have been impossible to integrate with the canonical state transition model, in which the state 2-light phosphorylates both the PSII and LHCII proteins, concomitantly enhancing the excitation of PSI (transfer to state 2) whereas the state 1-light dephosphorylates both groups of phosphoproteins and enhances PSII excitation (transfer to state 1) (Fig 1). Thus, the artificial state transitions induce strong imbalance in excitation energy distribution to PSII and PSI in the thylakoid membrane, which does not occur in fluctuating light conditions despite strong changes in phosphorylation of both the LHCII and PSII core proteins (Fig 4 and Tikkanen, et al., 2010).

Availability of the stn7 (Depege, et al., 2003; Bellafiore, et al., 2005; Bonardi, et al., 2005) and the stn8 (Bonardi, et al., 2005) kinase mutants enabled investigations of the physiological roles of thylakoid protein phosphorylation with respect to changes in light intensity. It rapidly turned out that the STN7-dependent phosphorylation of thylakoid proteins is a low light effect (Bellafiore, et al., 2005, Tikkanen et al., 2006). Although, it was known already before that LHCII phosphorylation and state transitions is are LL acclimation mechanisms (Walters & Horton 1991, Finazzi, et al., 2004). STN7 kinase was shown to be required for sufficient excitation energy transfer to PSI under low light and redox balance, occurring in connection with extremely low thermal dissipation of excitation energy and thus maximal light energy capture in PSII (Tikkanen, et al., 2010 and 2011). Conversely, a shift of plants to high light causes rapid induction of the PSBS-protein-dependent thermal dissipation of excitation energy, restoring the redox imbalance caused by the lack of the STN7 kinase (Tikkanen, et al., 2010; Grieco, et al., 2012). Despite the above described facts indicating that the redox state of the ETC is not affected by the phosphorylation status of LHCII in HL, prolonged HL illumination nevertheless induces dephosphorylation LHCII (Rintamaki, et al., 1997; Rintamaki, et al., 2000) with no obvious physiological reason.

The discovery that the tap38/pph1 phosphatase mutant (Pribil, et al., 2010; Shapiguzov, et al., 2010) lacks the capacity for LHCII dephosphorylation in shift of plants to high light (Fig 3
and Fig 4), provided an excellent tool to address the enigmatic physiological role of LHCII dephosphorylation in high light (Rintamaki, et al., 1997; Rintamaki, et al., 2000). TAP38/PPH1 phosphatase was previously shown responsible for dephosphorylation of LHCII in “state 1” light and in darkness (Pribil, et al., 2010; Shapiguzov, et al., 2010), the conditions that deactivate the STN7 kinase (Vener, et al., 1997). Here we demonstrate that TAP38/PPH1 is responsible for dephosphorylation of LHCII in high light as well (Fig 3 and Fig 4). Thus, in theory, it is possible that not only the STN7 kinase but also the TAP38/PPH1 phosphatase is redox regulated. Nevertheless, in practice such a redox regulation of TAP38/PPH1 would need a sophisticated regulation mechanism that would allow an increase in phosphatase activity under oxidizing conditions both in darkness and in “state 1” light but additionally also under strongly reducing high light conditions. On the other hand, although the thylakoid protein kinases and phosphatases have preferred substrates, some substrate overlap has also been reported (Bonardi, et al., 2005; Vainonen, et al., 2005; Samol, et al., 2012). The STN8 kinase cannot noticeably phosphorylate LHCII in the stn7 mutant or in WT when STN7 is inhibited upon HL illumination (Fig 3). There is, however, a possibility that in the absence of the TAP38/PPH1 phosphatase, the STN8 might show enough unspecific activity to keep LHCII phosphorylated. In the future, this possibility should be tested by using the stn8 tap38/pph1 double mutant.

Intriguingly, the tap38/pph1 mutant increases the PSII core protein phosphorylation upon shift to high light, similar to WT, but cannot concomitantly dephosphorylate the LHCII proteins as WT does (Fig 3 and Fig 4). This leads to a strong simultaneous phosphorylation of both the PSII and LHCII phosphoproteins in tap38/pph1, closely resembling the traditional “state 2” light-induced phosphorylation pattern of WT thylakoids (Fig. 1), whereas WT, stn8 and pbcp do dephosphorylate LHCII in high light and stn7 keeps LHCII always dephosphorylated.

The PBCP phosphatase has been shown to be involved in phosphorylation balance of PSII core proteins in constant growth light (Samol, et al., 2012) as we also demonstrate here (Fig 2). Moreover, PBCP is required to dephosphorylate PSII core protein in traditional “state 1” and has also been linked to dephosphorylation of PSII core protein in photoinhibitory conditions (Puthiyaveetil, et al., 2014). Despite of such an obvious role of PBCP phosphatase in dephosphorylation of PSII core proteins (Samol, et al., 2012; Puthiyaveetil, et al., 2014 and Fig 2), it was surprising that the pbcp mutant normally dephosphorylates the PSII core
proteins upon decrease in light intensity (Fig 3). This indicates that there is still an unidentified phosphatase that is responsible for PSII core protein dephosphorylation upon lowering of the light intensity. In the future, it will be crucial to identify this still unknown phosphatase, because it would allow investigating the physiological role of PSII core protein dephosphorylation in LL that may be crucial in enhancing the light harvesting efficiency by increasing the connectivity between the PSII complexes (See the schematic model in Fig 8).

Here, the crucial physiological role of LHCII dephosphorylation in HL was resolved by comparison of the thylakoid protein phosphorylation status with the excitation energy distribution between PSII and PSI (77K fluorescence emission spectra) in tap38/pph1 to that in WT, stn7, stn8 and pbcp (See the schematic model in Fig 8). In case of WT, stn7, stn8 and pbcp, the absorption cross section (i.e. the excitation) of the two photosystems remains unchanged upon transfer of plants from growth light to high light. The tap38/pph1 mutant behaves completely differently. Transfer of tap38/pph1 to high light leads to “state 2”-type simultaneous phosphorylation of both the PSII core and LHCII proteins and this is reflected in strongly enhanced relative excitation of PSI (Fig 4), similarly to that occurring in WT under the “state 2” condition (Fig 1 A). Thus, it is not only the behavior of a single kinase or a simple reciprocity between one kinase and the respective phosphatase that determines the excitation energy distribution between the two photosystems. Instead, the kinetics and regulation of both the kinases, one specific for the PSII core and the other for LHCII proteins, in concert with the respective phosphatases, at least the TAP38/PPH1 phosphatase, altogether control the distribution of excitation energy to PSII and PSI. Upon fluctuations in the light intensity, this regulation mechanism is directed towards even distribution of excitation energy to both photosystems and in preventing the occurrence of state transition.

It is worth noting that the tap38/pph1 mutant is not more susceptible to high light than WT (Pribil, et al., 2010; Shapiguzov, et al., 2010). Indeed, as long as the light harvesting antenna is in quenched state, LHCII phosphorylation seems not to play any role in the control of the photosynthetic machinery (Tikkanen, et al., 2010, Grieco, et al., 2012). Moreover, PSI is a very efficient quencher of excitation energy and can handle the excitation energy if the electron transfer is in control (Tikkanen, et al., 2014). This raises a question, why should plants avoid state transition upon increase in light intensity? It seems likely that the large relative light harvesting capacity is not harmful to PSI in high light, but the problem would arise upon subsequent decrease in light intensity, inducing excitation imbalance and energy...
losses in PSII. This would occur because upon shift to LL, PSII with a small antenna would not have enough excitation energy to satisfy the electron need of PSI with large antenna, leading to energy losses in non-photochemical processes by PSI. Indeed, plants do not only need to keep ETC optimally oxidized to prevent the damage of PSI upon increase in light intensity (Suorsa, et al., 2012; Grieco, et al., 2012; Tikkanen, et al., 2014), but also to keep ECT optimally reduced to avoid photochemical losses upon sudden decrease in light intensity.

Screening of the phosphorylation pattern of various photosynthesis regulation mutants revealed that not only tap38/pph1 but also the pgr5 mutant exhibits a strong simultaneous phosphorylation of both the PSII core and LHCII proteins at high light (Fig 5 A and 6 A), again resembling WT under “state 2 light” (Fig 1). The pgr5 mutant has however rather normal amount of STN7 kinase and TAP38/PPH1 phosphatase (Fig 5 B), indicating that the required dephosphorylation capacity is present, but the regulation of the reversible LHCII phosphorylation is not working in the mutant. PGR5 is important in maintaining the proton gradient across the thylakoid membrane (Munekage, et al., 2002). Upon high light intensity it is required to control electron transfer and to induce thermal dissipation of excess energy thus protecting PSI from photodamage. (Munekage, et al., 2002; Nandha, et al., 2007; Joliot and Johnson, 2011; Suorsa, et al., 2012; Tikkanen, et al., 2014). Apparently, the photodamage of PSI, combined with low thermal dissipation in high light, leads to strong reduction of the PQ-pool in pgr5 combined with oxidation of the PSI electron acceptors. This resembles the traditional “State 2” condition where STN7 and STN8 kinase activities dominate and the antagonistic TAP38/PPH1 and PBCP phosphatases cannot dephosphorylate the PSII and LHCII proteins.

It is notable that only those light conditions, either artificial ones for WT or natural ones for the tap38/pph1 and pgr5 mutants, which induce a strong simultaneous phosphorylation of both the PSII core proteins and the LHCII proteins demonstrate a distinct change in relative excitation of PSII and PSI. On the contrary, the WT and all the mutants with functional LHCII dephosphorylation at high light, clearly make use of dynamic regulation of the thylakoid membrane to prevent any major light intensity-dependent changes in the distributions of excitation energy to the two photosystems. It is, however, surprising how similarly the PSII and LHCII proteins are phosphorylated in GL acclimated plants as compared to the “state 2” light (Fig 1 A) and the “state 2” mimicking HL in tap38/pph1 (Fig
3 and Fig 4) and pgr5 (Fig 5 and Fig 6). Indeed, it seems obvious that protein phosphorylation is not a sole driving force of the state transition, but rather a factor allowing it to happen. Obviously, state transition occurs only when strong phosphorylation of both PSII and LHCII proteins is combined with high excitation pressure towards PSII (HL or PSII light). It can be speculated that reaction is triggered by something dependent on the high reduction state of ETC and able to alter the attraction and repulsion forces between PSII, LHCII and PSI. In fact, it was proposed already 1977 that state transitions are based on thylakoid surface charges but may also involve conformational changes of protein(s) (Ried and Reinhardt, 1977). It should also be noted that the reorganizations of the thylakoid protein complexes required for efficient degradation of D1 in photoinhibitory conditions seem not to be dependent on the phosphorylation, but phosphorylation facilitates the vital unpacking and mobility of the PSII-LHCII complexes (Tikkanen, et al., 2008a; Goral, et al., 2010).

What could be the underlying mechanism behind reversible but opposite phosphorylation of the PSII core and LHCII proteins in controlling the excitation energy distribution between PSII and PSI upon changes in light intensity? To address this question we solubilised the thylakoid membranes of GL and HL acclimated WT and tap38/pph1 plants by using two different detergents (Fig 7). DM solubilisation, used to reveal photoinhibition-related and PSII core protein-dependent changes in the PSI-LHCII supercomplexes (Tikkanen, et al., 2008a), did not show any difference between WT and tap38/pph1. On the contrary, digitonin solubilisation, addressing only the loosely packed stroma lamellae and grana margins, revealed distinct differences between WT and tap38/pph1 in HL. Both WT and tap38/pph1 had the PSI-LHCl state transition complex (Pesaresi et al. 2009; Wientjes et al. 2013b) in GL but only WT was capable of disassembling the complex upon shift to HL. The preservation of the complex, however, cannot explain the increased relative excitation of PSI upon increase in light intensity. Indeed, another distinct difference between WT and tap38/pph1 is a higher amount of large PSII-LHCII and PSI-LHCI containing megacomplexes in tap38/pph1 (Fig 7). These complexes have been described before (Järvi, et al., 2011), but the molecular organization and energy transfer properties of these complexes are still unclear. The appearance of the complexes, however, clearly indicates that the dynamics of the PSI-LHCII complex is not the only light-induced phosphorylation-dependent rearrangement in the grana margin region as also demonstrated in (Tikkanen et al. 2008b). It is highly conceivable that HL induces rearrangements of the thylakoid pigment protein complexes that enhance the
energy transfer to PSI, and this unfavorable situation needs to be compensated by LHCII dephosphorylation in order to maintain the excitation balance between PSII and PSI.

Concluding remarks

Efficient use of light for photochemical reactions and fluent electron transfer in the thylakoid membrane require excitation balance between PSII and PSI under all light conditions. Light acclimation and the maintenance of the photosynthetic machinery, however, involve structural changes in the thylakoid membrane, like lateral migration of the photosynthetic pigment protein complexes or smaller conformational changes in photosynthetic complexes. Such dynamic structural reorganizations of thylakoid protein complexes disturb the balanced excitation energy distribution to the two photosystems, and require active mechanisms to selectively limit and facilitate thylakoid reorganizations in order to maintain the excitation balance between photosystems. The maintenance of excitation balance between PSII and PSI upon changes in light intensity is shown to correlate with reversible but opposite PSII core and LHCII protein phosphorylation. Thus, extensive redox regulated and co-ordinated PSII core and LHCII protein phosphorylation-dephosphorylation-dependent network together with the proton gradient across the thylakoid membrane assist the dynamics of thylakoid protein complexes and concomitantly allows the maintenance of the excitation balance between PSII and PSI. Protein phosphorylation may, however, not be the sole driving force of the thylakoid rearrangements. Indeed, extensive coordination between many regulatory mechanisms of thylakoid electron transfer network should not be neglected, as demonstrated here by a crucial involvement of trans-thylakoid proton gradient in proper distribution of excitation energy to both photosystems in HL. Altogether, the presented model integrating both the reversible but opposite PSII core and LHCII protein phosphorylation and the trans-thylakoid proton gradient into regulation of excitation energy distribution to the two photosystems is one further step towards understanding how the thylakoid membrane functions and is regulated as one single entity.

Materials and methods

Plant material, growth conditions and light treatments
Wild-type (WT) *Arabidopsis thaliana* ecotype Columbia and *stn7* (SALK_073254) (Bellafiore, et al., 2005), *stn8* (SALK_060869) (Bonardi, et al., 2005), *tap38* (SALK_025713) (Pribil, et al., 2010), *pbc* (SALK_127920) (Samol, et al., 2012) and *pgr5* (AT2G05620) (Munekage, et al., 2002)) mutant plants were grown in controlled environmental chambers for 5-6 weeks at 120 µmol photons m\(^{-2}\)s\(^{-1}\), with 8/16h light/dark cycle and relative humidity of 70%. OSRAM PowerStar HQIT 400/D Metal Halide Lamps were used as light source for both plant growth and LL and HL treatments.

In LL and HL treatments, plants were placed in a temperature-controlled chamber at 23 °C with light passed through a heat filter and exposed to 20 and 1000 µmol photons m\(^{-2}\)s\(^{-1}\) in 20 min intervals for total duration of 80 min. In HL treatment, plants were exposed to 1000 µmol photons m\(^{-2}\)s\(^{-1}\) for 1 and 2 hours. To induce state transitions wild-type plants were exposed to light favoring the excitation of PSII (state 2 light) and that of PSI (state 1 light) for 60 min. A fluorescent tube (GroLux F58W/GROT8; Sylvania) covered with an orange filter (Lee 105; Lee Filters) served as state 2 light, and state 1 light was obtained from halogen lamps (500 W) covered with an orange filter (Lee 105; Lee Filters) and a median blue filter (Roscolux 83; Rosco Europe). See for details in (Piippo, et al., 2006). Temperature was maintained at 23°C by a water-cooled glass chamber between the fluorescence tube and the plants.

**Isolation of thylakoid membranes**

Thylakoid membranes were isolated according to Suorsa, et al., 2004 and re-suspended in buffer containing 100 mM sorbitol, 50 mM HEPES-KOH (pH 7.5), 10 mM NaF and 10 Mm MgCl\(_2\). Chlorophyll concentration was determined according to Porra et al. (Porra, et al., 1989).

**SDS-PAGE and immunoblotting**

Thylakoid membrane proteins were separated on 15% SDS-polyacrylamide gels with 6M urea (Suorsa, et al., 2004). After electrophoresis, the polypeptides were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and the membrane was blocked with 5% fatty acid-free bovine serum albumin (Sigma-Aldrich). For phosphoprotein detection samples equivalent to 0.5 µg of Chl were loaded in each well and the phosphoproteins were visualized with anti-phosphothreonine antibody (New England Biolabs) and subsequent Phototope-Star Chemiluminescence detection (New England
Biolabs). The amounts of the STN7 kinase and TAP38/PPH1 phosphatase were determined by using antibodies raised against these proteins (Agrisera, Vännäs, Sweden). Samples equivalent to 2 µg of Chl were loaded in each well for kinase and phosphatase detection.

77K Chl a Fluorescence Measurements

Fluorescence emission spectra were measured from frozen suspension at 77 K by using an Ocean Optics QE Pro spectrometer. Isolated thylakoid membranes were diluted to 1µg Chl ml\(^{-1}\) in storage buffer containing 100 mM sorbitol, 50 mM HEPES (pH 7.5), 10 mM NaF and 10 Mm MgCl\(_2\) and excited at 480 nm. The raw spectra were normalized at 685 nm for comparison of fluorescence emission bands from PSI.

Native gel electrophoresis

Thylakoid membrane solubilization by 1 % dodecyl maltoside or 1 % digitonin and the subsequent separation of protein complexes by large pore native gel were performed according to Järvi et al. 2011.

Acknowledgements

We thank Dr. Dario Leister for the tap38 mutant, Toshiharu Shikanai for the pgr5 mutant and Dr. Michel Goldschmidt-Clermont for the pbcp mutant.

Tables

Table 1. A summary of the previously published characterization of the light acclimation mutants used in this study as well as the chlorophyll a/b ratios of these mutants under the growth conditions used here, see Material and Methods

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primary defect</th>
<th>Direct and indirect consequences of the mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>stn7</td>
<td>Lack of the STN7 kinase (Bellafiore, et al., 2005)</td>
<td>stn7 lacks LHCII phosphorylation (STN7 kinase) and therefore suffers from inefficient energy transfer from LHCII to PSI (Bellafiore, et al. 2005; Bonardi, at al. 2005, Tikkanen, et al. 2006). stn7 compensates this by increasing the amount of PSI complexes in constant growth light (Tikkanen, et al. 2006 and Grieco, et al. 2012). Slightly decreased chlorophyll a/b ratio despite decreased PS to LHCII ratio (Tikkanen, et al. 2006). Chl a/b decreased in all different PSII-LHCII complexes (Grieco, et al. 2012). Chlorophyll a/b ratio in our growth condition...</td>
</tr>
<tr>
<td>Gene</td>
<td>Lack of the enzyme (Reference)</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>stn8</td>
<td>Lack of the STN8 kinase (Bonardi, et al., 2005)</td>
<td><em>stn8</em> has strongly reduced phosphorylation of PSII core protein especially when shifted to high light (Bonardi, et al. 2005; Vainonen, et al. 2006). Delayed D1 degradation due to problems in unpacking of photodamaged PSII-LHCII complexes in photoinhibitory conditions (Tikkanen, et al. 2008a; Fristedt, et al., 2009; Goral, et al., 2010; Kirchhoff, et al., 2011). Rigidity and packing of the grana membranes increased (Fristedt, et al., 2009). No reported distortion in excitation energy or electron transfer. No reported changes in thylakoid protein composition or chlorophyll a/b ratio. Chlorophyll a/b ratio in our growth is about 3.3</td>
</tr>
<tr>
<td>pgr5</td>
<td>Unfunctional PGR5 protein (Munekage, et al., 2002)</td>
<td><em>pgr5</em> is impaired in generation of trans-thylakoid proton gradient upon increase in light intensity. Low thermal dissipation of excitation energy and high reduction level of intersystem electron transfer chain. Decreased amount of PSI in constant growth light and high susceptibility of PSI to photodamage upon increase in light intensity. (Munekage, et al., 2002; Nandha, et al., 2007; Joliot and Johnson, 2011; Suorsa, et al., 2012; Tikkanen, et al., 2014). Chl a/b ratio is about 3.4 despite decreased amount of PSI (see <em>stn7</em> above).</td>
</tr>
</tbody>
</table>
**Figure 1.** Comparison of thylakoid protein phosphorylation with excitation energy distribution between PSII and PSI in growth light (GL) and lights preferentially exciting either PSI (“State 1”) or PSII (“State 2”). A. Immunoblot demonstrating the phosphorylation of the PSII core proteins CP43 (P-CP43), D2 (P-D2) and D1 (P-D1) and the LHCII proteins LHCBI and LHCBIII (migrate in the same band assigned as P-LHCII) in wild-type. B. 77 K Chl a fluorescence emission spectra. WT samples were collected from growth light (GL) (120 μmol photons m⁻² s⁻¹) and after subsequent one hour exposure to state 1 light (far red light 30 μmol photons m⁻² s⁻¹) and to “state 2” light (red light 50 μmol photons m⁻² s⁻¹) (see for details in Piippo, et al., 2006). CBB = Coomassie Brilliant Blue; a loading control. Gels were loaded on equal chl basis (0.5 μg chl / well).
Figure 2. Thylakoid protein phosphorylation by the STN7 and STN8 kinase and TAP38/PPH1 and PBCP phosphatase pathways in constant growth light (GL) (120 μmol photons m\(^{-2}\) s\(^{-1}\)). A. P-thr immunoblot demonstrating the phosphorylation of the PSII core proteins CP43, D2 and D1 and the LHCII proteins (LHCBI and LHCBI) in wild-type (WT) plants and the \textit{stn7}, \textit{stn8}, \textit{tap38/pph1}, \textit{pbcp} and \textit{pgr5} mutants in GL 4 h after the beginning of 8 h light period. B. Dilution series of WT thylakoids demonstrating the response of the antibody to different protein amounts. CBB = Coomassie Brilliant Blue; a loading control. Gels were loaded on equal chl basis (0.5 μg chl / well).
**Figure 3.** Regulation of thylakoid protein phosphorylation by the STN7 and STN8 kinase and TAP38/PPH1 and PBCP phosphatase pathways. A. Immunoblot demonstrating the phosphorylation of the PSII core proteins CP43, D2 and D1 and the LHClI proteins (LHCBI and LHCBI2) in the wild-type (WT) plants and in the *stn7, stn8, tap38/pph1* and *pbcp* mutants after repeated exposure to 20 (low light, LL) and 1000 (high light, HL) μmol photons m⁻² s⁻¹ in 20 min intervals. CBB = Coomassie Brilliant Blue; a loading control. Gels were loaded on equal chl basis (0.5 μg chl / well).
Figure 4. Relationship between PSII core and LHCII protein phosphorylation and the excitation energy distribution between PSII and PSI. P-thr immunoblots demonstrating the phosphorylation pattern of thylakoid proteins (A) and the 77 K Chl a fluorescence emission spectra (B) were recorded from wild-type (WT) and from the \textit{stn7}, \textit{stn8}, \textit{tap38/pph1} and \textit{pbcp} mutant plants exposed first to growth light (GL) (120 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) for two hours and then to high light (HL) (1000 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) for 1 and 2 hours. CBB = Coomassie Brilliant Blue; a loading control. Otherwise as in Figure 1.
Figure 5. Immunoblots of thylakoid phosphoproteins in WT and the pgr5 mutant and of the amounts of the STN7 kinase and TAP38 phosphatase in WT, stn7, tap38 and pgr5. A. Phosphorylation pattern of the PSII and LHCII proteins after low light (LL) and high light (HL) treatments of wild type and the pgr5 mutant plants as described in the legend for Fig.3. B. The amounts of the STN7 kinase and TAP38/PPH1 phosphatase in pgr5, WT, stn7 and tap38/pph1. Gels were loaded on equal chl basis (2 μg chl / well). CBB = Coomassie Brilliant Blue; a loading control.
Figure 6. Comparison of the phosphorylation pattern of thylakoid proteins (A) with the 77 K chl a fluorescence emission spectra (B) of WT and the pgr5 mutant treated as described in the legend for Fig. 4.
Figure 7. The lpBN gel demonstrating the composition of thylakoid protein complexes in growth light (GL) and high light (HL) acclimated *Arabidopsis*. Thylakoid membranes were isolated from GL acclimated (120 μmol photons m$^{-2}$ s$^{-1}$) and 1 h HL treated WT and *tap38/pph1*, solubilized with dodecyl maltoside (DM) or digitonin (Dig) and separated with lpBN gel electrophoresis. Gel was stained with Coomassie brilliant blue.
**Dynamics of protein complexes:**

- Enhanced attraction between PSII-LHCII and PSI-LHCl complexes
- Suppressed attraction between PSII-LHCII and PSI-LHCl complexes
- Enhanced repulsion between PSII-LHCII complexes
- Enhanced attraction between PSII-LHCII complexes

**Regulation of kinases and phosphatases:**

- Activation
- Inactivation
- Inhibition
- XXX Enzyme needed for the response
- Electron density in the PQ pool and the acceptor side of PSI

---

**Acclimation to darkness and "State 1"**

**Short-term acclimation upon turning light on**

**Light acclimated state**

**Decrease in light intensity**

**Increase in light intensity**

**Special cases: tap38/pph1 mutant in HL, any mutant keeping PSI acceptor side oxidised in HL or WT in "State 2" light**
Figure 8. A schematic model presenting how the concerted regulation of PSII core and LHCII protein phosphorylation maintains the excitation balance in ETC. Kinases and phosphatases required for the response and the redox regulation of the kinases are presented. A. In darkness and in “State 1” condition, the STN7 and STN8 kinases are inactive and TAP38/PPH1 and PBCP phosphatases are active. All thylakoid proteins (sometimes with exception of CP43) are dephosphorylated. Fully dephosphorylated state maximizes the lateral segregation of PSII and PSI and minimizes the area where PSI can interact with LHCII and PSII. B. Turning the light on activates both STN7 and STN8 kinases leading to increased phosphorylation of both LHCII and PSII core proteins. PSII core protein phosphorylation loosens the tight packing of PSII-LHCII rich thylakoid grana domain and LHCII phosphorylation enhances the attraction between PSI and LHCII. C. In constant light, there is a homeostasis between the kinase and phosphatase activities and PSII core and LHCII proteins are moderately phosphorylated in all thylakoid domains (Tikkanen, et al., 2008b; Grieco, et al., 2012; Wientjes, et al., 2013b). Moderate phosphorylation is needed to provide the grana membranes with sufficient fluidity as well as sufficient energy transfer from LHCII to PSI. The former allows proper turnover of PSII (Tikkanen, et al., 2008a; Fristedt, et al., 2009; Goral, et al., 2010; Kirchhoff, et al., 2011) and the latter the maintenance of optimal oxidation of ETC (Bellaﬁore, et al., 2005; Grieco, et al., 2012; Tikkanen, et al., 2010; Tikkanen, et al., 2006) thus preventing the damage of the photosynthetic apparatus (Grieco, et al., 2012; Tikkanen, et al., 2014). D. Decrease in light intensity leads to dephosphorylation of the PSII core proteins, by inactivation of the STN8 kinase and activation of a still unknown phosphatase (PBCP unknown), thus inducing higher packing of PSII-LHCII in the grana (Tikkanen, et al., 2008a; Fristedt, et al., 2009; Goral, et al., 2010). This likely enhances the light absorption efficiency by increasing the connectivity between different PSII-LHCII and LHCII complexes (Haferkamp and Kirchhoff, 2008; Haferkamp, et al., 2010). High packing of PSII-LHCII complexes in the grana segregates PSI from the LHCII system. The STN7-induced increase in LHCII phosphorylation is required to increase the affinity between LHCII and PSI (Tikkanen, et al., 2008b; Grieco, et al., 2015), allowing sufﬁcient energy transfer to PSI and maintaining the excitation balance. E. Increase of PSII core protein phosphorylation in high light facilitates the unpacking and mobility of PSII-LHCII, thus alleviating the strict segregation between PSII-LHCII and PSI. This would lead to uncontrolled excitation energy transfer from PSII-LHCII to PSI without concomitant dephosphorylation of LHCII by the TAP38/PPH1 phosphatase and the inhibition of STN7, which prevents PSI over excitation. F. In case of failure to dephosphorylate LHCII


Copyright © 2015 American Society of Plant Biologists. All rights reserved.
by reversible phosphorylation. Biochemistry 34: 16022-16029


spinach thylakoid protein heterogeneity offers insights into the photosystem II repair cycle. Biochim Biophys Acta 1837: 1463-1471


Wientjes E, van Amerongen H, Croce R (2013b) LHCII is an antenna of both photosystems after long-term acclimation. Biochim Biophys Acta 1827: 420-426
