Plants Actively Avoid State Transitions upon Changes in Light Intensity: Role of Light-Harvesting Complex II Protein Dephosphorylation in High Light[1][OPEN]

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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 PSII core phosphatases). Shift of plants from growth light to high light results in an increase of PSII core phosphorylation, whereas 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 photosystem I remains unchanged. This differs drastically from the canonical-state transition model induced by artificial states 1 and 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, *tap38/pph1* mutant revealed strong concomitant phosphorylation of both the PSII core and LHCII proteins upon transfer to high light, thus resembling the wild type 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 *pgr5* mutant mimics phenotypes of *tap38/pph1*. This shows that there is a close cooperation between the redox- and proton gradient-dependent regulatory mechanisms for proper function of the photosynthetic machinery.

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 PSII and 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 b_6f, 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 (light-harvesting complex I [LHCI] and LHCII). The majority of the light-absorbing pigments are bound to LHCII trimers that can serve the light harvesting of both photosystems (Galka et al., 2012; Kouřil et al., 2013; Wientjes et al., 2013b). Energy distribution from LHCII is regulated by protein phosphorylation (Bennett, 1979; Bennett et al., 1980; Allen et al., 1981) under control of the STN7 and STN8 kinases (Depège et al., 2003; Bellafiore et al., 2005; Bonardi et al., 2005; Vainonen et al., 2005) and the TAP38/PPH1 and Photosystem II Core Phosphatase (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, the protein composition of the LHCII trimers also affects 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 PSII- and PSI-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 (Pesaresi et al., 2009; Järvi et al., 2011; Caffarri et al., 2014). The 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 Suc gradient centrifugation or in native gel analyses (Caffarri et al., 2009; Järvi et al., 2011), 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 77-K fluorescence

[1] This work was supported by the Academy of Finland (project nos. 271832, 273870, and 260094).

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Eva-Mari Aro (evaaro@utu.fi).

N.R.M. performed most of the experiments; M.S., M.R., and M.T. provided technical assistance to N.R.M.; M.T. designed the experiments and analyzed the data; M.T. conceived the project and wrote the article with E.-M.A.

[OPEN] Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.15.00488

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 (for review, see 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; Leoni et al., 2013; Wientjes et al., 2013a)—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-LHCI to different thylakoid domains. It, however, seems likely that PSII and PSI are energetically connected through a 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 the state transition complex (Pesaresi et al., 2009; Järvi 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 show 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 (Järvi 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 (Rintamäki et al., 1997, 2000) in the timescale of 5 to 30 min. When light intensity increases, the PSII core protein phosphorylation increases, whereas 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 (Rintamäki et al., 1997, 2000). The presence and absence of the PSI-LHCII state transition complex correlate with LHCII phosphorylation (similar to the state transitions; Pesaresi et al., 2009; Wientjes et al., 2013b). Despite all of 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 behaviors of PSII core and LHCII protein phosphorylation, as described above, has been known for more than 15 years (Rintamäki et al., 1997, 2000), but 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 PSII core protein 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 are dephosphorylated 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 states 1 and 2 lights induce state transitions, mechanisms that change 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 (Rintamäki et al., 1997, 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. However, it is worth noting that LHCII phosphorylation under state 2 light is not enough to induce the state 2 transition but that 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; Depège et al., 2003; Bellafiore et al., 2005; Bonardi 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 (Vener et al., 1997; Rintamäki et al., 2000; 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., 2005; Tikkanen et al., 2006). In addition to this, STN7 takes part in the phosphorylation of PSII core proteins (Vainonen et al., 2005), especially in LL (Tikkanen et al., 2008b, 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., 2005; 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 the two phosphatase (*tap38/pph1*and *pbcp*) mutants of Arabidopsis (*Arabidopsis thaliana*) to elucidate the individual roles of these enzymes in reversible thylakoid protein phosphorylation and distribution of excitation energy between PSII and PSI upon changes in light intensity. It is shown 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 Compared with the States 1 and 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, the P-threonine (P-Thr) immunoblot in Figure 1A is used to show the phosphorylation levels of LHCII phosphoproteins LHCB1 and LHCB2 (migrated together and designed as P-LHCII) and the PSII core phosphoproteins P-D1, P-D2, and P-CP43 in growth light (GL) and 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 compared with GL (Fig. 1A). Despite strong change in thylakoid protein phosphorylation upon transfer of plants from GL to state 1 light (Fig. 1A), only a slight decrease occurred in the relative PSII to PSI absorption cross section, which was deduced from the 77-K fluorescence emission spectrum, revealing the PSII peaks at 685 and 695 nm and the PSI peak at 733 nm (Fig. 1B). Transfer of plants from GL to state 2 light, in turn, strongly increased the relative excitation of PSI (Fig. 1B), despite the fact that the changes in PSII core and LHCII protein phosphorylation were rather minor (Fig. 1A).

Phosphorylation of LHCII and PSII Core Proteins in the Wild Type and the stn7, stn8, tap38/pph1, pbcp, and proton gradient regulation5 Mutant Plants

With the purpose of exploring the individual physiological roles of the thylakoid protein kinases and phosphatases, the thylakoid phosphorylation pattern of the wild type 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) steady-state GL-acclimated plants, (2) plants subjected to 20-min shifts between LL and HL,

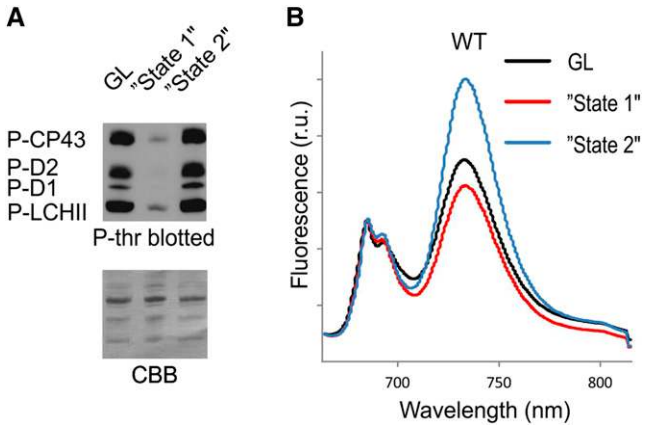

Figure 1. Comparison of thylakoid protein phosphorylation with excitation energy distribution between PSII and PSI in GL and lights preferentially exciting either PSI (state 1) or PSII (state 2). A, Immunoblot showing the phosphorylation of the PSII core proteins CP43 (P-CP43), D2 (P-D2), and D1 (P-D1) and the LHCII proteins LHCB1 and LHCB2 (migrate in the same band assigned as P-LHCII) in the wild type. B, A 77-K Chl *a* fluorescence emission spectra. Wild-type samples were collected from GL (120 μmol photons m^{-2} s^{-1}) and after subsequent 1-h exposure to state 1 light (far red light: 30 μmol photons m^{-2} s^{-1}) and state 2 light (red light: 50 μmol photons m^{-2} s^{-1}; for details, see Piippo et al., 2006). Gels were loaded on equal Chl basis (0.5 μg of Chl per well). CBB, Coomassie Brilliant Blue (a loading control); WT, wild type.

and (3) plants shifted from GL to HL for 1 and 2 h. Previously, by screening the light intensity-dependent phosphorylation of thylakoid proteins in different mutant plants, we had found that the *proton gradient regulation5* (*pgr5*) mutant, unable to generate transthylakoid 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 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 I. Table I also contains the chlorophyll (Chl) *a/b* ratios of the wild type and mutant plants in GL conditions.

First, the phosphorylation levels of LHCII and the PSII core phosphoproteins in wild-type 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, whereas the phosphorylation levels of D1, D2, and CP43 were clearly higher compared with the wild type. The *stn8* mutant had strongly decreased phosphorylation of D1 and D2, but the phosphorylation level of CP43 was not significantly different from that of the wild type. In *tap38/pph1*, the phosphorylation levels of all thylakoid phosphoproteins were very similar to those in the wild type. In turn, the lack of the PBCP phosphatase caused slightly higher phosphorylation level of PSII core proteins than observed in the wild type. All of the results on kinase and phosphatase mutants described above are in line with several earlier studies (Bellafiore et al., 2005; Bonardi 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) showed similar phosphorylation levels of LHCII and PSII core proteins at steady-state GL as the wild type (Fig. 2A), indicating no drastic difference in the kinase and phosphatase system or the redox status of ETC in constant GL.

Second, the plants from GL (with thylakoid phosphorylation as shown in Fig. 2) were exposed to repetitive 20-min LL and HL periods. As shown in Figure 3, in the wild type, the LHCII proteins were highly phosphorylated upon shift of plants to LL, and the PSII core proteins became more phosphorylated upon shift of plants from LL to HL (Tikkanen et al., 2010). On the contrary, the *stn7* mutant lost its capacity to keep PSII core proteins phosphorylated upon shift to HL, whereas 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 showed that STN7 phosphorylates not only the LHCII proteins but also, in LL, PSII core proteins, especially CP43 (Vainonen et al., 2005), whereas STN8 is rather specific only for the PSII core proteins and most active in HL (Rintamäki et al., 1997; Bonardi et al., 2005; Vainonen et al., 2005; Tikkanen et al., 2008a, 2010). 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 the wild type (Fig. 3; Tikkanen et al., 2008a, 2010), results from the fact that the reduction state of ETC increases in *stn7* upon shift of plants from HL to LL (Bellafiore et al., 2005; Tikkanen et al., 2006, 2010), whereas upon a similar shift of wild-type plants, the ETC becomes oxidized (Tikkanen et al., 2010; Grieco et al., 2012). Importantly, the *tap38/pph1* mutant showed 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 darkness (Pribil et al., 2010; Shapiguzov et al., 2010). Interestingly, the *pbcp* mutant behaved in light intensity shifts like the wild type (Fig. 3), despite the facts that the PBCP phosphatase has been shown to be responsible for PSII core protein dephosphorylation in state 1 light (Samol et al., 2012) and that the mutant also showed slightly higher PSII core protein phosphorylation than the wild type in steady-state GL (Samol et al., 2012; 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 the wild type 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 wild-type and kinase mutant plants between LL, GL, and HL (Tikkanen et al., 2010). Because the major aim of this 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 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 the wild type, the HL treatment led to a high phosphorylation of the PSII core proteins and 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 compared with the wild type upon shift to HL (Fig. 4).

Table I. *A summary of the previously published characterizations of the light acclimation mutants used in this study as well as the Chl a/b ratios of these mutants under the growth conditions used here*

Mutant	Primary Defect	Direct and Indirect Consequences of the Mutation
stn7	Lack of the STN7 kinase (Bellafiore et al., 2005)	*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 for this by increasing the amount of PSI complexes in constant GL (Tikkanen et al., 2006; Grieco et al., 2012); slightly decreased Chl *a/b* ratio, despite a decreased PS to LHCII ratio (Tikkanen et al., 2006); Chl *a/b* decreased in all different PSII-LHCII complexes (Grieco et al., 2012); the Chl *a/b* ratio in our growth condition in the wild type is about 3.3, and it is about 3.2 in *stn7*.
stn8	Lack of the STN8 kinase (Bonardi et al., 2005)	*stn8* has strongly reduced phosphorylation of PSII core protein, especially when shifted to HL (Bonardi et al., 2005; Vainonen et al., 2005). Delayed D1 degradation caused by 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 Chl *a/b* ratio; Chl *a/b* ratio in our growth is about 3.3.
tap38/pph1	Lack of the TAP38 (Pribil et al., 2010)/PPH1 (Shapiguzov et al., 2010) phosphatase	*tap38/pph1* cannot dephosphorylate LHCII in darkness or state 1 condition (Pribil et al., 2010; Shapiguzov et al., 2010); no obvious difference from the wild type in the phosphorylation PSII and LHCII in constant GL but still enhanced excitation of PSI and oxidation of the intersystem ETC; reported to grow faster than the wild type in LL (Pribil et al., 2010); no reported changes in the protein composition of the thylakoid membrane (Pribil et al., 2010; Shapiguzov et al., 2010). Chl *a/b* ratio in our growth condition is about 3.5 for unknown reasons (see *stn7* above).
pbcp	Lack of the PBCP phosphatase (Samol et al., 2012)	*pbcp* has impaired dephosphorylation of PSII core proteins in state 1 condition and slightly increased phosphorylation of PSII core proteins in GL and darkness (Samol et al., 2012) as well as in photoinhibitory light condition (Puthiyaveetil et al., 2014); reported to have minor modulations in the thylakoid structure (Samol et al., 2012), thylakoid protein composition, and PSII function (Puthiyaveetil et al., 2014); delayed D1 degradation in photoinhibitory conditions (Puthiyaveetil et al., 2014). Chl *a/b* ratio in our growth condition is about 3.3.
pgr5	Not functional PGR5 protein (Munekage et al., 2002)	*pgr5* is impaired in generation of transthylakoid proton gradient upon increase in light intensity; low thermal dissipation of excitation energy and high reduction level of intersystem ETC; decreased amount of PSI in constant GL 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., 2014a, 2014b). Chl *a/b* ratio is about 3.4, despite decreased amount of PSI (see *stn7* above).

Despite clear differences in the behavior of thylakoid protein phosphorylation upon shift of the wild type, 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 of dephosphorylating LHCII and therefore, keeping both the PSII core and LHCII proteins strongly phosphorylated in HL, behaved completely differently from the wild type, the kinase mutants, and the *pbcp* phosphatase mutant. Indeed, the 77-K fluorescence spectrum of the *tap38/pph1* mutant showed strong increase in relative excitation of PSI already after a 1-h shift to HL (Fig. 4), resembling the artificial state 2 condition with respect to both protein phosphorylation pattern and excitation energy distribution (Fig. 1). Thus, the HL exposure induced the state 2 transition in the *tap38/pph1* mutant because of the fact that it cannot regulate reversible LHCII phosphorylation in the same way as the wild type upon changes in light intensity. It is worth noting that these changes in the *tap38/pph1* mutant were more distinct after 1-h HL exposure than after 2-h 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. 5A). 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 through the cytochrome b_6f complex, leading to damage of PSI upon shift to HL; Suorsa et al., 2012). This most likely leads to an incapability to properly reduce the electron acceptors of PSI that, in turn, are required for inhibition of the

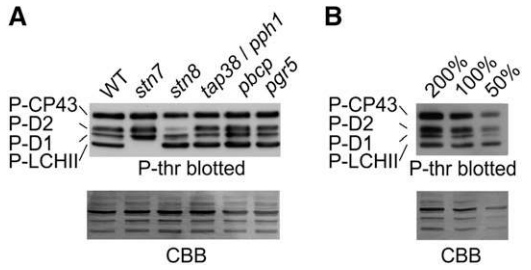

Figure 2. Thylakoid protein phosphorylation by the STN7 and STN8 kinases and TAP38/PPH1 and PBCP phosphatase pathways in constant GL (120 μmol photons m^{-2} s^{-1}). A, P-Thr immunoblot showing the phosphorylation of the PSII core proteins CP43, D2, and D1 and the LHCII proteins (LHCB1 and LHCB2) in wild-type plants and the *stn7*, *stn8*, *tap38/pph1*, *pbcp*, and *pgr5* mutants in GL 4 h after the beginning of the 8-h light period. B, Dilution series of thylakoids showing the response of the antibody to different protein amounts. Gels were loaded on equal Chl basis (0.5 μg of Chl per well). CBB, Coomassie Brilliant Blue (a loading control); WT, wild type.

LHCII kinase in HL (Rintamäki et al., 2000). The amounts of the TAP38/PPH1 phosphatase and the STN7 kinase were, however, also estimated by immunoblotting from the wild type and the *pgr5* mutant (Fig. 5B). Although minor differences were observed in the amounts of the STN7 kinase and the TAP38/PPH1 phosphatase between the wild type and the *pgr5* mutant, they were not regarded as big enough to explain the missing regulation of LHCII phosphorylation in the *pgr5* mutant upon changes in light intensity.

As shown in Figure 6A, the *pgr5* mutant also behaved similar to the *tap38/pph1* mutant (Fig. 4) with respect to the failure in dephosphorylation of the LHCII proteins during 1- 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. 6B) observed in the 77-K fluorescence spectrum, thus mimicking the transition to state 2 (Fig. 1).

In case of both the *tap38/pph1* (Fig. 4) and *pgr5* (Fig. 6A) mutants, prolonged exposure of leaves to HL (2 h) already started diminishing the superior PSI fluorescence emission at 77 K. This is an indication of initiation of a general acclimation strategy of plants toward 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-h 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 Organization 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 wild-type and *tap38/pph1* plants and then subjected to solubilization with two different detergents and separation of the pigment-protein complexes by blue native gel electrophoresis (Fig. 7). Dodecyl maltocide (DM) was used to solubilize the entire thylakoid membrane to explore the packing of PSII-LHCII supercomplexes (Tikkanen et al., 2008a). In this treatment, no clear differences in pigment-protein complexes between GL and HL and between the wild type 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 solublize the highly packed PSII-LHCII complexes of grana core, but it 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, and the PSI-LHCII state transition complex in addition to the PSII and PSI monomer and free LHCII trimer and monomer complexes. The digitonin solubilization experiment showed that the LHCII-PSI-LHCI complex (state transition complex) was similarly present in the wild type and *tap38/pph1* under GL but disappeared only from the wild type 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 the wild type. 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 rearrangements of pigment protein complexes in grana margin regions compared with the wild type.

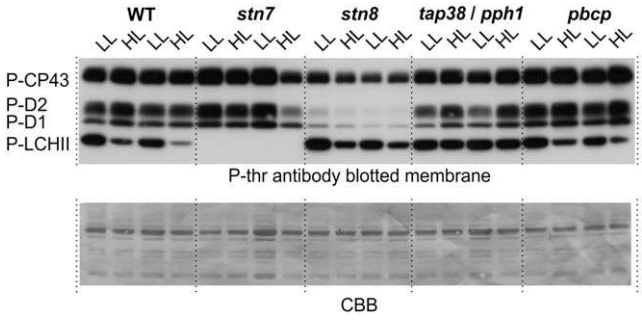

Figure 3. Regulation of thylakoid protein phosphorylation by the STN7 and STN8 kinases and TAP38/PPH1 and PBCP phosphatase pathways. Immunoblot showing the phosphorylation of the PSII core proteins CP43, D2, and D1 and the LHCII proteins (LHCB1 and LHCB2) in the wild-type plants and the *stn7*, *stn8*, *tap38/pph1*, and *pbcp* mutants after repeated exposure to 20 (LL) and 1,000 (HL) μmol photons m^{-2} s^{-1} in 20-min intervals. Gels were loaded on equal Chl basis (0.5 μg of Chl per well). CBB, Coomassie Brilliant Blue (a loading control); WT, wild type.

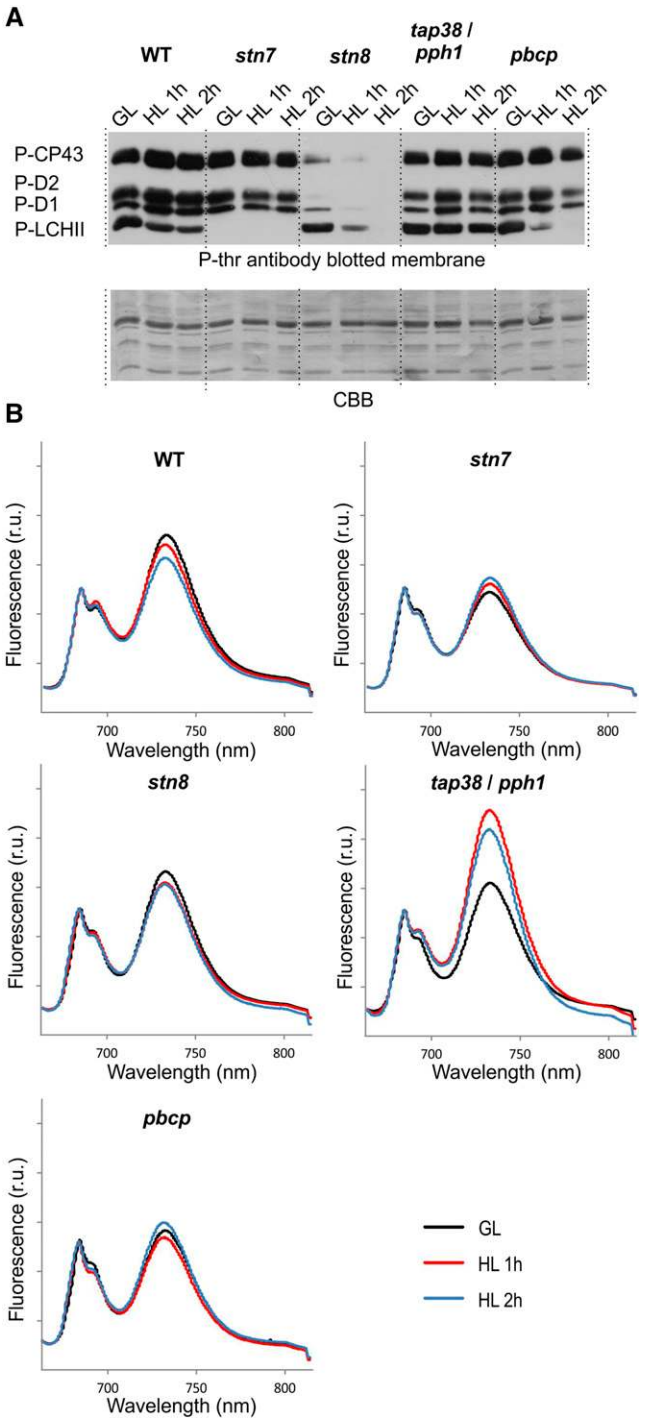

Figure 4. Relationship between PSII core and LHCII protein phosphorylation and the excitation energy distribution between PSII and PSI. P-Thr immunoblots showing the phosphorylation pattern of thylakoid proteins (A) and the 77-K Chl *a* fluorescence emission spectra (B) were recorded from the wild type and the *stn7*, *stn8*, *tap38/pph1*, and *pbcp* mutant plants exposed first to GL (120 μmol photons m^{-2} s^{-1}) for 2 h and then, HL (1,000 μmol photons m^{-2} s^{-1}) for 1 and 2 h (otherwise as in Fig. 1). CBB, Coomassie Brilliant Blue (a loading control); WT, wild type.

DISCUSSION

Evidence has accumulated during the past 10 years indicating that reversible thylakoid protein phosphorylation is one important component in the multifaceted and highly integrated regulatory network for optimal harnessing of light energy and its transduction into chemical energy in the thylakoid membrane (for 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 reinvestigation. Instead of traditional views assigning reversible LHCII phosphorylation a unique role in state transitions (for review, see Haldrup et al., 2001; Allen and Forsberg, 2001; Rochaix, 2007) and the PSII core protein phosphorylation in fluent PSII turnover in HL (for review, see Aro et al., 1993; Tikkanen and Aro, 2012), we provide here compelling evidence emphasizing that, instead of such individual roles, the cooperation 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 (Figs. 4 and 6).

The crucial early observation was the fact that HL illumination leads to not only increased phosphorylation of PSII core proteins but also, dephosphorylation of the LHCII proteins in wild-type plants (Rintamäki et al., 1997). These facts have been impossible to integrate with

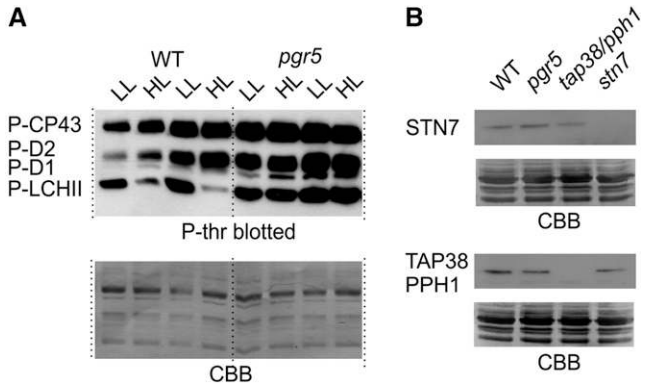

Figure 5. Immunoblots of thylakoid phosphoproteins in the wild type and the *pgr5* mutant and the amounts of the STN7 kinase and TAP38 phosphatase in the wild type, *stn7*, *tap38*, and *pgr5*. A, Phosphorylation pattern of the PSII and LHCII proteins after LL and HL treatments of wild-type and *pgr5* mutant plants as described in Figure 3. B, The amounts of the STN7 kinase and TAP38/PPH1 phosphatase in *pgr5*, the wild type, *stn7*, and *tap38/pph1*. Gels were loaded on equal Chl basis (2 μg of Chl per well). CBB, Coomassie Brilliant Blue (a loading control); WT, wild type.

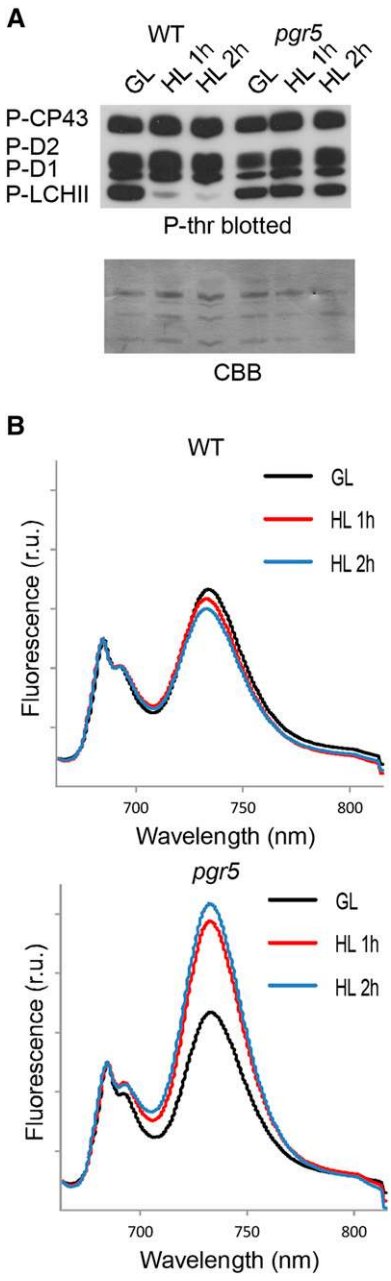

Figure 6. Comparison of the phosphorylation pattern of thylakoid proteins (A) with the 77-K Chl *a* fluorescence emission spectra (B) of the wild type and the *pgr5* mutant treated as described in Figure 4. CBB, Coomassie Brilliant Blue (a loading control); WT, wild type.

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; Tikkanen et al., 2010).

Availability of the *stn7* (Depège 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 an LL effect (Bellafiore et al., 2005; Tikkanen et al., 2006), although it was known already before that LHCII phosphorylation and state transitions are LL acclimation mechanisms (Walters and Horton, 1991; Finazzi et al., 2004). STN7 kinase was shown to be required for sufficient excitation energy transfer to PSI under LL 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, 2011). Conversely, a shift of plants to HL causes rapid induction of the PSBS protein-dependent thermal dissipation of excitation energy (Li et al., 2000), 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 (Rintamäki et al., 1997, 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 the shift of plants to HL (Figs. 3 and 4) provided an excellent tool to address the enigmatic physiological role of LHCII dephosphorylation in HL (Rintamäki et al., 1997, 2000). TAP38/PPH1 phosphatase was previously shown to be 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 show that TAP38/PPH1 is responsible for dephosphorylation of LHCII in HL as well (Figs. 3 and 4). Thus, in theory, it is possible that not only the STN7 kinase but also, the TAP38/PPH1 phosphatase are 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 in both darkness and state 1 light but additionally, under strongly reducing HL conditions. However, 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 the wild type 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 HL, similar to the wild type, but cannot concomitantly dephosphorylate the LHCII proteins like the wild type (Figs. 3 and 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 wild-type thylakoids (Fig. 1); however, the wild type, *stn8*, and *pbcp* do dephosphorylate LHCII in HL, and *stn7* keeps LHCII always dephosphorylated.

The PBCP phosphatase has been shown to be involved in phosphorylation balance of PSII core proteins in constant GL (Samol et al., 2012), which we also show 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 such an obvious role of PBCP phosphatase in dephosphorylation of PSII core proteins (Samol et al., 2012; Puthiyaveetil et al., 2014; 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 investigation of the physiological role of PSII core protein dephosphorylation in LL, which may be crucial in enhancing the light-harvesting efficiency by increasing the connectivity between the PSII complexes (the schematic model is 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 (77-K fluorescence emission spectra) in *tap38/pph1* with that in the wild type, *stn7*, *stn8*, and *pbcp* (the schematic model is in Fig. 8). In the case of the wild type, *stn7*, *stn8*, and *pbcp*, the absorption cross section (i.e. the excitation) of the two photosystems remains unchanged upon transfer of plants from GL to HL. The *tap38/pph1* mutant behaves completely differently. Transfer of *tap38/pph1* to HL 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), similar to that occurring in the wild type under the state 2 condition (Fig. 1A). 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 of the kinases, one specific for the PSII core and one specific 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 toward even distribution of excitation energy to both photosystems and prevention of the occurrence of state transition.

It is worth noting that the *tap38/pph1* mutant is not more susceptible to HL than the wild type (Pribil et al., 2010; Shapiguzov et al., 2010). Indeed, as long as the light-harvesting antenna is in the 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., 2014b). 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 HL, 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 a large antenna, leading to energy losses in nonphotochemical 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., 2014a, 2014b) but also, need to keep ETC 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 exhibit a strong simultaneous phosphorylation of both the PSII core and LHCII proteins at HL (Figs. 5A and 6A), again resembling the wild type under state 2 light (Fig. 1). The *pgr5* mutant has, however, rather normal amounts of STN7 kinase and TAP38/PPH1 phosphatase (Fig. 5B),

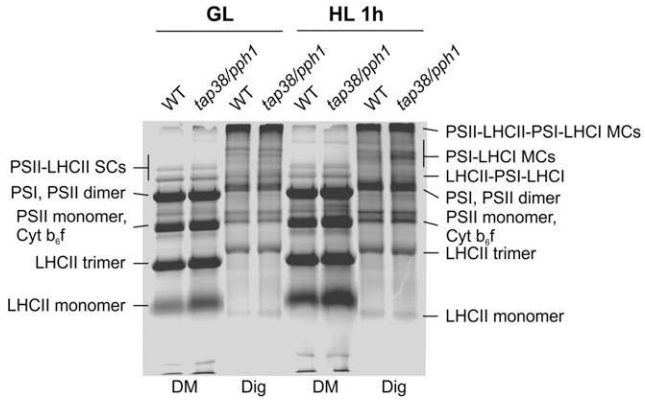

Figure 7. The large pore blue native (lpBN) gel showing the composition of thylakoid protein complexes in GL- and HL- acclimated Arabidopsis. Thylakoid membranes were isolated from GL-acclimated (120 μmol photons m^{-2} s^{-1}) and 1-h HL-treated wild type and *tap38/pph1*, solubilized with DM or digitonin, and separated with lpBN gel electrophoresis. Gel was stained with Coomassie Brilliant Blue. Dig, Digitonin; WT, wild type; Cyt b_6f, cytochrome b_6f.

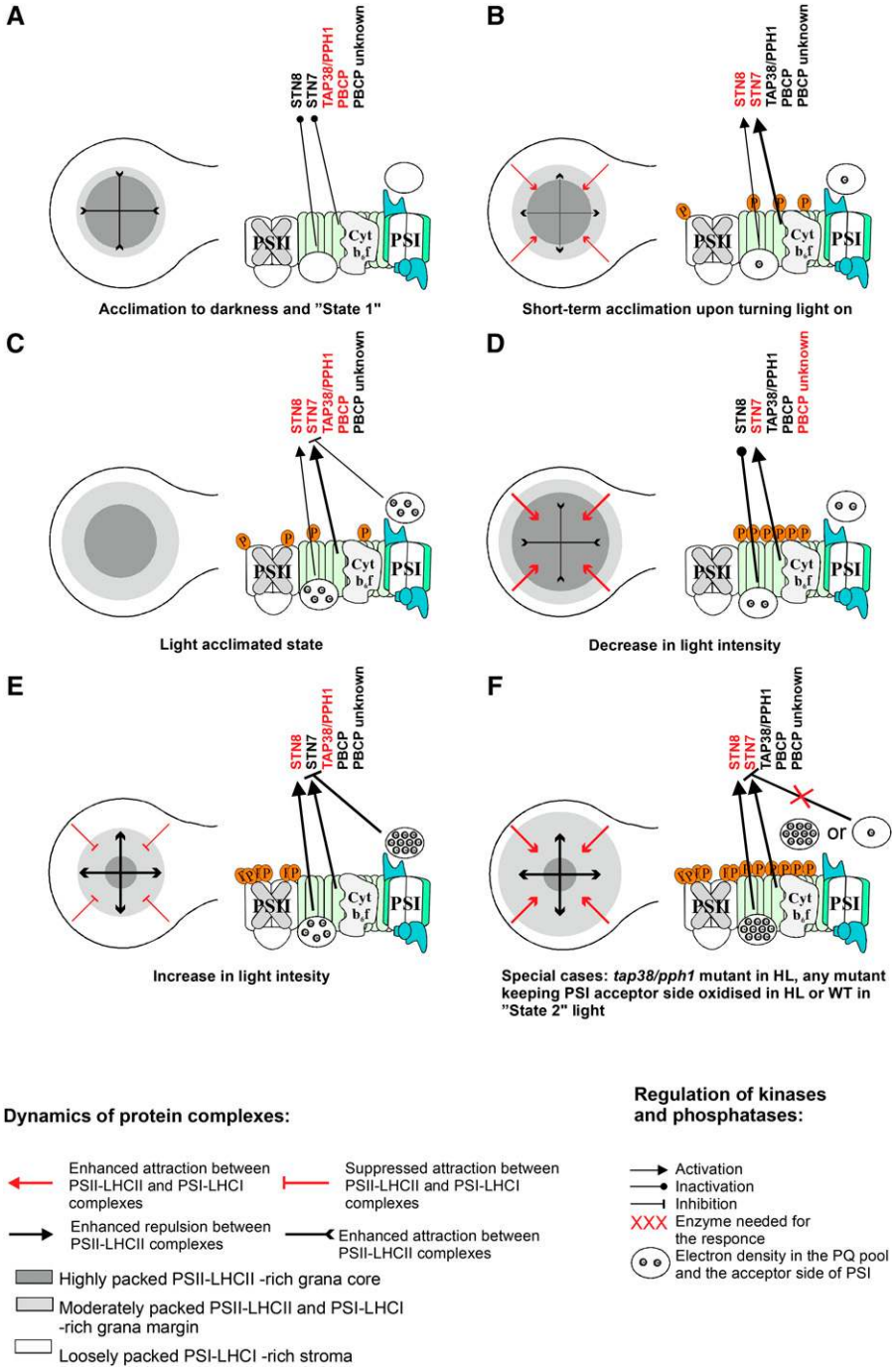

Figure 8. A schematic model presenting how the concerted regulation of PSII core and LHCII protein phosphorylation maintains the excitation balance in ETC. Kinases and phosphatases required for the response and the redox regulation of the kinases are presented. A, In darkness and state 1 condition, the STN7 and STN8 kinases are inactive, and the TAP38/PPH1and PBCP phosphatases are active. All thylakoid proteins (sometimes with the exception of CP43) are dephosphorylated. The 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 the 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 allows the

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 HL intensity, it is required to control electron transfer and 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., 2014a, 2014b). Apparently, the photodamage of PSI combined with low thermal dissipation in HL lead to strong reduction of the plastoquinone 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 the wild type or natural ones for the *tap38/pph1* and *pgr5* mutants, that induce a strong simultaneous phosphorylation of both the PSII core proteins and the LHCII proteins show a distinct change in relative excitation of PSII and PSI. On the contrary, the wild type and all of the mutants with functional LHCII dephosphorylation at HL 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 compared with the state 2 light (Fig. 1A) and the state 2-mimicking HL in *tap38/pph1* (Figs. 3 and 4) and *pgr5* (Figs. 5 and 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 toward PSII (HL or PSII light). It can be speculated that the 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 in 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 do not seem to be dependent on the phosphorylation but that 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 solubilized the thylakoid membranes of GL- and HL-acclimated wild-type and *tap38/pph1* plants by using two different detergents (Fig. 7). DM solubilization, 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 the wild type and *tap38/pph1*. On the contrary, digitonin solubilization, addressing only the loosely packed stroma lamellae and grana margins, revealed distinct differences between the wild type and *tap38/pph1* in HL. Both the wild type and *tap38/pph1* had the PSI-LHCI state transition complex (Pesaresi et al., 2009; Wientjes et al., 2013b) in GL, but only the wild type 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 the wild type 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

Figure 8. (*Continued.*)
maintenance of optimal oxidation of ETC (Bellafiore et al., 2005; Tikkanen et al., 2006, 2010; Grieco et al., 2012), thus preventing the damage of the photosynthetic apparatus (Grieco et al., 2012; Tikkanen et al., 2014a, 2014b). 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 HL 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 overexcitation. F, In case of failure to dephosphorylate LHCII (*tap38/pph1* and *pgr5*), the high concomitant phosphorylation of both the PSII core and LHCII proteins leads to a high amount of PSII-LHCII in the PSI-rich grana margin regions and a high attraction between PSII-LHCII and PSI complexes, resulting in uncontrolled excitation energy transfer to PSI. This situation is analogous to the so-called state 2 condition, which also induces strong concomitant phosphorylation of both the PSII core and LHCII proteins. WT, Wild type; Cyt b_6f, cytochrome b_6f; PQ, plastoquinone.

margin region, which was also shown 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 to maintain the excitation balance between PSII and PSI.

CONCLUSION

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 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, the extensive redox-regulated and -coordinated PSII core and the LHCII protein phosphorylation-dephosphorylation-dependent network together with the proton gradient across the thylakoid membrane assist the dynamics of thylakoid protein complexes and concomitantly, allow 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, which is shown here by a crucial involvement of transthylakoid proton gradient in proper distribution of excitation energy to both photosystems in HL. Altogether, the presented model (Fig. 8) integrating both the reversible but opposite PSII core and LHCII protein phosphorylation and the transthylakoid proton gradient into regulation of excitation energy distribution to the two photosystems is one further step toward 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 Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 and *stn7* (SALK_073254; Bellafiore et al., 2005), *stn8* (SALK_060869; Bonardi et al., 2005), *tap38* (SALK_025713; Pribil et al., 2010), *pbcp* (SALK_127920; Samol et al., 2012), and *pgr5* (AT2G05620; Munekage et al., 2002) mutant plants were grown in controlled environmental chambers for 5 to 6 weeks at 120 μmol photons m^{-2} s^{-1} with a 8-h-light/16-h-dark cycle and relative humidity of 70%. OSRAM PowerStar HQIT 400/D Metal Halide Lamps were used as the 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 1,000 μmol photons m^{-2} s^{-1} in 20-min intervals for a total duration of 80 min. In HL treatment, plants were exposed to 1,000 μmol photons m^{-2} s^{-1} for 1 and 2 h. 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). Details are 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 resuspended in buffer containing 100 mM sorbitol, 50 mM HEPES-KOH (pH 7.5), 10 mM NaF, and 10 mM $MgCl_2$. Chl concentration was determined according to Porra et al. (1989).

SDS-PAGE and Immunoblotting

Thylakoid membrane proteins were separated on 15% (w/v) SDS-polyacrylamide gels with 6 M urea (Suorsa et al., 2004). After electrophoresis, the polypeptides were transferred to a polyvinylidene difluoride membrane (Millipore), and the membrane was blocked with 5% (w/v) 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-P-Thr 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). Samples equivalent to 2 μg of Chl were loaded in each well for kinase and phosphatase detection.

77-K 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 1 μg of 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 florescence emission bands from PSI.

Native Gel Electrophoresis

Thylakoid membrane solubilization by 1% (w/v) DM or 1% (w/v) digitonin and the subsequent separation of protein complexes by large pore native gel were performed according to Järvi et al. (2011).

ACKNOWLEDGMENTS

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

Received March 31, 2015; accepted April 21, 2015; published April 22, 2015.

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