Title: **Thylakoid Protein Phosphorylation in Chloroplasts**

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Abstract

Because of their abundance and extensive phosphorylation, numerous thylakoid proteins stand out amongst the phosphoproteins of plants and algae. In particular, subunits of Light Harvesting Complex II (LHCII) and of Photosystem II (PSII) are dynamically phosphorylated and de-phosphorylated in response to light conditions and metabolic demands. These phosphorylations are controlled by evolutionarily conserved thylakoid protein kinases and counteracting protein phosphatases, which have distinct but partially overlapping substrate specificities. The best characterized are the kinases STATE TRANSITION 7 (STN7 / STT7) and STATE TRANSITION 8 (STN8), and the antagonistic phosphatases PROTEIN PHOSPHATASE 1/THYLAKOID ASSOCIATED PHOSPHATASE 38 (PPH1/TAP38) and PHOTOSYSTEM II CORE PHOSPHATASE (PBCP). The phosphorylation of LHCII is mainly governed by STN7 and PPH1/TAP38 in plants. LHCII phosphorylation is essential for state transitions, a regulatory feedback mechanism that controls allocation of this antenna to either PSII or PSI, and thus maintains the redox balance of the electron transfer chain. Phosphorylation of several core subunits of PSII, regulated mainly by STN8 and PBCP, correlates with changes in thylakoid architecture, the repair cycle of PSII after photo-damage as well as regulation of light harvesting and of alternative routes of photosynthetic electron transfer. Other kinases, such as the PLASTID CASEIN KINASE II (pCKII), also intervene in thylakoid protein phosphorylation and take part in the chloroplast kinase network. While some features of thylakoid phosphorylation were conserved through the evolution of photosynthetic eukaryotes, others have diverged in different lineages possibly as a result of their adaptation to varied environments.

Keywords:

LHCII, PSI, PSII, kinase, phosphatase, state transitions

Introduction

In plant and algal cells, the most prominent phospho-proteins are embedded in the thylakoid membrane. This reflects the fact that these thylakoid proteins are amongst the most abundant in green tissues and cells, and furthermore that under certain conditions, several of them are phosphorylated to a high degree. In most thylakoid proteins this reflects extensive phosphorylation at only one - or a few - phosphosites. A prime example is LHCII, one of the most prevalent protein complexes in green tissues, and in particular its LHCB2 subunit, 70% of which can be in a phosphorylated state after a shift from far-red to blue light in seedlings of *Arabidopsis thaliana* (hereafter Arabidopsis) (Longoni et al. 2015). Other subunits of LHCII, as well as several components of the PSII core can also be prominently phosphorylated depending on the conditions. There are many more phosphoproteins in the thylakoid membrane, some of which will be discussed below. Non-targeted, "shotgun" proteomic approaches have identified numerous phosphoproteins in the chloroplast, and in particular in the thylakoid membrane (reviewed by Grieco et al. 2016). In Arabidopsis, this review lists more than 50 phosphorylated thylakoid proteins involving approximately 200 phosphosites, identified with high confidence by mass spectrometry (Grieco et al. 2016). There are numerous other phosphoproteins and phosphosites identified with lower confidence, some of which may well turn out to be significant. However it is also possible that some of these phosphosites represent off-target, collateral phosphorylation due to the imperfect specificity of protein kinases. Thus it is a somewhat overwhelming challenge to address the physiological significance of all protein phosphorylation events in the thylakoid membrane.

Some criteria may be used to narrow down on the most relevant phosphoproteins and phosphosites. One criterion is the evolutionary conservation of the phosphosites (Grieco et al. 2016; Grouneva et al. 2013), which applies well to evolutionarily conserved proteins like the subunits of the photosystems, but less so to more divergent ones like the light-harvesting antennae. Another is the extent of phosphorylation and its response to changing environmental conditions. While extensive and dynamic phosphorylation of photosynthetic proteins may reflect regulation, proteins with low phosphorylation should not necessarily be dismissed, considering for example that phosphorylation could be more extensive in other untested conditions, or that the modification could be a signal for degradation, and thus be short-lived by essence. Nevertheless, to have a physiological impact on photosynthetic electron flow, a significant extent of phosphorylation is probably needed. The dynamics of phosphorylation of a protein can readily be determined by comparing the relative levels of phosphorylation under the different conditions of interest, using many methods (Table 1). However evaluating the absolute extent of phosphorylation, or in other words what proportion of a protein is phosphorylated, is more difficult. In some cases, the phospho-protein migrates differently from the non-phospho form during poly-acrylamide gel electrophoresis (PAGE), as for example CP29 in monocots (Bergantino, E. et al. 1995; Betterle et al. 2015), or D2 (PsbD) and LHCSR3 in *Chlamydomonas reinhardtii* (hereafter

Chlamydomonas) (Bonente et al. 2011; de Vitry et al. 1991; Scholz et al. 2019). Phos-tag PAGE will resolve many phosphoproteins from their non-phosphorylated forms, and also allow absolute quantifications of the fraction of the protein that is phosphorylated with respect to its total amount (Crepin and Caffarri, 2015; Longoni et al. 2015). Furthermore, in the case of proteins with multiple phosphorylation sites, this technique may discriminate between different phosphorylation states. A 2-D combination of conventional SDS PAGE in the first dimension and Phos-tag PAGE in the second revealed more than 30 proteins in the thylakoid membrane that appear to be extensively phosphorylated (Figure 1) (Nishioka et al. 2021). Mass spectrometry will also allow an estimation of the absolute level of phosphorylation, in cases where both a phosphopeptide and the corresponding non-phosphorylated peptide can be monitored (Samol et al. 2012).

Two paralogous protein kinases, STN7 (STATE TRANSITION 7) and STN8 (STATE TRANSITION 8) are essential for phosphorylation of LHCII and the PSII core subunits, as well as many other thylakoid proteins. In Chlamydomonas, the corresponding kinases are named STT7 (STATE TRANSITION 7) and STL1 (STN7 LIKE 1). The kinases are counteracted by two protein phosphatases, PPH1/TAP38 (PROTEIN PHOSPHATASE 1 / THYLAKOID ASSOCIATED PHOSPHATASE 38) and PBCP (PHOTOSYSTEM II CORE PHOSPHATASE) (reviewed by Goldschmidt-Clermont and Bassi, 2015; Rochaix et al. 2012). Although these kinases and phosphatases show some overlap in their substrate specificities, STN7 and PPH1/TAP38 mainly target LHCII while STN8 and PBCP mainly affect the PSII core in Arabidopsis. The nomenclature of the kinases and phosphatases, along with their main targets, are summarised in Table 2. It is often assumed that the kinases act directly on their thylakoid targets, but it cannot be excluded that they function indirectly through a kinase cascade. The phosphatases do act directly, as shown by their activity *in vitro* towards their respective protein targets or corresponding phosphopeptides (Liu et al. 2019; Pribil et al. 2010; Wei et al. 2015). Furthermore the X-ray crystallographic structures of PPH1/TAP38 and PBCP show how they interact with their respective phosphopeptide substrates, and provide a molecular rationale for their substrate specificity (Liu et al. 2019; Wei et al. 2015).

Protein phosphorylation intervenes in the regulation of photosynthetic electron flow in the thylakoid membrane at numerous steps, which will be discussed below. These include regulation of light harvesting (state transitions, dissipation of excess light energy), repair of PSII after photodamage, formation of PSII supercomplexes, operation of cyclic electron flow (CEF), and the architecture of thylakoid grana.

LHCII phosphorylation

A major fraction of the proteins embedded in the thylakoid membrane is composed of pigment-protein complexes devoted to light capture and transfer of the chlorophyll excitation energy: the light harvesting complexes (LHC). There are two LHC complexes in eukaryotic photosynthetic organisms: LHCI, tightly bound to PSI, and LHCII, which can be dynamically associated to both PSI and PSII (Caffarri et al. 2009; Kouřil et al. 2005; Wientjes et al. 2013)

(reviewed in Goldschmidt-Clermont and Bassi, 2015). The dynamic association of LHCII is, at least partially, dependent on its phosphorylation status. Under optimal light conditions a large portion of LHCII is phosphorylated, so that LHCII is the most abundant phosphoprotein of the thylakoid membrane. Amongst the proteins composing LHCII, it is possible to distinguish major LHCII proteins, which are the most abundant and are usually organized into LHCII trimers, and "minor" monomeric LHCII proteins, which are usually found associated to a LHCII trimer or bridging the association of the trimers to the PSII core (reviewed by Crepin and Caffarri, 2018).

State transitions

The kinase primarily responsible for LHCII phosphorylation in Chlamydomonas, STT7, was discovered by screening for mutations suppressing the redox-dependent reallocation of LHCII between the two photosystems (Depège et al. 2003; Fleischmann et al. 1999). A homologous kinase was found in Arabidopsis, STN7, showing that the state transition kinase is conserved across the eukaryotic photosynthetic lineage (Bellafiore et al. 2005). Knock-out mutants of the STT7/STN7 kinase brought the confirmation that it is only upon phosphorylation that LHCII can efficiently associate with PSI. The association of LHCII to PSI is reversible, and the process is known as "state transitions''. In the state transitions model, State1 (St1) is defined as the state when most of the LHCII is associated with PSII; this association correlates with a very low level of LHCII phosphorylation (Bennett, 1980). In State 2 (St2), upon phosphorylation of certain LHCII subunits, a portion of the trimeric LHCII associates to PSI, forming a stable PSI-LHCI-LHCII complex (Drop et al. 2014; Huang et al. 2021; Kouřil et al. 2005; Pan et al. 2018, 2021; Takahashi et al. 2006). A decrease of the LHCII associated with PSII results in diminished PSII excitation, and hence PSII fluorescence, for this reason state transitions are part of PSII non-photochemical quenching mechanisms (NPQ), referred to as "qT" (Goldschmidt-Clermont and Bassi, 2015). State transitions represent a homeostatic short term response that occurs within minutes and maintains the redox balance of the photoactive PQ pool under low or moderate light. On the longer term, adjustments of the stoichiometry of the photosystems and of their antennae maintain the electron transport chain in equilibrium. Once plants are acclimated to the prevailing light conditions, the system is at an intermediate state between St1 and St2, and LHCII serves as an efficient antenna for both photosystems (Wientjes et al. 2013). The portion of LHCII involved in "state transitions'' varies from species to species. In higher plants such as Arabidopsis only part of the trimeric ("major") LHCII can shuttle between the two photosystems, however, in some species such as Chlamydomonas the monomeric ("minor") LHCII protein LHCB4 (CP29) can also be found associated to PSI in some purified preparations (Drop et al. 2014; Takahashi et al. 2006, Takahashi et al. 2014). In Arabidopsis, the stable PSI-LHCI-LHCII complex that can be purified after solubilization of thylakoid membranes with digitonin, contains one LHCII trimer, bound on the PsaH-PsaL-PsaO side of PSI. The atomic structure of this complex, determined by cryo-EM, shows the molecular recognition of the pThr on LHCB2 by residues belonging to the PSI subunits PsaH and PsaL (Pan et al. 2018). There is evidence that a second LHCII trimer may bind on the opposite side of PSI through the LHCI subunits (Benson et al. 2015; Yadav et al. 2017). The PSI-LHCI-LHCII supercomplexes that are stable enough to withstand purification do not

entirely represent the complexes in which LHCII functionally interacts with PSI *in vivo*: spectroscopic analyses indicate that up to three LHCII trimers can efficiently transfer energy to PSI, depending on the plant species (Bell et al. 2015; Bos et al. 2017; Chukhutsina et al. 2020). In Chlamydomonas, the purified PSI-LHCI-LHCII complexes contain two LHCII trimers bound on the PsaH-PsaL-PsaO side of PSI as in plants, and some preparations also contain LHCB4 (CP29) and a lesser amount of LHCB5 (CP26) (Drop et al. 2014; Takahashi et al. 2006, Takahashi et al. 2014). High-resolution structures of Chlamydomonas PSI-LHCI-LHCII complexes were obtained recently by cryo-electron microscopy (Huang et al. 2021; Pan et al. 2021), showing PSI associated with two LHCII trimers, but not with LHCB4 or LHCB5.

Equilibration of excitation between PSII and PSI is the best accepted physiological role of state transitions in plants. A disequilibrium in the excitation of the two photosystems may occur at certain wavelengths as the excitation spectra of PSII and PSI do not match entirely. This is particularly true when plants experience far-red light (730-800nm) because PSI contains some "red shifted" chlorophylls. These pigments allow PSI excitation by far-red light, which does not excite PSII to the same extent. The state transition kinase STN7/STT7 detects and responds to imbalances between the excitation levels of the two photosystems. The molecule allowing a readout of the status of linear electron transport is plastoquinol (PQH2), the reduced form of the lipophilic electron carrier plastoquinone (PQ). A pool of PQ molecules participates in the electron transport chain (Figure 1). A disequilibrium between the photosystems will be reflected in the redox poise of the pool of PQ molecules. When the excitation level of PSII is higher than that of PSI, more electrons are injected in the PQ pool than withdrawn by PSI, therefore the pool of PQ will accumulate reduced molecules of PQH_2 which occupy the Qo site of the cytochrome b_6f complex and activate the STT7/STN7 kinase and thus the transition to St2 mediated by the LHCII phosphorylation (Vener et al. 1997; Zito et al. 1999). The STT7/STN7 kinase is associated with the cytochrome b_6f complex, interacting with subunit IV and the RIESKE protein (Dumas et al. 2017; Lemeille et al. 2009; Shapiguzov et al. 2016; Singh et al. 2016). However, this is not the only site of interaction between the kinase and the cytochrome b_6f complex, and also the stromal interaction with subunit IV has been shown to be crucial for STT7 activity (Dumas et al. 2017). The head domain of the Rieske subunit moves during the catalytic cycle of the b_6f complex. This movement, which is induced by binding of PQH2, may play a role in the activation of the kinase (Finazzi *et al.*, 2001). In a recently proposed model, the interactions of STT7 with the b_6f complex bring a pair of conserved cysteines of the kinase close to the PQH_2 binding site Qo. The binding of PQH_2 promotes the dynamic, transient formation of intermolecular disulfide bridges by these two cysteines, thus regulating the kinase activity (Shapiguzov et al. 2016; Wunder, et al. 2013). An alternative model proposes that the conserved cysteines of inactive STT7 form a stable disulphide bridge. Superoxide, produced at the b_6f complex upon oxidation of PQH2, would reduce the disulphide and thus activate the kinase (Singh et al. 2016). The authors of this model argue, based on midpoint redox potentials, that reduction of the disulphide bond (-0.12 to -0.47 V) by superoxide (-0.14V) could be possible.

In Chlamydomonas, the preferential excitation of PSI by far-red light is not as pronounced as in plants. As a consequence, the state transitions induced by changes in the spectrum of light

have a lesser amplitude than those induced by the depletion of cellular ATP (Bulté et al. 1990). Anaerobiosis or uncouplers of mitochondrial respiration induce fermentative starch degradation and the production of excess reducing equivalents, leading to the reduction of the PQ pool and strong transitions to St2.

Further regulation of STN7 and STT7

Besides being regulated by the redox status of the photosynthetic electron transfer chain, the STN7 kinase is subjected to multiple layers of regulation, so that it has been suggested to be a main responder also implicated in chloroplast retrograde signaling to the nucleus (Pesaresi et al. 2009; Tikkanen et al. 2012). Part of STN7 regulation is mediated by thioredoxins (TRXs), which play a central role in detecting and signaling the redox status of the chloroplast, leading to different responses at the level of the photosynthetic apparatus (Nikkanen and Rintamäki, 2014). STN7 is one of the targets of the TRXs regulation network (Rintamäki et al. 2000). Negative regulation by TRX-m has a major impact on STN7 activity as was shown by overexpressing TRXs in tobacco, which results in an almost complete loss of LHCII phosphorylation, implying a strong inactivation of the STN7 kinase (Ancín et al. 2019).

A third layer of STN7/STT7 regulation is based on the protein amount and stability. A true over-accumulation of STN7 kinase is difficult to obtain *in vivo* as reported for an STN7-OE line in Arabidopsis; in this case the limiting factor for kinase accumulation becomes the level of cytochrome b_6f complex (Wunder et al. 2013). The amount of STN7 protein can be regulated by protein degradation. This occurs, for instance, in conditions in which LHCII phosphorylation has to remain constantly very low, such as prolonged far-red light. This degradation depends on the de-phosphorylation of the kinase itself, so that the degradation of a constitutively phospho-mimic protein, generated by substitution of the target amino acids with aspartate residues, is hampered (Willig et al. 2011). In Chlamydomonas, STT7 is also degraded during prolonged conditions that promote St1 (Lemeille et al. 2009). The phosphorylation of STN7 correlates, at least partially, with the kinase activity suggesting that some sites may be subjected to an autophosphorylation (Trotta et al. 2016). However, not all phosphosites are modified along with STN7 activity; therefore, STN7 may also be a substrate of other kinases (see below).

The phosphatases that counteract STN7 and STT7

Further to regulation of the kinase, the level of phosphorylation of LHCII is also controlled by a counteracting phosphatase, or in Chlamydomonas by two phosphatases that act coordinately on the LHCII system (Cariti et al. 2020). In Arabidopsis, knock-out mutation of the counteracting phosphatase PPH1/TAP38 does not lead to an increase in LHCII phosphorylation under standard light condition (Longoni et al. 2015). This suggests that the amount of this phosphatase is not a major determinant of the LHCII phosphorylation level, or that the overlapping activity of the second phosphatase, PBCP, is sufficient to control the extent of phosphorylation (Longoni et al. 2019; Samol et al. 2012). However, loss of PPH1/TAP38 results in the persistent phosphorylation of the major LHCII even in St1 conditions, thus blocking the plants in “St2” (Pribil et al. 2010; Shapiguzov et al. 2010). A lower

amount of PPH1/TAP38 was reported in the *psaL* mutant of Arabidopsis, which is defective in the main docking site for LHCII on PSI. This mutant is characterized by a very high level of LHCII phosphorylation, suggesting that a specific regulation of phosphatase stability and protein amount may become relevant under extreme conditions (Rantala et al. 2016). So far, the commonly accepted working model assumes that the PPH1/TAP38 phosphatase activity is constitutive; however, to the best knowledge of the authors, there is little experimental evidence for this lack of regulation. This assumption is based on the observation that overexpression of PPH1/TAP38 in Arabidopsis leads to a constantly lower level of LHCII phosphorylation independently from the light condition (Pribil et al. 2010).

Different roles for the phosphorylation of LHCII subunits

In higher plants, three isoforms compose the trimeric ("major") LHCII: LHCB1, LHCB2 and LHCB3. These three isoforms have different relative abundances so that the LHCII trimers do not necessarily contain all the three isoforms but are composed of different combinations of these three proteins. The main phosphorylation of trimeric LHCII subunits occurs at a threonine located in the N-terminal portion of the mature protein. The peptide containing this threonine is highly conserved across plant species in LHCB2 while it is more variable for LHCB1, with certain LHCB1 isoforms having a variable number of aminoacids composing the N-terminal domain after the putatively phosphorylated threonine, while others lack the threonine entirely (Crepin and Caffarri, 2018). Finally, LHCB3 does not have a threonine close to the N-terminus of the mature protein so that the phospho-peptides of this isoform mostly detected in proteomic approaches in Arabidopsis belong to the signal peptide (Al-Momani et al. 2018; Grieco et al. 2012). However, LHCB3 has been found to be phosphorylated, at least at a low level, in the moss *Physcomitrella patens* suggesting that this isoform may have a direct regulatory role in certain species (Gerotto et al. 2019).

The strong evolutionary sequence conservation of the LHCB2 N-terminal peptide indicates that this phosphorylated protein has a specific functional role. In fact, it has been shown that the phosphorylated threonine of LHCB2 is a crucial component of the molecular recognition between the LHCII trimer and PSI (Pan et al. 2018). Consistently, biochemical studies have shown that LHCB2 present in the trimer strongly associated to PSI in the purified PSI-LHCI-LHCII complex is almost completely phosphorylated while, in contrast, LHCB1 phosphorylation is extremely low in this complex (Crepin and Caffarri, 2015; Leoni et al. 2013; Longoni et al. 2015). The phosphorylation of the LHCB2 isoform is favored relatively to the phosphorylation of the more abundant LHCB1 isoform in pea and Arabidopsis *in vivo*, resulting in a higher degree of phosphorylation (Larsson et al. 1987; Leoni et al. 2013; Longoni et al. 2015). As such, the phosphorylation status of LHCB2 is a major driver of "state transitions". However, in certain higher plants the scenario is more complex. In the monocots maize and barley, the LHCII antenna connected to PSI *in vivo*, measured by biophysical methods, shows on average more than 2 LHCII trimers associated with each PSI. These data, when compared with the level of LHCB2 phosphorylation, suggest that certain trimers can connect to PSI independently of the phosphorylation of LHCB2 (Chukhutsina et al. 2020).

Therefore, the phosphorylation of trimeric LHCII can lead to different physiological effects depending on which isoform is phosphorylated. An even more complex scenario prevails in Chlamydomonas; in this eukaryotic alga the trimeric LHCIIs is composed of eight different antenna isoforms, LHCBM1 to LHCBM9, encoded by nine genes with LHCBM2 and LHCBM7 sharing an identical amino acid sequence (reviewed by Crepin and Caffarri, 2018). In this organism, LHCBM1 (type IV) is important for thermal dissipation of excess energy (Elrad et al. 2002; Ferrante et al. 2012). Nevertheless, LHCBM1 has a clear difference in phosphorylation induced in St2 and dependent on the STT7 kinase (Cariti et al. 2020). LHCBM2/7 (type III) is required for state transition, but whether this isoform can be phosphorylated is not clear (Cariti et al., 2020; Iwai et al., 2008). In any case, LHCBM2/7 is apparently not phosphorylated when it is part of the PSI-LHCI-LHCII complex (Drop et al., 2014). LHCBM5 is required for efficient state transitions and is heavily phosphorylated in St2 (Pan *et al.*, 2021). The other trimeric LHCII isoforms are also phosphorylated in conditions inducing St2, as shown by the detection of a phosphorylated peptide corresponding to LHCBM3 (type I), as well as a phosphopeptide shared by LHCBM4/6/8 and 9 (type I) (Cariti et al. 2020; Drop et al. 2014; Lemeille et al. 2009, 2010). Furthermore, several of the LHCBM isoforms, as well as the monomeric isoforms LHCB4 and LHCB5, can undergo multiple phosphorylations, some of which are independent of STT7. Two phosphatases counteract the phosphorylation of LHCII in Chlamydomonas: the type I isoforms are mainly de-phosphorylated by CrPBCP, the type IV isoform by CrPPH1, and the type II isoform, as well the monomeric antennae, are putative substrates of both phosphatases.

In Chlamydomonas, high-resolution structures of the PSI-LHCI-LHCII complex show that the two trimers, LHCII-1 and LHCII-2, that are associated to PSI contain one LHCBM3/4 (type I) isoform, one LHBM2/7 (type III), and either LHCBM1 (trimer LHCII-1) or LHCBM5 (trimer LHCII-2) (Pan *et al.*, 2021). The position of LHCII-1 is similar to that of the single trimer that associates with PSI in plants. The phosphorylated threonine of LHCBM1 and the two preceding residues specifically interact with the PSAH and PSAL subunits of PSI, in a configuration that is highly similar to the binding of LHCB2 to PSI in plants (Huang *et al.*, 2021; Pan *et al.*, 2021). For LHCBM5 of LHCII-2, the phosphorylated threonine and the surrounding residues in the extended N-terminal domain interact with PSAH. Phosphorylated LHCBM1 is key to the binding of LHCII-1 to PSI, but it is replaced by LHCBM3 in the *Δlhcbm1* mutant, explaining why LHCBM1 is not essential for state transitions (Elrad *et al.*, 2002; Ferrante *et al.*, 2012; Pan *et al.*, 2021). The central role of phosphorylated LHCM5 for binding of LHCII-2 to PSI is consistent with the delayed state transitions observed in the *Δlhcbm5* mutant (Pan *et al.*, 2021).

LHCII is also composed of monomeric (“minor”) isoforms. These isoforms are found associated with the PSII-LHCII complexes and bridge the core subunits of the photosystem and the trimeric LHCII (reviewed by Rantala et al. 2020). In higher plants there are commonly three minor antennae: LHCB4 (CP29), LHCB5 (CP26) and LHCB6 (CP24). These antennae are also subject to phosphorylation. Amongst them LHCB4 (CP29) is the best investigated in terms of its phosphorylation (Bergantino et al. 1998) and is the minor antenna most commonly found to be phosphorylated in higher plants (Chen et al. 2013). Phosphorylation of this

antenna protein was first shown in *Zea mays* under cold stress (Bergantino et al. 1995). In Arabidopsis the phosphorylation level of these isoforms appears to be lower than that of the trimeric LHCII, at least in terms of threonine phosphorylation. However, in a recent report based on Phos-tag separation combined with mass spectrometry a high level of phosphorylation was reported for LHCB4 (CP29) (Nishioka et al. 2021). The phosphorylation status of LHCB4 correlates with the disassembly of the PSII-LHCII supercomplexes under high light or during senescence (see below) (Fristedt and Vener, 2011; Poudyal et al. 2020). In Chlamydomonas, phosphorylated LHCB4 is found in purified preparations of PSI-LHCI-LHCII complexes, and is thus part of the mobile antenna involved in state transitions (Drop et al. 2014; Takahashi et al. 2006, Takahashi et al. 2014).

Another minor antenna found to be phosphorylated is LHCB6 (CP24). Phosphorylation of this LHCII isoform has been reported to occur in maize under low light (Fristedt et al. 2012), and investigated in the lycophytes *Selaginella martensii* and *Lycopodium squarrosum* where, conversely, LHCB6 appears to be more phosphorylated upon exposure to high light (Ferroni et al. 2014). Interestingly, in *S. martensii* the phosphorylation of LHCB6 correlates with antenna association to PSI suggesting that it may play a specific role in acclimation to high light in the lycophytes, possibly favoring energy spillover towards PSI and thus generating an "extra qT" (Ferroni et al. 2018). In a recent investigation based on phos-tag gel electrophoresis, phosphorylated forms of both LHCB6 (CP24) and LHCB5 (CP26) were tentatively identified in Arabidopsis (Nishioka et al. 2021). In a mass-spectrometric comparison of protein phosphorylation at the end of the day with the end of the night, Lhcb6 was found to be more phosphorylated at the end of the day (Reiland et al. 2009). Further investigations are required to elucidate the role of minor antenna phosphorylation in acclimation.

In summary, the different isoforms composing LHCII play different roles in photosynthetic acclimation. Their amount and extent of phosphorylation, along with the sequence of amino acids surrounding the phosphorylation site, may be the features that define their specific roles. The minor antennae, although evolutionarily more conserved than the major trimeric subunits, show a high degree of functional diversification and differential regulation by phosphorylation among the photosynthetic organisms. This supports a key role of the "minor antenna" in allowing adaptation to different ecological niches.

PSII phosphorylation

In Arabidopsis, STN8 is the protein kinase mainly responsible for the phosphorylation of the PSII subunits PsbA (D1), PsbD (D2), PsbC (CP43) and PsbH (Bonardi et al. 2005; Reiland et al. 2011; Vainonen et al. 2005) while PBCP is the counter-acting protein phosphatase most involved in their de-phosphorylation (Samol et al. 2012). The phosphorylation of these PSII subunits is elevated under high light (HL), and decreases under far red (FR) (Rintamäki et al. 1997; Samol et al. 2012), but the molecular basis of this regulation is not fully understood. In Chlamydomonas, the homolog of STN8 is called STL1 (Depège et al. 2003), and the closest

homolog of PBCP is CrPBCP (Cre06.g257850) (Cariti et al. 2020). STL1 has to our knowledge not been characterized in any detail, and a paralog of CrPBCP, encoded close-by on chromosome VI (Cre06.g256300), also remains to be analyzed in depth (Cariti, 2019; Samol et al. 2012) (Cariti, PhD Thesis). In Arabidopsis, there is some overlap in the targets of the kinases STN7 and STN8, but the degree of phosphorylation of PSII subunits is mainly determined by the balance between the counteracting activities of the STN8 kinase and the PBCP phosphatase. Thus, an increase in the phosphorylation of PSII could reflect regulation by (i) activation of the STN8 kinase (ii) inactivation of the PBCP phosphatase (iii) or a combination of both.

Regulation of STN8

In a line over-expressing STN8, the phosphorylation levels of CP43 as well as D1 and/or D2 increases in low light compared to the wild type, and phosphorylation is further enhanced in HL (Wunder, Xu, et al. 2013). Phosphorylation is much lower after FR treatment, albeit still higher in the STN8 over-expressor than in the wild type. These observations suggest that the activity of STN8 and/or PBCP is regulated by HL and FR, but that this is not under the control of a strict feedback loop that would limit PSII phosphorylation when the response is excessive. This differs from STN7 overexpression, which does not result in LHCII over-phosphorylation in the light (Wunder, Liu, et al. 2013), presumably because of negative feedback on kinase activity, through oxidation of the PQ pool. In a triple mutant retaining only STN8 (*stn7/pph1/pbcp*), phosphorylation of PsbA/D1 is not changed under light conditions that favor either oxidation (FR) or reduction (blue light) of the PQ pool (Longoni et al. 2019). This observation suggests that the decreased PSII phosphorylation under FR is not mediated through a regulation of STN8 by the redox state of the PQ pool, in contrast to the regulation of STN7. The total amount of STN8 is not affected by treatment in HL (Wunder, Xu, et al. 2013), again at variance with STN7 whose abundance decreases under FR (Willig et al. 2011). Thus whether and how STN8 is regulated remains rather elusive at this time. Little is known on the regulation of STL1 in Chlamydomonas, although it was observed that phosphorylation of STL1 depends on STT7 in St2, under anaerobiosis or in high light (Bergner et al. 2015; Lemeille et al. 2010).

Regulation of PBCP

The activity of recombinant PBCP *in vitro* was initially reported to be moderately enhanced by the reducing agent dithiothreitol (Samol et al. 2012), suggesting a possible involvement of the thioredoxin pathway. However in tobacco transplastomic plants overexpressing the plastidic thioredoxins TRX-m or TRX-f , the phosphorylation of D1 (PsbA) and D2 (PsbD) was not significantly affected under moderate light, unlike the phosphorylation of LHCII which is strongly decreased in the TRX-m over-expressing lines (Ancín et al. 2019). The recombinant phosphatase is strongly inhibited *in vitro* by glutathione (GSH) plus H_2O_2, which leads to S-glutathionylation of Cys residues (Liu et al. 2019). In the atomic structure of PBCP from rice,

three Cys residues are positioned in proximity to the active site (Liu et al. 2019). Thus under high light, which causes an increased production of ROS, the activity of PBCP may be down-regulated by S-modification of Cys residues near its active site by GSH or other redox-active agents (Liu et al. 2019).

Thylakoid architecture

Several physiological functions, some of them partially overlapping, have been ascribed to STN8 and PBCP. One of the main ones is in the control of thylakoid architecture. In the *stn8* mutant, and the double mutant *stn7/stn8*, electron micrographs show grana domains of larger diameter (Fristedt et al. 2010), while the *pbcp* mutant has a reduced number of membrane layers in the grana stacks (Samol et al. 2012). Phosphorylation of PSII and of LHCII exposes negative charges on the stromal side of the thylakoid membrane. These charges engage in electrostatic interactions and, depending on the concentrations of monovalent and divalent cations, may change the balance of attractive and repulsive forces between the membranes across the stromal gap (Barber, 1982; Fristedt et al. 2010; Puthiyaveetil et al. 2017). Thus, changes in the phosphorylation of PSII and LHCII could influence grana stacking through electrostatic effects, in combination with other post-translational modifications such as N-terminal acetylation, proteolytic trimming, or lysine acetylation (Albanese et al. 2020; Koskela et al. 2018).

Members of the CURT1 protein family, which have membrane-bending properties *in vitro*, localize to the margins of grana and may thus contribute to the sharp bending that is required for tight stacking of the thylakoid membrane (Armbruster et al. 2013; Pribil et al. 2018). The organization of thylakoid grana is affected in *curt1* mutants, which have grana of increased diameter but with fewer layers. The most dramatic effect is in the quadruple mutant *curt1abcd* with its continuously appressed membranes that extend over the entire length of the chloroplast. Conversely, over-expressors of CURT1A exhibit grana of small diameter but with a high number of layers. The activity of CURT1 proteins correlates with their degree of oligomerization (Pribil et al. 2018). Interestingly, CURT1 family members are subject to STN8-dependent protein phosphorylation (Reiland et al. 2011; Trotta et al. 2019). How CURT1 phosphorylation would affect thylakoid architecture remains to be determined.

Repair of PSII after light-induced damage

Another proposed function of STN8 and PBCP is in the repair cycle of damaged PSII during photoinhibition. Upon exposure to excess light, the D1 subunit of PSII (PsbA) is a primary site of damage. The repair cycle involves migration of the damaged PSII complex from the grana to stroma-exposed domains of the thylakoid membrane where the complex is partly disassembled (reviewed by Järvi et al. 2015). The damaged D1 subunit is degraded and replaced by a newly synthesized one, allowing the re-assembly of a functional complex. The repair cycle is thought to involve a cycle of phosphorylation and de-phosphorylation (Koivuniemi et al. 1995), where phosphorylation of D1 may retard its degradation (Kato and

Sakamoto, 2014). Consistently, the phosphorylated form of D1 is enriched in stromal lamellae, where the repair of PSII is proposed to take place (Nishioka et al. 2021). Lack of PSII core phosphorylation, as in *stn8* mutants of Arabidopsis and rice, results in delayed repair of PSII. This may be due to increased grana diameter and stacking, hampering the lateral migration of PSII to the stromal lamellae (Nath et al. 2013; Tikkanen et al. 2008). Furthermore, PSII phosphorylation may facilitate its partial disassembly. A more elusive effect on repair was also reported in *pbcp* mutants (Puthiyaveetil et al. 2014). These requirements for both the kinase and the phosphatase are consistent with the proposed phosphorylation and de-phosphorylation steps in the repair of D1. FTSH is a thylakoid protease that plays a major role in the degradation of damaged D1. It is noteworthy that FTSH is phosphorylated, independently of STN7 or STN8, by a yet unknown protein kinase (Kato and Sakamoto, 2019).

Chlamydomonas *stl1* mutants have not yet been analyzed in detail, but the role of PSII phosphorylation was investigated in site-directed mutants of specific subunits. In the alga, the D1 subunit is not a major phosphoprotein, although its phosphorylation was detected by mass spectrometry (Delepelaire 1984, de Vitry 1991, Turkina 2006). D2 is phosphorylated at Thr2, but mutations of this residue to Ala or Gly (non-phosphorylatable) do not have phenotypic consequences on PSII activity, nor on photoinhibition and recovery (Andronis *et al.*, 1998; Fleischmann and Rochaix, 1999). PsbH can be phosphorylated at two of three sites, either Thr3 and Thr5, or Thr3 and Ser6 (Lemeille et al. 2010). Replacement of Thr3 with Ala has only a minor effect on PSII activity or repair (O'Connor *et al.*, 1998). A preliminary report indicates that the simultaneous substitution of both Thr3 and Thr5 to Ala may cause a delay in PSII repair after photoinhibition (Riché *et al.*, 2019). This effect appears more pronounced in a multiple mutant defective for phosphorylation of both PsbH and CP43 (PsbC). STT7 is not necessary for PsbH phosphorylation, while CrPBCP is involved in its dephosphorylation (Lemeille et al, 2010; Cariti et al. 2020). From these diverse data on Chlamydomonas, it tentatively appears that phosphorylation of multiple subunits may play a role in the PSII repair cycle.

Supercomplexes of PSII

In association with LHCII, PSII forms supercomplexes composed of a dimer of the photosystem core subunits associated with various complements of LHCII antenna proteins, depending on the light conditions (Albanese et al. 2016). The supercomplexes contain the "minor" monomeric antenna proteins LHCB4 (CP29), LHCB5 (CP26) and LHCB6 (CP24), as well as the "major" trimeric antenna proteins (LHCB1, LHCB2, LHCB3). PSII-LHCII complexes of different sizes can be resolved, containing a dimer of the core (C_2) and up to 2 trimers S (for strongly associated) and 2 trimers M (moderately associated) denoted as C_2S, C_2S_2, C_2S_2M or $C_2S_2M_2$ (Caffarri et al. 2009; Su et al. 2017; Wei et al. 2016). Several loosely associated (L) trimers are also connected to PSII. In Arabidopsis, while the large supercomplexes accumulate under light that favors PSI, they tend to dissociate under light

that favors PSII (Dietzel et al. 2011). This dissociation depended redundantly on the activities of STN8 and STN7, and correlates with the phosphorylation of CP43 (PsbC). The disassembly of PSII supercomplexes in high light is affected in an *stn8* mutant of rice (Nath et al. 2013) and in the double mutant *stn7/stn8* of Arabidopsis (Tikkanen et al. 2008). In Arabidopsis, disassembly of PSII supercomplexes is also correlated with the phosphorylation of CP29, which is under the control of STN7 (Fristedt and Vener, 2011). In the grana of plants exposed to blue light, phosphorylation of LHCB1 and LHCB2, controlled by STN7 and PPH1, is more extensive in the smaller PSII supercomplexes (Longoni et al. 2015). However when the supercomplexes are examined in the dark (St1) versus moderate light (St2), no dissociation is apparent, suggesting that release of M trimers is not necessary for state transitions, and supporting the role of L trimers in this process (Wientjes et al. 2013). In Chlamydomonas, conditions that promote State 1 favor the formation of supercomplexes and multimeric megacomplexes, while conditions that induce State 2 lead to the dissociation of the larger complexes (Iwai et al. 2008). In contrast, under high light the *stt7-1* mutant shows dissociation of PSII-LHCII complexes with concomitant over-phosphorylation of the PSII subunits D2 (PsbD) and CP43 (PsbC) (Bergner et al. 2015). From these disparate observations, a general picture emerges that the phosphorylation of LHCII, of PSII or of both may under certain environmental conditions favor the partial dissociation of the PSII supercomplexes and facilitate state transitions.

Further regulation of the photosynthetic electron transfer chain

Dissipation of excess energy

In rice (*Oryza sativa*), the kinase OsSTN8 and the phosphatase OsPBCP contribute to the response to excess light by controlling the phosphorylation of LHCB4 (CP29) (Betterle et al. 2015). In isolated chloroplasts, treatment with a kinase inhibitor that blocks phosphorylation of LHCB4 causes a decrease of energy-dependent quenching (the qE component of NPQ) (Betterle et al. 2015). Thus in monocots such as rice and maize, phosphorylation of antenna proteins seems to control not only state transitions, but also energy dissipation by qE. In Chlamydomonas, LHCSR3 is one of the major effectors of qE, and it is transcriptionally induced by high light through the PHOT blue-light receptor (Peers et al. 2009; Petroutsos et al. 2016). LHCSR3 is synergistically phosphorylated at several residues in its N-terminal part, under the control of STT7 (Bergner et al. 2015; Scholz et al. 2019). Intriguingly LHCSR3 phosphorylation in turn influences the phosphorylation of LHCB4.

Cyclic electron flow

The transition from cyclic electron flow (CEF) to linear electron flow (LEF) during a dark to light transition is influenced by STN8 in Arabidopsis. A large-scale proteomic survey identified PGRL1A, a protein implicated in CEF, as a target of STN8 (Reiland et al. 2011). In Chlamydomonas under anoxia, which induces CEF, PGRL1 is phosphorylated (albeit at a

different site) (Bergner et al. 2015). Unlike phosphorylation of PGRL1 under high light, phosphorylation of PGRL1 under anoxia does not require STT7, but whether it depends on STL1 is not known. PETO, a thylakoid protein also implicated in the regulation of CEF in the alga, is a major phosphoprotein (Hamel et al. 2000; Takahashi et al. 2016). The phosphorylation of PETO is controlled by STT7 and both CrPBCP and CrPPH1 (Bergner et al. 2015; Cariti et al. 2020). In Chlamydomonas, CAS is involved in the calcium-dependent regulation of CEF (Terashima et al. 2012). In Arabidopsis, phosphorylation of CAS is regulated by STN8 and also in a calcium-dependent manner (Stael et al. 2012; Vainonen et al. 2005). In the alga, conditions or mutations that increase the reducing status in the chloroplast enhance CEF (Cardol et al. 2009; Finazzi et al. 2002; Takahashi et al. 2016; Terashima et al. 2012). The disputed role of STT7 in this regulation could be to enlarge the antenna of PSI in St2 and thus enhance CEF, while another kinase (possibly STL1) could regulate CEF activity more directly in response to the redox status. In summary, boldly assuming some evolutionary conservation of CEF regulation in algae and plants, one could speculate that CEF is under control of both STN8/STL1 and a calcium-dependent pathway involving CAS, and that CEF is enhanced in St2 because the PSI antenna is larger, due to LHCII phosphorylation by STT7/STN7.

Other phosphoproteins and other kinases of the thylakoid membrane

Numerous other thylakoid phosphoproteins and part of their phosphosites have been identified by mass spectrometry (reviewed by Grieco et al. 2016). Some of them were shown to be phosphorylated to a significant extent (Nishioka et al. 2021), suggesting that the post-translational modification may be relevant to photosynthesis, and this inference is reinforced if the phosphosite is evolutionarily conserved (Grieco et al. 2016; Grouneva et al. 2013). However in most cases the physiological consequences of phosphorylation have not been elucidated. Several subunits of PSI and its LHCI antenna are phosphorylated, at phosphosites that are only partly evolutionarily conserved (Grieco et al. 2016). Amongst these proteins, it was shown that PSAE and LHCA4 are extensively phosphorylated, and this phosphorylation is retained in the double mutant *stn7/stn8* , implying that another kinase is involved (Nishioka et al. 2021). The phosphorylation of PSAN is calcium-dependent *in vitro*, illustrating a cross-talk between Ca^{2+} signalling and phosphorylation (Stael et al. 2012). Ferredoxin NADP Reductase (FNR1), which transfers electrons from Ferredoxin to NADP+, may also be phosphorylated, independently of STN7 and STN8 (Nishioka et al. 2021; Yang et al., 2013), although there is also evidence to the contrary (Lehtimäki *et al.*, 2014). Several subunits of ATP synthase are also strongly phosphorylated (Nishioka et al. 2021), and in the case of AtpB and AtpC, one of the phosphosites is conserved between dicots and Chlamydomonas (Grieco et al. 2016). In Arabidopsis, the phosphorylation of AtpB is higher at the end of the night than at the end of the day (Reiland et al. 2009). AtpB is a potential substrate of pCKII (Kanekatsu et al. 1998), and its phosphorylation (as well as that of several other ATPsynthase subunits) is independent of STN7 and STN8 (Nishioka et al. 2021).

The comparison of sequences surrounding the phosphosites of Arabidopsis chloroplast proteins identified an acidic motif typical of casein kinase substrates (Reiland et al. 2009). The chloroplast kinase pCKII is involved in phosphorylation of the transcription machinery and of RNA-binding proteins (Lisitsky and Schuster, 1995). As mentioned above, AtpB is a putative substrate of pCKII, and one of the phosphosites in STN7 matches its recognition motif (Reiland et al. 2009).

Studies on stripe rust resistance in wheat led to the discovery of another kinase localized at the thylakoid membrane: YR36/WKS1. This kinase, which has no obvious homolog in Arabidopsis, phosphorylates PSBO, an extrinsic subunit of PSII. The phosphorylation of PSBO weakens its association to the PSII core, thus regulating its activity and photosynthetic ROS production (Wang et al. 2019). Yet another family of three thylakoid-associated kinases (TAK 1-3) was discovered in Arabidopsis (Snyders and Kohorn, 1999). TAK1 is active on LHCII proteins *in vitro*, however the physiological role of the TAKs as not been elucidated yet (Snyders and Kohorn, 2001).

It is worth mentioning that other kinases appear to be also part of the acclimatory network involving protein phosphorylation. Recent advances show an interaction between the atypical kinases associated with the plastoglobules (ABC1Ks) and the regulation of photosynthetic electron transport (Lundquist *et al.*, 2013; Martinis *et al.*, 2014). Plastoglobules are lipid droplets, contiguous to the thylakoid membrane, containing a variety of lipidic compounds and in particular a pool of non-photoactive PQ (Austin et al. 2006). Noteworthy, it was reported that ABC1K1 activity is important to maintain the homeostasis of the photoactive pool of PQ and thus its mutation indirectly affects the phosphorylation of thylakoid proteins (Pralon et al. 2019; 2020). Furthermore, PGL35, which is an abundant structural component of plastoglobules, shows extensive phosphorylation (Nishioka et al. 2021).

Concluding remarks

Because STN7 and STN8 have many protein targets other than subunits of the PSII core (Bergner et al. 2015; Reiland et al. 2009; Schönberg et al. 2017), it is difficult to discriminate the physiological functions of the kinases. Any phenotype of *stn8* mutants will obviously correlate with a loss of PSII phosphorylation, but the primary cause of the phenotype could in fact lie - at least in part - with other targets of the kinase. For example, effects of the loss of STN8 or PBCP on thylakoid architecture may be due to the changes in PSII phosphorylation, but could also involve phosphorylation of the CURT1 protein family. Likewise for photoinhibition caused by excess light, defects in the repair of PSII may relate to the cycle of phosphorylation and dephosphorylation of the D1 subunit (PsbA), but could also be the result of altered accessibility of the repair machinery to damaged PSII, due to changes in thylakoid architecture (Fristedt et al. 2012). Furthermore, when phosphorylation of PSII is investigated, the focus is usually on CP43, D1 and D2, but the status of PsbH is

often neglected even though this small protein is prominently phosphorylated. This may be because PsbH is not resolved with the acrylamide concentrations usually used for SDS-PAGE. Mutants of STN7 show changes in the expression of nuclear genes, which imply retrograde signalling from the chloroplast to the nucleus. This could reflect a direct role of the kinase in long-term responses, through the phosphorylation of unidentified targets. However there is also a possibility that it is the altered redox state of the chloroplast in the mutant which is sensed and relayed to the nucleus (Fey et al. 2005; Pesaresi et al. 2009; Tikkanen et al. 2012).

Thylakoid protein phosphorylation has long been considered in bulk, with broad classes such as LHCII phosphorylation or PSII phosphorylation. However recent developments have shown that phosphorylation of specific subunits or phosphosites can have different roles. A prime example is the specific molecular recognition of P-LHCB2 and the exclusion of P-LHCB1 in the formation of the PSI-LHCI-LHCII complex in St2 (Crepin and Caffarri, 2015; Longoni et al. 2015; Pan et al. 2018). Bulk phosphorylation may be relevant for electrostatic interactions that are crucial for example to thylakoid stacking, but specific molecular recognition of phosphorylation may be more important than previously recognized (Allen and Forsberg, 2001). Classical genetics with mutants defective in the protein kinases and phosphatases, which all have numerous substrates, have favored the in-bulk perspective on phosphorylation. Targeted mutagenesis of chloroplast or nuclear genes (now enhanced by Crispr-CAS technology) offers opportunities to shed more light on the specific function of individual phosphoproteins and phosphosites (Scholz et al. 2019).

A striking feature of the regulation of photosynthesis by protein phosphorylation is that the key kinases and phosphatases have been conserved through evolution of photosynthetic eukaryotes. However with the evolution of multicellularity, the conquest of land and the adaptation of the organisms to different environments, many interesting differences have arisen in the targets of phosphorylation, or in the substrate specificities of the enzymes (Grouneva et al. 2013). The most obvious example is the central role of LHCII phosphorylation in the regulation of light harvesting (state transitions) which has been conserved, while the divergent evolution of the LHCB and LHCBM families (in plants and green algae respectively) came with differences in the phosphosites that are mobilized in this regulation (Crepin and Caffarri, 2018; Grieco et al. 2016). Likewise, the main phosphatase involved in this regulation is PPH1/TAP38 in plants, while both CrPPH1 and CrPBCP intervene in Chlamydomonas. As the panel of organisms that are investigated enlarges, this “eco-evo-physio” perspective on thylakoid protein phosphorylation will gain in scope and relevance (Betterle et al. 2015; Ferroni et al. 2014; Gerotto et al. 2019; Grouneva et al. 2013).

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Table 1

Method	Principle	Pros and cons	Quantifcation	References
***In vitro* isotope labelling**	Incubation with radioactive ATP (γ-^{32}P-ATP or γ-^{33}P-ATP).	Applicable to isolated chloroplasts or thylakoids. Detects phosphorylation activity during the incubation, not pre-existing phosphorylation.	Relative	(Bennett, J. 1977)
***In vivo* isotope labelling**	Incubation with radioactive phosphate (^{32}P or ^{33}P).	Applicable to algal cells, detached leaves. Detects phosphorylation activity during the incubation, not pre-existing phosphorylation.	Relative	(Owens and Ohad, 1982)
ProQ Diamond	Staining of electrophoresis gel with fluorescent dye.	Can be combined with total protein staining. Some cross-reactions to non-phosphorylated proteins.	Relative	(Steinberg et al. 2003)
Anti-P-Thr antibodies	Imunoblotting after gel electrophoresis.	Batch-to-batch variability. Unequal detection depending on protein environment of P-Thr. Do not detect P-Ser.	Relative	(Rintamäki et al. 1997)
Phospho-specific antibodies (P-D1; P-Lhcb1; P-Lhcb2)	Imunoblotting after gel electrophoresis	Specific to protein of interest.	Relative	(Leoni et al. 2013)
Phos-tag gel electrophoresis	Phos-tag crosslinked to acrylamide matrix retards migration of P-proteins during gel electrophoresis	Detection of multiply phosphorylated forms of target proteins. Not all proteins readily amenable to separation. Phos-tag hinders blotting to membrane.	Absolute	(Crepin and Caffarri, 2015; Kato and Sakamoto, 2019; Kinoshita et al. 2015; Longoni et al. 2015)
Mass-spectrometry	Identification of P-peptides by MS-MS	Identification of P-sites.	Relative or absolute	(Bergner et al. 2015; Reiland et al. 2011; Vener, Alexander V. et al. 2001)

Table 1. The table summarizes the major methods used to analyze thylakoid protein phosphorylation. Abbreviations: P-proteins, phosphoproteins; P-peptides, phosphopeptides; P-sites, phosphosites. In the 4th column, “Relative” qualifies methods that allow relative comparisons of phosphorylation in different conditions, while “Absolute” refers to methods that allow determining the absolute extent of phosphorylation of a protein.

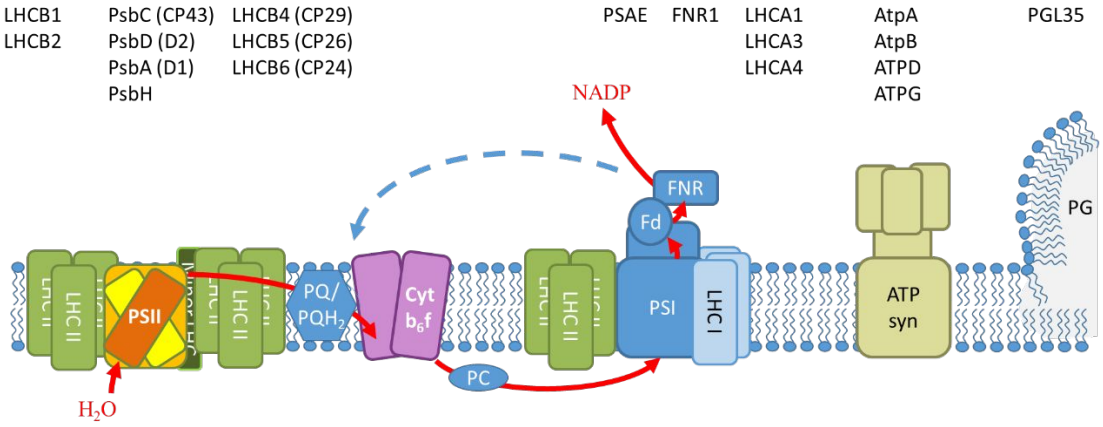
Table 2. Thylakoid protein kinases and phosphatases

Kinases		Main known targets[a]	
Arabidopsis	**Chlamydomonas**	**Arabidopsis**	**Chlamydomonas**
STN7 (AT1G68830)	STT7 (Cre02.g120250)	Major LHCII subunits: LHCB1, LHCB2	LHCBM1 (type IV) LHCBM3/4/6/8/9 (type I) LHCBM5 (type II) LHCB4, LHCB5
STN8 (AT5G01920)	STL1[b] (Cre12.g483650)	PSII core: PsbA, PsbD, PsbC, PsbH	Unknown
pCKII (AT2G23070)		Transcription machinery and RNA-binding proteins, AtpB	
Phosphatases		**Arabidopsis**	**Chlamydomonas**
Arabidopsis	**Chlamydomonas**		
PPH1/TAP38 (AT4G27800)	CrPPH1 (Cre04.g218150)	Major LHCII subunits: LHCB1, LHCB2	LHCBM1 (type IV), LHCBM5 (type II) PETO
PBCP (AT2G30170)	CrPBCP (Cre06.g257850)	PSII core: PsbA, PsbD, PsbH	LHCBM3/4/6/8 (type I) LHCBM5 (type II) PsbH PETO
-	PBCP-LIKE[c] (Cre06.g256300)	-	Unknown

a) The targets reported here are the most obvious and abundant proteins missing in knock-out lines. Further targets (not shown) were identified by phospho-proteomic approaches.
b) STL1 is the closest homolog to STN8, but has not yet been characterized.
c) Cariti 2019

For details and references, please refer to the main text.

Figure 1.



Major phosphoproteins of the thylakoid membrane in higher plants.

In the linear mode of electron flow (LEF), PSII and PSI work in series to energize the chain using the energy of photons. The light harvesting antenna LHCI is associated with PSI, while LHCII can transfer energy to either photosystem, under the regulation of state transitions. Electrons (red arrow) are extracted from water at PSII, transferred to the membrane-soluble carrier plastoquinone (PQ) which is reduced to PQH2 with the uptake of two protons from the stroma. PQH2 shuttles to the cytochrome *b*$_6$*f* complex where it is oxidized with the release of protons to the thylakoid lumen. In the Q-cycle at the *b*$_6$*f* complex, half of the PQ molecules are re-reduced, allowing a net enhancement of proton transfer from the stroma to the thylakoid lumen. Electrons are transferred from the *b*$_6$*f* complex to a soluble carrier protein in the lumen, usually plastocyanin (PC) but in some cases a cytochrome. They are then transferred to PSI where light energy allows the reduction of ferredoxin (Fd). The electrons from reduced ferredoxin (Fd) can be used by Ferredoxin NADP Reductase (FNR) for the reduction of NADP to NADPH, or directly in other metabolic pathways. The proton gradient across the thylakoid membrane drives ATP synthase (ATP syn). In the cyclic mode of electron flow (CEF), electrons from PSI return to the *b*$_6$*f* complex, so that ATP is produced but no net reducing power.

The abundant and extensively phosphorylated proteins of higher plant thylakoid membranes and plastoglobules (PG) are listed above the complex to which they belong. Their acronyms follow the recommended General Guidelines that distinguish between chloroplast-encoded proteins, with only first and last capital letters (*e.g.* PsbA), and nuclear encoded proteins, all in capital letters (*e.g.* LHCB1).

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LHCB1
LHCB2

PsbC (CP43)
PsbD (D2)
PsbA (D1)
PsbH

LHCB4 (CP29)
LHCB5 (CP26)
LHCB6 (CP24)

PSAE FNR1

LHCA1
LHCA3
LHCA4

AtpA
AtpB
ATPD
ATPG

PGL35