

Very rapid phosphorylation kinetics suggest a unique role for Lhcb2 during state transitions in Arabidopsis

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Received 31 October 2012; revised 28 June 2013; accepted 9 July 2013; published online 25 July 2013.

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SUMMARY

Light-harvesting complex II (LHCII) contains three highly homologous chlorophyll-*a/b*-binding proteins (Lhcb1, Lhcb2 and Lhcb3), which can be assembled into both homo- and heterotrimers. Lhcb1 and Lhcb2 are reversibly phosphorylated by the action of STN7 kinase and PPH1/TAP38 phosphatase in the so-called state-transition process. We have developed antibodies that are specific for the phosphorylated forms of Lhcb1 and Lhcb2. We found that Lhcb2 is more rapidly phosphorylated than Lhcb1: 10 sec of 'state 2 light' results in Lhcb2 phosphorylation to 30% of the maximum level. Phosphorylated and non-phosphorylated forms of the proteins showed no difference in electrophoretic mobility and dephosphorylation kinetics did not differ between the two proteins. In state 2, most of the phosphorylated forms of Lhcb1 and Lhcb2 were present in super- and mega-complexes that comprised both photosystem (PS)I and PSII, and the state 2-specific PSI-LHCII complex was highly enriched in the phosphorylated forms of Lhcb2. Our results imply distinct and specific roles for Lhcb1 and Lhcb2 in the regulation of photosynthetic light harvesting.

Keywords: Arabidopsis, Lhcb2, LHCII, phosphorylation, state transitions.

INTRODUCTION

Protein phosphorylation regulates many important biological processes in plants. One of the first plant processes found to be regulated by protein phosphorylation was the so-called 'state transition'. State transitions, which were first described by Bonaventura and Myers (1969) and Murata and Sugahara (1969), and subsequently by, for example, Bennett (1977) and Allen (1992), alleviate the imbalance in the photosynthetic machinery during unequal excitation of two photosystems, PSI and PSII. This imbalance is sensed by changes in the redox state of the plastoquinone (PQ) pool that activate a specific kinase, which phosphorylates an amino acid close to the N-termini of the Lhcb1 and Lhcb2 polypeptides of LHCII (light-harvesting complex of PSII; see (Jansson, 1994) for review). As a result, part of LHCII becomes functionally detached from PSII and acts as an external antenna of PSI. The name 'state transition' originates from the technique used to

detect the changes; the fluorescence properties of intact leaves or algal cells can be reversibly shifted from 'state 1' to 'state 2' simply by changing the spectrum of incident light. The kinetics of the process are typically in the order of minutes. Ten minutes are often long enough to reach a steady state of fluorescence and the process follows first-order kinetics (Islam, 1987; Larsson *et al.*, 1987). In higher plants, only some of the Lhcb proteins, ca. 20–25%, are thought to participate in state transitions (Vener, 2007). Although the two photosystems have different excitation spectra – this is the reason why changes in light quality can induce excitation imbalance – the vast majority of photons is absorbed equally well by the two photosystems. Nevertheless, photons with wavelengths >680 nm are preferentially used by PSI, although recently it has been suggested that PSII electron transfer can also be driven by far-red light of wavelengths up to 800 nm (Thapper *et al.*,

2009). State transitions are important for plants that live under a canopy, where the majority of the visible light has already been absorbed by the photosynthetic apparatus of other leaves, but where a large proportion of the photons with wavelengths around 700 nm are transmitted. In addition to changes in light quality, variations in light quantity can also provoke reversible LHCII protein phosphorylation (Rintamaki *et al.*, 2000), and it is likely that, under natural conditions, state transitions may be more important for acclimation of the photosynthetic apparatus to fluctuations in light quantity than for adjustments to changes in light quality (Tikkanen and Aro, 2011; Tikkanen *et al.*, 2012).

In the last few years, the molecular details of state transitions have been elucidated. A crucial breakthrough was the identification of a kinase responsible for the phosphorylation of LHCII, first in *Chlamydomonas* (where it is named Stt7; Depege *et al.*, 2003) and later in Arabidopsis, in which it is named STN7 (Bellafiore *et al.*, 2005). Subsequent studies, using for example mutants that lacked STN7, have produced much important information regarding the function and regulation of the kinase. A homologous kinase, STN8, primarily phosphorylates PSII core subunits (Bonardi *et al.*, 2005; Vainonen *et al.*, 2005). PPH1/TAP38 phosphatase, which catalyzes the reverse reaction, has also been identified (Pribil *et al.*, 2010; Shapiguzov *et al.*, 2010). The elucidation of the three-dimensional structures of different supercomplexes of PSI and PSII with LHCII has also been important for a better understanding of the phosphorylation and subsequent lateral migration of LHCII in the membrane. There is, however, one area in which progress has been limited: the importance of substrate specificity in the regulatory phosphorylation of Lhcb proteins. The major LHCII complex, which consists of the highly homologous Lhcb1, Lhcb2 and Lhcb3 gene products, has long been known to occur as trimers in the thylakoid membrane, although monomeric forms may also exist. Lhcb1 is typically found in large excess compared with the other subunits, so Lhcb1₃ trimers are abundant; most of the Lhcb2 and Lhcb3 subunits are probably in Lhcb1₂/Lhcb2 and Lhcb1₂/Lhcb3 trimers but Lhcb1/Lhcb2₂ trimers are also found (Standfuss and Kuhlbrandt, 2004), and there are also reports of Lhcb1/Lhcb2/Lhcb3 heterotrimers (Caffarri *et al.*, 2004; Jackowski, 2004). It is conceivable that additional kinds of LHCII trimers exist and that the relative abundance of each trimer is a simple consequence of the relative stoichiometries of the Lhcb1, Lhcb2 and Lhcb3 polypeptides. Different LHCII subpopulations have different phosphorylation properties (Bassi *et al.*, 1988a,b). It should be noted that Lhcb1 and Lhcb2 are also phosphorylated at other sites, but this phosphorylation does not seem to be catalyzed by STN7 or STN8 (Engelsson and Vener, 2012).

Lhcb3 differs from Lhcb1 and Lhcb2 as it lacks the 'state-transition phosphorylation site', a Thr (or, in some

species, a Ser) residue very close to the N-terminus of the protein. Lhcb1₂/Lhcb3 trimers appear also to have a specific binding pocket on PSII (Damkjaer *et al.*, 2009) and not to participate in state transitions. In Arabidopsis that lack Lhcb3, the binding pocket is instead occupied by trimers that lack Lhcb3, and state transitions in such plants are slightly faster than in wild-type plants, presumably because there are more substrates available for the STN7 kinase, as all remaining LHCII trimer subunits have three potential phosphorylation sites. However, to date, it has not been possible to investigate any putative differences in the function between the Lhcb1 and Lhcb2 subunits using molecular genetics. The Lhcb1 and Lhcb2 gene products are extremely similar; only 14 amino acids consistently distinguish them from each other (Jansson and Gustafsson, 1990). Yet both protein sequences have been conserved for over 300 million years (MY) and they are thus likely to possess specific functions (Jansson and Gustafsson, 1990). Arabidopsis Lhcb1 is encoded by five genes and Lhcb2 by three genes. Some of these genes lie in close proximity on the chromosomes and it has not been possible to generate quintuple Lhcb1 or triple Lhcb2 knockout plants, only antisense plants that lack both Lhcb1 and Lhcb2 (Andersson *et al.*, 2003; Ruban *et al.*, 2003).

So far, all quantitative studies on LHCII phosphorylation have been performed using either radioactively labelled phosphate, or antibodies against phosphothreonine (P-Thr) that are unable to distinguish between phosphorylated forms of Lhcb1 and Lhcb2, hereafter named P-Lhcb1 and P-Lhcb2, at least not in Arabidopsis in which Lhcb1 and Lhcb2 have the same electrophoretic mobility. Moreover, the antibodies also naturally react with the other ca. 25 thylakoid proteins that can be phosphorylated (Aro *et al.*, 2004; Pesaresi *et al.*, 2009). In addition, different brands – even different batches – of P-Thr antibodies show significant differences in specificity towards the different thylakoid phosphoproteins (see e.g. Martinsuo *et al.*, 2003; Engelsson and Vener, 2012). The ProQ phospho-staining method combined with the Sypro total protein stain has also been used to quantify thylakoid protein phosphorylation (e.g. Tikkanen *et al.*, 2006; Fristedt *et al.*, 2010), but the difficulty in separating the Lhcb1 and Lhcb2 proteins remains a problem. Mass spectrometry methods, on the other hand, have limited quantitative power (Vainonen *et al.*, 2005). Overall, carrying out quantitative studies on the localization of the P-Lhcb1 and P-Lhcb2 in different supermolecular complexes and thylakoid domains is not a trivial undertaking.

To overcome these limitations we have generated antibodies that specifically recognize Arabidopsis P-Lhcb1 or P-Lhcb2 and describe their use for investigating LHCII phosphorylation kinetics and dynamics in wild-type and mutant plants.

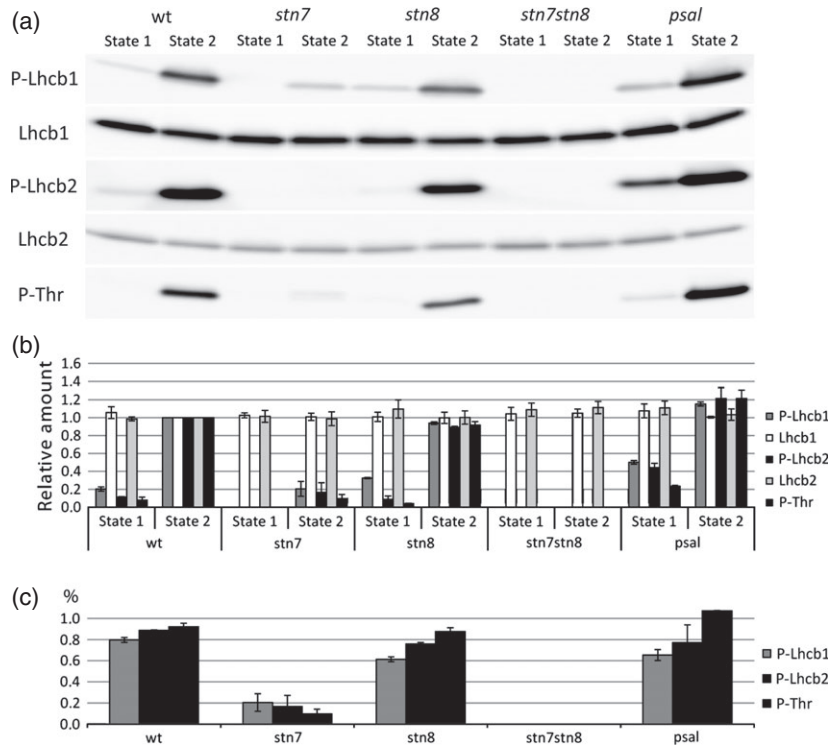


Figure 2. Specificity of P-Lhcb1 and P-Lhcb2 antibodies.

Arabidopsis wild-type (wt), *stn7* (SALK_073254) and *psal* (SALK_010554), *stn8* (SALK_060869), *stn7stn8* plants were subjected to state 1 and state 2 light conditions, and then proteins were extracted and immunoblotted with antibodies specific against phosphorylated and non-phosphorylated Lhcb1 and Lhcb2 forms.

(a) Immunoblots. The specificity of antibodies is indicated on the left side.

(b) Densitometric analysis of immunoblots. Intensity of signals has been normalized respect to the value in wild-type state 2.

(c) Difference in phosphorylation between state 1 and state 2.

Lhcb2 showed very rapid phosphorylation kinetics

To study the phosphorylation kinetics of Lhcb1 and Lhcb2, wild-type plants were first treated for 60 min with red light, supplemented with far-red light, to induce state 1 and then exposed to red light alone for different time intervals, to bring plants into state 2. The kinetics of phosphorylation was very different for the two proteins: Lhcb2 was more rapidly phosphorylated than Lhcb1 (Figure 4a). When expressed on a scale where 0% corresponds to the phosphorylation level in state 1 and 100% is the maximum phosphorylation level in state 2 of the wild-type (measured after 60 min when the reaction has reached steady state), within 10 sec after the light shift, Lhcb2 phosphorylation reached about 30% of the maximum level and after 10 min it was approximately 85% of the maximum. In contrast, Lhcb1 was phosphorylated to only 15% of the maximum level after 30 sec, and to only slightly more than 60% after 10 min. Again, P-Thr antibody was used for confirmation. In this case, two bands in the Lhcb1/2 region were recognized by the P-Thr antibody. The phosphorylation kinetics of both Lhcb1 and Lhcb2 during the transition from state 1 to state 2 were dependent only on the substrate specificity of the LHCII kinase; the kinetics in the KO mutant that lacked the phosphatase *pph1/tap38* (SALK_025713C, (Pribil

et al., 2010; Shapiguzov *et al.*, 2010)) were similar to that described in wild-type plants (ca. 60–80% phosphorylation after 10 min). We also compared phosphorylation/dephosphorylation kinetics when state transitions were induced by shifts from dark to low light and *vice versa*. Although the changes in phosphorylation levels were less pronounced (Figure S2) Lhcb2 phosphorylation appeared also under these conditions to have faster kinetics.

In order to study dephosphorylation kinetics, plants were treated with red light for 40 min to induce state 2 and then exposed to additional far-red light for different time intervals to induce dephosphorylation and return the plants to state 1. The dephosphorylation of both Lhcb1 and Lhcb2 was much slower than phosphorylation; after 10 min only 10–20% of both Lhcb1 and Lhcb2 was dephosphorylated (Figure 4b).

Lhcb1 and Lhcb2 phosphorylation kinetics were influenced by PSII antenna protein composition

We then studied the phosphorylation and dephosphorylation kinetics of Lhcb1 and Lhcb2 proteins in mutant and transgenic lines with modified PSII antenna composition. To this end, we compared wild-type Arabidopsis with knockout mutants that lacked Lhcb3 (Damkjaer *et al.*,

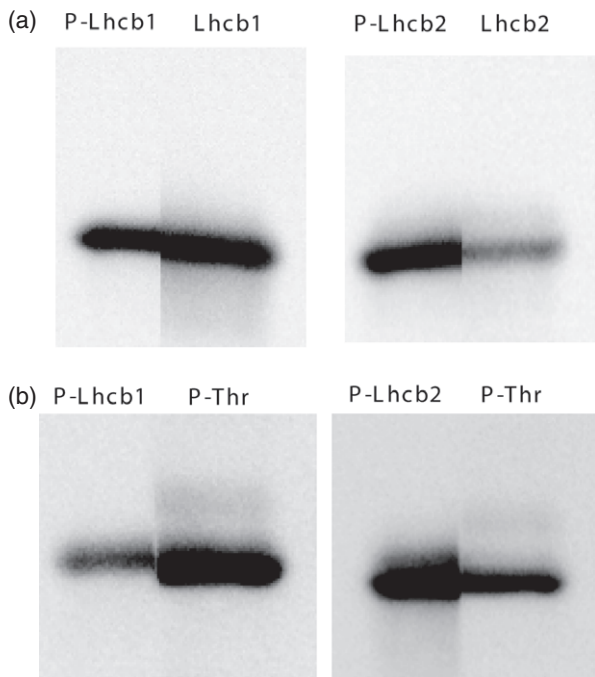


Figure 3. Comparison of gel mobilities of Lhcb1 and Lhcb2 proteins. (a) Immunoblot analysis with P-Lhcb1/Lhcb1 and P-Lhcb2/Lhcb2 antibodies obtained by cutting the membrane through the middle of the electrophoretic lane. (b) Immunoblot analysis of P-Lhcb1/P-Thr antibodies and P-Lhcb2/P-Thr antibodies.

2009), Lhcb5 (SALK_014869C) or Lhcb6 (SALK_035761C, (Kovacs *et al.*, 2006)) and the *npq4-1* mutant that lacked PsbS (Li *et al.*, 2000). We also included a Lhcb2 antisense line (*asLhcb2-12*) that has been shown to be devoid of both Lhcb1 and Lhcb2 proteins (Andersson *et al.*, 2003). However, some of the plants generated from seeds harvested from the *asLhcb2-12* did not express such a pale phenotype as we expected. When several *asLhcb2-12* plants were

analysed for chlorophyll, Lhcb1 and Lhcb2 levels (Figure S1), there was a significant variation in the amount of, in particular, Lhcb1 between the individual plants, and this correlated with the chlorophyll content. Apparently, although the *asLhcb2* construct consistently repressed the accumulation of Lhcb2 proteins, repression of Lhcb1 was variable. Only those *asLhcb2-12* plants with a pale phenotype, as described before (Andersson *et al.*, 2003) showed strong suppression of Lhcb1 and only these were used for further experiments.

Most mutants did not show significant differences from wild-type in the phosphorylation levels of either Lhcb1 or Lhcb2 after either 30 sec, 10 min or 60 min (Figure 5a,b). Similar results were also obtained using P-Thr antibodies (Figure 5c). We have previously shown that LHCII phosphorylation is more rapid in *koLhcb3* mutants (Damkjaer *et al.*, 2009). However, in order to pick up this subtle difference, more time points, and possibly more replicates, would be needed. The aim of this experiment was to carry out a broader screen to see whether absence of any antenna protein had a particularly large effect on the phosphorylation kinetics. The *koLhcb6* and *npq4* lines showed kinetics similar to those of wild-type plants, a finding that indicated that the absence of CP24 and PsbS did not significantly influence the rate of phosphorylation and, hence, state transitions. Phosphorylation of Lhcb1 was faster in *koLhcb5* mutants, which showed significantly higher Lhcb1 phosphorylation after 30 sec than WT, and reached maximum phosphorylation within 10 min. This situation may indicate that the lack of CP26 either increases activation of the kinase or allows for better access to the substrate (LHCII).

The residual amount of Lhcb1 in *asLhcb2* plants had a lower phosphorylation level after 30 sec compared with that in wild-type, but after 10 min no difference was recorded (Figure 5a).

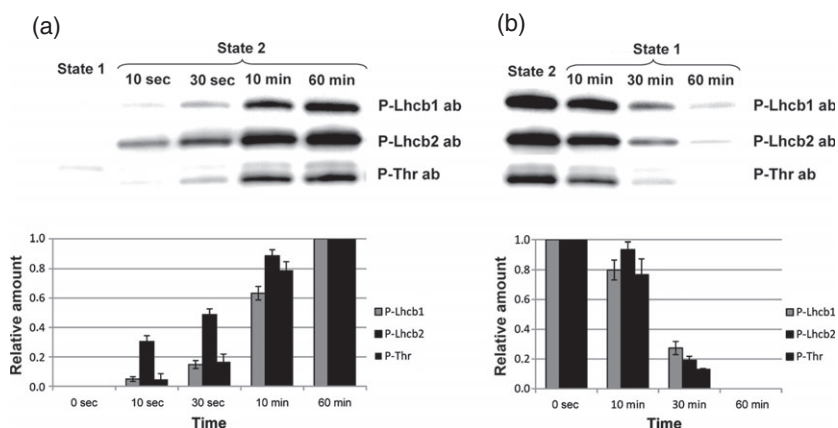


Figure 4. Phosphorylation (a) and dephosphorylation (b) kinetics of Arabidopsis Lhcb1 and Lhcb2 during state transitions, analysed using P-Lhcb1 and P-Lhcb2 antibodies. Immunoblot analysis of phosphorylated proteins during State 1 and State 2 are shown at the top, while the lower graphs show quantification of the immunoblot data.

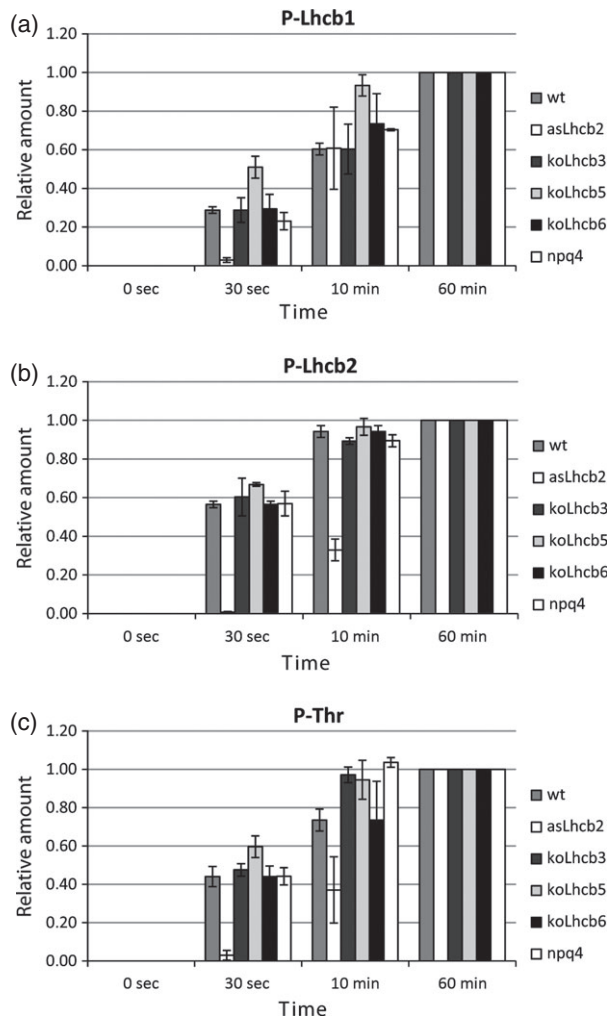


Figure 5. Phosphorylation kinetics for wild-type (wt) and *asLhcb2*, *koLhcb3*, *koLhcb5*, *koLhcb6* and *npq4-1* mutants with altered PSII antenna composition. Quantification of immunoblot data for samples incubated with P-Lhcb1 (a), P-Lhcb2 (b) and P-Thr (c) antibodies. Values for wild-type (wt) have not been included, and letters A, B, and C are not present in the figure.

P-Lhcb1 and P-Lhcb2 are located in both LHCII trimers and large complexes

In order to study the distribution within supermolecular complexes of P-Lhcb1 and P-Lhcb2 in state 1 and 2, we subjected wild-type and *psal* thylakoids to large pore Blue Native (IpBN) electrophoresis and subsequent immunoblotting. Before thylakoid preparation, the plants were subjected for 60 min to far-red light or red light conditions in order to induce state 1 and 2, respectively. We used digitonin to solubilize the thylakoid membrane. Digitonin is a mild detergent and is known to preferably solubilize the stroma-exposed lamellae and grana margins, allowing preservation of weak protein–protein interactions (Järvi *et al.*, 2011). This method thus allows for better visualiza-

tion of, for example, less abundant state 2-specific complexes such as the LHCII–PSI complexes (Zhang and Scheller, 2004; Galka *et al.*, 2012) and the PSI–PSII–LHCII mega-complexes (Järvi *et al.*, 2011), which are found in these parts of the thylakoid membranes. To separate these high molecular mass complexes, the membranes solubilized with 1% digitonin were subjected to IpBN electrophoresis (Figure 6). The polypeptide composition of each complex was determined by 2D-electrophoresis (Figure S3).

Most of both the phosphorylated and the non-phosphorylated forms of the Lhcb1 and Lhcb2 proteins were found in the high molecular mass mega-complexes. However, free LHCII trimers also contained P-Lhcb1 and P-Lhcb2. In state 1, Lhcb1 and Lhcb2 phosphorylation was very low. Only in *psal* mutants some phosphorylation was observed in large complexes. In state 1, the PSI–LHCII complexes were, as expected, absent. Induction of state 2 by turning off the far-red light resulted in an increase in the PSI–LHCII complex. Interestingly, this complex (absent in *psal* mutants, as described by Pesaresi *et al.* (2009)) was highly enriched in P-Lhcb2 but virtually lacked P-Lhcb1.

A yet undescribed complex, denoted ‘LHCII complex’ in Figure 6, and that migrated between *cytb₆f* and LHCII trimer, contained only P-Lhcb1, not P-Lhcb2. Protein complexes from *psal* plants in state 2 were hyperphosphorylated (Figures 2 and 6) as observed by Lunde *et al.* (2000). The overall amount of super- and mega-complexes was higher in state 2; this is visible both in the stained gels and in the immunoblots using antibodies against Lhcb1 and Lhcb2 (Figure S3). As digitonin preferentially solubilizes stroma-exposed thylakoids and grana margins, this result shows that these complexes accumulate in these areas of the chloroplast in state 2, or alternatively that grana stacks in state 2 are easier to solubilize. The majority of P-Lhcb1 or P-Lhcb2 was associated with mega-complexes. Moreover, it seemed that P-Lhcb1 was more enriched than P-Lhcb2 in these mega-complexes.

DISCUSSION

Although the primary photosynthetic process is very similar in all plants, including green algae, there are significant differences in the way it is regulated. Different proteins have been employed to regulate the so-called qE quenching: PsbS is used in higher plants (Li *et al.*, 2000), L1818/LHCSR in *Chlamydomonas* (Peers *et al.*, 2009) and diatoms (Depauw *et al.*, 2012), while both systems are operational in the moss *Physcomitrella patens* (Alboresi *et al.*, 2010). The kinases that regulate state transitions are conserved between *Chlamydomonas* and Arabidopsis (Depege *et al.*, 2003; Bellafiore *et al.*, 2005), but their substrate polypeptides differ; the split between the genes coding for Lhcb1, Lhcb2 and Lhcb3 polypeptides occurred after the radiation of green algae and higher plants so there are no direct counterparts of Lhcb1, Lhcb2 and Lhcb3 in green

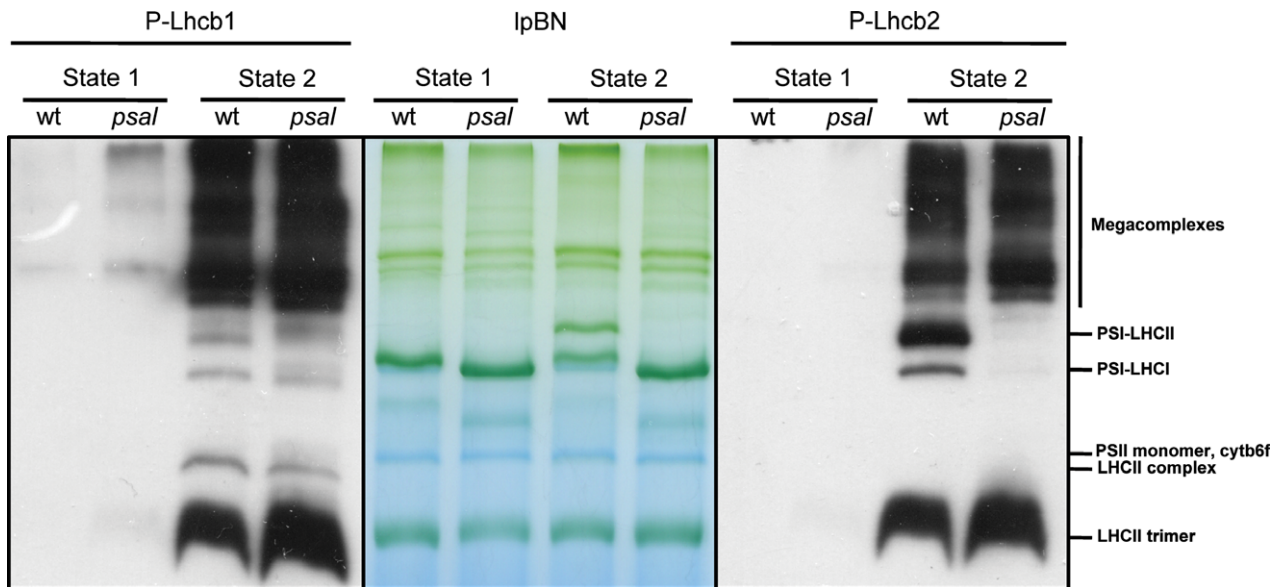


Figure 6. Immunolocalization of phosphorylated and non-phosphorylated forms of Lhcb1 and Lhcb2 in thylakoid membrane complexes of Arabidopsis wild-type and *psal* knockout (KO) mutant.

Thylakoids from plants in state 1 and state 2 conditions were separated by large pore Blue Native (IpBN) gel electrophoresis. From left: Immunoblot with P-Lhcb1 antibody; IpBN; immunoblot with P-Lhcb2 antibody.

algae (Elrad and Grossman, 2004). Therefore, results from *Chlamydomonas* (Ferrante *et al.*, 2012) cannot be directly used to inform about the situation in higher plants. Lhcb3 does not carry a phosphorylation site and therefore probably does not play a direct role in state transitions, but as plants respond to the lack of Lhcb3 by increasing the amount of (phosphorylatable) Lhcb1 and Lhcb2 subunits, Lhcb3 KO mutants have slightly faster state transitions (Damkjaer *et al.*, 2009).

We have been able to generate antibodies specific for the phosphorylated forms of Lhcb1 and Lhcb2, which we believe will be important tools for gaining a better understanding of the biochemistry of state transitions and, in the longer term, of the true physiological role of LHCII phosphorylation in natural environments. By using these antibodies, we have obtained information that sheds new light on the state-transition process in Arabidopsis. Our key findings are: (i) Lhcb2 is more rapidly phosphorylated than Lhcb1 after a shift from 'state 1 light' to 'state 2 light'; within only 10 sec, Lhcb2 phosphorylation reached ca 1/3 of the maximum level, (ii) STN8 shows, at least in *stn7* mutants, some activity towards both Lhcb1 and Lhcb2, (iii) there is no difference in gel mobility between the phosphorylated and non-phosphorylated forms of the proteins, (iv) there is no difference in the kinetics of dephosphorylation between Lhcb1 and Lhcb2, in either the presence or the absence of the PPH1/TAP38 phosphatase, in other words the PPH1/TAP38 phosphatase is not selective, (v) in neither case is phosphorylation of the protein strongly dependent on the presence of Lhcb3, Lhcb5, Lhcb6 or PsbS, (vi) in plants with very little Lhcb1 – and no Lhcb2 – Lhcb1

phosphorylation is slower, (vii) some, but by no means all, P-Lhcb1 and P-Lhcb2 associate with PSI complexes, (viii) the PSI-LHCII complex formed in state 2 is highly enriched in the phosphorylated forms of Lhcb2, (ix) a large proportion of the Lhcb1 and P-Lhcb1 proteins is associated with mega-complexes comprising both PSI and PSII, and finally (x) we visualized a new LHCII complex enriched in P-Lhcb1.

Interestingly, early studies using isolated spinach chloroplasts or thylakoids identified the so-called 25 kDa peptide (later found to correspond to Lhcb2, Jansson *et al.*, 1990) as having three times faster kinetics of phosphorylation than the so-called 27 kDa peptide (Ljungberg *et al.*, 1986), which was later found to be encoded by Lhcb1 genes (Jansson *et al.*, 1990). At this time the third LHCII protein, Lhcb3, had not yet been discovered, which complicates interpretation of the early data, but it appears that in both Arabidopsis and spinach Lhcb2 has more rapid phosphorylation kinetics than Lhcb1. To induce state transitions we mainly employed light conditions that cause a rapid and drastic shift in light quality as an effective mean of studying the underlying mechanistic details of the state-transition process. Our results suggest that the STN7 kinase is localized in close proximity to its substrate. Whether or not it is the structure of the substrate itself, i.e. the difference in the primary sequences of Lhcb1 and Lhcb2, that makes the Lhcb2 protein a better substrate for STN7, or whether Lhcb2 has a location in the thylakoid that makes it more accessible to the kinase, is a question that cannot yet be answered.

It has been suggested that 20–25% of LHCII is engaged in state transitions in higher plants (Allen, 1992) while in *Chlamydomonas* up to 80% is engaged (Delosme *et al.*, 1996). There are very few data on absolute phosphorylation levels of LHCII in plants, but a few reports (e. g. Islam, 1987) have estimated that about one-third of LHC could be phosphorylated. It is evident that not all P-Lhcb1 and P-Lhcb2 are associated with PSI under state 2, as revealed by our IpBN gel electrophoresis, with much of the P-Lhcb1 and P-Lhcb2 also being found in the LHCII trimer fraction. It is of course possible that most of the phosphorylated LHCII trimers are functionally attached to PSI *in vivo*, but dissociated during solubilization. Alternatively, the amount of phosphorylated LHCII may not be directly correlated with the extent of state transition, as suggested by studies of *Arabidopsis* plants that lacked Lhcb3 (Damkjaer *et al.*, 2009) and analyses of purified PSI–LHCII complexes isolated with digitonin (Zhang and Scheller, 2004). P-Lhcb2 was enriched in the PSI–LHCII complex that appears rapidly after a shift to state 2 light, suggesting that Lhcb2 may have a major role in the initial stage of state transitions; interestingly, the non-phosphorylated form of Lhcb2 was also enriched in that complex (Figure S3). This finding is in accordance with earlier observations on the purified PSI–LHCII complex, which also contained non-phosphorylated LHCII (Zhang and Scheller, 2004). It should also be mentioned that we saw no evidence for any phosphorylation-induced monomerisation of LHCII trimers. Moreover, the distribution of the Lhcb2 protein responded to the state transition, as more Lhcb2 was found in the PSI–LHCII supercomplexes in state 2 as compared with state 1. This situation is consistent with a remodelling of the PSII–LHCII supercomplexes in the context of state transitions, as recently documented by Dietzel *et al.* (2011). It must, however, be borne in mind that our solubilization protocol has, like other methods, a bias for different thylakoid compartments. The thylakoid membrane is remodelled during state transitions; during state 1 membranes are tightly connected forming grana stacks, while upon shift to state 2 membranes undergo unstacking (Chuartzman *et al.*, 2008). This solubilization bias complicates comparisons of data generated by different methods, but we believe that the P-Lhcb1 and P-Lhcb2 antibodies will become useful tools for analysing the composition of LHCII using different techniques.

As mentioned above, if the Lhcb1.4 gene product, which lacks the STN7-phosphorylatable Thr residue, is instead phosphorylated at the Ser residue close to its N-terminus we would not be able to quantify it with our antibodies, but phosphoproteomics have failed to detect phosphorylation at this residue (Ingelsson and Vener, 2012). Even if Lhcb1.4 were phosphorylated by STN7, we think it is highly unlikely that this could affect our conclusions, as Lhcb1.4 is not the major Lhcb1 gene product in *Arabidopsis* (Jansson, 1999). Lhcb1.4 is phosphorylated (Wang

et al., 2012), but apparently only at the Ser48 residue – marked with an asterisk in the sequence GPSGS*PWYGSDR (Ingelsson and Vener, 2012) where the other four *Arabidopsis* Lhcb1 gene products are also phosphorylated. To our knowledge, there are no data linking the STN7- and STN8-independent phosphorylations of this residue (Ingelsson and Vener, 2012) to state transitions, so we believe that Lhcb1.4 has no direct role in state transitions. It should be pointed out that some *Lhcb1* gene products from other species may also lack the phosphorylatable Thr residue. However, there is no subgroup among *Lhcb1* gene sequences that would suggest the existence of two functionally distinct types of Lhcb1, one phosphorylated and participating in state transitions and one not, so it appears that it is not necessary for plants with multiple *Lhcb1* gene copies to have all of their products subject to phosphorylation by STN7.

In this work, we have mainly used illumination of intact plants with red/far-red light to induce state transitions in order to reveal the mechanistic consequences of differential phosphorylation kinetics of the Lhcb1 and Lhcb2 proteins. This approach does not mean that we regard LHCII phosphorylation as a way of regulating PSI/PSII imbalances in excitation due to fluctuations in light intensity as being less important, merely that light quality fluctuations have certain experimental advantages. However, after a light intensity shift, Lhcb2 seemed also to have more rapid phosphorylation kinetics.

We have made no attempt to quantify exactly the differences in relative stoichiometries of Lhcb1, P-Lhcb1, Lhcb2 and P-Lhcb2 in the different super- and mega-complexes of PSI and/or PSII, but we have provided indications that most, if not all, of the large complexes that could be separated by IpBN-PAGE contain phosphorylated LHCII subunits. This situation indicates that the mega-complexes are prone to dynamically regulated reversible LHCII phosphorylation, and are thus functionally involved in the regulation of photosynthesis. Our antibodies provide a useful tool for studying protein complexes isolated under different conditions in order to better understand the relationships between state transitions and the variety of different thylakoid membrane super- and mega-complexes.

To conclude, we have shown that in *Arabidopsis* the two major LHCII proteins Lhcb1 and Lhcb2 differ substantially in the kinetics of STN7-dependent phosphorylation. The implication of this finding is that the two proteins probably have unique roles in the state-transition process, despite their very high degree of sequence conservation.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) and the mutant lines *asLhcb2-12* (Andersson *et al.*, 2003), *koLhcb3* (SALK_02314C)

(Damkjaer *et al.*, 2009), *koLhcb5* (SALK_014869C), *koLhcb6* (SALK_035761C) (Kovacs *et al.*, 2006), *npq4-1* (SALK_095156), *stn7* (SALK_073254) (Bellafiore *et al.*, 2005), *stn8* (SALK_060869) (Vainonen *et al.*, 2005), *stn7stn8* (Bonardi *et al.*, 2005), and *psal* (SALK_010554) were grown for 6 weeks under 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (or, for lpBN, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$) light irradiance and an 8 h light (23°C)/16 h dark (18°C) photoperiod. The humidity was maintained at 75%.

State-transition induction

To induce state 1 and state 2 artificially, Arabidopsis plants were treated with a customized light-emitting diode (LED) Light Source (SL 3500-R-D) from Photon System Instruments using the Light Studio program. To trigger phosphorylation, plants were subjected for 60 min to 100% far-red light supplemented with 15% red light to develop state 1, and then the far-red light was switched off for another 60 min to induce a shift to state 2. The reverse light regime was used to study dephosphorylation kinetics. Leaves for thylakoid isolation were sampled at different time intervals. To induce state 1 and state 2 by changes in light intensity wild-type plants were transferred from the growth light into either the dark (D) or low light (LL) to induce state 1 and state 2 respectively, and then shifted to LL or D for phosphorylation and dephosphorylation induction. Leaves samples were collected at the same time intervals as for LED lights treatment.

Thylakoid extraction and immunoblot analysis

Thylakoids were extracted as previously described by Järvi *et al.* (2011). For the (de)phosphorylation assays, 6-week-old leaves were homogenized together with ice-cold grinding medium [0.45 M sorbitol, 20 mM tricine pH 8.4, 10 mM EDTA, 10 mM NaHCO₃, 0.1% (w/v) BSA] and, after filtration through two layers of muslin, centrifuged at 4000 *g* for 10 min. The pellet was resuspended in 15 ml of hypotonic buffer (20 mM tricine pH 7.8, 5 mM MgCl₂), followed by the addition of 15 ml of osmoticum medium (0.66 M sorbitol, 20 mM tricine pH 7.8, 5 mM MgCl₂), and re-centrifuged for 10 min at 4000 *g*. The pelleted thylakoid proteins were then resuspended in resuspension buffer (0.33 M sorbitol, 20 mM tricine pH 7.8, 5 mM MgCl₂). All the buffers contained 10 mM NaF to inhibit the activity of phosphatases and maintain the *in vivo* phosphorylation state (Bellafiore *et al.*, 2005). All the stages in thylakoid protein preparation were performed on ice or in a cold room (4°C). Protein extracts were used for chlorophyll quantification according to Porra *et al.* (1989).

To generate antibodies specific for phosphorylated Lhcb1 and Lhcb2, synthetic peptides with the sequences RKT*VAKPKGP and RRT*VKSTPQS, where T* indicates phospho-Thr, were synthesized by standard Fmoc peptide synthesis by Innovagen (Lund, Sweden) and injected into rabbits. For immunopurification, 5–6 mg of each peptide was coupled to 2 ml Ultralink iodoacetyl gel (Pierce). Antibodies from 20 ml of serum was first immunopurified with the phosphopeptides used for immunization bound to the gel essentially according to the Vendors manual. After elution, a second step was performed where instead the Ultralink with corresponding non-phosphorylated peptide coupled was used, to remove all antibodies recognizing the non-phosphorylated form. The flowthrough from this step consisted of the purified antibodies. Immunoblotting analysis was performed, using 1 μg of thylakoid proteins, essentially as described by Ganeteg *et al.* (2001), with slight modifications according to the instructions

provided by the antibody manufacturer. Chemiluminescence was detected after 1 and 5 min using a LAS-3000 cooled CCD camera. Images were recorded, processed and quantified according to Damkjaer *et al.* (2009).

Three biological replicates for each state-transition condition and genotype were used for detection of Lhcb1 and Lhcb2 phosphorylated proteins. Statistical analysis was performed using the R application package and Student's *t*-test and data normality test (Ihaka and Gentleman, 1996).

Large pore Blue Native (lpBN) gel electrophoresis and 2-D immunoblot analysis

Samples were prepared and run as described previously (Sirpiö *et al.*, 2011). Thylakoid membranes were resuspended in ice-cold 25BTH20G buffer [25 mM BisTris-HCl, pH 7.0, 20% (v/v) glycerol and 0.25 mg ml⁻¹ Pefabloc] with the addition of 10 mM NaF, to a chlorophyll concentration of 1 $\mu\text{g} \mu\text{l}^{-1}$, and an equal volume of digitonin solution was added to give a final concentration of 1%. Samples were solubilized for 8 min in darkness with gentle mixing. Insoluble material was removed by centrifugation at 18000 *g* at 4°C for 20 min. 1/10 volume of Serva Blue G buffer (100 mM BisTris-HCl, pH 7.0, 0.5 M amino-n-caproic acid, 30% sucrose and 50 mg ml⁻¹ Serva Blue G) was added to each sample prior to loading to introduce a negative charge and to increase the solubility of the sample. lpBN gels were run as described in Sirpiö *et al.* (2011) and Järvi *et al.* (2011).

For immunoblotting, the whole lpBN gels were electroblotted on to a polyvinylidene fluoride (PVDF) membrane (Millipore, Watford Herts, UK). For two-dimensional analyses, the gel strips from the lpBN were excised and incubated with gentle shaking in Laemmli buffer [138 mM Tris-HCl, pH 6.8, 6 M urea, 22.2% (v/v) glycerol, 4.3% (w/v) sodium dodecyl sulphate (SDS), 5% (v/v) 2-mercaptoethanol] for 1 h. Thereafter, the gel strips were transferred to the top of an SDS-PAGE gel [15% (w/v) polyacrylamide, 6 M urea] and sealed with 0.5% agarose in SDS-PAGE running buffer [25 mM Tris base, 190 mM glycine, 0.1% (w/v) SDS]. After electrophoretic separation, the 2D gels were stained with silver as described in Blum *et al.* (1987).

ACKNOWLEDGEMENTS

This work was supported by the European Union project FP7-PEOPLE-ITN-2008 'HARVEST: Control of Light Use Efficiency in Plants and Algae From Light to Harvest', the Swedish Research Council (VR), the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas), the Academy of Finland (project Nos 118637 and 138703) and C. Leoni by a 1-year fellowship from the University of Bari (Rectoral Decree No. 1598). The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Immunoblot analysis of Lhcb1 and Lhcb2 in individual *aslhcb2* plants.

Figure S2. Phosphorylation (a) and dephosphorylation (b) kinetics of Arabidopsis Lhcb1 and Lhcb2 during state transitions in induced by dark to LL shift (and back) analyzed using P-Lhcb1 and P-Lhcb2 antibodies.

Figure S3. Polypeptide composition of thylakoid protein complexes from leaves in state 1 and state 2 separated by large pore Blue Native (lpBN) gel electrophoresis (top).

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