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Abstract

Reversible protein phosphorylation plays a major role in the acclimation of the photosynthetic apparatus to changes in light. Two paralogous kinases phosphorylate subsets of thylakoid membrane proteins. STATE TRANSITION7 (STN7) phosphorylates LHCII, the light-harvesting antenna of photosystem II (PSII), to balance the activity of the two photosystems through state transitions. STN8, which is mainly involved in phosphorylation of PSII core subunits, influences folding of the thylakoid membranes and repair of PSII after photodamage. The rapid reversibility of these acclimatory responses requires the action of protein phosphatases. In a reverse genetic screen, we identified the chloroplast PP2C phosphatase, PHOTOSYSTEM II CORE PHOSPHATASE (PBCP), which is required for efficient dephosphorylation of PSII proteins. Its targets, identified by immunoblotting and mass spectrometry, largely coincide with those of the kinase STN8. The recombinant phosphatase is active in vitro on a synthetic substrate or on isolated thylakoids. Thylakoid folding is affected in the absence of PBCP, while its overexpression alters the kinetics of [...]
Identification of a photosystem II phosphatase involved in light acclimation in Arabidopsis

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Synopsis:

Thylakoid protein phosphorylation plays a central role in the dynamic acclimation of the photosynthetic electron transfer chain to changing light conditions. This work identifies a protein phosphatase that is required for de-phosphorylation of photosystem II subunits, and counteracts the activity of the protein kinase STN8.

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ABSTRACT

Reversible protein phosphorylation plays a major role in the acclimation of the photosynthetic apparatus to changes in light. Two paralogous kinases phosphorylate subsets of thylakoid membrane proteins. STN7 phosphorylates LHCII, the light harvesting antenna of photosystem II (PSII), to balance the activity of the two photosystems through state transitions. STN8, which is mainly involved in phosphorylation of PSII core subunits, influences folding of the thylakoid membranes and repair of PSII after photo-damage. The rapid reversibility of these acclimatory responses requires the action of protein phosphatases. In a reverse genetic screen we have identified the chloroplast PP2C phosphatase, PBCP (PHOTOSYSTEM II CORE PHOSPHATASE), which is required for efficient de-phosphorylation of PSII proteins. Its targets, identified by immunoblotting and mass spectrometry, largely coincide with those of the kinase STN8. The recombinant phosphatase is active in vitro on a synthetic substrate or on isolated thylakoids. Thylakoid folding is affected in the absence of PBCP, while its over-expression alters the kinetics of state transitions. PBCP and STN8 form an antagonistic kinase and phosphatase pair whose substrate specificity and physiological functions are distinct from those of STN7 and the counteracting phosphatase PPH1 (TAP38), but their activities may overlap to some degree.
INTRODUCTION

The primary reactions of plant and algal photosynthesis occur in the thylakoid membranes of chloroplasts. These membranes harbor large multimeric pigment-protein complexes called photosystems that convert light energy into chemical energy with high efficiency, a process that ultimately fuels most of the biosphere. In the linear mode of electron transfer through the photosynthetic chain, photosystem II (PSII) and photosystem I (PSI) are connected in series through plastoquinone, the cytochrome b₆f complex and plastocyanin (Eberhard et al., 2008). The activities of PSII and PSI must be balanced for efficient electron transfer, which requires an appropriate redox poise of the intermediate electron carriers. Under low light, the activity of the two photosystems is tuned through a mechanism called state transitions (Allen, 1992; Wollman, 2001; Aro and Ohad, 2003; Rochaix, 2007; Lemeille and Rochaix, 2010; Minagawa, 2011). A mobile part of the LHCII light-harvesting antenna is dynamically allocated to PSII or PSI depending on the redox state of the plastoquinone (PQ) pool. Binding of reduced plastoquinol to the Qₒ site of the b₆f complex activates the thylakoid kinase STN7, which phosphorylates components of the LHCII antenna and causes their dissociation from PSII and at least in part their association with PSI (state 2) (Vener et al., 1997; Zito et al., 1999; Bellafiore et al., 2005; Bonardi et al., 2005; Minagawa, 2011; Tikkanen and Aro, 2012). This process is reversible – when the PQ pool is oxidized, dephosphorylation by the recently identified PPH1/TAP38 phosphatase (Pribil et al., 2010; Shapiguzov et al., 2010) results in the association of the LHCII components with PSII (state 1). In the green alga Chlamydomonas reinhardtii, a large fraction of the antenna is implicated in state transitions, which are controlled by the protein kinase Stt7, the ortholog of STN7 (Depege et al., 2003). In the alga, transition to state 2 induces cyclic electron flow around PSI and favors the production of ATP (Finazzi et al., 2002).

The thylakoid membranes of plant chloroplasts form a continuous network of flattened membrane vesicles organized into regular stacks with many tightly appressed layers, called grana, interconnected by non-appressed stromal lamellae (Dekker and Boekema,
The two photosystems are unevenly distributed in the thylakoid membranes. PSII is confined to grana, while PSI is enriched in stromal lamellae as well as in the margins and ends of grana stacks. State transitions involve changes in thylakoid membrane stacking that become strikingly apparent when de-enveloped chloroplasts are examined under the electron microscope (Chuartzman et al., 2008). In vivo, phosphorylation of thylakoid proteins such as the core subunits of PSII by the kinase STN8, a paralog of STN7, favors changes in membrane folding (Vainonen et al., 2005; Fristedt et al., 2009a). In vitro, stacking is strongly promoted by increasing cation concentrations, and this can counteract the effect of thylakoid protein phosphorylation (Barber, 1980, 1982; Fristedt et al., 2010).

PSII has the remarkable ability to extract electrons from water using energy from light. Photo-oxidation of the reaction center chlorophyll (P680+) creates one of the strongest oxidants in biology. This powerful photochemistry can cause collateral damage to the PSII complex itself, particularly to its D1 subunit (Edelman and Mattoo, 2008). Moreover, molecular oxygen can react with light-activated chlorophyll molecules or reduced cofactors of the electron transfer chain to produce highly reactive oxygen species, which can be damaging to the photosynthetic machinery and to other components of the chloroplast. Thus photosynthetic organisms, which are subjected to large fluctuations in their light environment, must continuously adapt to maximize photosynthetic activity under low light and to minimize photo-oxidative damage under excess light (Eberhard et al., 2008; Li et al., 2009). Photo-oxidative damage occurs at all light intensities, but when the rate of damage exceeds the capacity for repair, photoinhibition is manifested as a decrease in photosynthetic activity and in the proportion of active PSII reaction centers (Edelman and Mattoo, 2008). Under conditions when the absorbed light excitation energy exceeds the capacity of the photosynthetic electron transfer chain or the metabolic demands of the cell, a number of different mechanisms intervene to down-regulate light harvesting and alleviate photo-oxidative damage. Alternative routes of electron flow, which counteract the excessive reduction of electron carriers, include cyclic electron flow around PSI, the chlororespiratory pathway involving plastid terminal oxidase, as well as the Mehler reaction and the water-water cycle (Eberhard et al., 2008). Non-photochemical
quenching (NPQ) is induced in the light harvesting antenna of PSII (LHCII) to dissipate excess captured light energy as heat (Li et al., 2009). The induction of NPQ limits the reduction of the PQ pool by decreasing the activity of PSII. Thus in high light NPQ takes over a major role instead of state transitions in maintaining the redox poise of the electron transfer chain (Tikkanen et al., 2011; Tikkanen and Aro, 2012). Whereas phosphorylation of LHCII by the STN7 kinase is inhibited under high light, most likely through the thioredoxin pathway (Rintamaki et al., 2000; Martinsuo et al., 2003), phosphorylation of the PSII core subunits is enhanced (Tikkanen et al., 2008; Tikkanen et al., 2010).

The D1 subunit of PSII is the primary target of photo-oxidative damage, and an active cycle of repair functions to selectively replace the damaged subunit. Because PSII is mainly localized in the tightly appressed membranes of the thylakoid grana, the repair cycle requires its migration to membrane domains that are exposed to the stroma, allowing the selective degradation of the damaged D1 subunit and the synthesis, maturation and assembly of a new subunit (Baena-Gonzalez and Aro, 2002; Takahashi and Murata, 2008). This process is facilitated by the protein kinase STN8 (Tikkanen et al., 2008), which shows specificity towards proteins of the PSII core (Bonardi et al., 2005; Vainonen et al., 2005). The action of the STN8 kinase favors unfolding of thylakoid membranes and the ability of the degradation and repair machinery to access damaged D1 (Fristedt et al., 2009a).

The kinase STN7 and the phosphatase PPH1/TAP38 form a pair with opposing effects on phosphorylation of the LHCII antenna, an antagonism that allows the reversible regulation of state transitions to maintain the redox poise of the electron transfer chain. Similarly, it is expected that chloroplast phosphatase(s) will counterbalance the activity of the STN8 kinase. Here we identify a protein phosphatase that is required for efficient de-phosphorylation of several core subunits of PSII in vivo, and show that the purified recombinant protein is active in vitro. Mutants lacking this phosphatase show changes in the folding of thylakoid membranes. In the mutants state transitions are not affected, but they are altered in transgenic lines over-expressing the phosphatase.
RESULTS
Efficient de-phosphorylation of the PSII core requires the protein phosphatase PBCP

We designed a genetic screen for chloroplast phosphatases involved in de-phosphorylation of thylakoid proteins in Arabidopsis (Shapiguzov et al., 2010). In brief, we first searched genomic databases for putative catalytic or regulatory subunits of protein phosphatases, and then selected those that were predicted to be targeted to the chloroplast (Suba II (Heazlewood et al., 2007)). They were ranked by the strength of these predictions and the degree of correlated co-expression of their mRNAs with those of the protein kinases STN7 and STN8 in the Genevestigator microarray database (Zimmermann et al., 2005). Homozygous mutants for these predicted phosphatases were obtained from the SALK collection (Alonso and Ecker, 2006). Seedlings were systematically screened for defective de-phosphorylation of thylakoid proteins after a shift from white to far-red light, using a streamlined immunoblot assay with anti-phosphothreonine antibodies (Rintamaki et al., 1997). The PPH1/TAP38 phosphatase previously identified in this screen is required for efficient de-phosphorylation of LHCII antenna proteins and transition from state 2 to state 1 (Pribil et al., 2010; Shapiguzov et al., 2010).

Using the same screen we have now identified a different phosphatase that is required for efficient de-phosphorylation of PSII core subunits. In moderate white light many thylakoid proteins are phosphorylated by the kinases STN7 and STN8, including components of the light harvesting complex LHCII and of PSII, as well as other thylakoid proteins such as TSP9 and CaS (Vener, 2007; Vainonen et al., 2008; Fristedt et al., 2009b; Reiland et al., 2011). Upon exposure to far-red light, which favors PSI excitation, the plastoquinone pool is oxidized, a transition to state 1 is induced, and several thylakoid proteins are de-phosphorylated. In the wild type, strong de-phosphorylation of the core subunits of PSII and of the LHCII antenna is apparent within 20 minutes in immunoblots with anti-phosphothreonine antibodies (from Zymed, Fig 1A). In contrast, phosphorylation of the PSII subunits CP43, D2 and D1 is partly retained even after 40 minutes in the mutant affecting PBCP (photosystem II core
phosphatase, At2g30170), while the de-phosphorylation of the LHCII antenna proceeds as efficiently as in the wild type. It is interesting to contrast the specificity of the pbcp-1 phenotype with that of pph1-3, where de-phosphorylation of the PSII subunits proceeds normally while the LHCII antenna remains phosphorylated (Fig 1A). Thus the two phosphatases seem to have complementary roles: PBCP is primarily required for efficient de-phosphorylation of the PSII core, while PPH1/TAP38 is required for efficient de-phosphorylation of the LHCII antenna.

Mass-spectrometric analysis of PBCP-dependent thylakoid protein de-phosphorylation

To measure the phosphorylation of thylakoid proteins specifically and quantitatively, we used two methods based on high performance liquid chromatography coupled to electrospray ionization mass spectrometry (LC-MS). Thylakoid membranes were isolated from the leaves of mutant and wild-type plants that had been exposed to blue light or far-red light for 30 minutes. The surface-exposed peptides were then released by extensive proteolytic shaving with trypsin. In the first method of analysis, the peptide samples from the wild type and the mutant were differentially labeled by esterification of carboxylic groups with hydrogen- or deuterium-containing methanol respectively (Vener et al., 2001; Ficarro et al., 2002; Shapiguzov et al., 2010). A 1:1 mixture of these two preparations was subjected to IMAC (immobilized metal ion affinity chromatography) to retain the phosphorylated peptide methyl esters. These were then subjected to LC-MS, which allowed simultaneous measurements of light- and heavy-isotope labeled phosphopeptide pairs and thus the determination of the ratio of phosphorylation of specific peptides in the mutant and the wild type (Table 1). In the second method of analysis, the surface-exposed peptides released by trypsin were directly analyzed by high resolution LC-MS. Both the phosphorylated and de-phosphorylated forms of peptides from the core subunits of PSII were identified and quantified as described previously (Fristedt et al., 2010). The values for phosphorylation of individual peptides were corrected for the difference in the signal intensities of the phosphorylated and de-phosphorylated forms, and the percentage of phosphorylation of each peptide was calculated (Table 2).
Strikingly, after exposure to far-red light the ratio of phosphorylation of the D1 and D2 polypeptides of PSII in the pbcp-1 mutant versus the wild type was approximately two (Table 1), in agreement with the results of immunoblotting. In the wild type, the percentage of phosphorylation of D1 and D2 was two-fold lower after far-red light treatment compared to blue light (Table 2). In contrast, in the pbcp-1 mutant phosphorylation decreased only slightly compared to the decrease in the wild type. We also noted that in blue light D1 and D2 were phosphorylated to a higher extent in the mutant than in the wild type (Tables 1 and 2).

We could also detect and quantify two phosphorylated forms of PsbH. After far-red treatment, the isoform of PsbH that is mono-phosphorylated at Thr2 was over two-fold more abundant in the mutant than in the wild type, reflecting its higher degree of phosphorylation in the pbcp-1 mutant (Table 2). The form that is di-phosphorylated at Thr2 and Thr4 showed a different behavior. It was present in similar proportions in the mutant and the wild type after far-red treatment, even though in the mutant it may have been slightly over-phosphorylated in blue light (Table 2). The proportion of di-phosphorylated PsbH in the wild type and in pbcp-1 diminished to a similar extent in far-red compared to blue, suggesting that PBCP is not required for de-phosphorylation of P-Thr at position 4.

After far-red treatment, there was no significant difference between the mutant and the wild type in the level of phosphorylation of the LHCII antenna proteins Lhcb1 and Lhcb2 (Table 1), in agreement with the results obtained by immunoblotting with anti-phosphothreonine antibodies (Fig 1A). Under blue light, the mutant showed reduced levels of Lhcb1 phosphorylation compared to the wild type. One interpretation of this observation is that compensatory changes in the activity of other phosphatases could occur when PBCP is deficient. The phosphorylation levels of the CaS protein, a presumptive target of STN8, were also unaffected in the mutant (Vainonen et al., 2008). Phosphopeptides from CP43 were not detected in this mass-spectrometric analysis, and conversely PsbH was not analyzed by immunoblotting. Taken together the data from the two methods indicate that PBCP is required for efficient de-phosphorylation of
the core subunits of PSII (CP43, D1, D2, and PsbH-Thr2) but not of the LHCII proteins such as Lhcb1 and Lhcb2.

**PBCP belongs to the family of PP2C phosphatases**

We have obtained two alleles of PBCP: pbcp-1 carries a SALK T-DNA insertion in the fourth exon and pbcp-2 contains a WiscDs-Lox insert in the 5’ UTR (Supplemental Fig 1) (Alonso and Ecker, 2006; Woody et al., 2007). Both homozygous mutants show similar delays in de-phosphorylation of the core proteins of PSII (Fig 1B). In this panel, an anti-phosphothreonine antibody from a different commercial source (Cell Signaling) was used for comparison with Figure 1A (antibody from Zymed). Different antibodies have distinct recognition specificities, as demonstrated by contrasting the relative signal intensities of the main phospho-proteins detected in Figure 1 panels A and B (Vainonen et al., 2005). In particular, the antibodies from Zymed detect phosphorylation of the PSII subunits more efficiently, and conversely those from Cell Signaling recognize LHCII more strongly.

We used Agrobacterium to transform the homozygous pbcp-1 mutant with a wild-type copy of the PBCP cDNA tagged with a triple-HA epitope and placed under the control of the CaMV 35S promoter. Amongst the pbcp-1;35S:PBCP:HA transformed lines, one was selected in which PBCP accumulates to levels only slightly higher than the wild type (dubbed “rescued”). As shown in Figure 1C, rapid de-phosphorylation of the PSII core subunits, induced by a transfer from white to far-red light, was restored to a large extent in the rescued line. Taken together, these data show that the defect in de-phosphorylation of the PSII core is indeed caused by a mutation in PBCP. A second pbcp-1;35S:PBCP:HA line was also obtained, in which PBCP is strongly overexpressed (“pbcpOE”, Fig 1D). In this line, de-phosphorylation of the core subunits of PSII was more rapid than in the wild type. Unexpectedly, the LHCII antenna was also somewhat less phosphorylated compared to the wild type in white light or after 20 minutes of far-red treatment. Since phosphorylation of LHCII is not elevated in the pbcp mutants, it is not clear whether de-phosphorylation of LHCII in pbcpOE indicates that the antenna is a target of PBCP, or whether over-expression induces a loss of specificity and a promiscuous activity of the phosphatase towards LHCII.
The PBCP polypeptide deduced from the cDNA sequence has 298 residues and a molecular mass of 32 kDa, and is predicted to carry an amino-terminal transit peptide for chloroplast targeting by the algorithms TargetP (Emanuelsson et al., 2000), Wolfpsort (Horton et al., 2007) and Multiloc (Hoglund et al., 2006). The PBCP protein is not predicted to contain any trans-membrane domains. The mature protein belongs to the family of PP2C protein phosphatases, which comprises approximately 80 members in plants (Kerk et al., 2008; Xue et al., 2008) and also includes the PPH1/TAP38 phosphatase. PBCP and its orthologs belong to a phylogenetic clade that extends from monocots and dicots to the lycopod Selaginella moellendorfii, the moss Physcomytrella patens and the green algae Chlamydomonas reinhardtii and Volvox carteri (Supplemental Figure 2). This phylogenetic analysis thus shows that PBCP has been conserved throughout the evolution of plants (Viridiplantae). PBCP shares only ~16 % sequence identity with PPH1/TAP38, and is more closely related to other PP2C phosphatases such as At4G33500 which is part of a neighboring phylogenetic clade. However insertion mutants of the latter did not show any detectable phenotype in a de-phosphorylation assay with far-red light similar to those presented in Figure 1.

A polyclonal antiserum raised against PBCP recognizes a polypeptide of approximately 32 kDa in the wild type (Fig 1C and D, Supplemental Figure 1C). This is approximately the predicted molecular mass of the mature PBCP protein after removal of the putative transit peptide. The protein is not detectable in pbcp-1 and pbcp-2 (Supplemental Figure 1C), indicating that both are strong or null alleles and confirming that the 32 kDa band recognized by the antiserum is indeed the PBCP protein. A slightly retarded migration of PBCP, which can be ascribed to the presence of the triple-HA tag, is observed in the rescued mutant and in the pbcpOE line (Fig 1C and D).

**PBCP is a chloroplast protein**

The deduced amino acid sequence of PBCP carries a predicted amino-terminal transit peptide for chloroplast targeting. Indeed D. Leister and coworkers (Schliebner et al., 2008) have observed that the fluorescent protein dsRed fused to the transit peptide of PBCP (At2g30170) clearly localizes to the chloroplasts in transfected Arabidopsis protoplasts. This protein was also identified in purified chloroplast fractions by mass
spectrometry (Zybailov et al., 2008). To confirm the localization of PBCP, we prepared chloroplasts from rosette leaves, and further fractionated them by centrifugation into a stromal supernatant and a membrane pellet. These fractions were analyzed by immunobloting using antiserum against PBCP, and the quality of the fractionation was assessed with antisera against the thylakoid protein D1, the stromal enzyme phosphoribulokinase (PRK) and the cytoskeletal protein actin (Fig 2). The PBCP protein was largely found in the chloroplast stromal fraction and its distribution closely paralleled the stromal marker PRK. A minor amount of PBCP was also observed in the membrane fraction.

**PBCP de-phosphorylates subunits of PSII in vitro**

As described above, PBCP belongs to the family of PP2C phosphatases, and thylakoid protein de-phosphorylation is affected in the pbcp mutants in planta. To confirm that PBCP indeed has phosphatase activity and to gain insight regarding whether its action on the core subunits of PSII is direct, we purified recombinant PBCP (rPBCP) and assayed its activity in vitro. The mature form of PBCP, without the predicted transit peptide but with a C-terminal poly-histidine tag, was expressed in *E. coli*. The enzyme was purified under denaturing conditions by Ni-affinity chromatography, allowed to refold by dialysis into non-denaturing conditions, and then further purified by size exclusion chromatography (Fig 3A). A His-tagged bacterial maltose-binding protein (MBP) was expressed and purified in parallel as a negative control. The phosphatase activity of rPBCP was first demonstrated with the chromogenic substrate, para-nitrophenyl phosphate (pNPP) (Fig 3B). Consistent with some similar phosphatases of the PP2C class, the activity of rPBCP was much higher with Mn$^{2+}$ than with Mg$^{2+}$ as a cofactor in the assay (Pullen et al., 2004; Wehenkel et al., 2007), and the activity was strongly inhibited by the addition of EDTA as a chelator. Surprisingly, the activity was almost unaffected by the addition of 10 mM NaF, a general inhibitor of thylakoid protein phosphatases (Bennett, 1980; Hammer et al., 1995; Vener et al., 1999). Addition of the reducing agent DTT stimulated the activity, raising the possibility that the phosphatase could be regulated by the redox state of its cysteine residues. To test the activity of rPBCP on its natural substrates, thylakoid membranes were purified from the *pbcp-1*
mutant and exposed to white light for 15 minutes in the presence of ATP to maximize thylakoid protein phosphorylation. The membranes were then incubated in the dark in the presence of purified rPBCP, using as controls the buffer or the purified MBP protein. De-phosphorylation was monitored by immunoblotting with anti-phosphothreonine antibodies. After 5 minutes of incubation, the de-phosphorylation activity of rPBCP in the presence of Mn\(^{2+}\) was apparent as a decrease in the phosphorylation of CP43, D1 and D2 (Fig 3C, lanes 5 versus lane 1). Phosphorylation decreased by 40-50 % for D1 and CP43, and approximately 70 % for D2. The activity was strongly inhibited by the addition of EDTA (lane 7). It was much lower in the presence of Mg\(^{2+}\), and was not significantly inhibited by NaF (Supplemental Figure 3). The addition of DTT did not have a detectable effect on the activity (Fig 3C, lane 6), but the sensitivity of the assay may be insufficient to detect the effect observed with pNPP as a substrate. Some de-phosphorylation of the LHCII proteins was apparent in this in vitro assay, similar to what was observed in vivo for the pbcpOE over-expressor.

Role of PBCP in thylakoid membrane organization

In the stn8 mutant and the stn7 stn8 double mutant, which are deficient in thylakoid protein phosphorylation, the membranes tend to form extended grana stacks (Fristedt et al., 2009a), suggesting that thylakoid protein phosphorylation affects membrane folding and grana size. We examined the ultrastructure of the thylakoid membranes in leaf chloroplasts of the pbcp mutants, the pbcpOE overexressor and the wild-type by transmission electron microscopy. We compared 11-day old seedlings treated with blue light for three hours, to favor thylakoid protein phosphorylation, and further treated for one-and-a-half hours with far-red light to promote de-phosphorylation (Fig 4). To avoid any differences due to cell-type specific differentiation of the plastids, we always selected chloroplasts in the sub-epidermal cell layer on the adaxial side, the presumptive palisade mesophyll cells of these developing leaves. There was a striking difference between the wild type and the pbcp mutants, which showed many regions where only a few membranes were appressed and thus a concomitant reduction in the number of layers in the grana stacks (Fig 4A-D). Thylakoid membrane folding in the pbcpOE was similar to the wild type (Fig 4E and F). To represent these differences
quantitatively, we measured the total length of thylakoid membranes that were found in single stromal lamellae or in grana stacks with different number of layers. The resulting histogram (Fig 4G) shows that in the wild type the broad distribution extended to stacks with 10 or more layers, while in the mutants the distribution was clearly displaced towards fewer layers and showed only very few grana stacks with 8 layers or more. Thus the activities of the PBCP phosphatase and of the STN8 kinase seem to affect the folding of the thylakoid membrane in opposing ways. The distribution in the pbcpOE was similar to the wild type, as might be expected after prolonged far-red treatment when thylakoid protein phosphorylation in the wild type and the over-expressor reaches comparable low levels (Fig 1).

**State transitions in the pbcp mutants and pbcpOE over-expressor**

In the different pbcp-1 transgenic lines transformed with the 35S:PBCP:HA construct, PBCP was expressed at different levels (Fig 1C and D). It was thus possible to compare different lines forming an allelic series with increasing amounts of PBCP, from the pbcp mutants devoid of PBCP, through the wild type or the rescued line with slightly elevated levels of PBCP, to the pbcpOE over-expressor with strongly increased accumulation of the phosphatase. We observed a consistent trend in the levels of PSII phosphorylation and the kinetics of de-phosphorylation. In the pbcp-1 mutant exposed to white or blue light, phosphorylation of the thylakoid proteins was somewhat elevated compared to the wild type (Fig 1, Tables 1 and 2), and de-phosphorylation upon transfer to far-red light was strongly retarded. The rescued line showed normal phosphorylation patterns in white light and restored de-phosphorylation upon transfer to far-red light (Fig 1C). In pbcpOE, we observed slightly reduced phosphorylation in white light, and rapid de-phosphorylation upon transfer to far-red light (Fig 1D). This is consistent with the presumed antagonistic effects of the STN8 kinase and the PBCP phosphatase in white or blue light, where the kinase is active, and the role of PBCP in de-phosphorylation in far-red light, where the kinase is thought to be down-regulated. However, these observations are also compatible with the possibility that the activity of PBCP is regulated.
State transitions, which involve phosphorylation of the LHCII antennae and their dynamic allocation to PSII or PSI, are regulated by the STN7 kinase and the PPH1/TAP38 phosphatase. Blue or white light induces a transition to state 2 through the activation of the STN7 kinase and the association of phosphorylated LHCII with PSI. This is reversed in far-red light, which induces de-phosphorylation of the LHCII antennae under the action of the PPH1/TAP38 phosphatase and allocation of LHCII to PSII. While the STN8 kinase has substrate specificity distinct from STN7, the activities of the two kinases may partly overlap. This is suggested by the residual phosphorylation of LHCII and PSII subunits, which is observed in the single mutants of the kinases, and is more extensively abolished in the double mutant \textit{stn7 stn8} (Bonardi et al., 2005; Tikkanen et al., 2008). It was therefore of interest to investigate whether the PBCP phosphatase mutants were affected in state transitions, which were measured by monitoring chlorophyll fluorescence in two sets of experiments, either at room temperature or at 77 K.

At room temperature, chlorophyll fluorescence emanates almost exclusively from PSII and its light harvesting antennae. Under white light, which favors state 2, part of the LHCII antenna is dissociated from PSII so the maximal fluorescence \(F_{m2}\) induced by a saturating flash is lower than the maximal fluorescence \(F_{m1}\) in far-red light, which induces state 1 and the association of LHCII with PSII (Fig 5A). The state transition parameter, \(q_T = \frac{(F_{m1} - F_{m2}) \times 100}{F_{m1}}\) was approximately +3 in the wild-type seedlings but decreased to a negative value of -2 in the \textit{stn7} mutant and was also strongly affected in the \textit{pph1-3} mutant, both of which were used as controls that are known to affect LHCII phosphorylation and state transitions (Fig 5B). \(q_T\) was comparable to the wild type in the \textit{stn8} and in the rescued line, as well as in the \textit{pbcp-1} and \textit{pbcp-2} mutants. Thus state transitions are not significantly affected in the absence of PBCP. Interestingly, \(q_T\) was strongly reduced in the \textit{pbcpOE} over-expressor line, an indication that state transitions may be affected by the increased activity of PBCP. This was also apparent when the kinetics of state transitions were monitored by following the fluorescence parameter \(F_s\), which is influenced by the redox state of the plastoquinone pool (PQ). When the far-red light is turned off, \(F_s\) increases abruptly, reflecting the over-reduction of the PQ pool, and then \(F_s\) gradually decreases as
transition to state 2 allows re-oxidation of the pool (Fig 5C). Compared to the wild type, this decrease was blocked in the stn7 mutant, was strongly retarded in the pph1-3 mutant, and was not affected in the stn8, pbcp-1 and pbcp-2 lines. The kinetics of the Fs decrease in the transition from state 1 to state 2 were strikingly slower in the pbcpOE over-expressor, suggesting that state transitions are affected by the increased activity of the phosphatase. A small effect was also apparent in the rescued line, which has slightly elevated levels of PBCP (Fig 1C).

State transitions can also be monitored by measuring the fluorescence emission spectra of chlorophyll at 77 K. Emission at 685 nm can be ascribed to PSII, and emission at 732 nm to PSI. As the LHCII antenna is reallocated by state transitions, changes in the cross section of the two photosystems are reflected by changes in the relative size of the respective fluorescence emission peaks (Supplemental Figure 4). When seedlings were transferred from far-red light to blue light, the amplitude of the transition to state 2 was reduced in the pbcpOE line compared to the wild-type, but not as strongly as in the stn7 mutant. Conversely, when the seedling were returned to far-red light, the transition to state 1 was more pronounced in the pbcpOE line than in the wild type. Consistent with the qT and Fs measurements at room temperature, this indicates that over-expression of PBCP affects state transition. These results can be correlated with the observation that the LHCII proteins are less phosphorylated in pbcpOE compared to the wild type (Fig 1D).

DISCUSSION

While PPH1/TAP38 was shown to be necessary for de-phosphorylation of LHCII subunits (LHCB 1, LHCB 2) (Pribil et al., 2010; Shapiguzov et al., 2010), here we demonstrate that PBCP is required for efficient de-phosphorylation of the core subunits CP43, D1, D2 and PsbH of PSII. The substrate specificities of the two phosphatases are similar to those of the two kinases, STN7 and STN8 (Bellafiore et al., 2005; Bonardi et al., 2005; Vainonen et al., 2005; Fristedt and Vener, 2011; Reiland et al., 2011). The PBCP phosphatase and the STN8 kinase thus seem to form an antagonistic pair, whose reciprocal activities on phosphorylation of PSII core subunits could allow a balanced acclimatory response. This is similar to the pair formed by the STN7 kinase
and the PPH1/TAP38 phosphatase, which have opposing effects on phosphorylation of the LHCII antenna to regulate state transitions and maintain a balanced redox state of the electron transfer chain.

Although the two phosphatases appear to have distinct specificities, over-expression of PBCP perturbed state transitions and the phosphorylation of LHCII, which are the major roles ascribed to PPH1/TAP38. The converse effects were not obvious in the pbcp mutants, which did not show elevated levels of LHCII phosphorylation in white or blue light (Fig 1, Table 1) or changes in the kinetics of state transitions (Fig 5 and Supplemental Figure 4). Thus caution should be used in interpreting the phenotype of the pbcpOE line, which is a dominant gain-of-function mutant, since over-expression of PBCP might cause a loss of its normal specificity. Nevertheless, it is interesting to consider three more possible interpretations, which are not entirely mutually exclusive, of these observations. One is that PBCP may have some overlapping activity towards LHCII, which only becomes apparent upon over-expression, and that its specificity may not be entirely restricted to the PSII core. A similar situation seems to prevail for the two kinases, whose substrate specificity appears to overlap since the residual phosphorylation that is apparent in the single kinase mutants, stn7 or stn8, is abolished in the double mutant stn7 stn8 (Tikkanen et al., 2008; Fristedt et al., 2009a). An alternative interpretation is that PBCP may have functional interactions with other members of a regulatory network, for example PPH1 or STN7, and influence their activity directly or indirectly. A different interpretation could be that phosphorylation of the core subunits influences the mobility of proteins within the thylakoid membrane, which in turn may influence the rate of state transitions. Because of steric hindrance, PSI and ATP synthase are excluded from the PSII-enriched appressed membranes of grana stacks. This segregation is thought to minimize spillover of energy between the photosystems (Dekker and Boekema, 2005). Changes in protein phosphorylation may affect the function of the photosynthetic electron transfer chain by changing the distribution and interactions of the photosynthetic complexes, and in particular may influence state transitions by limiting the transfer of LHCII between PSI and PSII. Phosphorylation by the STN7 and STN8 kinases was shown to influence the mobility of thylakoid proteins within the membrane (Goral et al., 2010).
Phosphorylation of PsbH reveals another aspect of the antagonism between STN8 and PBCP. There are two phosphorylation sites at the N-terminus of PsbH, at Thr2 and Thr4. We find that in the pbcp mutant it is Thr2 that retains phosphorylation in far-red light, whereas Thr4 is de-phosphorylated as in the wild type. However, mass-spectrometric analysis of phosphorylation in the stn8 mutant has shown that the STN8 kinase is responsible for phosphorylation at Thr4, and that this requires prior phosphorylation at Thr2 (Vainonen et al., 2005; Fristedt and Vener, 2011). Thus in the case of PsbH, antagonism of PBCP and STN8 might involve the de-phosphorylation of Thr2 by PBCP, which in turn would prevent phosphorylation by STN8 at Thr4. Little is known on the physiological role of phosphorylation at the individual threonine residues in PsbH, or in D2 and CP43. In Chlamydomonas, a mutation of PsbH that prevents phosphorylation of Thr2 by replacing it with Ala had no apparent phenotypic consequence (O’Connor et al., 1998). Mutation of the D2 subunit changing Thr2 to Ala allowed normal photosynthetic activity, but mutations to Asp or Glu which mimic phosphorylation strongly affected PSII accumulation (Fleischmann and Rochaix, 1999).

What then may be the function of STN8 and PBCP in light acclimation? In the stn8 mutant, phosphorylation of PSII is reduced and the size of the thylakoid membrane grana increases. Conversely, we have observed in the pbcp mutants that phosphorylation of PSII is retained upon exposure to far-red light and that the proportion of large grana stacks decreases. Furthermore STN8 may facilitate the repair cycle of PSII by promoting unfolding of the membranes and allowing easier access of membrane proteases such as FtsH and co-translational integration of D1 (Tikkanen et al., 2008; Fristedt et al., 2009a). However our attempts to determine whether photoinhibition and D1 turnover are affected in the pbcp mutants did not give consistent results.

The rPBCP purified from E. coli showed phosphatase activity in vitro using the artificial substrate pNPP. Since rPBCP promoted de-phosphorylation of the core subunits of PSII in isolated thylakoids, it seems likely that these are its direct substrates. However, we cannot entirely exclude the possibility that PBCP is only indirectly required for de-phosphorylation of PSII. As with some other PP2C phosphatases, PBCP is Mn$^{2+}$-
dependent and has negligible activity with Mg\textsuperscript{2+} as the metal cofactor (Pullen et al., 2004; Wehenkel et al., 2007). The activity of the phosphatase is strongly inhibited by the addition of EDTA. Surprisingly NaF, which is a general inhibitor of thylakoid phosphatases (Bennett, 1980; Vener et al., 1999), has only a relatively minor effect on PBCP activity \textit{in vitro}, both when assayed with pNPP or with thylakoid membranes. This observation is puzzling, because in pumpkin leaves NaF could prevent de-phosphorylation of the PSII core subunits and retard degradation of D1 (Rintamaki et al., 1996). It will thus be interesting to determine whether in Arabidopsis the PBCP phosphatase is directly involved in the repair cycle of D1, or whether another phosphatase is involved.

The PP2C family of protein phosphatases comprises approximately 80 members in Arabidopsis or rice (Xue et al., 2008). The number is much larger than in animals, and this expansion in plants suggests that PP2C phosphatases have acquired distinct substrate specificities or novel functions (Kerk et al., 2008). Indeed our data indicate that for the de-phosphorylation of thylakoid membrane proteins, PPH1/TAP38 and PBCP have different substrate preferences. The presence of different phosphatases is one possible explanation for the various kinetic classes which were discerned in assays of endogenous phosphatase activity with isolated thylakoid membranes from pea chloroplasts (Silverstein et al., 1993). The strong preference of PBCP for Mn\textsuperscript{2+} and its insensitivity to NaF distinguish it from both the stromal and the membrane phosphatases active on LHCII phosphopeptides which were previously purified from pea chloroplasts (Hammer et al., 1995; Hammer et al., 1997). It is expected that further phosphatases involved in regulating thylakoid membrane structure and function remain to be identified.
METHODS

Plant material

*Arabidopsis thaliana* mutant lines in Col-0 background were obtained from NASC (European Arabidopsis Stock Center, *pbcp-1* is SALK_127920.31.10.N, *pbcp-2* is WiscDsLox_359F02. They were genotyped as described in Supplemental Fig 1. The *stn7* (Bellafore et al., 2005), *stn8* (Vainonen et al., 2005; Fristedt et al., 2009a) and *pph1-3* mutants (Shapiguzov et al., 2010) were described.

To rescue the *pbcp-1* mutant with the wild-type cDNA, the complete coding sequence of PBCP (At2g30170), excluding the stop codon, was amplified by PCR from a cDNA clone (pda00938) obtained from the Riken Institute (Japan). The forward primer (5′ATCTCGAGCGAGAGAGCAAGCCATTGCAG) and reverse primer (5′GTCGACTGAGCTAACAACTTTGGCAACG) included XhoI and SalI sites, respectively. The PCR product was inserted in the XhoI and SalI sites of pCF399 under the control of the CaMV 35S promoter and the NOS terminator with a triple HA epitope tag at the C-terminus (Lorrain et al., 2008). Plants of *Arabidopsis thaliana* were transformed by floral dipping using *Agrobacterium tumefaciens* GV3101 strain. T1, T2 and T3 progeny seedlings were selected on MS medium supplemented with 1.5 % sucrose and 10 µg mL⁻¹ Basta (Pentanal).

De-phosphorylation *in vivo*

For the genetic screen, seedlings were grown for 10 days on Murashige-Skoog agar in a growth chamber (Percival CU36L5) in long days (16 h light and 8 h dark) at 22 °C under fluorescent white light (50-60 µE m⁻² s⁻¹). De-phosphorylation was monitored as described (Shapiguzov et al., 2010) by placing seedlings under far-red LEDs (L735; Epitex) for the designated time. Seedlings were snap-frozen in liquid nitrogen and ground twice for 10 s with glass beads in a triturator (Silamat S5; Ivoclar Vivadent AG). Protein extracts were prepared by incubating in lysis buffer containing 100 mM Tris-HCl, pH 7.7, 2 % SDS, 50 mM NaF, and 2X Protease Inhibitor Mixture (Sigma Aldrich) for 30
min at 37 °C. The samples were separated by Tris-glycine SDS-PAGE in 12 % acrylamide gels supplemented with 6 M urea, which allowed of better separation of the D1 and D2 phosphoproteins. Immunoblotting was performed using anti-phosphothreonine antibodies (Zymed or Cell Signaling) or anti-actin as a control (Sigma Aldrich).

**Analyses of in vivo protein phosphorylation by mass spectrometry**

Thylakoids for mass-spectrometric (MS) analyses were isolated from 15 day-old seedlings grown on Murashige-Skoog agar plates. The plants were dark-adapted for 1 hour then placed under far-red or blue LEDs for 30 min to induce state 1 or state 2, respectively. Thylakoid isolation was performed in the dark (under green light) in the cold room as described (Fristedt et al., 2009a). For trypsin treatment thylakoids were resuspended in 25 mM NH₄HCO₃ and 10 mM NaF at a concentration of 2 mg mL⁻¹ of chlorophyll and incubated with sequencing-grade modified trypsin (Promega) for 3 h at room temperature. Peptide extracts were cleared by ultracentrifugation, aliquoted and dried in a SpeedVac.

For relative quantitative MS studies, the collected peptides from trypsin-treated wild-type and *pbcp-1* mutant thylakoids were subjected to esterification by either d0-methyl methanol or d3-methyl methanol (Sigma Aldrich) prior to enrichment of phosphopeptides by IMAC. Both esterification of peptides and the subsequent phosphopeptide enrichment by IMAC were done as described before (Shapiguzov et al., 2010). The esterified samples were mixed 1:1, subjected to IMAC and analyzed by nano-liquid chromatography (LC) electrospray ionization MS, which allowed simultaneous measurements of intensities for light and heavy isotope-labeled phosphopeptide pairs and quantitative comparison of the phosphorylation differences in the mutant and wild-type proteins. The reverse labeling of peptides from wild type and *pbcp-1* was performed as an internal control. For analysis of the peptides, an on-line nano-flow HPLC system (EASY-nLC; Bruker Daltonics) was used and MS analysis with the HCTultra PTM Discovery System (Bruker Daltonics) was performed using the same settings as before (Shapiguzov et al., 2010). The automated, online, tandem MS
analyses were made using alternating collision-induced dissociation/electron transfer
dissociation fragmentation of peptide ions.

Label-free characterization of the phosphorylation status for the PSII core proteins was
done by LC-MS analyses of the peptides obtained from the proteolytic treatment of wild-
type and pbcp-1 thylakoids without further enrichment as described (Fristedt et al.,
2010). LC-MS separation and detection of the phosphorylated peptides and their non-
phosphorylated counterparts was used for determination of the phosphorylation
stoichiometry for PSII proteins. The level of phosphorylation for the PSII core proteins
was calculated from the ratios of phosphorylated to non-phosphorylated peptide
intensities in each LC-MS chromatogram using normalization for the difference in the
signal intensities of the phosphorylated and de-phosphorylated peptide ions from the
D1, D2 and PbsH proteins as determined in (Fristedt et al., 2010).

Purification of recombinant PBCP

The cDNA sequence of the predicted mature PBCP protein was cloned into pET28a
(Invitrogen) to include a C-terminal 6x-Histidine tag and expressed in E. coli strain
BL21. Following cell lysis and centrifugation at 7 600 g, the pellet containing
recombinant PBCP was solubilized in 6 M guanidine with phosphate buffer at pH 8.0.
Cell debris was pelleted at 23 000 g and the denatured proteins were loaded onto Ni-
NTA resin (Qiagen). The Ni-NTA was washed with 4 M urea containing 35 mM
imidazole and rPBCP was eluted with 2 M urea containing 300 mM imidazole and 200
mM NaCl. 10 mM EDTA and 20 mM 2-mercaptoethanol were added to the eluate.
Denatured PBCP was refolded by dialyzing the eluate with 300 mM NaCl and 50 mM
HEPES, pH 8.0. Following dialysis the rPBCP eluate was centrifuged at 23 000 g and
further purified by size exclusion chromatography on a preparative grade Superdex-200
column (GE Healthcare) with a mobile phase of 300 mM NaCl and 50 mM HEPES, pH
8.0. Recombinant PBCP eluted at the expected globular particle size of approximately
30 kDa and was concentrated to stocks of 1 mg mL⁻¹. As a source of antigen to raise a
rabbit anti-PBCP serum, the recombinant protein was purified by Ni-NTA
chromatography as recommended by the manufacturer (Quiagen) followed by
preparative SDS-PAGE.
Activity assays of recombinant PBCP

Recombinant PBCP phosphatase activity was assayed in vitro by monitoring the dephosphorylation of para-nitrophenylphosphate (pNPP). The reaction rate was calculated by the increase in absorbance at 405 nm as a function of time across the linear portion of the curve. The reaction mixture contained 300 mM NaCl, 50 mM HEPES, pH 8.0, 5 mM MnCl$_2$ or MgCl$_2$, 10 mM pNPP, and 5 µg mL$^{-1}$ PBCP (~170 nM). Stocks of pNPP, NaF, DTT, and EDTA were prepared in aqueous solution containing 300 mM NaCl, 50 mM HEPES, pH 8.0, and 5 mM MnCl$_2$ or MgCl$_2$.

Plants for thylakoid extraction were cultivated for 3 weeks on soil in growth chambers at 24 °C under white light (100 µE) in short days (8 h light, 16 h dark). Thylakoids were prepared as described (Fristedt et al., 2009a) without addition of NaF, washed in a buffer containing 25 mM Tricine-NaOH, pH 7.8, 100 mM sorbitol, 10 mM KCl and either 5 mM MgCl$_2$ or 5 mM MnCl$_2$. Thylakoids (containing 0.17 mg mL$^{-1}$ chlorophyll) were supplemented with ATP to a concentration of 0.4 mM and incubated in white light (100 µE m$^{-2}$·s$^{-1}$) for 15 min. Recombinant PBCP, maltose binding protein (MBP) or buffer were added to the thylakoids, and where indicated supplemented with 10 mM DTT or 50 mM NaF, and incubated in the dark for the designated times before the reaction was stopped by the addition of the 4 x gel-loading lysis buffer (10 % SDS, 40 % sucrose, 1 mM EDTA, 50 mM Tris-HCl, pH 6.8, 20 % β-mercaptoethanol). Thylakoid protein phosphorylation was analyzed by immunoblotting with anti-phosphothreonine antibodies, and quantified by comparison to a dilution series of an untreated thylakoid sample.

Isolation of intact chloroplasts and fractionation

Intact chloroplasts were isolated from mature plants grown on soil in long days (12 h light and 12 h dark). The procedure was performed on ice. The leaves were ground in 20 mM HEPES-KOH, pH 8.0, 5 mM EDTA, 330 mM sorbitol with a Polytron mixer (Kinematica). The homogenate was filtered through 2 layers of Miracloth (Calbiochem) and centrifuged at 1 000 g at 4 °C for 5 min. The chloroplast pellet was resuspended in 0.5 mL of the same buffer and intact chloroplasts were isolated on a Percoll gradient as
described in (Aronsson and Jarvis, 2002). Washed intact chloroplasts were lysed by hypo-osmotic shock in 10 mM Tricine-NaOH, pH 7.8, 5 mM MgCl₂ and 10 mM NaF. The insoluble fraction containing thylakoid membranes was pelleted by centrifugation (21 000 g for 10 minutes). The soluble fraction enriched in chloroplast stromal proteins was cleared by an additional centrifugation step (21 000 g for 30 minutes). The samples were treated with 10 % trichloracetic acid to precipitate protein.

**Transmission electron microscopy**

Seedlings were grown on Murashige-Skoog agar plates (16 h light, 8 h dark, 50-60 µE m⁻² s⁻¹) for 11 days. Four hours after the onset of light, they were exposed for 3 h to blue LEDs and then for 1.5 h to far-red LEDs (L470 and L735, Epitex). Cuttings from the first pair of leaves, taken in the middle of the lamina, were immediately immersed in 2.5 % glutaraldehyde in 100 mM cacodylate sodium buffer (pH 7.0) containing 0.01 % Tween 20. The leaf pieces were fixed overnight at 4 °C under agitation. They were then rinsed sequentially (30 min/step) in cacodylate sodium buffer containing (1) 0.1 % Tween, (2) 0.05 % Tween and (3) three times in cacodylate alone. The samples were post-fixed in 1.5 % OsO₄ for 2 h, washed 3 times in cacodylate buffer, then once in milliQ water and further fixed in 1 % aqueous uranyl acetate. Sample preparation was performed at 4 °C. After washing in distilled water the tissue was dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin cross sections (85 nm thick) were cut and stained with 2.5 % uranyl acetate and then Reynolds lead citrate. The sections were viewed in a FEI Tecnai G2 Sphera transmission electron microscope at 120 kV. Care was taken to always select plastids located in adaxial subepidermal cells (presumptive palisade parenchyma cells). The lengths of membranes from electron micrographs were measured using ImageJ software (http://rsbweb.nih.gov/ij/). Data presented in Fig 4G are from 30 images of the wild type and 22 of pbcp-1 (from 5 independent experiments), 12 of pbcp-2 (2 experiments), and 9 of pbcpOE (1 experiment).

**Chlorophyll fluorescence analysis**

Room temperature chlorophyll fluorescence was monitored with a FluorCam 800MF kinetic imaging fluorimeter (PhotoSystems Instruments) essentially as described in
Seedlings grown on MS agar for 15 days were dark-adapted for 30 min. White actinic light (8 %) was then switched on for 17 min to induce state 2. Far-red light (100 %) was then turned on against the background of continuing white light for 17 min to stimulate transition from state 2 to state 1. Finally, far red light was turned off to initiate the reverse transition. Measuring flashes continued throughout the protocol to record F_s. Saturating light flashes were applied at the end of the dark, at the end of the 17-min white, and at the end of the 17-min white plus far-red light periods to measure F_m, F_m2 and F_m1 respectively. The fluorescence signal was averaged for 26 seedlings. Chlorophyll fluorescence at 77 K was measured on seedlings essentially as described (Shapiguzov et al., 2010). Seedlings were frozen in liquid nitrogen, ground and resuspended in ice-cold buffer containing 50 mM HEPES-KOH, pH 7.5, 100 mM sorbitol, 10 mM MgCl_2, and 10 mM NaF. The samples were filtered and frozen in liquid nitrogen, and chlorophyll fluorescence at 77 K was recorded on a Jasco FP-750 spectrofluorometer. Excitation was at 480 nm (slit width 5 nm) and emission was recorded in the 600-800 nm range (slit width 5 nm). Curves were normalized for the PSII peak at 685 nm.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AT2G30170.1, GenBank: AY063046.1.

**Supplemental data**

The following materials are available in the online version of this article.

Sup. Fig 1. Two mutant alleles of PBCP

Sup. Fig 2 Phylogenetic analysis of PBCP homologs

Sup. Fig 3 PBCP activity *in vitro* in the presence of Mg^{2+} or NaF

Sup. Fig 4. Analysis of state transitions by chlorophyll fluorescence at 77 K
Supplemental Dataset 1. Text file of alignment used for phylogenetic analysis in Supplemental Figure 2.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

Designed the research: MGC, JDR, AV, IS, AS, GF, BI, MC
Performed research: IS, AS, GF, BI, MC
Analyzed data: MGC, JDR, AV, IS, AS, GF, BI, MC
Wrote the paper: MGC, JDR
FIGURE LEGENDS

Figure 1  PSII de-phosphorylation is deficient in pbcp mutants

A) Wild-type, pph1-3 and pbcp-1 seedlings were grown in white light for 10 days and then transferred to far-red light (FR) for 20 or 40 minutes. Total protein extracts were analyzed by immunoblotting with anti-phosphothreonine antibodies from Zymed, and then with anti-actin antibodies as a loading control.

B) Wild-type, pbcp-1 and pbcp-2 seedlings were treated and analyzed as in panel A with anti-phosphothreonine antibodies (Cell Signaling). The relative intensities of the phosphoprotein signals differ with different sources of anti-phosphothreonine antibodies (compare to panel A, where the antibody from Zymed was used).

C) Wild-type, pbcp-1 and rescued (“resc”: pbcp-1;35S:PBCP:HA) seedlings were treated and analyzed as in A with anti-phosphothreonine antibodies from Zymed. Anti-PBCP serum was also used to monitor the level of expression of the transgenic protein in the rescued line. Its slower migration is due to the presence of the triple HA epitope, but the nature of the doublet band that is observed is not known.

D) Wild-type, pbcp-1 and pbcpOE over-expressor seedlings were analyzed as in C. Minor bands that co-migrate with PBCP:HA in the wild-type and pbcp-1 samples (FR) are due to artefactual spill-over of the samples from the adjacent lanes.

Figure 2  PBCP is a chloroplast protein

Chloroplasts from wild-type plants were isolated and further fractionated into a membrane pellet and a stromal supernatant. Equal amounts of protein from each fraction (10 µg) were analyzed by immunoblotting with antibodies against PBCP, the stromal protein phosphoribulokinase (PRK), the thylakoid membrane protein D1 of PSII, and the cytoskeletal protein actin.

Figure 3  Recombinant PBCP has phosphatase activity in vitro

A) SDS-PAGE of purified rPBCP loaded under reducing and denaturing conditions.
B) *In vitro* spectrophotometric assay of PBCP activity. The dephosphorylation of para-nitrophenylphosphate (pNPP) was measured by the increase in absorption at 405 nm as a function of time in 1 mL reaction volumes containing 5 µg recombinant PBCP, 300 mM NaCl, 50 mM HEPES, pH 8.0, 10 mM pNPP, and the additives indicated. Error bars represent the standard deviation of triplicate measurements.

C) Thylakoid membranes (containing 0.16 µg chlorophyll) isolated from the *pbcp-1* mutant, exposed to white light for 15 minutes and then incubated for 5 minutes in the dark in the presence of purified recombinant PBCP (5 µg), or with protein buffer or recombinant maltose-binding protein (MBP) as controls, in a final reaction volume of 100 µL containing 5 mM MnCl₂. The effect of DTT (10 mM) and EDTA (5 mM) were tested as indicated. The samples were analyzed by immunoblotting with anti-phosphothreonine antibodies from Zymed.

**Figure 4** Thylakoid folding is altered in *pbcp* mutants

Electron micrographs of thin sections from leaves of 11-day old seedlings exposed to blue light for three hours and then shifted to far-red light for one-and-a-half hours to induce de-phosphorylation of the PSII core subunits. All scale bars represent 500 nm, panels B, D and F show larger magnifications of panels A, C and E respectively.

A) and B): wild type
C) and D): *pbcp-2*
E) and F): *pbcpOE*

G) The length of thylakoid membranes in stromal lamella and in grana stacks with different numbers of layers were measured in electron micrographs of the wild type (n=30), the *pbcp-1* mutant (n=22), the *pbcp-2* mutant (n=12) and the *pbcpOE* over-expressor (n=9). The histograms show the length of membranes in domains with the indicated numbers of layers (1 layer for stromal lamellae, 2 or more layers in grana stacks) as a fraction of the total length of membranes measured.
Figure 5  State transitions in the pbcp mutants and pbcpOE over-expressor.

A) The kinetics of state transitions were analyzed by continuously monitoring chlorophyll fluorescence with the FluorCam (PhotoSystems Instruments). After a pre-incubation of 30 min in darkness, actinic light (represented below as a white bar) was turned on for 17 minutes to induce state 2 before maximal fluorescence (Fm2) was measured with a saturating flash (arrow). Far-red light was then turned on for 17 minutes (represented below as a red bar) to induce state 1 and maximal fluorescence (Fm1) was again measured with a saturating flash (arrow) before the far-red light was turned off to return to state 2. The curves (normalized to Fo) are the average of 26 seedlings of each genotype from two experiments. Line breaks indicate that the Fm peaks are out of scale.

B) The state transition parameter qT was calculated from the experiments in panel A (qT= (Fm1-Fm2) x 100/Fm1). The bars represent the mean and SD of 26 seedlings of each genotype from two experiments. The asterisks indicate P<0.001 in Student’s t-test.

C) Chlorophyll fluorescence was normalized for the value of Fs immediately after turning the far-red light off to allow comparisons of the kinetics of the transition to state 2.
Table 1. Ratios of phosphopeptides in *pbc*-*p* 1 versus wild-type leaves

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Identified phosphopeptide</th>
<th>At Gene identifier</th>
<th>pbc-1 / WT ratio</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Blue</td>
</tr>
<tr>
<td>D1</td>
<td>Ac-tAILER</td>
<td>AtCg00020</td>
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<td>AtCg00270</td>
<td>1.3±0.1 **</td>
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Ac denotes N-terminal acetylation and t represents P-Thr
Results are from four IMAC experiments with differential stable isotope labelling,
** marks statistically significant difference at *P* < 0.01 in Student’s t-test.
Table 2. *In vivo* phosphorylation stoichiometry (% of phosphorylation) for the PSII core proteins from wild-type and *pbcp-1* leaves.

<table>
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<th>Protein</th>
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<th>Far red light</th>
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<tr>
<td></td>
<td>WT</td>
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<tr>
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<td>------------</td>
<td>---------------</td>
</tr>
<tr>
<td>P-D1</td>
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<tr>
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<tr>
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<tr>
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<td>37±4 **</td>
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</table>

* and ** represent statistically significant differences between the *pbcp-1* mutant and the wild type at *P* < 0.05 and *P* < 0.01 respectively in Student’s *t* test.
REFERENCES


Figure 2

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<td>anti-actin</td>
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Figure 5

A

B

C

qT

WT

pbcp-1

pbcp-2

resc

pbcpOE

stn7

stn8

pph1-3

WT

pbcp-1

pbcp-2

min

43 44 45 46 47 48 49 50 51 52
Supplemental Figure 1. Two mutant alleles of *PBCP*

**A** The diagram depicts the structure of the PBCP gene (At2g30170): exons are indicated by black boxes, 5' and 3'UTRs by open boxes and introns by thin lines. The positions of the T-DNA insertions in *pbcp-1* and *pbcp-2* are indicated with white triangles.

**B** Genotyping by PCR was performed with DNA from the wild type (Col-0), *pbcp-1* (SALK_127920.31.10.N), *pbcp-2* (WiscDsLox_359F02) with the primers indicated. The products were analyzed by agarose gel electrophoresis and detected by fluorescence after ethidium bromide staining. M indicates size markers (Smart Ladder, Eurogentec).

Oligonucleotide primers:

85 f-1: 5’-CACACACAAAGTACGCACCAC
85 r-1: 5’-AGCAGACCCCTCAGAGCTTAG
LB: 5’- TGGTTCACGTAGTGGGCCATCG
85 f-2: 5’-TCCAAAAAGGGAACAAAAACC
85 r-2: 5’- GCCGAGATGCTTTATTTCAAG
p745: 5’-AACGTCCGCAATGTGTTATTAAGTTGTC

**C** A polyclonal rabbit serum against PBCP was used to decorate immunoblots of total protein extracts of wild-type, *pbcp-1* and *pbcp-2* seedlings. The blot was reprobed with anti-actin antibodies as a loading control.
Supplemental Figure 2. Phylogenetic analysis of PBCP homologs

Amino acid sequences were retrieved from Phytozome (http://www.phytozome.net/) and aligned in MEGA5 using ClustalW and manual editing. The tree was generated using the Neighbor Joining algorithm with the Poisson correction method assuming uniform rates of variation and partial deletions (40 %) in MEGA5 (Saitou and Nei, 1987; Tamura et al., 2011). The scale bar indicates the number of amino acid substitutions per site. Bootstrap confidence scores ≥ 70 are indicated at branch points. An orthologous cluster of PBCP sequences is highlighted in blue. An orthologous cluster of PBCP-like sequences is highlighted in green. The outgroup contains bacterial and plant PP2C type-A sequences. At; Arabidopsis thaliana, Al; Arabidopsis lyrata, Cr; Chlamydomonas reinhardtii, Cs; Cucumis sativa, Eg; Eucalyptus grandis, Mt; Medicago truncatula, Os; Oryza sativa, Pp; Physcomitrella patens, Pt; Populus trichocarpa, Re; Ralstonia eutropha, Sb; Sorghum bicolor, Sm; Selaginella moellendorffii, Vc; Volvox carteri, Vv; Vitis vinifera, Zm; Zea mays.
Supplemental Figure 3. PBCP activity in vitro in the presence of Mg^{2+} or NaF

**A)** Thylakoid membranes (containing 0.16 µg chlorophyll) isolated from the *pbcp-1* mutant, exposed to white light for 15 minutes and then incubated for 20 minutes in the dark in the presence of purified recombinant PBCP (1 or 5 µg), or with protein buffer or recombinant maltose-binding protein (MBP) as controls, in a final reaction volume of 100 µL containing 5 mM MnCl₂. The effect of NaF (10 mM) was tested as indicated. The samples were analyzed by immunoblotting with anti-phosphothreonine antibodies from Zymed.

**B)** As in A but with 5 mM MgCl₂ instead of MnCl₂. No activity of PBCP is detectable in these conditions.
Supplemental Figure 4. Analysis of state transitions by chlorophyll fluorescence at 77 K

Fluorescence emission spectra were measured with excitation at 480 nm on crude extracts frozen in liquid nitrogen. Seedlings grown in white light for 10 days were placed under far-red light for 90 min, transferred to blue light for 40 minutes to induce state 2, and returned to far-red light for 20 minutes to return to state 1. The fluorescence emission peak at 685 nm corresponds to PSII, at 732 nm to PSI. The curves were normalized to the PSII emission at 685 nm. The transition from state 1 to state 2 is apparent as an increase in the relative intensity of the PSI peak. The amplitude and the kinetics of this transition in pbcpOE are intermediate between the wild type and the stn7 mutant.