Identification of a Photosystem II Phosphatase Involved in Light Acclimation in Arabidopsis

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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 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 PROTEIN PHOSPHATASE1/THYLAKOID-ASSOCIATED PHOSPHATASE38, 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 (PQ), the cytochrome b6f complex, and plastocyanin (Eberhardt 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; Lemelle and Rochaix, 2010; Minagawa, 2011). A mobile part of the light-harvesting complex II (LHCII) light-harvesting antenna is dynamically allocated to PSII or PSI depending on the redox state of the 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 PROTEIN PHOSPHATASE1/THYLAKOID-ASSOCIATED PHOSPHATASE38 (PPH1/TAP38) phosphatase (Pribil et al., 2010; Shapiguzov et al., 2010) results in the association of the LHCII components with PSI (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 (Depège 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 nonappressed stromal lamellae (Dekker and Boekema, 2005). 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. Photooxidation 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 photooxidative damage under excess light (Eberhard et al., 2008; Li et al., 2009). Photooxidative 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 downregulate light harvesting and alleviate photooxidative 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; Tikkkanen and Aro, 2012). Whereas phosphorylation of LHCII by the STN7 kinase is inhibited under high light, most likely through the thioredoxin pathway (Rintamäki et al., 2000; Martinsuo et al., 2003), phosphorylation of the PSII core subunits is enhanced (Tikkanen et al., 2008, 2010).

The D1 subunit of PSII is the primary target of photooxidative 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-González and Aro, 2002; Takahashi and Murata, 2008). This process is facilitated by the protein kinase STN8 (Tikkanen et al., 2008), which shows specificity toward 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 dephosphorylation 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 overexpressing the phosphatase.

RESULTS

Efficient Dephosphorylation of the PSII Core Requires the Protein Phosphatase PBCP

We designed a genetic screen for chloroplast phosphatases involved in dephosphorylation of thylakoid proteins in Arabidopsis thaliana (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 coexpression 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 dephosphorylation of thylakoid proteins after a shift from white to far-red light, using a streamlined immunoblot assay with antiphosphothreonine antibodies (Rintamäki et al., 1997). The PPH1/TAP38 phosphatase previously identified in this screen is required for efficient dephosphorylation of LHCII antenna proteins and transition from state 2 to state 1 (Fribil et al., 2010; Shapiguzov et al., 2010).

Using the same screen, we identified a different phosphatase that is required for efficient dephosphorylation 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 LHClI and of PSII, as well as other thylakoid proteins, such as THYLAKOID SOLUBLE PROTEIN9 and CALCIUM SENSING (CaS) (Vener, 2007; Vainonen et al., 2008; Fristedt et al., 2009b; Reiland et al., 2011). Upon exposure to far-red light, which favors PSII excitation, the PQ pool is oxidized, a transition to state 1 is induced, and several thylakoid proteins are dephosphorylated. In the wild type, strong dephosphorylation of the core subunits of PSII and of the LHCII antenna is apparent within 20 min in immunoblots with antiphosphothreonine antibodies (from Zymed; Figure 1A).

By contrast, phosphorylation of the PSII subunits CP43, D2, and D1 is partly retained even after 40 min in the mutant affecting PBCP (for photosystem II core phosphatase; At2g30170), while the dephosphorylation of the LHClI antenna proceeds as efficiently as in the wild type. It is interesting to contrast the specificity of the pbcp-1 phenotype with that of ppb1-3, where dephosphorylation of the PSII subunits proceeds normally while the LHClI antenna remains phosphorylated (Figure 1A). Thus, the two phosphatases seem to have complementary roles:
PBCP is primarily required for efficient dephosphorylation of the PSII core, while PPH1/TAP38 is required for efficient dephosphorylation of the LHCII antenna.

Mass Spectrometry Analysis of PBCP-Dependent Thylakoid Protein Dephosphorylation

To measure the phosphorylation of thylakoid proteins specifically and quantitatively, we used two methods based on HPLC 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 min. 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 immobilized metal ion affinity chromatography (IMAC) 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 dephosphorylated 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 dephosphorylated 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 \textit{pbcp-1}
The two methods indicate that PBCP is required for efficient dephosphorylation 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 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’ untranslated region (see Supplemental Figure 1 online) (Alonso and Ecker, 2006; Woody et al., 2007). Both homozygous mutants show similar delays in dephosphorylation of the core proteins of PSII (Figure 1B). In this panel, an antiphosphothreonine 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 phosphoproteins detected in Figures 1A and 1B (Vainonen et al., 2005). In particular, the antibodies from Zymed detect phosphorylation of the PSII subunits more efficiently; conversely, those from Cell Signaling recognize LHCII more strongly.

### Table 1. Ratios of Phosphopeptides in pbcp-1 versus Wild-Type Leaves

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Identified Phosphopeptide</th>
<th>At Gene Identifier</th>
<th>pbcp-1/Wild Type Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>Ac-tAILER</td>
<td>AtCG00020</td>
<td>1.4 ± 0.1**</td>
</tr>
<tr>
<td>D2</td>
<td>Ac-tlALGK</td>
<td>AtCG00270</td>
<td>1.3 ± 0.1**</td>
</tr>
<tr>
<td>PsbH</td>
<td>AtOVTQEDSSR</td>
<td>AtCG00710</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>PsbH</td>
<td>AtOQVEDSSR</td>
<td>AtCG00710</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>LHCb 1.1</td>
<td>Ac-RKIVAKPK</td>
<td>At1g29910</td>
<td>0.7 ± 0.1**</td>
</tr>
<tr>
<td>LHCb 1.2</td>
<td></td>
<td>At1g29920</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>LHCb 1.3</td>
<td></td>
<td>At1g29930</td>
<td></td>
</tr>
<tr>
<td>LHCb 1.5</td>
<td></td>
<td>At2g34420</td>
<td></td>
</tr>
<tr>
<td>LHCb 2.1</td>
<td>Ac-RRtVK</td>
<td>At2g05100</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>LHCb 2.2</td>
<td></td>
<td>At2g05070</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>LHCb 2.4</td>
<td></td>
<td>At3g27690</td>
<td></td>
</tr>
<tr>
<td>CaS</td>
<td>SGKFLPSSD</td>
<td>At5g23060</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>

Some members of the LHCb family share a common tryptic peptide as shown; phosphorylation cannot be ascribed to a specific gene product. “Ac” denotes N-terminal acetylation and “t” represents P-Thr. Results are from four IMAC experiments with differential stable isotope labeling. Asterisks indicate a statistically significant difference at P < 0.01 in the Student’s t test.

We could also detect and quantify two phosphorylated forms of PsbH. After far-red treatment, the isoform of PsbH that is monophosphorylated at Thr-2 was over twofold 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 diphasphorylated at Thr-2 and Thr-4 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 overphosphorylated in blue light (Table 2). The proportion of diphasphorylated PsbH in the wild type and in pbcp-1 diminished to a similar extent in far-red compared with blue, suggesting that PBCP is not required for dephosphorylation 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 antiphosphothreonine antibodies (Figure 1A). Under blue light, the mutant showed reduced levels of Lhcb1 phosphorylation compared with 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 spectrometry (MS) analysis; conversely, PsbH was not analyzed by immunoblotting. Taken together, the data from the two methods indicate that PBCP is required for efficient dephosphorylation of the core subunits of PSII (CP43, D1, D2, and PsbH-Thr2) but not of the LHCII proteins, such as Lhcb1 and Lhcb2.

### Table 2. In Vivo Phosphorylation Stoichiometry (Percentage of Phosphorylation) for the PSII Core Proteins from Wild-Type and pbcp-1 Leaves

<table>
<thead>
<tr>
<th>Protein</th>
<th>Wild Type</th>
<th>pbcp-1</th>
<th>Blue Light t Test</th>
<th>Wild Type</th>
<th>pbcp-1</th>
<th>Far-Red Light t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>42 ± 4</td>
<td>60 ± 6</td>
<td>**</td>
<td>20 ± 3</td>
<td>45 ± 4</td>
<td>**</td>
</tr>
<tr>
<td>D2</td>
<td>42 ± 7</td>
<td>56 ± 9</td>
<td>*</td>
<td>17 ± 4</td>
<td>32 ± 2</td>
<td>**</td>
</tr>
<tr>
<td>PsbH</td>
<td>35 ± 6</td>
<td>40 ± 2</td>
<td></td>
<td>20 ± 3</td>
<td>42 ± 3</td>
<td>**</td>
</tr>
<tr>
<td>PP-PsbH</td>
<td>23 ± 9</td>
<td>37 ± 4</td>
<td>**</td>
<td>12 ± 6</td>
<td>19 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

A single asterisk and a double asterisk represent statistically significant differences between the pbcp-1 mutant and the wild type at P < 0.05 and P < 0.01, respectively, in the Student’s t test.
We used Agrobacterium tumefaciens to transform the homozygous pbcp-1 mutant with a wild-type copy of the PBCP cDNA tagged with a triple-haemagglutinin (HA) epitope and placed under the control of the cauliflower mosaic virus 35S promoter. Among 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 dephosphorylation 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 dephosphorylation 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; Figure 1D). In this line, dephosphorylation of the core subunits of PSII was more rapid than in the wild type. Unexpectedly, the LHCII antenna was also somewhat less phosphorylated compared with the wild type in white light or after 20 min of far-red treatment. Since phosphorylation of LHCII is not elevated in the pbc mutants, it is not clear whether dephosphorylation of LHCII in pbcpOE indicates that the antenna is a target of PBCP or whether overexpression induces a loss of specificity and a promiscuous activity of the phosphatase toward LHCII.

The PBCP polypeptide deduced from the cDNA sequence has 298 residues, a molecular mass of 32 kD, and is predicted to carry an N-terminal transit peptide for chloroplast targeting by the algorithms TargetP (Emanuelsson et al., 2000), Wolfpsort (Horton et al., 2007), and Multiloc (Höglund et al., 2006). The PBCP protein is not predicted to contain any transmembrane domains. The mature protein belongs to the family of PP2C phosphatases, which comprises ~80 members in plants (Kerk et al., 2008; Xue et al., 2008) and includes the PPH1/TAP38 phosphatase. PBCP and its orthologs belong to a phylogenetic clade that extends from monocots and dicots to the lycophytes Selaginella moellendorffii, the moss Physcomitrella patens, and the green algae C. reinhardtii and Volvox carteri (see Supplemental Figure 2, Supplemental References 1, and Supplemental Data Set 1 online). 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 dephosphorylation assay with far-red light similar to those presented in Figure 1.

A polyclonal antiserum raised against PBCP recognizes a polypeptide of ~32 kD in the wild type (Figures 1C and 1D; see Supplemental Figure 1C online). 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 pbc-1 and pbcp-2 (see Supplemental Figure 1C online), indicating that both are strong or null alleles and confirming that the 32-kD 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 (Figures 1C and 1D).

**PBCP Is a Chloroplast Protein**

The deduced amino acid sequence of PBCP carries a predicted N-terminal transit peptide for chloroplast targeting. Indeed, D. Leister and coworkers (Schliebner et al., 2008) 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 MS (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 immunoblotting 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 (Figure 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 Dephosphorylates Subunits of PSII in Vitro**

As described above, PBCP belongs to the family of PP2C phosphatases, and thylakoid protein dephosphorylation is affected in the pbc mutants in plants. 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-His tag, was expressed in Escherichia coli. The enzyme was purified under denaturing conditions by Ni-affinity chromatography, allowed to refold by dialysis into nondenaturing conditions, and then further purified by size exclusion chromatography (Figure 3A). A His-tagged bacterial maltose binding protein (MBP) was expressed and purified in parallel as a negative control. The phosphatase
The activity of rPBCP was much higher with Mn$^{2+}$ than with Mg$^{2+}$ as consistent with some similar phosphatases of the PP2C class, the substrate, para-nitrophenyl phosphate (pNPP), and the additives indicated. Error bars represent the SD of triplicate measurements. Activity of rPBCP was first demonstrated with the chromogenic substrate, para-nitrophenyl phosphate (pNPP) (Figure 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 Cys 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 min 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. Dephosphorylation was monitored by immunoblotting with antiphosphothreonine antibodies. After 5 min of incubation, the dephosphorylation activity of rPBCP in the presence of Mn$^{2+}$ was apparent as a decrease in the phosphorylation of CP43, D1, and D2 (Figure 3C, lane 5 versus lane 1). Phosphorylation decreased by 40 to 50% for D1 and CP43, and ~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 (see Supplemental Figure 3 online). The addition of DTT did not have a detectable effect on the activity (Figure 3C, lane 6), but the sensitivity of the assay may be insufficient to detect the effect observed with pNPP as a substrate. Some dephosphorylation of the LHCII proteins was apparent in this in vitro assay, similar to what was observed in vivo for the pbcpOE overexpressor.

**Figure 3.** Recombinant PBCP Has Phosphatase Activity In Vitro.

(A) SDS-PAGE of purified rPBCP loaded under reducing and denaturing conditions. The sizes of the markers in lane M are indicated on the left (kD).

(B) In vitro spectrophotometric assay of PBCP activity. The dephosphorylation of 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 SD of triplicate measurements.

(C) Thylakoid membranes (containing 0.16 µg chlorophyll) isolated from the pbcp-1 mutant, exposed to white light for 15 min, and then incubated for 5 min in the dark in the presence of purified recombinant PBCP (5 µg), or with protein buffer or recombinant MBP as controls, in a final reaction volume of 100 µL containing 5 mM MnCl$_2$. The effects of DTT (10 mM) and EDTA (5 mM) were tested as indicated. The samples were analyzed by immunoblotting with antiphosphothreonine antibodies from Zymed.

**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 overexpressor, and the wild type by transmission electron microscopy. We compared 11-d-old seedlings treated with blue light for 3 h to favor thylakoid protein phosphorylation and further treated for 1.5 h with far-red light to promote dephosphorylation (Figure 4). To avoid any differences due to cell type—specific differentiation of the plastids, we always selected chloroplasts in the subepidermal 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 (Figures 4A to 4D). Thylakoid membrane folding in the pbcpOE was similar to the wild type (Figures 4E and 4F). 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 (Figure 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 toward fewer layers and showed only very few grana stacks with eight 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
Figure 4. Thylakoid Folding Is Altered in pbc mutants.

Electron micrographs of thin sections from leaves of 11-d-old seedlings exposed to blue light for 3 h and then shifted to far-red light for 1.5 h to induce dephosphorylation of the PSII core subunits. (B), (D), and (F) show larger magnifications of (A), (C), and (E), respectively. WT, the wild type. Bars = 500 nm.

(A) and (B) The wild type.

(C) and (D) pbc-2.

(E) and (F) pbcOE.
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 Fm2 induced by a saturating flash is lower than the maximal fluorescence Fm1 in far-red light, which induces state 1 and the association of LHCII with PSII (Figure 5A). The state transition parameter, qT = (Fm1 − Fm2) × 100/Fm1 was approximately +3 in the wild-type seedlings but decreased to a negative value of −2 in the stn7 mutant and was also strongly affected in the pph1-3 mutant, both of which were used as controls that are known to affect LHCII phosphorylation and state transitions (Figure 5B). qT was comparable to the wild type in the stn8 mutant and in the rescued line, as well as in the pbcp-1 and pbcp-2 mutants. Thus, state transitions are not significantly affected in the absence of PBCP. Interestingly, qT was strongly reduced in the pbcpOE overexpressor 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 F5, which is influenced by the redox state of the PQ pool. When the far-red light is turned off, F5 increases abruptly, reflecting the overreduction of the PQ pool, and then F5 gradually decreases as transition to state 2 allows reoxidation of the pool (Figure 5C). Compared with 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 F5 decrease in the transition from state 1 to state 2 were strikingly slower in the pbcpOE overexpressor, 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 (Figure 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 (see Supplemental Figure 4 online). 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 with the wild type, but not as strongly as in the stn7 mutant. Conversely, when the seedlings 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 F5 measurements at room temperature, this indicates that overexpression of PBCP affects state transition. These results can be correlated with the observation that the LHCII proteins are less phosphorylated in pbcpOE compared with the wild type (Figure 1D).

Figure 4. (continued).

(G) The length of thylakoid membranes in stromal lamellae 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 overexpressor (n = 9). The histograms show the length of membranes in domains with the indicated numbers of layers (one layer for stromal lamellae and two or more layers in grana stacks) as a fraction of the total length of membranes measured.
DISCUSSION

While PPH1/TAP38 was shown to be necessary for dephosphorylation of LHCII subunits (LHCB1 and LHCB2) (Pribil et al., 2010; Shapiguzov et al., 2010), here, we demonstrate that PBCP is required for efficient dephosphorylation 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, overexpression 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 (Figure 1, Table 1) or changes in the kinetics of state transitions (Figure 5; see Supplemental Figure 4 online). Thus caution should be used in interpreting the phenotype of the pbcpOE line, which is a dominant gain-of-function mutant, since overexpression 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 toward LHCII, which only becomes apparent upon overexpression, 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

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 a FluorCam (PhotoSystems Instruments). After a preincubation of 30 min in darkness, actinic light (represented below as a white bar) was turned on for 17 min to induce state 2 before maximal fluorescence (Fm2) was measured with a saturating flash (arrow). Far-red light was then turned on for 17 min (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. WT, the wild type.

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

Figure 5. State Transitions in the pbcp Mutants and pbcpOE Over-expressor.

(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.
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 Thr-2 and Thr-4. We find that in the pbcp mutant, it is Thr-2 that retains phosphorylation in far-red light, whereas Thr-4 is dephosphorylated as in the wild type. However, MS analysis of phosphorylation in the stn8 mutant has shown that the STN8 kinase is responsible for phosphorylation at Thr-4 and that this requires prior phosphorylation at Thr-2 (Vainonen et al., 2005; Fristedt and Vener, 2011). Thus, in the case of PsbH, antagonism of PBCP and STN8 might involve the dephosphorylation of Thr-2 by PBCP, which in turn would prevent phosphorylation by STN8 at Thr-4. Little is known on the physiological role of phosphorylation at the individual Thr residues in PsbH or in D2 and CP43. In C. reinhardtii, a mutation of PsbH that prevents phosphorylation of Thr-2 by replacing it with Ala had no apparent phenotypic consequence (O’Connor et al., 1998). Mutation of the D2 subunit changing Thr-2 to Ala allowed normal photosynthetic activity, but mutations to Asp or Glu, which mimic phosphorylation, strongly affected PSI accumulation (Fleischmann and Rochaix, 1999).

What then may be the function of STN8 and PBCP in light acclimation? In the stn8 mutant, phosphorylation of PSI is reduced and the size of the thylakoid membrane grana increases. Conversely, we observed in the pbcp mutants that phosphorylation of PSI 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 PSI by promoting unfolding of the membranes and allowing easier access of membrane proteases such as FtsH and cotranslational integration of D1 (Tikkonen et al., 2009; 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 dephosphorylation of the core subunits of PSI 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 dephosphorylation of PSI. As with some other PP2C phosphatases, PBCP is Mn²⁺ dependent and has negligible activity with Mg²⁺ 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 in vitro, both when assayed with pNPP or with thylakoid membranes. This observation is puzzling because in pumpkin (Cucurbita maxima) leaves NaF could prevent dephosphorylation of the PSII core subunits and retard degradation of D1 (Rintamäki 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 ~80 members in Arabidopsis or rice (Oryza sativa; 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 dephosphorylation of thylakoid membrane proteins, PP1H/TAP38 and PBCP have different substrate preferences. The presence of different phosphatases is one possible explanation for the various kinetic classes that were discerned in assays of endogenous phosphatase activity with isolated thylakoid membranes from pea (Pisum sativum) chloroplasts (Silverstein et al., 1993). The strong preference of PBCP for Mn²⁺ and its insensitivity to NaF distinguish it from both the stromal and the membrane phosphatases active on LHCII phosphoproteins, which were previously purified from pea chloroplasts (Hammer et al., 1995, 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 Columbia-0 background were obtained from the European Arabidopsis Stock Center (pbcp-1 is SALK_127920.31.10.N, and pbcp-2 is WiscDsLox_359F02). They were genotyped as described in Supplemental Figure 1 online. The stn7 (Bellaire 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’-ATTCGAGCGAGA-GAGCAAGCATGGCAG-3’) and reverse primer (5’-GTCGAGTCGACACAAT TTGGCAAC-3’) included XhoI and SalI sites, respectively. The PCR product was inserted in the XhoI and SalI sites of pCF399 under the control of the cauliflower mosaic virus 35S promoter and the NOS terminator with a triple HA epitope tag at the C terminus (Lorain et al., 2008). Plants of Arabidopsis were transformed by floral dipping using Agrobacterium tumefaciens GV3101 strain. T1, T2, and T3 progeny seedlings were selected on Murashige and Skoog medium supplemented with 1.5% Suc and 10 μg mL⁻¹ Basta (Pentanal).

**Dephosphorylation in Vivo**

For the genetic screen, seedlings were grown for 10 d on Murashige and 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 to 60 μE m⁻² s⁻¹). Dephosphorylation was monitored as described (Shapiguzov et al., 2010) by placing seedlings under far-red light-emitting diodes (LEDs; L735; Epix) for the designated time. Seedlings were snap-frozen in liquid nitrogen and ground twice for 10 s with glass beads in a triturator (Silamat 55; Ivozar Vivadent).

Protein extracts were prepared by incubating in lysis buffer containing 100 mM Tris-HCl, pH 7.7, 2% SDS, 50 mM NaF, and 2× Protease Inhibitor Mixture (Sigma-Aldrich) for 30 min at 37°C. The samples were separated by Tris-Gly SDS-PAGE in 12% acrylamide gels supplemented with 6 M urea, which allowed of better separation of the D1 and D2 phosphoproteins. Immunodetection was performed using antiphosphothreonine antibodies (Zymed or Cell Signaling) or antiactin as a control (Sigma-Aldrich).

**Analyses of in Vivo Protein Phosphorylation by MS**

Thylakoids for MS analyses were isolated from 15-d-old seedlings grown on Murashige and Skoog agar in a growth chamber (Percival CU36LSL) in long days (16 h light and 8 h dark) at 22°C under fluorescent white light (50 to 60 μE m⁻² s⁻¹). Dephosphorylation was monitored as described (Shapiguzov et al., 2010) by placing seedlings under far-red light-emitting diodes (LEDs; L735; Epix) for the designated time. Seedlings were snap-frozen in liquid nitrogen and ground twice for 10 s with glass beads in a triturator (Silamat 55; Ivozar Vivadent).

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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 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 the wild type and pbcp-1 was performed as an internal control. For analysis of the peptides, an online 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 nonphosphorylated 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 nonphosphorylated peptide intensities in each LC-MS chromatogram using normalization for the difference in the signal intensities of the phosphorylated and dephosphorylated peptide ions from the D1, D2, and PbsH proteins as determined (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-His tag and expressed in Escherichia coli strain BL21. Following cell lysis and centrifugation at 7600g, the pellet containing recombinant PBCP was solubilized in 6 M guanidine with phosphate buffer at pH 8.0. Cell debris was pelleted at 23,000g, and the denatured proteins were loaded onto Ni-nitrotriacetic acid (Ni-NTA) resin (Qiagen). The Ni-NTA was washed with 4 M urea (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. EDTA (10 mM) 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 23,000g 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 ~30 kD 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 (Qiagen) followed by preparative SDS-PAGE.

Activity Assays of Recombinant PBCP

Recombinant PBCP phosphatase activity was assayed in vitro by monitoring the dephosphorylation of 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 MnCl₂ or MgCl₂, 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 MnCl₂ or MgCl₂.

Plants for thylakoid extraction were cultivated for 3 weeks on soil in growth chambers at 24°C under white light (100 µE m⁻² s⁻¹) 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 MgCl₂ or 5 mM MnCl₂. 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, MBP, or buffer was 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× gel-loading lysis buffer (10% SDS, 40% Suc, 1 mM EDTA, 50 mM Tris-HCl, pH 6.8, and 20% β-mercaptoethanol). Thylakoid protein phosphorylation was analyzed by immunoblotting with antiphosphothreonine 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, 12 h dark). The procedure was performed on ice. The leaves were ground in 20 mM HEPES-KOH, pH 8.0, 5 mM EDTA, and 330 mM sorbitol with a Polytron mixer (Kinematica). The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 1000g 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 (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 (21,000g for 10 min). The soluble fraction enriched in chloroplast stromal proteins was cleared by an additional centrifugation step (21,000g for 30 min). The samples were treated with 10% trichloroacetic acid to precipitate protein.

Transmission Electron Microscopy

Seedlings were grown on Murashige and Skoog agar plates (16 h light, 8 h dark, 50 to 60 µE m⁻² s⁻¹) for 11 d. 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 postfixed in 1.5% OsO₄ for 2 h, washed three 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 plasids located in adaxial subepidermal cells (presumptive palisade parenchyma cells). The lengths of membranes from electron micrographs were measured using ImageJ software (http://
Chlorophyll Fluorescence Analysis

Room temperature chlorophyll fluorescence was monitored with a FluorCam 800MF kinetic imaging fluorimeter (PhotoSystems Instruments) essentially as described (Willig et al., 2011). Seedlings grown on Mura-shige and Skoog agar for 15 d 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 Fs. 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 Fm, Fm2, and Fm1, respectively. The fluorescence signal was averaged for 26 seedlings. Chlorophyll fluorescence at 77K 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 MgCl2, and 10 mM NaF. The samples were filtered and frozen in liquid nitrogen, and chlorophyll fluorescence at 77K was recorded on a Jasco FP-750 spectrofluorometer. Excitation was at 480 nm (slit width 5 nm) and emission was recorded in the 600- to 800-nm range (slit width of 5 nm). Curves were normalized for the PSIi peak at 685 nm.

Supplemental Data

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

Supplemental Figure 1. Two Mutant Alleles of PBCP.
Supplemental Figure 2. Phylogenetic Analysis of PBCP Homologs.
Supplemental Figure 3. PBCP Activity in Vivo in the Presence of Mg2+ or NaF.
Supplemental Figure 4. Analysis of State Transitions by Chlorophyll Fluorescence at 77K.
Supplemental Data Set 1. Text File of Alignment Used for Phylogenetic Analysis in Supplemental Figure 2.
Supplemental References 1. Supplemental References for Supplemental Figure 2.

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AUTHOR CONTRIBUTIONS

M.G.-C., J.-D.R., A.V.V., I.S., A.S., G.F., B.I., and M.C. designed the research. I.S., A.S., G.F., B.I., and M.C. performed the research. M.G.-C., J.-D.R., A.V.V., I.S., A.S., G.F., B.I., and M.C. analyzed the data. M.G.-C. and J.-D.R. wrote the article.

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