PsbP Protein, But Not PsbQ Protein, Is Essential for the Regulation and Stabilization of Photosystem II in Higher Plants

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PsbP and PsbQ proteins are extrinsic subunits of photosystem II (PSII) and participate in the normal function of photosynthetic water oxidation. Both proteins exist in a broad range of the oxygenic photosynthetic organisms; however, their physiological roles in vivo have not been well defined in higher plants. In this study, we established and analyzed transgenic tobacco (Nicotiana tabacum) plants in which the levels of PsbP or PsbQ were severely down-regulated by the RNA interference technique. A plant that lacked PsbQ showed no specific phenotype compared to a wild-type plant. This suggests that PsbQ in higher plants is dispensable under the normal growth condition. On the other hand, a plant that lacked PsbP showed prominent phenotypes: drastic retardation of growth, pale-green-colored leaves, and a marked decrease in the quantum yield of PSII evaluated by chlorophyll fluorescence. In PsbP-deficient plant, most PSII core subunits were accumulated in thylakoids, whereas PsbQ, which requires PsbP to bind PSII in vitro, was dramatically decreased. PSII without PsbP was hypersensitive to light and rapidly inactivated when the repair process of the damaged PSII was inhibited by chloramphenicol. Furthermore, thermoluminescence studies showed that the catalytic manganese cluster in PsbP-deficient leaves was markedly unstable and readily disassembled in the dark. The present results demonstrated that PsbP, but not PsbQ, is indispensable for the normal PSII function in higher plants in vivo.

Plants, algae, and cyanobacteria are unique in their ability to catalyze the oxidation of water to molecular oxygen by using light energy (for review, see Barber, 2004). This process occurs in a PSII protein complex embedded in thylakoid membranes (Hillier and Babcock, 2001). In the luminal side of PSII, a cluster of three inorganic ions, manganese (Mn), calcium (Ca), and chloride (Cl), catalyzes water oxidation (Debus, 2000). Since ancestral cyanobacteria are the progenitors of chloroplasts in plants and algae, many PSII subunits are conserved between plants, algae, and cyanobacteria (Hankamer et al., 2001). However, the compositions of the PSII extrinsic proteins, which are associated with the luminal side of PSII and involved in oxygen evolution, are significantly different among these organisms (Seidler, 1996): Higher plants and green algae have a set of three extrinsic proteins (PsbO [33 kD], PsbP [23 kD], and PsbQ [17 kD]; Murata and Miyao, 1985), whereas cyanobacteria have a different set of proteins (PsbO, PsbU [12 kD], and PsbV [cytochrome c550]; Shen and Inoue, 1993; Enami et al., 2000). In addition, recent genomic and proteomic studies have demonstrated the existence of PsbP and PsbQ homologs in cyanobacteria (Kashino et al., 2002; De Las Rivas et al., 2004; Thornton et al., 2004). Therefore, it is reasonable to assume that five extrinsic proteins, PsbO, PsbP, PsbQ, PsbU, and PsbV, existed in ancestral cyanobacteria, but PsbU and PsbV were lost from the chloroplasts of higher plants during evolution.

The molecular functions of PSII extrinsic proteins have been studied in both higher plants and cyanobacteria. In higher plants, the functional characteristics of each protein in oxygen evolution in vitro have been extensively analyzed by release-reconstitution experiments using isolated oxygen-evolving PSII preparations. PsbO is responsible for the stable binding of the Mn cluster by facilitating Cl− retention in PSII (Miyao and Murata, 1984a). PsbP is involved in Ca2+ and Cl− retention in PSII (Ghanotakis et al., 1984a), and PsbQ mainly participates in Cl− retention (Akabori et al., 1984; Miyao and Murata, 1985). Finally, PsbP and PsbQ protect the Mn cluster from a bulky reductant (Ghanotakis et al., 1984b). The functions of cyanobacterial extrinsic proteins, elucidated by both in vitro and in vivo studies (Philbrick et al., 1991; Shen et al., 1998; Debus, 2000), are similar to those in higher plants. However, the protein nature of PsbP and PsbQ is...
greatly different between species, which suggests that they have changed considerably during evolution (Thornton et al., 2004).

Although in vitro studies have demonstrated the mechanistic roles of higher-plant PsbP and PsbQ under artificial conditions and their crystal structures have recently been determined to high resolution (Calderone et al., 2003; Ifuku et al., 2004), there is almost no information available on the physiological roles of these proteins in vivo. In previous studies, a *Chlamydomonas reinhardtii* mutant lacking PsbP showed decreased oxygen-evolving activity (Mayfield et al., 1987), and an increased concentration of Cl⁻ was required to restore oxygen evolution in isolated thylakoid membranes (Rova et al., 1994). However, the in vivo photosynthetic properties of the mutant cells were not well characterized in these studies. Furthermore, algal PsbP and PsbQ are different from those in higher plants in many respects, including primary sequence, binding features, and functional contributions to oxygen evolution (Suzuki et al., 2003, 2004), and these differences presumably reflect differences in ionic conditions in the thylakoid lumen between land plants and aqueous algae. Thus, the physiological functions of PsbP and PsbQ need to be determined in higher plants.

To address the above issues, we produced two transgenic tobacco (*Nicotiana tabacum*) lines, ΔPsbP and ΔPsbQ, in which the level of accumulation of PsbP or PsbQ was severely decreased by the RNA interference technique (RNAi). ΔPsbQ plants did not show any distinguishable differences from wild-type plants under the conditions examined, whereas ΔPsbP showed distinct phenotypes, such as a drastic reduction in growth rate and pale-green-colored leaves. In ΔPsbP leaves, photochemical reaction was severely impaired and PSI was hypersensitive to light. Thermoluminescence (TL) studies showed that in intact leaves of ΔPsbP, the Mn cluster was released from PSI in the dark and reassembled in the light. This study demonstrated that higher-plant PsbP, but not PsbQ, is essential for the normal function of PSI and plays a crucial role in stabilizing the Mn cluster in vivo.

**RESULTS**

The *psbP* and *psbQ* Genes Were Effectively Silenced by RNAi

In tobacco, PsbP is encoded by four nuclear iso- genes, and all four are expressed in plants (Hua et al., 1992). In the initial trials to obtain tobacco plants lacking PsbP protein (ΔPsbP), a cDNA fragment of psbP (350 bp) was used as an RNAi trigger. However, we could not obtain ΔPsbP lines. Then we created a new RNAi vector, pBE-psbP37 bpir, which was designed to express a hairpin RNA with a 37-bp self-complementary region that contained a 35-bp perfect match among all of the *psbP* isogenes (Fig. 1A). After transformation, two of 17 kanamycin-resistant lines showed a marked decrease in the amount of all of the *psbP* transcripts.

To obtain a tobacco plant that lacked PsbQ protein (ΔPsbQ), we first cloned the *psbQ* cDNA in tobacco by reverse transcription-PCR with the degenerated primers. All five clones sequenced contained an identical 687-bp open reading frame encoding a product consisting of 228 amino acids (GenBank accession no. AB188569), indicating that the obtained *psbQ* gene was mainly expressed in tobacco. A 347-bp fragment of *psbQ* cDNA was then used as an RNAi trigger in an RNAi vector, pHELLSGATE8-psbQir (Fig. 1A). After transformation, 12 of 15 kanamycin-resistant lines showed a marked decrease in the amount of *psbQ* transcripts.

The ΔPsbP and ΔPsbQ lines, which showed strong and stable gene silencing, were then selected and used for the subsequent analyses. In these lines, accumulation of the silenced gene could scarcely be detected by northern analysis (Fig. 1B). Quantitative analyses using real-time PCR showed that the amount of total *psbP* transcripts in ΔPsbP was decreased to 3% ± 1% of that in the wild type, whereas in ΔPsbQ the amount of

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**Figure 1.** RNAi constructs and suppression of the *psbP* and *psbQ* genes. A, RNAi vectors used to down-regulate the *psbP* and *psbQ* genes. El2, Doubled enhancer-like elements; 35S p, cauliflower mosaic virus 35S promoter; Ω, Ω sequence of *Tobacco mosaic virus*; NOST, nopaline synthase terminator; OCSt, octopine synthase terminator. B, Northern analysis for high-molecular-weight (HMW) and low-molecular-weight (LMW) RNAs. RNAs (5 μg) extracted from leaves of wild type and transformants were separated on agarose/formaldehyde gel and hybridized with the indicated [32P]-labeled DNA probes.
psbQ transcripts was below the level of detection (data not shown). The accumulation of short interfering RNA (siRNA), 21 to 23 bp of double-stranded RNA with a silenced-gene sequence, confirmed that the silenced phenotypes of the selected lines were caused by the normal RNAi pathway (Fig. 1B).

The plants were then transferred to soil, cultivated until they flowered, and self-fertilized. Since the segregation ratio for silenced: nonsilenced plants in T1 seedlings was approximately 3:1, our selected lines of ΔPsbP and ΔPsbQ seem to have a transgene(s) in a single locus. The T1 seedlings of ΔPsbP hardly grew in soil, so plants transferred to soil from in vitro culture (T0 generation) were used in subsequent analyses.

PSII Activity and Photoautotrophic Growth Were Impaired in ΔPsbP But Not in ΔPsbQ Tobacco

The ΔPsbQ plant grew normally under the conditions examined (25°C, 100 μE m⁻² s⁻¹), whereas ΔPsbP showed distinct phenotypes, such as a drastic reduction in growth rate and pale-green-colored leaves (Fig. 2). The chlorophyll (chl) content and the chl a/b ratio were lower in leaves of the ΔPsbP plant than in those of the wild-type and ΔPsbQ plants (Table I). The effects of RNAi on photosynthetic activity were evaluated by measuring chl fluorescence, and the results are summarized in Table I. The ΔPsbP plants showed much lower Fv/Fm values (an indicator of the potential PSII activity [quantum yield of PSII at the maximum level]) than wild-type and ΔPsbQ plants (approximately 0.82), although the values fluctuated within a range from 0 to 0.6, depending on leaf development and light intensity during growth. The calculated quantum yield of PSII (ΦPSII) under these growth conditions also was low (0.0–0.2) in ΔPsbP. The PSII activity of isolated thylakoid membranes was then measured using an oxygen electrode with p-phenylbenzoquinone and K₃Fe(CN)₆ as an artificial electron acceptor. The thylakoid membranes isolated from the wild-type and ΔPsbQ leaves showed high O₂ activities of around 250 μmol O₂ (mg chl)⁻¹ h⁻¹, whereas those from ΔPsbP showed very low activity (approximately 30 μmol O₂ [mg chl]⁻¹ h⁻¹; Table II). Further addition of Ca²⁺ and Cl⁻ scarcely enhanced the activity of ΔPsbP membranes. The Mn content in thylakoid membrane was relatively lower in ΔPsbP than in wild type and ΔPsbQ (Table II).

ΔPsbP Tobacco Was Hypersensitive to Light

The low value of Fv/Fm indicates the susceptibility of the ΔPsbP plant to light-induced damage. In fact, exposure of ΔPsbP leaves to light at 100 μE m⁻² s⁻¹ resulted in a marked decrease in the Fv/Fm value by 90% within 150 min when chloramphenicol was added to prevent the recovery of PSII by inhibiting the de novo synthesis of the reaction center D1 protein (Fig. 3A). In contrast, under these conditions, the Fv/Fm value decreased less than 20% in wild-type and ΔPsbQ leaves. In the absence of inhibitor, the decrease was limited to approximately 20% in ΔPsbP leaves, indicating that light-induced damage is largely compensated by a repair process in ΔPsbP. Therefore, the hypersensitivity of ΔPsbP tobacco to light is caused by enhanced photodamage to PSI rather than by damage to a repair process, which has been proposed as a mechanism of photoinhibition in cyanobacteria (Nishiyama et al., 2001). ΔPsbP plants developed symptoms of photodamage, as manifested photobleached leaves after 1 to 2 weeks of growth and exposure to light (150 μE m⁻² s⁻¹), and the Fv/Fm values decreased to <0.2 (Fig. 3B).

While Most PSII Subunits Were Accumulated, PSI Was Markedly Decreased in ΔPsbP Tobacco

The accumulation of PSII subunits in wild type, ΔPsbP, and ΔPsbQ was analyzed by immunoblotting with protein-specific antibodies to clarify the biochemical consequences of these genetic modifications. All of the PSII subunits except for PsbQ were accumulated in ΔPsbQ as in wild-type plants (Fig. 4). On the other hand, PSI core proteins and PsbO were accumulated in ΔPsbP to an almost similar extent as in wild type. This result was quite different from the observation that PSI was not assembled in a PsbO-RNAi plant of

![Figure 2. Phenotypes of wild-type, ΔPsbP, and ΔPsbQ plants. Tobacco plants precultivated on 0.5 × LS agar medium supplemented with 1.5% Suc were transplanted into soil and grown at 25°C under continuous light (100 μE m⁻² s⁻¹) for 3 weeks.](image-url)
Arabidopsis (Yi et al., 2005). Similar to a PsbO-RNAi plant of Arabidopsis, PsbQ was almost completely absent in \( \Delta \text{PsbP} \) tobacco. Instead of PsbQ, a protein band with a faster migration rate in gel, a probable degradation fragment of PsbQ, was detected in \( \Delta \text{PsbP} \) leaves.

The amounts of PSI subunit (PsaC) and PSI antenna (Lhca1) were markedly decreased in \( \Delta \text{PsbP} \). The amount of spectrophotometrically active PSI centers (P700) was significantly decreased in \( \Delta \text{PsbP} \) leaves (approximately 30% of the wild type; data not shown). Although the mechanism that underlies this phenomenon is unknown, it might be the result of acclimation to a state of low PSI pressure (for review, see Walters, 2005). The amount of PSI antenna (light-harvesting complex II) and an ATPase subunit (AtpB) was not different between transgenic and wild type, whereas the amounts of PsbS, cytochrome \( f \), and an NAD(P)H dehydrogenase subunit (Ndh-H) were significantly increased in \( \Delta \text{PsbP} \). The increased amount of PsbS, which plays a central role in nonphotochemical energy dissipation in PSI, indicates that the mechanism for dissipating light energy was activated in \( \Delta \text{PsbP} \). The up-regulation of NAD(P)H dehydrogenase in \( \Delta \text{PsbP} \) may be caused by accumulated oxidative stress, as suggested by Martin et al. (1996).

### Table II. PSII activity and Mn content of thylakoid membranes isolated from wild-type, \( \Delta \text{PsbP} \), and \( \Delta \text{PsbQ} \) tobacco plants

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>( \Delta \text{PsbP} )</th>
<th>( \Delta \text{PsbQ} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( O_2 ) evolutiona (( \mu )mol O(_2) [mg chl](^{-1}) h(^{-1}))</td>
<td>241 ± 10</td>
<td>26 ± 3</td>
<td>242 ± 12</td>
</tr>
<tr>
<td>+ 5 mM CaCl(_2)</td>
<td>245 ± 15</td>
<td>53 ± 3</td>
<td>240 ± 9</td>
</tr>
<tr>
<td>Mn (ng [mg chl](^{-1})(^{-1}))</td>
<td>0.66 ± 0.04</td>
<td>0.44 ± 0.01</td>
<td>0.64 ± 0.03</td>
</tr>
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</table>

*The buffer for measurement was 50 mM HEPES/NaOH, pH 7.6, containing 0.4 M Suc, 10 mM NaCl, 5 mM MgCl\(_2\), and 5 mM NH\(_4\)Cl. As electron acceptors for PSII, 0.5 mM K\(_3\)Fe(CN)\(_6\) and 0.5 mM p-phenylbenzoquinone were used. *Five millimolar CaCl\(_2\) was added to the buffer for measurements instead of 10 mM NaCl. *The Mn content was determined using an atomic absorption spectrophotometer.

Another marked difference between wild type and \( \Delta \text{PsbP} \) was the slower decay of chl fluorescence after a saturating pulse (Fig. 5A). The slower decay indicates a slower oxidation of the reduced quinone acceptor \( Q_A^- \) due to forward electron transfer to subsequent acceptors in \( \Delta \text{PsbP} \) compared to wild-type thylakoid membranes. A very similar observation was reported in a tobacco mutant that lacked PsbJ protein, in which PsbP and PsbQ were both lost (Regel et al., 2001). The substantial accumulation of \( Q_A^- \) in \( \Delta \text{PsbP} \) (determined by the value 1 – \( q_P \)) was also observed under continuous light even at low intensity (approximately 50 \( \mu \)E m\(^{-2}\) s\(^{-1}\); Fig. 5B). These results suggest that not only PSII activity but also subsequent electron transfer was inefficient in the thylakoid membrane of \( \Delta \text{PsbP} \), consistent with the low PSI level in \( \Delta \text{PsbP} \).

**Mn Cluster Was Unstable in the Dark in Leaves of \( \Delta \text{PsbP} \) Tobacco**

To investigate the redox properties of the donor and acceptor of PSI in vivo, we performed TL measurements...

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**Forward Electron Flow on Thylakoid Membranes Was Slowed in \( \Delta \text{PsbP} \) Tobacco**

PSII activity in vivo was further studied by analyzing induction curves of chl fluorescence. The \( F_v \) value, the minimum yield of chl fluorescence in dark-adapted leaves, was 3 times higher in \( \Delta \text{PsbP} \) than in wild-type plants, resulting in a low \( F_v/F_m \) value in \( \Delta \text{PsbP} \) (Fig. 5A). The high \( F_v \) level in \( \Delta \text{PsbP} \) was not affected by additional far-red light illumination, which specifically excites PSI and leads to the oxidation of the plastoquinone pool (data not shown). This indicates that the high \( F_v \) level in \( \Delta \text{PsbP} \) was not due to the reduction of the plastoquinone pool in the dark but rather to inactive PSII centers, which show a high chl fluorescence yield.

![Figure 3](image-url)

**Figure 3.** Hypersensitivity of \( \Delta \text{PsbP} \) tobacco to light. A, Time course of photoinactivation in wild-type (circle), \( \Delta \text{PsbP} \) (square), and \( \Delta \text{PsbQ} \) (triangle) leaves exposed to 100 \( \mu \)E m\(^{-2}\) s\(^{-1}\) for 150 min with (black symbols) or without (white symbols) the vacuum infiltration of chloramphenicol (200 \( \mu \)g mL\(^{-1}\)). The results are expressed as percentage of the initial \( F_v/F_m \) values (0.82 for wild type and \( \Delta \text{PsbP} \), and 0.53 for \( \Delta \text{PsbQ} \)) prior to light incubation. B, Chronic photodamage observed in \( \Delta \text{PsbP} \). The \( \Delta \text{PsbP} \) plants were grown under moderate light conditions (approximately 50 \( \mu \)E m\(^{-2}\) s\(^{-1}\)) for 2 months. The plant on the right was then transferred to standard light conditions (150 \( \mu \)E m\(^{-2}\) s\(^{-1}\)) and grown for another week.
in leaf segments of ΔPsbP plants. TL originates from a PSII reaction center that is re-excited by a charge recombination with an increase in the temperature of samples, where light-induced charge pairs in PSII are freeze-trapped (Vass and Inoue, 1992). The charge pairs involved can be identified by their emission temperatures. The B-band, which we investigated here, arises from a recombination of the S2 state of the Mn cluster with the semiquinone Q$_{B}$ (Rutherford et al., 1982). A single-flash excitation of the wild-type leaves after a short incubation (10 min) in the dark gave a prominent B-band (Fig. 6A). The ΔPsbP leaves also generated a B-band; however, the intensity of the flash-induced band gradually decreased during incubation in the dark, and only a very small residual band was induced after 162 min (Fig. 6B). No TL band was detected in ΔPsbP leaves that had been adapted to the dark for 5 h (data not shown). Incubation in the dark for 5 h did not affect either the intensity or peak temperature of the flash-induced B-band in wild-type leaves (data not shown). This indicates that dark incubation would cause the loss of the functional Mn cluster in PsbP leaves. Alternatively, dark incubation might cause the reduction of Tyr-D radical on D2 protein in PsbP leaves. The reduced Tyr-D was shown to reduce rapidly the S$_2$ state of the Mn cluster to the S$_1$ state after a single-flash excitation (Vass and Styring, 1991). In both cases, no TL signal would be observed.

Interestingly, the incubation of dark-adapted ΔPsbP leaves under dim light (0.5 μE m$^{-2}$ s$^{-1}$) resulted in a gradual restoration of the capacity for B-band formation within approximately 10 min (Fig. 6C). The observed kinetics of recovery of TL signal suggests that it would be due to the photoassembly of the functional Mn cluster (Tamura and Cheniae, 1987) but not due to the reoxidation of the reduced Tyr-D, which is known to occur in the nanosecond or microsecond time scale (Faller et al., 2001). A similar phenotype was reported in ΔPsbO cells of the cyanobacterium Synechocystis 6803, in which the Mn cluster was destabilized due to the absence of the Mn-stabilizing PsbO protein, to be disassembled in the dark and photoassembled in the light (Burnap et al., 1996). This phenotype has not been reported in any mutants in eukaryotes including higher plants. Therefore, the present results indicate that in ΔPsbP leaves, the functional Mn cluster was readily dissociated from PSII in the dark, although it could be reassembled under dim light. This view apparently accounts for the lower Mn content in thylakoids from ΔPsbP leaves, as shown in Table II. Presumably, some Mn was lost during the isolation of thylakoids, after which Mn clusters were continuously destroyed but the released Mn ions were preserved in the fraction. This situation may be responsible for the very low O$_2$ evolution activity in ΔPsbP thylakoid preparations (Table II), and...
also partly for the low $F_v/F_m$ (Table I) since chl fluorescence was measured after 2 h of incubation in the dark. The peak temperatures of the B-bands tended to decrease and increase during dark disassembly (Fig. 6B) and light assembly (Fig. 6C), respectively. This may be ascribed to the changes in the pH of the thylakoid lumen upon the inactivation and activation of water oxidation.

**DISCUSSION**

This work is the first to describe the properties of tobacco plants that are deficient in PsbP and PsbQ ($\Delta$PsbP and $\Delta$PsbQ, respectively). Down-regulation of PsbQ has not been reported in any eukaryotes. Interestingly, down-regulation of $psbP$ in the obtained $\Delta$PsbP plants was not perfect (approximately 5% of wild type) compared to that of $psbQ$ in $\Delta$PsbQ (<0.5% of wild type), suggesting that the complete down-regulation of $psbP$ genes would cause fatal damage in higher plants. In our previous screening for high-$F_v$ chl fluorescence mutants of Arabidopsis, we could obtain the $psbo1$ mutant (Murakami et al., 2002), whereas we could not obtain a mutant that has a defect in the $psbP$ gene. Arabidopsis has two $psbO$ genes ($psbo1$ and $psbo2$), and both of them are expressed. In the $psbo1$ mutant, the presence of $psbO2$ partially compensates the defect of $psbo1$ and allows plant survival (Murakami et al., 2005). However, Arabidopsis has a single $psbP$ gene that is expressed (At1g06680), making it difficult to obtain knock-out or knock-down plants. Therefore, we used tobacco that has four $psbP$ genes and successfully obtained $\Delta$PsbP tobacco lines by RNAi. Although $\Delta$PsbP tobacco could grow photoautotrophically, it showed very severe phenotype especially in the seedling stage, and such a knock-down plant will be very difficult to obtain Arabidopsis. Our $\Delta$PsbP and $\Delta$PsbQ tobacco clearly showed that the lack of PsbP severely impaired the photochemical reaction of PSII in the light and led to disassembly of the Mn cluster in the dark, whereas the lack of PsbQ did not alter the plant phenotype. Thus, we concluded that PsbP, but not PsbQ, is indispensable for plant survival and normal PSII functions in higher plants in vivo.

**Higher-Plant PsbP Is Required to Maintain the Active Mn-Ca-Cl Cluster in Vivo**

The $\Delta$PsbP plant accumulated most of the PSII subunits, whereas its PSII activity in vivo was considerably low, as indicated by chl fluorescence. PsbP has been shown to be involved in the high-affinity binding of Ca$^{2+}$ and Cl$^-$ in PSII (Ghanotakis et al., 1984c; Miyao and Murata, 1984b, 1985). The cyanobacterial mutant that lacked PsbP was shown to require Ca$^{2+}$ and Cl$^-$ in the growth media for optimal oxygen evolution (Thornton et al., 2004). Therefore, it is reasonable to assume that a shortage of these ions is responsible for the impairment of PSII function in $\Delta$PsbP tobacco. Ca$^{2+}$ has been proposed to be a metal constituent of the Mn-Ca-Cl cluster and to participate in water-oxidation chemistry (Debus, 1992). In vitro experiments indicate that Ca$^{2+}$ bound to PSII is not equilibrated with bulk ions in solution in the presence of PsbP, but the absence of PsbP makes the Ca$^{2+}$ site exchangeable with the bulk ions with an affinity on the order of several millimolar (Ghanotakis et al., 1984c; Miyao and Murata, 1984b; Homann, 1988). The concentration of free Ca$^{2+}$ in the stroma is very low (2–6 $\mu$M; Kreimer et al., 1988; Johnson et al., 1995), and the Ca$^{2+}$/H$^+$ antiporter increases its concentration in the thylakoid lumen using light-induced $\Delta$pH formed across the thylakoid membranes (Ettinger et al., 1999). However, acidification of the thylakoid lumen...
upon ΔpH generation facilitates the release of Ca$^{2+}$
from PSII (Homann, 1988; Krieger and Weis, 1993),
presumably via the partial dissociation of PsbP. Thus,
the balance between the Ca$^{2+}$ concentration and the
pH inside the thylakoid lumens would regulate the PSII
function in vivo. The absence of PsbP would push this
balance toward a shortage of Ca$^{2+}$ in PSII. On the other
hand, higher plants usually contain the Cl$^{-}$ in abun-
dance, and its concentration in stroma is reported to
range from 30 to 60 mM (Demming and Gimmler, 1983).
Considering the relatively high permeability of thy-
laufoid membranes to Cl$^{-}$ (Schuldiner and Avron, 1971),
Cl$^{-}$ deficiency in PSII is less likely to be the main con-
tributor to the effects due to the absence of PsbP.

TLC studies in intact leaves of ΔPsbP indicated that
the Mn cluster was dissociated from PSII in the dark
and reassembled in the light (Fig. 6, B and C). In vitro
the Mn cluster was dissociated from PSII in the dark
activation could rapidly occur without PsbP in vivo.

Previous studies suggested that H$_2$O$_2$ is generated
from PSII by reducing Mn ions in higher oxidation
states to Mn$^{2+}$ (Ghanotakis et al., 1984b). Very recently,
PsbP was reported to have the ability to bind Mn and
facilitate the light-dependent ligation of the Mn cluster
to PSII, a process called photoactivation (Bondarava
et al., 2005). However, our results showed that photo-
activation could rapidly occur without PsbP in vivo.

Although we could not identify the endogenous
reductant that reduced Mn in ΔPsbP leaves, H$_2$O$_2$
may be a candidate of such a reductant. Reductants such as
ascorbate or ferrocyanide do not release Mn from PSII
depleted of PsbP and PsbQ (Ghanotakis et al., 1984b).
Previous studies suggested that H$_2$O$_2$ is generated
from the PSII acceptor side when the oxidation of Q$_{A}$
is perturbed (Schröder and Akerlund, 1990), or from
the PSII donor side when the function of the Mn
cluster is impaired (Schröder and Akerlund, 1986; Fine
and Frasch, 1992; Hillier and Wydrzynski, 1993;
Klimov et al., 1993; Arató et al., 2004). PSII depleted
of the Mn cluster has also been reported to generate
Q$_{A}$ efficiently (Chen et al., 1995). Since both the
acceptor side and donor side reactions of PSII were
impaired in ΔPsbP leaves, H$_2$O$_2$ may present around
PSII and attack the Mn cluster. Further studies were
obviously required to address this issue.

What Is the Physiological Role of PsbQ in Higher Plants?

The present data clearly demonstrate that PsbQ was
not necessary for either PSII function or growth under
normal conditions. We also grew ΔPsbQ tobacco in a
greenhouse (28°C, 700–1,000 μE m$^{-2}$ s$^{-1}$ in the day-
time), and no significant difference was observed
between ΔPsbQ and wild type (data not shown). In
vitro studies, PsbQ has been shown to be required
for optimum PSII activity under low Cl$^{-}$ conditions
(Akabori et al., 1984; Miyao and Murata, 1985) or
under conditions in which the function of PsbP is
impaired by truncation of its N terminus (Ifuku and
Sato, 2002). However, such conditions are unlikely to
occur naturally in vivo.

Although the physiological function of PsbQ in
higher plants is still unclear, our results and recent
publications suggest that the function and relationship
of PsbP and PsbQ in higher plants have evolved
differently from those in cyanobacteria and green
algae. (1) The function of PsbQ requires PsbP in both
higher plants (Miyao and Murata, 1985) and green
algae (Suzuki et al., 2003), but not in cyanobacteria
(Thornton et al., 2004). (2) The binding of PsbQ to PSII
requires PsbP in higher plants (Miyao and Murata,
1983, 1989), but not in cyanobacteria (Thornton et al.,
2004) and green algae (Suzuki et al., 2003, 2004).
In addition, PsbQ was degraded in ΔPsbP leaves, which
suggests that unassembled PsbQ is degraded by a pro-
tease. Consistent with this observation, a protease that
specifically degrades PsbQ has been reported in spin-
ach (Spinacia oleracea) leaves (Kuwabara and Suzuki,
1994), and a degradation fragment of PsbQ was
detected in ΔPsbP leaves by immunoblotting (Fig. 4).
These observations were in contrast to those reported
in cyanobacteria and green algae, where PsbQ can
accumulate and bind to PSII in the absence of PsbP
(Mayfield et al., 1987; de Vitry et al., 1989; Suzuki et al.,
2003; Thornton et al., 2004). Therefore, we assume that
PsbQ acts as an auxiliary, as if PsbQ is an accessory
subunit to support the function of PsbP in higher
plants. Alternatively, PsbQ could be required in vivo
under some extreme conditions. In fact, cyanobacterial
PsbQ has been shown to be essential for photoauto-
 trophic growth under conditions of limited Ca$^{2+}$, Cl$^{-}$,
and iron (Summerfield et al., 2005).

MATERIALS AND METHODS

Plant Material

Tobacco plants (Nicotiana tabacum cv Samsun NN) were grown on agar-
solidified 0.5× Linsmaier-Skoog (LS) medium supplemented with 1.5% Suc
under continuous light (10 μE m$^{-2}$ s$^{-1}$) at 25°C. For analytical purposes,
tobacco plants (T$_0$ generation) precultivated on LS agar medium were trans-
planted into soil and grown under continuous light (50–100 μE m$^{-2}$ s$^{-1}$)
at 25°C for 2 to 3 weeks. Fully developed leaves (fourth and fifth leaves from the
top) were used in all experiments.

RNAi Vector Construction and Transformation

To down-regulate the expression of the psbP gene, the RNAi vector pBE-
pbP/GUS, which has a fragment containing sense and antisense sequences
of the psbP gene (37 bp, respectively) separated by a 20-bp fragment of
the β-glucuronidase gene, was prepared. The binary vector pBE2113-GUS
(Mitsuhara et al., 1996) was used as a backbone. To down-regulate
the expression of the psbQ gene, the cDNA encoding tobacco PsbQ protein
was cloned by reverse transcription-PCR to produce the RNAi vector pHELLS-
GATE8-psbQir, which has a fragment containing sense and antisense sequences
of the psbQ gene (347 bp, respectively). The binary vector pHELLSGATE8
(Hellwell and Waterhouse, 2003) was used for construction. The experimental
details of the vector construction were described elsewhere (Yamamoto et al.,
2005). The above RNAi vectors were introduced into Agrobacterium tumefaciens
LBA4404, and tobacco plants were transformed by the leaf-disc method.
Isolation and Analysis of RNA

Total RNA was extracted from leaves with TRI-reagent (Sigma). High-molecular-weight RNA (rRNA and mRNA) and low-molecular-weight RNA, including siRNA, were isolated and separated by the method described by Hamilton et al. (2002). High-molecular-weight RNA was analyzed on a form-aldehyde-denaturing-agarose gel in 1X MOPS buffer (20 mM MOPS-KOH, pH 7.0, 5 mM sodium acetate, 1 mM EDTA) and blotted onto a charged nylon membrane (Hybond N+; Amersham). siRNA was analyzed as described by Hamilton and Baulcombe (1999). cDNA fragments of psbP or psbQ were labeled with ^32P and used as probes.

SDS-PAGE and Immunoblot Analysis of Protein

To isolate thylakoid membranes, leaves were chopped in a blender with the ice-cold buffer (50 mM HEPES-NAOH, pH 7.6, containing 0.4 mM Suc, 10 mM NaCl, 5 mM MgCl2, and 5 mM sodium ascorbate). The mixture was then filtrated and centrifuged, and the pellet was washed and resuspended in the same buffer without sodium ascorbate. The chl contents of thylakoid membranes were determined as described by Arnon (1949). Proteins corresponding to 3 µg chl were separated on 15% SDS-polyacrylamide gels. To analyze membrane-embedded protein, 6 µm urea was included in the gels. PsAc was analyzed by a Tricine-SDS-PAGE system (Schagger and von Jagow, 1987). Separated proteins were transferred to a polyvinylidene difluoride membrane using a semidry blotting system (Bio-Rad). Immunoblot detection was performed using an enhanced chemiluminescence system (ECL; Amersham Biosciences).

Photosynthetic Measurements

Oxygen evolution from thylakoid membranes was measured at 25°C with a Clark-type oxygen electrode (Hanzatech) with 0.5 mM K3Fe(CN)6 and 0.5 mM aldehyde-denaturing-agarose gel in 1X MOPS buffer (Hanzatech) with 0.5 mM K3Fe(CN)6 and 0.5 mM aldehyde-denaturing-agarose gel in 1X MOPS buffer (Hanzatech). The change in the absorbance of P700 at 820 nm was measured with a PAM-fluorometer (Walz). The minimum chl fluorescence at an open PSII center (Fm) and the steady-state P700 measurements. We thank Dr. T. Endo, Kyoto University, for his technical assistance in P700 measurements. We thank Dr. T. Matoh and Dr. N. Ochiai, Kyoto University, for their help in measuring Mn using an atomic absorption spectrophotometer. We thank all of the investigators who kindly provided specific antibodies as listed in “Materials and Methods.” Finally, we thank Mr. Y. Watanabe, Kyoto University, for his excellent technical assistance throughout this research.

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