

# PsbP Protein, But Not PsbQ Protein, Is Essential for the Regulation and Stabilization of Photosystem II in Higher Plants<sup>1</sup>

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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<sup>-</sup> retention in PSII (Miyao and Murata, 1984a). PsbP is involved in Ca<sup>2+</sup> and Cl<sup>-</sup> retention in PSII (Ghanotakis et al., 1984a), and PsbQ mainly participates in Cl<sup>-</sup> 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

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greatly different between species, which suggests that they have changed considerably during evolution (Thornton et al., 2004).

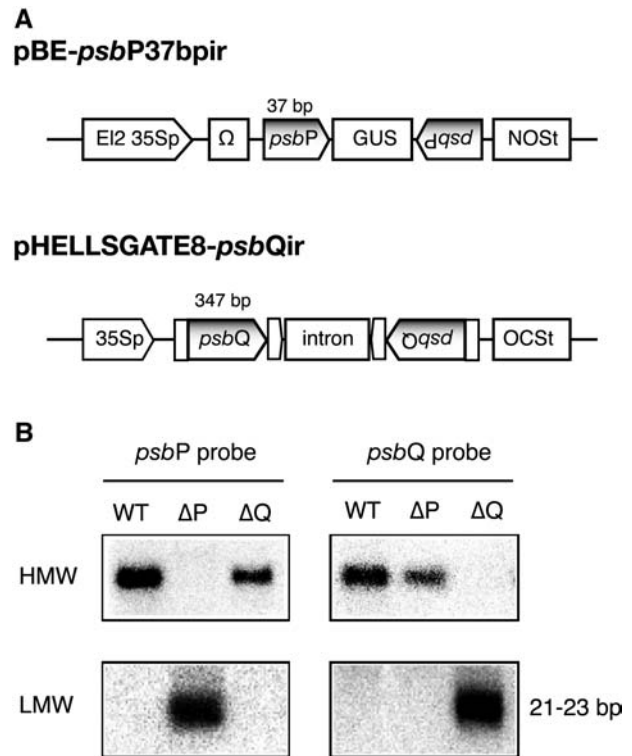
Although *in vitro* studies have demonstrated the mechanistic roles of higher-plant PsbP and PsbQ under artificial conditions and their crystal structures have recently been determined to high resolution (Calderone et al., 2003; Ifuku et al., 2004), there is almost no information available on the physiological 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  $\text{Cl}^-$  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,  $\Delta\text{PsbP}$  and  $\Delta\text{PsbQ}$ , in which the level of accumulation of PsbP or PsbQ was severely decreased by the RNA interference technique (RNAi).  $\Delta\text{PsbQ}$  plants did not show any distinguishable differences from wild-type plants under the conditions examined, whereas  $\Delta\text{PsbP}$  showed distinct phenotypes, such as a drastic reduction in growth rate and pale-green-colored leaves. In  $\Delta\text{PsbP}$  leaves, photochemical reaction was severely impaired and PSII was hypersensitive to light. Thermoluminescence (TL) studies showed that in intact leaves of  $\Delta\text{PsbP}$ , the Mn cluster was released from PSII 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 PSII 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 isoforms, and all four are expressed in plants (Hua et al., 1992). In the initial trials to obtain tobacco plants lacking PsbP protein ( $\Delta\text{PsbP}$ ), a cDNA fragment of *psbP* (550 bp) was used as an RNAi trigger. However, we could not obtain  $\Delta\text{PsbP}$  lines. Then we created a new RNAi vector, pBE-*psbP*37 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* isoforms (Fig. 1A). After



**Figure 1.** RNAi constructs and suppression of the *psbP* and *psbQ* genes. A, RNAi vectors used to down-regulate the *psbP* and *psbQ* genes. E12, Doubled enhancer-like elements; 35Sp, cauliflower mosaic virus 35S promoter;  $\Omega$ ,  $\Omega$  sequence of *Tobacco mosaic virus*; NOST, nopaline synthase terminator; OCSst, octopine synthase terminator. B, Northern analysis for high-molecular-weight (HMW) and low-molecular-weight (LMW) RNAs. RNAs (5  $\mu\text{g}$ ) extracted from leaves of wild type and transformants were separated on agarose/formaldehyde gel and hybridized with the indicated [ $^{32}\text{P}$ ]-labeled DNA probes.

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 ( $\Delta\text{PsbQ}$ ), 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-*psbQ*ir (Fig. 1A). After transformation, 12 of 15 kanamycin-resistant lines showed a marked decrease in the amount of *psbQ* transcripts.

The  $\Delta\text{PsbP}$  and  $\Delta\text{PsbQ}$  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  $\Delta\text{PsbP}$  was decreased to  $3\% \pm 1\%$  of that in the wild type, whereas in  $\Delta\text{PsbQ}$  the amount of

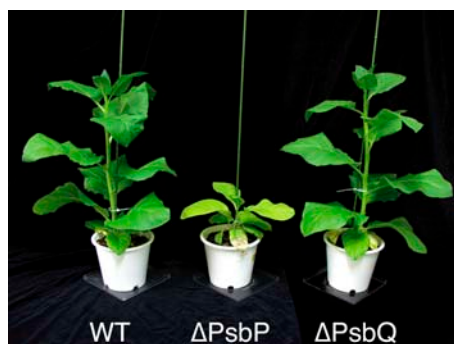
*psbQ* transcripts was below the level of detection (data not shown). The accumulation of short interfering RNA (siRNA), 21 to 23 bp of double-stranded RNA with a silenced-gene sequence, confirmed that the silenced phenotypes of the selected lines were caused by the normal RNAi pathway (Fig. 1B).

The plants were then transferred to soil, cultivated until they flowered, and self-fertilized. Since the segregation ratio for silenced:nonsilenced plants in  $T_1$  seedlings was approximately 3:1, our selected lines of  $\Delta$ PsbP and  $\Delta$ PsbQ seem to have a transgene(s) in a single locus. The  $T_1$  seedlings of  $\Delta$ PsbP hardly grew in soil, so plants transferred to soil from in vitro culture ( $T_0$  generation) were used in subsequent analyses.

### PSII Activity and Photoautotrophic Growth Were Impaired in $\Delta$ PsbP But Not in $\Delta$ PsbQ Tobacco

The  $\Delta$ PsbQ plant grew normally under the conditions examined ( $25^\circ\text{C}$ ,  $100 \mu\text{E m}^{-2} \text{s}^{-1}$ ), whereas  $\Delta$ PsbP 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  $\Delta$ PsbP plant than in those of the wild-type and  $\Delta$ PsbQ 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  $\Delta$ PsbP plants showed much lower  $F_v/F_m$  values (an indicator of the potential PSII activity [quantum yield of PSII at the maximum level]) than wild-type and  $\Delta$ PsbQ 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 ( $\Phi_{\text{PSII}}$ ) under these growth conditions also was low (0–0.2) in  $\Delta$ PsbP.

The PSII activity of isolated thylakoid membranes was then measured using an oxygen electrode with *p*-phenylbenzoquinone and  $\text{K}_3\text{Fe}(\text{CN})_6$  as an artificial electron acceptor. The thylakoid membranes isolated from the wild-type and  $\Delta$ PsbQ leaves showed high  $\text{O}_2$  activities of around  $250 \mu\text{mol O}_2 (\text{mg chl})^{-1} \text{h}^{-1}$ , whereas those from  $\Delta$ PsbP showed very low activity



**Figure 2.** Phenotypes of wild-type,  $\Delta$ PsbP, and  $\Delta$ PsbQ plants. Tobacco plants precultivated on  $0.5 \times$  LS agar medium supplemented with 1.5% Suc were transplanted into soil and grown at  $25^\circ\text{C}$  under continuous light ( $100 \mu\text{E m}^{-2} \text{s}^{-1}$ ) for 3 weeks.

**Table I.** Chl fluorescence and content of wild-type,  $\Delta$ PsbP, and  $\Delta$ PsbQ tobacco plants

Data presented are mean  $\pm$  SD of three measurements.

	Wild Type	$\Delta$ PsbP	$\Delta$ PsbQ
$F_v/F_m^a$	$0.82 \pm 0.01$	$0.47 \pm 0.03$	$0.81 \pm 0.01$
$\Phi_{\text{PSII}}^b$	$0.73 \pm 0.02$	$0.22 \pm 0.09$	$0.73 \pm 0.01$
Total chl (mg/g fresh weight)	$0.69 \pm 0.14$	$0.42 \pm 0.05$	$0.72 \pm 0.12$
Chl <i>a/b</i> ratio	$3.25 \pm 0.05$	$2.12 \pm 0.11$	$3.24 \pm 0.03$

<sup>a</sup>The  $F_v/F_m$  value was measured after incubation in the dark for 2 h. <sup>b</sup>The  $\Phi_{\text{PSII}}$  was measured under illumination in a growth chamber ( $100 \mu\text{E m}^{-2} \text{s}^{-1}$ ).

(approximately  $30 \mu\text{mol O}_2 [\text{mg chl}]^{-1} \text{h}^{-1}$ ; Table II). Further addition of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  scarcely enhanced the activity of  $\Delta$ PsbP membranes. The Mn content in thylakoid membrane was relatively lower in  $\Delta$ PsbP than in wild type and  $\Delta$ PsbQ (Table II).

### $\Delta$ PsbP Tobacco Was Hypersensitive to Light

The low value of  $F_v/F_m$  indicates the susceptibility of the  $\Delta$ PsbP plant to light-induced damage. In fact, exposure of  $\Delta$ PsbP leaves to light at  $100 \mu\text{E m}^{-2} \text{s}^{-1}$  resulted in a marked decrease in the  $F_v/F_m$  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  $F_v/F_m$  value decreased less than 20% in wild-type and  $\Delta$ PsbQ leaves. In the absence of inhibitor, the decrease was limited to approximately 20% in  $\Delta$ PsbP leaves, indicating that light-induced damage is largely compensated by a repair process in  $\Delta$ PsbP. Therefore, the hypersensitivity of  $\Delta$ PsbP tobacco to light is caused by enhanced photodamage to PSII rather than by damage to a repair process, which has been proposed as a mechanism of photoinhibition in cyanobacterium (Nishiyama et al., 2001).  $\Delta$ PsbP plants developed symptoms of photodamage, as manifested photobleached leaves after 1 to 2 weeks of growth and exposure to light ( $150 \mu\text{E m}^{-2} \text{s}^{-1}$ ), and the  $F_v/F_m$  values decreased to  $<0.2$  (Fig. 3B).

### While Most PSII Subunits Were Accumulated, PSI Was Markedly Decreased in $\Delta$ PsbP Tobacco

The accumulation of PSII subunits in wild type,  $\Delta$ PsbP, and  $\Delta$ PsbQ 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  $\Delta$ PsbQ as in wild-type plants (Fig. 4). On the other hand, PSII core proteins and PsbO were accumulated in  $\Delta$ PsbP to an almost similar extent as in wild type. This result was quite different from the observation that PSII was not assembled in a PsbO-RNAi plant of

**Table II.** PSII activity and the Mn content of thylakoid membranes isolated from wild-type,  $\Delta$ PsbP, and  $\Delta$ PsbQ tobacco plants

Data presented are mean  $\pm$  SD of three measurements.

	Wild Type	$\Delta$ PsbP	$\Delta$ PsbQ
O <sub>2</sub> evolution <sup>a</sup> ( $\mu$ mol O <sub>2</sub> [mg chl] <sup>-1</sup> h <sup>-1</sup> )	241 $\pm$ 10	26 $\pm$ 3	242 $\pm$ 12
+5 mM CaCl <sub>2</sub> <sup>b</sup>	245 $\pm$ 15	53 $\pm$ 3	240 $\pm$ 9
Mn (ng [mg chl] <sup>-1</sup> ) <sup>c</sup>	0.66 $\pm$ 0.04	0.44 $\pm$ 0.01	0.64 $\pm$ 0.03

<sup>a</sup>The buffer for measurement was 50 mM HEPES/NaOH, pH 7.6, containing 0.4 M Suc, 10 mM NaCl, 5 mM MgCl<sub>2</sub>, and 5 mM NH<sub>4</sub>Cl. As electron acceptors for PSII, 0.5 mM K<sub>3</sub>Fe(CN)<sub>6</sub> and 0.5 mM *p*-phenylbenzoquinone were used. <sup>b</sup>Five millimolar CaCl<sub>2</sub> was added to the buffer for measurements instead of 10 mM NaCl. <sup>c</sup>The Mn content was determined using an atomic absorption spectrophotometer.

Arabidopsis (Yi et al., 2005). Similar to a PsbO-RNAi plant of Arabidopsis, PsbQ was almost completely absent in  $\Delta$ PsbP tobacco. Instead of PsbQ, a protein band with a faster migration rate in gel, a probable degradation fragment of PsbQ, was detected in  $\Delta$ PsbP leaves.

The amounts of PSI subunit (PsaC) and PSI antenna (Lhca1) were markedly decreased in  $\Delta$ PsbP. The amount of spectrophotometrically active PSI centers (P700) was significantly decreased in  $\Delta$ 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 PSII pressure (for review, see Walters, 2005). The amount of PSII 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  $\Delta$ PsbP. The increased amount of PsbS, which plays a central role in nonphotochemical energy dissipation in PSII, indicates that the mechanism for dissipating light energy was activated in  $\Delta$ PsbP. The up-regulation of NAD(P)H dehydrogenase in  $\Delta$ PsbP may be caused by accumulated oxidative stress, as suggested by Martín et al. (1996).

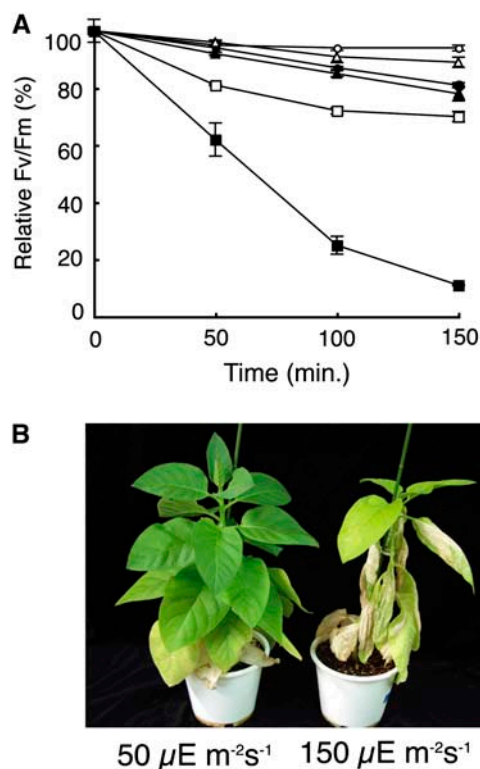
#### Forward Electron Flow on Thylakoid Membranes Was Slowed in $\Delta$ PsbP Tobacco

PSII activity in vivo was further studied by analyzing induction curves of chl fluorescence. The  $F_0$  value, the minimum yield of chl fluorescence in dark-adapted leaves, was 3 times higher in  $\Delta$ PsbP than in wild-type plants, resulting in a low  $F_v/F_m$  value in  $\Delta$ PsbP (Fig. 5A). The high  $F_0$  level in  $\Delta$ 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  $F_0$  level in  $\Delta$ 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.

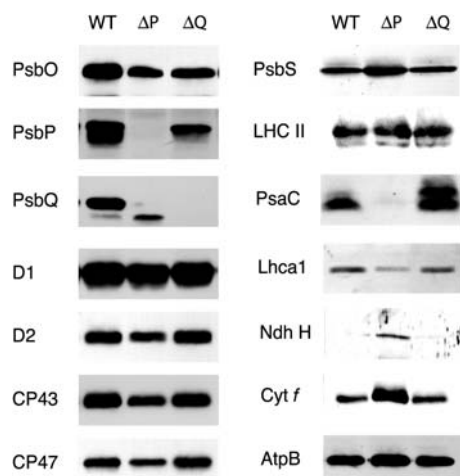
Another marked difference between wild type and  $\Delta$ PsbP was the slower decay of chl fluorescence after a saturating pulse (Fig. 5A). The slower decay indicates a slower oxidation of the reduced quinone acceptor  $Q_A^-$  due to forward electron transfer to subsequent acceptors in  $\Delta$ PsbP 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  $\Delta$ PsbP (determined by the value  $1 - qP$ ) 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  $\Delta$ PsbP, consistent with the low PSI level in  $\Delta$ PsbP.

#### Mn Cluster Was Unstable in the Dark in Leaves of $\Delta$ PsbP Tobacco

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



**Figure 3.** Hypersensitivity of  $\Delta$ PsbP tobacco to light. A, Time course of photoinactivation in wild-type (circle),  $\Delta$ PsbP (square), and  $\Delta$ 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  $\Delta$ PsbQ, and 0.53 for  $\Delta$ PsbP) prior to light incubation. B, Chronic photodamage observed in  $\Delta$ PsbP. The  $\Delta$ 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.

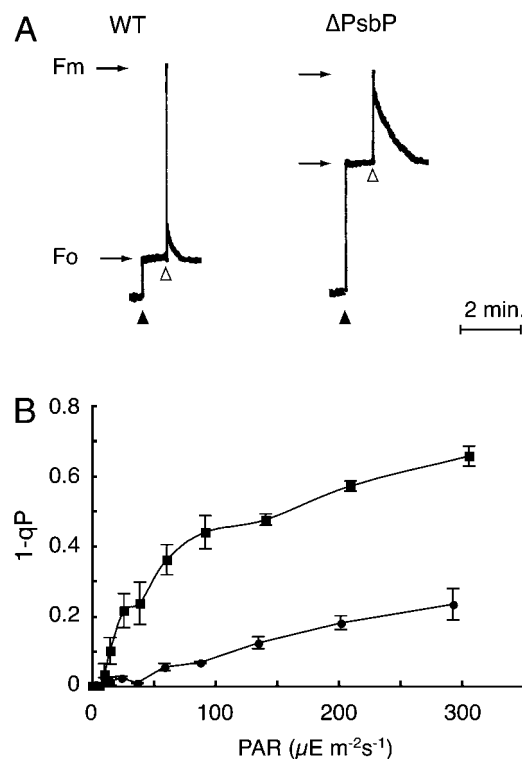


**Figure 4.** Immunoblot analyses of thylakoid membrane proteins. Proteins prepared from leaves and thylakoid membranes of tobacco plants grown under moderate light conditions (approximately  $50 \mu\text{E m}^{-2} \text{s}^{-1}$ ) were subjected to SDS-PAGE or Tricine-SDS-PAGE, and then detected by immunoblotting using specific antibodies. To detect degraded fragments, total proteins from leaves were used to analyze the extrinsic proteins (PsbO, PsbP, and PsbQ). Each lane contained  $5 \mu\text{g}$  of chl. WT, Wild-type tobacco;  $\Delta\text{P}$ ,  $\Delta\text{PsbP}$  tobacco;  $\Delta\text{Q}$ ,  $\Delta\text{PsbQ}$  tobacco.

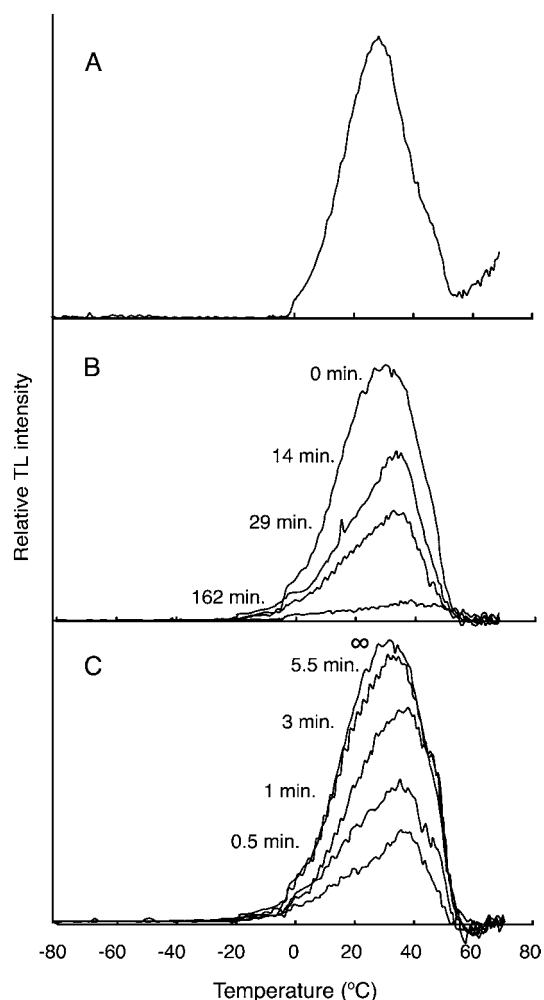
in leaf segments of  $\Delta\text{PsbP}$  plants. TL originates from a PSII reaction center that is re-excited by a charge recombination with an increase in the temperature of samples, where light-induced charge pairs in PSII are freeze-trapped (Vass and Inoue, 1992). The charge pairs involved can be identified by their emission temperatures. The B-band, which we investigated here, arises from a recombination of the  $\text{S}_2$  state of the Mn cluster with the semiquinone  $\text{Q}_\text{B}^-$  (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  $\Delta\text{PsbP}$  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  $\Delta\text{PsbP}$  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  $\text{S}_2$  state of the Mn cluster to the  $\text{S}_1$  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  $\Delta\text{PsbP}$  leaves under dim light ( $0.5 \mu\text{E m}^{-2} \text{s}^{-1}$ ) 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 Chenaie, 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  $\Delta\text{PsbO}$  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  $\Delta\text{PsbP}$  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  $\Delta\text{PsbP}$  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  $\text{O}_2$  evolution activity in  $\Delta\text{PsbP}$  thylakoid preparations (Table II), and



**Figure 5.** Chl fluorescence induction and parameters obtained from wild-type and  $\Delta\text{PsbP}$  plants. A, Fluorescence decay kinetics in wild type (WT) and  $\Delta\text{PsbP}$ . The black and white arrowheads indicate the application of measuring light and a saturating light pulse, respectively. B, Values of  $1 - \text{qp}$  under different light intensities in wild type (circle) and  $\Delta\text{PsbP}$  (square). The value of  $1 - \text{qp}$  represents the accumulation of the reduced quinone acceptor ( $\text{Q}_\text{A}^-$ ) within PSII and was determined 2 min after the application of actinic light at different intensities. The  $F_0'$  level was determined with the application of far-red light after each saturating light pulse. The plants were adapted to the dark for 2 h before measurements.



**Figure 6.** TL glow curves for the wild-type and  $\Delta$ PsbP leaves. A, The TL band observed in the wild-type leaves. B, The ability of the TL band to decay in the dark in  $\Delta$ PsbP leaves. The leaves were incubated in the dark at 20°C for the indicated period and then illuminated by a single flash at 0°C. B, The development of TL B-band capability during weak light illumination in dark-adapted  $\Delta$ PsbP leaves.  $\Delta$ PsbP leaves were dark adapted for 5 h and then incubated under dim light ( $0.5 \mu\text{E m}^{-2} \text{s}^{-1}$ ) at 20°C for the indicated period. The sample leaves were excited with a single flash at 0°C after 10 min of incubation in the dark. The TL B-band originates from recombination of the  $\text{Q}_\text{B}^-/\text{S}_2$  charge pair in PSII.

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- $F_o$  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  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  in PSII (Ghanotakis et al., 1984c; Miyao and Murata, 1984b, 1985). The cyanobacterial mutant that lacked PsbP was shown to require  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  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.  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  bound to PSII is not equilibrated with bulk ions in solution in the presence of PsbP, but the absence of PsbP makes the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  in the stroma is very low ( $2\text{--}6 \mu\text{M}$ ; Kreimer et al., 1988; Johnson et al., 1995), and the  $\text{Ca}^{2+}/\text{H}^+$  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  $\Delta$ pH generation facilitates the release of  $\text{Ca}^{2+}$  from PSII (Homann, 1988; Krieger and Weis, 1993), presumably via the partial dissociation of PsbP. Thus, the balance between the  $\text{Ca}^{2+}$  concentration and the pH inside the thylakoid lumen would regulate the PSII function *in vivo*. The absence of PsbP would push this balance toward a shortage of  $\text{Ca}^{2+}$  in PSII. On the other hand, higher plants usually contain the  $\text{Cl}^-$  in abundance, and its concentration in stroma is reported to range from 30 to 60 mM (Demming and Gimmler, 1983). Considering the relatively high permeability of thylakoid membranes to  $\text{Cl}^-$  (Schuldiner and Avron, 1971),  $\text{Cl}^-$  deficiency in PSII is less likely to be the main contributor to the effects due to the absence of PsbP.

TL studies in intact leaves of  $\Delta$ PsbP indicated that the Mn cluster was dissociated from PSII in the dark and reassembled in the light (Fig. 6, B and C). *In vitro* studies demonstrated that the removal of PsbP and PsbQ increased the susceptibility of the Mn cluster to exogenous reductants, such as  $\text{NH}_2\text{OH}$ , hydroquinone,  $\text{H}_2\text{O}_2$ , and  $\text{Fe}^{2+}$ , which cause the release of Mn from PSII by reducing Mn ions in higher oxidation states to  $\text{Mn}^{2+}$  (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 photoactivation could rapidly occur without PsbP *in vivo*. Therefore, the function of PsbP would protect the Mn cluster from exogenous reductants or retain reduced Mn ( $\text{Mn}^{2+}$ ) in the vicinity of the PSII reaction center.

Although we could not identify the endogenous reductant that reduced Mn in  $\Delta$ PsbP leaves,  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  is generated from the PSII acceptor side when the oxidation of  $\text{Q}_\text{A}^-$  is perturbed (Schröder and Åkerlund, 1990), or from the PSII donor side when the function of the Mn cluster is impaired (Schröder and Åkerlund, 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  $\text{O}_2^-$  efficiently (Chen et al., 1995). Since both the acceptor side and donor side reactions of PSII were impaired in  $\Delta$ PsbP leaves,  $\text{H}_2\text{O}_2$  may be 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  $\Delta$ PsbQ tobacco in a greenhouse ( $28^\circ\text{C}$ ,  $700\text{--}1,000 \mu\text{E m}^{-2} \text{s}^{-1}$  in the daytime), and no significant difference was observed between  $\Delta$ PsbQ and wild type (data not shown). In *in vitro* studies, PsbQ has been shown to be required for optimum PSII activity under low  $\text{Cl}^-$  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  $\Delta$ PsbP leaves, which suggests that unassembled PsbQ is degraded by a protease. Consistent with this observation, a protease that specifically degrades PsbQ has been reported in spinach (*Spinacia oleracea*) leaves (Kuwabara and Suzuki, 1994), and a degradation fragment of PsbQ was detected in  $\Delta$ PsbP 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 photoautotrophic growth under conditions of limited  $\text{Ca}^{2+}$ ,  $\text{Cl}^-$ , 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 $\times$  Linsmaier-Skoog (LS) medium supplemented with 1.5% Suc under continuous light ( $10 \mu\text{E m}^{-2} \text{s}^{-1}$ ) at  $25^\circ\text{C}$ . For analytical purposes, tobacco plants ( $T_0$  generation) precultivated on LS agar medium were transplanted into soil and grown under continuous light ( $50\text{--}100 \mu\text{E m}^{-2} \text{s}^{-1}$ ) at  $25^\circ\text{C}$  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-*psbP*37 bp, which has a fragment containing sense and antisense sequences of the *psbP* gene (37 bp, respectively) separated by a 20-bp fragment of the  $\beta$ -glucuronidase gene, was prepared. The binary vector pBE2113-GUS (Mitsuhashi 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 pHELLSGATE8-*psbQ*, which has a fragment containing sense and antisense sequences of the *psbQ* gene (347 bp, respectively). The binary vector pHELLSGATE8 (Hellmuth 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* LBA404, 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 formaldehyde-denaturing-agarose gel in 1× 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 <sup>32</sup>P 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 M Suc, 10 mM NaCl, 5 mM MgCl<sub>2</sub>, 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 5 μg chl were separated on 15% SDS-polyacrylamide gels. To analyze membrane-embedded protein, 6 M 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 K<sub>3</sub>Fe(CN)<sub>6</sub> and 0.5 mM *p*-phenylbenzoquinone as electron acceptors under saturating red light with an R-60 red long-pass filter (Kenko).

Chl fluorescence parameters were measured using a PAM-2000 chl fluorometer (Walz). The minimum chl fluorescence at an open PSII center ( $F_o$  and  $F_o'$ ) was determined using light (655 nm) at an intensity of 0.05 to 0.1 μE m<sup>-2</sup> s<sup>-1</sup>. A saturation pulse of white light (2,500 μE m<sup>-2</sup> s<sup>-1</sup> for 0.8 s) was applied to determine the maximum chl fluorescence at closed PSII centers in the dark ( $F_m$ ) and during actinic light illumination ( $F_m'$ ). The steady state of the chl fluorescence level ( $F_s$ ) was recorded during actinic light illumination (3–300 μE m<sup>-2</sup> s<sup>-1</sup>). The  $F_v/F_m$  and during steady-state photosynthesis ( $\Phi_{PSII}$ ) were calculated as  $(F_m - F_o)/F_m$  and  $(F_m' - F_s)/F_m'$ , respectively. Photochemical quenching  $qP$  was calculated as  $(F_m' - F_s)/(F_m' - F_o)$ .

The change in the absorbance of P700 at 820 nm was measured with a PAM-2000 chl fluorometer equipped with an emitter-detector unit (ED 800T; Walz; Schreiber et al., 1988). The change in absorbance induced by saturating far-red light represents the relative amount of photo-oxidizable P700.

TL was recorded with a home-built apparatus, as described elsewhere (Ono and Inoue, 1986). Leaf segments 10 mm in diameter were cooled to 0°C, illuminated with a saturating single turnover flash, and then quickly frozen in liquid N<sub>2</sub>. Light emission during sample warming was recorded against sample temperature.

## Antisera

Rabbit antibodies against PsbP and D1 were produced by the authors. Rabbit antibodies against PsbO and PsbQ were provided by the late Dr. A. Watanabe, Tokyo University. Rabbit antibodies against CP47 and light-harvesting complex II were provided by Dr. A. Tanaka, Hokkaido University. Rabbit antibodies against D2 and CP43 were gifts from Dr. Y. Kashino, Hyogo Prefectural University. Rabbit antibodies against Ndh-H were produced by Mr. A. Takabayashi, Kyoto University. Rabbit antibodies against AtpB (TF1-B) were a gift from Dr. T. Hisabori, Tokyo Industrial University. Rabbit antibodies against Psac and Lhc1a and hen antibodies against PsbS were purchased from AgriSera.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AB188569.

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