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Xiaoping Yi
Louisiana State University

Stefan R. Hargett
Louisiana State University

Haijun Liu
Louisiana State University

Laurie K. Frankel
Louisiana State University

Terry M. Bricker
Louisiana State University

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The PsbP Protein Is Required for Photosystem II Complex Assembly/Stability and Photoautotrophy in *Arabidopsis thaliana*[*]

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Xiaoping Yi, Stefan R. Hargett, Haijun Liu, Laurie K. Frankel, and Terry M. Bricker[1]

From the Division of Biochemistry and Molecular Biology, Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803

Interfering RNA was used to suppress the expression of the genes *At1g06680* and *At2g30790* in *Arabidopsis thaliana*, which encode the PsbP-1 and PsbP-2 proteins, respectively, of photosystem II (PS II). A phenotypic series of transgenic plants was recovered that expressed intermediate and low amounts of PsbP. Chlorophyll fluorescence induction and Q_A^- decay kinetics analyses were performed. Decreasing amounts of expressed PsbP protein led to the progressive loss of variable fluorescence and a marked decrease in the fluorescence quantum yield (F_V/F_M). This was primarily due to the loss of the J to I transition. Analysis of the fast fluorescence rise kinetics indicated no significant change in the number of PS II$_\beta$ centers present in the mutants. Analysis of Q_A^- decay kinetics in the absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea indicated a defect in electron transfer from Q_A^- to Q_B, whereas experiments performed in the presence of this herbicide indicated that charge recombination between Q_A^- and the oxygen-evolving complex was seriously retarded in the plants that expressed low amounts of the PsbP protein. These results demonstrate that the amount of functional PS II reaction centers is compromised in the plants that exhibited intermediate and low amounts of the PsbP protein. Plants that lacked detectable PsbP were unable to survive in the absence of sucrose, indicating that the PsbP protein is required for photoautotrophy. Immunological analysis of the PS II protein complement indicated that significant losses of the CP47 and D2 proteins, and intermediate losses of the CP43 and D1 proteins, occurred in the absence of the PsbP protein. This demonstrates that the extrinsic protein PsbP is required for PS II core assembly/stability.

In higher plants, algae, and cyanobacteria, at least six intrinsic proteins appear to be required for oxygen evolution by PS II[2] (1–3). These are CP47, CP43, the D1 and D2 proteins, and the α and β subunits of cytochrome b_{559}. Insertional inactivation or deletion of the genes for these components results in the disassembly of the PS II complex and the complete loss of oxygen evolution activity (for review, see Ref. 4). Additionally, a number of low molecular mass, intrinsic membrane protein components are associated with PS II (5–7), although the functions of many of these proteins remain obscure. Although PS II complexes containing only these intrinsic components can evolve oxygen *in vitro*, they do so at low rates (~25–40% of control), are extremely susceptible to photoinactivation, and require high, nonphysiological levels of calcium and chloride for maximal activity (1, 3).

In higher plants and green algae, three extrinsic proteins, with apparent molecular masses of 33, 24, and 17 kDa, are required for high rates of oxygen evolution at physiological inorganic cofactor concentrations. The 33-kDa component, the PsbO protein, has been termed the manganese-stabilizing protein due to its stabilization of the manganese cluster during exposure to low chloride concentrations or to exogenous reductants. *In vitro*, the 24- and 17-kDa proteins (termed the PsbP and PsbQ proteins, respectively) appear to modulate the calcium and chloride requirements for efficient oxygen evolution. The precise roles of these proteins in oxygen evolution and PS II assembly/stability *in vivo*, however, remain unclear. These three extrinsic components interact with intrinsic membrane proteins and possibly with each other to yield fully functional oxygen-evolving complexes.

The mature PsbP protein is highly conserved (8) in higher plants. In *Arabidopsis*, there are two putative genes, *At1g06680* and *At2g30790*, which encode PsbP-1 and PsbP-2, respectively. It should be noted that initially only PsbP-1 was observed in *Arabidopsis* (9, 10) using two-dimensional IEF-SDS-PAGE. Recently, however, PsbP-2 has been detected during two-dimensional difference gel electrophoresis (11). In the cyanobacterium *Synechocystis* 6803, mutants in which the homologue of the *psb*P gene had been deleted exhibited reduced photoautotrophic growth as well as decreased water oxidation activity under $CaCl_2$-limiting conditions (7, 12), whereas in *Chlamydomonas*, a mutant which did not accumulate PsbP was deficient in photoactivation (13).

RNAi is a post-transcriptional gene-silencing process in which double-stranded RNA induces the degradation of homologous mRNA sequences (14). RNAi has been successfully applied as a powerful gene-silencing tool in a variety of organisms, including *Caenorhabditis elegans* and *Drosophila melanogaster*, and in mouse oocytes. It has also become a pop-

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[1] To whom correspondence should be addressed: Dept. of Biological Sciences Biochemistry and Molecular Biology Section, Louisiana State University, 206 LIfe Sciences Bldg., Baton Rouge, LA 70803. Tel.: 225-578-1555; Fax: 225-578-7258; E-mail: btbric@lsu.edu.

[2] The abbreviations used are: PS I and II, photosystems I and II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LiDS, lithium dodecyl sulfate; Tricine, *N*-tris[hydroxymethyl]methyl glycine.

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ular research methodology for investigating the physiological functions of target genes in plants (15). With respect to PS II membrane proteins, RNAi has been used to study the function of the PsbO and PsbQ proteins in *Arabidopsis* (16, 17) as well as the PsbP and PsbQ proteins in tobacco (18). The studies performed in tobacco indicated that RNAi suppression of the PsbQ proteins led to no observable phenotype, but RNAi suppression of the PsbP proteins led to retardation of photoautotrophic growth, lower quantum yield of PS II, loss of PsbQ, and an unstable manganese cluster that disassembled in the dark. Accumulation of the intrinsic PS II core proteins and the extrinsic PsbO protein in PsbP-suppressed tobacco plants was similar to that in wild type (18). Studies performed in *Arabidopsis* showed that the RNAi suppression of PsbQ led to the impaired assembly/stability of PS II in low light growth conditions, resulting in the loss of photoautotrophy (16). In our current study, we report on the RNAi suppression of PsbP expression in *Arabidopsis*. Our studies differ from those performed in tobacco (18) in that we find, in *Arabidopsis*, the PsbP protein appears to be required for photoautotrophy and that, in its absence, significant decreases in the amounts of the PS II core proteins D2 and CP47 occur. We provide fluorescence yield data, immunological data, and growth characterization to support the hypothesis that PsbP is required for PS II assembly/stability and photoautotrophic growth in *Arabidopsis*.

MATERIALS AND METHODS

RNA Interference Construct and Transformation—The pHANNIBAL vector (19) was used to construct an intron-spliced hairpin RNA (RNAi construct). The sequence (+261 to +528) of the *psb*P gene (*At1g06680*) was chosen to suppress the expression of both PsbP-1 and PsbP-2. This construct will be referred to as *psb*P-RNAi. The primers for *psb*P-RNAi were 5′-CCGAATTCGAAGCCAAAGACGAACAGA-3′ and 5′-CAGGTACCAGAGGCAGTCTCACCGA-3′ for the sense fragment and 5′-AGGATCCGAAGCCAAAGACGAACAGA-3′ and 5′-CCATCGATCAGAGGCAGTCTCACCGA-3′ for the antisense fragment of *psb*P. PCR was performed on a RapidCycler (Idaho Technology, Inc.) in thin-walled microcentrifuge tubes in 50-μl reactions containing 5 μl of 10× PCR reaction buffer, 1.5 μl of 50 mM $MgCl_2$, 1.5 μl of 2.5 mM dNTP mixture, 3 μl of 10 pM/μl primer mixture, 0.25 μl of 5 units/μl platinum *Taq* polymerase High Fidelity (Invitrogen), and 25 ng of *Arabidopsis* genomic DNA in purified water. Cycling parameters were a pre-denaturation step at 95 °C for 5 min followed by 35 amplification cycles (denaturing at 94 °C for 25 s, annealing at 55 °C for 25 s, and extension at 72 °C for 45 s) and a final extension at 72 °C for 7 min. The amplified sequence was cloned in both forward and reverse orientations flanking the Pdk intron of the pHANNIBAL vector. After construction and verification by sequencing, the expression region was excised from pHANNIBAL with NotI and then subcloned into pART27 for transformation of the *Agrobacterium* strain GV3101 by the freeze-thaw method (20). Four-week-old *Arabidopsis* plants (Col-0) were transformed by the floral dip method as described previously (21). Harvested seeds were surface-sterilized with 50% ethanol and 0.5% Tween 20 for 3 min, washed briefly with 95% ethanol, and then 70% ethanol for 3 min followed by washing three times with sterile water. Seeds were spread on solid MS medium containing 0.7% agar, 2% sucrose, 50 mg/liter kanamycin, and 400 mg/liter carbenicillin and then incubated for 2 days at 4 °C in the dark. Germination and the first 10 days of growth occurred under lighted conditions at 28 °C in Petri dishes, and then the plants were transplanted into culture boxes containing solid MS medium with 2% sucrose, 50 mg/liter kanamycin, and 400 mg/liter carbenicillin at a temperature of 23 °C under ~50 μmol photons/m^2/s continuous light. To test for photoautotrophic growth, plants were transplanted onto medium from which sucrose was omitted.

Screening—The presence of the RNAi construct in the kanamycin-resistant plant lines was confirmed by the use of PCR with primers designed to amplify the cauliflower mosaic virus 35S promoter and the targeted gene of the introduced DNA. All of the plants that exhibited the kanamycin-resistant phenotype also exhibited the expected 1.0-kbp amplification product, which was absent in the wild-type plants (data not shown). Individual kanamycin-resistant plants were screened for the presence of the PsbP protein by "Western" blotting. One leaf was placed in a 1.5-ml microcentrifuge tube and ground to a powder in the presence of liquid nitrogen. After evaporation of the liquid nitrogen, a protein isolation buffer (20 mM Tricine-NaOH, pH 8.4, 10 mM EDTA, 450 mM sorbitol, 0.1% bovine serum albumin) was added, followed by the addition of LiDS-PAGE solubilization buffer, and the samples were incubated on ice for at least 15 min. The samples were then centrifuged at 14,000 × *g* for 5 min before loading onto a 12.5–20% gradient polyacrylamide gel. PAGE, gel blotting, blocking, primary and secondary antibody probing, and chemiluminescent peroxidase substrate were used followed by exposure to x-ray film and photographic development. An antibody directed against the mature spinach PsbP was found to cross-react with the PsbP protein from *Arabidopsis* and was used in these studies. Other antibodies that were used to investigate the stability of the Photosystem II complex were the kind gifts from many investigators.

Fluorescence Measurements on Leaves—Fluorescence induction was monitored with a Photon Systems Instruments FL3000 dual modulation kinetic fluorometer (commercial version of the instrument described in Ref. 22). Both measuring and saturating flashes were provided by computer-controlled photodiode arrays. The flash profile exhibited a square shape for low power measuring flashes and deviated only 5% from an ideal square shape for saturating actinic flashes. For all of the fluorescence experiments, single leaves from wild-type and PsbP-suppressed plants grown on sucrose were excised and dark-incubated for 5 min before initiation of the experiments. In the standard fluorescence induction experiments (Kautsky experiments), data were collected in a logarithmic time series between 1 ms and 4 s after the onset of strong actinic light. In the flash fluorescence induction experiments, the kinetics of the rapid fluorescence rise following a single saturating flash delivered by light-emitting diodes were examined for 50 μs with 1-μs time resolution in the presence of DCMU. Data were collected at a frequency of 10 MHz with 12-bit resolution. The proportions of $PS\ II_\alpha$ and $PS\ II_\beta$ centers were calculated using proprietary Photon Systems Instruments software (22). In the fluorescence decay experiments, the kinetics of the electron transfer between Q_A^- and Q_B were examined in the absence of DCMU, while the recombination reactions of Q_A^- with PS II donor-

side components were examined in the presence of DCMU. For these experiments data were collected between 150 μs to 60 s following a single saturating flash. Data were analyzed using the equations outlined previously (23). In this mathematical treatment, three exponential decay components and a long-lived ($\tau > 10$ s) residual component were included. In the DCMU treatment experiments, the leaves were immersed in 40 μM DCMU, 0.1% Tween 20 in water for 30 min prior to performing the fluorescence experiments. Data were analyzed using the Origin 6.1 software package and proprietary software provided by Photon Systems Instruments.

Immunological Characterization of Thylakoid Proteins—For a more in-depth analysis of the protein complement of the thylakoid membranes, chloroplasts were isolated from wild-type and four mutant plant lines that expressed low levels of PsbP. These lines, selected from the initial screening, were RNAi-P1, -P8, and -P11. Leaves were ground in a glass homogenizer with a chloroplast isolation buffer (300 mM sorbitol, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 20 mM HEPES/KOH, pH 8.0, 10 mM $NaHCO_3$). The homogenate was then passed through two layers of Miracloth® (Calbiochem), and the chloroplasts were pelleted by centrifugation at 6000 $\times$ *g* for 5 min. The chloroplasts were then resuspended in a small amount of isolation buffer, and the chlorophyll concentration was determined by the method of Arnon (24). LiDS-PAGE was performed on a 12.5–20% gradient gel with 3 μg of chlorophyll loaded/lane. "Western" blotting, blocking, and probing with primary and secondary antibodies were as described above. For detection of the immobilized antibodies, a chemiluminescent substrate (SuperSignal® West Pico chemiluminescent substrate, Pierce) was used, and the x-ray film was exposed to the blots. After development, the x-ray films were scanned with a UMax PowerLook III scanner at 600-d.p.i. resolution and an 8-bit color depth.

RESULTS AND DISCUSSION

Screening of Transgenic Plants—Seeds from wild type and from plants transformed with the *psb*P-RNAi construct were distributed onto agar plates containing Murashige and Skoog medium supplemented with 50 μg/ml each of kanamycin and carbenicillin. Although wild-type seeds could not germinate successfully on medium containing these antibiotics, a few antibiotic-resistant seedlings were recovered from plants transformed with the RNAi construct. When, however, seeds from the treated plants were sown onto agar plates containing Murashige and Skoog medium plus antibiotics plus sucrose, many more antibiotic-resistant seedlings were recovered (data not shown). The presence of the *psb*P-RNAi construct in 10 antibiotic-resistant plants was confirmed by PCR amplification of the cauliflower mosaic virus 35S promoter region. All of the plants that exhibited antibiotic resistance also exhibited the 1-kbp PCR amplification product, indicating the presence of the cauliflower mosaic virus 35S promoter region of the RNAi construct (data not shown).

To screen individual transgenic plants for the presence of the PsbP proteins, "Western" blot analysis with a polyclonal antibody that recognizes the PsbP proteins was performed. The results from a typical screening experiment are shown in Fig. 1. In wild-type plants, two immunoreactive bands were observed

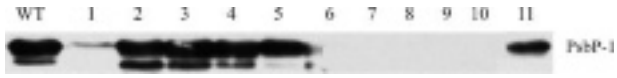

FIGURE 1. **Immunological screening for the presence of PsbP proteins in eleven transgenic plants.** Proteins from whole leaf extracts of wild-type (*WT*) and eleven transgenic plants (*1* through *11*) were resolved by LiDS-PAGE followed by "Western" blotting, probing with an anti-PsbP antibody, and subsequent chemiluminescent detection. Individual RNAi-P plants exhibited variable amounts of the PsbP protein.

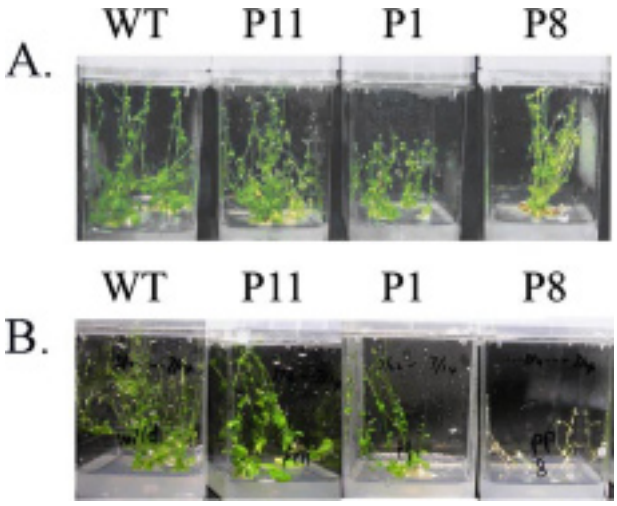

FIGURE 2. **Growth of wild type and RNAi-P transgenic plants on solid MS medium.** *WT*, Columbia wild type; *P1*, *P8*, and *P11* represent RNAi-PsbP1, -P8, and -P11 transgenic plant lines, respectively. *A*, growth on sucrose-containing solid Murashige and Skoog medium; *B*, photoautotrophic growth on solid Murashige and Skoog medium without sucrose. Please note that the medium of the transgenic plants contained kanamycin and carbenicillin in addition to salts.

that bound the anti-psbP protein antibody. The nature of the minor band is unclear at this time, although it may represent a migrational variant of the PsbP-1 protein. It should be noted that this minor band is not the PsbP-2 protein, because this component migrates at a significantly lower apparent molecular mass (11). No immunoreactive band was detected at the PsbP-2 location in the RNAi-suppressed plants, which we analyzed in this communication (data not shown). Most plants transformed with the *psb*P-RNAi construct showed either a partial or complete loss of the PsbP-1 band. In total, 14 plants were screened for the presence of the PsbP protein. These results indicated that 28% of the plants had expression levels similar to that of wild type for the PsbP-1 protein, 14% exhibited an intermediate level of expression, and 28% of the transgenic plants exhibited almost complete loss of the PsbP-1 protein. These results are consistent with the results obtained in other RNAi studies targeting other proteins. In almost all instances, different RNAi-containing plant lines exhibit varying degrees of suppression of the protein targets (20), often allowing the isolation of a graded phenotypic series of mutants deficient in the targeted component.

Plants That Express Low Levels of PsbP Cannot Grow Photoautotrophically—Plants containing *psb*P-RNAi that were germinated on Murashige and Skoog medium plus antibiotics plus sucrose were transplanted onto MS medium plus antibiotics. Prior to transplantation, the plant that lacked PsbP-1 (Fig. 2*A*, *plant P8*) was lighter green than the wild-type plant or plants that accumulated detectable amounts of PsbP-1 (Fig. 2*A*,

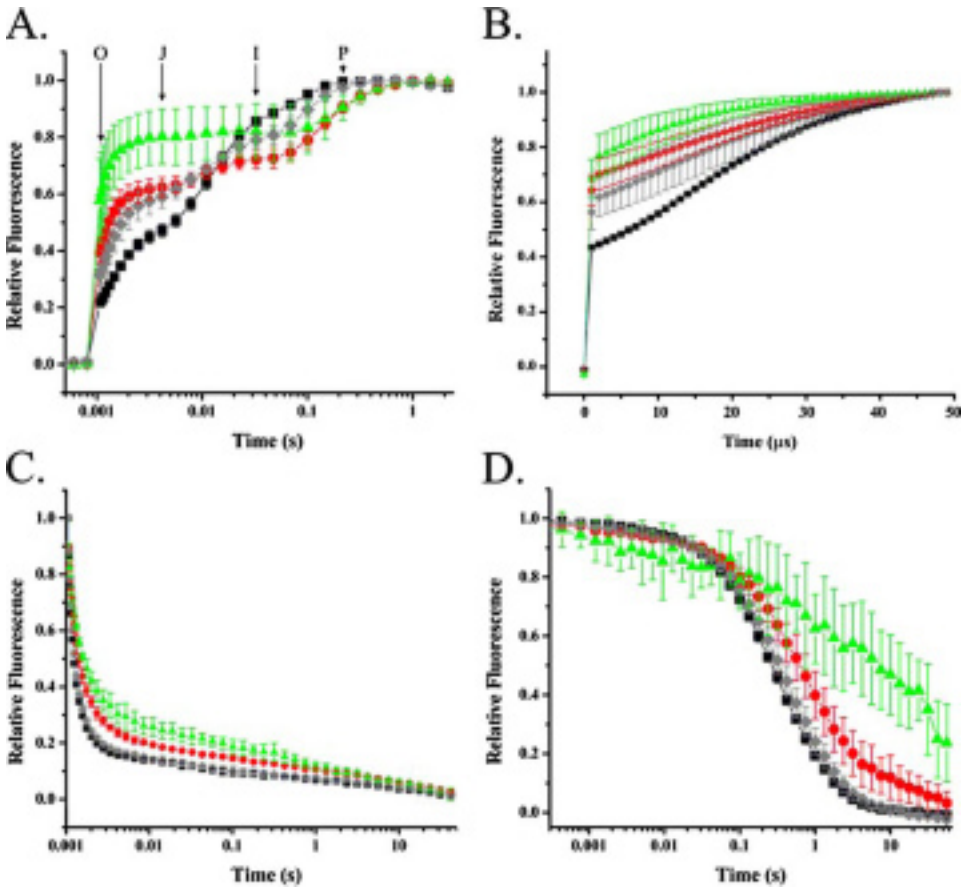

FIGURE 3. **Chlorophyll fluorescence of wild type and PsbP-deficient plants.** *A*, the standard fluorescence induction rise following continuous illumination (Kautsky curves). The location of the O, J, I, and P transitions for wild type are indicated. *B*, the fluorescence rise following a single saturating flash. Note different time scales for *A* and *B*. *C* and *D* illustrate the fluorescence decay following a single saturating flash in the absence and presence of 40 μM DCMU, respectively. ■, wild type; ◆, RNAi-P11; *orange dot*, RNAi-P1; *green triangle*, RNAi-P8. $n = 2–6$, *error bars*, ± 1.0 S.D.; in some instances the *error bars* are *smaller than the symbols*.

TABLE 1
Fluorescence induction characteristics of wild type and the RNAi-P mutants

Strain	F_V/F_M	PS II$_\alpha$	PS II$_\beta$	α/β
		%		
Wild type	0.78 ± 0.01[a]	56 ± 6	45 ± 6	1.2
RNAi-P11	0.68 ± 0.04[b]	48 ± 2	52 ± 2	0.9
RNAi-P1	0.61 ± 0.03[b]	55 ± 33	45 ± 33	1.2
RNAi-P8	0.42 ± 0.15[b]	43 ± 29	56 ± 29	0.8

[a] Values given are means ± 1.0 S.D., $n = 3–6$.
[b] $p < 0.05$ as compared with wild type using Student's *t*-test.

plants P11 and *P1*). After transplantation, the plant that lacked detectable levels of PsbP-1 yellowed and died (Fig. 2*B*, *plant P8*), whereas the other PsbP-deficient plants, which expressed small but detectable amounts of PsbP, grew somewhat more slowly than wild type (Fig. 2*B*). These results indicate that PsbP is required for photoautotrophic growth in *Arabidopsis*.

Changes in Fluorescence Induction Caused by Suppressed Expression of PsbP—Fig. 3*A* shows the chlorophyll *a* fluorescence rise (Kautsky curves) observed in *Arabidopsis* wild-type and in the RNAi-P mutant plants. The normalized fluorescence yield is shown. From these curves it is possible to calculate the quantum yield of variable fluorescence (F_V/F_M). F_V, the variable fluorescence, is equal to F_M minus F_O. For any given Kautsky curve of dark-adapted samples, F_O is the minimal fluorescence observed upon onset of illumination and F_M is the maximal amount of fluorescence observed. In Fig. 3*A*, F_O and F_M correspond to the points labeled O and P, respectively, for wild-type. These results are summarized in Table 1. Wild type exhibits a high quantum yield, whereas the RNAi-P mutants exhibit progressively lower quantum yields (WT > P11 > P1 > P8). In *Synechocystis* 6803, a strong correlation exists ($r = 0.94$) between the quantum yield of variable fluorescence (F_V/F_M) and the PS II content (25) of wild-type and mutant strains. Because such a correlation has never been directly established in either green algae or higher plants, we view such an analysis as being only semiquantitative when applied to the higher plant system. With this caveat in mind, our results indicate that there appears to be a dramatic decrease in the quantity of fully functional PS II reaction centers in the phenotypic series of PsbP-deficient plants. This finding is fully consistent with the results obtained by Ifuku *et al.* (18) in tobacco.

Additionally, using a logarithmic timing series, a polyphasic fluorescence rise exhibiting the O-J-I-P transients was observed for the wild-type sample (26, 27). An inspection of the curves shown in Fig. 3*A* reveals several interesting features. First, the O to J transition occurs with higher fluorescence yield in the RNAi-P mutants than in wild type. A phenotypic series is evident, with RNAi-P11 and -P1 being least affected and RNAi-P8 being most affected. This initial O-J transition constitutes the photochemical phase of the chlorophyll *a* fluorescence rise. This result indicates that electron transfer from Q_A^- to Q_B is slowed in the mutant. This would result in an increased accumulation of Q_A^- and consequently an increased fluorescence yield. This result is similar to, but less extreme than the effects of treatment with DCMU on the fluorescence induction curve (28). Second, in wild type the J and I transients appeared at 3 ms and 30 ms, respectively; however, in the RNAi-P mutants the J transient occurred earlier (~2 ms), and there was a progressive loss of the I transient throughout the phenotypic series. In wild type the J to I transition accounted for 30% of the total fluorescence yield. However, in the RNAi-P plants there is a progressive loss of the J to I transition, with RNAi-P1 and -P11 being the least affected and RNAi-P8 being the most affected. Indeed, this latter plant exhibited essentially no J to I transition. This result may indicate a defect in the oxygen-evolving complex. A similar decrease in the magnitude of the J to I transition has been observed in treatments that damage the oxygen-evolving com-

TABLE 2
Q_A^- reoxidation kinetics of wild type and RNAi-P mutants in the absence and presence of DCMU

	Strain	Fast phase		Middle phase		Slow phase		Residual
		τ	Amplitude	τ	Amplitude	τ	Amplitude	
		ms	%	*ms*	%	*s*	%	%
−DCMU[a]	Wild type	0.28 ± 0.02[b]	67.5 ± 1.4	7.3 ± 3.7	18 ± 2.0	1.57 ± 0.39	7.4 ± 1.4	5.0 ± 1.0
	RNAi-P11	0.28 ± 0.05	62.8 ± 4.2	6.2 ± 3.9	18.9 ± 1.6	1.28 ± 0.87	10.5 ± 2.7	5.9 ± 0.8
	RNAi-P1	0.45 ± 0.09[c]	52.8 ± 3.2[c]	7.9 ± 3.7	21.8 ± 2.4	0.93 ± 0.23[c]	13.1 ± 1.3[c]	10.2 ± 1.2[c]
	RNAi-P8	0.47 ± 0.05[c]	46.0 ± 5.5[c]	7.0 ± 2.8	21.1 ± 2.3	0.85 ± 0.29[c]	18.9 ± 3.6[c]	11.2 ± 0.6[c]
+DCMU[a]	Wild type	156 ± 32	18.9 ± 6.7	612 ± 126	48.9 ± 4.5	2.41 ± 0.57	30.5 ± 9.3	1.1 ± 1.3
	RNAi-P11	2.8 ± 0.8[c]	1.9 ± 0.9[c]	330 ± 39[c]	42.8 ± 8.2	1.50 ± 0.23	53.4 ± 5.9[c]	1.8 ± 1.5
	RNAi-P1	20.6 ± 34.8[c]	3.7 ± 1.8[c]	537 ± 164	29.6 ± 7.8[c]	2.81 ± 1.15	47.1 ± 4.7[c]	19.0 ± 8.0[c]
	RNAi-P8	2.8 ± 1.0[c]	8.0 ± 7.1	437 ± 53	24.3 ± 6.6[c]	3.98 ± 0.28[c]	30.2 ± 16.5	36.6 ± 2.5[c]

[a] The data were fit to a model containing three exponential decaying components plus a residual long-lived ($\tau > 10$ s) component (23). The curve fits in the absence of DCMU exhibited average X^2/DoF values between 0.00017 and 0.00022 for the different strains, whereas in the presence of DCMU the average X^2/DoF values were between 0.00001 and 0.00059.
[b] Values given are means ± 1.0 S.D., $n = 2–6$.
[c] $p < 0.05$ as compared with wild type using Student's *t*-test.

plex, such as mild Tris or heat treatment (29), and in plants that possess only the defective PsbO-2 protein in *Arabidopsis* (30). Alternatively, the absence of the J to I transition may indicate a defect in the ability to form the $Q_A^-Q_B^-$ state (27). It should be noted that these two explanations are not mutually exclusive, and both could, in principal, contribute to the loss of the J to I transition in the mutants. In both wild type and the RNAi-P mutants, the fluorescence signal started to rise at 30–40 ms, with all strains reaching the P level in 200–300 ms. Overall, the total fluorescence yield of the thermal phase (J to P transition) is progressively reduced (wild type > P11 = P1 > P8) in the RNAi-P mutants.

Flash Fluorescence Induction—Fig. 3*B* shows the flash-induced fluorescence induction at a 1-μs time resolution in the absence of DCMU. The observed fluorescence rise is the result of the re-reduction by the oxygen-evolving complex of Y_Z, which is in equilibrium with the primary electron donor P_{680}^+ (40). The shape of this fluorescence rise contains information bearing on the amount of PS II_α and PS II_β reaction centers present in the sample. Exponential fluorescence rise kinetics indicate that the antennae of individual PS II centers are not coupled (*i.e.* a characteristic of PS II_β centers), while sigmoidal fluorescence rise kinetics indicate a high degree of interconnectivity between the antennae of PS II centers (*i.e.* a characteristic of PS II_α centers) (41). This is true under conditions of both continuous relatively weak light illumination (slow fluorescence induction) and under single saturating flash conditions (fast fluorescence induction) as demonstrated by Nedbal *et al.* (22). This experiment allows the determination of the relative proportions of PS II_α and PS II_β reaction centers present (22) (Table 1). In addition to the differences noted above, PS II_β centers have a smaller antenna size, are enriched in chlorophyll *a*, and are depleted of the light-harvesting chlorophyll proteins. They appear to be principally located in the stromal thylakoid membranes (31) and at the grana margins (32). Additionally, PS II_β centers appear to be defective in their ability to transfer electrons from Q_A^- to Q_B (33). In wild type, we find the ratio of PS II_α to PS II_β to be 1.2. This value is similar to those obtained in other studies (34–36). In the PsbP mutants we find that the PS II_α to PS II_β ratio is slightly decreased, although none of the values observed for the mutants was statistically significant at the $p = 0.05$ level. The trend toward a slightly higher proportion of PS II_β centers in the RNAi-P mutants may indicate a slightly increased rate of metabolic turnover of PS II in the mutant, as conditions that increase photoinactivation also lead to increases in the amount of PS II_β centers observed (33). This is in marked contrast to a mutant that contains only the PsbO-2 protein. In this mutant, loss of PsbO-1 leads to a dramatic increase in the number of PS II_β centers present (30).

Analysis of the Q_A^- Decay Kinetics in Leaves of PsbP-deficient Plants—Additional information concerning the electron transfer characteristics on both the reducing and oxidizing side of the photosystem was obtained by the examination of Q_A^- reoxidation kinetics in either the absence (Fig. 3*C*) or presence of DCMU (Fig. 3*D*), respectively. In the absence of DCMU, the fluorescence decay after a single saturating flash can be resolved into three exponential components and a residual component with a $\tau > 10$ s (23). The fastest (and dominant) exponential decay component that we observed for wild type is related to the electron transfer from Q_A^- to Q_B (280 μs, 67%) (Table 2). The middle exponential decay component (7.3 ms, 18%) is associated with electron transfer from Q_A^- to Q_B in reaction centers that have to bind plastoquinone to the Q_B site before Q_A^- oxidation can occur. The slowest decay component (1.6 s, 7%) is related to a charge recombination reaction in which the reoxidation of Q_A^- occurs with donor-side components. Finally, a residual fraction of the fluorescence yield (around 5%) is very long-lived and may result from the equilibrium between Q_A^- and Q_B (37). For the RNAi-P mutants, the time constant for the fast phase increased significantly throughout the phenotypic series while the amplitude of the fast component decreased. This indicates that electron transport from Q_A^- to Q_B is slowed in the mutant. The time constant for the middle exponential decay component and its amplitude were very similar in wild type and the RNAi-P mutants. Additionally, the time constant for the slow phase significantly decreased throughout the phenotypic series while its amplitude increased. Finally, the residual amplitude (for the decay component(s) with $\tau > 10$ s) also increased significantly, perhaps indicating a change in the $Q_A^-Q_B$ equilibrium in favor of Q_A^-. Overall, these results indicate that the principal modification on the reducing side of the photosystem observed in the mutants was a significant slowing of electron transfer between Q_A^- and Q_B and an increased rate of charge recombination between Q_A^- and oxidizing side component(s).

In the presence of DCMU, which prevents electron transfer from Q_A^- to Q_B, the decay of fluorescence following a saturating flash is dominated by charge recombination between Q_A^- and the oxidizing-side components of the photosystem. Fig. 3*D* illustrates the fluorescence decay kinetics of wild type and the RNAi-P mutants in the presence of 40 μM DCMU. The observed fluorescence decay curves were fit to the same model described above (38). Significant alterations are observed in the mutants. It should be noted that alternative models containing two exponential decay components and a hyperbolic component (38), or only two exponential decay components, failed to adequately fit the data. Table 2 shows the kinetic parameters obtained for the fluorescence decay in the presence of DCMU. The fastest decaying component observed in wild type exhibited a time constant of 156 ms and is attributed to a fraction of PS II reaction centers that lack a functional manganese cluster (39), in which Q_A^- recombines with oxidized Y_Z. The slowest decay component observed for wild type exhibited a time constant of 2.4 s and is attributed to charge recombination between Q_A^- and the S_2 and, possibly, the S_3 states (40). The origin of the intermediate decay component observed in wild type (612 ms) is unclear, although it may represent a sub-fraction of reaction centers in which the charge recombination rate between Q_A^- and the S_2 state is 4- to 5-fold faster than the 2.4-s component (23). Finally, a small proportion (~1%) of wild-type reaction centers exhibited a time constant of >10 s. It should be noted that, in the presence of DCMU, which displaces Q_B, no equilibrium between Q_A^- and Q_B exists. In this case the residual amplitude is the result of slow charge recombination between Q_A^- and oxidizing-side components. In the mutant RNAi-P8, the slowest decay component was observed to have a time constant of nearly 4 s. This result indicates that the S_2 and possibly the S_3 states are more stable in this mutant than in wild type. Little change was observed for the time constant of the intermediate decay component for the RNAi-P mutants, although the amplitude generally decreased throughout the phenotypic series. The amplitude of the residual decay component ($\tau > 10$ s) increased dramatically throughout the phenotypic series, becoming the dominant decay component in RNAi-P8. This result indicates that a major disruption in the water-oxidizing complex has occurred in the RNAi-P mutants and that charge recombination with Q_A^- and oxidizing-side components is dramatically slowed. A large difference was also observed for the rapidly decaying component. The time constant for this decay component decreased from 156 ms in wild type to 3 ms in RNAi-P8, and its amplitude dropped from 18.9% to 8%. The origin of these changes is unclear at this time; however, a similar change was observed in an *Arabidopsis* mutant that lacked the PsbO-1 protein and exhibited similar dramatic changes in a variety of fluorescence induction and decay parameters (30).

In an earlier study, the effects of PsbP and PsbQ depletion on the kinetic properties of isolated PS II membrane preparations were reported (54). In this study, the extrinsic proteins were removed from spinach membranes by treatment with 2 M NaCl, which elicited changes in both the oxidizing side and reducing side of the photosystem. These investigators observed a gradual loss in the coupling between the oxygen-evolving complex and $Y_Z^{\bullet}$, which occurred in the light and which could be reversed by the addition of $CaCl_2$ or reconstitution with the PsbP and PsbQ proteins. They also observed changes that could not be reversed by these additions. These include a slowing of the $S_3 \Rightarrow (S_4) \Rightarrow S_0$ transition, changes in the Kok parameters and loss of the period two oscillations associated with the two electron gating mechanism for quinone reduction. The authors attributed these changes to irreversible alterations in PS II engendered by the high NaCl concentrations used in their studies. At this time we cannot comment on their observed alterations in the rate of the S state transition or changes in the Kok parameters. However, our observation that the loss of PsbP (and, consequently, PsbQ, see below) leads to alterations in Q_A^- to Q_B electron transfer may indicate that their earlier observation was not an artifact. Overall, the results that we have obtained *in vivo* are consistent with the *in vitro* results reported in Ref. 54.

Suppressed Expression of PsbP Leads to Alterations of the Thylakoid Protein Complement—To determine whether decreased expression of PsbP led to a loss of other PS II components, immunological analysis using chemiluminescent detection was performed. The relative amounts of selected PS II and control proteins present were analyzed in chloroplast preparations of wild-type *Arabidopsis* and the transgenic plants in the phenotypic series (Fig. 4). Four proteins that are present in the intrinsic core of PS II (CP47, CP43, D1, and D2) and the three extrinsic proteins (PsbO, PsbP, and PsbQ) were examined. In this particular gel, the PsbP protein was not detected in the RNAi-P1 and P8 lanes. In other immunoquantification experiments, when compared with wild-type plants, RNAi-P11 exhibited 5–10% of the PsbP protein while RNAi-P1 exhibited barely detectable, and RNAi-P8 exhibited no detectable amounts of this protein. The expression of the extrinsic PsbO and PsbQ extrinsic proteins was also assessed. In wild-type and PsbP-deficient plants, two immunoreactive bands were observed that bound the anti-PsbO antibody. These were previously identified to be PsbO-1 and PsbO-2 (17). The PsbO components were only modestly affected by the loss of PsbP. A markedly different pattern was observed for the PsbQ protein, because PsbP-deficient plants exhibited a complete loss of this extrinsic component. Even in the RNAi-P11 plant, in which PsbP was only partially suppressed, PsbQ could not be detected. This result is consistent with the earlier tobacco study (18). It is unclear at this time whether the loss of the PsbQ protein in *Arabidopsis* was due to decreased synthesis or increased degradation of this component due to weak binding to PSII. It should be noted, however, that large pools of unassembled mature PsbQ protein can exist in the thylakoid lumen without being degraded either in the presence (41, 42) or in the absence (43, 44) of assembled and functional PS II reaction centers. Consequently, it is possible that the loss of the PsbQ protein may be the direct result of the loss of expression of PsbP and not a consequence of the reduced levels of PS II reaction centers resulting from decreased PsbP expression. Indeed, the RNAi-P11 plant exhibits a complete loss of the PsbQ protein even though the amount of PS II reaction centers is near normal. The previous studies performed in tobacco indicated that RNAi suppression of the PsbP protein led to a dramatic decrease in PsbQ, whereas accumulation of PS II core proteins and PsbO

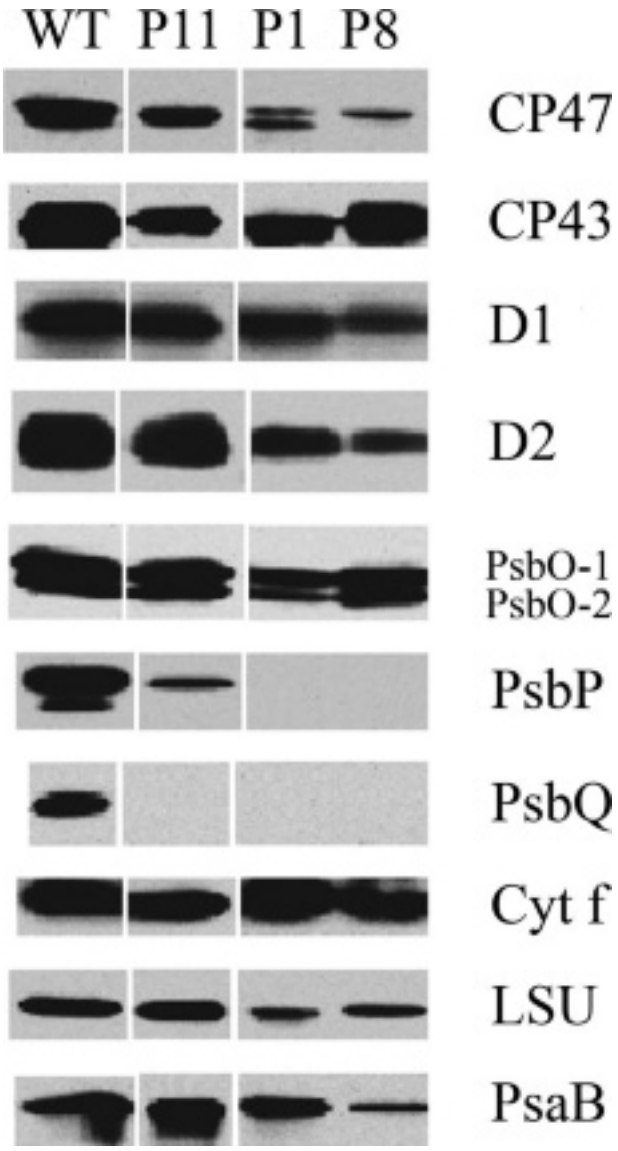

FIGURE 4. **Immunological analysis of selected thylakoid membrane proteins of wild-type and RNAi-P transgenic plants.** Wild-type (*WT*) plants and RNAi-P plants (*P11*, *P1*, and *P8*) were examined by LiDS-PAGE followed by "Western" blotting, probing with various antibodies, and subsequent chemiluminescence detection. Individual proteins are labeled to the *right*. All immunoblots for a given protein were performed on the same gel; the order, however, was rearranged to reflect the phenotypic series. Please note that, although on this gel no PsbP protein is visible in the *P1 lane*, the protein is detected on gels that were overloaded. No PsbP protein was ever detected in the P8 plant.

was similar to that of wild type (18). An earlier report on a PsbP-deficient *Chlamydomonas* mutant showed that it synthesized and accumulated the PSII core proteins and extrinsic PsbO and PsbQ proteins with no visible differences from wild type (43, 45).

Two of the intrinsic PS II components examined, D2 and CP47, also exhibited significant losses upon the loss of PsbP expression, whereas the D1 and CP43 proteins were more modestly affected (Fig. 4). This result differs substantially from the earlier tobacco study (18) in which PsbP-suppressed plants showed a wild type-like accumulation of all of the PS II core proteins examined. It should be noted that, in our study, small amounts of accumulated PsbP (for instance, in RNAi-P11) supported near wild-type levels of the CP47 and D2 proteins. In the tobacco study, the authors state that small amounts (~5% of wild type) of the PsbP proteins were present (18). This small amount, approximately equivalent to plant RNAi-P11 in our study, appears sufficient to maintain the number of assembled PS II reaction centers in near normal amounts. This assertion is supported by the near wild-type fluorescence characteristics exhibited by RNAi-P11. In plant RNAi-P8 we were not able to detect any PsbP protein. Under these conditions the loss of the D2 and CP47 components, and consequently fully assembled PS II core complexes, is exacerbated. It is clear that the absence of the PsbP protein leads to a profound alteration in the PS II protein complement. These results indicate that the absence of the PsbP protein leads to the loss of the assembly/stability of PS II.

In addition to these PS II proteins, three control proteins were also examined. As expected, the amount of cytochrome *f*, a component of the cytochrome b_6/f complex, was not affected by decreased expression of the PsbP protein. The PsaB protein, a core component of PS I, was somewhat decreased in the RNAi-P1 and P8 plants. It should be noted that this could not be the result of a general loss of thylakoid membranes, because such a situation would also lead to a loss of the cytochrome b_6/f complex. The mechanism for this phenomenon is not well established but is similar to the effects observed in other studies in which the steady-state amounts of PS I reaction centers were decreased by loss of PS II components (46). Similar results were obtained for the nuclear mutant *hcf*136, which is deficient in the HCF136 protein and does not assemble PS II reaction centers. The loss of PS II reaction centers apparently led to decreased accumulation of PS I but not the cytochrome b_6/f complex. These plants also accumulate lower steady-state amounts of RuBp carboxylase.

Earlier, it had been (41, 42) demonstrated that there was a large pool of unassembled PS II extrinsic proteins comprising 20–50% of the total extrinsic proteins present in the thylakoid lumen and that these proteins were competent in binding to the photosystem. They suggested that this unbound pool participated in the maintenance of homeostasis with respect to turnover and assembly of PS II. Our observation that a lower level of PsbP in PsbP-deficient plants led to the loss of PS II reaction center proteins suggests that maintenance of this unassembled pool is essential for the normal expression levels of PS II reaction center proteins, supporting the hypothesis of Hashimoto *et al.* (42).

In an earlier study, we examined the effect of RNAi suppression of the expression of the PsbO-1 and PsbO-2 proteins in *Arabidopsis* (17). In that study, we determined that loss of these PsbO proteins also led to a marked decrease in the number of functional PS II reaction centers and a loss of photoautotrophic growth. Because, at least *in vitro*, it appears that the PsbO protein is required for binding of the PsbP component (47–49), it is formally possible that the detrimental effects we observed for the PsbO protein were, in fact, due to the loss of the association of PsbP with the photosystem. Several observations, however, appear to indicate that this is not the case. First, the loss of PsbO leads to a marked decrease in the amounts of the CP43 intrinsic component (17). In contrast, RNAi suppression of PsbP led to a substantial loss of the CP47 component with the CP43 protein

being modestly affected. These differential patterns of protein loss in plants lacking PsbO and PsbP may indicate different roles for these components in the stability/assembly of the photosystem. Second, in the case of the phenotypic series resulting from RNAi suppression of PsbO expression, incremental loss of the PsbO protein was paralleled by the incremental loss of PS II functionality and PS II assembly/stability (17). In the case of the phenotypic series resulting from RNAi suppression of PsbP, which we report here, even very small amounts of PsbP could support near normal levels of PS II function and accumulation (for example, the phenotype of RNAi-P11). In this regard, it is interesting to note that, in cyanobacteria, a PsbP homologue is necessary to maintain fully functional PS II even though it is present in substoichiometric amounts (50). Finally, we have recently demonstrated that the *Arabidopsis* mutant *psbo1*, which lacks the major PsbO isoform PsbO-1, accumulates large quantities of PS II_β reaction centers (30) exhibiting a PS II_α to PS II_β ratio of 0.3. In PsbP-suppressed plants, which we have examined in this communication, no significant change in the PS II_α to PS II_β ratio was observed. In light of these observations, we conclude that the detrimental effects observed on PS II function and assembly/stability observed upon RNAi suppression of the PsbO protein are not due directly to the concomitant loss of PsbP from the photosystem.

It should be noted that the pattern of PS II core protein loss in both the RNAi-suppressed PsbP plants and the RNAi-suppressed PsbO plants (17) is quite interesting. Structural studies from cyanobacteria (51, 52) indicate that the D1 protein associates closely with CP43 and that the D2 protein associates closely with CP47. Additionally, the cyanobacterial PsbO protein appears to interact primarily with CP43 (although CP47 also contains binding determinants for PsbO (53)). Although there are certainly differences between cyanobacteria and higher plant PS II (8), these structural studies on cyanobacteria provide an initial framework for our understanding of the structure of the higher plant photosystem. Our observation, that loss of PsbO in higher plants leads primarily to the loss of CP43 (17) while the loss of PsbP (this study) leads primarily to the loss of CP47, may indicate specific roles for these two extrinsic proteins in maintaining the assembly/stability characteristics of these intrinsic membrane components.

CONCLUSIONS

Our results show that the use of RNAi methodology has proven a useful tool in probing the effects of a reduction of PsbP in *Arabidopsis*. Decreased amounts of PsbP led to a loss of photoautotrophic growth, the progressive loss of variable fluorescence and a marked decrease in the fluorescence quantum yield (F_V/F_M), fewer functional PS II reaction centers, and a significant loss of PS II reaction center components, with D2 and CP47 being strongly affected. We conclude that the PsbP protein is required for PS II core complex assembly/stability and photoautotrophic growth in *Arabidopsis*.

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