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Xiaoping Yi
Louisiana State University

Stefan R. Hargett
Louisiana State University

Laurie K. Frankel
Louisiana State University

Terry M. Bricker
Louisiana State University

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The PsbQ Protein Is Required in *Arabidopsis* for Photosystem II Assembly/Stability and Photoautotrophy under Low Light Conditions*

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

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

RNA interference was used to simultaneously suppress the expression of the two genes that encode the PsbQ proteins of Photosystem II (PS II) in *Arabidopsis thaliana*, *psbQ-1* (At4g21280) and *psbQ-2* (At4g05180). Two independent PsbQ-deficient plant lines were examined. These plant lines produced little detectable PsbQ protein. Under normal growth light conditions, the wild type and mutant plants were visually indistinguishable. Additionally, analysis of steady state oxygen evolution rates and chlorophyll fluorescence characteristics indicated little alteration of photosynthetic capacity in the mutant plants. No loss of other PS II proteins was evident. Interestingly, flash oxygen yield analysis performed on thylakoid membranes isolated from the mutant and wild type plants indicated that the oxygen-evolving complex was quite unstable in the mutants. Furthermore, the lifetime of the S_2 state of the oxygen-evolving complex appeared to be increased in these plants. Incubation of the wild type and mutant plants under low light growth conditions led to a significantly stronger observed phenotype in the mutants. The mutant plants progressively yellowed (after 2 weeks) and eventually died (after 3–4 weeks). The wild type plants exhibited only slight yellowing after 4 weeks under low light conditions. The mutant plants exhibited a large loss of a number of PS II components, including CP47 and the D2 protein, under low light conditions. Additionally, significant alterations of their fluorescence characteristics were observed, including an increased F_O and decreased F_V, yielding a large loss in PS II quantum efficiency (F_V/F_M). Analysis of Q_A^- decay kinetics in the absence of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea indicated a defect in electron transfer from Q_A^- to Q_B, whereas experiments performed in the presence of this herbicide indicated that the recombination rate between Q_A^- and the S_2 state was strongly retarded. These results indicate that the loss of the PsbQ protein induces significant changes in Photosystem II function, particularly in low light-grown plants, and that the PsbQ protein is required for photoautotrophic growth under low light conditions.

In higher plants and cyanobacteria, at least six intrinsic proteins appear to be required for oxygen evolution by Photosystem II (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 complete loss of oxygen evolution activity. Additionally, a number of low molecular mass, intrinsic membrane protein components are associated with PS II (4–6); however, the functions of many of these proteins remain obscure. Although PS II complexes containing only these intrinsic components can evolve oxygen, they do so at low rates (~25–40% of control), are extremely susceptible to photoinactivation, and require high, non-physiological levels of calcium and chloride for maximal activity (1, 3).

In higher plants, 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 (for review, see Ref. 7). The 33-kDa component, PsbO protein, is required for stabilization of the manganese cluster during exposure to low chloride concentrations or to exogenous reductants. The 24- and the 17-kDa proteins (termed the PsbP and PsbQ proteins, respectively) appear to modulate the calcium and chloride requirements for efficient oxygen evolution. These three extrinsic components interact with intrinsic membrane proteins and possibly with each other to yield fully functional oxygen-evolving complexes. It is unclear, however, whether the PsbP and PsbQ proteins act in concert in modulating the cofactor requirement or whether each has individual, discrete functions within the photosystem. Miyao and Murata (8) demonstrate that the PsbQ protein enhances oxygen evolution at chloride concentrations of <3 mM. Additionally, they show that the PsbQ protein, in concert with the PsbP component, slow the inactivation of oxygen evolution during chloride depletion and the activation of oxygen evolution during chloride reconstitution. As these authors point out, however, these experiments were performed under non-physiological conditions. It had been demonstrated earlier that the thylakoid membrane is highly permeable to chloride (9) and that the stromal chloride concentration is 30–60 mM (10). Consequently, it is unclear what role the PsbQ component plays under physiological conditions. It should be noted that

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

[2] The abbreviations and trivial names used are: PS II, photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; Tricine, *N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine; RNAi, RNA interference; DCBQ, 2,6-dichloro *p*-benzoquinone.

homologues of the PsbP and PsbQ proteins also exist in cyanobacteria. Deletion of these components in the cyanobacterial system leads to significant alterations in PS II activity (11). *Arabidopsis* contains two expressed PsbQ proteins (12, 13). These proteins are encoded by two genes, *psbQ-1* (At4g21280) and *psbQ-2* (At4g05180). The mature proteins are highly conserved among all higher plant species (7).

RNA interference (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 popular research methodology for investigating the physiological functions of target genes in plants (15). With respect to PS II proteins, RNAi has been used to investigate the *in vivo* function of the PsbO proteins in *Arabidopsis* (16) and the PsbP and PsbQ proteins in tobacco (17). The studies performed in tobacco indicate that RNAi suppression of the PsbQ protein leads to no observable phenotype (17).

In this study, we have reported the use of RNAi technology to simultaneously suppress the expression of both *psbQ* genes in *Arabidopsis*. Our results indicate that the PsbQ protein is required for the stabilization of oxygen-evolving PS II complexes under normal illumination conditions and for PS II function/stability and photoautotrophic growth under low light conditions.

MATERIALS AND METHODS

RNA Interference Construct and Transformation—The pHANNIBAL vector (18) was used to construct an intron-spliced hairpin RNA (RNAi construct). Comparison of the nucleotide sequences of the *psbQ-1* and the *psbQ-2* genes allowed the identification of one region of relatively high nucleotide identity (+183 to +563 in *psbQ-1*), which was chosen to silence both the *psbQ-1* and *psbQ-2* genes simultaneously. This construct will be referred to as *psbQ*-RNAi. The primers for RNAi-Q were 5′-GCGATCGATGCTGGTTTAGCTGGTGGCT-3′ and 5′-GCGGGATCCGGTATCGAAGAGCTTGGT-3′ for the sense fragment and 5′-GCGGGTACCTGGTTTAGCTGGTGGCT-3′ and 5′-GCGCTCGAGGTATCGAAGAGCTTGT-3′ for the antisense fragment of *psbQ-1*.

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 *Taq* polymerase (Invitrogen), and 25 ng of *Arabidopsis* genomic DNA in purified water. Cycling parameters were a predenaturation step at 96 °C for 2 min followed by 30 amplification cycles (denatured at 95 °C for 20 s, annealed at 53 °C for 20 s, and extended at 72 °C for 40 s) and a final extension at 72 °C for 7 min. The amplified sequence was cloned into both forward and reverse orientations flanking the Pdk intron of the pHANNIBAL vector (18). 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 (19). Four-week-old *Arabidopsis* plants (Col-0) were transformed by the floral dip method as described previously (20). Harvested seeds were surface-sterilized with 50% ethanol and 0.5% Tween 20 for 3 min, washed briefly with 95% ethanol, and then soaked in 70% ethanol for 3 min followed by washing three times with sterile water. The seeds were spread on solid Murashige and Skoog 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 to culture boxes containing solid Murashige and Skoog medium with 2% sucrose, 50 mg/liter kanamycin, and 400 mg/liter carbenicillin. To test for photoautotrophic growth, the plants were transplanted onto medium from which the sucrose was omitted and then transplanted onto soil to obtain seeds. The plants were grown under continuous light at an intensity of 25–40-μmol photons/m^2/s.

Screening—The presence of the RNAi construct in the kanamycin-resistant plant lines was confirmed by PCR with primers designed to amplify the cauliflower mosaic virus 35S promoter and target gene region of the introduced DNA. All of the plants that exhibited the kanamycin-resistant phenotype also exhibited the presence of the 1-kbp cauliflower mosaic virus 35S promoter and its conjugated gene region, which was absent in the wild type plants (data not shown). Individual kanamycin-resistant plants were screened for the presence of the PsbQ 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, and 0.1% bovine serum albumin) was added followed by the addition of lithium dodecyl sulfate-PAGE solubilization buffer, and the samples were incubated on ice for at least 15 min. The samples were then centrifuged at 16,000 × *g* for 5 min before running on a 15% polyacrylamide gel. Western blotting and antibody probing were performed as previously described (16) followed by visualization with a chemiluminescent peroxidase substrate (Super-Signal® West Pico chemiluminescent substrate, Pierce). X-ray film was exposed by the blot and developed photographically. The monoclonal antibody FCC4 directed against the mature spinach PsbQ protein (21) was found to cross-react with both the PsbQ-1 and the PsbQ-2 proteins from *Arabidopsis* and was used in these studies.

To characterize the RNAi-Q plants in which expression of the PsbQ protein was much lower than in the non-transgenic plants, second generation plants were grown under continuous illumination at a light intensity of 25–40-μmol photons/m^2/sec, and a subset of these plants was transferred to low light growth conditions (4–5-μmol photons/m^2/s) for varying lengths of time.

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 two mutant plant lines that expressed low levels of the PsbQ protein. These lines were designated RNAi-Q7 and RNAi-Q30, and these plant lines are collectively referred to as PsbQ-deficient plants. Leaves were ground in a glass homoge-

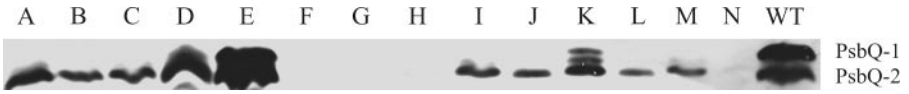

FIGURE 1. **Immunological screening for the presence of PsbQ proteins in 14 transgenic Plants.** Proteins from whole leaf extracts of wild type (*WT*) and 14 transgenic plants (*A–N*) were resolved by lithium dodecyl sulfate-PAGE followed by Western blotting and detection by chemiluminescence. Individual plants exhibited variable amounts of the two PsbQ proteins. The minor immunoreactive band(s) located between PsbQ-1 and PsbQ-2 are probably the result of a small amount of proteolysis.

nizer 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, and 10 mM $NaHCO_3$), the homogenate was then passed through two layers of Miracloth (Calbiochemcal Co.), and the chloroplasts pelleted by centrifugation at 6,000 × *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 (22). Lithium dodecyl sulfate-PAGE was performed on a 12.5–20% gradient gel with 3 μg of chlorophyll loaded/lane. Western blotting, blocking, probing with antibodies, and x-ray film exposure were as described above. After development, the x-ray films were scanned with a UMax PowerLook III scanner at 300 dots/inch resolution and an 8-bit color depth.

Fluorescence Measurements on Leaves—The fluorescence characteristics of wild type and PsbQ-deficient plants were examined on either a Walz 101/103 pulsed amplitude fluorometer or a Photon Systems Instruments FL3000 dual modulation kinetic fluorometer. It should be noted that, for these and other functional studies, the data from the RNAi-Q7 and RNAi-Q30 plants were combined. Essentially no functional differences were observed between these two mutant plant lines. Single leaves were excised from the plants and dark-incubated for 5 min before illumination with the weak measuring beam. In some experiments, the leaves were treated with 40 μM DCMU and 0.1% Tween 20 in water for 30 min prior to performing the fluorescence experiments. Q_A^- reoxidation was measured after a single saturating flash, either in the absence or presence of 40 μM DCMU. Single leaves were excised from the plants and dark-incubated for 5 min before illumination with a 4-μs saturating flash. Data were analyzed using Origin 6.1 software.

Steady State and Flash Oxygen Yield Analysis—Oxygen evolution studies were performed on thylakoid membrane fragments isolated from wild type and PsbQ-deficient plants. Thylakoid fragments were isolated by grinding 2–5 leaves in a glass homogenizer with 1 ml of a buffer containing 0.45 M sorbitol, 10 mM EDTA, 0.1% bovine serum albumin, 1% polyvinylpyrrolidone, and 20 mM tricine-NaOH, pH 8.4. The homogenate was filtered through 1 layer of Miracloth, and the thylakoid fragments were pelletted by centrifugation at 4 °C at 16,000 × *g* in a microcentrifuge. Steady state measurements were performed in a Hansatech oxygen polarograph. The chlorophyll concentration for these experiments was between 4 and 13 μg of chlorophyll/ml assayed in 0.3 M sorbitol, 5 mM $MgCl_2$, and 20 mM Tricine-NaOH, pH 7.6, with 200 μM DCBQ added as an electron acceptor. The light intensity for these experiments was 2000-μmol photons/m^2/s.

Flash oxygen yield measurements were performed on a bare platinum electrode (Artesian Scientific Co., Urbana IL). Flashes were supplied by an integrated, computer-controlled Xenon flash lamp (20 μs at one-half height). For the measurements of S-state distributions and S-state parameters, thylakoid fragments were pelleted and applied to the electrode as a thin paste. The thylakoids were then incubated for 5 min in the dark, the electrode was polarized at 0.73 V for 10 s, and a series of saturating flashes was applied. Data points were collected at 500-μs intervals during the duration of the flash train. The data were analyzed using a four-step, homogeneous model (23). Five- and six- state models that incorporated either an S_{-1} state or S_{-1} and S_{-2} states, respectively, uniformly failed to fit the data acquired either from the wild type or mutant thylakoids. For experiments that examined the stability of the oxygen-evolving complex, the thylakoid fragments were applied to the electrode as described above and incubated on the electrode (in the absence of polarizing voltage) at room temperature (24 °C) for various lengths of time prior to initiation of the flash yield experiment, either in the dark or under low light conditions (5-μmol photons/m^2/sec).

RESULTS AND DISCUSSION

Seeds from wild type and from plants transformed with the RNAi-Q construct were distributed on agar plates containing Murashige and Skoog medium supplemented with 50 μg/ml kanamycin. The presence of the RNAi-Q construct in 10 kanamycin-resistant plants was confirmed by PCR amplification of the cauliflower mosaic virus promoter region. All of the plants that exhibited kanamycin resistance also exhibited the 1-kbp PCR amplification product, indicating the presence of the cauliflower mosaic virus promoter region of the RNAi construct (data not shown).

To screen individual transgenic plants for the presence of the PsbQ proteins, Western blot analysis with a monoclonal antibody that recognizes both the PsbQ-1 and PsbQ-2 proteins was performed. The results from a typical screening experiment are shown in Fig. 1. In the wild type plants, two major protein bands reacted strongly with the monoclonal reagent and represent the putative PsbQ-1 and PsbQ-2 proteins. These two bands migrated with apparent molecular masses of 15.4 and 14.2 kDa, which are comparable to the masses previously observed for these proteins (13). Additionally, plants transformed with RNAi constructs designed to suppress the *psbQ-1* gene lost the upper band, and plants transformed with constructs designed to suppress the *psbQ-2* gene lost the lower band (data not shown). In the current study, individual transgenic plants exhibited different degrees of suppression of the expression of the two PsbQ proteins. In total, 56 plants were screened for the presence of the PsbQ proteins. The results showed that 42% of the plants had expression levels similar to wild type for the PsbQ-1 and PsbQ-2 proteins, 39% exhibited an intermediate level of expression, and ~19% of the transgenic plants exhibited almost complete loss of the PsbQ proteins. 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 different degrees of suppression of the protein targets (16, 24, 25).

Photosynthetic Competence of Plants Grown under Normal and Low Light Conditions—When grown under normal light intensities, the PsbQ-deficient plants exhibited growth rates, leaf color, and overall morphology visually indistinguishable from that of wild type. To assess the effect of the loss of the expression of PsbQ proteins on the functional competence of PS II, chlorophyll fluorescence characteristics were examined in wild type and transgenic plant leaves. Under normal light growth conditions, fluorescence analysis indicated that the PsbQ-deficient plants were very similar to wild type. Few differences were observed during the initial fluorescence rise following a single saturating flash (Fig. 2*A*), during a fluorescence induction experiment (Fig. 2*C* and Table 1), or in the fluorescence decay following a single saturating flash either in the absence (Fig. 3*A*) or presence (Fig. 3*C*) of 40 μM DCMU. The latter two experiments probed the efficiency of the electron transfer from Q_A^- to Q_B or the charge recombination between Q_A^- and the S_2 state of the oxygen-evolving complex, respectively. These results indicated that, with respect to a number of fluorescence parameters, wild type and the PsbQ-deficient plants were very similar. Additionally, steady state oxygen evolution measurements (Table 1) indicated that the wild type and PsbQ-deficient plants exhibited similar oxygen evolution capabilities. These results verify and extend the observations by Ifuku *et al.* (17) on transgenic RNAi-containing tobacco plants that were deficient in PsbQ. These authors concluded that, under normal conditions, the absence of the PsbQ protein yielded no observable phenotype.

In contrast to these studies, we observed that, upon transfer from normal light intensities to low light conditions, the PsbQ-deficient plants began yellowing (after 2 weeks) and eventually died (after 3–4 weeks). This phenotype was observed for the PsbQ-deficient plants either in the presence or absence of the selective antibiotic. Wild type plants, on the other hand, exhibited little visible color change after 2–3 weeks and only slight yellowing after 4 weeks under low light growth conditions. The visible changes observed in the PsbQ-deficient plants after 2–3 weeks under low light growth conditions were mirrored in changes in their fluorescence and oxygen evolution characteristics. In Fig. 2*B*, marked changes were observed in the fluorescence rise after a single saturating flash. Although the wild type plants exhibited the typical sigmoidal rise from F_O to F_M ob-

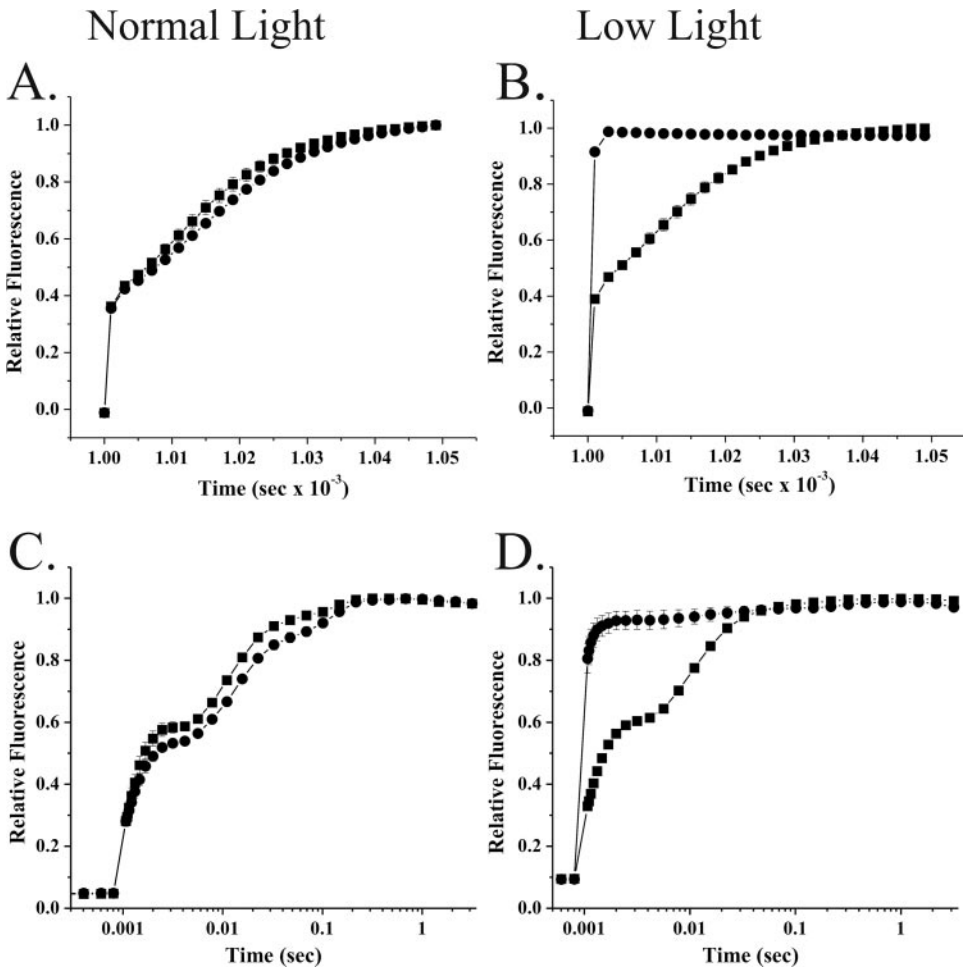

FIGURE 2. **Chlorophyll fluorescence induction of wild type and PsbQ-deficient plants grown under normal and low light conditions.** Plants grown under normal light conditions are shown to the *left*, whereas plants incubated under low light conditions for 2 weeks are shown to the *right*. For clarity, every other data point was removed. *A* and *B*, fluorescence rise following a single saturating flash. *C* and *D*, fluorescence induction curves. Note different time scales for *A* and *B versus C* and *D*. ■, wild type; ●, PsbQ-deficient (n = 3–5). *Error bars* are ±1 S.D. In some instances, the *error bars* are smaller than the *symbols*.

TABLE 1

Fluorescence and steady state oxygen evolution characteristics of wild type and PsbQ-deficient *Arabidopsis* grown under normal and low light conditions

Plant type	Illumination conditions	F_O	F_M	F_V	F_V/F_M	Oxygen evolution
						μmoles O_2 /mg chl/hr
Wild type[a]	Normal	0.45 ± 0.08[b]	1.87 ± 0.33	1.42 ± 0.26	0.76 ± 0.02	249 ± 57
	Low	0.58 ± 0.10	1.84 ± 0.33	1.26 ± 0.28	0.68 ± 0.05	227 ± 54
PsbQ-deficient[a]	Normal	0.51 ± 0.03	1.90 ± 0.26	1.40 ± 0.25	0.73 ± 0.04	272 ± 98
	Low	1.2 ± 0.39	1.73 ± 0.49	0.52 ± 0.28	0.26 ± 0.15	94 ± 21

[a] n = 3–5.
[b] ±1.0 S.D.

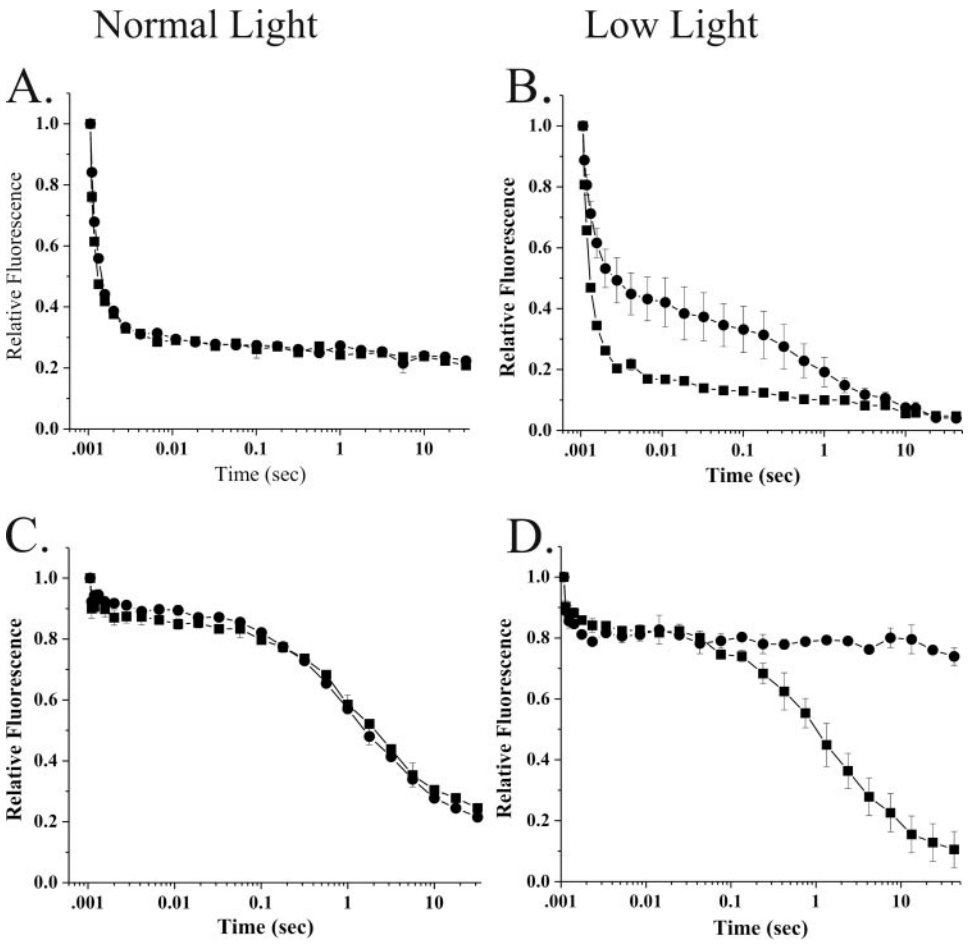

FIGURE 3. **Chlorophyll fluorescence decay following a single saturating flash of wild type and PsbQ-deficient plants grown under normal and low light conditions.** Plants grown under normal light conditions are shown to the *left*, whereas plants incubated under low light conditions for 2 weeks are shown to the *right*. *A* and *B*, fluorescence decay in the absence of DCMU. *C* and *D*, fluorescence decay in the presence of 40 μM DCMU. ■, wild type; ●, PsbQ-deficient (n = 3–5). *Error bars* are ±1 S.D. In some instances, the *error bars* are smaller than the *symbols*.

served in Fig. 2*A*, the mutant plants exhibited a very rapid rise (<1 μs) to a near maximal fluorescence level. This rapid rise is indicative of the fluorescence emission from disconnected light-harvesting chlorophyll proteins. Large changes were also observed in the fluorescence induction experiment (Fig. 2*D* and Table 1). Again, although the wild type plants exhibited little alterations in the development of variable fluorescence compared with plants grown under normal light conditions, the PsbQ-deficient plants exhibited markedly increased F_O and decreased F_M. This result indicates that the PS II quantum yield (F_V/F_M) was significantly decreased in the mutant plants (Table 1). Additionally, the normal polyphasic fluorescence rise exhibiting the O-J-I-P transient (26) was absent in PsbQ-deficient plants under low light growth conditions (Fig. 2*D*). Finally, the PsbQ-deficient plants also exhibited a loss in the ability to evolve oxygen when grown under low light conditions (Table 1). In *Synechocystis* 6803, a strong correlation exists (r = 0.94) between the total yield of variable fluorescence (F_M-F_O) and the PS II content (27) of wild type and mutant strains. Because such a correlation has never been 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 PsbQ-deficient plants incubated under low light conditions.

The PS II reaction centers that were present in the low light-grown PsbQ-deficient plants were defective. This is illustrated in Fig. 3, *B* and *D*. In Fig. 3*B*, the decay of fluorescence was monitored after a single saturating flash. Fluorescence decay under these conditions principally monitors the transfer of electrons from Q_A^- to Q_B and is a measure of the intactness of the reducing side of PS II. The results shown indicate that the PsbQ-deficient plants exhibit a defect in the ability to transfer electrons to Q_B.

In Fig. 3*D*, the decay of fluorescence was monitored after a single saturating flash in the presence of 40 μM DCMU. Because under these conditions electron transfer cannot occur beyond Q_A, fluorescence decay principally monitors charge recombination between Q_A^- and the S_2 state of the oxygen-evolving complex. Our results indicate that this charge recombination in the PsbQ-deficient plants is strongly retarded. The results from these fluorescence decay experiments (minus and plus DCMU) demonstrate that both the reducing and oxidizing sides of the photosystem are compromised in the PsbQ-deficient plants that have been grown at low light intensities.

Our observations on the growth of the PsbQ-deficient plants, their fluorescence characteristics, and their steady state oxygen evolution capability indicate that, in the absence of the PsbQ protein, the plants cannot maintain PS II in a functional state under low light conditions. Ifuku *et al.* (17) characterize their transgenic PsbQ-deficient tobacco plants under normal and high light conditions. The conditions under which Ifuku *et al.* conducted their examination would not have allowed them to note the phenotype that we observed.

Alterations of the Thylakoid Protein Complement in PsbQ-deficient Plants—Fig. 4 presents an analysis of the thylakoid proteins isolated from wild type and the two PsbQ-deficient plant lines RNAi-Q7 and RNAi-Q30. In Fig. 4*A*, the Coomassie Blue protein-staining profile is shown for plants that had been grown under normal light conditions. Essentially no visible differences were evident. In Fig. 4*B*, immunostaining was used to examine a number of PS II components and control proteins. Under normal illumination conditions, the PsbQ protein was absent from the RNAi-Q7 and RNAi-Q30 plants. None of

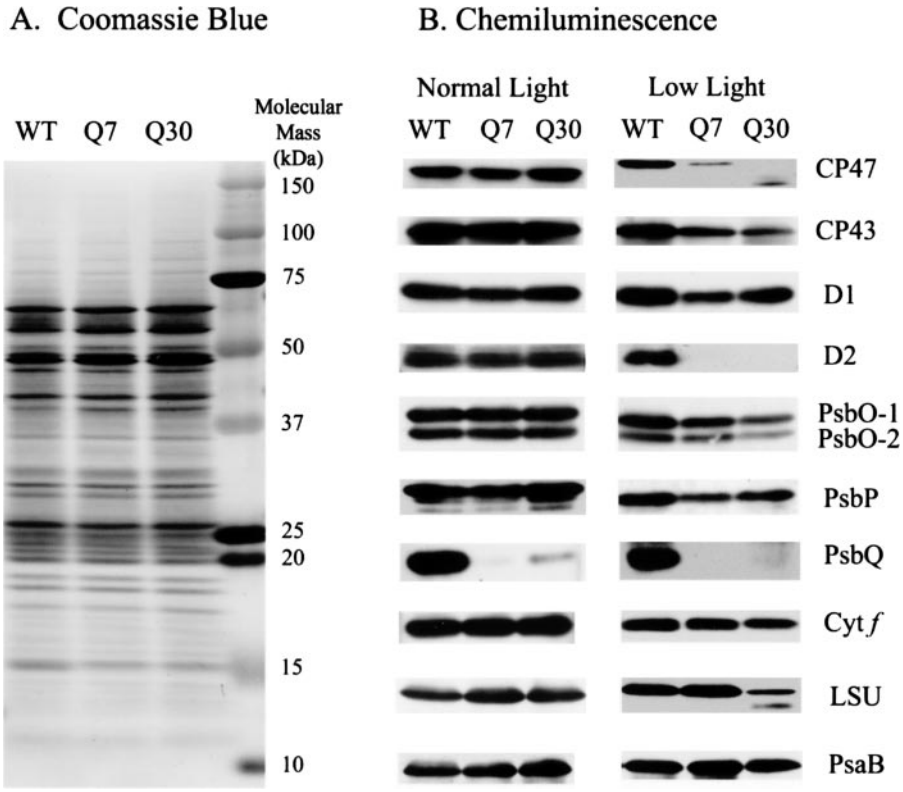

FIGURE 4. **Immunological analysis of the protein complement of the thylakoid membranes of wild type and PsbQ-deficient plants.** Wild type (*WT*) plants and PsbQ-deficient plants (*Q7* and *Q30*) were examined by lithium dodecyl sulfate-PAGE followed by Western blotting. *A*, blot stained with Coomassie Blue. Molecular mass standards are shown to the *left*. *B*, blot probed with various primary antibodies and appropriate secondary antibody-peroxidase conjugates followed by detection with chemiluminescence. Proteins detected with the various antibody probes are indicated. Plants grown under normal light conditions are to the *left*, whereas plants incubated for 2 weeks under low light conditions are to the *right*.

the other PS II components examined (CP47, CP43, D1, D2, the PsbOs, and PsbP) or the control proteins (cytochrome *f*, the large subunit of ribulose-1,5-bis-phosphate carboxylase and PsaB) was affected. Upon low light incubation, however, a markedly different protein pattern was observed. Although the thylakoids isolated from wild type plants exhibited few changes when compared with the plants grown under normal light, the two PsbQ-deficient plant lines exhibited profound alterations in their protein complement. The CP47 and D2 proteins were substantially decreased while the CP43, D1, the PsbOs, and PsbP proteins were reduced by varying degrees. Although cytochrome *f* and the PsaB protein were unaffected, the large subunit of ribulose-1,5-bis-phosphate carboxylase was significantly reduced in one of the PsbQ-deficient plant lines (RNAi-Q30). The significance of this latter observation is unclear at this time.

It should be noted that these immunological observations should be treated as only semiqualitative in nature. Due to differences in antibody titer, avidity, and the non-linear nature of the antibody binding and response, strict quantification experiments require a significantly greater number of controls than presented in this current study. For instance, the D2 protein appears to be nearly completely lost in the PsbQ-deficient plants. However, further experiments indicated that the limit of detection of the D2 protein under the conditions utilized in this study was 25–30% of the control amount of D2. In contrast, the minimum amount of the PsbQ protein that was detectable was 1–5% (data not shown).

In any event, it is clear that the absence of the PsbQ protein leads to a profound alteration in the PS II protein complement when plants are incubated under low light conditions. These results, when coupled with the functional measurements described in the previous section, indicate that the absence of the PsbQ protein leads to the loss of the assembly/stability and function of PS II under low light growth conditions. The low light sensitivity of the PsbQ-deficient mutants is quite unusual. Only plants deficient in the PsbR protein, which appears to be an assembly factor for PS II, exhibit a similar phenotype, although at higher light intensities than reported here (28).

Alterations of the Oxygen-evolving Complex in PsbQ-deficient Plants Grown under Normal Light Intensities—Although PsbQ-deficient plants grown at normal light intensities exhibited normal growth, color, fluorescence, and oxygen evolution characteristics, we hypothesized that loss of the PsbQ protein could result in defects in the oxygen-evolving complex that would not necessarily be evident in fluorescence or steady state oxygen evolution experiments. Consequently, we examined the stability and function of the oxygen-evolving complex in plants grown under normal light conditions. Our initial attempts to determine S_2 and S_3 state lifetimes in thylakoids isolated from the PsbQ-deficient plants failed. This was puzzling, because these experiments were normally quite easy to perform with thylakoids isolated from wild type plants. These measurements require the oxygen-evolving complex to be stable for 20–40 min at 22 °C. We therefore examined the stability of the oxygen-evolving complexes in thylakoids isolated from wild type and the PsbQ-deficient plants. In these experiments, we monitored the flash oxygen yield arising from the third flash in a series of 16 saturating flashes. Between these flash series, the thylakoids were incubated on the platinum electrode (polarizing current off), either in the dark or at 5-μmol photons/m^2/s for varying lengths of time. The results obtained are shown in Fig. 5*A*. Within the limits of error, the thylakoids isolated from wild type plants contained oxygen-evolving complexes that were quite stable and exhibited little change during either light or dark incubation. The oxygen-evolving complexes contained in the thylakoids isolated from the PsbQ-deficient plants, however, were very unstable. These lost their ability to evolve oxygen, with half-times of 12.5 and 5 min in the dark and light, respectively. These results indicate that, even though the PsbQ-deficient plants appeared to assemble normal

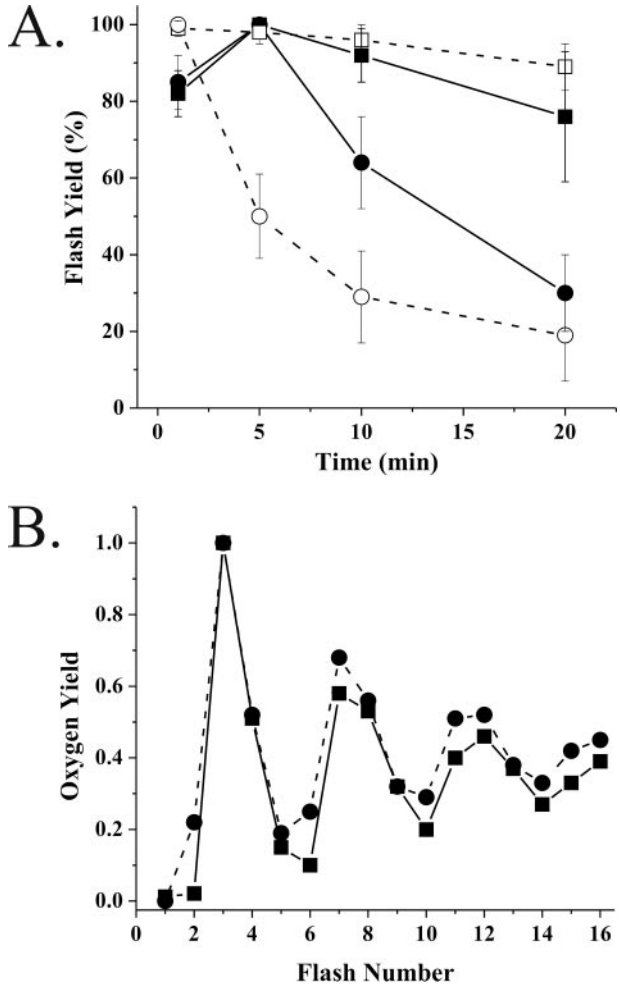

FIGURE 5. **Flash oxygen yield analysis of thylakoid membranes isolated from wild type and PsbQ-deficient plants grown under normal light conditions.** *A*, stability of the flash oxygen yield at room temperature (22 °C during dark (*closed symbols*) and light conditions (5-μmol photons/m^2/sec, *open symbols*). ■ and □, wild type; ○ and ●, PsbQ-deficient ($n = 5$). *Error bars* are ±1 S.D. *B*, typical flash oxygen yield pattern obtained during a train of 16 saturating flashes. *Symbols* are as described for *A*.

amounts of PS II reaction centers (Fig. 4), these centers are quite unstable with respect to their ability to carry out oxygen evolution.

When incubated in the dark, both the wild type and the PsbQ-deficient thylakoids exhibited their maximal third flash oxygen yield at 5 min. This is because, in the dark, the S states redistribute so that the majority of PS II centers are in S_1, with most of the remainder being in S_0. Few centers are normally found in the S_2 or S_3 states. Typical flash oxygen yield patterns for thylakoids isolated from wild type and the PsbQ-deficient mutant are shown in Fig. 5*B*. Examination of these curves demonstrated that, after 5 min of dark incubation, the PsbQ-deficient thylakoids exhibited significant oxygen yield on the second flash. This indicated qualitatively that the mutant contained PS II centers in the S_2 state. When the flash oxygen yield pattern is analyzed using a four-state homogeneous model (23), the distribution of S states and the associated S-state parameters can be quantified (Table 2). After 5 min of dark incubation, essentially all of the oxygen-evolving complexes in wild type thylakoids were in the S_0 and S_1 states (8). The PsbQ-deficient mutant thylakoids, however, exhibited significantly

TABLE 2
S-state distributions and parameters for wild type and PsbQ-deficient *Arabidopsis* grown under normal light conditions

	Wild type	PsbQ-deficient
S-state distribution[a]		
S_0	14.8 ± 7.1[b]	18.1 ± 6.2
S_1	90.7 ± 12.3[c]	70.8 ± 9.4[c]
S_2	−6.6 ± 8.4[c]	9.9 ± 13.2[c]
S_3	1.0 ± 0.9	1.1 ± 0.7
S-state parameters[a]		
Misses	13.1 ± 2.2[c]	10.3 ± 1.1[c]
Single hits	81.7 ± 4.5	85.6 ± 0.9
Double hits	4.1 ± 2.7	1.4 ± 3.5
Deactivations	−0.1 ± 1.5	1.4 ± 1.5

[a] $n = 5$.
[b] ±1.0 S.D.
[c] $p < 0.05$, Student's t test.

fewer centers in the S_1 state and significantly more centers in the S_2 state. The mutant also exhibited significantly fewer misses than did wild type, although the meaning of this latter observation is unclear. Overall, these results indicated that the S_2 state in the PsbQ-deficient plants appeared to be more stable than in wild type.

The PsbQ protein has been implicated in the maintenance of chloride at the active site of PS II (8). It is also known that chloride depletion can lead to increased S_2 state lifetimes (29). Consequently, it is possible that the loss of the PsbQ protein could lead to increased S_2 state lifetimes under chloride-limiting conditions. It should be noted, however, that the chloride concentration in our flash yield experiments was 10 mM. This concentration is significantly higher than the 3 mM chloride concentration below which effects were observed in the absence of the PsbQ protein (8). Consequently, we believe that chloride depletion effects on the S_2 state lifetimes are unlikely to occur in the types of experiments we performed. We hypothesize that the increased S_2 state lifetimes that we observed were directly due to the loss of the PsbQ component.

CONCLUSIONS

Our results indicate that the loss of the PsbQ protein leads to profound changes in the oxygen-evolving apparatus of PS II. Under normal illumination conditions, the oxygen-evolving complex was observed to be quite unstable in isolated thylakoids of the mutants. Additionally, loss of the PsbQ protein led to a marked stabilization of the S_2 state in these plants. Under low light growth conditions, the phenotype observed for the PsbQ-deficient plants was much more extreme, leading to a loss of photoautotrophy. Profound alterations in the fluorescence characteristics, significant loss of oxygen evolution capability, and loss of a number of other PS II components were observed under these conditions.

We hypothesize that, under normal growth conditions, PS II repair mechanisms (possibly photoactivation) can compensate for the observed instability of the oxygen-evolving complex. However, under low light conditions, the repair rate is insufficient to compensate for this defect, and PS II complexes are ultimately lost from the thylakoid membrane. We are currently testing this hypothesis in our PsbQ-deficient plant lines.

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