THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 1, pp. 145–150, January 6, 2006

PsbR, a Missing Link in the Assembly of the Oxygen-evolving Complex of Plant Photosystem II*

Received for publication, September 28, 2005, and in revised form, November 3, 2005 Published, JBC Papers in Press, November 9, 2005, DOI 10.1074/jbc.M510600200

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The oxygen-evolving complex of eukaryotic photosystem II (PSII) consists of three extrinsic nuclear-encoded subunits, PsbO (33 kDa), PsbP (23 kDa), and PsbQ (17 kDa). Additionally, the 10-kDa PsbR protein has been found in plant PSII and anticipated to play a role in water oxidation, yet the physiological significance of PsbR has remained obscure. Using the *Arabidopsis psbR* mutant, we showed that the light-saturated rate of oxygen evolution is strongly reduced in the absence of PsbR, particularly in low light-grown plants. Lack of PsbR also induced a reduction in the content of both the PsbP and the PsbQ proteins, and a near depletion of these proteins was observed under steady state low light conditions. This regulation occurred post-transcriptionally and likely involves a proteolytic degradation of the PsbP and PsbQ proteins in the absence of an assembly partner, proposed to be the PsbR protein. Stable assembly of PsbR in the PSII core complex was, in turn, shown to require a chloroplast-encoded intrinsic low molecular mass PSII subunit PsbJ. Our results provided evidence that PsbR is an important link in the PSII core complex for stable assembly of the oxygen-evolving complex protein PsbP, whereas the effects on the assembly of PsbQ are probably indirect. The physiological role of the PsbR, PsbP, and PsbQ proteins is discussed in light of their peculiar expression in response to growth light conditions.

Photosystem II (PSII)[2] is a supramolecular pigment-protein complex embedded in the thylakoid membrane. It catalyzes light-induced electron transfer from water to plastoquinone, with concomitant evolution of oxygen occurring on the luminal side of PSII. The oxygen-evolving complex (OEC) of eukaryotic PSII is composed of three extrinsic nuclear-encoded subunits, PsbO (33 kDa), PsbP (23 kDa), and PsbQ (17 kDa), which protect the $CaMn_4$ cluster bound to D1 and CP43 proteins (1). Cyanobacterial OEC, on the other hand, is composed of the PsbO protein, together with PsbU and PsbV (the cytochrome c_{550}) (2). Despite these differences in the protein composition of OEC, the functional features of the manganese cluster and the requirement of two inorganic ions, calcium and chloride, in photosynthetic water oxidation have remained similar during the evolution of higher plants.

Recent genome and proteome analyses have, however, revealed that homologs of both PsbQ and PsbP are present in cyanobacteria (3–5) and are essential for regulation of PSII assembly and/or activity, particularly under calcium or chloride deficiency (5, 6). The PsbQ homolog was found to be present as one molecule for each cyanobacterial PSII core, whereas the PsbP homolog was suggested to be present only in substoichiometric amounts (5). On the other hand, it was recently found that in total membrane preparations and in whole cell extracts, the PsbP homolog is present in amounts comparable with other PSII proteins, suggesting that the protein becomes depleted by detergents used during the isolation of PSII complexes (7) Moreover, it has been reported that the 20-kDa extrinsic protein, which belongs to the OEC proteins of red algae, shows sequence similarity with the PsbQ protein of green algae and higher plants as well as with the PsbQ homolog of cyanobacteria, and it was proposed that the red algae 20-kDa protein is an ancestral form of the PsbQ protein (8).

The assembly process of the 33-kDa PsbO protein to the luminal side of the PSII core complex has been reported to take place in stroma-exposed thylakoid membranes of higher plants (9). Structural studies have revealed that the luminal loops of the PSII core proteins D2 and CP47 are essential for docking the PsbO protein (10), and the presence of CP43 is also necessary since no stable assembly of the PsbO protein was found in the PSII monomer lacking CP43 (11). The 23- and 17-kDa OEC proteins PsbP and PsbQ have been found to associate with PSII only in the grana thylakoids (9). Moreover, the low molecular mass (LMM) PSII core protein PsbJ was recently reported to be a prerequisite for the association of PsbP with the PSII core (11, 12). It has been suggested that PsbO provides a docking site for PsbP, and both PsbO and PsbP together are needed for the binding of PsbQ (13, 14). Unlike in higher plants, the PsbP and PsbQ homologs in cyanobacteria contain a lipid moiety in their N terminus, which probably anchors these proteins to the thylakoid membrane (5).

Besides the OEC proteins, the PSII core complex in higher plants and cyanobacteria also differs with respect to the necessity of several intrinsic LMM proteins to the function and assembly of the complex (15, 16). PsbJ is a representative example of such proteins. In cyanobacteria, the deletion of PsbJ allows slow photoautotrophic growth, whereas in tobacco plants, the corresponding deletion leads to a complete heterotrophy (11, 12, 17–19). Moreover, higher plant PSII contains the nuclear-encoded LMM subunits PsbW and PsbR, which are completely lacking from cyanobacterial PSII.

The functional role and properties of PsbR, also known as the 10-kDa PSII polypeptide, have remained poorly characterized. It has been proposed that PsbR is located on the luminal side of PSII and is involved in binding the OEC proteins (20), although no direct *in vivo* experimental proof is available. Here we have characterized the *Arabidopsis* T-DNA insertion mutant *psbR* and demonstrated the requirement of the PsbR protein for stable association of the OEC subunit PsbP with the PSII core.

* This work was supported by grants from the Academy of Finland, the Finnish Ministry of Agriculture and Forestry (Nordiskt Kontaktorgan för Jordbruksforskning Project), the Biological Interactions Graduate School of the Finnish Ministry of Education, and the Swedish Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: PSII, photosystem II; PSI, photosystem I; Chl, chlorophyll; LHC, light-harvesting complex; LMM, low molecular mass; OEC, oxygen-evolving complex; WT, wild type; LL, low light; HL, high light; GL, growth light.

MATERIALS AND METHODS

Plant Material and Growth Conditions—*Arabidopsis thaliana* ecotype Columbia T-DNA insertion mutant (SALK_114469) was obtained from the Salk collection (the Salk Institute Genomic Analysis Laboratory) (21) via the Nottingham Arabidopsis Stock Centre. Three plants were obtained lacking the PsbR protein, as analyzed by Western blotting. Furthermore, PCR analysis of these three plants and the wild type (WT) (ecotype Columbia) with primers specific to the flanking sequences of both the left (LP, 5′-ACCGGATCTTCTCTCCCTCGT-3′) and the right (RP, 5′-TCTTGTGCATGGAAATCATAAAGGA-3′) sides of the PsbR gene together with a primer inside the T-DNA insert (LB, 5′-TGGTTCACGTAGTGGGCCATCG-3′) confirmed the three plants to be homozygous mutants. PCR amplification of homozygous psbR mutant DNA resulted in a 780-bp product, whereas amplification of the WT DNA with the same primers produced a 960-bp fragment. Both psbR and WT plants were grown in a growth chamber under a photon flux density of 125 μmol of photons m^{-2} s^{-1} in 8-h light/16-h dark cycles at a temperature of 25 °C. For some experiments, plants were also grown under very low (15 μmol of photons m^{-2} s^{-1}), low (50–60 μmol of photons m^{-2} s^{-1}), and high (450 μmol of photons m^{-2} s^{-1}) light. Fully grown rosettes were used for studies. For short-time regulation studies, WT plants were, after turning the lights on, first kept under growth light for 2 h and then exposed for 5 h to darkness, very low light (15 μmol of photons m^{-2} s^{-1}), growth light (125 μmol of photons m^{-2} s^{-1}), and high light (450 or 750 μmol of photons m^{-2} s^{-1}).

The details of transformation, selection, and growth of the tobacco (*Nicotiana tabacum*) *psbEFLJ* operon mutants were described earlier (11, 18, 19). WT and "RV" (which has an *aadA* gene cassette in a neutral EcoRV site in the 3′-untranslated region of the operon) plants, grown under the same conditions as the mutants, were used as controls.

Northern Blotting—Total RNA was isolated with TRIzol (Invitrogen) according to Tri reagent total RNA isolation protocol (Sigma), and Northern blotting was performed with standard techniques (22).

Isolation of Thylakoid Membranes—Leaves were briefly homogenized with a blender in 50 mM HEPES-KOH, pH 7.5, 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 5 mM ascorbate, 0.05% bovine serum albumin, and 10 mM NaF, filtered through Miracloth, and centrifuged for 4 min, 2500 × *g*, and at 4 °C. The pellet was resuspended in 50 mM HEPES-KOH, pH 7.5, 5 mM sorbitol, 10 mM NaF and centrifuged for 4 min at 2500 × *g*, 4 °C. The thylakoid pellet was resuspended in 50 mM HEPES-KOH, pH 7.5, with 100 mM sorbitol, 10 mM $MgCl_2$, and 10 mM NaF, centrifuged for 3 min at 2500 × *g*, 4 °C, and finally resuspended in the same buffer. Chlorophyll (Chl) was extracted in 80% (v/v) buffered acetone (2.5 mM HEPES-NaOH, pH 7.5) and quantified as described (23).

Protein Analysis—Proteins were separated in SDS-PAGE (15% acrylamide, 6 M urea) (24). After electrophoresis, proteins were electroblotted to a polyvinylidene fluoride membrane (Millipore). Western blotting with chemiluminescence detection was performed with standard techniques using protein-specific antibodies or an antibody raised against the PSI complex. The PsbR antibody was produced against synthetic peptides (Eurogentec). Antibodies against the Lhcb and Lhca proteins were purchased from Agrisera, and the phosphothreonine antibody was from New England Biolabs. 0.5 μg of Chl was loaded, except for the detection of Lhca and Lhcb subunits (0.3 μg), PsbQ (2 μg), and the phosphothreonine antibody (1 μg). These amounts of protein have been tested in our laboratory to give a linear immunoresponse with respective antibodies. Immunoblots were quantified with FluorChem Image Analyzer (Alpha Innotech Corp.). Blue native gel electrophoresis was performed as described earlier (25).

TABLE 1

Chlorophyll (Chl) *a*/*b* ratio, rate of oxygen evolution and the PSII excitation pressure (1- qP) of the *psbR* and WT plants

For measurements of light saturated oxygen evolution, the plants were grown under 125 μmol photons m^{-2} s^{-1} (GL) and 50 μmol photons m^{-2} s^{-1} (LL). The oxygen evolution rate of *psbR* as a percentage value of that measured in WT is given in parentheses. For PSII excitation pressure, the plants were grown under 125 μmol photons m^{-2} s^{-1} (GL), and the measurements were performed under the same light and temperature conditions. The values are means ± S.D.

	WT	*psbR*
Chl *a*/*b*	3.15 ± 0.13	3.08 ± 0.13
O_2 evolution (μmol O_2/mg Chl h^{-1})		
GL	313 ± 8.9	216 ± 11.2 (69%)
LL	216 ± 12.4	126 ± 8.3 (54%)
PSII excitation pressure	0.065 ± 0.009	0.147 ± 0.020

Functional Measurements—Light-saturated steady state rates of oxygen evolution were measured at 25 °C with Hansatech DW1 O_2 electrode using 1 mM 2,6-dimethyl-*p*-benzoquinone as an artificial electron acceptor. Isolated thylakoid membranes were suspended in 50 mM Hepes-NaOH (pH 7.0), 15 mM NaCl, 5 mM $MgCl_2$, and 400 mM sucrose at a Chl concentration of 10 μg ml^{-1}. PSII excitation pressure (1-qP) was measured with a PAM fluorometer (PAM 101/103, Heinz Walz) according to Ref. 26, and the PSII efficiency was measured as a ratio of variable to maximal fluorescence (F_v/F_{max}), detected from intact leaves with a Hansatech Plant Efficiency Analyzer fluorometer (King's Lynn, UK) after dark incubation of 30 min.

Room temperature EPR was measured with a Bruker ELEXSYS E500 spectrometer equipped with a standard Bruker 4102 cavity as described (27). The relative amount of photosystem I (PSI) in the thylakoid membrane was estimated from the intensity of non-saturated EPR spectra from chemically oxidized $P700^+$. The relative amount of PSII was estimated from the intensity of non-saturated EPR spectra from the dark stable radical form $Y_D^{\cdot}$ (tyrosine 161 on the D2 protein). The intensities of the Y_D and P700+ were determined by double integration of the spectra.

RESULTS

We have characterized the T-DNA insertion mutant line SALK_114469, having an insert located in the third exon of the *PsbR* gene (At1g79040). *psbR* mutant plants showed no phenotype when grown under normal light conditions (125 μmol of photons m^{-2} s^{-1}), under high light (450 μmol of photons m^{-2} s^{-1}), or low light (15 or 50 μmol of photons m^{-2} s^{-1}). The Chl *a*/*b* ratio was slightly lower in the mutant thylakoids as compared with WT (Table 1), although no statistically significant difference could be detected.

Functional Properties of PSII in the Absence of the PsbR Protein—The steady state oxygen evolution was markedly lower in the thylakoids of the *psbR* mutant as compared with WT. 30% reduction was measured for *psbR* plants grown under standard growth light conditions, and over 50% reduction was measured under low light conditions (Table 1). PSII photochemical efficiency, deduced from the F_v/F_{max} measurements, was, however, not compromised in the absence of PsbR. Moreover, the lack of PsbR did not make plants more susceptible to donor site photoinhibition (28) during high light illumination of the leaves (data not shown). On the other hand, the *psbR* mutant exhibited over 2-fold higher PSII excitation pressure as compared with WT plants (Table 1). Such defects in the electron transfer chain were reflected as reduced steady state phosphorylation of both the PSII core and the LHCII phosphoproteins as compared with WT (Fig. 1*A*).

PSI/PSII Ratio in the psbR Mutant and WT Plants—By using room temperature EPR measurements of the dark stable $Y_D^{\cdot}$ radical and the

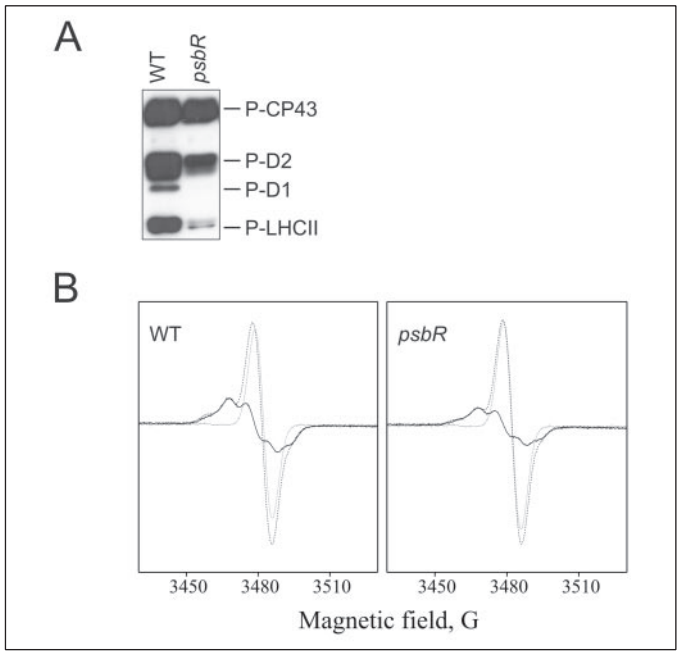

FIGURE 1. **Steady state phosphorylation of PSII-associated proteins and the PSI/PSII ratio in *psbR* and WT thylakoid membranes.** *A*, a phosphothreonine immunoblot demonstrating a steady state phosphorylation of the PSII core and LHCII proteins in WT and the *psbR* mutant thylakoid membranes. Proteins were separated by SDS-PAGE, electroblotted onto a polyvinylidene fluoride membrane, and probed with phosphothreonine antibody. 1 μg of Chl was loaded. *B*, EPR spectra recorded from the WT and the *psbR* mutant thylakoid membranes. EPR spectra were recorded in the dark after preillumination of the sample to oxidize Y_D (1 spin per PSII reaction center, *solid line*) and after oxidation of the sample with 5 mM ferricyanide, representing both Y_D and $P700^+$ radicals (*dashed line*). Ferricyanide-oxidized minus dark difference spectra representing pure $P700^+$ (1 spin per PSI reaction center) are indicated by *dotted lines*. The EPR conditions are as follows: room temperature, microwave frequency 9.78 GHz, microwave power 8 milliwatt, modulation amplitude 5 G. Plants grown under 125 μmol of photons m^{-2} s^{-1} were used for the experiments.

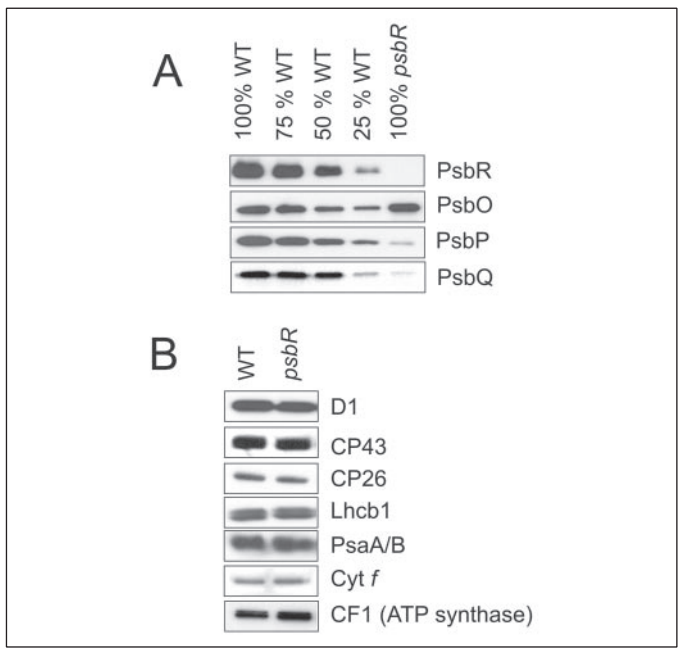

FIGURE 2. **Immunoblots of thylakoid membrane proteins in the WT plants and the *psbR* mutant.** *A*, the presence of the PsbR and the OEC proteins PsbO, PsbP, and PsbQ. *B*, D1, CP43, Lhcb1, PsaA/B, cytochrome *f* (*Cyt f*), and the CF1 α/β subunits. Proteins were separated by SDS-PAGE, electroblotted on to a polyvinylidene fluoride membrane, and probed with antisera against different thylakoid membrane proteins. The equivalent of 0.5 μg of Chl (100%) was loaded, except for the detection of PsbQ (2 μg = 100%) and Lhcb1 (0.3 μg = 100%). Plants grown under 125 μmol of photons m^{-2} s^{-1} were used for the experiments.

$P700^+$ radical, recorded from the ferricyanide-oxidized samples, we obtained the values of 0.71 and 1.01 for the PSI/PSII ratios in WT and *psbR* thylakoids, respectively (Fig. 1*B*). This clearly indicates that the WT thylakoids in our growth conditions (125 μmol of photons m^{-2} s^{-1}) had fewer PSI centers than PSII centers, whereas in the *psbR* mutants, the amounts of PSI and PSII centers were nearly equal. On the basis of measurements of Y_D^-, we found that the amount of PSII (on a Chl basis) in the *psbR* mutant was 72% of that in WT, whereas the amount of PSI was quite unaffected by the mutation.

Protein Composition of the psbR Mutant and WT Thylakoid Membranes—As expected, the T-DNA insert in the *PsbR* gene resulted in a complete loss of the PsbR protein, determined by immunoblotting (Fig. 2*A*). Of the OEC proteins, PsbO was present in near equal amounts in the *psbR* mutant and in the WT thylakoids, whereas the amounts of PsbP and PsbQ were drastically reduced in the *psbR* mutant, PsbP being only 37 ± 3% (mean ± S.D., $n = 3$) of that in the WT plants (Fig. 2*A*). As to the PSII core proteins, the amount of D1 in the *psbR* mutant was slightly lowered (78 ± 5%, $n = 3$) as was also true of the other tested PSII core protein, the internal antenna protein CP43 (Fig. 2*B*). The amounts of the LHCII antenna proteins Lhcb1, Lhcb5 (CP26), and Lhcb4 (CP29) (data not shown) were similar in *psbR* and the WT thylakoid membranes (Fig. 2*B*). Opposite to PSII core proteins, the amounts of the PSI reaction center heterodimer PsaA/B and α/β subunits of CF1 were higher (113 ± 8%, $n = 3$ and 136 ± 10%, $n = 3$, respectively) in *psbR* (Fig. 2*B*), whereas no distinct differences were observed in the amounts of LHCI antenna proteins Lhca1, Lhca2, Lhca3, or Lhca4 between the mutant and WT plants (not shown).

The cytochrome b_6f complex seemed to be unaffected by the lack of PsbR, deduced from the equal presence of the cytochrome *f* subunit (Fig. 2*B*). It was also tested by blue native gel electrophoresis that the oligomeric structures of the membrane protein complexes in *psbR* thylakoids were not altered (data not shown).

Expression of the OEC Proteins and the PsbR Protein in Mutant and WT Plants Grown under Low Light Intensity—When WT and the *psbR* mutant plants were grown under low light (50 μmol of photons m^{-2} s^{-1}), extremely low amounts of both the PsbP and the PsbQ proteins were present in the *psbR* mutant as compared with WT, PsbP being only 6 ± 2% ($n = 3$) and PsbQ 31 ± 8% ($n = 3$) of that in WT plants as determined by immunoblotting (Fig. 3*A*). Similar low amounts of PsbP and PsbQ were evident from Coomassie Brilliant Blue-stained gels of WT and *psbR* thylakoid proteins (Fig. 3*B*). Interestingly, the PsbO protein was present in equal amounts in the *psbR* mutant and WT, independently of the growth light conditions (Fig. 3*A*).

When WT plants grown under medium light (125 μmol of photons m^{-2} s^{-1}) and low light (50 μmol of photons m^{-2} s^{-1}) conditions were compared, it was observed that the amounts of the OEC proteins PsbP and PsbQ were much higher in the thylakoids from low light-grown plants (Fig. 3*A*). Also, the amount of the PsbR protein was somewhat higher in low light-grown than in control light-grown WT plants, although not as much increased as the PsbP and PsbQ proteins. No low light-induced increase was detected for the PsbO protein (Fig. 3*A*).

Because drastic differences were found in the amounts of the OEC proteins PsbP and PsbQ between the *psbR* mutant and the WT, we next investigated whether the regulation takes place at the level of transcription. Northern blots revealed that $PsbP_2$ and $PsbQ_2$ as well as $PsbO_1$ transcripts were present in both the WT and the *psbR* mutant plants in more or less equal amounts (Fig. 3*C*). Moreover, albeit clear increases at the protein level were observed in WT plants grown under low light, the

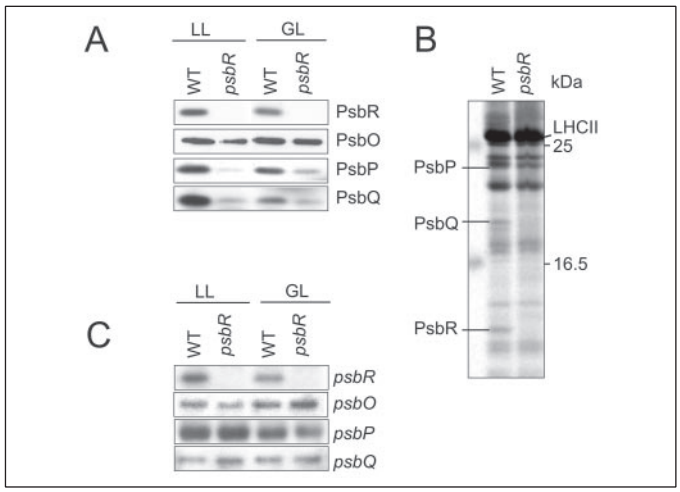

FIGURE 3. **Changes in the contents of the OEC proteins and PsbR, and respective transcripts, after acclimation of *psbR* and WT plants to low light conditions.** *A*, immunoblots representing the PsbR, PsbO, PsbP, PsbQ, and D1 protein levels in the *psbR* mutant and the WT plants grown under LL (50 μmol of photons m^{-2} s^{-1}) and GL (125 μmol of photons m^{-2} s^{-1}) conditions. 0.5 μg of Chl was loaded, except for detection of PsbQ (2 μg). *B*, Coomassie Blue-stained gel of thylakoid proteins of WT and *psbR* plants grown under low light (50 μmol of photons m^{-2} s^{-1}). *C*, Northern blots demonstrating the transcript levels of the *PsbR*, *PsbO*, *PsbP*, *PsbQ*, and *psbA* genes in the *psbR* mutant and the WT plants grown under low light conditions and growth light conditions. 15 μg of RNA was loaded.

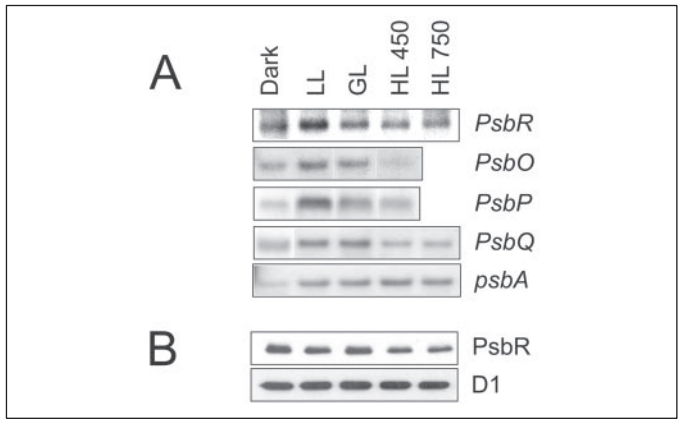

FIGURE 4. **Response of the *PsbR*, *PsbO*, *PsbP*, *PsbQ*, and *psbA* mRNAs and the PsbR and D1 proteins to short term changes in light conditions.** The WT plants were exposed to growth light for 2 h, after which they were transferred to darkness (*Dark*), very LL (15 μmol of photons m^{-2} s^{-1}), and HL (450 or 750 μmol of photons m^{-2} s^{-1}) for 5 h. *A*, transcripts of *PsbR*, *PsbO*, *PsbP*, *PsbQ*, and *psbA*. 15 μg of RNA was loaded. *B*, immunoblot of the D1 and PsbR proteins. 0.5 μg of Chl was loaded. Plants grown under 125 μmol of photons m^{-2} s^{-1} were used for the experiments.

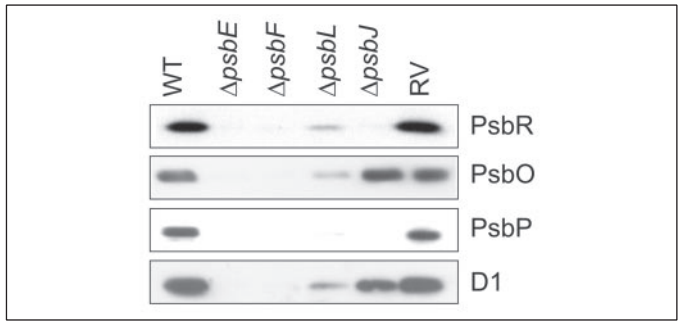

FIGURE 5. **Immunoblots demonstrating the contents of PsbR, the OEC proteins PsbO and PsbP, and the D1 protein in four tobacco *psbEFLJ* operon mutants and the controls, WT and RV.** Proteins were separated by SDS-PAGE, electroblotted on to a polyvinylidene fluoride membrane, and probed with antisera against different thylakoid membrane proteins. 0.5 μg of Chl was loaded, except for the detection of D1 (1 μg).

amounts of *PsbP* and *PsbQ* mRNAs in low light- and growth light-grown plants were nearly equal.

Short Term Light Regulation of the OEC Proteins and the PsbR Protein—Short term light regulation of genes encoding the OEC proteins and PsbR was also tested by exposing WT plants for 5 h to darkness, low light (LL) (here 15 μmol of photons m^{-2} s^{-1}), two different intensities of high light (HL), 450 μmol of photons m^{-2} s^{-1} and 750 μmol of photons $m^{-2}s^{-1}$, and as a control, growth light (125 μmol of photons m^{-2} s^{-1}). As shown in Fig. 4*A*, the *PsbR* transcripts were clearly up-regulated in LL as compared with growth light (GL). The same trend was detected for the expression of *PsbP* and, to a lesser extent, also for *PsbO* and *PsbQ*. Moreover, a slight decrease occurred in these mRNAs at high light, especially for intensities $\geq$450 μmol of photons m^{-2} s^{-1} (Fig. 4*A*). For comparison, the amounts of the *psbA* transcripts were recorded under similar conditions, and contrary to the OEC transcripts, a slight up-regulation under high light was detected for the *psbA* mRNA.

In parallel with mRNA studies, we addressed the possible protein changes of PsbR after the 5-h exposure of plants to different light quantities. PsbR protein contents of the WT plants treated for 5 h at different light conditions were more stable than the respective transcripts. Nevertheless, a decrease in the PsbR protein content was observed after exposing the plants to high light for 5 h (Fig. 4*B*), being 82 $\pm$ 7% and 70 $\pm$ 5% of the control light values after the 5 h of treatment at 450 and 750 μmol of photons m^{-2} s^{-1}, respectively. No decrease in the quantity of the D1 protein occurred during the 5-h treatment of plants under elevated irradiances (Fig. 4*B*).

Occurrence of the PsbR Protein in psbEFLJ Operon Mutants of Tobacco—To gain insights into the interaction of PsbR with the PSII core proteins, we tested the presence of PsbR in the thylakoid membranes of tobacco *psbEFLJ* operon mutants (11, 18, 19). PsbR was missing from the thylakoids of the Δ*psbE* and Δ*psbF* mutants (Fig. 5), which do not show assembly of PSII core complexes (11). In the absence of PsbL, only a small amount of the PsbR protein was accumulated in the thylakoid membranes, corresponding to the contents of the D1 protein and the amount of assembled PSII complexes in this mutant. This straightforward comparison between the D1 protein and assembled PSII core complexes is possible because the D1 protein was never detected as a free protein in the thylakoid membrane. Thylakoids of the Δ*psbJ* mutant, on the contrary, completely missed the PsbR protein, whereas these thylakoids have high amounts of assembled PSII complexes, although inactive in oxygen evolution (11). Intriguingly, the OEC protein PsbP was also completely missing from the Δ*psbJ* thylakoids, whereas PsbO was present in amounts comparable with those in WT thylakoids. Moreover, although the Δ*psbL* mutant had only traces of assembled PSII centers, we also found traces of PsbP protein in Δ*psbL* thylakoids (Fig. 5). An immunoblot of the D1 protein is included in Fig. 5 to indicate the overall contents of the PSII complexes in *psbEFLJ* mutants (Fig. 5) (for details, see Ref. 11).

DISCUSSION

PsbR has long been regarded as a "mystery" subunit of PSII implicated to reside on the luminal side of PSII in a close association with the OEC proteins. Since the *PsbR* gene is missing from cyanobacterial genomes, the high resolution structures of PSII complexes have not been helpful in elucidation of the location and the functional roles of PsbR in the PSII complex. As we have shown here, the absence of PsbR clearly diminishes the oxygen evolution capacity of thylakoid membranes, indicating that PsbR is essential for optimization of photosynthetic water splitting and electron transfer in PSII. Based on the high PSII excitation pressure,

which is an indicator of a high redox state of Q_A, together with a low phosphorylation state of both the PSII core and the LHCII proteins in the *psbR* mutant, it can be deduced that the electron transfer from PSII to the plastoquinone pool is hampered in the *psbR* mutant. Moreover, the higher PSI/PSII ratio in *psbR* is likely to keep the plastoquinone pool more oxidized than in WT under the steady state growth conditions.

The PsbR Protein Enables the Stable Assembly of the OEC Protein PsbP to the PSII Core—Among plants grown under low light intensity (50 μmol of photons m^{-2} s^{-1}), the *psbR* mutant showed a near complete loss of the OEC protein PsbP and extremely low amounts of PsbQ. Under moderate growth light (125 μmol of photons m^{-2} s^{-1}), the PsbP and PsbQ proteins were clearly observable yet in much smaller quantities as compared with WT thylakoids. It is thus conceivable that in higher plants, the PsbR protein is a prerequisite for a stable assembly of these two OEC proteins. The exact location of PsbR in the PSII core is not known, and it remains to be investigated whether PsbR offers a direct docking site for PsbP (and probably PsbP, in turn, for PsbQ, see below) or whether PsbR only fine-tunes the structure of PSII on the luminal side, making the association of PsbP and PsbQ more feasible. Cyanobacteria lack the PsbR protein and also the proper PsbP and PsbQ proteins. However, homologs of *PsbP* and *PsbQ* are found in cyanobacterial genomes, and their gene products have been localized to PSII complexes. Anchoring of cyanobacterial PsbP and PsbQ homologs to the thylakoid membrane, however, takes place via a lipid moiety (5) and probably does not need docking to a protein, like PsbR in higher plants.

Our experimental results corroborate with an early suggestion of PsbR being important for the assembly/docking of PsbP to the PSII complex (20, 29). On the other hand, the characterization of *PsbR* antisense mutants of potato resulted in a contradictory conclusion of the PsbR protein having no effect on OEC proteins (30). However, a closer look at the Western blots of this latter study reveals a high variation in the amounts of PsbP, particularly depending on the extent of silencing of the PsbR protein in different antisense plants.

Low amounts of the PsbP and PsbQ proteins in the *psbR* mutant under steady state growth conditions were apparently due to post-transcriptional regulation since the *PsbP* and *PsbQ* transcripts were present in WT levels. In theory, the low amounts of the PsbP and PsbQ proteins in the *psbR* mutant could result from malfunctions of polyribosome loading, translation initiation or elongation, processing of the preproteins in cytosol, protein import into chloroplasts and to the lumen, and finally, from the capability of proteins to stably associate with the PSII core. Although at present we do not have experimental data to exclude any of these phases, it is reasonable to conclude that the primary reason for low steady state amounts of the PsbP and PsbQ proteins in the *psbR* mutant are likely to be found from improper assembly of these proteins to PSII in the absence of PsbR.

PsbJ, a PSII Core Protein, Is Required for the Assembly of Both the PsbR and the PsbP Proteins—The location of PsbR in the PSII core complex has been difficult to study because it easily drops out from the thylakoid membrane together with the oxygen-evolving proteins. The requirement of specific PSII proteins for the assembly of PsbR is, however, possible to investigate using the knock-out plants of various PSII LMM subunits. Strong *in vivo* association of PsbR with the PSII core is emphasized by the lack of PsbR from thylakoid membranes in the absence of stable PSII assembly in the Δ*psbE* and Δ*psbF* mutants. PsbR was also found to be practically missing in a tobacco Δ*psbJ* mutant (Fig. 5). Interestingly, this mutant has a complete set of all other PSII core proteins studied so far and also the PsbO protein (19), but it specifically lacks the PsbP protein (Fig. 5) (11,12). These results provide evidence that the association of these three proteins, PsbP, PsbR, and PsbJ, to the PSII core is highly dependent on each others.

PsbJ has one putative transmembrane helix (31) and is stably located in PSII core in a close vicinity to cytochrome b_{559} and CP43 (1). It is important to note that PsbJ is an evolutionarily conserved PSII protein and present in both cyanobacteria and higher plants. PsbR, which also has one putative transmembrane helix and is missing from cyanobacterial PSII, as well as from higher plant PSII if the PsbJ protein is knocked out, apparently requires the PsbJ protein for proper assembly to the PSII core. PsbP, on the other hand, is an extrinsic protein located on the luminal side of PSII, and it seems that a proper assembly of both PsbJ and PsbR to the PSII core is a prerequisite for docking of PsbP. Our recent studies on sequential assembly steps of PSII polypeptides corroborate this conclusion. PsbR was found to assemble to the PSII core monomer complex before the internal core antenna protein CP43 (25), whereas PsbP assembles only later in the grana thylakoids (9), probably after a PSII-LHCII supercomplex is formed.

The PsbR Protein Is Not Required for the Assembly of the PsbO Protein and Only Indirectly for the Assembly of the PsbQ Protein—Although no differences in the content of the PsbO protein was observed between *psbR* and WT, the amount of the 17-kDa OEC protein PsbQ was much lower in *psbR* thylakoids. It has been previously suggested that the presence of both PsbO and PsbP is needed for the binding of PsbQ (14). At present, we cannot make a distinction whether the significant decrease in the amount of PsbQ in *psbR* is a direct consequence from the loss of PsbR or whether it is due to the loss of the PsbP protein, an assumed docking site for PsbQ.

Higher plant PsbP and PsbQ have several features in common, which distinctively differ from those of PsbO. PsbO is the only subunit of the OEC complex that is present in all oxyphotoautotrophs and directly involved in water splitting. The recently found PsbP and PsbQ homologs of cyanobacteria most probably have a regulatory role in PSII assembly (5). Functionally, PsbO stabilizes the manganese cluster, whereas PsbP and PsbQ, as well as PsbU and PsbV in cyanobacteria, are involved in optimizing the calcium and chloride binding to the OEC. In addition, PsbO already associates with the PSII core in the stroma lamellae via a Sec-dependent pathway, whereas PsbP and PsbQ utilize the ΔpH-dependent pathway for translocation (Ref. 9 and references therein) and assemble to PSII only after migration to grana thylakoids. PsbO is located toward the D2/CP47 side of the PSII core, whereas PsbP and PsbQ are positioned toward the N terminus of the D1 protein (10). It has also been reported that virus infection of tobacco (*Nicotiana benthamiana*) plants typically reduces the contents of only the PsbP and PsbQ proteins, whereas PsbO remains unaffected (32, 33).

Peculiar Regulation of the OEC Proteins and the PsbR Protein, Acclimation to Low Light Enhances the Expression of the PsbR, PsbP, and PsbQ Proteins but Not of the PsbO Protein—To elucidate the possible role of light-induced chloroplast signals in the expression of nuclear genes encoding the OEC proteins, we applied short-time light treatments of varying light intensity to WT plants. Shift of plants to low light induced a clear up-regulation in *PsbR, PsbO, PsbP*, and *PsbQ* transcripts, which is consistent with our recent report on enhanced synthesis of nuclear encoded thylakoid proteins upon shift of plants to low light (25). It is highly conceivable that those short term processes demonstrate the trigger phenomena, which in long term low light acclimation lead to accumulation of the PsbP, PsbQ, and PsbR proteins in thylakoids of WT plants (Fig. 3*A*). At high light, on the contrary, the synthesis and steady state accumulation of these proteins declined (Fig. 4*B*). Intriguingly, PsbO did not respond to changes in light intensity at the protein level,

suggesting that a stoichiometric relationship between PsbO and the PSII core is maintained independently of light conditions.

It is known that the general rule of "quality control," *i.e.* a rapid degradation of subunits of the membrane protein complexes not capable for assembly (34–36), does not always hold true for the OEC proteins. Indeed, a pool of free, unassembled OEC proteins capable for assembly has been found in the thylakoid lumen (9, 37, 38). It was suggested that these unassembled subunits function as a store to ensure rapid reassembly of functional, oxygen-evolving PSII complexes during the PSII repair cycle (9, 37, 38). This explanation is, however, very unlikely considering the low light conditions tested here, in which the damage and repair of PSII occur very slowly (39, 40). Quite conversely, it was recently speculated that, in addition to their role in water splitting and oxygen evolution, the higher plant OEC proteins might also have structural roles, PsbP and PsbQ possibly being important for the stacking of grana thylakoids (41), which is known to become more pronounced upon acclimation of plants to low light (42). In line with this suggestion, the PSII complexes in non-grana bundle sheet chloroplasts of maize and *Sorghum* are depleted in PsbR, PsbP, and PsbQ proteins as compared with mesophyll chloroplasts with differentiated grana and stroma thylakoids (43). From different light treatments of *Arabidopsis* WT and *psbR* plants, it is clear that the stoichiometry of the PsbR, PsbP, and PsbQ proteins with respect to the PSII core and the PsbO protein can be highly variable, and novel functions for these proteins are likely to be discovered.

Concluding Remarks—Taken together, our results have provided the first direct experimental proof for the role of the PSII LMM protein PsbR in binding the OEC protein PsbP. Moreover, another LMM protein PsbJ is, in turn, required for the assembly of the PsbR protein, thus explaining the earlier reported importance of PsbJ for the assembly of PsbP (11, 12). An interesting adaptation to constant low light conditions was detected; in WT plants, the OEC proteins PsbP and PsbQ as well as PsbR were up-regulated, whereas the PsbO amounts were not dependent on light intensity. This is hypothesized to be connected with enhanced grana stacking at low light.

Acknowledgments—We thank the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutants, Dr. R. Herrmann for providing the tobacco psbEFLJ operon mutants, and Dr. R. Barbato, Dr. T. Hundahl, Dr. C. Spetea, and Dr. F.-A. Wollmann for antibodies.

REFERENCES

1. Ferreira, K. N., Iverson, T., Maghlaoui, K., Barber, J., and Iwata, S. (2004) *Science* **303,** 1831–1838
2. Seidler, A. (1996) *Biochim. Biophys. Acta* **1277,** 35–60
3. Kashino, Y., Lauber, W. M., Carrol, J. A., Wang, Q., Whitmarsh, J., Satoh, K., and Pakrasi, H. B. (2002) *Biochemistry* **41,** 8004–8012
4. De Las Rivas, J., Balsera, M., and Barber, J. (2004) *Trends Plant Sci.* **9,** 18–25
5. Thornton, L. E., Ohkawa, H., Roose, J. L., Kashino, Y., Keren, N., and Pakrasi, H. B. (2004) *Plant Cell* **16,** 2164–2175
6. Summerfield, T. C., Shand, J. A., Bentley, F. K., and Eaton-Rye, J. J. (2004) *Biochemistry* **44,** 805–815
7. Ishikawa, Y., Schröder, W. P., and Funk, C. (2005) *Photosynth. Res.* **84,** 257–262
8. Ohta, H., Suzuki, T., Ueno, M., Okumura, A., Yoshihara, S., Shen, J.-R., and Enami, I. (2003) *Eur. J. Biochem.* **270,** 4156–4163
9. Hashimoto, A., Ettinger, W. F., Yamamoto, Y., and Theg, S. M. (1997) *Plant Cell* **9,** 441–452
10. Nield, J., Orlova, E., Morris, E., Gowen, V., van Heel, M., and Barber, J. (2000) *Nat. Struct. Biol.* **7,** 44–47
11. Suorsa, M., Regel, R. E., Paakkarinen, V., Battchikova, N., Herrmann, R. G., and Aro, E.-M. (2004) *Eur. J. Biochem.* **271,** 96–107
12. Hager, M., Hermann, M., Biehler, K., Krieger-Liszkay, A., and Bock, R. (2002) *J. Biol. Chem.* **277,** 14031–14039
13. Andersson, B., Larsson, C., Jansson, C., Ljungberg, U., and Åkerlund, H.-E. (1984) *Biochim. Biophys. Acta* **766,** 21–28
14. Miyao, M., and Murata, N. (1989) *Biochim. Biophys. Acta* **977,** 315–321
15. Baena-Gonzalez, E., and Aro, E.-M. (2002) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **357,** 1451–1459
16. Shi, L.-X., and Schröder, W. P. (2004) *Biochim. Biophys. Acta* **1608,** 75–96
17. Lind, L. K., Shukla, V. K., Nyhus, K. J., and Pakrasi, H. B. (1993) *J. Biol. Chem.* **268,** 1575–1579
18. Regel, R. E., Ivleva, N. B., Zer, H., Meurer, J., Shestakov, S. V., Herrmann, R. G., Pakrasi, H. B., and Ohad, I. (2001) *J. Biol. Chem.* **276,** 41473–41478
19. Swiatek, M., Regel, R. E., Meurer, J., Wanner, G., Pakrasi, H. B., Ohad, I., and Herrmann, R. G. (2003) *Mol. Gen. Genet.* **268,** 699–710
20. Ljungberg, U., Åkerlund, H.-E., and Andersson, B. (1986) *Eur. J. Biochem.* **158,** 477–482
21. Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C. C., and Ecker, J. R. (2003) *Science* **301,** 653–657
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 7.37–7.42; 7.46–7.48; 7.52, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) *Biochim. Biophys. Acta* **975,** 384–394
24. Laemmli, U. K. (1970) *Nature* **227,** 680–685
25. Rokka, A., Suorsa, M., Saleem, A., Battchikova, N., and Aro, E.-M. (2005) *Biochem. J.* **388,** 159–168
26. Pursiheimo, S., Mulo, P., Rintamäki, E., and Aro, E.-M. (2001) *Plant J.* **26,** 317–327
27. Danielsson, R., Albertsson, P.-Å., Mamedov, F., and Styring, S. (2004) *Biochim. Biophys. Acta* **1608,** 53–61
28. Aro, E.-M., Virgin, I., and Andersson, B. (1993) *Biochim. Biophys. Acta* **1143,** 113–134
29. Ljungberg, U., Åkerlund, H.-E., Larsson, C., and Andersson, B. (1984) *Biochim. Biophys. Acta* **767,** 145–152
30. Stochaus, J., Höfer, M., Renger, G., Westhoff, P., Wydrzynski, T., and Willmitzer, L. (1990) *EMBO J.* **9,** 3013–3021
31. Hankamer, B., Morris, E., Nield, J., Gerle, C., and Barber, J. (2001) *J. Struct. Biol.* **135,** 262–269
32. Rahoutei, J., Garcia-Luque, I., and Baron, M. (2000) *Physiol. Plant.* **110,** 286–292
33. Perez-Bueno, M., Rahoutei, J., Sajnani, C., Garcia-Luque, I., and Baron, M. (2004) *Proteomics* **4,** 418–425
34. Luzikov, V. N. (1986) *FEBS Lett.* **200,** 259–264
35. Mullet, J. E., Klein, P. G., and Klein, R. R. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4038–4042
36. Yamamoto, Y. (2001) *Plant Cell Physiol.* **42,** 121–128
37. Ettinger, W. F., and Theg, S. M. (1991) *J. Cell Biol.* **115,** 321–328
38. Hashimoto, A., Yamamoto, Y., and Theg, S. M. (1996) *FEBS Lett.* **391,** 29–34
39. Aro, E.-M., McCaffery, S., and Anderson, J. M. (1993) *Plant Physiol.* **103,** 835–843
40. Aro, E.-M., McCaffery, S., and Anderson, J. M. (1994) *Plant Physiol.* **104,** 1033–1041
41. Dekker, J. P., and Boekema, E. J. (2005) *Biochim. Biophys. Acta* **1706,** 12–39
42. Anderson, J. M., and Aro, E.-M. (1994) *Photosynth. Res.* **41,** 315–326
43. Meierhoff, K., and Westhoff, P. (1993) *Planta* **191,** 23–33