Deletion of PsbM in Tobacco Alters the $Q_B$ Site Properties and the Electron Flow within Photosystem II*§

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Photosystem II, the oxygen-evolving complex of photosynthetic organisms, includes an intriguingly large number of low molecular weight polypeptides, including PsbM. Here we describe the first knock-out of psbM using a transplastomic, reverse genetics approach in a higher plant. Homoplasmotic ΔpsbM plants exhibit photoautotrophic growth. Biochemical, biophysical, and immunological analyses demonstrate that PsbM is not required for biogenesis of higher order photosystem II complexes. However, photosystem II is highly light-sensitive, and its activity is significantly decreased in ΔpsbM, whereas kinetics of plastid protein synthesis, reassembly of photosystem II, and recovery of its activity are comparable with the wild type. Unlike wild type, phosphorylation of the reaction center proteins D1 and D2 is severely reduced, whereas the redox-controlled phosphorylation of photosystem II light-harvesting complex is reversely regulated in ΔpsbM plants because of accumulation of reduced plastoquinone in the dark and a limited photosystem II-mediated electron transport in the light. Charge recombination in ΔpsbM measured by thermoluminescence oscillations significantly differs from the 2/6 patterns in the wild type. A simulation program of thermoluminescence oscillations indicates a higher $Q_B/Q_A^-$ ratio in dark-adapted mutant thylakoids relative to the wild type. The interaction of the $Q_A/Q_B$ sites estimated by shifts in the maximal thermoluminescence emission temperature of the Q band, induced by binding of different herbicides to the $Q_A$ site, is changed indicating alteration of the activation energy for back electron flow. We conclude that PsbM is primarily involved in the interaction of the redox components important for the electron flow within, outward, and backward to photosystem II.

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Photosystem II (PSII), a supramolecular pigment-protein complex of photosynthetic organisms, utilizes absorbed light energy to oxidize water, releasing dioxygen and electrons that serve as the major source of reducing power in photosynthetic activity. The mechanisms of this process have been studied extensively. Based on biochemical (reviewed in Ref. 1), biophysical (2, 3), and structural analysis, including electron microscopy (4, 5) and x-ray diffraction (6–9), comprehensive knowledge of the basic steps of the water oxidation mechanism, electron transfer reactions, and organization of the individual components involved in these processes has been delineated. Information is also available on the structure of LHCII and the energy transfer to the inner antenna and the photochemical reaction center (10, 11), as well as on the components involved in the biogenesis of PSII (12–14). Most if not all genes encoding PSII subunits have been identified in cyanobacteria and higher plants, and their function, expression, and regulation have been studied extensively (15–17).

PSII is the most intricate assembly of thylakoid membrane systems consisting of more than 30 subunits. It assembles as a dimer together with the minor light-harvesting antenna CP24, CP26, and CP29 for each monomer and is further surrounded by trimeric LHCII protein complexes (5). Based on the similarity of subunit sequences, composition, and activity of PSII, it is generally accepted that the structure of the PSII core in eukaryotes is basically similar to that of cyanobacteria, for which x-ray diffraction structures between 3.8 and 3.0 Å resolution have been obtained (8, 9). However, despite sustained attempts to obtain a higher structural resolution for the PSII of higher plants (10), the heterogeneity of the photochemical center of PSII caused by light-induced changes has so far prevented the formation of crystals allowing a higher resolution of the complex.

One of the most intriguing features of the PSII core is the presence of 16 bitopic, intrinsic, or peripheral low molecular weight proteins. Knowledge about their roles in the overall photo-
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Chloroplasts indeed this has been established for PsbI, PsbT, the α and β subunits of the two-chain cytochrome b559, PsbE and PsbF, respectively, as well as for PsbL and PsbJ, which fulfill crucial structural and functional roles (20–28). However, only limited information is available on the function and exact position of the other LMWs within the PSII complex. Moreover, studies on distinct LMWs from various organisms have indicated different roles (reviewed in Refs. 16, 17) or control of the same function, albeit to a different degree, as is the case for PsbJ in Synecocystis sp. PCC 6803 and tobacco (23). Apparently, the management of PSII electron flow in terms of energy dissipation and avoiding generation of oxygen radicals has to cope with different requirements in various organisms. Such differences may depend on the eco-physiological conditions under which different organisms thrive and the divergence of the outer antenna. It is therefore conceivable that the properties of the LMWs of PSII vary in different organisms.

The study of all chloroplast-encoded LMWs in one organism offers a better chance to understand the roles for each one of them in the same assembly. Therefore, we have inactivated plastid genes encoding LMWs of PSII using a transplastomic approach in tobacco, and we reported on the roles of six of them, including PsbE, PsbF, PsbL, PsbJ, PsbI, and PsbZ (23–28).

The PsbM polypeptide has been detected in PSII complexes isolated from Chlamydomonas reinhardtii (29), Synecocystis sp. PCC 6803 (30), and Synechococcus vulcanus (31), and its presence in Synecocystis sp. PCC 6803 (32) and pea (33) has been confirmed recently using proteomics approaches. However, mutation studies of PsbM have not yet been reported from any organism. Moreover, the exact position of PsbM within the PSII assembly as well as its function remains to be established (8, 17).

The analyses of the first PsbM knock-out presented here demonstrate that the biogenesis of PSII is not significantly altered in the absence of PsbM. However, properties of the Qb site and its interaction with QA and charge recombination within PSI are specifically impaired in ΔpsbM resulting in a shift of thermoluminescence (TL) B band oscillations, a decreased rate of oxygen evolution and forward electron flow, and thus an increased light-induced photoactivation as well as dephosphorylation of LHCII. Because of a high plastoquinol (PQH2) content in dark-adapted mutant plants, levels of LHCII phosphorylation are significantly elevated as compared with the wild type. Phosphorylation of the reaction center proteins D1/D2 is faintly detectable presumably because of conformational changes induced by loss of PsbM proteins.

EXPERIMENTAL PROCEDURES

Clone Construction to Inactivate the psbM Gene in Tobacco Chloroplasts—The recombinant plasmid B20 (tobacco plastome clone bank) (34) containing a 17,235-bp insertion (nucleotide positions 26,191–43,426 in the plastid chromosome, accession number Z00044) in the vector pBR322 was digested with BamHI, and the resulting 2,096-bp fragment containing psbM was subcloned into the singular BamHI restriction site of pBluescript II KS− (Stratagene Inc., La Jolla, CA). Inactivation of the psbM gene (nucleotide position 30,861 (N) to 30,757 (C) bp, accession number Z00044) was achieved by insertion of the adaA cassette, including a terminator signal (35) at a unique BsgI restriction site in the N-terminal part of the gene (nucleotide position 30,844). Transformation of Nicotiana tabacum cv. Petit Havanna, the selection procedure, and in vitro propagation of transformants were carried out essentially as described (25). Isolation of plastid chromosomes by orthogonal pulse-field gel electrophoresis was carried out as described (36). Tobacco lines carrying the adaA cassette in a neutral insertion site and referred to as RV plants were used as wild type (WT) control plants (25).

Preparation and Handling of Thylakoid Membranes—Thylakoid membranes isolated from 4-week-old plants grown under greenhouse conditions were chosen for immunoblot analysis, phosphorylation experiments, and analysis of their composition by the blue native gel (BN) method as described (25). For separation of photosynthetic chlorophyll-protein complexes by sucrose gradient centrifugation, 4-week-old in vitro grown (10–20 μE m−2 s−1 light intensity; 12-h photoperiod) plants were used.

Isolated thylakoid membranes (final chlorophyll concentration 1 mg/ml) were partially lysed during an incubation of 45 min on ice in darkness in bis-Tris/n-dodecyl-β-d-maltoside buffer (final concentrations of 20 mM bis-Tris/HCl, pH 6.5, 5 mM NaCl, 5 mM MgCl2, and 1.0% n-dodecyl-β-d-maltoside). After centrifugation at 18,000 × g and 4°C for 12 min, the solubilized fraction was layered onto a linear (0.1–1.0 M) sucrose gradient in bis-Tris/n-dodecyl-β-d-maltoside buffer. The gradient was run at 4°C for 18 h in a Beckman SW40Ti rotor at 200,000 × g.

Blue native-PAGE (BN-PAGE) was performed as described earlier with modifications (25). The appearing spots were sequenced by mass spectrometry and assigned accordingly (37).

For TL measurements, thylakoids were prepared by grinding a few leaves in a buffer containing 20 mM Tris/HCl, pH 7.4, 5 mM MgCl2, 20 mM NaCl, and 100 mM sorbitol. The material homogenized at 0°C was filtered through nylon micromeshes and used immediately for measurements.

Chlorophyll a Fluorescence Induction Kinetics—Chlorophyll a fluorescence induction kinetics was measured using a pulse amplitude-modulated fluorimeter (PAM-101, Waltz, Effeltrich, Germany) (38). Prior to measurements, leaves were dark-adapted for 5 min. The potential maximum quantum yield of PSII was measured as (Fm′−Fm)/Fm′ = Fv′/Fm′. Red actinic light (650 nm, 20 and 250 μE m−2 s−1) was used for measurements of fluorescence quenching. Photochemical (qP) and nonphotochemical (NPQ) quenching were determined by repetitive saturation pulses. The quenching coefficients, NPQ and qP, were calculated as (Fm′−Fm)/Fm′ and (Fm′−F)/Fm′, respectively (38).

State Transition and Thylakoid Protein Phosphorylation—State transition in intact leaves was calculated using the PAM-101 fluorimeter as (Fm′−F)/Fm′ (39). Protein phosphoryla-
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The deletion of the PsbM gene was carried out using isolated thylakoids as described (25, 40). All buffers used during thylakoid preparation contained 10 mM NaF. Detection of the phosphorylation level of thylakoid membrane proteins was carried out by immunoblotting using anti-phosphothreonine antibodies (New England Biolabs) as described earlier (25).

Low Temperature Fluorescence Measurements—Thylakoid suspensions (20 μg of chlorophyll/ml) of WT and mutant leaves were frozen by immersing a liquid nitrogen-cooled glass rod (4 mm diameter) into the thylakoid suspension and rapidly returning it to the Dewar vessel of the sample holder of the fluorometer filled with liquid nitrogen (Fluoromax-3, Horiba Jobin-Yvon, France). Fluorescence emission spectra were recorded using 430 nm excitation and 1.5 nm slits for both excitation and emission monochromators.

Photosystem I (PSI) Redox State—The redox state of PSI was measured on leaves using the PSI attachment of PAM101 (Walz, Effeltrich, Germany). The oxidation status of PSI at the light intensities indicated was expressed as the ratio ΔA/ΔA_max (41).

Thermoluminescence Measurements—TL of thylakoid suspensions was measured using a homemade apparatus as described (42). Samples (400 μl) of 40 μg of chlorophyll/ml were placed on the TL stage, dark-adapted at 20 °C for 3 min, and rapidly frozen to −20 °C by a stream of liquid nitrogen. The sample was then excited by saturating flashes delivered by a xenon arc discharge lamp (0.05 microfarad capacitor, charged at 1000 V, 3 xenon arc discharge lamp (0.05 μF capacitor, charged at 1000 V, 300-ms interval between flashes) was given at 0 °C followed by single turnover excitations, a train of flashes (1–6 flashes, about 0.5 s apart) was given at 0 °C, and 1.5 nm slits for both excitation and emission monochromators.

Inactivation of psbM—Nine independent transformants with an identical phenotype were initially obtained, and three lines were used for further studies. Their homoplastic status was confirmed by sequencing of the insertional site and by PCR analysis using isolated plastid chromosomes as template (Fig. 1A). Northern analysis with a strand-specific probe containing the coding region of the psbM gene demonstrated that not even traces of psbM transcripts were detectable confirming the homoplastic state of the mutant (Fig. 1B).

Levels and Compositions of Thylakoid Membrane Complexes in ΔpsbM Resemble Those of the WT—Inactivation of psbM in tobacco caused a quite distinct phenotype. Different from several other LMW mutants of the chloroplast, such as ΔpsbE, ΔpsbF, ΔpsbL, and ΔpsbI, ΔpsbM plants are capable of phototrophic growth on soil. However, mutant leaves appeared bleached if the light intensity exceeded ~200 μE m⁻² s⁻¹, thus indicating increased light sensitivity of the photosynthetic apparatus. To elucidate the function of PsbM, homoplastic ΔpsbM mutants in tobacco were analyzed by biochemical and biophysical approaches.

The relative amounts and sizes of pigment-containing thylakoid membrane complexes in sucrose gradients of ΔpsbM did not differ significantly from those of the WT (Fig. 2A). Only PSII-LHCII supercomplexes were faintly diminished, and consequently trimeric LHCII antennae complexes showed a slight increase in ΔpsbM. Moreover, a significant increase in the LHCII-CP24-CP29 complex was observed in the mutant compared with WT. These results demonstrate that PSII can be assembled in the absence of PsbM and that its deletion does not
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The measurement of chlorophyll fluorescence kinetics of photoautotrophically grown mutants showed a reduced quantum yield (Fv/Fm) of PSI (0.78 ± 0.012 versus 0.81 ± 0.003 in WT). Fluorescence quenching analysis uncovered at low light intensity (20 μE m⁻² s⁻¹) that the mutant exhibits qP values comparable with those of WT (0.93 ± 0.009 versus 0.96 ± 0.006 in WT), but at higher light intensities (250 μE m⁻² s⁻¹) this value remained much lower (0.34 ± 0.041 versus 0.54 ± 0.052 in WT), which could be due to a lower rate of PSII-dependent electron transport causing an increase in the ratio QA⁻/QA in the mutant as compared with the WT. Lower NPQ values were also detected in the mutant at both light intensities indicating that mutant PSI is more susceptible to thermal dissipation (Table 1). Taken together, these results indicated that although PSII levels are comparable in mutant and WT, the quantum yield of PSI is significantly lower in ΔpsbM. The results above are also supported by our findings that PSI activity measured as O₂ evolution under saturating light was lowered to about 52% in ΔpsbM as compared with WT thylakoids (Table 1).

Energy Transfer to PSI—The 77 K fluorescence emission of ΔpsbM samples exhibited the same peaks as the WT at 685, 695, and 735 nm attributed to the major pigment-protein core complexes of PSII, CP43, CP47, and the PSI-LHCI complex, respectively (Fig. 3). However, a slight lowering of the PSII-related fluorescence emission bands compared with that of PSI was noted in ΔpsbM. This indicated an exiguous disconnection of the outer antenna of PSI and is consistent with the above finding that only levels of PSII supercomplexes are slightly decreased in the mutant.

The Oxidation State of PSI Is Increased in Light-exposed ΔpsbM Plants—Absorption measurements showed a significantly higher oxidation state of PSI in ΔpsbM as compared with WT at both 20 and 250 μE m⁻² s⁻¹ actinic light intensities (Table 1). These results indicate a lower rate of electron flow from PSI to the PQ pool, relative to that of PQH₂ oxidation via PSI activity, and support the conclusion that PSI activity is significantly lower in the mutant as compared with the WT. This conclusion was also supported by measurements of steady state fluorescence levels (Fv/Fm) elicited by 650 nm actinic light at an intensity of 34 μE m⁻² s⁻¹ on background of far-red light (12 watts m⁻²). The ratio Fv/Fm in ΔpsbM/WT was 0.59 ± 0.06, demonstrating that the electron flow to PQ is much lower than that from the reduced quinol to the electron sink via PSI.

State Transition and Phosphorylation of LHCl II and D1/D2 Are Affected in ΔpsbM—Phosphorylation/dephosphorylation of LHClII resulting in transition to state 2 or state 1 was induced by exposing leaves to PSI or PSII light for 15 min, respectively (39). The percentage of state transition was 8.4 for WT and 4.6 for ΔpsbM, corresponding to 55% of the WT activity in ΔpsbM.

FIGURE 1. Inactivation of psbM by introduction of the aadA cassette in nucleotide position 30844 of the tobacco plastid chromosome. A, PCR analysis was performed using plastome-specific primers (5’ CGGACTGAA-AATCCACATTG3’ and 5’ GTTAATTTAATGGTGTGACT3’) flanking the insertion site. The PCR only amplified an expected product of 1,671 bp in the mutant and a 259-bp product in the WT (24) indicating homoplastomy of the psbM gene demonstrates that insertion of the aadA cassette prevents expression of psbM. The amounts of 25S rRNA show equal loading.

alter significantly the assembly of thylakoid membrane complexes in vivo, or during the solubilization process. This finding was substantiated in two ways, by BN-PAGE and sucrose gradient centrifugation (Fig. 2, A and B) followed by denaturing SDS-PAGE (supplemental S2). In the first electrophoretic dimension, the separation profile of the native thylakoid membrane complexes in ΔpsbM was close to that of WT, but again a very slight decrease in the amounts of PSI-LHCII supercomplexes was noticed (Fig. 2B). Comparably, immunoblot analyses showed no difference in the presence of the relative amounts of the intrinsic PSII subunits D1, D2, CP43, CP47, PsbE, PsbI, and PsbW, the extrinsic proteins PsbO, PsbP, PsbQ, and PsbR, involved in water oxidation, as well as the antenna proteins CP29, CP26, CP24, and LHClII (supplemental S3).

Levels of PSII relative to PSI were estimated by quantitative immunoblot analysis using D1 and PsAD antibodies and the AIDA software. We repeatedly used dilution series of WT and mutant thylakoids based on chlorophyll amounts to precisely quantify levels of D1 and PsAD (Fig. 2C). Based on this analysis, we calculated that D1 accumulates to 103.4 ± 3.6% and PsAD to 100.7 ± 5.5% in the mutant relative to the WT. Therefore, PSII and PSI levels are comparable in mutant and WT.

Levels of other thylakoid complexes, i.e., PSI, cytochrome b₆f complex, ATP synthase, and antenna proteins remained unal-
It has been demonstrated that activation of the protein kinase phosphorylating LHCII is related to the process of plastoquinol oxidation by the cytochrome b₆f complex (44). Thus, both light-dependent reduction of the PQ pool or addition of duroquinol in darkness induce phosphorylation of LHCII in the WT (Fig. 4A) (40). However, unlike WT, LHCII in ΔpsbM was unexpectedly highly phosphorylated in darkness even in absence of duroquinol. LHCII phosphorylation decreases significantly in light-exposed mutant samples in the absence or presence of DCMU but still increases upon incubation with duroquinol in darkness (Fig. 4A).

A phosphorylation pattern identical to that of isolated thylakoids was obtained when measuring LHCII phosphorylation in vivo. As in isolated thylakoids, LHCII was significantly phosphorylated in dark-adapted mutant leaves. Exposure of such leaves to red light and far-red light of low intensity preferentially exciting PSI caused dephosphorylation of LHCII indicating that the PQ pool in the mutant is reduced in darkness (Fig. 4B). Interestingly, different from WT the phosphorylation level of D1/D2 was strongly reduced under all experimental conditions, whereas that of CP43 was unchanged in the mutant (Fig. 4).

Thermoluminescence Emission Reveal an Increased Ratio for Q₉/Q₉ in Dark-adapted Mutant Plants—Although PSII complexes in ΔpsbM accumulate at levels comparable with the WT, the data presented so far indicated a significant impairment of PSII activity. To check disturbances of the forward/back electron flow within the PSII complex, thermoluminescence emission was recorded (45). Excitation by a single turnover flash of dark-adapted thylakoids advances the S-state cycle by one step, and the quinone or semiquinones bound to the Q₉ site are fur-
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The difference between the patterns of the TL signal oscillation intensity as a function of the number of exciting flashes could be due to deviation from the predicted S0/S1 ratio in the dark adapted state (1/3) and their respective \( Q_B^-/Q_B^- \) occupancies (1/1) (45). To test this possibility, a simulation program allowing the prediction of the occupancy of the S-states by semiquinone or oxidized quinone, respectively, was employed using the oscillation pattern measured as a function of the number of exciting flashes (supplemental S1).

The results indicate that the quinone occupancy of the \( Q_B^-/Q_B^- \) and the S-state charge recombining pair is designated as the B TL emission band. In tobacco, this band occurs at \(-35 \, ^\circ C\) as is vascular plants in general (24, 47, 48).

Because the emission temperature of the B band at \(35 \, ^\circ C\) was found to be the same in both mutant and WT, the activation energy for back electron flow and recombination process is not changed in \( \Delta psbM \). The fraction of recombining pairs, \( Q_B^-/S_2^-S_3^- \) and the B band emission intensities oscillate with a period of four flashes showing a maximum emission at the 2nd and 6th flash in the WT (24). However, \( \Delta psbM \) showed various oscillation patterns. In each case the first flash resulted in a higher B band emission than that induced by the second flash. A 1/5 oscillation pattern predominantly appeared in young and fast-expanding leaf material (Fig. 5), whereas binary and zigzag oscillations were rarely and almost exclusively observed in mature and slow growing mutant leaves (supplemental S4).

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oxygen evolving complex in darkness can vary significantly in ΔpsbM.

When electron flow from QA to QB is inhibited by ligands binding to the QB site, such as urea or phenolic type herbicides (DCMU and ioxynil, respectively), light excitation results only in the reduction of the QA quinone. In this case, the related TL band temperature is downshifted, compatible with a lower energy required for back electron transfer to the manganese cluster. The resulting emission band is referred to as Q band (49–51). Binding of the herbicides to the QB pocket may slightly modify its structure, which in turn affects the redox potential gap between the QA and QB electron acceptors. This was shown to be the case for binding of DCMU that alters the QA:QA/QB−:QB potential (52, 53). The emission temperature of the Q band in ΔpsbM was significantly downshifted in the presence of both DCMU (10 °C versus 15 °C in the WT) and ioxynil (∼6 to 0 °C versus 3 °C in the WT) (Fig. 6, A and B). Furthermore, the intensity of the Q band emission increases with increasing ioxynil concentrations (5, 10 and 20 μM, respectively), whereas the B band emission persisted in the presence of low ioxynil concentrations and decreased significantly at 20 μM (Fig. 6A). These results indicate the presence of functionally distinct populations of PSII centers with altered QB sites in mutant thylakoids.

The variable and different B band oscillation patterns and the increased QB/QB− ratio in the dark-adapted state of ΔpsbM could be due to a slower back electron flow during the dark adaptation process. This could be caused by an insufficient dark adaptation time of the sample at 20 °C prior to light excitation and thus residual QA−:S1, QA−:S2, or even QB−:S2 populations may still be present. This in turn could affect the final ratio of the S1/S0 states and their occupancy by semiquinol or oxidized quinone. To check this possibility, the time course of the B band decay was measured at 20 °C in darkness. The results demonstrate a complete decay of the B band emission after 3 min of dark adaptation (Fig. 6C). The dark adaptation in all experiments was carried out at 20 °C for 3 min prior to application of single turnover flashes and thus was sufficient to allow complete decay of the S2,3 states. Therefore, loss of QB−:S2 or QB−:S3 recombination after dark adaptation cannot account for the observed departure from the normal TL oscillation pattern in thylakoids of ΔpsbM.

Photoinhibition and PSII Recovery—The alteration of PSII electron flow and activation energy of recombining pairs within PSII could increase the light sensitivity in ΔpsbM because of singlet oxygen formation accompanied by a loss of variable fluorescence and degradation of the D1 protein (reviewed in Refs. 54 and 55). Therefore, repair of PSII activity requires de novo D1 protein synthesis. To compare the photosensitivity, WT and ΔpsbM leaves were exposed to 500 μE m−2 s−1 after treatment with chloramphenicol, an inhibitor of chloroplast translation activity. After 180 min of illumination, ∼84% of PSII quantum yield was lost in ΔpsbM as compared with only 55% in WT leaves. However, exposure to the same light intensity in the absence of chloramphenicol resulted in 70% loss of PSII activity in the mutant as compared with only 27% in WT (Fig. 7A).

FIGURE 6. Thermoluminescence emission of thylakoids treated with herbicides. A, thermoluminescence emission because of QA−:S3 state recombination (Q band) generated by the addition of ioxynil (5 μM) for the wild type (WT, solid line) and curves a–c for ΔpsbM thylakoids dark-adapted in the presence of 5, 10, and 20 μM ioxynil, respectively. Note the presence and changes of the Q band (52, 53). B, the curves show representative Q band emissions of wild type and mutant samples in the presence of 10 μM DCMU. BL, base line of dark-adapted, unexcited mutant sample showing an after-glow band at 45 °C (46). C, time course of the B band decay in ΔpsbM thylakoids in darkness. Excitation was given at 25 °C, and the sample temperature was maintained for times as indicated prior to fast cooling (10 s) to −20 °C before starting the measurements.
The PSII quantum yield measured in high light-exposed leaves is the result of a balance between the rate of PSII protein degradation (56) and the rate of de novo protein synthesis, reassembly, and photoactivation of the complex. To estimate the ability of the mutant to repair photoinactivated PSII, WT and ΔpsbM plants were irradiated with light of higher intensity (1,500 μE m⁻² s⁻¹) to induce significant photoinhibition followed by incubation of the leaves at low light intensity to allow recovery of PSII activity. Before the photoinhibitory treatment, values of $F_v/F_m$ were typical for WT and mutant leaves used in this experiment. Light exposure was continued until PSII was inactivated to a similar degree ($F_v/F_m = 0.17$). PSII activity recovered with the same kinetics and reached almost the initial value of $F_v/F_m$ within 6 h in both mutant and WT (Fig. 7B), indicating that the capacity of de novo synthesis of the degraded protein(s), reassembly of the complex, and its photoinactivation are not notably impaired by the mutation.

DISCUSSION

PsbM Is Not Required to Maintain the Assembly or Recovery of PSII—PsbM is a conserved hydrophobic LMW subunit of the PSII assembly. The sequence of higher plants PsbM shares ∼54% identity with that of Synechocystis sp. PCC 6803 (16). The interface between the PSII core monomers forming the dimer that houses PsbM in the cyanobacterial PSII structure as resolved by x-ray diffraction at 3.5 Å (8) and 3.0 Å (9) also contains the helix PsbTc, and both are close to helix PsbL. PsbM, PsbTc, and PsbL form a protein domain of six helices at the interface of the two PSII monomers. As such, inactivation of $psbM$ results in the loss of two transmembrane helices in the dimeric axis of PSII. Based on the above, two prominent functions could be ascribed to PsbM as follows: (i) involvement in the interaction/binding of the two monomers, and (ii) a possible role in the controlled association/dissociation of the PSII dimer during the biogenesis and/or the photoinhibition/repair process. If so, loss of PsbM could weaken the dimer interconnection and possibly impair PSII repair during photoinhibitory illumination. However, deletion of PsbM does not prevent formation of dimers as well as assembly of active PSII supercomplexes and photautotrophic growth under appropriate greenhouse conditions. This implies that PsbM is dispensable for the biogenesis of PSII. The results presented here clearly demonstrate that none of the proposed functions according to the localization within the complex can be assigned to PsbM in tobacco.

LHCII Dark Phosphorylation in ΔpsbM—Our data indicate that loss of PsbM does not affect the accessibility of the corresponding kinase to LHCII nor its redox-regulated phosphorylation in the mutant. Normally, the PQ pool is relatively reduced under state 2 conditions (57). The unexpectedly high level of LHCII phosphorylation in dark-adapted mutant leaves could be due to persistence of a reduced PQ pool in the dark. This might have resulted from chlororespiration and/or NAD(P)H dehydrogenase activity (58, 59). On the other hand, an impaired PSII-mediated re-oxidation of PQH₂ in dark-adapted mutant leaves could be responsible for the elevated PQ reduction level and consequently phosphorylation of LHCII (see below).

Because application of a weak far-red light was already sufficient to induce dephosphorylation of LHCII, the accessibility of the phosphatase seems to be unaltered. Alternatively, the presence of increased LHCII trimer levels in the mutant may cause aggregation of the trimers during illumination and, as a result, hinder the accessibility of the phosphorylation sites to the protein kinase (40, 60).

Loss of PsbM Impairs Electron Transport Within, Outward, and Backward to PSII—Inactivation of the intrinsic PsbM protein caused a reduced PSII activity increasing P700 oxidation and thus maintaining mutant plants in state 1 as revealed by fluorometric analysis and the phosphorylation status of LHCII in the light. This effect could be due to an increase in the redox potential gap between the QA and QB quinones. Furthermore, $ΔpsbM$ exhibits a departure from the 2/6 oscillation pattern of the TL B band emission (25).

Unlike WT, mutant plants display quite different patterns of TL oscillations with the number of exciting flashes (supplemental S4). The computational simulation of the dark-adapted PSII
states leading to these oscillation patterns indicates that the three different types observed in mutant plants can be generated by a higher $Q_b/Q_A^−$ ratio in dark-adapted thylakoids (Fig. 5 and see supplemental S4 and S5).

A residual B band emission persisted even in the presence of 20 $μm$ oxynil in mutant thylakoids, whereas the emission of that band was already abolished by 5 $μm$ oxynil in the WT. Moreover, the emission temperature of the Q band induced by DCMU was downshifted by 5 °C in $ΔpsbM$ as compared with the WT, implying a structural change in the Q$_B$ site upon binding of DCMU inducing an effect on the activation energy of the recombination process from Q$_A^−$.

These results indicate the presence of a residual PSII population in which the Q$_B$ sites after oxynil treatment and dark adaptation are still occupied by PQ that can be reduced following light excitation to form a Q$_A^−$. Possibly, the Q$_B$ site properties are progressively altered with the development of mutant leaves. This process varied among mutant leaves as indicated by the TL oscillations and the alteration of the $Q_A/Q_B^−$ ratio as compared with the WT. Oxynil binding at the Q$_B$ site in $ΔpsbM$ also suggests conformational changes in the structure of the Q$_B$ binding pocket and its interaction with the Q$_A$ site. This was shown by changes because of DCMU binding at the Q$_B$ site and possible alteration of the activation energy for the back electron flow from Q$_A$ semiquinone (52, 53). Thus, we propose that the Q$_B$ site may also have variability in interacting or exchanging bound semiquinone with PQH$_2$ from the plastoquinol pool. The back electron flow during dark adaptation via deactivation of S$_{2/3}$ in the S$_{2/3}$Q$_B$ centers may need reduction of the Q$_B$ quinone in darkness by a reduced plastoquinol to reach the predicted 1/3 ratio of S$_0$/S$_1$ and 1/1 ratio of Q$_B$/Q$_B^−$ (45, 49). Thus it is possible that the alteration of the Q$_B$ site interferes with this process resulting in more oxidized Q$_B$ sites than semiquinone sites in the dark-adapted PSII populations of the mutant. This again would cause a shift to the 1/5 B band oscillations observed in the mutant. The elevated PQ reduction level and phosphorylation of LHCII in dark-adapted mutant plants may be a consequence of the impaired PSII-mediated reoxidation of PQH$_2$ involving the Q$_B$ pocket. A similar function has also been proposed for the LMW subunit PsbI (25). Unlike $ΔpsbI$, dimeric PSII supercomplexes accumulate in $ΔpsbM$. Therefore, loss of PSII dimerization in $ΔpsbI$ does not necessarily account for the observed effect on the 1/5 oscillation pattern and phosphorylation of LHCII in darkness.

To validate the ability of the simulation program to detect changes in the back electron flow, we have simulated the oscillation pattern of the $ΔpsbI$ mutant as well. The oscillation patterns with the number of exciting flashes also fit with the measured values as reported for this mutant (25) when using the parameters in the simulation program as used in this work (supplemental S4). To obtain a good fit of the measured and simulated data, it is necessary to assume the presence of a residual amount of S$_0$ following dark adaptation. However, an increase in the ratio $Q_A/Q_B^−$ of the dark-adapted thylakoids is suggested also for the $ΔpsbI$ mutant, in which the Q$_B^−$/Q$_A$ sites interaction is altered as well. Considering the binding properties at the Q$_B$ site, these results reflect the most striking functional differences between $ΔpsbM$ and WT.

The results of this work are in agreement with observations that the structure of the PSII core complex exhibits a certain degree of flexibility of the Q$_A$ site conformation. This is indicated by the different effect of herbicide binding at this site on the midpoint potential of Q$_A$ (53). Furthermore, alteration of the D-de loop of the D2 protein affects the properties of the Q$_B^−$-binding site of the D1 protein (61). It is thus conceivable that the structures of the quinone-binding sites harbored by the D1/D2 heterodimer and their interactions with surrounding protein helices are not rigid and are subject to fluctuations required for optimizing electron flow outward and within the PSII. Hence, removal of the PsbM helix pair from the interface region could cause a local conformational change that may affect the adjacent regions of the quinone-binding site(s) as well as D1/D2 phosphorylation. The phosphorylation level of CP43 is comparable in mutant and WT implying that its confirmation is unaltered in $ΔpsbM$ allowing access of the corresponding kinase. The LMWs of PSII may play an important role in stabilizing the plasticity of these quinone-binding sites. In their absence, changes may occur in the PSII core that may be detrimental to its activity and light sensitivity.

We conclude that the lower oxygen evolution rate, the reduced photosynthetic quantum yield, the increased light sensitivity, the shift of the Q$_A$ midpoint potential, an alteration of the Q$_B$-binding site, and the modified oscillation pattern of the TL signals are related to the loss of PsbM. Our data suggest that the PsbM protein in tobacco plays an important role in ensuring an efficient and functional PSII-mediated electron transport rather than in maintaining the assembly, structure, and stability of the PSII complex.

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