PsbI Affects the Stability, Function, and Phosphorylation Patterns of Photosystem II Assemblies in Tobacco*

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Photosystem II (PSII) core complexes consist of CP47, CP43, D1, D2 proteins and of several low molecular weight integral membrane polypeptides, such as the chloroplast-encoded PsbE, PsbF, and PsbI proteins. To elucidate the function of PsbI in the photosynthetic process as well as in the biogenesis of PSII in higher plants, we generated homoplastomic knock-out plants by replacing most of the tobacco psbI gene with a spectinomycin resistance cartridge. Mutant plants are photoautotrophically viable under green house conditions but sensitive to high light irradiation. Antenna proteins of PSII accumulate to normal amounts, but levels of the PSII core complex are reduced by 50%. Bioenergetic and fluorescence studies uncovered that PsbI is required for the stability but not for the assembly of dimeric PSII and supercomplexes consisting of PSII and the outer antenna proteins of PSII-LHCII. Thermoluminescence emission bands indicate that the presence of PsbI is required for assembly of a fully functional Qo binding site. We show that phosphorylation of the reaction center proteins D1 and D2 is light and redox-regulated in the wild type, but phosphorylation is abolished in the mutant, presumably due to structural alterations of PSII when PsbI is deficient. Unlike wild type, phosphorylation of LHCII is strongly increased in the dark due to accumulation of reduced plastoquinone, whereas even upon state II light phosphorylation is decreased in ΔpsbI. These data attest that phosphorylation of D1/D2, CP43, and LHCII is regulated differently.

Analyses of the polypeptide composition of the oxygen-evolving PSII,4 the most complex assembly of the thylakoid system, have uncovered the presence of the intriguing number of 16 low molecular weight proteins (LMWs) that are generally conserved from cyanobacteria to higher plants (for review, see Refs. 1–3). In photosynthetic eukaryotes, the majority, namely PsbE, F, H, I, J, K, L, M, N, Tc, and Z, are plastome-encoded; the remaining five, PsbR, Tn, W, X, and Y1/Y2, are encoded by nuclear genes (4). The high homology between the plastome-encoded and cyanobacterial LMWs suggests conserved roles, as has been proposed for PsbE and PsbF, the α and β subunits of the two-chain cytochrome b6f. Cytochrome b6f is a mandatory constituent of PSII that plays a major role in PSII function and biogenesis (5–7).

The LMW components of PSII are generally bitopic, i.e. harbor a single transmembrane helix, and in all members, except for PsbK and PsbTc, the N-terminal domain has been suggested to be exposed to the stromal face of the membrane complex (PsbE, PsbF, PsbH, PsbI, PsbJ, PsbL, and PsbM) (for review, see Ref. 2). PsbH is the only known LMW component, which is phosphorylated in the chloroplast, but its phosphorylation is missing in cyanobacteria (8). Apart from PsbH, the other major thylakoid phosphoproteins are those of the light-harvesting protein complex (LHCII) and of the PSII core (CP43 and the reaction center proteins D1 and D2) (9–11).

Biochemical approaches and x-ray crystallography have been instrumental in determining the localization of the LMW proteins within the PSII assembly. For the thermophile cyanobacterial PSII core complex, the location of these proteins has been specified with increasing precision (12–18). These components are either located at the periphery of the PSII core monomers or, centrally, at the interface of the two PSII monomers forming a dimer (17). However, besides the psbE/psbF operon products (19, 7), relatively little is known about the function of the other LMWs, their interactions with PSII core components, or their relevance for lipid-protein interactions for the assembly and the energy transfer processes.

The majority of LMWs of PSII in cyanobacteria define the boundary between the dimeric complex and the surrounding lipids of the thylakoid membrane, whereas in the chloroplast many of these proteins form the border between the core complex and the minor light-harvesting antenna system, CP29, CP26, and CP24, and presumably also the trimeric LHCII, constituting the mobile antenna of the complex (3). Consequently, the LMWs in the chloroplast may be involved in processes different from those of their cyanobacterial counterparts and, for instance, affect regulation of state transition or antenna-related processes like light-trapping or non-photochemical quenching (20–21). Because of an evolutionary functional divergence, inactivation of homologous LMWs in cyanobacteria and green algae/higher plants can lead to quite different phenotypes, as

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has recently been shown for PsbJ and PsbL (22). Although the loss of these proteins has only a minor effect in *Synechocystis* sp. PCC6803, relatively dramatic changes of PSII function and stability have been noted in the corresponding tobacco mutants. Inactivation of PsbI and PsbL in plants affects the $Q_{A}$ re-oxidation kinetics and the back-electron flow from plastoquinol to $Q_{A}$, respectively (19, 22). Similarly, inactivation of PsbH, PsbI, and PsbK in cyanobacteria and chloroplasts of algae resulted in quite diverse phenotypes (for details and review, see Refs. 1 and 2). It is, therefore, conceivable to conduct comparative studies on different evolutionary lineages in a phylogenetic context. Moreover, the biogenesis of PSII components differs in cyanobacteria and higher plants. Because of the dual genetic origin of the thylakoid system in higher plants, the biogenesis depends largely on factors encoded by the nuclear genome. For instance, HCF136, one of these factors, is essential for the biogenesis of the PSII core complex in higher plants, but its homologous protein appears to be largely dispensable in cyanobacteria (23, 24).

The assignment of several LMW subunits including PsbI (4.8 kDa) to the x-ray structure of PSII still remains to be settled (25, 26). Earlier studies of the cyanobacterial PSII x-ray structure placed the transmembrane helix of PsbI opposite to the dimerization axis and close to Chl$z$ D2 (13), whereas the crystallographic studies located the PsbI protein at the periphery of PSII core, in close proximity to Chl$z$ D1, the helices A and B of the D1 protein, and the helix VI of CP43 (17, 18). PsbI was present in PSII reaction center (RC) preparations of both spinach and cyanobacteria (27). No knock-out strains for *psbI* are currently available from higher plants. Mutants lacking PsbI have been generated from *Chlamydomonas reinhardtii* (28) and *Synechocystis* (29). Inactivation of *psbI* in *Synechocystis* and *Thermosynechococcus elongatus* strain BP-1 caused some reduction of PSII activity, resulting in decreased oxygen evolution to 70–80% of WT levels, but the mutants grew photoautotrophically. The *Synechocystis* *ΔpsbI* mutant was found to be slightly more sensitive to light than the corresponding WT (29). Deletion of *psbI* in *C. reinhardtii* caused a more severe effect compared with that in cyanobacteria (28). Although the *C. reinhardtii* *ΔpsbI* mutant grows photoautotrophically under low light, its growth rate was quite sensitive to high light. Both the amounts of PSII and the oxygen evolution activity in the mutant were found to be only 10–20% of WT levels.

In an attempt to evaluate the roles as well as biogenetic and phylogenetic aspects of the enigmatic LMW PSII subunits, we have systematically inactivated individual genes using a transplastomic approach in tobacco. Here we demonstrate that PsbI exerts a crucial role in the stability of dimeric PSII and the intrinsic electron flow as well as in the phosphorylation of proteins of the core complex and the light-harvesting antenna of PSII in tobacco.

**EXPERIMENTAL PROCEDURES**

**Knock-out Construct Strategy for ΔpsbI**—In the tobacco plastome the *psbI* gene is located downstream of *psbK*, which also encodes a LMW PSII polypeptide (30–32). In a transplastomic approach, the *psbI* gene of *Nicotiana tabacum* cv. Petit Havanna, 110 bp in length (nucleotide position 8398–8508 on the plastid chromosome; GenBank™/EBI accession number Z00044), was inactivated by replacing most of the gene with the terminator-less, chimeric amino glycoside 3’ adenylation transferase (*aadA*) cassette conferring resistance to spectinomycin in reading frame orientation (7). For this, two PCR reactions were performed with primers *psbl-1* (5’-GGA TCC AAA ATG CAA TTA TCT CTC C-3’) and *psbl-2* (5’-AAG CTT GCA GCT GAA TTC TAC ACA ATC TTC ACA TTC CCG GAC GTA ATC CTG G-3’) and *psbl-4* (5’-ATC TCG AGA TTA CAA CTA TAA CAG GC-3’). *Psbl-2* and *psbl-3* generated overlapping products and introduced a diagnostic EcoRI restriction site. The third reaction was performed with primers *psbl-1* and *psbl-4* using the first PCR product as a template. Primers *psbl-1* and *psbl-4* introduced two flanking restriction sites, BamHI and XhoI, used for cloning into the vector pBluecript II KS− (Stratagene Inc., La Jolla, CA). The presence of a unique restriction site, BtrI, within the disrupted *psbl* gene allowed the insertion of the *aadA* cassette between the EcoRI and BtrI restriction sites. The details of the map construction, location of restriction sites, and primer annealing positions are shown in Fig. 1 A and B. The resulting construct (carrying the *aadA* cassette in the same polarity as *psbl*) was sequenced to verify correct copying of the gene and used for plastid transformation employed in a biolistic approach (33). Selection and culture conditions of the transformed material as well as the check for homoplasy were carried out as described (7).

Independent transformants were obtained displaying an identical phenotype (data not shown). The transformed material was first grown (12-h photoperiod at 25 °C, 10–20 μmol m$^{-2}$ s$^{-1}$ light intensity) for 4–5 weeks on Murashige and Skoog (34) medium supplemented with 3% sucrose, 0.8% agar, and 500 mg/liter spectinomycin. Before transferring the plants to the greenhouse, they were kept for 4–5 weeks on Murashige and Skoog medium without sucrose. If not otherwise indicated, all analyses were carried out with young leaves of ~2-month-old plants grown *in vitro* and under greenhouse conditions (day 27 °C, night 20 °C), respectively. Tobacco lines carrying the *aadA* cassette in a neutral insertion site and referred to as RV plants were used as WT control plants (22).

**SDS-PAGE and Immunoblot Analysis**—Thylakoid membrane proteins of 3–4-week-old plants were isolated and solubilized for SDS-PAGE as described (7). Proteins separated by SDS-Tris-glycine-PAGE (15% acrylamide) (35) were electroblotted to polyvinylidene difluoride membranes (Amersham Biosciences), incubated with monospecific polyclonal antisera against *psbI* gene product (5) and with prim-
BN-PAGE analysis was performed as described earlier (37). All solutions were supplemented with 10 mM NaF. Thylakoid membranes equivalent to 30 μg of chlorophyll were solubilized with dodecyl-β-D-maltoside (1% final concentration) and separated on a 4–12% acrylamide gradient. After electrophoresis, the gels were silver-stained and run in the second dimension in SDS-PAGE with 15% acrylamide. Subsequently, the gels were stained, and the spots containing proteins of interest were excised, denatured, and resolved by SDS-PAGE. The extent of phosphorylation was determined by mass spectrometry (38).

**Chlorophyll a Fluorescence Induction Kinetics**—Chlorophyll a fluorescence induction kinetics of tobacco WT and mutant leaves was measured using a pulse-modulated fluorimeter (PAM101, Walz, Effeltrich, Germany) (22). Leaves were dark-adapted for 5 min before the fluorescence measurements. The minimal (Fo) and maximal (Fm) fluorescence yield and the variable fluorescence (Fv), calculated as (Fm – Fo) as well as the ratio Fv/Fm, which reflects the potential yield of the photochemical reaction of PSII (41), were recorded at room temperature. Photochemical and non-photochemical quenching (qP and NPQ, respectively) were calculated as (Fm – Fm’)/Fm’ and (Fm’ – F)/Fm’, respectively (42).

**Measurements of PSI Activity**—Photosystem I activity was measured on leaves as absorption changes at 830 nm induced by far red light (ΔAmax) (730 nm; 12 watts m⁻²) and in the absence or presence of actinic light (ΔA) (650 nm, 20 and 250 μmol of photons m⁻² s⁻¹ using the PSI attachment of PAM101 (Walz, Effeltrich, Germany) (43). The oxidation status of PSI at the light intensities indicated was expressed as the fraction ΔA/ΔAmax.

**Thermoluminescence (TL) Measurements**—TL measurements were performed using a home built apparatus (22). Thylakoid fractions were prepared by grinding leaves in a buffer containing 20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0 mM NaCl, and 100 mM sorbitol. Homogenized material was filtered through nylon micromesh and used immediately for measurements. Samples (200 μl; 10–15 μg chlorophyll/sample) were dark-adapted on the TL stage at 20 °C for 3 min and then rapidly frozen to −20 °C. The samples were then excited with saturating flashes delivered by a xenon arc discharge lamp (EG&G, 0.05-microfarad capacitor, charged at 1000 V, 3 μs at 70% light emission). TL was recorded upon heating the sample at a constant rate of 0.6 °C s⁻¹. The herbicides DCMU (3-3’4’-dichlorophenyl)-1,1-dimethylurea) or Ioxynil (4-hydroxy-3,5-di-iodobenzonitrile) were added at concentrations of 10 and 5 μM, respectively, to inhibit the electron transfer from Qₐ to Qₐ. For measuring the B band (Qₐ/S₂ and Sₐ/S₈ recombination) oscillations, the dark-adapted samples were slowly cooled, and consecutive flashes (1–6 flashes, time interval 300 ms) were applied between 1 and 0 °C followed by rapid cooling to −10 °C.

**Low Temperature Fluorescence Measurements**—Low temperature (77 K) emission spectra were recorded with thylakoids prepared from young dark-adapted leaves of WT and Δpsbl plants (44). Thylakoid membranes (40 μg chlorophyll/ml) were transferred into a glass tube (0.7-mm internal diameter) for fluorescence measurements and immediately frozen in liquid nitrogen. Fluorescence was excited at 440 nm, and the emission was recorded between 650 and 800 nm. All spectra were recorded with a Jobin Yvon Spex Fluorolog spectrophurometer (Horiba, France) equipped with a photomultiplier (R 374, Hamamatsu, Japan). Slits of 1 nm were used.

**Photoinhibition**—To determine the sensitivity of PSII to oxidative stress, leaves of WT and Δpsbl plants grown under greenhouse conditions were exposed to 500 μmol of photons m⁻² s⁻¹, and the photoinactivation of PSII was measured as Δ(Fv/Fm)/time. To estimate the PSII recovery process during the exposure to the high light treatment, leaf discs were exposed to a similar light treatment after preinfiltration with a
solution of d-threo-chloramphenicol (200 µg ml⁻¹) for 30 min before the light exposure. For control purposes leaf discs were incubated in water.

To assess the photoinhibition and the capacity to recover from photoinhibition, leaves were also exposed to 1500 µmol of photons m⁻² s⁻¹ until a similar loss of activity was reached in both WT and ΔpsbI (measured as Fv/Fm = 0.17), and subsequently incubated at low light (3 µmol photons m⁻² s⁻¹) for up to 6 h, measuring the Fv/Fm level every 1 h.

RESULTS

Disruption of psbI in the tobacco plastid chromosome caused an increased light sensitivity, but young leaves appeared normal green in independent transformants. Comparable with ΔpsbZ but different from ΔpsbE - F, - L, or - J (19, 7), ΔpsbI was capable of growing photoautotrophically on soil under greenhouse conditions. Initial fluorescence kinetic data of ΔpsbI suggested a defect in PSII (see below).

ΔpsbI Mutants Exhibit a Low Photosystem II Quantum Yield—Measurements of PSII quantum yield of ΔpsbI plants grown autotrophically exhibited an increased minimal fluorescence, Fo, causing a reduced ratio Fv/Fm between 0.25 and 0.74 depending on the leaf age as compared with a stable ratio of 0.81 ± 0.02 in the WT. The lower value was found exclusively in older leaves, indicating a gradual loss of quantum efficiency with aging of the leaf. The average Fv/Fm value in rapidly expanding young leaves detached from the upper part of the plants was in the range of 0.66 – 0.74. Fluorimetric measurements indicated a higher NPQ (0.24 ± 0.06 versus 0.17 ± 0.03 in WT) elicited at low actinic light intensity (20 µE/m² s⁻¹) and a lower NPQ (0.77 ± 0.25 versus 1.21 ± 0.21 in WT) when the actinic light intensity was raised to 250 µmol of photons m⁻² s⁻¹. The photochemical quenching (qP) part in ΔpsbI remained almost unchanged at both light regimes (0.99 ± 0.02 versus 0.94 ± 0.01 in WT and 0.78 ± 0.09 versus 0.76 ± 0.12 in WT at 20 and 250 µmol of photons m⁻² s⁻¹, respectively) (Table 1).

To compare the efficiency of the electron flow between PSII and PSI with that between PSI and its final electron acceptors in intact leaves, the extent of PSI oxidation expressed as ΔA/ΔAmax was monitored using absorption changes at 830 nm in the background of different intensities of actinic light in the steady state (20 and 250 µmol of photons m⁻² s⁻¹, 650 nm) (Table 1). The results showed significantly higher levels of oxidized PSI in the steady state excited by both actinic light intensities in the mutant as compared with the WT (Table 1). The results are indicative of a significantly lower rate of electron flow from PSII to the plastoquinone pool in the mutant relative to plastoquinol oxidation activity via PSI.

Levels of PSII Core Components Are Specifically Reduced in the ΔpsbI Mutant—To corroborate that inactivation of psbI did not affect the expression of genes for PSII antenna and other photosynthetic membrane complexes, immunoblotting using specific antiseras against distinct thylakoid proteins was performed. The data obtained confirmed that the stationary protein levels of PSII antenna (LHCBI, CP29, CP26, and CP24), PSI (PsAF and LHCI), ATP synthase (α and β subunits), and cytochrome b₆f complex (cytochrome f) were comparable with those of the WT (Fig. 2 and data not shown). Levels of the PSII core proteins D1, CP43, and CP47 as well as of the oxygen evolving complex protein PsbO were reduced to about 50% compared with the WT, indicating that the relative content of the PSII RC is lower in ΔpsbI (Fig. 2).

The Qₐ Midpoint Potential Is Affected in ΔpsbI as Measured by Charge Recombination—Charge recombination between the different oxidation states (S_i – S_j) of the Mn₅Ca complex at the electron donor side and reduced primary and secondary semiquinone acceptors of PSI Qₐ or Qₐ₂, respectively, serves as an indicator of the forward and back electron flow activity within photosystem II (45, 46). During the recombination process (S_i – S_j) is generated that is reduced by electron flow from Qₐ or Qₐ₂, a process accompanied by luminescence (46, 47). Back
electron flow uphill the redox potential requires energy input that can be supplied by heat and thus the glow generated by the charge recombination in darkness is termed thermoluminescence (TL) (48). The temperature at which the luminescence is maximal is related to the energy gap between the recombining pairs. The maximal TL signal generated by recombination of $Q_B$/H$_{11002}$/S$_{2,3}$ pairs, the B band emission, occurs in tobacco thylakoids at about 35 °C (22, 49). To measure the recombination of the $Q_A$/S$_2$ pair, one has to block reduction of the $Q_A$ quinone during the excitation of the sample by a single turnover flash. This can be achieved by addition of electron flow inhibitors binding specifically at the $Q_B$ site such as the urea or phenol derived herbicides, DCMU or Ioxynil, respectively. Because back electron flow from $Q_A$/H$_{11002}$/P$_{680}$/H$_{11001}$ presents a lower energy gap, the resulting TL signal, termed Q band, occurs at a lower temperature. In WT tobacco, the Q band in presence of DCMU occurs at about 15 °C, whereas that obtained in presence of Ioxynil occurs at about 3 °C (Fig. 3) (22, 50, 51). This difference between the Q band temperature resulting from the recombination of the same charge separated pair ($Q_A$ to P$_{680}$) is ascribed to changes in the conformation of the $Q_B$ site upon binding of DCMU that affects the midpoint potential of the $Q_A$ site. Conversely, binding of Ioxynil to the $Q_B$ site is supposed not to affect the midpoint potential of the $Q_A$ (50). Thus, use of these herbicides may give information not only on their efficiencies to bind to the $Q_B$ site but also on the effect of their binding on the conformation of this site and the resulting effect on its interaction with the $Q_A$ site expressed as alterations in the midpoint potential $Q_B$/H$_{11002}$/S$_3$:QB/QA:QA/H$_{11002}$.

Measurements of the TL emission of $Δpsbl$ thylakoids showed that the peak temperatures of the B band and that of the Q band induced by the addition of Ioxynil were the same, 35 and 3 °C, respectively, for both mutant and WT control (Figs. 3, A and B). However, the Q band was downshifted to 10 °C in DCMU-treated mutant samples (Fig. 3B).

In light-exposed thylakoids, the $Q_B$ site quinone exhibits a binary oscillation between the quinone and semiquinone reduced states. The double-reduced quinone is protonated to quinol that leaves the site and is replaced by a quinone molecule from the plastoquinone pool. Upon transition from light to darkness, half of the PSII population is in the $Q_B^-$ and half in the $Q_B^+$ states. In illuminated thylakoids, the stable S-states of the Mn$_4$Ca complex exhibit a four steps oscillation, S$_0$ and three increasing oxidation steps, S$_1$ to S$_3$. The states S$_4$ and S$_4'$ are highly unstable, extract 4 electrons from water, releasing dioxygen and returning to the S$_0$ state (52).

Upon transition to darkness, the population of PSII consists of the S$_0$ to S$_3$ states. However, during dark adaptation at 25 °C for 3 min back electron flow from the $Q_B^-$ population to that of the oxidized states of the Mn$_4$Ca complex and, thus, charge recombination, will occur driven by the thermal energy and potential difference between the oxidized S$_3$ and S$_4$ states and $Q_B^-$. The S$_0$ state practically does not recombine (47). Under the experimental condition used, this will result in a final ratio of 75% S$_3$, 25% S$_0$ and practically equal amounts of $Q_B^-$ and $Q_B^+$ (47). Consecutive single turnover excitations of dark-adapted thylakoids leads to oscillations of the ratio of recombining $Q_B^-$/S$_3$:Q$_B^-$/S$_2$ pairs and respective light emission with a higher

![FIGURE 3. Thermoluminescence Q and B band emissions and oscillations of the B band in $Δpsbl$ mutant and WT. Shown are thylakoids of WT (A) and mutants (B) were dark-adapted on the TL stage at 20 °C for 3 min. Before cooling to −20 °C, two saturating single turnover flashes were applied at 0 °C to induce the B band emission. Q band emission was induced by flashing at −20 °C in the presence of the inhibitors Ioxynil (5 μM) and DCMU (10 μM). Peak temperature positions for the TL glow Q and B band are indicated. C, the maxima of the oscillating B band emissions are expressed relative to consecutive flashes (1–6) at 0 °C as indicated. Emission is expressed as number of photons per second (cps).](https://www.jbc.org/content/281/45/34231)
emission for the recombination of the QB/S3 pair. Thus, the TL signal intensity of the B band oscillates with the number of single turnover exciting flashes given to dark-adapted thylakoids, with a period of 4, the maxima being at the second and sixth flash (46, 47, 52).

The changes in the properties of the QB site of the Δpsbl mutant may be exhibited not only in the TL measurements elicited by a single turnover flash in presence of DCMU but also under conditions of multiple excitations sustaining forward electron flow. Therefore, we tested whether the mutation affects the oscillation pattern of the TL signal with the number of excitation flashes due to a limited forward electron flow. Indeed, the WT exhibits the normal 2/6 oscillation pattern, whereas the mutant shows an unusual 1/5 oscillation type, indicating an interference with the electron transfer on either the donor or the acceptor side of PSII (Fig. 3C). These results indicate possible changes in both back and forward electron flow in the mutant, suggesting that the mutation affects the interaction between the QA/QB sites and, thus, possibly resulting in alteration of the QA midpoint potential under continuous forward electron flow.

The Ratio PSII/PSI and Energy Transfer to the PSII RC Are Reduced in the Δpsbl Mutant—The ratio of PSI to PSII (F_{PSII}/F_{PSII}) and the functional connection of the LHCII antenna to the RC were monitored by 77 K fluorescence spectroscopy (Fig. 4, A and B). Thylakoid suspensions prepared from young mutant leaves, which displayed a relatively high ratio Fv/Fm of 0.70, were used. A significantly increased ratio F_{PSII}/F_{PSII} appeared in all mutant samples tested as compared with WT (Fig. 4A). The data indicate an excess of PSI relative to PSII. When the signal intensities of the low temperature emission spectra were normalized to the CP43-related peak at 688 nm, leaves of the Δpsbl mutant yielded a higher CP47-related fluorescence at 697 nm as compared with the WT (Fig. 4B). This is indicative of a decreased energy transfer to CP43 and a favored fluorescence emission from CP47 in the mutant. Mutant leaves exhibited an emission shoulder at 680 nm, which is missing in the wild type, indicating a partial dissociation of the outer LHCII antenna from the PSII RC (Fig. 4B). This may also reflect
FIGURE 6. **BN gel electrophoresis of thylakoid membrane complexes and analysis of the second dimension.**

A, thylakoids were solubilized with 1% n-dodecyl-β-D-maltoside, and electrophoresis was performed in the first (BN-PAGE) and second (SDS-PAGE) dimension. Individual protein spots were silver-stained.

B, immunological analysis of the D1 protein was performed of the second dimension. An overexposure could not even detect traces of PSII-LHC supercomplexes but low amounts of the dimer in Δpsbl samples.

C, the patterns of A were selectively stained in red (WT) and in blue (mutant) (Photoshop Version 8.0.1), and the figures were subsequently merged. Note that the PSII dimer and the supercomplexes are missing in Δpsbl. Individual spots, which appear in the second dimension of solubilized thylakoid membrane complexes, were sequenced by mass spectrometry and are labeled accordingly (38).
the reduced amount of the core components relative to the antenna of PSII (Fig. 2).

**Effect of the Δpsbl Mutation on the Stability of Dimeric PSII and Higher Order PSII-LHCII Complexes**—The presence and the relative content of chlorophyll-protein complexes have been investigated by separation of solubilized thylakoid membrane complexes in sucrose density gradients. The chlorophyll-protein banding patterns showed that the PSI-LHCII supercomplexes were below the limit of detection, whereas levels of the PSII monomer as well as of trimeric LHCII complexes associated with CP29 and CP24 antenna were predominant in mutant thylakoids (Fig. 5). A moderate increase was noted in the relative intensity of free LHCII monomers to that of the WT control sample. These results indicated the presence of unstable dimeric PSII-LHCII supercomplexes in the psbl mutant. To confirm these data native membrane complexes were separated by BN-PAGE followed by SDS-PAGE in the second dimension. In accordance with the data described, trimeric LHCII complexes, monomeric PSII and monomeric RC47 complexes lacking CP43, accumulated to higher levels at the expense of PSII dimers and PSII-LHCII supercomplexes in Δpsbl (Fig. 6, A–C). The potential of Δpsbl to assemble higher order PSII complexes was checked by immunological detection of the D1 protein; however, only minute amounts of dimeric PSII complexes could be detected in Δpsbl upon prolonged exposure (Fig. 6B). Interestingly, when de novo synthesized native complexes were investigated by in vivo radiolabeling, substantial amounts of dimers and higher order PSII-LHCII supercomplexes were found to assemble in the mutant (Fig. 7). We, therefore, conclude that Psbl is not essential for an efficient assembly process but rather for the stability of dimeric PSII and PSII-LHCII supercomplexes. Other thylakoid protein complexes, such as PSI, ATP synthase, and cytochrome b6f complex, were found to be unaltered in size and abundance (data not shown and Fig. 6, A and C).

**Phosphorylation of PSII Core Proteins Is Remarkably Decreased and That of LHCII Is Reversely Regulated in psbl Mutants**—Light-induced phosphorylation of the LHCII is mediated by the redox state of the plastoquinone pool, whereas that of the RC has not been studied extensively. Photoautotrophically grown tobacco WT plants showed an increased phosphorylation of D1, D2, and CP43 with increasing light intensity from 30 to 200 μmol m⁻² s⁻¹ and with increasing incubation time from 5 to 15 min.

DCMU treatment inhibits the light-induced phosphorylation of the RC proteins in the WT and, thus, resulted in a phosphorylation status similar to that of dark-adapted plants (Fig. 8A). Phosphorylation of PSII-RC proteins was almost equally distributed in WT supercomplexes, dimers, monomers, and RC47 complexes separated by BN-PAGE (Fig. 8B). However, in Δpsbl plants phosphorylation of D1 and D2 was close to the limit of detection and that of CP43 was reduced to ≤5% in the dark and under all chosen light treatments (Fig. 8, A–C). We conclude that the responsible kinase does not phosphorylate RC proteins efficiently in the psbl mutants.

Phosphorylation of the LHCII antenna is barely detectable in darkness (state I) but increases with increasing light intensity at 650 nm from 10 to 40 μmol photons m⁻² s⁻¹ (state II) in the WT (Fig. 8, C and D). High light treatment (500 μmol of photons m⁻² s⁻¹) caused a decrease in LHCII phosphorylation in the WT (39). Strikingly, phosphorylation of LHCII was high in state I (dark) in the mutant and decreased already under low light (650-nm light at 10 μmol m⁻² s⁻¹). Therefore, it is evident that the phosphorylation of LHCII is reversely regulated in Δpsbl compared with the wild type upon dark/light changes. Phosphorylation of LHCII in the dark induced a re-distribution of light energy in favor of PSI in the mutant as revealed by calculation of the Fₚₚₚₚ/Fₚₚₚ phosphorimaging (BAS2000 software package and the AIDA software package Version 3.25 beta; Raytest, Straubenhardt, Germany). Substantial amounts of PSII-LHCII supercomplexes and dimers were detectable.
FIGURE 8. Redox- and light-dependent phosphorylation of PSII reaction center and antenna proteins. A, immunoblot analysis of thylakoid membrane proteins were performed using an anti-phosphothreonine antibody from Zymed Laboratories Inc. (A) or New England Biolabs (B–E). In agreement with previous reports, the antiserum from Zymed Laboratories Inc. hardly recognizes phosphorylated LHCII proteins (11). Phosphorylation of reaction center proteins CP43, D1, and D2 is induced by light (heterochromatic) in the WT but is almost absent in ΔpsbI. The immunoblot performed with antisera raised against the ATP synthase α and β subunits demonstrates equal loading. B, immunological analysis of the second dimension demonstrates that only traces of CP43 are detectable and that LHCII proteins are highly phosphorylated in dark-adapted mutants. C, a dilution series of the WT was chosen to estimate the lowered amount of phosphorylation of reaction center and antenna proteins in the mutant in the dark and in the light. D, the phosphorylation of LHCII in thylakoids of dark-adapted and red light-incubated plants showed a reverse regulation of the phosphorylation pattern in the mutant (state I dark, state II = 40 μmol of photons m⁻² s⁻¹ at 650 nm). E, phosphorylation of LHCII and reaction center proteins of dark-adapted thylakoids is significantly induced by reduced duroquinone in the WT. Duroquinol activates phosphorylation of the LHCII but only traces of CP43 in the mutant. F, oxidation of the plastoquinone pool was achieved by applying PSI-specific far-red (FR) light (6 watts m⁻²) for 15 min to leaves. This treatment induced dephosphorylation of LHCII in the mutant, indicating that the reduced plastoquinone pool caused phosphorylation of LHCII in the dark.
Psbl Affects Phosphorylation, Stability, and Function of PSII

**DISCUSSION**

Transplastomic psbl Knock-out Mutants in Tobacco Grow Photoautotrophically—Stability of the PSII core complex in higher plants depends not only on the presence and subsequent assembly of D1, D2, CP43, and CP47 but also on several LMW proteins such as psbE, psbF, psbL, and psbl, which are all encoded by the plastid chromosome (19, 7, 22). This is consistent with the succession of subunit integration during the assembly process that has been settled for the order given: cytochrome b559, D2, D1, CP47, and CP43 (57–59). It is, therefore, not surprising that deletion of either of those early assembled as well as structurally and functionally crucial components is detrimental to the photosynthetic process, since no functional PSII complexes are formed.

Limited information is available on LMWs (4, 7), including Psbl, which is also present in the PSII core complex (60). Unlike other components, which are present in core preparations (1–2), Psbl is not essential for photoautotrophic growth in *Synechocystis* and *C. reinhardtii* (28–29). To understand the biogenetic and structural as well as functional aspects of PSII in higher plants more profoundly, we have generated tobacco knock-out plants lacking psbl and characterized the mutant using biochemical, spectroscopic, and fluorimetric approaches. The data obtained show that different from all other core components, Psbl is dispensable for the assembly of the RC core in higher plant thylakoids. Moreover, its loss even allows photoautotrophic growth, but its requirement is disclosed only under distinct light regimes. This differs from other LMWs, such as PsbE, F, L, and J (7), the latter two involved primarily in governing the redox potential of cofactors ligated by the D1/D2 heterodimer to ensure efficient charge separation and the following forward electron transfer through and out of PSII (19, 22).

Psbl Confers Stability to Dimeric PSII-LHCII Supercomplexes—Analysis of PSII assemblies illustrates that only traces of PSII dimer and supercomplexes could be found in the absence of Psbl (Figs. 6 and 7). However, *in vivo* labeling experiments revealed that Δpsbl mutants possess the potential to form various PSII-LHCII supercomplexes (Fig. 7D). Therefore, it is evident that the Psbl protein is essential for the stability of dimeric PSII and, depending on it, of PSII-LHCII complexes. In addition, Psbl is crucial for an efficient forward electron transport within photosystem II. In summary, Psbl exerts a dual function. It is less important for the basic assembly of this photosystem; however, it is required for the stability of higher order complexes of PSII and the proper functioning of PSII.

Although the precise localization of Psbl in the PSII assembly is still a matter of debate based on the crystal structure of the cyanobacterial PSII dimer and the proposed location of the individual LMW subunits, Psbl was assigned toward the outer side of the monomeric core (17–18). At this position it may be involved in binding the antenna protein Lhcb4 (CP29), which in turn could interact with the LHCII trimers M and/or S (3). If this were true, dimer stability could be influenced by low mass subunits residing at two positions, centrally at the monomer interphase, such as Psbl (22), and peripherally, such as PsbL, with its interaction along the pseudo 2-fold axis of symmetry with the other monomeric core (17). Thus, Psbl could “bracket” two monomers forming a PSII dimer. Its absence would destabilize the dimer and, as a consequence, the interaction between PSII and the CP29-LHCII. This could explain the effects on the energy transfer to the core complex in the mutant. The low temperature fluorescence (77 K) analysis is consistent with a

**FIGURE 9.** Photoinhibition of PSII and recovery kinetics in WT and mutant leaves. A, measurement of photoinhibition was based on the recording of the Fv/Fm ratio using 1,500 μmol m⁻² s⁻¹ of irradiance (△ and ▼, WT and mutant without chloramphenicol, respectively; ■ and ●, WT and mutant with chloramphenicol, respectively). B, the restoration of Fv/Fm was recorded for 6 hours after photoinhibition (Fv/Fm = 0.17) at 3 μmol of photons m⁻² s⁻¹ in WT (○) and Δpsbl (△).

and mutant plants were exposed to high irradiance (1,500 μmol of photons m⁻² s⁻¹), and PSII photoinactivation was measured as a function of exposure time with and without the chloroplast translational inhibitor chloramphenicol. The results indicate that PSII photoinactivation is faster in the mutant as compared with the WT (Fig. 9A). To evaluate the cause of light sensitivity in Δpsbl, the recovery rate of photo-damaged PSII was measured after photoinactivation. Within the first hour the recovery rate was almost identical in both cases. The mutant restored 75% of its original PSII quantum yield as compared with 88% for that of the WT after 6 h of recovery, indicating that Δpsbl is primarily light-sensitive but is basically able to assemble photodamaged PSII complexes and, therefore, to recover from photoinhibitory injury (Fig. 9B).
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Psbl is required for phosphorylation of PSII core proteins and a reduced plastoquinone pool in the dark causes phosphorylation of LHCII in the dark in Δpsbl—Phosphoproteins of the RC, i.e. D1, D2, and CP43, have been reported to depend on the protein kinase STN8 in Arabidopsis, whereas those of the LHCII depend on STN7 and STT7 in Arabidopsis and Chlamydomonas, respectively (9–11). However, the direct targets of the kinases and whether the orthologous proteins in tobacco exert the same functions remain elusive.

Strikingly, phosphorylation of LHCII was regulated in response to light in the mutant, but regulation was reverse to the WT behavior when dark and light adapted probes were compared. Irrespective of chosen light conditions (10, 40 (state II), and 500 μmol of photons m⁻² s⁻¹, 650 nm), LHCII phosphorylation was abolished by light, indicating an imbalance of electron transport causing excitation re-distribution between the two photosystems as also shown by 77 K measurements (Table 1).

It has been suggested that cytochrome b₅₅₉ functions in the re-oxidation of the plastoquinone pool in dark-adapted leaves (65). Therefore, it is conceivable that Psbl also supports this function and that the plastoquinone pool remains predominantly reduced in the dark (Table 1). This may cause a redox-regulated activation of the corresponding kinase(s) in the dark in Δpsbl. Our data unequivocally demonstrate that phosphorylation of the LHCII in the dark is not due to structural changes of PSII in the mutant but due to the reduced plastoquinone pool (Fig. 8F). Unlike WT, the increased re-oxidation rate of the plastoquinone induced by PSI as compared with the lower PSII-dependent reduction rate in Δpsbl leads to oxidation of the plastoquinone pool under any light conditions. This explains the light-induced inactivation of the kinase and the dephosphorylation of LHCII.

Remarkably, Δpsbl lost its capability to efficiently phosphorylate PSII-RC proteins. Phosphorylation of LHCII proteins is regulated and induced by duroquinol, but that of D1 and D2 cannot be induced under all chosen conditions. Therefore, we conclude that physiological responses cause the reverse regulation of LHCII phosphorylation but that structural alterations of PSII result in the loss of D1/D2 phosphorylation in the muta-

Reduced rate of energy transfer to the core, especially to CP43, in the Δpsbl mutant. Monomeric cores attached to LHCII proteins have never been observed in mixed populations of disrupted grana as recognized in cryoelectron micrographic images after quick and mild solubilization of the membranes (for review, see Ref. 3). Furthermore, antenna proteins tend to readily dissociate from monomerized PSII core complexes.

Thus, it is conceivable that disruption of psbl affects primarily the stability of the dimer and only secondarily, the formation of supercomplexes.

Examination of the PSII₂ LHCII₄₊₂ model (3) suggests that one of the functions of Psbl is to provide a flexible interface between the rather rigid structure of the PSII core, the fixed antennae, and the mobile LHCII. Psbl may serve as a structural buffer between the ever-changing positions and interaction strength of the peripheral antennae and the photochemical core. It may also play a direct role in stabilization of the dimeric form of PSII. Inactivation of Psbl not only alters energy transfer from the major antenna but also stability of structure/function of the PSII core dimer. The relatively pronounced effects in Δpsbl attest the flexibility of the connection of the photosynthetic machinery with the “outer world.”

Increased Light Sensitivity of PSII in Psbl Mutants—Accumulation of monomeric PSII complexes in Δpsbl may generate β-like PSII centers impaired in plastoquinone reduction (61) and, thus, be responsible for the slow reduction of oxidized P700, an increased sensitivity to photoactivation, and a somewhat slower re-assembly of photodamaged PSII.

Inhibition of electron flow from the primary (Qₐ) to the secondary (Qₐ) quinone acceptor and the resulting accumulation of reduced Qₐ species are the principal events that initiate damage of PSII by photooxidative stress (62, 54). Under high light conditions, the primary cause for photoactivation of PSII is thought to be due to damage of the D1 protein (for review, see Ref. 55). Therefore, the sensitivity of PSII to high light and its ability to restore photochemical efficiency under conditions of low light was analyzed in mutant and WT. It appears that PSII is more rapidly degraded than repaired in Δpsbl with increasing light intensity. Three hours of photoinhibitory light treatment were required to lose 75% of PSII quantum yield in the WT; however, a similar loss was noted already after 2 h in Δpsbl, reinforcing an increased light sensitivity of its PSII (data not shown). Although the initial recovery rate is identical in mutant and WT, the overall capability to recover was lower in the WT.

The Δpsbl Mutation Destabilizes the Qₐ Midpoint Potential—TL measurements, performed to check the effect of the mutation on the electron flow within PSII, indicated an alteration in the properties of the Qₐ binding site that affects the binding of ligands, resulting in changes of the midpoint potential of the Qₐ/Qₐ site. The effect of the mutation is expressed not only in the presence of DCMU occupying the Qₐ site but also in its absence as indicated by the alteration from the normal 2/6 (63) to the unusual 1/5 oscillation pattern. The above changes may result from an aberration of the theoretical 3:1 ratio of S₂: S₀ states and/or the 1:1 occupancy of the state populations Qₐ⁻Qₐ⁺ in dark-adapted samples (51, 64). This in turn may reflect alterations in the midpoint potential of the Qₐ site.

The down-shift in the emission temperature of the DCMU-induced Q band indicates an accelerated charge recombination from Qₐ⁻ to S₂ and, thus, a partial inhibition of back electron flow from Qₐ⁻ and charge recombination via P₆₈₀⁺ that will affect the synchronization of the Qₐ⁻/S₂ transition of the PSII population. In conclusion, in the absence of Psbl the structural dynamics of the Qₐ binding site during light excitation may be destabilized, possibly affecting the Qₐ⁻/Qₐ⁺ midpoint potential and, thus, back and forward electron flow of PSII. Therefore, the properties of electron transport appear modified by alterations in catalytic rather than changes in regulatory characteristics.

Psbl Is Required for Phosphorylation of PSII Core Proteins and a Reduced Plastoquinone Pool in the Dark Causes Phosphorylation of LHCII in the Dark in Δpsbl—Phosphoproteins of the RC, i.e. D1, D2, and CP43, have been reported to depend on the protein kinase STN8 in Arabidopsis, whereas those of the LHCII depend on STN7 and STT7 in Arabidopsis and Chlamydomonas, respectively (9–11). However, the direct targets of the kinases and whether the orthologous proteins in tobacco exert the same functions remain elusive.

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tion. It is reasonable to assume that the Psbl protein allows a close contact of the kinase to the RC presumably by a direct interaction. It is also likely that the lack of phosphorylation of the core complex subunits D1, D2, and CP43 in Δpsbl partially causes the increased light sensitivity since nuclear Arabidopsis mutants defective in phosphorylation of reaction center proteins show a slightly pale phenotype and a somewhat increased photosensitivity (10–11). The fact that phosphorylation of CP43 can be induced by duroquinol but that of D1 and D2 cannot either implies that an earlier unidentified protein kinase could be involved in the phosphorylation of the PSII-RC and/or that access of the kinase to CP43 is favored as compared with D1 and D2.

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