Impact of PsbTc on Forward and Back Electron Flow, Assembly, and Phosphorylation Patterns of Photosystem II in Tobacco1[W][OA]

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Photosystem II (PSII) of oxygen-evolving cyanobacteria, algae, and land plants mediates electron transfer from the Mn4Ca cluster to the plastoquinone pool. It is a dimeric supramolecular complex comprising more than 30 subunits per monomer, of which 16 are bitopic or peripheral, low-molecular-weight components. Directed inactivation of the plastid gene encoding the low-molecular-weight peptide PsbTc in tobacco (Nicotiana tabacum) does not prevent photoautotrophic growth. Mutant plants appear normal green, and levels of PSII proteins are not affected. Yet, PSII-dependent electron transport, stability of PSII dimers, and assembly of PSII light-harvesting complexes (LHCII) are significantly impaired. PSII light sensitivity is moderately increased and recovery from photoinhibition is delayed, leading to faster D1 degradation in ΔpsbTc under high light. Thermoluminescence emission measurements revealed alterations of midpoint potentials of primary/secondary electron-accepting plastoquinone of PSII interaction. Only traces of CP43 and no D1/D2 proteins are phosphorylated, presumably due to structural changes of PSII in ΔpsbTc. In striking contrast to the wild type, LHCII in the mutant is phosphorylated in darkness, consistent with its association with PSI, indicating an increased pool of reduced plastoquinone in the dark. Finally, our data suggest that the secondary electron-accepting plastoquinone of PSII site, the properties of which are altered in ΔpsbTc, is required for oxidation of reduced plastoquinone in darkness in an oxygen-dependent manner. These data present novel aspects of plastoquinone redox regulation, chlororespiration, and redox control of LHCII phosphorylation.

PSII, the oxygen-evolving pigment-protein complex of the thylakoid membrane system, is present in photosynthetic organisms from cyanobacteria to vascular plants. The proteins of the photochemically active reaction center (RC), the heterodimeric polypeptides PsbA (D1) and PsbD (D2) and the low-molecular-weight (LMW) subunits PsbE and PsbF, and the α- and β-chains of cytochrome b559 coordinate the redox cofactors necessary for primary PSII photochemistry. Electron flow following charge separation occurs via several electron carriers of the RC, including pheophytin α, as well as the primary and secondary quinones, QA and QB. The striking similarities between cyanobacterial and land plant PSII core components, including the intrinsic light-harvesting antennae, the chlorophyll-binding proteins CP43 and CP47, and their interactions during photosynthetic charge separation, suggest a high degree of structural and functional conservation. Thus, much of the information obtained from crystallization of a cyanobacterial PSII can be applied to that of higher plants (Zouni et al., 2001; Kamiya and Shen, 2003; Ferreira et al., 2004; Thornton et al., 2005; Müh et al., 2008). PSII cores of higher plants and cyanobacteria form a functional dimer. In chlorophyll a/b lineages, the additional chlorophyll proteins light-harvesting complex II (LHCII),
forming trimers CP24, CP26, and CP29, are associated with dimeric PSII complexes and, except for CP24, partially with PSI, depending on the energy balance between the two photosystems (Dekker and Boekema, 2005; Takahashi et al., 2006). This process is regulated by reversible phosphorylation of these chlorophyll-binding proteins. Excess light excitation of PSII relative to the accessibility of the electron sink induces back electron flow and charge recombination that generates singlet oxygen species, considered one of the causes of photoinactivation and related light-dependent degradation of the D1 protein (Vass et al., 2007) as well as of a genetically programmed stress response (Wagner et al., 2004). Another primary event of photodamage to PSII may be a direct inactivation of the oxygen-evolving complex (Hakala et al., 2005; Westhoff and Herrmann, 1988) as well as of a genetically programmed stress response (Wagner et al., 2004). In this context, it is relevant to mention that the effect of each in the same organism. Here, the first study (to our knowledge) of the effect of psbTc inactivation on PSII function in a higher plant is presented. Our data show that QA and QB site properties, forward and back electron flow, phosphorylation, assembly, and stability of PSII proteins are affected in the mutant. In addition, experiments performed with the wild type reveal that in vivo the QA site of PSII is responsible for the oxidation of reduced plastoquinone and the reduction of oxygen in the dark, presumably to keep the acceptor side of PSII oxidized. This sheds new light on the nature of a terminal oxidase working during chlororespiration.

RESULTS

Inactivation of psbTc

To selectively inactivate psbTc, we inserted a terminatorless amino glycose 3′ adenyln transferase (aadA) cassette into a newly introduced restriction site in the 5′ region of the gene to avoid expression of a functional or truncated protein (Fig. 1A). Homoplasmity of transformed lines was confirmed by PCR analysis of isolated chloroplast chromosomes (Fig. 1B). Primers used for PCR did not amplify the 1,021-bp product obtained for the wild type but exclusively amplified the aadA-containing product of 1,952 bp in ΔpsbTc. Insertion of the selection cassette into a pentacistronic operon could affect the expression, processing, and/or stability of transcripts originating from the same operon or, in this case, from the oppositely transcribed psbN. Therefore, the integrity and levels of well-known processed RNA species (Westhoff and Herrmann, 1988) were estimated by northern-blot analyses using strand-specific probes for psbT, psbH, and psbN (Regel et al., 2001; Ohad et al., 2004; Schwenkert et al., 2006). Detailed analyses of knockout mutants of PSII LMW peptides in tobacco (Nicotiana tabacum) have revealed specific roles for PsbE, PsbF, PsbL, and PsbJ (Regel et al., 2001; Swiatek et al., 2003b; Ohad et al., 2004) as well as for PsbZ (Swiatek et al., 2001), PsbI (Schwenkert et al., 2006), and PsbM (Umata et al., 2007).

Two different LMW peptides associated with PSII were originally designated PsbT. PsbTc of 4 kD originates from a chloroplast gene and the unrelated protein PsbTn of 11 kD originates from a nuclear gene (for review, see Shi and Schröder, 2004; Müh et al., 2008). PsbTc is an intrinsic, bitopic LMW protein harboring a single transmembrane helix and, different from most LMW peptides, exposes its N-terminal tail to the luminal phase of the thylakoid membrane (Shi and Schröder, 2004). The psbTc gene is located in a highly conserved operon, together with the PSII genes psbB and psbH, the cytochrome b,f complex genes petB and petD, encoding cytochrome b, and subunit IV, and psbN on the opposite strand. The operon is transcribed into a primary product of 5.7 kb that is processed in a complex way (Barkan, 1988; Kohchi et al., 1988; Westhoff and Herrmann, 1988).

As part of an ongoing project, individual thylakoid LMW peptides were systematically disrupted in tobacco to elucidate the effect of each in the same organism. Here, the first study (to our knowledge) of the effect of psbTc inactivation on PSII function in a
to photosynthetic parameters, such as reduction of the potential maximum quantum yield of PSII ($F_v/F_m$), to about $0.68 \pm 0.03$ compared with $0.82 \pm 0.01$ in the wild type using at least 20 individuals from each cross. This unequivocally indicates that loss of PsbTc accounts exclusively for the phenotype. Three chosen mutant lines, ΔpsbTc-1 to -3, were used for detailed studies.

Composition of Thylakoid Membrane Proteins in ΔpsbTc-1

The protein composition of thylakoids isolated from ΔpsbTc and wild-type plants was estimated by immunoblot analysis using antisera raised against individual thylakoid proteins (Schwenkert et al., 2006). Levels of PSII proteins D1, D2, CP47, and PsbI and the extrinsic polypeptide PsbO of the oxygen-evolving complex in ΔpsbTc-1 were comparable with those of the wild type (Fig. 2A). Also, no notable differences were found for the major LHCII (Lhcb1), LHCI (Lhca1), and minor antenna apoproteins CP29, CP26, and CP24 or for components of PSI, the cytochrome $b_6f$ complex, and ATP synthase (Fig. 2A; data not shown). This corroborates that translation and accumulation of petB and petD transcripts downstream of psbTc-1 are not affected by the aadA insertion.

Assembly of PSII-LHCII Supercomplexes and Stability of PSII Dimers Are Affected in ΔpsbTc-1 to -3

To gain information on the assembly and stability of PSII in ΔpsbTc plants, controlled partial lysates of isolated thylakoids solubilized with 1% β-dodecylmaltoside were resolved by blue native (BN)-PAGE and subsequent SDS-PAGE. The protein patterns uncovered that PSII-LHCII supercomplexes were almost missing and levels of dimeric PSII complexes were reduced, whereas trimeric LHCII complexes, as well as monomeric PSII RC complexes lacking CP43, called RC47, were increased in ΔpsbTc-1 (Supplemental Fig. S2, A and B). Levels and sizes of all other detectable thylakoid membrane complexes were not significantly affected. To estimate a possible loss of unstable PSII supercomplexes in the mutant during sample preparation, milder separation of thylakoid lysates by Suc density gradient centrifugation and BN-PAGE using 1.5% digitonin (Schwenkert et al., 2007) was applied. Separation profiles confirmed reduced levels of PSII-LHCII supercomplexes in the mutant (Supplemental Fig. S1A). Levels of all other detectable thylakoid membrane complexes were not significantly affected. To estimate a possible loss of unstable PSII supercomplexes in the mutant during sample preparation, milder separation of thylakoid lysates by Suc density gradient centrifugation and BN-PAGE using 1.5% digitonin (Schwenkert et al., 2007) was applied. Separation profiles confirmed reduced levels of PSII-LHCII supercomplexes in the mutant (Supplemental Fig. S1A). Levels of all other detectable thylakoid membrane complexes were not significantly affected. To estimate a possible loss of unstable PSII supercomplexes in the mutant during sample preparation, milder separation of thylakoid lysates by Suc density gradient centrifugation and BN-PAGE using 1.5% digitonin (Schwenkert et al., 2007) was applied. Separation profiles confirmed reduced levels of PSII-LHCII supercomplexes in the mutant (Supplemental Fig. S1A). Levels of all other detectable thylakoid membrane complexes were not significantly affected. To estimate a possible loss of unstable PSII supercomplexes in the mutant during sample preparation, milder separation of thylakoid lysates by Suc density gradient centrifugation and BN-PAGE using 1.5% digitonin (Schwenkert et al., 2007) was applied. Separation profiles confirmed reduced levels of PSII-LHCII supercomplexes in the mutant (Supplemental Fig. S1A). Levels of all other detectable thylakoid membrane complexes were not significantly affected. To estimate a possible loss of unstable PSII supercomplexes in the mutant during sample preparation, milder separation of thylakoid lysates by Suc density gradient centrifugation and BN-PAGE using 1.5% digitonin (Schwenkert et al., 2007) was applied. Separation profiles confirmed reduced levels of PSII-LHCII supercomplexes in the mutant (Supplemental Fig. S1A). Levels of all other detectable thylakoid membrane complexes were not significantly affected.

Figure 1. A, Strategy for inactivation of the chloroplast psbTc gene. The organization of the psbB operon with the genes psbB, psbTc, psbH, petB, petD, and psbN on the opposite strand is indicated. Arrows designate the direction of their transcription. The aadA selection cassette without termination signal and its 16S rDNA promoter (P) and ribosome-binding site (R) were inserted into the introduced HpaI restriction site (plastome position 76,694 bp) of the psbTc coding frame (plastome position 76,683–76,787 bp). B, PCR products of the psbTc region in the wild type (WT) and mutant indicate the homoplasmatic state of the insertion. C, Northern analysis of the wild type and ΔpsbTc-1 using double-strand (petB and psbB) and single-strand (psbTc, psbH, and psbN) probes. Arrowheads indicate shifted psbTc-containing transcripts, confirming the homoplasmatic state of the aadA insertion. Arrowheads 1 to 3 correspond to the up-shifted aadA containing primary spliced and unspliced transcripts in ΔpsbTc-1. Arrows 4 and 5 indicate the up-shifted psbB-psbTc-aadA and the appearing psbTc-aadA transcript in the mutant, respectively. Ribosomal RNA (rRNA) was stained with methylene blue as a loading control.
readily isolated in ΔpsbTc thylakoids. Therefore, a third approach was chosen to discern whether the formation or stability of PSII-LHCII supercomplexes and dimeric complexes is affected in ΔpsbTc-1. Leaves were labeled with [35S]Met, and isolated thylakoids were analyzed by two-dimensional BN-SDS-PAGE (Fig. 2C). This approach showed that the mutant is capable of assembling RC47, PSII monomers, and dimers efficiently but not PSII-LHCII supercomplexes compared with the wild type. Since steady-state levels of dimers are severely reduced, we conclude that the stability of dimeric PSII complexes is affected by the mutation, presumably due to conformational changes induced by the absence of PsbTc. The relative amount of assembled PSII-LHCII complexes even after a short 5-min pulse resembles the pattern after a pulse of 40 min (Fig. 2C), indicating that the supercomplexes are not lost because of severe instability during the labeling period in ΔpsbTc-1. Improper assembly of LHCII may result secondarily from conformational changes of the PSII dimer (Fig. 2C).

**Light Sensitivity: Photoinactivation and Recovery of PSII Activity**

Upon excessive illumination, D1 protein is damaged and degraded, resulting in impaired PSII activity. Therefore, the extent of PSII light sensitivity and its ability to recover from photoinactivation were studied by measuring $F_v/F_m$ as an estimation of the potential PSII quantum yield in wild-type and mutant plants. In order to distinguish between PSII damage and repair (de novo D1 synthesis) processes, the effect of the chloroplast protein synthesis inhibitor D-threo-chloramphenicol (CAP) was examined. For this, we chose saturating concentrations of CAP (200 μg mL$^{-1}$) in darkness and incubation times (30 min) sufficient to completely block D1 synthesis (Supplemental Fig. S1B). Wild-type and ΔpsbTc-1 to -3 leaf discs were illuminated at 1,200 μE m$^{-2}$ s$^{-1}$ after pretreatment with CAP. In the absence of CAP (control conditions), $F_v/F_m$ was reduced to about 50% of the initial value in all three mutant lines compared with only 66% ± 1.2% in the wild type after 4 h of illumination (Fig. 3A; Supplemental Fig. S1C). Addition of CAP induced a more pronounced PSII inactivation in both wild-type and ΔpsbTc-1 plants to 33% ± 1.3% and 20% to 23% of their initial values, respectively (Fig. 3A; Supplemental Fig. S1C). This shows that recovery principally takes place and that PSII is more sensitive to high light exposure in the mutants than in wild-type plants. To further analyze the efficiency of the recovery process, wild-type and ΔpsbTc-1 leaf disc samples were exposed to 1,200 μE m$^{-2}$ s$^{-1}$ until the PSII quantum yield was reduced by about 50%.
yield reached 0.17 in all samples and then were allowed to recover PSII activity under very low light intensity (3 \mu E m^{-2} s^{-1}; Fig. 3B). After 2 h, recovery was about two times faster in the wild type compared with \Delta psbTc-1. Mutant leaf samples finally recovered 70.4\% \pm 8\% of their initial PSII activity compared with 84.2\% \pm 7\% in the wild type within 6 h, indicating a delayed recovery of PSII activity from photoinhibition in \Delta psbTc-1. To further analyze photosensitivity at the protein level, quantitative immunological analysis with D1 proteins of high light-treated wild-type and mutant plants was performed in the absence and presence of CAP (Fig. 3C). We calculated that D1 accumulates to about 85\% in the wild type and to 70\% in the mutant at 3 h after photoinhibition. After CAP and high light treatment, D1 was reduced to approximately 45\% in the wild type and to 15\% in the mutant. These data confirmed that the photoinhibitory effect in \Delta psbTc-1 was indeed due to D1 degradation and that CAP induced a more pronounced degradation of D1 in the mutant compared with the wild type, again indicating an increased light sensitivity of PSII in \Delta psbTc-1 (Fig. 3C).

Activities of PSII and PSI

The maximum quantum yield of PSII, \( F_v/F_m \), was decreased in all independently selected and backcrossed mutant lines (\( \Delta psbTc-1 \) to -3) and ranged from 0.65 to 0.71 versus 0.82 \pm 0.01 in the wild type, which was due to an increased \( F_o \) and/or a decreased \( F_v \) again indicating dissociation of the outer PSI antenna and/or malfunction of PSII electron transport activity. Further photosynthetic parameters have been measured in the three lines (Fig. 4). The photochemical quenching parameter (qP) as well as the effective PSII activity (\( \Phi_{psii} \)) were decreased at 5 and 45 \mu E m^{-2} s^{-1} red actinic light in \Delta psbTc-1 to -3 mutant plants (Fig. 4, A and C), indicating a partial loss of PSII function. Nonphotochemical quenching (NPQ) was also significantly lower at both light intensities, amounting to only about 50\% in the mutants compared with the wild type at red light intensity of 45 \mu E m^{-2} s^{-1}, indicating a decreased transmembrane proton gradient due to lower PSII activity (Fig. 4B). The PSI oxidation state at 5 and 45 \mu E m^{-2} s^{-1} red light intensities was about 7-fold and 4-fold higher, respectively, in \Delta psbTc-1 to -3 compared with the wild type (Fig. 4D). Since NPQ is significantly decreased at both light intensities, the increased oxidation level of P700 can be solely attributed to a lower capacity of electron transfer from PSII to the plastoquinone pool in the mutants. This inference is supported by steady-state fluorescence measurements (\( F_{o}\)') elicited by 650-nm actinic light at an intensity of 34 \mu E m^{-2} s^{-1} on far-red background light (12 W m^{-2}). The ratio \( F_{o}'/F_{o}' \) in \Delta psbTc-1 to \( F_{o}' \) in wild type was 0.77, illustrating that the rate of electron flow toward plastoquinone is lower than that from plastoquinol to the electron sink via PSI in the mutant. These results are also supported by a lowered PSII activity ranging

![Figure 3](https://www.plantphysiol.org)
from 65% to 76% in three mutant plants compared with wild-type thylakoids, measured as oxygen evolution under saturating light conditions in the presence of uncouplers.

Electron Flow within PSII as Measured by Thermoluminescence

Thermoluminescence (TL) measurements were performed to further investigate the effect of psbTc inactivation on electron flow within PSII. Activation energy required for back electron flow from QB \(^2\) to the S2,3 states of the Mn4Ca donor side of PSII via P680\(^+\) charge recombination is supplied by temperature rise and accompanied by light emission. The temperature at which recombination and light emission are maximal is related to the redox potential difference between the recombining charge-separated pairs and thus to the energy input required to drive back electron flow (Krieger-Liszkay and Rutherford, 1998; Rutherford and Krieger-Liszkay, 2001). Charge recombination between QB\(^2\) and oxidized states of S2 or S3 occurs at about 35\(^\circ\)C. The luminescence emission appearing at this temperature is designated the B band (Schwenkert et al., 2006; Ducruet et al., 2007). Blocking electron flow to the QB site results in back electron flow from QA\(^2\) and charge recombination with the S2/3 state, resulting in the Q band emission. Due to the lower energy gap between these recombining pairs, the Q band temperature occurs at about 3\(^\circ\)C to 5\(^\circ\)C (Krieger-Liszkay and Rutherford, 1998; Rutherford and Krieger-Liszkay, 2001; Ohad et al., 2004).

The B band emission temperature was up-shifted to about 38\(^\circ\)C to 42\(^\circ\)C (Fig. 5A), while the Q band temperature emitted in the presence of DCMU was strongly down-shifted to about 3\(^\circ\)C in mutant thylakoids (Fig. 5A). The TL emission temperature in the presence of Ioxynil also showed a severe down-shift of the Q band emission temperature to \(-10^\circ\)C in \(\Delta psbTc\) and \(\Delta psbTc\) (Fig. 5B). Strikingly, the concentration of Ioxynil (5 \(\mu M\)) that is sufficient to saturate the QB site in wild-type thylakoids, resulting only in emission of the Q band at 3\(^\circ\)C, does not completely abolish the B band emission in the mutant, which shows an increase in the Q band and a decrease of the B band emission intensity with increasing Ioxynil concentration up to 20 \(\mu M\) (Fig. 5B). These results demonstrate that the absence of PsbTc induces significant conformational changes with respect to the QA and QB site properties.

The oxidation/reduction of the Mn4Ca cluster usually occurs with a period of four stable steps (S0–S3) during oxygen evolution (Haumann et al., 2005), while that of the QB site occurs in two steps (QB and QB\(^2\)). Following transition from light to darkness, back electron flow from the reduced electron carriers of PSII (pheophytin, QA\(^-\), and QB\(^-\)) occurs, resulting in the accumulation of about 25% S0 and 75% S1 states and a 1:1 ratio of QB\(^-\)/QB (Rutherford et al., 1982, 1984; 1,1-dimethylurea (DCMU) affects the QB site conformation and thus alters its interaction with the QA site, resulting in a decrease of the QA/QA\(^-\)/QB\(^-\)/QB redox potential gap and thus an up-shift of the required energy input to drive back electron flow that occurs at 12\(^\circ\)C to 15\(^\circ\)C (Krieger-Liszkay and Rutherford, 1998; Rutherford and Krieger-Liszkay, 2001; Ohad et al., 2004).

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Krieger-Liszkay and Rutherford, 1998; Umate et al., 2007). The intensity of TL B band emission of dark-adapted tobacco thylakoids, following single-turnover excitations (one to six flashes), is proportional to the number of recombining Q_B^2/S_{2,3} pairs and oscillates with the number of exciting single-turnover flashes with maximal emission at flashes 2 and 6 in the wild type (Umate et al., 2007). Since deletion of PsbTc shows drastic effects on the Q_A/Q_B site interactions and charge recombination, one would expect changes also in the oscillation pattern of the B band emission with the number of excitation flashes. Results of experiments addressing this question indeed showed an alteration of the TL B band oscillation patterns for ΔpsbTc-1 and ΔpsbTc-2, exhibiting emission maxima at flashes 1 and 5 (Fig. 5C). Using a simulation program for oscillation patterns (Umate et al., 2007), the ratio Q_B/Q_B^2 present in dark-adapted thylakoids prior to the flash excitations was significantly increased in ΔpsbTc-1 (1.68 versus 0.90 in the wild type; Supplemental Table S1). These results support the conclusion that the back electron flow within PSII is altered in the mutant, presumably because of alterations of the Q_B binding site properties.

Effect of the Mutation on Redox-Controlled Phosphorylation of LHII and PSII Core Proteins

Plants have developed several mechanisms to efficiently adjust their energy conversion to different light qualities and quantities. Shifting their antenna from one photosystem to the other (state transition) allows optimal adaptation to changing light conditions. Thus, phosphorylation and dephosphorylation of a mobile pool of CP29, CP26, and LHII are induced under light, which preferentially excites PSII (state II) and PSI (state I), respectively (Rochaix, 2007). Regulation of phosphorylation of the D1, D2, and CP43 proteins is less understood. Therefore, phosphorylation and antenna association experiments were performed (Fig. 6, A and B). Strikingly, in the absence of PsbTc, the PSII core proteins D1/D2 were not phosphorylated and CP43 was only marginally phosphorylated under both state I and state II conditions in all three mutant lines (Fig. 6A; Supplemental Fig. S1D). On the other hand, in contrast to the wild type, LHII was already highly phosphorylated in darkness and dephosphorylated under state II light conditions in ΔpsbTc-1 to -3. To check whether phosphorylation in the mutants results from a reduced plastoquinone pool in darkness, state I conditions were adjusted with far-red light. This light treatment abolished LHII phosphorylation in both wild-type and mutant plants (Fig. 6A). Mild solubilization of thylakoids with digitonin and separation of lysates by BN-PAGE allows the detection of LHII-PSII supercomplexes under state II light conditions in the wild type (Schwenkert et al., 2007). In contrast, in the mutant, LHII-PSII supercomplexes were formed only in darkness, consistent with the phosphorylation pattern and the reduced state of the plastoquinone pool.

**Figure 5.** TL measurements of wild-type (WT) and ΔpsbTc-1 thylakoids. A, The B band occurring at 35°C in the wild type is slightly up-shifted to 38°C in the mutant. The Q band emission in the presence of DCMU (10 μM) occurs at 15°C in the wild type and at 3°C in ΔpsbTc. BL indicates the baseline signal generated from dark-adapted, unexcited samples. B, Q band emission of wild-type (α = 5 μM ioxynil) and ΔpsbTc-1 (b = 5 μM, c = 10 μM, and d = 20 μM ioxynil) thylakoid samples. The Q band emission temperature is down-shifted to −10°C at all ioxynil concentrations compared with 3°C to 5°C in the wild type. Note that curves b, c, and d depict a persistent B band emission in the presence of ioxynil. BL, Baseline. C, The B band oscillation pattern shows a 1/5 oscillation type in ΔpsbTc-1 compared with the typical 2/6 type for the wild type. All SD values are below 0.5%. Comparable results were obtained with ΔpsbTc-2 plants.
PsbTc Affects Function of PSII and Redox State of Plastoquinone

DISCUSSION

Effect of PsbTc Deletion on Assembly, Stability, and Structure of PSII

Studies on PsbTc deletion mutants were reported from Synechocystis 6803 (Iwai et al., 2004) and two Thermosynechococcus species (Henmi et al., 2008) representing eubacterial organisms and from Chlamydomonas reinhardtii (Monod et al., 1994; Ohnishi and Takahashi, 2001; Ohnishi et al., 2007). Data obtained with Thermosynechococcus suggested that PsbTc is involved in the assembly of dimeric PSII complexes. However, since deletion of psbTc in the Chlamydomonas mutant resulted only in a weakly impaired PSII, it was concluded that this protein is not essential for the organization and function of PSII. Solubilization studies of proteins labeled in vivo indicate that PsbTc in tobacco is not required for the assembly but rather for the stability of the dimeric PSII core (Fig. 2, B and C).

The absence of PSII-LHCII assemblies in ΔpsbTc-1 to -3 is intriguing, since PsbTc has been localized to the region interconnecting PSII monomers next to the central 2-fold axis (Ferreira et al., 2004; Loll et al., 2005). From its outlined function and position, PsbTc could be involved in the attachment of the outer antenna, possibly through interaction with adjacent proteins, like CP47; therefore, it exerts a long-distance effect.

The proposed positions of PsbTc, PsbL, and PsbM close to the central axes of the PSII dimer previously

(Fig. 6B, top). The composition of the bands representing the LHCII-PSI supercomplex was also demonstrated by immunoblot analysis of the native gel using antibodies raised against LHCII and PsaaA/B (Fig. 6B, bottom). These data indicate a role of the PSII protein PsbTc in the oxidation of reduced plastoquinone in the dark.

The QB Binding Site Is Required for Oxidation of PQH₂ in the Dark

Reduction of plastoquinone takes place via several nonphotochemical pathways, and reoxidation often depends on oxygen (for review, see Rumeau et al., 2007). A prominent functional effect of the mutation seems to be an alteration in the role of the QB binding site important for efficient forward electron flow in the light and presumably reoxidation of the plastoquinone pool, which is nonphotochemically reduced in darkness. Were this true, specific inhibition of the QB site could be involved in the attachment of the outer antenna, possibly through interaction with adjacent proteins, like CP47; therefore, it exerts a long-distance effect.
Photosensitivity is more striking with translation instead of photoinhibition in plants. Lack of PsbTc results in increased D1 degradation and related oxidative damage in ΔpsbTc.

Effect of PsbTc Deletion on QA and QB Site Properties and PSII Electron Flow

The conformational changes in the organization of PSII induced by the loss of PsbTc affect QA and QB site properties and/or interaction, resulting in an increased ratio Q8/QB and alterations in the PSII charge recombination, as judged from TL measurements. This indicates that there are more oxidized Q8 sites than semiquinones in the mutant, causing an impaired back electron flow and reduction of the plastoquinone pool in dark-adapted PSII populations. However, it still remains controversial which plastoquinone-binding site is largely or directly affected by the absence of PsbTc, because both peaks of Q and B bands in transformant plants were shifted compared with those of wild-type plants. Due to the closer vicinity of PsbTc to QA, it is conceivable that conformational changes at the Q8 site may affect its interaction with the Q8 site. Therefore, the effect on the Q8 site could represent a secondary effect of the mutation, as has also been implied for the long-distance effect on the assembly of PSII-LHCII supercomplexes and the stability of PSII dimers in ΔpsbTc.

The increase in the B band emission temperature and the down-shift of the Q band also imply an increase in the redox potential gap between the QA/Q8 sites and a decrease of the pheophytin/QA redox potential gap, thus lowering the forward electron flow and promoting charge recombination by back electron flow from QA. This is supported by a decreased PSII-dependent electron transport capability, measured as oxygen evolution rate, in ΔpsbTc to about 72% of wild-type levels.

PsbTc Deletion Alters the Redox State of the Plastoquinone Pool and Phosphorylation of PSII Proteins

It is well established that the association of LHCII, CP26, and CP29 with PSII and partially with PSI (state I and state II, respectively) is regulated via reversible phosphorylation of the chlorophyll a/b-binding apoproteins (Kargul and Barber, 2008). Activation of the respective kinase(s) is regulated by the interaction of reduced plastoquinone with the oxidation site of the cytochrome b6f complex (Vener et al., 1997). The orthologous protein kinases, STT7 and STN7 in Chlamydomonas and Arabidopsis (Arabidopsis thaliana), respectively, are involved in the regulation of phosphorylation of LHCII, CP26, and CP29, although the primary targets of the kinases still remain unknown (Rochaix, 2007).

In this study, it could be shown that accumulation of reduced plastoquinone leads to phosphorylation of LHCII in darkness in ΔpsbTc mutant plants. Moreover, BN-PAGE analysis revealed that LHCII is not only phosphorylated but also attached to PSI in dark-
adapted mutant plants. Apparently, no light-induced signals cause migration of the LHCII antenna to PSI in the mutant. Therefore, the data uncover a new level of control of LHCII phosphorylation in darkness.

Although the related protein kinase, STN8, triggers phosphorylation of all three PSII core proteins D1, D2, and CP43 (for review, see Vener, 2006), phosphorylation of D1/D2 proteins is considerably more responsive than that of CP43 to redox regulation in tobacco wild-type plants (Fig. 6A; Umate et al., 2007), indicating that additional kinases could be involved in PSII core protein phosphorylation. Phosphorylation of outer antenna proteins and presumably of PSII core proteins is affected not only by activation/deactivation of the respective protein kinase(s) but also by the phosphorylation sites at thylakoid membrane surfaces and, thus, on their accessibility to the active site of the respective protein kinase(s) (Zer et al., 1999, 2003; Vink et al., 2000). Phosphorylation of the core proteins CP43, D1, and D2 is almost completely abolished in a plastid psbTc gene was inactivated by targeted disruption with a selectable markerless chimeric aadA cassette conferring spectinomycin resistance (Koop et al., 1996; the cassette was inserted in the orientation of the open reading frame [Fig. 1]. Using a PCR-based site-directed mutagenesis approach, a diagnostic HpaI restriction site was generated at the 5’ region of psbE with the oligonucleotides psbE/Emul2for (5’-ATCATGGAACGATTTGTTAACAACATT-CTCTTGATCTGCAGC-3’) and psbE/Emul1rev (5’-GTCGAGAATAGGAGATGTGTGTTACAACATGCTGATGA-3’). The DNA fragment amplified using the oligonucleotides psbE/Emul1and psbE/Emul2 (5’-5’-TCTACGCGGTAGAATACCCAGTAA-3’) was inserted into a pDrive cloning vector (Qiagen) to produce plasmid pD17A (Fig. 1A). The chimeric aadA gene cassette was excised as a 916-bp Smal-HindIII fragment, blunted, and ligated into the HpaI site of pAIt. Tobacco (Nicotiana tabacum ‘Petit Havanna’) plastsids were transformed essentially as described (Swab et al., 1990). Selection, culture conditions of the transformed material, and assessment of the homoplastic state of transformed lines were carried out as described (Swiatek et al., 2003a) using the oligonucleotides 5’-TACCT-GAAATCCGATGTTGC-3’ and 5’-CTCTAATAGATAAGAAATAATACCCAGTAA-3’ (Fig. 1B). All seven independent transformants obtained showed the same phenotype with respect to the Fv/Fm ratio ranging between 0.65 and 0.71. Three lines, ΔpsbTc-1 to -3, were selected for further studies on photosynthetic performance, assembly, photosensitivity, and phosphorylation patterns of PSII proteins (Figs. 2–6; Supplemental Fig. S1).

Northern-Blot Analysis

Northern-blot analysis was performed as described (Lezhneva and Meurer, 2004) using either radiolabeled DNA probes or end labeling of specific oligonucleotides with T4 polynucleotide kinase (New England Biolabs). Strand-specific end labeling was performed for psbTc, psbH, and psbN with the oligonucleotides 5’-GCCAGTCGTAAGGAATACCGCA-3’ and 5’-GCCAGTATTATCCCTCCTAACAACCAGGCGTAATG-3’, respectively (Fig. 1C). The DNA fragment amplified using the oligonucleotides psbH/Emul1 and psbH/Emul2 (5’-GCCAGTATTATCCCTCCTAACAACCAGGCGTAATG-3’) was inserted into a pDrive cloning vector (Qiagen) to produce plasmid pD17A (Fig. 1A). The chimeric aadA gene cassette was excised as a 916-bp Smal-HindIII fragment, blunted, and ligated into the HpaI site of pAIt. Tobacco (Nicotiana tabacum ‘Petit Havanna’) plastsids were transformed essentially as described (Swab et al., 1990). Selection, culture conditions of the transformed material, and assessment of the homoplastic state of transformed lines were carried out as described (Swiatek et al., 2003a) using the oligonucleotides 5’-TACCT-GAAATCCGATGTTGC-3’ and 5’-CTCTAATAGATAAGAAATAATACCCAGTAA-3’. Tobacco plastids were transformed essentially as described (Swab et al., 1990). Selection, culture conditions of the transformed material, and assessment of the homoplastic state of transformed lines were carried out as described (Swiatek et al., 2003a) using the oligonucleotides 5’-TACCT-GAAATCCGATGTTGC-3’ and 5’-CTCTAATAGATAAGAAATAATACCCAGTAA-3’. Tobacco plastids were transformed essentially as described (Swab et al., 1990). Selection, culture conditions of the transformed material, and assessment of the homoplastic state of transformed lines were carried out as described (Swiatek et al., 2003a) using the oligonucleotides 5’-TACCT-GAAATCCGATGTTGC-3’ and 5’-CTCTAATAGATAAGAAATAATACCCAGTAA-3’. Tobacco plastids were transformed essentially as described (Swab et al., 1990). Selection, culture conditions of the transformed material, and assessment of the homoplastic state of transformed lines were carried out as described (Swiatek et al., 2003a) using the oligonucleotides 5’-TACCT-GAAATCCGATGTTGC-3’ and 5’-CTCTAATAGATAAGAAATAATACCCAGTAA-3’.

Preparation of Thylakoid Membranes, SDS-PAGE, and Immunoblot Analysis

Thylakoid membrane proteins were isolated, separated by SDS-PAGE, and subjected to immunoblot analysis as described (Umate et al., 2007). Quantitative immunoblot analysis was performed using AIDA software (Raytest).

Suc Density Gradients

Thylakoid membrane complexes were separated by Suc gradient centrifugation using 4-week-old in vitro-grown plants (light intensity of 10–20 μmol m−2 s−1, 12-h photoperiod) as described (Schwenkert et al., 2006).

BN-PAGE

BN-PAGE was performed as described earlier (Schwenkert et al., 2007). Thylakoid fractions were solubilized with either 1% β-dodecylmaltoside or...
Thylakoid Protein Phosphorylation Assay

State I conditions were adjusted by either keeping plants in darkness for 24 h or treatment with light at 728 nm (PSI light) of 20 μE m⁻² s⁻¹ for 30 min. For state II conditions, leaves were illuminated with light at 650 nm (PSII light) of 45 μE m⁻² s⁻¹ for 30 min. All buffers used for thylakoid preparations contained 10 mM NaF. Phosphorylated proteins were detected using antiphosphothreonine antiserum (New England Biolabs). Anaerobic conditions were generated by placing leaves in a humid screw-cap vial flushed with 10 min with pure argon at room temperature.

PSII Quantum Yield and Fluorescence Quenching Analysis

A pulse amplitude-modulated fluorimeter (PAM-101; Walz) was used to study chlorophyll a fluorescence kinetics. Leaves dark adapted for not less than 5 min were used for measurements. Red actinic light (650 nm, 5 and 45 μE m⁻² s⁻¹) was used for measurement of fluorescence parameters. The maximum quantum yield of PSII was determined as (Fm − Fv)/Fm = Fm/Fm and the effective quantum yield of PSII, ΦPSII, was expressed as Fm′ − Fv/Fm′ (Genty et al., 1989). qP and NPQ were determined by repetitive saturation pulses. The quenching coefficients, NPQ and qP, were calculated as (Fm − Fm)/Fm and (Fm′ − Fv)/Fm′, respectively (van Kooten and Snel, 1990).

Oxygen Evolution Measurements

PSII electron transport activity was determined using a Clark-type oxygen electrode as described (Umate et al., 2007). In order to measure exclusively PSII-dependent electron transport, the PSII-specific electron acceptor p-benzoquinone as well as the uncouplers NH₄Cl (5 mM) and gramicidin (3 μM) were used under saturating light conditions. PSII activity of wild-type thylakoids was 198 ± 19 μmol oxygen mg⁻¹ chlorophyll h⁻¹.

TL Measurements

TL measurements of thylakoids were measured using a home-built apparatus as described (Schwenker et al., 2006). The Q band emission resulting from Qₐ → S₁ charge recombination was measured by blocking electron flow to QA with the addition of DCMU (10 μM) or lipoxylin (5, 10, and 20 μM) during dark adaptation. Concentrations of lipoxylin higher than 20 μM were avoided due to high fluorescence quenching induced by this herbicide.

A computer-based simulation program allowing the prediction of S-state ratio and the occupancy ratio Qb/Qa was employed. The program simulates predicted oscillation profiles and checks for the correlation between simulated and measured values (Umate et al., 2007).

PSII Photoinactivation and Recovery Kinetics

The sensitivity of PSII to oxidative stress was determined using leaf discs of wild-type and Δpsb/Te discs exposed to 1,200 μE m⁻² s⁻¹ heterochromatic light. Photoinactivation of PSII was measured as changes in the Fv/Fm parameter as a function of exposure time. To estimate the contribution of the PSII recovery process during treatment with high light, leaf discs were infiltrated with a solution of CAP (200 μg mL⁻¹) in darkness for 30 min prior to exposure to high light. To assess photoinhibition kinetics and capacity to recover PSII activity, leaf discs were exposed in the absence of CAP pretreatment to high light (1,200 μE m⁻² s⁻¹) until an Fv/Fm of 0.17 was reached in wild-type and Δpsb/Te discs and then allowed to recover PSII activity by lowering the light intensity to 3 μE m⁻² s⁻¹ for up to 6 h, measuring the Fv/Fm level every 1 h.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Studies on the assembly, photo inhibition, and phosphorylation patterns of the independent transformants Δpsb/Te-2 and Δpsb/Te-3.

Supplemental Figure S2. Separation of wild-type and Δpsb/Te-1 thylakoids by BN-SDS-PAGE using 1.5% digitonin.

Supplemental Table S1. Computer-based simulation for the prediction of the S-state ratios and the occupancy ratio Qb/Qa.

ACKNOWLEDGMENTS

We acknowledge Noam Adir for helpful discussions and Dario Leister for kindly reading the manuscript.

Received July 8, 2008; accepted September 12, 2008; published September 19, 2008.

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