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Deletion of the tobacco plastid *psbA* gene triggers an upregulation of the thylakoid-associated NAD(P)H dehydrogenase complex and the plastid terminal oxidase (PTOX)

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Summary

We have constructed a tobacco *psbA* gene deletion mutant that is devoid of photosystem II (PSII) complex. Analysis of thylakoid membranes revealed comparable amounts, on a chlorophyll basis, of photosystem I (PSI), the cytochrome *b6f* complex and the PSII light-harvesting complex (LHCII) antenna proteins in wild-type (WT) and $\Delta psbA$ leaves. Lack of PSII in the mutant, however, resulted in over 10-fold higher relative amounts of the thylakoid-associated plastid terminal oxidase (PTOX) and the NAD(P)H dehydrogenase (NDH) complex. Increased amounts of Ndh polypeptides were accompanied with a more than fourfold enhancement of NDH activity in the mutant thylakoids, as revealed by in-gel NADH dehydrogenase measurements. NADH also had a specific stimulating effect on P700⁺ re-reduction in the $\Delta psbA$ thylakoids. Altogether, our results suggest that enhancement of electron flow via the NDH complex and possibly other alternative electron transport routes partly compensates for the loss of PSII function in the $\Delta psbA$ mutant. As mRNA levels were comparable in WT and $\Delta psbA$ plants, upregulation of the alternative electron transport pathways (NDH complex and PTOX) occurs apparently by translational or post-translational mechanisms.

Keywords: chloroplast electron transfer, D1 protein, photosystem II, plastome transformation.

Introduction

In oxygen-evolving organisms, the reaction center of photosystem II (PSII) consists of the D1 protein, the D2 protein, cytochrome *b₅₅₉*, and additional small proteins (Nanba and Satoh, 1987). The entire PSII complex, consisting of around 25 subunits, acts as a light-driven water-plastoquinone oxidoreductase and, together with cytochrome *bf* complex, photosystem I (PSI), and the ATP synthase, is responsible for production of NADPH and ATP (for a review, see Barber, 1998). As a crucial component of the chloroplast redox chemistry, PSII contributes to the regulation of soluble enzyme activities and possibly also to the regulation of both chloroplast and nuclear gene expression (Bruick and Mayfield, 1999; Roderick, 2001; Surpin *et al.*, 2002). During recent years, it has become clear that the electron transfer routes and membrane complexes

involved in chloroplast redox chemistry are much more versatile than previously thought, and a number of new components participating in alternative electron transport pathways have been identified (for reviews, see Bendall and Manasse, 1995; Carol and Kuntz, 2001; Nixon, 2000; Nixon and Mullineaux, 2001).

Efficient plastid transformation techniques have been developed for the higher plant tobacco (Svab and Maliga, 1993; Svab *et al.*, 1990). Based on the homologous recombination system, the tobacco plastome can thus be targeted for manipulation and employed in generating mutants for studies of metabolic and regulatory processes taking place in plant chloroplasts. Plastid reverse genetics has made it possible, for example, to investigate the physiological role of the chloroplast NDH complex (Endo *et al.*, 1997;

Guedeney *et al.*, 1996; Quiles *et al.*, 1996; Sazanov *et al.*, 1998), and a number of novel tobacco mutants with inactivated *ndh* genes (*ndhA-C*, *H-K*) have been constructed (Burrows *et al.*, 1998; Kofer *et al.*, 1998; Shikanai *et al.*, 1998). Depletion of the NDH complex in these plants did not result in a specific phenotype under standard growth conditions. Nevertheless, the absence of the NDH complex led to a loss of post-illumination rise in chlorophyll fluorescence, considered to originate from the dark reduction of the plastoquinone (PQ) pool (Asada *et al.*, 1993; Feild *et al.*, 1998; Groom *et al.*, 1993), and thus provided direct evidence for an *in vivo* role of the NDH complex in PQ reduction with stromal reductants. This prompted the suggestion that the NDH complex participates in PSII-independent electron transport around PSI, as well as in a putative respiratory chain (chlororespiration) resembling the one found in cyanobacteria (Burrows *et al.*, 1998).

We constructed a *psbA* deletion mutant of tobacco to test how a complete inhibition of PSII activity, caused by lack of the *psbA*-encoded reaction center protein D1, affects the structure and function of chloroplast thylakoid membranes. Depletion of the *psbA* gene resulted in complete loss of all PSII subunits whereas the assembly and biogenesis of PSI and Cyt *bf* complexes were not affected. Moreover, free LHCII complexes accumulated in the thylakoid membrane. Most strikingly, the NDH complex and the plastid terminal oxidase (PTOX, Carol *et al.*, 1999; Wu *et al.*, 1999) were more than 10-fold higher in thylakoid membranes of the $\Delta psbA$ mutant than in those of the wild type (WT). Increased NDH and PTOX accumulation was accompanied by a markedly enhanced NDH activity and by an increased capacity to oxidize NAD(P)H for P700⁺ reduction. These findings suggest the involvement of the NDH complex, and possibly other alternative routes, in sustaining thylakoid electron flow under conditions where the electron input by PSII is severely inhibited.

Results

Construction and phenotype of the tobacco $\Delta psbA$ mutant

Transplastomic shoots were selected by incubating the bombarded leaf segment on spectinomycin-containing (500 mg l⁻¹) shoot regeneration medium. Transformed, spectinomycin-resistant shoots were identified as green shoots on the bleached, bombarded leaf segments. The leaves on the shoots were variegated, containing light-green sectors. The light-green leaf sectors were excised aseptically and transferred to a plant regeneration medium. The light-green shoots regenerated from the sectors did not contain the *psbA* gene. The plastid genomes of these plants were the products of homologous recombination between

the promoter and 3'UTR of the native *psbA* gene and the promoter and 3'UTR of the chimeric *aadA* gene in the *psbA* cassette (Figure 1a). The *psbA* knockout shoots had a distinct phenotype. The youngest leaves (first and second) were green, whereas the older leaves gradually bleached out. This phenotype was maintained when the shoots were rooted and maintained on antibiotic-free plant maintenance medium. Uniform transformation of plastid genomes was confirmed by DNA gel blot analysis of *EcoRI*-digested total cellular DNA (Figure 1b). Hybridization was performed with a *psbA* probe to reveal the lack of this gene (Figure 1b) and with an *aadA* probe to check the size of the restriction fragments and verify that no additional deletions had occurred in the nearby genes (data not shown).

Figure 1. Construction of plastid *psbA* deletion mutants.

(a) Map of plastid transformation vector pZS162, the corresponding WT plastid genome region, and that of the *psbA* knockout plants ($\Delta psbA$). The spectinomycin resistance (*aadA*) gene coding region, and the *psbA* promoter (P) and 3'UTR (T) are marked. The hybridization probe is indicated as a solid bar under the *psbA* gene.

(b) DNA gel blot analysis of WT and $\Delta psbA$ tobacco using the probe indicated in (a). Total cellular DNA (8 μ g) was digested with the *EcoRI* restriction enzyme.

Figure 2. Wild-type and $\Delta psbA$ tobacco plants. (a) Phenotype of the plants grown under $30 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$. (b) Electron micrographs of WT and $\Delta psbA$ chloroplasts. Scale bars represent $1 \mu\text{m}$.

The $\Delta psbA$ mutants were unable to grow photoautotrophically and were therefore propagated by tissue culture on a medium containing MS salts and 3% sucrose. As a result of a sensitivity of $\Delta psbA$ mutants to light, they were grown in very dim light ($30 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$; Figure 2a); nevertheless, the leaves were light green. The experiments were carried out with the youngest and greenest expanded leaves. Examination of chloroplast ultrastructure by electron microscopy revealed drastically reduced size and number of grana stacks, and slightly swollen thylakoid membranes (Figure 2b).

77K fluorescence emission spectra of thylakoid membranes

We first determined the 77K fluorescence emission spectra of the mutant and WT thylakoids. This technique can distinguish between the characteristic emission bands belong-

Figure 3. 77K fluorescence emission spectra measured from isolated thylakoids of WT and $\Delta psbA$ tobacco plants.

ing to PSII (chlorophyll-binding proteins CP43 and CP47, 685 and 695 nm, respectively), and to PSI (735 nm) (Krause and Weis, 1991), and hence provides an estimate of the relative amounts of chlorophyll-protein complexes associated with PSII and PSI. As shown in Figure 3, the spectrum of the mutant clearly lacked the emission peaks characteristic of PSII (685 and 695 nm). In accordance with this result, no PSII activity could be inferred from thylakoid membranes when assayed with an oxygen electrode using 2,6-dichloro-*p*-benzoquinone (DCBQ) as an artificial electron acceptor (data not shown). Interestingly, the 77K fluorescence emission spectrum of the mutant revealed a clear emission peak at 680 nm, indicating that at least part of the PSII light-harvesting antenna (LHCII) existed as free pigment-protein complexes in the thylakoid membrane, and was not associated with PSI.

Polypeptide composition of thylakoid membranes

A general polypeptide pattern of thylakoid membranes of the WT and $\Delta psbA$ mutant, obtained by Coomassie staining after SDS-PAGE, is shown in Figure 4(a). The equal staining intensity of proteins in the gel loaded on a chlorophyll basis

Figure 4. Biochemical analysis of WT and $\Delta psbA$ thylakoids.

(a) Thylakoid polypeptides separated by SDS-PAGE and stained with Coomassie (2 μ g chlorophyll per lane; stars denote the main differences in the protein band pattern between the WT and the mutant).

(b) Immunoblots (1 μ g chlorophyll per lane, except for NdhH, NdhI, and PTOX, 15 μ g) of thylakoid proteins from WT tobacco and $\Delta psbA$ plants.

(c) Estimation of NdhI and PTOX content in $\Delta psbA$ thylakoids with regard to WT levels. For the 100% samples, 15 μ g chlorophyll (NdhI) or 9 μ g chlorophyll (PTOX) was loaded.

(d) Test of possible cross-reactions of antisera with homologous mitochondrial proteins. Left panel, anti-NdhI: T, $\Delta psbA$ thylakoids, 100 μ g protein; and M, purified mitochondrial membranes, 100 μ g protein. Right panel, anti-PTOX: T, $\Delta psbA$ thylakoids, 90 μ g protein; To1, protein extract of WT tomato, 50 μ g protein; To2, protein extract of a tomato *ghost* mutant lacking PTOX, 50 μ g protein; and M, purified mitochondria, 90 μ g of protein. The arrowheads point to the protein bands corresponding to NdhI and PTOX.

indicated similar chlorophyll/protein ratios in the two samples. Nevertheless, several differences in the polypeptide pattern were noted (Figure 4a). Closer examination of the polypeptide composition of thylakoid membranes by immunoblot analysis confirmed the lack of D1 protein in the $\Delta psbA$ mutant (Figure 4b). In the absence of the D1 protein, D2 and Cyt b_{559} were also missing from the thylakoid membranes and only traces of CP43, CP47, and the OEC33 protein could be detected in overexposed films. In contrast to other PSII proteins, the PsbW polypeptide was present at reduced levels in the $\Delta psbA$ mutant, which is in agreement with a recent report on the association of this protein also with PSI (Hiyama *et al.*, 2000). Despite the lack of PSII, the protein levels of the LHCII antenna appeared normal with regard to other thylakoid proteins, as was the case also for Cyt f , the PSI core, and the PSI light-harvesting chlorophyll (LHCI) antenna. In sharp contrast to other thylakoid proteins, the relative amounts of PTOX and two tested subunits of the NDH complex (NdhH and NdhI) were

strikingly increased in the $\Delta psbA$ mutant (Figure 4b). As judged from a dilution series, this relative increase was more than 10-fold for both PTOX and the Ndh subunits (Figure 4c). Importantly, this was not because of a cross-reaction of the antibodies with the mitochondrial counterparts of the respective proteins, as neither the NdhI nor the PTOX antibodies recognized any proteins of the same size from purified tobacco leaf mitochondria (Figure 4d). As the PTOX antibody recognized several protein bands, the right PTOX protein band of c. 32 kDa (Figure 4d) was identified by using control samples, which consisted of protein extracts of a tomato WT plant (To1; Figure 4d) and a tomato mutant (*ghost*; 32) lacking PTOX (To2; Figure 4d).

NDH activities of the thylakoid membrane

In order to check whether the relative increase in NdhH and NdhI polypeptides was accompanied by an upregulation of NDH activities, thylakoid complexes mildly solubilized with

Figure 5. NDH activities in WT and $\Delta psbA$ thylakoids.

(a) NADH and (b) NADPH dehydrogenase activities of thylakoid (WT and $\Delta psbA$) and mitochondrial (M) membranes. Membranes were solubilized with 1% DM, and the protein complexes were resolved by BN-PAGE. The identity of marked complexes (PSI and LHCII trimers) was established by immunoblotting assays after running of the gel strip in a second dimension (not shown). NDH activity assays were performed after BN-PAGE as described in Experimental procedures using NBT and either NADH (a) or NADPH (b) as electron donors. 100% corresponds to 15 μg chlorophyll (=200 μg protein). M, purified mitochondria, 100 μg protein. A1–A3: activity bands after NADH staining. B1–B3: activity bands after NADPH staining. Analysis of bands A1–A3 ($\Delta psbA$ mutant 50% sample and mitochondria, M) and B1–B3 ($\Delta psbA$ mutant 50% sample) by denaturing SDS-PAGE in the second dimension and immunodetection with an NdhI-specific antibody is shown in lower panels of (a) and (b), respectively.

n-dodecyl β -D-maltoside (DM) were resolved by Blue-Native (BN)-PAGE, and the NDH activity was localized using either NADH or NAD(P)H as an electron donor and nitro blue tetrazolium (NBT) as an electron acceptor (Figure 5a). This assay was performed using equal chlorophyll amounts for all the samples, and therefore the lanes in one zymogram are comparable, as also revealed from similar PSI and LHCII amounts in immunoblots of the same samples (data not shown). As can be seen in Figure 5(a), staining with NADH-NBT resulted in three activity bands (bands A1–A3), whose intensity was higher in a 25% mutant sample than in the 100% WT sample. As NADH can also be oxidized by the mitochondrial respiratory complexes, the mitochondrial complex I being very similar in size to the chloroplastic NDH complex (Buchanan *et al.*, 2000; Sazanov *et al.*, 1998), and as mitochondrial membranes might copurify with thylakoid membranes during the isolation procedure, we used, as a control, purified mitochondria from tobacco leaves that were solubilized with DM in a similar way and ran and stained in parallel with the thylakoid samples. The amount of mitochondrial proteins loaded in the well corresponded to the amount of protein present in the 50% $\Delta psbA$ thylakoid sample (Figure 5a). All three bands (A1–A3) were present also in the mitochondrial sample. However, immunoblotting analysis of a dilution series of thylakoid and mitochondrial samples with antisera against the mitochondrial marker protein Cyt *c* revealed

only a maximum of 14% mitochondrial contamination in the $\Delta psbA$ thylakoid samples (not shown). Thus, small mitochondrial contamination cannot explain the differences in NADH dehydrogenase activity observed between the WT and the mutant. Furthermore, examination of the polypeptide composition of the excised activity bands by SDS-PAGE and immunoblotting revealed the presence of the NDH subunit NdhI in bands A1 and A2 of the $\Delta psbA$ thylakoid sample (taken from 50% sample), whereas no NdhI immunosignal could be detected in the corresponding bands of a mitochondrial sample containing similar protein amounts (Figure 5a, lower panel, anti-NdhI). Interestingly, the NdhI protein was mainly located in band A1, despite the fact that A2 was the most intense activity band in the gel. Therefore, it is possible that band A1 represents the intact NDH complex whereas the faster mobility bands A2 and A3 might represent smaller NDH subcomplexes. Indeed, NdhI could not be detected in the thylakoid band A3, suggesting that the observed enzymatic activity might result from an NDH subcomplex from which NdhI had dissociated.

Having demonstrated that the NDH complex was at least partly responsible for the NADH-dependent differences in dehydrogenase activities between the WT and the mutant, we tested the ability of the NDH complex to also use NADPH as a substrate. As shown in Figure 5(b), staining with NADPH-NBT revealed three bands in the WT and $\Delta psbA$ mutant (bands B1–B3). However, in contrast to the NADH

assay (Figure 5a), the differences between the WT and the mutant were at most twofold, and no slow-mobility complex(es) (<PSI) was present. Furthermore, NdhI was not detected in any of the NADPH-dependent activity bands (B1–B3; Figure 5b, lower panel).

Oxido-reduction of the PSI reaction center chlorophyll P700

We next studied the oxidation and reduction states of the PSI primary electron donor, P700, to further characterize the NDH activities observed in the zymograms. In order to be able to compare the effect of added reductants in such different systems as WT and $\Delta psbA$ mutant thylakoids, the contribution of PSII to PQ reduction was eliminated

by using 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-poisoned thylakoids isolated from dark-adapted (16 h) plants. The maximum level of photo-oxidizable P700 was first measured by excitation of WT and $\Delta psbA$ mutant thylakoids by saturating single turnover flash in the presence of DCMU and reduced 2,6-dichlorophenol indophenol (DCPIP) as an artificial electron donor to PSI. Typical kinetic traces of the flash-induced absorbance changes at 810 nm in WT and $\Delta psbA$ mutant thylakoid membranes are shown as an inset in Figure 6(a,b). The maximum level of photo-oxidizable P700 in $\Delta psbA$ mutant thylakoids was slightly higher than that in WT samples, being in accordance with the oxygraph measurements of PSI electron transport activities, 338 ± 37 and $374 \pm 41 \mu\text{mol O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$, respectively. Moreover, the reduction kinetics

Figure 6. Oxidation and re-reduction of P700 in isolated thylakoids of WT and $\Delta psbA$ mutant. DCMU-poisoned thylakoids were illuminated for 30 sec under anaerobic conditions in the presence of exogenously added Fd followed by a 10-sec dark period. 30 consecutive cycles of light (1.2 sec; $1000 \mu\text{mol photons m}^{-2} \text{ sec}^{-1}$ of white light) and dark (8.8 sec) were applied to the sample and change in the redox state of P700 was monitored by absorbance changes at 810 nm (oxidation and increase in P700⁺ signal during the light phase and reduction of P700⁺ during the dark phase). The effect of NADH (2.4 mM, (a, b)) and NADPH (2.4 mM, (c, d)) on P700⁺ re-reduction was studied by adding these compounds to the sample after the DCMU curves had been recorded (DCMU + NADH and DCMU + NADPH curves; see Experimental procedures). Arrows indicate the starting (ON) and ending (OFF) points of the illumination period (1.2 sec). Each curve is an average of 15 repetitions. All measurements were carried out anaerobically. Insets in (a) and (b) show flash-induced maximum oxidation level of P700 and subsequent relaxation in darkness in WT and $\Delta psbA$ mutant, respectively.

of flash-induced $P700^+$ was nearly identical in both types of samples.

For the actual $P700$ oxido-reduction measurements, the thylakoid samples were first illuminated under anaerobic conditions with a long 30-sec pulse of white light ($1000 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$) in the presence of exogenously added ferredoxin (Fd), followed by a 10-sec dark period. Illumination period in anaerobic conditions resulted in reduction of the exogenously added Fd and photo-oxidation of $P700$, which, during the following 10-sec dark period, slowly relaxed either through back reactions or/and cyclic electron flow. Subsequent 15 cycles of light (1.2 sec of light followed by 8.8 sec of darkness) in the presence of pre-reduced Fd and DCMU induced only limited photo-oxidation of $P700$ (Figure 6). The fact that the amplitude of the $P700$ oxidation signal was higher in WT thylakoids (Figure 6a, DCMU) than in the corresponding mutant sample (Figure 6b, DCMU) possibly indicates an enhanced capacity for Fd-mediated cyclic electron flow (Munekage *et al.*, 2002) in the $\Delta psbA$ mutant.

To check the ability of the thylakoid membranes to utilize stromal reductants for reduction of the PQ pool and PSI, the $P700^+$ re-reduction in darkness was measured in the presence of NADH or NADPH by adding these substrates (2.4 mM) of the NDH complex(es) after the first 15 DCMU curves had been recorded. Addition of NADH to WT sample hardly changed the kinetics of $P700$ oxido-reduction (Figure 6a), while in the case of the mutant, an increase in the light-induced $P700$ oxidation level and a dramatic acceleration of $P700^+$ re-reduction rate in the dark phase were evident (Figure 6b). This indicates that the mutant thylakoids have an enhanced capacity to utilize NADH for the input of electrons to the intersystem electron transfer chain (IEC), which induced rapid re-reduction of $P700^+$ in darkness. Concomitant production of NAD^+ apparently enhanced the steady-state level of $P700^+$ (Figure 6b) by producing electron acceptors for PSI through oxidation of pre-reduced Fd and thus preventing rapid charge recombinations. Addition of NADPH also had a substantial accelerating effect on $P700^+$ re-reduction kinetics. Nevertheless, this effect was nearly similar in both the WT and the mutant (Figure 6c,d).

LHCII protein phosphorylation

As judged from immunoblot assays, the relative amount of the LHCII antenna polypeptides was normal in the mutant, despite the absence of PSII (Figures 4b and 7, lower panel, anti-LHCB2). We next analyzed the phosphorylation state of LHCII polypeptides in dark-adapted (kept for 18 h in total darkness) and light-treated WT and $\Delta psbA$ plants (leaves harvested 5 h after the onset of illumination) by immunoblotting thylakoid samples with a phosphothreonine antibody that specifically recognizes the phosphorylated forms

Figure 7. Phosphorylation of LHCII in thylakoids of dark-adapted (D) and illuminated (L) WT and $\Delta psbA$ tobacco leaves. The gel was loaded on a chlorophyll basis ($1 \mu\text{g}$ per lane). Immunodetection of the phosphorylated pool of PSII proteins (P-CP43, P-D2, P-D1, and P-LHCII) was performed with a phosphothreonine antibody and that of the total LHCII protein with an LHCB2-specific antibody. D, thylakoids isolated after an 18-h dark period; and L, thylakoids isolated after 5-h exposure to normal growth light.

of PSII proteins (Rintamäki *et al.*, 1997). As shown in Figure 7 (upper panel, anti-phosphothreonine), a clear phosphorylation of LHCII occurred in the light both in WT and $\Delta psbA$ thylakoids, indicating that the LHCII kinase could be activated in the mutant in spite of the loss of PSII activity. As reduced PQ in the Q_o site of the Cyt *b6f* complex is a prerequisite for activation of the LHCII kinase (Vener *et al.*, 1997; Zito *et al.*, 1999), this suggests that the PQ pool could be reduced by alternative electron transfer routes in the mutant. In WT control, low light normally maintained the phosphorylation of the LHCII proteins, which, however, remained strongly phosphorylated after the 18-h dark period, because of the sugar provided in the growth medium (Hou *et al.*, 2002). Noteworthy, despite the presence of sugar, the $\Delta psbA$ mutant oxidized the PQ pool in darkness, as suggested by a complete dephosphorylation of LHCII.

Transcript levels in the $\Delta psbA$ mutant

In order to check whether deletion of the *psbA* gene affected the accumulation of transcripts from other photosynthetic genes, we performed RNA gel blot analyses. In the case of *PTOX*, RT-PCR was used instead, as the amount of the *PTOX* transcripts is too low to be detected in RNA gel blots. As expected, no *psbA* (encoding D1) mRNA was detected in the mutant (Figure 8). This, in turn, did not appear to interfere with the accumulation of transcripts from the *psbD* (encoding the PSII D2 subunit) or the *psaA* genes (encoding the PSI A subunit). Also, the *ndhH* (encoding the NdhH subunit of the NDH complex) and the *PTOX* transcripts were present at comparable levels in WT and the mutant leaves (Figure 8). Comparable signals after probing for the plastid

Figure 8. Accumulation of various plastid mRNAs and *PTOX* mRNA in WT and $\Delta psbA$ tobacco leaves.

Total cellular RNA was isolated from plants previously exposed for 5 h to light. Two micrograms of RNA was loaded per lane to probe for all other transcripts but the *ndh* transcripts, for which 10 μ g RNA was loaded. Only the major transcripts identified by each probe are shown. However, when present, the minor transcripts (e.g. from *psaA* and *psbD*) also showed equal amounts in WT and the $\Delta psbA$ mutant. mRNA levels of *PTOX* were determined by RT-PCR amplification of total cellular RNA. Equal amounts of total RNA were used in each reaction. Amplification of globin mRNA (added to the RT reaction mix) was used as a control for the RT-PCR reaction.

16S rRNA and cytoplasmic 25S rRNA with the *rrn16* and *rrn25* gene probes indicate uniform loading of the lanes. As a result of the scarcity of *ndh* transcripts, mRNA levels of *ndhH* and *ndhL* were also determined by real-time quantitative RT-PCR. This approach confirmed the results obtained by RNA gel blot analyses, revealing no differences between the WT and $\Delta psbA$ mutant (data not shown). Lack of increase in *ndhH*, *ndhL*, and *PTOX* mRNA levels suggests that the enhanced amounts of their respective protein subunits detected by immunoblotting were because of translational or post-translational regulation of gene expression.

Discussion

Major thylakoid proteins and protein complexes in *psbA* deletion mutant

To test how a complete depletion of the PSII complex affects the biogenesis and structure of the thylakoid membranes, we knocked out the *psbA* gene, encoding the reaction center protein D1 of PSII, by biolistic tobacco chloroplast transformation (Svab and Maliga, 1993; Svab *et al.*, 1990). Chloroplast ultrastructure of the $\Delta psbA$ mutant, examined by electron microscopy, revealed drastically

reduced size and number of grana stacks, and slightly swollen thylakoid membranes (Figure 2b). The mutant thylakoids were nearly completely devoid of all PSII core subunits, which contrasts with the results obtained for $\Delta psbA$ mutants in cyanobacteria and *Chlamydomonas*, in which CP43, OEC33, and Cyt *b*₅₅₉ proteins are present at WT levels (Morais *et al.* 1998; Nilsson *et al.*, 1990, 1992). Free LHCII complexes however accumulated in the thylakoid membrane of $\Delta psbA$ tobacco, and the assembly and biogenesis of PSI and Cyt *bf* complexes took place normally (Figure 4b,c). Interestingly, absence of the entire PSII complex had no effect on transcript accumulation from other plastome-encoded photosynthetic genes (Figure 8), although, in WT plants, changes in the rates of plastid gene transcription upon illumination with light favoring PSI or PSII were attributed to regulation by the redox state of PQ pool (Pfannschmidt *et al.*, 1999). Thus, it appears that changes in the redox state, induced by depletion of PSII, do not directly affect plastid transcript levels in the *psbA* knockout plants, and that the absence of all other PSII core subunits, besides the D1 protein, is apparently because of operation of efficient translational and post-translational regulatory mechanisms preventing accumulation of PSII subunits in the absence of assembly partners (Baena-González and Aro, 2002; Zerges, 2002).

Complexes involved in alternative electron transfer pathways accumulate in thylakoids of the $\Delta psbA$ mutant

The artificial inactivation of PSII by deleting the gene for one of its core subunits resulted in an elevated activity of the alternative electron input pathways, which is in line with an apparent correlation in the literature between low or null functioning of PSII and upregulation of NAD(P)H-quinone reductases (NQRs, Casano *et al.*, 2000, 2001; Catalá *et al.*, 1997; Kubicki *et al.*, 1996; Martín *et al.*, 1996; Peltier and Schmidt, 1991). The NDH protein subunits were present in more than 10-fold higher amounts in the mutant than in the WT (Figure 4b,c), and the same was true for PTOX. Upregulation of the NDH subunits and PTOX in the $\Delta psbA$ mutant appeared to occur at a translational level, as the amounts of the corresponding mRNAs were comparable in WT and the $\Delta psbA$ mutant (Figure 8). Recently, it was reported that enhancement of NDH activity following photo-oxidative treatment is also under post-transcriptional control as an increase was detected only at protein level (Casano *et al.*, 2001).

NAD(P)H activity staining of thylakoid protein complexes in native gels revealed an over fourfold higher NADH-specific dehydrogenase activity in the mutant thylakoids (Figure 5a). This activity corresponded, at least partly, to the elevated level of the NDH complex, as evidenced by the presence of the NdhI subunit in two of the NADH activity bands (A1–A2; Figure 5a, lower panel). According to the

position of the bands in the native gel, the slower mobility band A1 possibly represents intact NDH complexes, whereas the faster mobility band A2 could correspond to an NDH subcomplex. As the NdhI protein is part of the peripheral module of the NDH complex (Friedrich and Scheide, 2000), it is conceivable that it easily dissociates during DM solubilization of thylakoid membranes. Therefore, the absence of NdhI from A3 does not rule out the possibility that this band also originates from the NDH complex, albeit in a state lacking the NdhI protein.

To test whether the increased NAD(P)H dehydrogenase activity of the mutant is also engaged in the flux of electrons to PSI, we measured $P700^+$ re-reduction kinetics in darkness under various conditions (Figure 6). Both NADPH and NADH had a stimulating effect on the rate of $P700^+$ reduction in DCMU-poisoned thylakoids, indicating that indeed the electrons provided by these reductants to the IEC are used to reduce $P700^+$ (Figure 6a,d). In the case of NADPH, the extent of this effect was similar in the WT and $\Delta psbA$ mutant (Figure 6c,d). Addition of NADH, in contrast, caused a much greater acceleration of $P700^+$ reduction in the mutant than in the WT (Figure 6a,b), in support of an upregulation of NADH-specific NQR activity in the absence of PSII (Figure 5). Strict conclusions on the identity of the enzyme(s) involved cannot, however, be drawn solely on the basis of the oxidized reductant, as the substrate specificity of these enzymes is still a matter of controversy, and in some cases, the enzymes have been reported to use both NADH and NADPH (Corneille *et al.*, 1998; Elortza *et al.*, 1999; Funk *et al.*, 1999; Guedeney *et al.*, 1996; Quiles and Cuello, 1998; Sazanov *et al.*, 1998).

Although the elevated amount of the NDH complex in the $\Delta psbA$ mutant appears to be involved in maintaining electron flux to PSI in the light, additional routes are known to be in operation as well. Two recent *in vivo* studies on WT and $\Delta ndhB$ plants point to an engagement of the NDH complex also in electron flow around PSI (Joët *et al.*, 2001, 2002). The need for the NDH complex was particularly evident under conditions of limited CO_2 availability induced by water stress (Joët *et al.*, 2001). The contribution of the NQRs to total electron flow is probably rather small under optimal growth conditions as reflected, for example, by the lack of phenotype of the various Δndh mutants in the absence of stress (Burrows *et al.*, 1998; Horváth *et al.*, 2000; Shikanai *et al.*, 1998). However, under strong inhibition of PSII activity or when the plants completely lack PSII function (as in the case of the $\Delta psbA$ mutant), these routes for electron input to the IEC are probably essential not only to support cyclic electron transport and ATP production in chloroplasts, but also to provide the chloroplast with reducing equivalents generated by PSI, particularly reduced Fd, which functions as an electron donor for several fundamental metabolic pathways in this organelle (Knaff, 1996).

Phosphorylation/dephosphorylation of LHCII in the $\Delta psbA$ mutant relies on alternative electron transfer pathways

Further evidence supporting the presence of alternative PQ-reducing activities in the $\Delta psbA$ thylakoids came from studies on LHCII protein phosphorylation, which became apparent in the mutant upon illumination of leaves (Figure 7). Phosphorylation of LHCII is known to be dependent on the binding of reduced PQ to the Q_o pocket of the Cyt *b6f* complex (Vener *et al.*, 1997; Zito *et al.*, 1999), with electrons being normally provided to PQ by PSII, as it is probably the case in the WT control. Indeed, blocking of PSII electron transfer with DCMU completely inhibits LHCII phosphorylation in the light (Rintamäki *et al.*, 2000). LHCII undergoes maximal phosphorylation at low light intensities, implying that a small amount of reduced PQ is sufficient for full activation of the kinase (Rintamäki *et al.*, 1997). In the case of the $\Delta psbA$ mutant, however, not even traces of PSII were present (77K fluorescence emission spectra, Figure 3; immunoblotting, Figure 4a; measurements of oxygen evolution, not shown). Hence, LHCII phosphorylation can be interpreted as an indication of PQ reduction by an NQR in the mutant.

On the other hand, in the dark, LHCII was totally dephosphorylated in $\Delta psbA$ plants grown on sucrose (Figure 7), which suggests an enhanced oxidation of PQH_2 in darkness as compared to the WT grown under similar conditions. The fact that WT plants grown on sucrose have LHCII protein phosphorylated in darkness, as opposed to WT plants grown on soil, results from alterations in the reduction state of PQ and the Cyt *b6f* complex caused by the sugar of the growth medium (Hou *et al.*, 2002). Efficient dephosphorylation of LHCII proteins in darkness in the $\Delta psbA$ plants is probably related to the increased PTOX amounts of the mutant (Figure 4b,c).

A question then arises why there is an over 10-fold increase in the relative amounts of PTOX in $\Delta psbA$ thylakoids as compared to the WT (Figure 4c). An explanation could be photo-oxidative stress generated by chlorophyll-binding free LHCII in the thylakoids. As revealed by 77K fluorescence emission spectra (Figure 3), at least part of LHCII is not connected to PSI in the $\Delta psbA$ mutant, and therefore the energy absorbed by the antenna chlorophylls is not expected to initiate an electron transfer chain. Instead, the excitation energy probably leads to the formation of chlorophyll triplets, which, if not quenched (e.g. by carotenoids), can result in the formation of extremely dangerous singlet oxygen (Foyer, 1997). As PTOX has been shown to be involved in carotenoid synthesis (Josse *et al.*, 2000), it is conceivable that it could have this function also in the $\Delta psbA$ mutant. In the light, PTOX cannot probably compete with PSI for oxidation of PQ, but its function in the $\Delta psbA$ mutant might be coupled with that of the NDH

complex in darkness for the synthesis of protective pigments.

In conclusion, in the $\Delta psbA$ mutant with complete loss of PSII centers, no alterations were observed in chloroplast gene expression at transcript level. Strikingly, in spite of equal protein levels of the PSI, Cyt *bf*, and LHCII complexes, the NDH and PTOX proteins were present in more than 10-fold higher amounts in the mutant than in the WT, apparently because of translational upregulation. Functional data obtained for the $\Delta psbA$ mutant reinforces the view that the upregulation of the NDH complex and probably also other NQRs in normal plants is crucial to support electron flow to PSI in thylakoids under conditions of PSII inactivation. Upregulation of PTOX under similar conditions is likely to exert protection against oxidative stress.

Experimental procedures

Construction of plastid transformation vector pZS162 and tobacco chloroplast transformation

Plasmid pZS160 is a pUC119 plasmid derivative, which carries a 2.4-kb *Pst*I fragment of the tobacco plastid genome (*Pst*I sites at nucleotides 154878 and 1334 in the plastid DNA, ptDNA) in which a *Bsp*HI site (nucleotide position 344 in the ptDNA) was converted to a *Sal*I site by linker ligation (5'-AAAGTACTTT-3'). Plasmid pZS162 was obtained by ligating the chimeric spectinomycin resistance gene (*PpsbA::aadA::TpsbA*) as an *Eco*RI/*Hind*III fragment into the *Sal*I site of plasmid pZS160. The chimeric *PpsbA::aadA::TpsbA* gene derives from plasmid pJS36, kindly provided by Jeffrey Staub. *PpsbA*, the chimeric *aadA* gene promoter, contains sequences between nucleotides 1735 (filled-in *Bgl*II site) and 1596 of the ptDNA. *TpsbA*, the 393-bp 3' region included for mRNA stability, is defined by a *Sau*3AI site (at bp 530) and a *Taq*I site (at bp 141) in the ptDNA.

Plastid transformation was carried out by the biolistic process. Transformation and regeneration of transgenic plants were carried out as described by Svab and Maliga (1993). Plants were grown aseptically on MS medium containing 3% sucrose (Murashige and Skoog, 1962) at 25°C and were illuminated for 16 h at a photo-synthetic photon flux density of 30 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$. WT tobacco plants were grown under the same conditions as the $\Delta psbA$ mutant.

Analysis of DNA and RNA

Total leaf DNA was isolated according to the hexadecyltrimethyl ammonium bromide (CTAB) extraction procedure of Rogers and Bendich (1985), with slight modifications. Uniform transformation of plastid genomes was assessed by standard DNA gel blot analysis (Sambrook and Russell, 2001) by digesting DNA (8 μg) with *Eco*RI and using a DNA probe against the *psbA* coding region. The fragment used for the synthesis of the probe was amplified with the following pair of primers: 5'-CTC TTG ACC GAA TCT GTA ACC-3' (plastome nucleotides 870–890), and reverse 5'-GGA TAA CTA GCA CTG AAA ACC-3' (complementary to plastome nucleotides 1517–1537).

RNA gel blot analysis was carried out according to Silhavy and Maliga (1998). Two micrograms of total cellular RNA was loaded

per lane to probe for *psbA*, *psaA*, *psbD*, *rrn16*, and *rrn25* transcripts and 10 μg RNA to probe for *ndhH* transcripts. DNA probes were prepared by PCR using the following primers: *psbA*, 5'-GGA TAA CTA GCA CTG AAA ACC-3' and 5'-CTC TTG ACC GAA TCT GTA ACC-3'; *psaA*, 5'-CGT TGT ACC GAG TAG TTG GAT C-3' and 5'-CGA TAG CCA TAC CAG TGA TTT G-3'; *psbD*, 5'-TTA TGG ATG ACT GGT TAC GG-3' and 5'-TAG ACC GAC TAC TCC AAG AG-3'; *rrn25*, 5'-TCA CCT GCC GAA TCA ACT AGC-3' and 5'-GAC TTC CCT TGC CTA CAT TG-3'; *ndhH*, 5'-CCA TCT CCA AGG AAA AAC GC-3' and 5'-GGA GAA AAT TGC GGA AAA CC-3'; and *ndhI*, 5'-CTG ACA TTG GTA AGC GAC CC-3' and 5'-CAA TAC GAG CCG CCA GAT AC-3'. The *rrn16* probe was an *Apal*/*Eco*RV fragment derived from plasmid pPRV1 (Zoubenko *et al.*, 1994). Double-stranded DNA probes were prepared by random-primed ^{32}P -labeling.

RT-PCR analysis of *PTOX* transcripts was carried out as described by Josse *et al.* (2000), using the following primers: 5'-GTG CAY TTT GCI GAR AGC TGG AAT G-3' and 5'-TCA TYG TIT TIC AAT GIT CTG CIT CRT CAT CTC-3', where Y = C + T, R = A + G, and I = deoxyinosine. The amplification consisted of 24 cycles of 30 sec at 94°C, 20 sec at 45°C, and 20 sec at 72°C.

Isolation of thylakoid membranes and chlorophyll determination

Leaves were homogenized in ice-cold buffer containing 50 mM Hepes (pH 7.5), 5 mM MgCl_2 , 1 mM EDTA, 0.33 M sorbitol, and 1% BSA. The homogenate was filtered through Miracloth and centrifuged for 5 min at 6000 *g*. The resulting pellet was washed with 10 mM Hepes (pH 7.5), 5 mM MgCl_2 , and 5 mM sorbitol and re-centrifuged, and the thylakoid membranes were finally suspended in 10 mM Hepes (pH 7.5), 10 mM MgCl_2 , 5 mM NaCl, and 0.1 M sorbitol. For analysis of thylakoid protein phosphorylation, leaves were harvested 5 h after the lights were turned on and in the end of an 18-h dark period, and thylakoids were isolated under very dim green light with buffers containing 10 mM NaF to prevent protein dephosphorylation.

Chlorophyll was extracted in 80% (v/v) buffered acetone (25 mM Hepes-NaOH, pH 7.5) and quantified as described by Porra *et al.* (1989).

Isolation of mitochondria

Mitochondria were purified from tobacco leaves according to the protocol of Day *et al.* (1985), except that PVP was omitted from the Percoll gradients.

Gel electrophoresis, protein gel blots, and in-gel NDH determination

SDS-PAGE and immunodetection of thylakoid proteins were performed as described earlier by Baena-González *et al.* (1999). The gels were loaded on a chlorophyll basis. Polyclonal antibodies against the DE loop of D1 and D2 were purchased from Research Genetics, Inc., and phosphothreonine antibodies were purchased from Zymed Laboratories, Inc. Polyclonal antibodies against recombinant PTOX were produced as described by Cournac *et al.* (2000), and the identity of the protein band was confirmed by parallel electrophoresis of protein extracts from WT and a tomato mutant (*ghost*), which lacks the PTOX protein (Josse *et al.*, 2000). Other antibodies were kindly provided as follows: LHCB2 by Dr S. Jansson (Sweden); OEC33 by Dr T. Hundal (Sweden); PsbW by Dr W. Schröder (Sweden); Cyt *f* by Dr F.-A. Wollman (France); PSI,

CP43, and CP47 by Dr R. Barbato (Italy); α -Cyt b_{559} by Dr R. Herrmann (Germany); NdhH by Dr G. Peltier (France) and NdhI by Dr P. Nixon (UK).

For NDH assays, freshly isolated thylakoid samples were run in a BN acrylamide gel. BN-PAGE was carried out as described by Cline and Mori (2001), with the following modifications. Washed thylakoids were suspended in the re-suspension buffer (20% (w/v) glycerol and 25 mM BisTris-HCl, pH 7.0) at 1.0 mg chlorophyll ml^{-1} . In the case of mitochondrial samples, washed membranes were suspended in the re-suspension buffer at 13 mg protein ml^{-1} . An equal volume of the re-suspension buffer supplemented with 2% DM was added to the thylakoid suspension in a dropwise manner. Samples were briefly vortexed and kept for 5 min on ice. Non-solubilized material was removed by centrifugation at 20 000 g for 20 min. The supernatant was combined with 1 : 10 volume of 5% Serva blue G (100 mM BisTris-HCl, pH 7.0, 0.5 M 6-amino-*n*-caproic acid, and 30% glycerol) and applied to 1.0-mm-thick 4–12.5% acrylamide gradient gels in a Hoefer Mighty Small vertical electrophoresis unit (Amersham Pharmacia Biotech, USA) connected to a cooling circulator (2–4°C). Electrophoresis was performed in the following manner: 75 V, 30 min; 100 V, 30 min; 125 V, 30 min; and 150 V, 30 min. At this point, when half of the gel was covered with dye, the cathode buffer was exchanged with buffer lacking the dye, and the electrophoresis was continued: 150 V, 30 min; 175 V, 30 min; and 200 V, 60 min. Activity staining was performed using NBT and NADH or NAD(P)H as substrates (Casano *et al.*, 2000).

Chlorophyll fluorescence measurements

Fluorescence emission spectra of thylakoid membranes were measured at 77K with a diode array spectrophotometer (S2000, Ocean Optics, Dunedin, FL, USA) equipped with a reflectance probe as described by Keränen *et al.* (1999). Fluorescence was excited with white light below 500 nm (defined with LS500S and LS700S filters (Corion Corp., Holliston, MA, USA) placed in front of a slide projector), and the emission was recorded between 600 and 780 nm. A 100- μl thylakoid sample (10 μg chlorophyll ml^{-1} in 0.1 M sorbitol, 10 mM Hepes (pH 7.4), 5 mM NaCl, and 10 mM MgCl_2) isolated from plants previously exposed for 5 h to light was used.

Measurements of PSII and PSI activities

Photosystem II and PSI activities of thylakoids were measured with a Hansatech DW1 oxygen electrode (Hansatech Instruments Ltd., UK) at 20°C. PSII activity was measured as oxygen evolution in a reaction mixture (1 ml) consisting of 5 mM NH_4Cl , 0.33 M sorbitol, 40 mM Hepes-KOH (pH 7.6), 5 mM NaCl, 5 mM MgCl_2 , 1 M glycine betaine, 1 mM KH_2PO_4 , and thylakoids equivalent to 20 μg chlorophyll. DCBQ (0.25 mM) was used as an electron acceptor. PSI activity was recorded as net oxygen consumption in a reaction mixture (1 ml) consisting of 40 mM Na-phosphate (pH 7.4), 1 mM NaCl, 0.6 mM NaN_3 , 0.12 mM methylviologen (MV), 0.3 mM DCPIP, 32 mM Na-ascorbate, and 0.01 mM DCMU.

Determination of P700 redox state

The redox state of P700 in thylakoids isolated from dark-adapted (16 h) plants was determined by absorbance changes at 810 nm, using A_{860} as a reference. Absorbance changes were monitored using an ED-P700DW unit attached to the PAM 101 fluorometer. Measurements were performed under anaerobic conditions in a

temperature-regulated cuvette (25°C) in 0.5 ml of buffer consisting of 50 mM Tricine (pH 7.5), 5 mM MgCl_2 , 6 mM glucose, 10 μM DCMU, 2 mM NH_4Cl , 400 U ml^{-1} catalase, and 50 μM Fd (Scheller, 1996). The mixture was thoroughly flushed with argon before 2 U of glucose oxidase and thylakoids (25 μg chlorophyll) were added. An initial illumination period (1000 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$) of 30 sec was applied to reduce Fd in the presence of DCMU. After that, the samples were kept in darkness for 10 sec followed by 30 cycles of actinic light (1.2 sec, 1000 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$) and darkness (8.8 sec). First, 15 curves were recorded from the sole DCMU-treated thylakoids. Thereafter, NADH or NAD(P)H (2.4 mM) was added to the mixture through a small hole on the side of the cuvette and 15 more curves were recorded. From the 15 curves of each treatment (DCMU, DCMU + NADH/NAD(P)H), an average curve was calculated and a minimum of two independent average curves were obtained for each treatment.

Maximum level of photo-oxidizable P700 in thylakoid membranes was determined by using 532-nm single turnover flash (4 nsec, and an energy of approximately 1.2 mJ cm^{-2}) provided from a Nd:YAG laser (Minilite, Continuum, Santa Clara, CA, USA) in the presence of 6 μM DCPIP and 10 mM Na-asc as an artificial electron donor and 20 μM DCMU. The 0.5-ml reaction mixture contained 25 μg chlorophyll in the buffer consisting of 50 mM Tricine (pH 7.5), 5 mM MgCl_2 , 10 mM NaCl, and 0.4 M sucrose.

Electron microscopy

For electron microscopy, the leaf was fixed with 3% glutaraldehyde in 0.1 M Na-phosphate buffer, pH 7.0, and post-fixed in 1% osmium tetroxide in the same buffer. After dehydration in an alcohol series, the samples were embedded in Epon. The thin sections, cut with KB ultramicrotome IV (LKB, Bromma, Sweden), were stained with uranyl acetate and Reynolds lead citrate, and examined with a Jeol Jem 1200EX (Tokyo, Japan) electron microscope.

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