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Effects of selective inactivation of individual genes for low-molecular-mass subunits on the assembly of photosystem II, as revealed by chloroplast transformation: the *psbEFLJ* operon in *Nicotiana tabacum*

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Abstract Photosystem (PSII) is a supramolecular polypeptide complex found in oxygenic photosynthetic membranes, which is capable of extracting electrons from water for the reduction of plastoquinone. An intriguing feature of this assembly is the fact that it includes more than a dozen low-mass polypeptides of generally unknown function. Using a transplastomic approach, we have individually disrupted the genes of the *psbEFLJ* operon in *Nicotiana tabacum*, which encode four such polypeptides, without impairing expression of downstream loci of the operon. All four mutants exhibited distinct phenotypes; none of them was capable of photoautotrophic growth. All mutants bleached rapidly in the light. Disruption of *psbE* and *psbF*, which code for the α and β apoproteins of cytochrome b_{559}, abolished PSII activity, as expected; Δ*psbL* and Δ*psbJ* plants displayed residual PSII activity in young leaves. Controlled partial solubilisation of thylakoid membranes uncovered surprisingly severe impairment of PSII structure, with subunit and assembly patterns varying depending on the mutant considered. In the Δ*psbL* mutant PSII was assembled primarily in a monomeric form, the homodimeric form was preponderant in Δ*psbJ*, and, unlike the case in Δ*psbZ*, the thylakoids of both mutants released some PSII supercomplexes. On the other hand, Photosystem I (PSI), the cytochrome b_6f complex, ATP synthase, LHCII, and CP24/CP26/CP29 antennae were present in near wild-type levels. The data are discussed in terms of their implications for structural, biogenetic and functional aspects of PSII.

Keywords Photosystem II assembly · Low-mass subunits · Cytochrome b_{559} · *psbL* and *psbJ* mutants · Chloroplast transformation

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Introduction

Photosystem II (PSII) is a unique molecular machine that houses the catalytic components that mediate light-driven oxidation of water to molecular oxygen, release of protons and electrons being coupled to the reduction of plastoquinone during photosynthesis. It is the most complex supramolecular polypeptide assembly found in oxygenic photosynthetic membranes, is predominantly located in the grana regions of the thylakoid membrane system, and consists of more than two dozen intrinsic and peripheral subunit species. Controlled partial solubilization and fractionation of photosynthetic membranes has demonstrated that PSII is made up of various subcomplexes which can complement each other functionally. These include an innermost reaction-center core complex, made up of the polypeptide species D1 (encoded by *psbA*), D2 (*psbD*), cytochrome b_{559} (*psbE* and *psbF*) and PsbI, which carries out the primary charge separation. Associated with the core is an ensemble of chlorophyll *a* and chlorophyll *a*/*b* assemblies which cooperate in light collection, energy transfer to the core complex and its thermal dissipation. The 51-kDa and 43-kDa chlorophyll *a*-binding proteins (CP47, CP43; PsbB and PsbC) act as the light-capturing

antenna for the core. They are intimately associated with the reaction center. The minor chlorophyll *a*/*b* antenna comprising CP24, CP26 and CP29, and the light-harvesting chlorophyll *a*/*b*-containing antenna LHCII, which is present in both mobile and bound forms at the periphery (Sandona et al. 1998; Andersson et al. 2001) are external to the core and chlorophyll *a* antenna, and are not essential for the primary photochemical charge-separation step, although they serve to enlarge the photochemically active cross-section. Recent structural data, obtained by X-ray crystallography and high-resolution electron microscopy, are basically consistent with this topography deduced from biochemical and biophysical work (Barber and Kühlbrandt 1999; Boekema et al. 1999; Hankamer et al. 1999; Nield et al. 2000a, 2000b; Zouni et al. 2001).

One of the most intriguing findings in PSII biology was the detection of a relatively large number (16) of intrinsic or peripheral polypeptide species of low mass (summarized in Hankamer et al. 2001; see also Baena-Gonzalez et al. 2001; Swiatek et al. 2001), which are not found in bacterial, non-oxygenic, quinone-reducing photosystems (Debus 2000). It is conceivable that an increased number of subunit species is required to regulate the electron flow in PSII, which is more complex than the cyclic flow that operates during anaerobic photosynthesis in bacterial cells. However, the functions of these polypeptides are not well understood, and even their association with, and/or localization within, PSII has not been rigorously demonstrated in all instances. Moreover, various transmembrane helices uncovered by structural studies could not be assigned to distinct polypeptides at the level of resolution presently available (Hankamer et al. 2001; Zouni et al. 2001). Low mass polypeptides such as PsbY (Gau et al. 1998) or PsbZ, a possible link between PSII and antenna (Baena-Gonzalez et al. 2001; Swiatek et al. 2001), obviously represent potential candidates to fill these gaps. Another set of such small polypeptides is encoded on most plastid chromosomes reported to date by the *psbEFLJ* operon. *psbE* and *psbF* encode the apoproteins α and β of cytochrome b_{559}, the only two-chain cytochrome known in nature (Herrmann et al. 1984). Cytochrome b_{559} is found in the minimal PSII subcomplex that is capable of light-induced charge separation (Nanba and Satoh 1987). Deletion of the corresponding genes, *psbE* and *F*, abolishes PSII activity (Pakrasi et al. 1990; Morais et al. 1998). Although the cytochrome can undergo light-induced redox reactions, it is not involved in the primary electron transfer reactions of PSII, leaving its role in the photosynthetic process uncertain. Recent proposals have focused on alternative electron transfer pathways involving cytochrome b_{559} that protect PSII against light-induced damage, either by dissipating excess energy through a cyclic electron flow within PSII or by deactivating potentially damaging redox states (Barry et al. 1994; Stewart and Brudvig 1998). Structural data deduced from a cyanobacterial PSII assembly support the idea that cytochrome b_{559} exists as an α/β heterodimeric structure (Zouni et al. 2001), with the heme molecule bound by single His residues in each of the chains, as predicted earlier from sequence and spectroscopic data (Herrmann et al. 1984; Babcock et al. 1985). The roles of PsbL and PsbJ remain unclear. *Synechocystis* 6803 cells in which *psbL* has been inactivated lack a functional PSII (Anbudurai and Pakrasi 1993; Kitamura et al. 1994; Ozawa et al. 1997); cyanobacterial Δ*psbJ* cells display altered oxygen evolution and the data suggest that the polypeptide is membrane associated (Lind et al. 1993). Little information is available on these components in thylakoids of vascular plants, which may differ in some respects from their cyanobacterial equivalents (Hager et al. 2002; Regel et al. 2001).

It is obvious that the low-mass polypeptides play an important role in photosynthesis, and hence knowledge of their structural and functional impact is essential for a full understanding of the photosynthetic process that occurs in PSII, and its biogenesis. This, together with the fact that the function and crucial structural details of cytochrome b_{559} are still enigmatic, has provided the incentive to use a transplastomic approach to disrupt and modify the genes of the *psbEFLJ* operon in the plastid chromosome of tobacco. The genes all encode small polypeptides of 38–83 amino acid residues (*psbL*, 38; *psbF*, 39; *psbJ*, 40; *psbE*, 83) that appear to share a common topology. They are known (E, F) or predicted (L, J) to be bitopic, i.e. each with a single central transmembrane segment of 18–22 residues flanked by relatively short hydrophilic stretches protruding from either side of the polypeptide chain into organelle stroma and thylakoid lumen, specifically by 18/39 (PsbE), 13/6 (PsbF), 17/1 (PsbL) and 9/9 (PsbJ) N-/C-terminal amino acid residues. In this communication we describe the phenotypes of mutants with defects in the genes for each of these proteins.

Materials and methods

Vector construction and transformation of tobacco chloroplasts

A 3675-bp *Sal*I- *Eco* 47III fragment of the plastid chromosome of *Nicotiana tabacum* cv. Bright Yellow 4 (nucleotide positions 65,310–68,985; EMBL Accession No. Z00044) containing the entire *psbEFLJ* operon and the species-specific *orf103* was inserted into the *Sma*I + *Sal*I-digested Bluescript KS$^-$ vector, and cloned in *E. coli* XL1-Blue. The four genes of the operon were inactivated individually by the insertion (in the correct polarity) of a terminator-less chimeric *aadA* gene fused to the homologous *psbA* promoter (Koop et al. 1996) into internal restriction sites for *Hin*dIII (*psbE* and *L*) or *Pst*I (*psbF* and *J*). These restriction sites had been introduced by site-specific mutagenesis (Kunkel 1985) using the synthetic oligonucleotides 5′-CTGGAAGCACAGGAGAAGCTTCGTTTGCTG-3′ (*psbE*), 5′-GGAGGCCCTAATGACTGCAGATCGAACCTATCC-3′ (*psbF*), 5′-CCGGAACG-AACAAAAGCTTGAATTGAATCGTACC-3′ (*psbL*) and 5′-GGGGTAAATGGCCGATACTGCAGGAAGGATTCCTC-3′ (*psbJ*). The *aadA* cassette with a heterologous (*Chlamydomonas reinhardtii*) *rbcL* terminator (Koop et al. 1996) was also placed in an *Eco*RV site located 3′ within the untranslated region of the operon (nucleotide position 66,053). The transplastomic line that carries this insertion is hereafter referred to as the RV line.

Chloroplasts of *N. tabacum* cv. Petit Havanna were transformed in one of two ways, by PEG treatment of protoplasts as described by Koop and Kofer (1995) or by particle bombardment of leaves (Svab and Maliga 1993). Selection and culture of transformed material, as well as the assessment of plastome segregation and of the homoplastomic state, were performed essentially as described by De Santis-Maciossek et al. (1999) and Swiatek et al. (2003). The material was grown on agar plates of MS medium (Murashige and Skoog 1962) containing 2% sucrose, and grown on 12 h dark/light cycles at 25°C with 20 μmol of photons/m^2 per s and, to maintain selective conditions, 500 μg/ml spectinomycin.

DNA and RNA analysis, protein overexpression and antisera

Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger et al. 1992). Both labeling with energy-transfer fluorochrome dideoxynucleotide (Rosenblum et al. 1997) with the ABI 377 system (Applied Biosystems) and fluorochrome primer labeling with the LI-COR 4200IR2 two-laser system (MWG Biotech) were used for detection of sequencing products (Hupfer et al. 2000).

Total RNA was isolated from leaves using the TRIazol reagent (Gibco/BRL), electrophoretically fractionated in 1% agarose-MOPS-formaldehyde gels (5 μg/lane), and transferred onto Hybond membranes (Pall Biodyne) for hybridization as outlined in Sambrook et al. (1989).

The individual genes of the *psbEFLJ* operon were amplified by PCR from the 3675-bp *Sal*I-*Eco*47III fragment mentioned above, using gene-specific primer pairs extended by *Xho*I and *Xba*I restriction sites at their 5′ and 3′ ends, respectively: 5′-GACTCGAG TCTGGAAGCACAGGAGAACG-3′ and 5′-GATCTAGAC TAAAACGATCTACTAAATTCATCG-3′ for *psbE*; 5′-TCCTC GAGACTATAGATCGAACCTATCC-3′ and 5′-ATTCTAGA TTATCGTTGGATGAACTGC-3′ for *psbF*; 5′-GACTCGAGA CACAATCAAACCCGAACG-3′ and 5′-GATCTAGATTAAT TGAAGAAATAATTGG-3′ for *psbL*; and 5′-GACTCGAGG CC GATACTACTGGAAGGATTCC-3′ and 5′-GATCTAGACTA GAGGGATGAACCCAATCC-3′ for *psbJ*. The resulting fragments were double-digested with *Xho*I and *Xba*I, inserted into Bluescript SK$^-$ or pThioHisA,band C vectors (Invitrogen), and cloned in *Escherihia coli* DH5α or BL21, respectively. Expression of the fusion products in the pThiolHis system was performed according to the manusfacturer's recommendations. The PsbF, PsbL and PsbJ fusion proteins were electrophoretically purified from inclusion bodies and used to elicit antisera by immunizing rabbits in the presence of TiterMax adjuvant (CytRX Corporation) according to Harlow and Lane (1988). Initial attempts to overexpress the small, bitopic proteins themselves in *E. coli* using the customary vectors outlined in Haehnel et al. (1994) failed. None of the proteins could be obtained in sufficient amounts. Antisera were stored at −20°C after addition of 0.01% sodium azide. Where titers were low, IgG fractions were purified on HiTrap rProtein A columns (Amersham Pharmacia Biotech) according to the manufacturer's protocol. The fractions were desalted on PD-10 columns (Amersham Pharmacia Biotech) and subsequently concentrated on Urifil-10 concentrators (Millipore).

Strand-specific riboprobes were prepared by in vitro transcription and [α-^{32}P]dUTP labeling of PCR products using the T7 and T3 promoters of *Xba*I- or *Xho*I-digested recombinant Bluescript vectors, respectively. Hybridization with double-stranded and single-stranded probes was carried out overnight at 65°C in Rapid Hybridization Buffer (Amersham).

Protein extraction, polyacrylamide gel electrophoresis and immunoblotting

Total leaf proteins were isolated and sucrose-gradient fractionation of partial thylakoid lysates was performed as described in Swiatek et al. (2001). Thylakoid membranes were prepared according to Zer et al. (1999) from leaves of 4-week-old, axenically grown plants. Aliquots were suspended in 50 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, 50% glycerol and stored at −70°C until use. Proteins were separated in 14% (w/v) polyacrylamide gels (Schägger and von Jagow 1987) in the presence of 6 M urea, or in the gel systems described in Westhoff et al. (1985). Samples were heated for 5 min at 80°C, mixed with 1/10 vol of glycerol/dye solution, immediately applied to the gel, and electrophoresed at 30 mA for 20 h at room temperature. Two-dimensional electrophoresis was performed using non-denaturing polyacrylamide gels (6–12% "blue native" gradient gels; Schägger and von Jagow 1991) for the first dimension and a 10–16% denaturing gradient gel in the second (Laemmli 1970). Individual bands in one-dimensional gels, and spots in 2-D gels were identified by immunodetection, after electrophoretic transfer to nitrocellulose filters (Schleicher and Schuell) or PVDF membranes (Pall Biodyne) and probing with monospecific, polyclonal antisera. Antibody binding was detected with horseradish peroxidase-conjugated α-rabbit IgG (Boehringer, Mannheim), and peroxidase activity was visualized by reaction with luminol and H_2O_2 (the enhanced chemiluminescence assay; ECL, Amersham). The antisera used were raised in rabbits against polypeptides from spinach, *Arabidopsis* or tobacco: D1 (*psbA*), D2 (*psbD*), CP47 (*psbB*), CP43 (*psbC*), α and β subunits of cytochrome b_{559} (*psbE*, *psbF*), PsbH (*psbH*), PsbL (*psbL*), PsbJ (*psbJ*), PsbS (*psbS*), PsbW (*psbW*), PsbZ (*psbZ*), the extrinsic 33-, 23- and 16-kDa polypeptides of the oxygen-evolving complex (*psbO*, *psbP* and *psbQ*) of PSII, CP24 (*lhcb4*), CP26 (*lhcb5*), CP29 (*lhcb6*), LHCII (*lhcb1-3*), LHCI (*lhca*), subunits Ia and b (*psaA* and *B*), II (*psaD*) and III (*psaF*) of PSI, cytochrome f (*petA*), cytochrome b_6 (*petB*) and Rieske FeS protein (*petC*) of the cytochrome b_6f complex, and subunits α (*atpA*) and b′ (*atpG*) of the thylakoid ATP synthase, plastocyanin (*petE*) and lumenal polyphenol oxidase (*ppo*).

Kinetic analysis of fluorescence and light absorption

Changes in chlorophyll fluorescence yield were measured using a commercial pulse-modulated fluorimeter (PAM-101, Walz, Effeltrich, Germany); the light guide that delivers the measuring and saturating light was held 5 mm from the upper surface of selected leaves. Leaves were dark adapted for 15–30 min prior to measurement of the induction fluorescence, and kept at 20°C. After recording the minimum fluorescence (Fo) induced by the measuring beam, saturating light flashes were provided in order to completely reduce the PSII acceptor side Q_A^- and to measure the maximum fluorescence yield (Fm). The intensity of the saturating light flash (800 ms) used for the measurements of Fm was 6000 μmol of photons/m^2 per s. The variable fluorescence (Fv) was calculated as Fm−Fo. The ratio Fv/Fm reflects the potential yield of the photochemical reaction of PSII (Krause and Weis 1991). Reoxidation of Q_A^- was recorded for 2 min. The actinic light intensity used for fluorescence induction was 20 μmol of photons/m^2 per s. The application of additional consecutive light pulses over a 5-min induction period served to calculate the amount of photochemical and non-photochemical quenching.

PSI activity in intact leaves was measured by detecting absorption changes resulting from P700 oxido-reduction using the dedicated PSI attachment of the PAM-101 instrument. For thylakoid preparations, 8–12 leaves exhibiting variable fluorescence were selected from different plants. Oxygen evolution by isolated thylakoid membranes was measured polarographically using dimethylbenzoquinone (DMBQ) or dichlorophenylindophenone as electron acceptors (Keren et al. 1997).

Other methods

Chlorophyll contents were measured in 80% acetone according to Porra et al. (1989). Samples for ultrastructural analysis and electron microscopy were prepared as described by De Santis-Maciossek et al. (1999).

Results

Disruption of the genes *psbE*, *psbF*, *psbJ* and *psbL*

For gene disruption, a terminator-less chimeric *aadA* cassette which confers spectinomycin resistance on chloroplasts was inserted in sense orientation into each of the four genes separately, using as targets restriction sites that had been generated by site-directed mutagenesis of each gene (Fig. 1). The vectors were designed to interrupt the ORFs near the N-terminus: between the codons for Glu7 and Arg8 in *psbE*, Thr3 and Ile4 in *psbF*, Asn10 and Val11 in *psbL*, and the two threonine residues at positions 4 and 5 in *psbJ*. Except in *psbL*, the insertions also caused a shift in the reading frame that prevents translational reinitiation in the interrupted gene but did not interfere with the decoding of the preceding and downstream genes in the operon. As controls, and to assess possible secondary effects of cassette insertion, the wild-type tobacco line and the RV line carrying the *aadA* gene in the transcribed intercistronic region downstream of *psbJ* were used. The latter insertion was previously shown to be phenotypically neutral (Bock et al. 1994), i.e. the transformed strain is functionally and ultrastructurally indistinguishable from wild-type.

The first resistant colonies or shoots formed approximately 3 weeks after transformation in all cases. The transformation efficiency varied between 18 and 74 independent lines per 10^6 treated protoplasts or 5 bombarded leaves, respectively. Correct integration of the *aadA* cassette, plastome segregation and the homoplastomic state were monitored by Southern analysis, nucleotide sequence analysis, and pulsed-field electrophoresis followed by PCR analysis of the band containing monomer plastid chromosomes (De Santis-Maciossek et al. 1999; Swiatek et al. 2003). The PFGE/PCR approach provides an efficient tool with which heteroplastomic lines can be recognized. Three cycles of shoot regeneration from leaf disks on selective medium were found to be sufficient for complete segregation of wild-type and mutant plastomes. In the case of Δ*psbE* and Δ*psbF*, which segregated relatively slowly, a clonal propagation step was included to speed up plastome separation, employing an efficient protocol for regeneration of plants from protoplasts, which allows shoot formation to occur within approximately 2 weeks (Dovzhenko et al. 1998). The mutant lines were maintained for several months on medium that induced the formation of multiple shoots. Individual plants were grown in jars for 4–6 weeks. With the exception of Δ*psbE*, all lines grew quite well and resembled the wild type morphologically (apart from the fact that leaves were occasionally smaller than normal).

Phenotypic characterization of the mutants

The material selected for analysis was homoplastomic (Swiatek et al. 2003) and displayed distinct phenotypes in all instances. All mutants, with the exception of Δ*psbL* and, to some extent, Δ*psbJ*., remained fairly green in dim light (Fig. 2), but bleached rapidly when exposed to light intensities of 100 μmol photons/m^2 per s or higher. The *psbJ* and *psbL* mutants displayed an unusual developmental phenotype in that older leaves senesced rapidly and turned off-white even at 10 μmol photons/m^2 per s (Fig. 2). None of the lines grew photoautotrophically, i.e. the plantlets were not capable of surviving on soil or growing axenically in the absence of sucrose, despite the fact that residual PSII activity (20–30%) could be detected in the Δ*psbJ* (Regel et al. 2001) and Δ*psbL* lines (see below).

The plastids of all four mutants were similar in size to those of the wild-type, but differed ultrastructurally from the typical lens-shaped wild-type and RV mutant chloroplasts, which show normal grana stacks interconnected by stroma thalykoids (Fig. 3A). The mutants Δ*psbE* and Δ*psbF* contained only stacked tylakoid membranes with 2–6 apposed membranes, which were unusually sectored and sometimes locally swollen (Fig. 3B). Generally, these membranes extended throughout the entire cross-section of the organelle. Similarly, Δ*psbL* and Δ*psbJ* were alike ultrastructurally, in that they exhibited densely stacked thylakoids, with bundles containing up to 20–30 stacked membranes in their central regions, and little, if any, stroma lamellae (Fig. 3C and D). All four mutants expressed normal

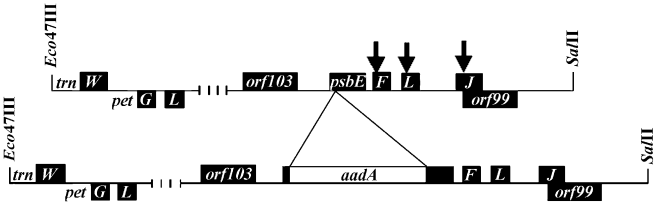


Fig. 1 Construction of *Nicotiana tabacum psbE*$^-$, *psbF*$^-$, *psbL*$^-$, and *psbJ*$^-$ plastome mutants. Each of the four genes was individually mutated by insertion of the *aadA* cassette into artifically generated, unique restriction sites within the respective reading frames of the genes, as described in Materials and methods. Only the disruption of *psbE* is shown in detail. The *arrows* mark the sites of insertion of the cassette into the other genes

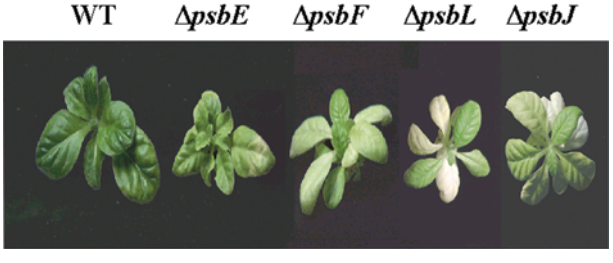


Fig. 2 Phenotypes of wild-type, and Δ*psbE*, Δ*psbF*, Δ*psbL*, and Δ*psbJ* mutant lines, respectively, grown axenically under dim light (10 μE/m^2 per s), which prevents bleaching of the mutant material. However, note the bleaching of older leaves of the Δ*psbL* mutant even under these conditions

levels of transcripts of the genes under study, and exhibited Northern patterns as expected. The general pattern of processing of the primary polycistronic transcript was unchanged, except for those RNA species that included the respective disrupted genes, which were enlarged by 906 bp (without terminator) and 1356 bp (with terminator) due to cassette insertion (data not shown).

Effects of gene disruption on the structure and function of PSII

Gel electrophoresis of total cellular proteins or of thylakoid proteins, as well as two-dimensional electrophoresis and high-resolution sucrose gradient centrifugation of dodecyl maltoside lysates of thylakoid fractions (Swiatek et al. 2001), combined with immunolabeling with specific antibodies, were performed in order to evaluate the effect of each of the mutations upon the status of the thylakoid system in general and of PSII in particular. Since the numerous low mass polypeptides of the thylakoid membrane system were difficult to resolve electrophoretically in a single gel system, high resolution electrophoresis on various gel systems, including that of Schägger and von Jagow (1987) and systems with and without urea (Westhoff et al. 1985), were employed to identify polypeptides in the low-molecular weight range. However, Western analysis with polyclonal, monospecific antisera raised against each of the components encoded by the *psbEFLJ* operon, as well as against PsbZ (Swiatek et al. 2001) and PsbW, was required to definitively identify the subunits of interest and clearly distinguish them from other comigrating thylakoid components (Fig. 4). No signals were found in stroma

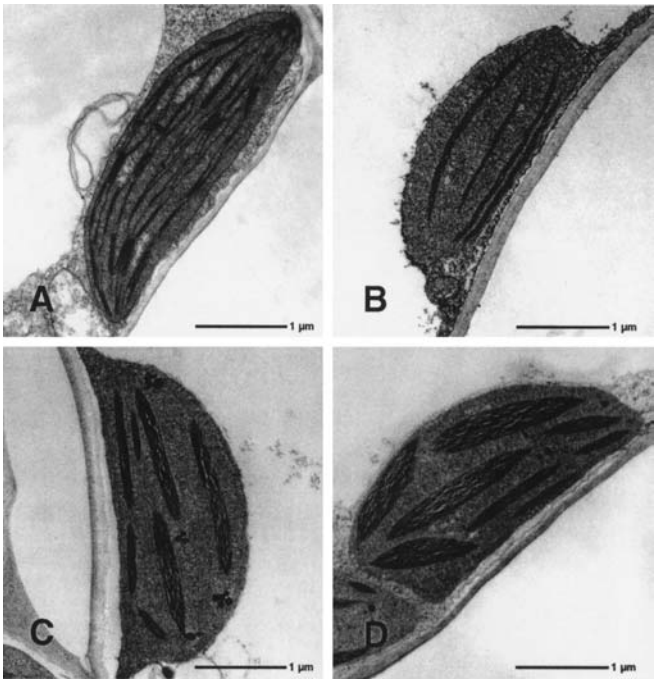
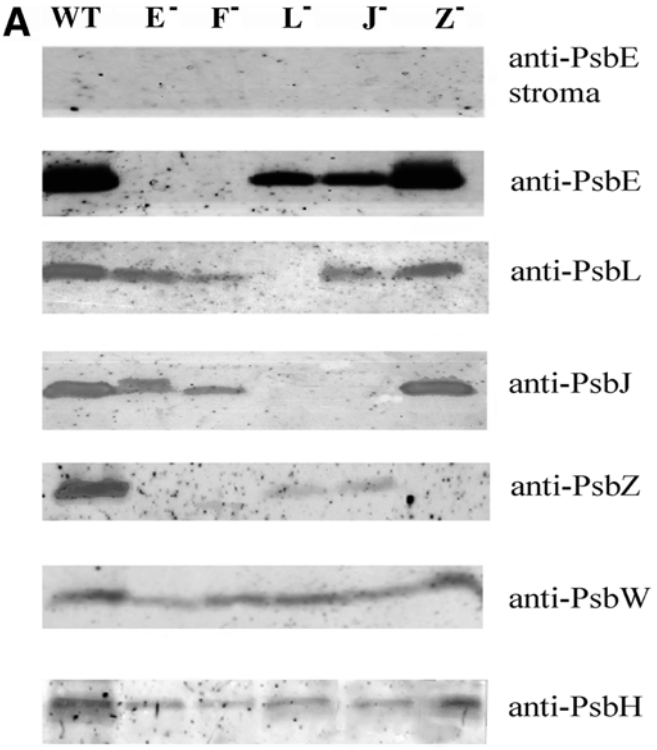


Fig. 3A–D Electron micrographs of ultra-thin sections of leaf tissue from wild-type tobacco and various PSII mutants. **A** Typical lens-shaped RV (and wild-type) chloroplast with well established grana and stroma thylakoids. The matrix is densely packed, except at nucleoids which are transparent. **b** Typical Δ*psbF* mutant plastid. Note the bundles of thylakoid layers without discernible stroma lamellae. Mutant Δ*psbE* plastids exhibit a similar phenotype. **C** and **D** Typical Δ*psbL* and Δ*psbJ* plastids, respectively, with disk-like, often locally swollen membrane stacks. Bar = 1 μm



B

Protein \ Mutant	Δ*psbE*	Δ*psbF*	Δ*psbL*	Δ*psbJ*	Δ*psbZ*
PsbE	-	-	+	+	-
PsbF	-	-	+	+	-
PsbL	+	+	-	+	+
PsbJ	+	+	-	-	+
PsbZ	+	+	+	+	-
PsbW	+	+	+	+	+
PsbH	+	+	+	+	+
PSII yield	-	-	+	+	+

Fig. 4A, B Immunoblot analysis of low-mass thylakoid membrane polypeptides of wild-type tobacco and *psbE*, *F*, *L*, *J* and *Z* mutants. A Lines were grown at 25°C under 10 $\mu E/m^2$ s, and thylakoid extracts were analyzed using the indicated monospecific polyclonal antisera. Since sera raised against PsbF, L and J had low titers, antibodies were purified and concentrated before use as described in Materials and methods. The blot with anti-F was qualitatively comparable to that of anti-E and is not shown. **b** Summary of the low-mass PSII subunits present in, and the PSII activity of, the mutants under study

fractions that would have indicated unincorporated polypeptides or their degradation products (Fig. 4). It is worth noting that the relative positions of the six low-molecular weight proteins (including PsbZ and PsbW) in the different gel systems did not coincide (see also Westhoff et al. 1985) nor did the respective molecular masses calculated from the sequence agree with those estimated from their electrophoretic migration velocities. Without the use of specific antisera, no unequivocal assignment of bands or meaningful interpretation of the data would have been possible.

One of the striking findings of this work was the variation in subunit patterns in the individual mutants, due to co-operative polypeptide loss or instability (Fig. 4). Probing thylakoid proteins of developing young leaves with anti-PsbE revealed that the α subunit of cytochrome b_{559} was missing, as expected, in the Δ*psbE* material, but it was lacking also in Δ*psbF*. In the absence of PsbF, PsbE obviously does not stably insert into the membrane. It is important to note that PsbZ was also undetectable in both mutants, but present in Δ*psbL* and Δ*psbJ*, albeit in reduced quantities. On the other hand, PsbL was only absent in the corresponding mutant, but detectable in all others, including PsbJ. PsbJ, missing in the corresponding mutant, was present in Δ*psbE*, Δ*psbF*, and Δ*psbZ*, but – strikingly – absent in Δ*psbL*. PsbW and PsbH were present in all mutants (Fig. 4). The findings just outlined were substantiated and refined in two ways.

Approximately 20 additional antisera, directed against plastid- and nucleus-encoded subunits of all major thylakoid assemblies, were chosen for Western analysis of the thylakoid systems of the mutant material under study. All subunits of PSI, ATP synthase, the cytochrome complex checked, and the lumenal proteins plastocyanin and polyphenoloxidase were present in isolated thylakoid membranes in amounts (per g fresh weight) comparable to those in the wild type (Fig. 5). In contrast, the pattern of PSII polypeptides differed markedly depending on the mutant and developmental stage of the leaves studied. Disruption of *psbE* and *psbF* caused the most serious effects (see below). Nevertheless a residual subunit spectrum of the PSII components was clearly present. In both mutants, the steady-state concentrations of D1 (*psbA*), D2 (*psbD*) and CP47 (*psbB*) were reduced to less than 5% of the wild-type level. On the other hand, approximately 15–30% of wild-type PSII polypeptide levels were found in Δ*psbL*, and up to 50% in Δ*psbJ* lines (Fig. 5). The detection of components of the oxygen-evolving complex, PsbO, PsbP and PsbQ, was strongly dependent upon the stage of leaf development examined. All three subunits were present in near-wild-type quantities in very young leaves, but greatly reduced or absent in older – but still green – leaf material (data not shown). Virtually no change was found in any of the mutants for PsbW, PsbH, and PsbS, for LHCII apoproteins and the minor antenna CP24, CP26 and CP29. All proteins of nuclear/cytosolic origin were terminally processed, including those located in the thylakoid lumen.

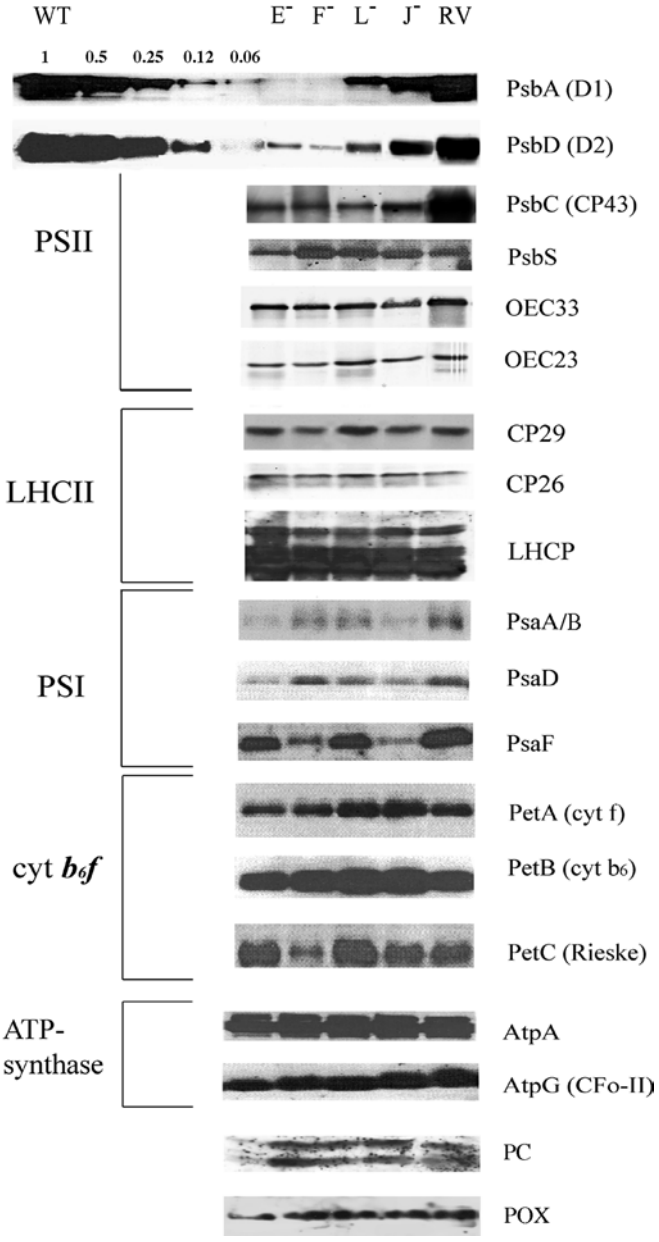


Fig. 5 Western analysis of thylakoid proteins from wild-type tobacco, *psbE*, *F*, *L*, *J* and *Z* mutants, and the RV control (lanes labeled at the *top*). The identities of the proteins detected are indicated on the *right*. The top two rows on the *left* provide a quantification scale

Controlled partial solubilization of thylakoid membranes with dodecyl maltoside (Westhoff et al. 1983) was chosen to evaluate the status of the principal membrane assemblies in the mutant thylakoids. The major photosynthetic complexes released were separated either by sucrose-gradient centrifugation or by electrophoresis in two-dimensional gels (data not shown; see Suorsa et al., submitted), and checked visually, photometrically and serologically for their assembly status and polypeptide composition, respectively. Figure 6 shows an example of solubilized Δ*psbL* and Δ*psbJ* thylakoids fractionated by the former approach, followed by electrophoretic

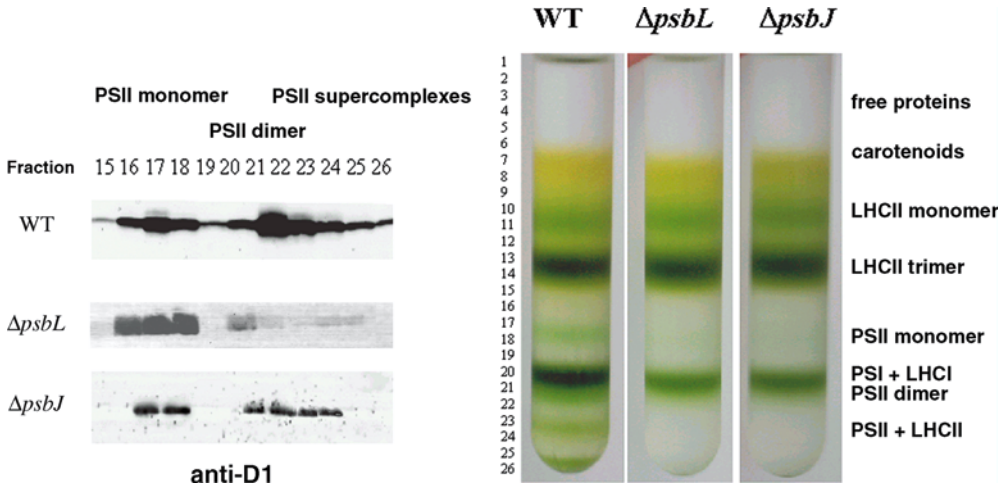


Fig. 6 Chlorophyll-protein complexes in wild-type, Δ*psbL* and Δ*psbJ* mutant thylakoids. *Right panel* Sucrose gradient fractionation of partially solubilized thylakoids obtained by treatment with β-dodecylmaltoside (gradient not overloaded; see Materials and methods). *Left panel* Immunodetection of various PSII assemblies with antiserum against D1 protein. Note that the higher-order PSII assemblies that occur in low amounts in the Δ*psbL* and Δ*psbJ* mutants can only be visualized in overloaded gradients. An equivalent pattern was obtained with anti-PsbE (not shown). The fraction numbers given at the *top* correspond to those given for the gradient on the *left*

separation of the polypeptides in relevant gradient fractions in a denaturing polyacrylamide gel, and immunological analysis for PSII components. This approach demonstrated that the subunits tested were assembled into dodecylmaltoside-stable PSII complexes in the mutants. Unlike the case in the wild-type, the monomer form of PSII was dominant in Δ*psbL*, but the dimer form was dominant in the Δ*psbJ* thylakoids, as in the wild type. Small amounts of PSII dimers were noted in Δ*psbL*. PSII supercomplexes (Boekema et al. 1995; Hankamer et al. 1997) could be detected as well, but their levels were extremely low in both mutants, and they were usually apparent in overloaded gradients only. In mutant thylakoid lysates, a minor fraction of core components, such as D1 or the α subunit of cytochrome b_{559}, was consistently found in the soluble protein fraction at the top of the gradients (data not shown). PSI, the cytochrome complex, ATP synthase, trimeric and monomeric LHCII and antenna with CP24, CP26 and CP29 (which comigrated with the LHC monomer) displayed banding and gel patterns that were essentially indistinguishable from those seen in solubilized wild-type membranes (Fig. 6).

No variable fluorescence nor oxygen evolution could be detected in Δ*psb*E and Δ*psb*F material (not shown). In contrast, Δ*psbL* and Δ*psbJ* displayed impaired though detectable PSII activity (for a more detailed analysis of PSII activity in Δ*psbJ* material, see Regel et al. 2001). Like Δ*psbJ*, the Δ*psbL* mutant exhibited PSII activity characterized by a relatively high Fo level and slow reoxidation of Q_A^-, compared to wild-type and RV control plants, after the application of saturating light pulses (Fig. 7). Unlike Δ*psbJ* (Regel et al. 2001), Δ*psbL*

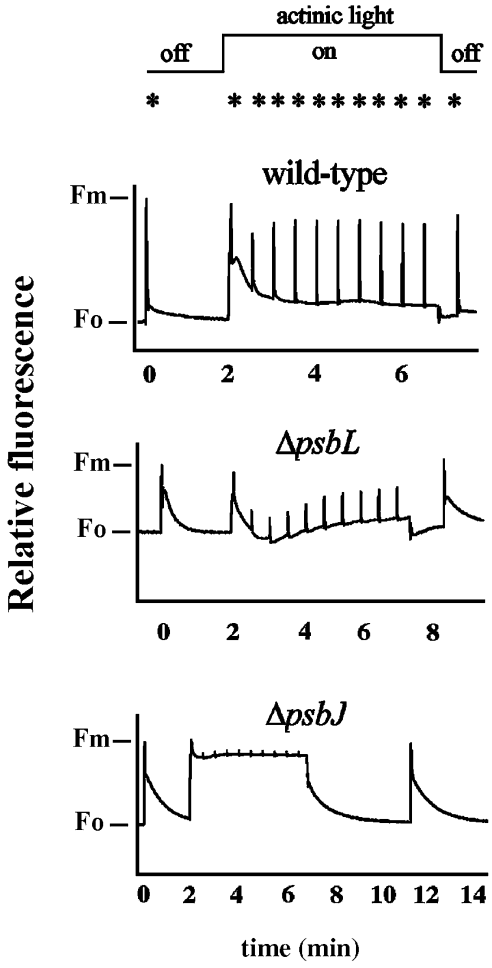


Fig. 7 Photosynthetic activity of the RV line (WT control) and representative Δ*psbL* and Δ*psbJ* mutants. Fluorescence emission traces were recorded by pulse-modulated fluorimetry as described in detail in Materials and methods. Saturating light pulses were administered at the points indicated by the *asterisks*. The respective levels of Fo and Fm are indicated. Reoxidation of Q_A^- was recorded after the application of saturating flashes to dark-adapted leaves. Actinic light was administered over a period of 5 min

also showed significant fluorescence quenching, which consists of photochemical and non-photochemical components. The Fv/Fm values were in the range of 0.74–0.76 for wild-type and RV controls, 0.25 ($\pm$0.1) for Δ*psbL*, and 0.37 ($\pm$0.1) for Δ*psbJ*. The large variation in the Fv/Fm parameter for the mutant leaves was due to progressive senescence in these leaves (Fig. 7, see also Fig. 2). This feature was not seen in the controls maintained under the same conditions for up to 6 weeks. Whereas the light pulse-induced fluorescence of Δ*psbL* returned to the Fo level within about 1 min., in Δ*psbJ* leaves Q_A^- exhibited a significantly longer half-life. The PQ pool was reduced by PSII in the *psbL* mutant, as indicated by the fluorescence kinetics (Fig. 7). The oxygen evolution activity of the control plants was in the range of 60–120 mmol of oxygen/mg chlorophyll per h, as compared to 20–35 μmol of oxygen/mg chlorophyll per h when DMBQ was used as an electron acceptor for Δ*psbJ* thylakoids (Regel et al. 2001).

All mutants exhibited P700 oxido-reduction activity, as measured with the PAM-101 PS I attachment. Under steady state conditions the oxidation state of P700, expressed as $\Delta A/\Delta A_{max}$ (Harbinson and Woodward 1987), was nearly at its maximal level (1) in both Δ*psbL* and Δ*psbJ*, whereas in wild-type it was 10 times lower (~0.1). Multiple turnover flashes, which are known to reduce the plastoquinone pool completely, induced complete reduction of P700 in background far-red light in wild-type, but led to only ~50% reduction in Δ*psbL* leaves.

Discussion

Photosystem II (PSII) is the most complex and most intriguing multisubunit assembly in the thylakoid system. Although, in recent years, the polypeptide complex has been dissected both biochemically and biophysically, crucial aspects of its structure, function, biogenesis and evolution have remained enigmatic – including the roles of the numerous low-mass subunits it contains (Herrmann 1996; Wollman et al. 1999; Hankamer et al. 2001).

Here, we demonstrate that all translation products of the *psbEFLJ* operon are present, and play a crucial role, in the assembly and activity of PSII, as well as contributing to the general structure of the thylakoids in tobacco. The complete or partial absence of PSII complexes in the mutants described here may perturb the segregation of thylakoid membrane complexes, and this may explain why stacking of membranes increases relative to the appearance of stroma lamellae (Kretzer et al. 1976). Northern and Western analysis of subthylakoid fractions with 30, generally monospecific, polyclonal antisera elicited against thylakoid proteins (including 19 components of PSII and LHCII) verified that the disruption of individual genes led to a monogenic lesion in each instance. It is important to note that the products of genes located downstream of a disrupted locus of the operon could be detected in the mutants, although the absence of one of them can interfere with the stability of others. This confirms that the individual lesions were monocistronic, and indicates that the remaining subunits are made but are rapidly degraded in the absence of others (see below), rather than arguing that epigenetic effects suppress the expression of their genes. Under non-destructive light regimes, the other major polypeptide complexes of the thylakoid system, Photosystem I, the cytochrome complex, ATP synthase, and even the formation of the LHCII trimer or CP24/CP26/CP29-containing LHC complexes remained largely unaffected (Fig. 5), substantiating the notion that there is no substantial reciprocal control between individual thylakoid assemblies during overall biogenesis.

Disruption of each of the four genes of the *psbEFLJ* operon caused quite serious effects. All four subunits are indispensable for the function of PSII, and essential for photoautotrophic growth. This is at variance with other low-mass components of PSII, such as PsbY1 and PsbY2 (Gau et al. 1998), PsbW (Thidholm et al. 2002), and PsbZ (Baena-Gonzalez et al. 2001; Swiatek et al. 2001), which are dispensable for basic photoautotrophic growth; the requirement for these components only becomes manifest when plants are cultivated under particular growth regimes. While disruption of the genes for the two-chain cytochrome b_{559}, *psbE* and *psbF*, abolishes PSII activity (Pakrasi et al. 1990; Morais et al. 2001), Δ*psbJ* and Δ*psbL* lines displayed residual activity of PSII. The increase in long-lived fluorescence in Δ*psbJ* could reflect an accumulation of Q_A^- due to alteration of the Q_A/Q_B potential (Regel et al. 2001). The decay kinetics of Q_A^- in Δ*psbL* following the excitation light pulse also follows a slower course than in Δ*psbJ*. However, the slow oxidation of Q_A^- in that case is due to changes in the accessibility of PSII to back electron flow from reduced plastoquinone (Chad et al., submitted).

PSII assemblies appear to exist as homodimers, which probably represent the functional form in vivo. In the supramolecular organization of PSII, the Lhcb proteins of the antenna dock with hydrophobic domains of the PSII core to form higher order PSII-antenna complexes with varying antenna composition (Boekema et al. 1995; Hankamer et al. 1997; Baena-Gonzalez et al. 2001; Swiatek et al. 2001). During the past few years, the composition and gross structures of several isolated PSII particles have been reported, including those of highly active dimeric PSII-LHCII supercomplexes. The loss of any of the low-mass subunits PsbE, F, L or J destabilizes PSII structures to a surprising extent, but at least the lack of the latter two does not destroy them. These are therefore the first mutants reported in higher plants in which PSII subunits have been lost *entirely* without complete loss of the ability to assemble appreciable amounts of relatively stable entities with residual photochemical activity. Assembled non-functional or impaired PSIIs have also been described recently from site-specific mutants of cytochrome b_{559} (Morais et al. 2001). Mutants of this sort will therefore not only allow the study of functions, but also of hitherto inaccessible

aspects of structure and assembly during the biogenesis or repair of photosynthetic activity following light stress. However, determination of the details of the processes and mechanisms by which PSII is formed, modified, and able to drive the highly oxidizing photochemical reactions will require substantially refined structural models. In this context, the data presented here have implications in three basic areas.

Aspects of biogenesis

Circumstantial evidence suggests that assembly and repair of PSII are highly regulated and occur gradually. So far, the sequence of subunit integration during PSII biogenesis has been studied only with the compounds that constitute the innermost reaction-center core, notably D1, D2, CP47 and CP43 and cytochrome b_{559} (e.g. Müller and Eichacker 1999; Zhang et al. 2001). No information is available on other, especially low mass, subunits, their relationships or impact on assembly kinetics. Also, it is not known whether free monomers exist in vivo, at what stage in assembly the unit dimerizes, whether this process depends upon the availability of new subunits, at what stage and how antenna complexes are added, whether the LHC trimer and minor antenna are made independently. Moreover, little is known about PSII dynamics and the components involved in the decomposition of the complexes, for instance, in the absence of their complete assembly. The results presented here indicate both a sequence, perhaps a hierarchy, of events, and co-operativity of subunit insertion/loss in the course of PSII assembly. The results presented in Figs. 4 and 5 demonstrate that the absence of the low-mass subunits studied does not prevent the core components and a few other subunits of unknown location within PSII from inserting into the membrane. Furthermore, three kinds of defect patterns for low molecular weight subunits became apparent from Western analyses and thylakoid fractionation data: PsbE, PsbF and PsbZ form one group, and PsbJ and PsbL a second that appears to integrate independently of the former. PsbW and PsbH represent a third group of small subunits (Fig. 4). Like PsbS, they are present in near wild-type amounts in all mutants.

PsbJ and PsbL, like PsbZ (Swiatek et al. 2001), severely influence the stability of higher-order PSII assemblies, but the generation of such assemblies is not hindered by the inactivation of PsbJ or PsbL. The patterns of subunit loss are consistent with the mutant phenotypes. Absence of PsbL, which also destabilizes PsbJ (but not conversely) has a more severe effect on PSII structure than the lack of PsbJ alone, which, in turn, is more deleterious than loss of PsbZ. Only the Δ*psbZ* mutant can grow photoautotrophically, albeit with reduced efficiency when compared to wild-type (Baena-Gonzalez et al. 2001; Swiatek et al. 2001). This is consistent both with the finding that all six low-mass subunits investigated assemble into PSII in Δ*psbZ* thylakoids and with the idea that this subunit is required relatively late in PSII biogenesis. In the absence of PsbJ, or of PsbZ, a relatively stable PSII dimer can be formed; thylakoids of the Δ*psbJ* and Δ*psbZ* mutants release this structure as the preponderant form. The Δ*psbL* mutant influences dimer stability more than the former, without notably affecting PsbE, F or Z. In that mutant, the PSII monomer is the dominant structure in sucrose gradients. It is important to note that overloaded gradients revealed minute amounts of both dimer (~2%) and supercomplex (~0.6%) in Δ*psbL*, and supercomplex in Δ*psbJ*, implying that even the loss of both subunits does not prevent the association of the mutant dimer complex with LHCII (Fig. 6). The release of core components, e.g. PsbE or D1 protein, that are found in soluble form in the top fractions of the gradient even after mild thylakoid lysis at low temperatures suggests that the different monomer, dimer and supercomplex ratios observed in Δ*psbL* and Δ*psbJ* thylakoids are due in large part to the instability of the higher-order assemblies. These polypeptides are not detectable in lysates from wild-type thylakoids nor in stroma fractions (Fig. 4), and hence must originate in the mutant membranes. It is therefore likely that even in Δ*psbL* the dimer is preponderant in vivo, but is not stable under the conditions of lysis used here. This differs from Δ*psbZ*, in which supercomplexes could not be detected, and indicates that both (stable) dimer formation and the presence of PsbZ are prerequisites for stable LHC attachment. Comparison of the Δ*psbL*, Δ*psbJ* and Δ*psbZ* phenotypes rules out the possibility that secondary effects in Δ*psbZ* result in the failure of LHCII and core to assemble, and argues that it is indeed the Z subunit, and not any of the other low-molecular-mass subunits studied, that is the real linker molecule between core complex, minor antenna and LHCII (Swiatek et al. 2001).

Western analyses of Δ*psbL* and Δ*psbJ* material show that the composition and assembly status of PSII can change with leaf maturation, premature senescence, and environmental factors, especially the light regime (Fig. 2). Of particular note are changes in composition of the extrinsic lumenal 33-kDa (PsbO), 23-kDa (PsbP) and 16-kDa (PsbQ) polypeptides of the oxygen-evolving system (see also Hager et al. 2002) which accompany the loss of PSII activity and are probably caused by secondary effects. These three polypeptides are easily detectable in very young leaves (Fig. 6), but are greatly reduced or even absent in older, still green leaves, notably PsbP and PsbP/PsbQ in Δ*psbL* and Δ*psbJ*, respectively. Although these findings correlate well with the assumed asymmetric arrangement of the extrinsic subunits in PSII (Nield et al. 2000a), the lack of effect on two other lumenal proteins (plastocyanin, polyphenoloxidase; Fig. 5), and PSII activity in leaves, it is not clear whether this instability reflects structural relationships or whether it is caused by a general release and degradation of these subunits from the ageing or photobleaching membrane. Substantially shorter half-lives have been noted for detached PsbO when compared

to its membrane-associated form (Palomares et al. 1993). The developmental and environmental effects on protein stability, the co-operative instability of small subunits and observations such as the finding that approximately 50% of cytochrome b_{559} or of the extrinsic subunits of the oxygen-evolving system may accumulate and persist in etiolated material or in off-white Δ*rpo* mutants, without any functional PSII assembly (Herrmann et al. 1992; DeSantis-Maciossek et al. 1999) reflect the operation of a quality control system, which is not just a mere scavenging system that recognizes and removes unassembled proteins, misassembled or incomplete structures (Herrmann 1996). A differentially operating quality control system renders unequivocal interpretations of the data difficult, until differences in intrinsic protein stability, and subtle aspects of proteolytic regulation and their impact for PSII are understood.

Structural aspects

A minimum of 36 (34) transmembrane helices that constitute PSII have been localized in the 3-D structure of a cyanobacterial PSII core preparation by X-ray crystallography (Zouni et al. 2001) and high-resolution electron microscopy with 2-D crystal diffraction (Barber und Kühlbrandt 1999; Hankamer et al. 1999), single-particle averaging (Boekema et al. 1999) and cryoelectron microscopy (Nield et al. 2000b). Of these, 24 have been assigned to D1, D2, CP43, CP47, PsbE and PsbF, 7 have been tentatively assigned (PsbH, I, K, L, W, T_c and X), and 5 helices could not be correlated with individual subunits (Hankamer et al. 2001; Zouni et al. 2001). It is difficult to relate low-molecular-mass subunits to structural elements, since the presently deduced structures display less helices than would be expected from known PSII subunits; assignments of PsbL and PsbK, for instance, rest on reasonable, but unproven assumptions, and PsbJ, PsbZ, PsbY1/Y2 and PsbS have not yet been considered or allocated to a PSII structure at all.

If co-operative subunit instability is structurally relevant, the finding that PsbH, PsbW and PsbS are present in near wild-type quantities suggests that they are not in direct spatial relation with the four low-mass subunits of PSII studied in this work. Due to the instability of CP26 in the Δ*psbZ* mutant, this subunit has been placed in the vicinity of CP26 (Swiatek et al. 2001). We tentatively assign PsbZ to either helix XII (which is conserved between cyanobacteria and higher plants) or XI according to Hankamer et al. (2001) which these authors have attributed to PsbH and/or X. The two helices reside close to cytochrome b_{559} (helices IX and X), which has been unambiguously localized in PSII by virtue of the heme iron (Zuoni et al. 2001). It may not be fortuitous that PsbZ (but not PsbL and PsbJ) is missing in Δ*psbE* and Δ*psbF* material, or that these helices reside in a region that is structurally variable between cyanobacterial and chloroplast PSIIs – which operate with different antenna systems. In spite of the phenotypic similarities between their respective mutants, several observations suggest that the positions and roles of PsbJ and PsbZ in LHCII attachment must be different: (1) the influence of PsbL upon the stability of PsbJ, but not of PsbZ; (2) the fact that loss of PsbL and PsbJ has no obvious effect on cytochrome b_{559}, (3) the differences in physiological and spectroscopic features between Δ*psbJ* and Δ*psbZ* mutants (Baena-Gonzalez et al. 2001; Hager et al. 2002; Regel et al. 2001; Swiatek et al. 2001), and (4) the finding that, unlike PsbZ, the stability of none of the three minor antenna apoproteins appears to be altered in Δ*psbJ*. Our data are compatible with a spatial relationship of the L and J subunits. By analogy, subunits K and L (and perhaps H or X) have been proposed (Hankamer et al. 2001) to be constituents of adjacent triple helices that form symmetry-related antipodes to PsbE, PsbF, and PsbZ, and are situated centrally at the monomer/monomer interface in the PSII homodimer. If this is correct, PsbJ, and not PsbH or X, could well be the third element. However, at this point, a firm structural assignment of subunits L and J must remain tentative, since effects may be indirect, and hence, loss of a subunit with concomitant dimer instability does not necessarily imply that this subunit is involved in dimer formation nor that it resides in the dimer groove. Also, the reduced or transient stability of PSII supercomplexes in Δ*psbL* and Δ*psbJ* thylakoids is at variance with a simple core-PsbZ-CP26 docking model of antenna association. It suggests topologically multiple association sites or (indirect) long-distance effects, comparable to those that have been inferred for CP29 in Δ*psbZ* (Swiatek et al. 2001).

Phylogenetic and ecophysiological aspects

It has been repeatedly noted that the loss of a particular gene can cause various, sometimes significantly different, phenotypes in different materials. That cyanobacterial Δ*psbJ*, Δ*psbH* or Δ*psbK* mutants, but not the corresponding mutants in *Chlamydomonas* and higher plants, are capable of reduced photoautotrophic growth (Ikeuchi et al. 1991; Lind et al. 1993; Mayers et al. 1993; Takahashi et al. 1994; Hager et al. 2001; Regel et al. 2001) reinforces the view that results obtained with different models, such as cyanobacteria, algae, or higher plants, cannot necessarily be generalized, in spite of the overall similarity in the basic photosynthetic process and membrane design. Thylakoids of widely different organisms have different phylogenetic histories and structural/functional details have evolved differently. PSII is an appealing model in which to probe into phylogenetic as well as ecophysiological aspects, to evaluate differences and subtleties in the regulation of corresponding processes among and within prokaryotes and eukaryotes, and to unravel the relationships between gene dispersal and the sequence of subunit integration, if any, in the overall process of PSII biogenesis and maintenance. However, the substantial effects seen with only a

few low mass subunits makes it obvious that a holistic approach will be indispensable if one wishes to derive a meaningful overall picture of PSII biology.

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