Lack of the Small Plastid-encoded PsbJ Polypeptide Results in a Defective Water-splitting Apparatus of Photosystem II, Reduced Photosystem I Levels, and Hypersensitivity to Light*

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Photiosystem II is a large pigment-protein complex catalyzing water oxidation and initiating electron transfer processes across the thylakoid membrane. In addition to large protein subunits, many of which bind redox cofactors, photosystem II particles contain a number of low molecular weight polypeptides whose function is only poorly defined. Here we have investigated the function of one of the smallest polypeptides in photosystem II, PsbJ. Using a reverse genetics approach, we have inactivated the psbJ gene in the tobacco chloroplast genome. We show that, although the PsbJ polypeptide is not principally required for functional photosynthetic electron transport, plants lacking PsbJ are unable to grow photoautotrophically. We provide evidence that this is due to the accumulation of incompletely assembled water-splitting complexes, which in turn causes drastically reduced photosynthetic performance and extreme hypersensitivity to light. Our results suggest a role of PsbJ for the stable assembly of the water-splitting complex of photosystem II and, in addition, support a control of photosystem I accumulation through photosystem II activity.

Photosystem II (PSII) is a large cofactor-protein complex consisting of at least 17 protein subunits (Ref. 1; for review, see e.g. Ref. 2). The PSII reaction center is formed by a heterodimer of two pigment-binding proteins, D1 and D2, which, in photosynthetic eukaryotes, are encoded by the chloroplast psbA and psbD genes, respectively. The photochemical reaction carried out by the reaction center converts the energy of a photon into a separation of charge and, in this way, initiates electron flow. Around the reaction center, the outer parts of PSII are assembled, the inner and outer antennae funneling absorbed light energy to the catalytic core and the oxygen-evolving complex splitting water into protons, electrons, and dioxygen (reviewed in Refs. 3 and 4).

In addition to the well-studied large protein subunits, purified PSII particles contain a number of low molecular weight polypeptides, many of which are encoded by the plastid genome of photosynthetically active eukaryotes (5). Most of these small subunits do not bind redox cofactors and, hence, are unlikely to participate directly in electron transfer reactions. It is generally assumed that they rather function as photosystem-assembling or stabilizing factors. However, in many cases, molecular evidence supporting such a structural role is largely lacking.

The successful development of transformation technologies for Chlamydomonas (6) and tobacco chloroplasts (7) has paved the way to functional characterizations of plastid genome-encoded genes by reverse genetics. Linked to a selectable marker gene, mutant alleles can be introduced into plastids by chloroplast transformation, which they replace the endogenous wild-type allele by homologous recombination. During the past decade, reverse genetics has become a powerful tool in plastid functional genomics (reviewed in Ref. 8).

Here, we have taken a reverse genetics approach to define the function of one of the smallest polypeptides in PSII, PsbJ. Using chloroplast transformation, we have generated tobacco plants lacking the PsbJ polypeptide. Physiological and biochemical analyses revealed that the PsbJ-deficient mutant plants accumulate incompletely assembled oxygen-evolving complexes, have reduced levels of PSI and are extremely sensitive to light.

MATERIALS AND METHODS

Plant Material and Growth Conditions—Sterile tobacco plants (Nicotiana tabacum cv. Petit Havana) were grown on agar-solidified MS medium containing 30 g/liter sucrose (9). Homoplasmic transplastomic lines were rooted and propagated on the same medium. Photoautotrophic growth was tested on MS medium without sucrose. For protein isolation and physiological measurements, wild type and transformed tobacco leaves were harvested at the age of only a few days in order to avoid interference with photodamaging damage in the mutant chloroplasts. Leaves referred to as "young leaves" were harvested at the age of 35 days and, in the case of the ΔpsbJ mutant, not yet photobleached.

Construction of a ΔpsbJ Plastid Transformation Vector—The region of the tobacco plastid genome containing the psbE operon was isolated as a 2383-bp SalI/SpeI fragment corresponding to nucleotide positions 65,313–67,695 (according to Ref. 10). The fragment was cloned into a pBluescript KS vector (Stratagene, La Jolla, CA) cut with SalI and SpeI. To remove the HincII site from the remaining polylinker, the plasmid was linearized with SalI and the recessed ends were filled in using the Klenow fragment of DNA polymerase I followed by religation. The psbE operon was subsequently excised by digestion with HincII and CiaI (restriction sites correspond to nucleotide positions 66,341 and 67,167) and replaced by a similarly cut PCR product carrying the psbJ deletion. The deletion of the psbJ coding region was introduced into a

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The abbreviations used are: PSII, photosystem II; PSI, photosystem I; Tricine, N,N′-dihydroxy-1,1-bis(hydroxymethyl)ethylylglycine.
PCR product by amplification with primers PT5′ (5′-GACGATCTCC-AAAATGAA-3′) and DJ1 (5′-TTTTTGTTGACGTACCATTCTAC-CCCACCTCCTCTCCA-3′). The 3′ portion of oligonucleotide DJ1 binds to the sequence immediately upstream of the psbD initiator codon and contains in its 5′ portion the nucleotide sequence downstream of the psbD operon 2 nucleotides to the HincII site at 66,341. Oligonucleotide PT5′ binds upstream of the CiaI site at 67,167. A chimeric aadA gene conferring resistance to aminoglycoside antibiotics (11) was cloned into the unique EcoRI site (position 66,054) to facilitate selection of chloroplast transfectants. A plasmid clone carrying the aadA gene in the same orientation as the psbD operon yielded the final transformation vector ( see Fig. 2 ).

Plastid Transformation and Selection of Homoplasmic Transformed Tobacco Lines—Young leaves from sterile tobacco plants were bombarded with plasmid pΔaadB-coated 1.1-μm tungsten particles using a biolistic gun (PDS1000He; Bio-Rad). Primary spectrominycin-resistant lines were selected on RMOP regeneration medium containing 50 mg/liter spectrominycin (7, 11). Plastid transformants were identified by PCR amplification according to standard protocols and using the primers pair P10 (5′-AAGCTTCTCATGACTAGGAGC-3′) complementary to the psbD 3′ untranslated region of the chimeric aadA gene and P11 (5′-AAGCTTCTCATGACTAGGAGC-3′) derived from the 3′ portion of the aadA coding region. Four independent transplastomic lines were subjected to four additional rounds of regeneration on RMOP/spectominycin medium to obtain homoplasmic tissue.

Isolation of Nucleic Acids and Hybridization Procedures—Total plant DNA was isolated by a rapid miniprep procedure ( 12 ). Total cellular RNA was extracted using the TRIzol reagent (Invitrogen). DNA samples were digested with EcoRI/DraI, separated on 1.2% agarose gels, and blotted onto Hybond-N nylon membranes (Amersham Biosciences). Total cellular RNA was electrophoresed on formaldehyde-containing 1% agarose gels and blotted onto Hybond N nylon membranes. For hybridization, [α-32P]dATP-labeled probes were generated by random priming (Multiprime DNA labeling system; Amersham Biosciences). A radiolabeled PCR product covering part of the psbE operon (obtained by amplification with primer pair P7652 (5′-CCGAGATCTCTGAAGAATCTT-3′)/P7355 (5′-GCTATAGTACGCAACTTACG-3′)) was used as probe for the restriction fragment length polymorphism analysis and for detection of psbE operon-specific transcripts. Hybridizations were carried out at 65–68 °C in rapid hybridization buffer (Amersham Biosciences). An NciI restriction fragment corresponding to nucleotide positions 41,487–42,385 in the tobacco chloroplast genome (10) was used as a specific probe for detection of transcripts from the plastid psaA/B operon. To control for equal loading, blots were stripped and reprobed with a cDNA probe amplified with primers (obtained by amplification with primers pair P5 18SSNT (5′-GTATATTATTTAAGTGTCGCACTT-3′)/P3 18SSNT (5′-AAACATTTGATTTCTCATAAGG-3′)). Transcript quantification was performed with a PhosphorImager using the Quantity One software (Bio-Rad).

Immunoblot Analysis of Proteins and SDS-PAGE—Thylakoid proteins from wild-type and mutant plants were isolated from total leaf material following published procedures (14). PSII-enriched membranes (BBY) were prepared according to a protocol originally developed for spinach (15). Following protein quantitation, equal amounts of thylakoid proteins were separated on Tricine-SDS-polyacrylamide gels (16) and stained with silver according to standard protocols (17). For Western blot analyses, electrophoretically separated proteins were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences) using the Trans-Blot Cell (Bio-Rad) and a standard transfer buffer (192 mM glycine, 25 mM Tris, pH 8.3). Immunoblot detection was performed using the enhanced chemiluminescence system (ECL; Amersham Biosciences).

Physiological Measurements—Determination of PSII activity was performed on dark-adapted leaves from wild-type and mutant plants grown under low light (2 μmol quanta m −2 s −1) and standard light (100 μmol quanta m −2 s −1) conditions, respectively. PSII-dependent chlorophyll fluorescence was recorded at 650-nm wavelength with a pulsed amplitude modulation fluorimeter (Walz, Effeltrich, Germany; Ref. 18) under illumination of intact leaf tissue with white actinic light (flux density 50 μmol quanta m −2 s −1 and 100 μmol quanta m −2 s −1; pulse frequency of measuring light, 1.6 kHz). For complete reduction of QA, leaves were exposed to a strong white light pulse (5, 50, or 200 ms; 6000 μmol quanta m −2 s −1). Oxygen evolution of young leaves from plants grown at 2 μmol quanta m −2 s −1 was measured with a Clark O2 electrode (Hansatech) at room temperature under saturating CO2 levels to minimize competing O2-consuming reactions. Oxygen evolution was monitored at flux densities of 30 μmol quanta m −2 s −1. Five independent measurements were performed to calculate average O2 evolution values. Thermoluminescence was measured on leaf segments with a homemade apparatus. Thermoluminescence was excited with single turnover flashes of 1 μs width and 1 ms interval. Samples were then heated with a heating rate of 20 °C/min to 60 °C, and the light emission was recorded. Graphical and numerical data analyses were performed according to Ducruet and Miranda (21).

RESULTS

Targeted Inactivation of the Chloroplast psbJ Gene—The psbJ gene is part of the plastid psbE operon, which comprises the four PSII genes psbE, psbF, psbL, and psbJ. This operon structure is conserved in all photosynthetically active multicellular plant species investigated to date but is not found in the unicellular green alga Chlamydomonas reinhardtii (22). The psbE operon is unique in that its tetracistrionic primary transcript does not undergo processing into monocistrionic or oligocistrionic units (23). Consequently, translation must initiate efficiently on all four cistrons of the polycistrionic mRNA. The resulting translation products are relatively small subunits of the PSII complex. The psbE and psbF genes specify the cytochrome b559 α and β subunits, which are essential for PSII assembly (24–26). The function of the other two polypeptides encoded by the psbE operon, PsbL and PsbJ, is much less clear. PsbL has recently been implicated in the stabilization of the PSII core complex and the dimeric form of PSII (27, 28). The PsbJ protein has been detected immunologically in thylakoid membranes of cyanobacteria (29, 30) and in purified PSI particles by MALDI-TOF mass spectrometry (1). Genetic analyses in cyanobacteria also support an association of PsbJ with PSII and, in addition, have established that, in Synechocystis, PsbJ is not essential for photosautotrophic growth (29).

The psbJ gene of photosynthetic eukaryotes specifies a hydrophobic polypeptide of only 40 amino acids (theoretical molecular mass: 4.1 kDa) which is evolutionarily highly conserved (Fig. 1). In order to define the function of the PsbJ polypeptide in PSII of higher plants, we have constructed a chloroplast transformation vector carrying a psbJ null allele (Fig. 2). The psbJ coding region was deleted from a cloned fragment of the tobacco plastid DNA using PCR-based mutagenesis techniques. A chimeric selectable marker gene aadA was inserted into the intergenic spacer in between the psbE operon and the petA gene (Fig. 2). Earlier work had established that this construct is a suitable site for the insertion of plastid transgenes (31, 32).

Biolistic bombardment of sterile tobacco leaves with plasmid pΔaadB-coated tungsten particles was followed by selection of spectinomycin-resistant cell lines. Successful chloroplast transformation was verified by tests for double resistance on medium containing two aminoglycoside antibiotics, spectinomycin

FIG. 1. PsbJ is a highly conserved hydrophobic polypeptide. An alignment of PsbJ amino acid sequences from the cyanobacterium Synechocystis and selected plant species (for references and accession numbers, see Refs. 29 and 53) is shown. Residues identical in all sequences listed here are denoted by asterisks; residues conserved in at least six out of the seven species are marked by dots.
and streptomycin (11), and further confirmed by PCR assays using aadA gene-specific primers (32). Chloroplast transformants were purified to homoplasm by passing them through additional regeneration cycles under antibiotic selection. Homoplasm of the transplastomic lines (i.e. absence of any residual copies of the wild-type chloroplast genome) was confirmed by restriction fragment length polymorphism analyses and Northern blots (Figs. 3 and 4) is also marked.

Since there is no absolute linkage between the introduced psbJ deletion and the selectable marker gene aadA in our transformation vector (Fig. 2), two different types of chloroplast transformants are obtained: (i) transformants carrying only the aadA but not the psbJ deletion and (ii) transformants having incorporated both the aadA gene and the psbJ null allele by homologous recombination upstream of psbJ (Fig. 2). Southern blot analyses confirmed the isolation of transplastomic lines from each of the two types (Fig. 3). As expected, lines belonging to the first type were phenotypically indistinguishable from the wild type (as shown earlier; Ref. 31), whereas lines of the second type all exhibited the same mutant phenotype (subsequently referred to as ΔpsbJ plants; see below).

Since the psbJ gene is part of an operon that is transcribed as a tetracistronic mRNA, it was important to verify that the deletion introduced into psbJ did not affect transcription of the psbE operon or stability of its mRNAs. We therefore comparatively analyzed accumulation of psbE operon transcripts in wild-type and transplastomic tobacco lines (Fig. 4). No significant difference was found in transcript pattern and mRNA accumulation levels between the transplastomic lines only having the aadA marker and those additionally carrying the psbJ deletion indicating that the deletion in the psbJ coding region does not negatively affect synthesis or stability of psbE operon transcripts. Faithful expression of the engineered psbE operon in ΔpsbJ plants was subsequently also confirmed at the protein level by assaying accumulation of the cytochrome b559 α-subunit, the psbE gene product (see below).

**Phenotype of Transplastomic Plants**—As established earlier for aadA transgenes inserted into the same genomic location (31, 32), transplastomic plants carrying the aadA selectable marker gene but not the psbJ deletion were phenotypically normal and indistinguishable from wild-type tobacco plants in all subsequent biochemical and physiological tests (see below). By contrast, homoplasmic psbJ knockout plants grown on synthetic medium under standard light conditions (100 μmol quanta m⁻² s⁻¹) displayed a clear mutant phenotype. While young leaves were almost normally green (Fig. 5), older leaves were completely white and showed strong symptoms of photo-bleaching. However, this phenotype was much less severe than that observed previously for tobacco photosynthesis null mu-
A Small Protein for Water Splitting

The ratio \( F_\text{m} - F_\text{n} \) can serve as a measure of the maximum quantum yield of PSII-driven photochemistry. In young leaves from \( \Delta \text{psbJ} \) plants, this ratio (and hence the maximum photochemical capacity of PSII) was found to be only slightly reduced as compared with wild-type leaves, suggesting that the absence of the PsbJ protein does not dramatically impair the electron transfer reactions in PSII. By contrast, mature leaves from \( \Delta \text{psbJ} \) plants were severely affected (Fig. 6), indicating that the PsbJ protein is directly or indirectly involved in the protection of PSII from light-induced damage or destabilization.

When wild-type leaves were exposed to continuous actinic light of low intensity, flashes of saturating white light superimposed on the actinic light resulted in a fluorescence rise, which approximately reached the initial value of \( F_\text{m} \), and strong photochemical fluorescence quenching occurred (Fig. 6). By contrast, in young leaves from \( \Delta \text{psbJ} \) plants, the fluorescence rise induced by the saturating flashes clearly did not reach the initial \( F_\text{m} \) value, and mature leaves from plants grown at 100 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) even lacked almost any PSII photochemistry. These results lend further support to the idea that, in the absence of the PsbJ protein, the photosynthetic apparatus is highly light-sensitive.

In order to measure the efficiency of electron transfer to downstream components of the photosynthetic electron transport chain, we next set out to determine the activity of PSI. PSI function in wild-type and mutant plants was deduced from absorption measurements at 830 nm. Absorption changes at 830 nm correlate with the redox state of the PSI reaction center chlorophyll, P700. In the dark, P700 is present in its reduced form \( (19) \). Illumination of dark-adapted leaves with far red light selectively excites PSI, thereby converting P700 in its oxidized form \( (7) \). This PSI-induced absorption shift is observed for the wild type as well as for all leaves from \( \Delta \text{psbJ} \) plants, including the mature leaves grown under normal light \( (100 \mu \text{mol quanta m}^{-2} \text{s}^{-1}) \), which had almost no measurable PSI activity, confirming that the knockout of \( \text{psbJ} \) primarily affects PSI. However, in all leaf samples from the mutant, the intensity of the absorption change was significantly lower than in the equivalent wild-type sample, suggesting that \( \Delta \text{psbJ} \) plants may have fewer active PSI units than the wild type \( (7) \).

When short pulses of white light are superimposed onto the continuous far red light, electrons are released from PSI and transferred to PSI, where they lead to a rereduction of P700, which again can be monitored as an absorption change at 830 nm \( (7) \). This PSI-dependent rereduction of P700 was observed for young mutant leaves but was significantly impaired in mature leaves from \( \Delta \text{psbJ} \) plants \( (7) \).

Altogether, these physiological data suggest that, while \( \Delta \text{psbJ} \) plants are capable of synthesizing principally functional photosystems, (i) PSI function appears to be highly light-sensitive in a leaf age-dependent manner and (ii) PSI levels or activity may be reduced in the absence of the PsbJ polypeptide.

Net Photosynthetic Capacity of \( \Delta \text{psbJ} \) Plants—Having established that the light reactions of photosynthesis still function to some extent in \( \Delta \text{psbJ} \) plants, we were interested to know whether or not the electron transfer capacity in the mutant was

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**Fig. 5. Phenotypic comparison of \( \Delta \text{psbJ} \) tobacco plants with E15 control plants.** Plants were grown on sucrose-containing synthetic medium under standard \( (100 \mu \text{mol quanta m}^{-2} \text{s}^{-1}) \) or extreme low light conditions \( (2 \mu \text{mol quanta m}^{-2} \text{s}^{-1}) \). Bleaching of mature leaves in \( \Delta \text{psbJ} \) plants under 100 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) but not under 2 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) indicates hypersensitivity to light and accumulation of photooxidative damage over time.

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Y. L. A. and E. H. F. observed that the lack of the PsbJ protein may reduce but does not completely abolish photosynthetic activity. The mutant phenotype was identical in all independently generated transplastomic lines and has remained stable during vegetative propagation, providing additional proof for the homoplasmy of the lines.

When grown under extreme low light conditions \( (2 \mu \text{mol quanta m}^{-2} \text{s}^{-1}) \), no bleaching of mature leaves occurred in \( \Delta \text{psbJ} \) plants (Fig. 5), confirming that the severe phenotype under standard light conditions is caused by the mutant’s high sensitivity to light, which appears to result in the accumulation of photooxidative damage over time.

**Photosynthetic Electron Transfer in \( \text{psbJ} \) Knockout Plants—**

The highly light-sensitive phenotype of the \( \text{psbJ} \) lines was indicative of inefficient electron transfer reactions, potentially leading to the production of free radicals that in turn cause photooxidative damage to chloroplast membranes and proteins. In order to determine the efficiency of photosynthetic electron transport, we comparatively analyzed PSI and PSI functions in wild-type and \( \Delta \text{psbJ} \) plants. In view of the light-sensitive phenotype of the \( \text{psbJ} \) knockout plants and the strong dependence of photobleaching on leaf age, we included young and mature leaves from plants grown at 2 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) as well as similar leaf material from plants raised at 100 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) \( (5-7) \). We define here “young” as the first (or at most the second, depending on the size of the youngest visible leaf) leaf from the tip of the plant and “mature” as a relatively expanded leaf (second, third, or fourth from the tip), which, in the case of the \( \Delta \text{psbJ} \) plants grown at 100 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) must not yet be photobleached.

We first measured PSI activity by chlorophyll fluorescence at room temperature. The minimum fluorescence \( F_0 \) was determined by exposure of dark-adapted leaves to measuring light of low intensity \( (6) \). Subsequently, maximum fluorescence \( F_\text{m} \) was obtained by illumination with two saturating light pulses each resulting in complete reduction of the primary quinone-type acceptor of PSI, \( Q_A \). High variable fluorescence \( (F_\text{var} = F_\text{m} - F_0) \) was detected for both young wild-type and young \( \Delta \text{psbJ} \) leaves (Fig. 6), strongly suggesting that \( \Delta \text{psbJ} \) plants synthesize functional PSII units capable of reducing the primary PSII acceptor \( Q_A \). In contrast, mature leaves from \( \Delta \text{psbJ} \) plants grown at 100 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) showed almost no variable fluorescence. \( F_\text{var} \) was also drastically reduced in mature leaves from \( \Delta \text{psbJ} \) plants grown under extreme low light conditions \( (6) \) mainly caused by a strong increase in minimum fluorescence, \( F_0 \). This may indicate a disproportion between the photon-capturing capacity of the PSI antenna and the transfer of absorbed light energy to the PSII reaction center resulting in dramatically enhanced chlorophyll fluorescence emission.
sufficient to sustain photoautotrophic growth. We therefore transferred cuttings from ΔpsbJ plants to sucrose-free synthetic medium. In this way, continued growth was made dependent on net photosynthetic carbon fixation. From our observations with the highly light-sensitive mutant phenotype (Fig. 5), we expected the light intensity to be the critical factor in these tests. We therefore assayed for photoautotrophic growth over a wide range of light intensities: 2, 10, 30, and 100 μmol quanta m⁻² s⁻¹. Under all of these light intensities, ΔpsbJ plants died after 2–4 weeks of incubation in the absence of an exogenously supplied organic carbon source (not shown), while all control plants survived and continued to grow. The phenotype at plant death was, however, slightly different. At 2 and 10 μmol quanta m⁻² s⁻¹, mutant plants showed severe wilting and turned brown, symptoms interpreted as death from carbon starvation. By contrast, under 30 and 100 μmol quanta m⁻² s⁻¹, while wilting similarly fast, turned white, showing strong evidence of photooxidation.

Low photosynthetic performance of ΔpsbJ plants was further supported by gas exchange measurements. While stomatal opening was not affected in the mutant, photosynthetic gas exchange was drastically reduced as compared with the wild type. Even in very young leaves from ΔpsbJ plants, the carbon dioxide-evolving processes exceeded the carbon dioxide-fixing processes; hence, no net carbon fixation occurs in ΔpsbJ plants: 0.2 μmol/m² s⁻¹, 0.2 μmol/m² s⁻¹, 0.2 μmol/m² s⁻¹, 0.2 μmol/m² s⁻¹. Under all of these light intensities, ΔpsbJ plants produce only about one-tenth of the oxygen evolved by an average wild-type leaf (wild type: 2 μmol/m² s⁻¹; ΔpsbJ plants: 0.2 μmol/m² s⁻¹).

Accumulation of Thylakoid Proteins in ΔpsbJ Plants—We next wanted to determine the molecular basis for the very low photosynthetic capacity in ΔpsbJ plants. To this end, we quantitatively assayed for the presence of the multiprotein complexes in the thylakoid membrane, which conduct the light reactions of photosynthesis. To get an overall impression of thylakoid protein accumulation levels in wild-type and ΔpsbJ plants, we first separated purified thylakoid proteins in high resolution SDS-polyacrylamide gels (16, 35) and stained the proteins with silver (Fig. 8). To facilitate unambiguous assignment of bands, we also loaded a PSI-deficient mutant (PSI−; Ref. 33) and a PSII null mutant (PSII−; produced by knockout of the PSI reaction center chlorophyll P700 results in a transition from its reduced to its oxidized state and hence reveals intact PSI photochemistry in both the wild type and the mutant. However, reoxidation of P700 by white light pulses and, hence, by electrons released in PSI reveals remarkable differences. The 200-ms light pulse on mature mutant leaves grown at 2 μmol quanta m⁻² s⁻¹ as well as all pulses on mature mutant leaves grown at 100 μmol quanta m⁻² s⁻¹ cannot reoxidize the PSI reaction center chlorophylls and instead result in an apparent overoxidation of the P700 pool. Note that plants grown at 100 μmol quanta m⁻² s⁻¹ generally possess more PSI than plants grown at 2 μmol quanta m⁻² s⁻¹, which is likely to reflect light-induced up-regulation of photosynthesis.
of the psbD/C operon). Unexpectedly, thylakoids from ΔpsbJ plants grown under low light conditions (2 μmol quanta m⁻² s⁻¹) contained wild-type levels of PSI proteins but had drastically reduced amounts of all PSI subunits (Fig. 8). The finding that a knockout of a small PSI subunit has a strong effect on PSI accumulation but no readily detectable effect on PSII accumulation was surprising. Subsequent analysis of thylakoid proteins from plants grown at standard light conditions (100 μmol quanta m⁻² s⁻¹) revealed that ΔpsbJ plants now also had significantly reduced PSI levels (Fig. 8), confirming that the lack of the PsbJ protein renders PSI light-sensitive and indicating that photoinduced protein degradation is indeed the cause of the highly light-sensitive phenotype of the ΔpsbJ mutant.

Having confirmed at the protein level that, in ΔpsbJ plants,
PSII performance caused by the defective water-splitting apparatus in the absence of PsbJ.

When identical immunoblot analyses were performed with thylakoids from young leaves grown under standard light conditions, two other PSII proteins were found to be present at reduced levels: PsbO, the manganese-stabilizing protein of the water-splitting complex, and PsbE, the α-subunit of cytochrome b$_{559}$ (Fig. 9). This may indicate that the cytochrome b$_{559}$ is one of the first targets of the light-induced destabilization of PSII as caused by the defective water-splitting apparatus in the ΔpsbJ mutant.

Immunoblot analyses with thylakoids from mature leaves revealed that now all PSII subunits were drastically reduced, including the two reaction center proteins D1 and D2 (PsbA and PsbD) as well as the inner antenna proteins CP43 and CP47 (Fig. 10). This observation confirms that light-induced PSII degradation occurs in ΔpsbJ plants and that photooxidative damage accumulates over time.

Taken together, the immunoblot analyses establish that the lack of PsbJ causes a defect in the water-splitting apparatus. This finding explains the light-sensitive phenotype of ΔpsbJ plants, confirms the results from our physiological measurements, and also explains why mutant plants grown under low light conditions are capable of conducting photosystem-dependent electron transport but evolve very little oxygen and cannot grow photoautotrophically.

In order to confirm that the loss of PsbP is the primary defect causing the mutant phenotype of the psbJ knockout, we measured flash-induced patterns of thermoluminescence in wild-type and ΔpsbJ plants. In thermoluminescence measurements, the emitted light originates from charge recombinations of trapped charge pairs (for a review, see Ref. 39). The charge pairs involved can be identified by their emission temperatures, which strongly depend on the redox potentials of the charge pairs. The most important thermoluminescence band for investigating the electron transfer within PS II is the B-band. Recombination of the S$_2$ or S$_1$ state of the oxygen-evolving complex at the donor side of PS II with the semiquinone Q$_B^-$ yields the B-band at $\sim$30 °C (40). Fig. 11 shows the oscillation of the B-band measured on dark-adapted leaf sections of the ΔpsbJ mutant and the wild type. In the wild type, a thermoluminescence curve is observed, which consists of two bands: the B-band and the afterglow band (21, 41). We focus here on the changes in the intensity of the B-band at 24 °C in dependence on the number of exciting single turnover flashes. As expected, the highest intensity of the B-band is observed after the first flash and the intensity of this band oscillates with a period of 4. In the mutant, the intensity of the B-band is lowered, while that of the afterglow band is comparable with the wild type. Additionally, the maximum temperature of the B-band decreased by about 5 K compared with wild type. The most striking observation is that the B-band shows no oscillation in dependence on the number of excitation flashes. It thus seems that the cycle of the S-states is perturbed after the formation of S$_1$ (Fig. 11). The S$_2$ state can clearly be formed, but subsequently, no longer positive charge equivalents can be accumulated. As for water oxidation, a complete S-cycle formation (S$_2$→S$_1$) is required, it is no longer surprising that the ΔpsbJ mutant evolves very little oxygen. These data confirm very recent thermoluminescence studies on psbJ-deficient cyanobacterial and tobacco mutants, which revealed that while the cyanobacterial mutant shows almost wild type-like signal oscillation, the tobacco mutant largely lacks detectable oscillation (30).

**Down-regulation of Photosystem I Is Not Transcriptional**—Having established that the low PSII capacity in ΔpsbJ plants results in drastically reduced PSI levels, we were interested to learn whether this PSI-mediated regulation of PSI was exerted through transcriptional or post-transcriptional control mechanisms. We therefore comparatively analyzed mRNA accumulation of the two large PSI reaction center polypeptides Psaa and Psab. These analyses revealed that the transcripts from these genes accumulate to identical levels in the wild type and in ΔpsbJ plants (data not shown). This suggests that the observed down-regulation of PSI levels in ΔpsbJ plants occurs neither transcriptionally nor at the level of mRNA stability but rather indicates that PSI dysfunction (as in the ΔpsbJ mutant) may result in a post-transcriptional down-regulation of PSI levels.

**DISCUSSION**

**Absence of the PsbJ Polypeptide Results in a Defective Water-splitting Complex of PSII**—The oxidation of water molecules in PSII is catalyzed by the luminal manganese cluster (believed to contain four manganese ions) and a redox-active tyrosine residue of the D1 protein, referred to as TyrE. In addition, at least three membrane-extrinsic proteins, PsbO, PsbP, and PsbQ, are important for maintenance of photosynthetic oxygen evolution.
in plants. These extrinsic proteins are assumed to be present in a 1:1:1 stoichiometry per PSII complex and have been implicated in stabilizing the manganese cluster and its obligatory cofactors Ca\(^{2+}\) and Cl\(^{-}\).

In the course of this work, we have shown that correct assembly of this extrinsic protein subcomplex of PSII is dependent on the presence of a small hydrophobic polypeptide, PsbJ. In the absence of PsbJ, the water-splitting complex of PSII lacks the PsbP subunit, which results in a drastically reduced photosynthetic oxygen evolution rate. Similar physiological data have been obtained previously for chloride ion-depleted PSII-enriched membrane fragments (42). Since maintaining the local concentration of chloride ions is believed to be a major function of the PsbP protein, we propose that the PSII complexes in the ΔpssbJ mutant are functionally equivalent to chloride ion-depleted PSI particles and that, in both cases, the water-splitting system is inhibited at the step of the S\(_2\) to S\(_3\) transition.

A recent study investigates photosynthetic electron flow in PsbJ-deficient mutants of *Synecocystis* PCC6803 and *Nicotiana tabacum* (30). The tobacco *pssbJ* knockout reported in this study was constructed in a different manner. The *aadA* gene was inserted directly into the *pssbJ* reading frame, whereas we avoided disruption of the *psbE* operon structure and placed the *aadA* in the downstream intergenic spacer (Fig. 2). However, similar physiological data were obtained; oxygen evolution was found to be reduced, and the oscillation of theromoluminescence signals with the number of exciting flashes turned out to be impaired. In addition, the lifetime of the reduced primary quinone acceptor Q\(_{b}\) was found to be increased more than 100-fold (and hence plastoquinone reduction was drastically slower than in the wild type (30)). The high chlorophyll fluorescence observed by the authors of this study in their fluorescence induction kinetics may be attributable to higher light intensities during plant growth (10 \(\mu\)mol quanta m\(^{-2}\)s\(^{-1}\) as compared with 2 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\) in our study) and/or to leaf age, which we have shown to be critical parameters for avoiding interference with photodamages (Figs. 5–10). Furthermore, in the present work, we provide new molecular and biochemical data that may explain many of the physiological observations made with PsbJ-deficient plants. Our finding that the knockout of *pssbJ* is associated with the loss of the extrinsic PsbP protein of the water-splitting complex provides a biochemical explanation for the observed low oxygen evolution capacity of the ΔpssbJ mutants. In addition, our molecular analyses have revealed an interesting regulatory connection between photosystem II activity and photosystem I synthesis (see below).

Remarkably, the phenotypic effects of *pssbJ* inactivation are much more dramatic in tobacco than in cyanobacteria (29, 30). Our finding that tobacco *pssbJ* deletion mutants are affected in water splitting and lack the extrinsic PsbP protein may explain this difference. The oxygen-evolving complexes of cyanobacteria and plants show remarkable structural differences, the most significant being that the cyanobacterial complex lacks the extrinsic oxygen evolution-enhancing PsbP and PsbQ proteins. Thus, the lack of the PsbP polypeptide is unlikely to have similarly dramatic consequences for cyanobacterial water splitting as it has for oxygen evolution in plant PSII.

How does the PsbJ protein mediate association of PsbP with PSII? Two possibilities can be envisaged: (i) PsbJ provides a docking site for PsbP binding, or (ii) PsbJ changes the conformation of PSII and, in this way, allows for stable binding of PsbP. In the former case, PsbJ would interact directly with PsbP; in the latter case, a direct physical interaction would not necessarily be involved. Thus far, our attempts to identify protein-protein interaction partners of PsbJ have remained unsuccessful, and further experimentation is required to define the exact position of PsbJ in relation to the PSII oxygen-evolving complex. It is noteworthy in this respect that the recently resolved X-ray structure of PSII crystals active in water splitting has revealed the presence of an unassigned helix in proximity to the extrinsic proteins and the manganese cluster of the water-splitting complex (1). However, which of the unassigned helices in PSII corresponds to PsbJ currently remains an open question.

**ΔpssbJ Tobacco Plants Are Incapable of Photoautotrophic Growth and Exhibit Hypersensitivity to Light**—In the absence of efficient water splitting, the electron transfer reactions in PSII are highly sensitive to light-induced inhibition, commonly referred to as donor-side photoinhibition. Photoinhibition is elicited by the failure to reduce PSI electron-transferring cofactors with electrons generated by oxidation of water molecules. This, in turn, results in the accumulation of highly oxidizing compounds, such as P680+ and oxidized Tyr\(^{2+}\), (for a review, see e.g., Ref. 44), which can severely damage surrounding macromolecules. Inhibition of electron transfer in PSII is ultimately followed by degradation of the PSII reaction center protein D1. Our finding that ΔpssbJ plants are impaired in photosynthetic water splitting, which makes them highly susceptible to irreversible donor-side photoinhibition, explains both the incapability of mutant plants to grow photoautotrophically and the accumulation of massive photooxidative damage leading to rapid photobleaching of mutant leaves at higher light intensities (Fig. 5). This is in line with the earlier finding that a mutant of *Chlamydomonas* (FUD 39), which lacks the extrinsic 23-kDa protein of PSII (PsbP; Ref. 43), shows chloride ion deficiency in PSII and is highly susceptible to photodamage (37, 45).

Interestingly, it has been shown recently that cytochrome *b*\(_{559}\) also undergoes light-induced degradation during photoinhibition of PSII (46). When analyzing early events in light-induced PSI degradation in *pssbJ* knockout plants (Fig. 9), we noticed that the α-subunit of cytochrome *b*\(_{559}\) (PsbE) was found to be reduced before a reduction of the reaction center proteins could be detected, possibly suggesting that, upon donor side photoinhibition, degradation of cytochrome *b*\(_{559}\) may precede D1 degradation. Being the first higher plant mutant affected in the unassigned helices in PSII corresponds to PsbJ currently remains an open question.

Reduced PSII Activity in *pssbJ* Knockout Plants Leads to Down-regulation of PSI—An interesting aspect of the *pssbJ* knockout plants was their significantly reduced PSI levels. At first sight, it may seem surprising that the lack of a small PSII protein subunit affects PSI. However, a growing body of evidence supports the idea that the expression of photosynthesis-related genes is influenced by redox signals originating from photosynthesis itself (Refs. 47 and 50; for a recent review, see e.g., Ref. 51). Our comparative analyses of protein and mRNA accumulation in the ΔpssbJ mutant and several other photosynthetic mutants (Figs. 8–10 and data not shown) provide genetic support for PSI accumulation being controlled by PSI activity. At present, we cannot yet define the level at which this regulation occurs. Translational regulation of gene expression in photosynthesis is well established (47, 50, 52) and has been suggested to employ redox signals generated in photosynthetic electron transport. Thus, it seems possible that, in ΔpssbJ plants, low PSII activity generates a redox signal that negatively regulates translation of PSI genes. Experiments are in

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progress that test this hypothesis and are hoped to provide insights into how photosystem stoichiometry is regulated.

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