PsbN Is Required for Assembly of the Photosystem II Reaction Center in *Nicotiana tabacum*\(^a\)

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The chloroplast-encoded low molecular weight protein PsbN is annotated as a photosystem II (PSII) subunit. To elucidate the localization and function of PsbN, encoded on the opposite strand to the *psbB* gene cluster, we raised antibodies and inserted a resistance cassette into *Psbn* in both directions. Both homoplastomic tobacco (*Nicotiana tabacum*) mutants Δ*psbN-F* and Δ*psbN-R* exhibit essentially the same PSII deficiencies. The mutants are extremely light sensitive and failed to recover from photoinhibition. Although synthesis of PSII proteins was not altered significantly, both mutants accumulated only ~25% of PSII proteins compared with the wild type. Assembly of PSII precomplexes occurred at normal rates, but heterodimeric PSII reaction centers (RCs) and higher order PSII assemblies were not formed efficiently in the mutants. The Δ*psbN-R* mutant was complemented by allotopic expression of the *Psbn* gene fused to the sequence of a chloroplast transit peptide in the nuclear genome. PsbN represents a bitopic trans-membrane peptide localized in stroma lamellae with its highly conserved C terminus exposed to the stroma. Significant amounts of PsbN were already present in dark-grown seedling. Our data prove that PsbN is not a constituent subunit of PSII but is required for repair from photoinhibition and efficient assembly of the PSII RC.

INTRODUCTION

Photosystem II (PSII) is a highly conserved multiprotein pigment-containing membrane complex that catalyzes light-driven water splitting, oxygen evolution, and plastoquinone reduction (Komenda et al., 2012; Pagliano et al., 2013). The complex contains at least two dozen subunits as well as antenna proteins. An intriguing feature of this photosystem is its high number of low molecular weight protein subunits, 11 of which have a molecular mass below 5 kD (Shi and Schröder, 2004; Shi et al., 2012). The role of low molecular weight subunits in electron flow, dimerization, stability, and phosphorylation patterns has been elucidated primarily in tobacco (*Nicotiana tabacum*), Chlamydomonas reinhardtii, and some cyanobacteria (Pagliano et al., 2013).

The structure of the dimeric PSII is phylogenetically highly conserved from cyanobacteria to vascular plants and has been reported at a resolution of 1.9 Å in *Thermosynechococcus vulcanus* (Boekema et al., 1995; Umena et al., 2011). It turns out that not only the composition but also the assembly of PSII is highly complex. Despite our advanced knowledge of the structure and function of PSII, along with the attached water-splitting apparatus, the molecular mechanisms underlying de novo PSII assembly are poorly understood (Suorsa and Aro, 2007; Nixon et al., 2010; Chi et al., 2012b; Komenda et al., 2012).

The functional PSII complex in vascular plants is predominantly localized in the grana regions of the thylakoid membrane, whereas insertion of newly synthesized D1, de novo PSII biogenesis, and assembly processes during the repair cycle are suggested to occur mainly in nonappressed stroma lamellar sheets (Danielsson et al., 2006; Tikkanen et al., 2008). This is supported by the localization of the PSII assembly factor HCF136 in stroma lamellae, polysome binding to exposed thylakoids, cotranslational assembly of D1, and the preferential appearance of distinct smaller PSII subcomplexes in stroma lamellae (Yamamoto et al., 1981; Meurer et al., 1998; Zhang et al., 1999). Initial assembly of thylakoid membrane complexes occurs through a number of intermediate steps, which follow a strict hierarchical and concerted order, and is to some extent also translationally regulated (Zhang et al., 2000; Choquet and Wollman, 2002). After independent formation of two precomplexes, pre-D1 containing pD1 and PsbI, and pre-D2 consisting of D2 together with PsbE and PsbF, these precomplexes are assembled to form the heterodimeric reaction center (RC) of PSII. Two additional precomplexes (pre-CP47 and pre-CP43) containing the inner antenna proteins CP47 and presumably several low molecular weight subunits, such as PsbH, PsbL, PsbM, PsbR, and PsbT, and CP43 together with PsbK and PsbZ, respectively, are assembled independently. Furthermore, several chaperones are thought to be associated with pre-CP43 and pre-CP47 (Nickelsen and Rengstl, 2013). Binding of pre-CP43 to the RC forms the RC47 (CP43-less monomer). Concomitant attachment of pre-CP43 leads to the assembly of the PSII monomer and enables attachment of the oxygen-evolving complex (Rokka et al., 2005; Boehm et al., 2012; Komenda et al., 2012; Nickelsen and Rengstl, 2013). Following dimerization of the monomer, the outer chlorophyll a/b binding antenna proteins CP29, CP26, and CP24 and finally trimeric light-harvesting complex II (LHClII) proteins are attached to form the PSII supercomplexes (Kouril et al., 2012). The nuclear-encoded low molecular weight PsbW...
protein has been shown to be important in this process in plants (Garcia-Cerdán et al., 2011). The precise sequential attachment of other low molecular weight subunits is still under debate (Rokka et al., 2005).

Over the past years, predominantly forward and reverse genetic approaches combined with biochemical and functional analyses have identified various auxiliary proteins that assist in distinct steps of the rather complicated PSII assembly process (Mulo et al., 2008; Rochaix, 2011; Chi et al., 2012b; Nickelsen and Rengstl, 2013). For instance, HCF136 was shown to be required primarily for accumulation of PSII (Meurer et al., 1998). Although translation rates of PSII proteins in hcf136 were unaffected, their stationary levels were barely detectable, causing seedling lethality. HCF136 is found in the nonappressed stroma lamellae and is essential for early assembly of the PSII RC (Meurer et al., 1998; Plücken et al., 2002). LPA19, a Psb27 homolog, is suggested to facilitate processing of the D1 precursor protein at the C terminus at the luminal side by the CtpA protease after insertion into the complex to generate mature D1 (Wei et al., 2010; Chi et al., 2012a). LPA1 interacts with D1 and is also required for efficient D1 synthesis and PSII assembly (Peng et al., 2006).

PAM68 was shown to associate with LPA1 and D1 (Armbruster et al., 2010). CYP38 was proposed to guide the folding of D1 and presumably CP43 into PSII, a prerequisite for the second codon of psbN (Kohchi et al., 1988). Transcripts of psbN were deleted in tobacco. Preliminary analysis of that mutant showed severely reduced levels of PSII and to a certain extent of photosystem I (PSI) proteins, leading to the assumption that PsbN is involved in the accumulation of both photosystems (Krech et al., 2013). However, its expression, localization, and topology within thylakoids as well as its precise function and the question of whether it represents a PSII subunit have remained elusive. This prompted us to generate antibodies for PsbN and to investigate PsbN knockouts in more detail.

Using a cotransformation approach, one nucleotide in the second codon of psbN was deleted in tobacco. Preliminary analysis of that mutant showed severely reduced levels of PSII and to a certain extent of photosystem I (PSI) proteins, leading to the assumption that PsbN is involved in the accumulation of both photosystems (Krech et al., 2013). However, its expression, localization, and topology within thylakoids as well as its precise function and the question of whether it represents a PSII subunit have remained elusive. This prompted us to generate antibodies for PsbN and to investigate PsbN knockouts in more detail. In order to exclude possible side effects caused by the orientation of the spectinomycin resistance cassette on the phenotype, we introduced the aminoglycoside-3'-adenylyltransferase (aad4) gene in both directions into the psbN coding frame. Based on comprehensive biochemical analysis, both types of homoplastomic mutants showed a comparable severe deficiency in the assembly of the heterodimeric PSII RC and also in their ability to recover from photoinhibition. This is supported by the localization of PsbN in stroma lamellae, the site where initial PSII assembly and reincorporation of newly synthesized D1 protein following photodamage is presumed to take place in chloroplasts (Fristedt et al., 2009). We therefore conclude that PsbN does not represent a constituent subunit of the functional PSII complex.

RESULTS

Localization and Topology of PsbN

PsbN is annotated as a PSII protein (http://www.ncbi.nlm.nih.gov). However, since its discovery, the molecular function of PsbN and its precise localization within chloroplasts have remained elusive. Most PsbN proteins in oxygenic photoautotrophs contain 43 amino acid residues with a less conserved hydrophobic N terminus and a hydrophilic C terminus, which is highly conserved from cyanobacteria to vascular plants (Supplemental Figure 1A). To investigate the localization and the
topology of PsbN, we raised antibodies against the short C-terminal part of this protein. PsbN was associated with thylakoid membranes and appears as an ~4-kD protein, as revealed by immunoblot analysis (Figures 1A to 1C). The question of whether PsbN contains a membrane-spanning helix was addressed by washing thylakoid membranes with different chaotropic and alkaline salt solutions. With increasing stringency of washing, the extrinsic PSII protein PsbO was released completely from membranes using 0.1 M NaOH, whereas PsbN still remained firmly associated with thylakoid membranes, indicating that the N-terminal part contains a membrane spanning helix (Figure 1A).

We isolated grana, stroma lamellae, and intermediate membranes from thylakoids to investigate the localization of PsbN within the thylakoid system (Figures 1B and 1C). Silver staining of the proteins shows the quality of fractionation (Figure 1B). To further demonstrate successful fractionation, we measured the chlorophyll $a$-chlorophyll $b$ ratio. The ratio was comparable in thylakoid (3.18 ± 0.04) and intermediate (2.99 ± 0.03) membranes. According to the higher chlorophyll $a$ amount in PSI compared with that of PSII, the ratio was 2.52 ± 0.02 and 5.54 ± 0.13 in grana and stroma lamellae, respectively. As expected, the PSI proteins PsbA and PsbO were enriched in the grana region, and the PSI protein PsaF was found mainly in stroma lamellae. To a certain extent, all proteins could be detected in intermediate membranes (Figure 1C). PsbN was highly enriched in the stroma lamellae, showing that this protein is not colocalized with PSII in appressed thylakoid membranes.

We also investigated the topology of PsbN and digested thylakoid membranes with thermolysin followed by immunodecoration (Figure 1D). The luminal PsbO protein was resistant against thermolysin treatment, whereas PsbN was digested within 60 s, demonstrating that its C terminus is exposed to the stroma. To reinforce this finding, thermolysin treatment was repeated after sonication of thylakoids, which generates inside-out vesicles (Figure 1D). This resulted in a partial digestion of PsbO and a partial resistance of PsbN, showing that the C terminus of PsbN is protected within vesicles. In summary, our data provide evidence that PsbN is not a constituent subunit of the functional PSII complex and that its conserved hydrophilic C terminus is exposed to the stroma in the nonappressed thylakoid membranes. The fact that the membrane anchor is less conserved between cyanobacteria and plants suggests that the functional part of PsbN is located in the stroma-exposed C terminus.

**Light-Induced Expression of PsbN**

We investigated light-induced expression of the PsbN protein in *Arabidopsis* compared with that of the thylakoid proteins PsaA, HCF136, D1, and PsbH (Figure 2). Subunits of both photosystems were first detectable 24 h after light induction. HCF136, a factor required for early PSI assembly (Meurer et al., 1998), was already found in the dark and accumulated continuously during light exposure. Significant amounts of PsbN were also present in the dark and increased rapidly within 4 h, reaching the maximum after 8 h of illumination. Levels of PsbN decreased after 24 h of illumination and remained constant thereafter (Figure 2). This indicates that the function of PsbN seems to already be important during early development of the thylakoid system.

**Figure 1.** Topology and Localization of PsbN in *Arabidopsis* Chloroplast Membranes.

(A) Thylakoid membranes treated with different salt-containing buffers were fractionated into pellet (P) and supernatant (S), separated by SDS-PAGE, and analyzed immunologically by probing with PsbN-specific antibodies. Antisera raised against the luminal PsbO protein, which is associated with PSI in nonappressed thylakoid membranes.

(B) Thylakoid membranes (T) were fractionated into grana (G), intermediate membranes (I), and stroma lamellae (S), and separated proteins were silver stained to judge the purity of the fractions. PSII proteins PsaA and PsaB were highly enriched in stroma lamellae, and only small amounts were present in grana membranes. PSI proteins CP47, CP43, D1, and D2 were predominantly present in grana fractions but hardly detectable in stroma lamellae.

(C) The fractions shown in (B) were tested for the presence of PsbN. Successful fractionation of grana and stroma lamellae was verified by immunodetection of PsaF, PsbO, and PsbA.

(D) Untreated and sonicated thylakoids were incubated with (+) and without (−) thermolysin for 1 min and subjected to immunodecoration using PsbN and PsbO antisera.

**Targeted Inactivation of the PsbN Gene**

To elucidate the precise molecular function of PsbN, transplastomic *psbN* knockout plants of tobacco were generated by inserting a resistance cassette into the 5′ region of the *PsbN* gene.
Expression of genes located farther upstream and downstream of the insertion site. Presumably, expression of the aadA gene on the opposite strand to the psbB gene cluster in \( \Delta psbN-F \) partially inhibits read-through by the plastid-encoded polymerase, resulting in downregulation of gene expression farther downstream of the insertion site.

**Figure 2.** Light-Induced Increase of PsbN Levels.

Levels of PsbN, PsaA, HCF136, D1, and PsbH in 8-d-old etiolated and subsequently illuminated seedlings with 50 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) at different time points during the first 96 h of light exposure were investigated by immunoblot analysis. Eight-day-old seedlings grown in light were used for comparison. Blots were stained with Coomassie blue (CBB).

**Figure 3.** Targeted Inactivation of the Plastid PsbN Gene.

(A) The aadA cassette was inserted in both orientations into the artificial HpaI site introduced into the 5’ region of the PsbN gene within the psbB gene cluster. The \( \Delta psbN-F \) and \( \Delta psbN-R \) constructs were developed with the aadA cassette cloned in the forward (F) or reverse (R) orientation to the psbN coding frame, respectively. Arrows indicate the position of primers.

(B) The homoplasmic state of \( \Delta psbN-F \) and \( \Delta psbN-R \) was confirmed by PCR analysis using primers psbTc-for, psbH-rev, and aadA-for shown in (A). WT, the wild type.

(C) Expression of genes within the psbB gene cluster was investigated by RNA gel blot analysis. Staining of rRNA using methylene blue is shown below. Eight and one microgram of total leaf RNA (1 and 1/8, respectively) was loaded from 6-week-old plants grown on medium.

**Transcript Profile of the psbB Operon in \( \Delta psbN-F \) and \( \Delta psbN-R \)**

Expression of genes located farther upstream and downstream of psbN within the psbB operon was estimated by RNA gel blot analysis using strand-specific probes (Figure 3C). psbB and psbTc are part of a prominent 2.0-kb dicistronic transcript that is not processed further to monocistronic transcripts in tobacco (Umate et al., 2008; Krech et al., 2013). Expression of this mRNA remained unchanged in \( \Delta psbN-F \) and \( \Delta psbN-R \) (Figure 3C). As expected, unlike in the wild type, the monocistronic psbN transcript was not detectable in both mutants. Instead, a strong signal of the aadA transcript of ~1.5 kb containing truncated psbN sequences was detected in \( \Delta psbN-F \) (Figure 3C). Levels of transcripts of the downstream located psbH and petB genes were reduced in \( \Delta psbN-F \) and severalfold increased in \( \Delta psbN-R \) (Figure 3C). This upregulation may have been caused by the introduction of an additional promoter into the psbB gene cluster in \( \Delta psbN-R \).
ΔpsbN-F and ΔpsbN-R Grow Efficiently in Semisterile Cultures and under State 1 Favored Light Condition

All transgenic lines reached maturity under low light but were extremely light sensitive and rapidly bleached when light intensities were increased above 40 µmol photons m⁻² s⁻¹. As reported recently, we also observed that the growth of young ΔpsbN seedlings was retarded and seedlings were more light sensitive compared with mature plants (Krech et al., 2013). Mutant seedlings were much less light sensitive under preferential PSI light condition (state I) at 20 µmol photons m⁻² s⁻¹ on soil when compared with heterochromatic light at the same light intensity. This primarily indicates that PSII is prone to photoinhibition even at low-light intensities in ΔpsbN-F and ΔpsbN-R. These findings show that both mutant plants possess the highest PSII yield at 10 µmol photons m⁻² s⁻¹ light intensity, which decreased rapidly with increasing light intensity (Table 1). To minimize the secondary effects caused from the highly detrimental impact of light due to the absence of PsbN, the plants were grown at 10 µmol photons m⁻² s⁻¹ for further analysis. Under this condition, the chlorophyll content was only slightly decreased in the mutants, which was therefore almost indistinguishable from the wild type (Table 1). However, the chlorophyll a/b ratio was significantly reduced in the mutants (Table 1).

In order to develop flowers rapidly and to backcross mutant plants with the wild type, a technique allowing semisterile growth of mutant plants was developed. For this, the stems of axenically grown plants were cut at the lower part and lower leaves were removed. The stem was then pressed through a sterile sponge fixed in the lid of the culture glass until the cut surface of the stem came in contact with the medium. This allowed the supply of Suc and development of roots within and growth of plants outside of the glass jar. It also avoided contaminations of the medium with bacteria or fungi and allowed rapid development of the stem, leaves, and fertile flowers outside of the glass (Figure 4), thus facilitating backcrossing experiments with wild-type pollen. Plants grown in this manner flowered only 1 month after transfer to the glass jar compared with 9 months when grown on soil (Krech et al., 2013). The collected seeds germinated efficiently both on medium and soil. Maternal ΔpsbN-F and ΔpsbN-R plants were backcrossed three times with the wild type, and all the resultant progeny showed the same phenotype as the parental lines, confirming that no background mutation contributed to the observed defects.

PSII Activity Is Reduced in ΔpsbN-F and ΔpsbN-R

The fact that ΔpsbN-F and ΔpsbN-R grew optimally under state I favored condition suggests that PSII activation causes increased light sensitivity in the mutants. Chlorophyll fluorescence analyses showed that F0 levels were 2-fold increased and that the maximum quantum yield of PSI Fv/Fm was reduced by about half in ΔpsbN-F and ΔpsbN-R compared with the wild type when plants were adapted to 10 µmol photons m⁻² s⁻¹ (Table 1). Fv/Fm of the wild type remained stable with increasing light intensity during growth but reached almost zero at 80 µmol photons m⁻² s⁻¹ growth light in the mutants, which re-emphasizes the notion that PSII is prone to photo-inhibition in the mutants. The chlorophyll fluorescence tended to drop below the F0 level upon induction (Supplemental Figure 2). The strength of this decrease in fluorescence appears to be a function of light intensity and is indicative of defective photosynthesis (Meurer et al., 1996; Peng et al., 2006; Armbruster et al., 2010). Non-photochemical quenching appeared not to be altered in the mutants (Table 1). The PSII yield (ΦPSII) was also severely reduced in both mutants compared with the wild type, showing that electron flow toward PSI is diminished most likely due to reduced PSII activity (Table 1). To estimate the rate-limiting step in photosynthetic electron transport, PSI yield (ΦPSI) and donor side limitation of PSI (ΦPSI ND) were measured (Table 1). This indicates that ΦPSI was only slightly reduced but ΦPSI ND was severalfold increased in the mutants compared with the wild type, again demonstrating that electron flow toward PSI is rate limiting, as also indicated by the reduced PSII activity.

Levels of PSII Proteins Were Reduced in ΔpsbN-F and ΔpsbN-R

To determine whether the impaired PSII activity found in the psbN mutants also reflects a reduction in the abundance of PSII subunits and to estimate the levels of representative members of other thylakoid membrane complexes, immunoblot analyses were performed with thylakoid membrane fractions (Figures 5A and 5B). PsbN could not be detected in ΔpsbN-F and ΔpsbN-R. Levels of PSII proteins D1, D2, CP43, CP47, PsbH, and PsbO were reduced to ~25% or below in both mutants (Figure 5A). Notably, PsbH amounts were reduced irrespective of the difference in psbH mRNA levels in both mutants (Figure 5A). This shows that down-regulation of PsbH is independent of the orientation of the adaA cassette and that secondary effects on gene expression caused by the adaA insertion can be excluded. Therefore, it is likely that the

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**Table 1. Photosynthetic Parameters of the Wild Type, ΔpsbN Mutants, and Complemented Lines**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type (n = 4)</th>
<th>ΔpsbN-F (n = 4)</th>
<th>ΔpsbN-R (n = 4)</th>
<th>ΔpsbN-Rcom (n = 4)</th>
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<tbody>
<tr>
<td>Chlorophyll a+b (µg/mg)</td>
<td>1.32 ± 0.13</td>
<td>1.03 ± 0.19</td>
<td>1.27 ± 0.22</td>
<td>1.22 ± 0.23</td>
</tr>
<tr>
<td>Chlorophyll a/b</td>
<td>3.10 ± 0.06</td>
<td>2.41 ± 0.11</td>
<td>2.43 ± 0.12</td>
<td>3.10 ± 0.11</td>
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<tr>
<td>Fv/Fm (10 µE)</td>
<td>0.81 ± 0.01</td>
<td>0.41 ± 0.02</td>
<td>0.46 ± 0.03</td>
<td>0.77 ± 0.02</td>
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<tr>
<td>Fv/Fm (40 µE)</td>
<td>0.79 ± 0.02</td>
<td>0.19 ± 0.03</td>
<td>0.12 ± 0.04</td>
<td>0.73 ± 0.03</td>
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<tr>
<td>bFv/bFm</td>
<td>0.76 ± 0.01</td>
<td>0.32 ± 0.03</td>
<td>0.35 ± 0.02</td>
<td>0.72 ± 0.02</td>
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<tr>
<td>NPQf</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>NPQg</td>
<td>0.78 ± 0.02</td>
<td>0.73 ± 0.06</td>
<td>0.70 ± 0.03</td>
<td>0.73 ± 0.04</td>
</tr>
<tr>
<td>NPQND</td>
<td>0.02 ± 0.01</td>
<td>0.10 ± 0.04</td>
<td>0.17 ± 0.04</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

*a in, number of plants measured.
*bFv/Fm, maximum quantum yield of PSII.
*cFv/Fm, effective quantum yield of PSII.
*dFv/Fm, quantum yield of nonphotochemical energy dissipation due to donor side limitation.

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observed deficiencies in proteins levels can be attributed solely to the loss of PsbN. The content of PSI subunits PsaA, PsaC, and PsaF was always around or above 50% in the mutants compared with the wild type (Figures 5A and 5B). Levels of outer antenna proteins of both photosystems, LHCa1 and LHCb1, as well as of the AtpC subunit of the ATP synthase were not reduced in both mutants (Figure 5A). According to the reduced petB mRNA levels (Figure 3C), the amounts of Cytb6 were also reduced in ΔpsbN-F but unchanged in ΔpsbN-R compared with the wild type (Figure 5A). Taken together, our results imply that disruption of psbN specifically affects accumulation of chlorophyll a–containing PSII core proteins irrespective and independent of the orientation of the aadA cassette within the psbB gene cluster. This is also consistent with the decreased chlorophyll a/b ratio in the mutants (Table 1).

**Effect of the Orientation of the aadA Cassette on Translation in ΔpsbN-F and ΔpsbN-R**

To test whether the changes in RNA expression of the psbB gene cluster in ΔpsbN-F and ΔpsbN-R are also reflected at the level of translation, in vivo labeling experiments were performed using [35S]Met (Figure 5C). Translation of the PSI proteins PsaA and PsaB as well as of the α- and β-subunits of the ATP synthase remained unaffected in the mutants. Labeling of the PSII proteins CP43, CP47, D1, and D2 was also comparable to that of the wild type (Figure 5C). According to the reduced levels of transcripts of genes located downstream of psbN, translation of PsbH and subunit IV of the cytochrome b_{6}f complex (PetD) was decreased in ΔpsbN-F. By contrast, in ΔpsbN-R, translation of PsbH and PetD was increased, although PsbH levels were

![Figure 4](Image101x365 to 245x693)

*Figure 4. Growth of Mutants in Semisterile Culture.*

Mutant plants were grown in semisterile conditions allowing rapid development of flowers for crossing experiments and biochemical analysis. Tobacco mutant plants rapidly grew to maturity in the glass jar. Bar = 5 cm.

![Figure 5](Image311x244 to 549x567)

*Figure 5. Accumulation and Translation of Thylakoid Membrane Proteins in ΔpsbN Mutants.*

(A) Immunoblot analyses of wild-type and ΔpsbN mutant thylakoid membrane proteins were performed using antisera for PsbN and representative PSII, PSI, cytochrome b_{6}f, and ATP synthase complexes. Proteins corresponding to 4, 2, and 1 µg chlorophyll/µL (1, 1/2, and 1/4, respectively) were loaded. CBB, Coomassie blue.

(B) Complemented mutant lines were subjected to immunoblot analysis. The star represents unspecific and/or degradation products of the PsbN-GFP fusion. The lower arrow shows the mature form of PsbN and the upper arrow the PsbN-GFP fusion protein.

(C) Young leaves were radiolabeled for 30 min using [35S]Met and subsequently thylakoid membrane proteins with equivalent amounts of radioactivity (100,000 cpm [the wild type and ΔpsbN mutants], 50,000 cpm [WT 1/2], and 25,000 cpm [WT 1/4]) were separated on polyacrylamide-SDS gels, blotted onto a nitrocellulose membrane, and analyzed by fluorography. Appearing bands are indicated (see text).
reduced to the same extent as in ΔpsbN-F (Figures 5A and 5C). This indicates that downregulation of PsbH is caused primarily by the lack of PsbN in both mutants. The elevated translation of these proteins can easily be explained by the use of the strong 16S promoter of the aadA cassette, which drives expression of all downstream located genes of the psbB gene cluster in ΔpsbN-R. Labeling of all other detectable bands of thylakoid membrane proteins was unaffected. In summary, the considerable reduction in PSII protein levels cannot be explained by changes in translation induced by the promoter of the aadA cassette, indicating that PsbN is required for stabilization of PSII proteins.

**Accumulation of Higher Order PSII Complexes Is Affected in psbN Mutants**

The reduced stability of PSII proteins observed could be attributed to a defect in efficient assembly of the complex. Thus, Blue-Native (BN) PAGE analysis was performed to investigate the effect of the psbN mutations on the assembly of thylakoid membrane complexes (Schwenkert et al., 2006). In the first dimension, PSII supercomplexes were clearly detectable in the wild type but not (or only in minute amounts) in ΔpsbN-F and ΔpsbN-R, indicating that assembly of higher order PSII complexes is severely abolished in psbN mutants (Figure 6A). Based on the second dimension, levels of RC47, PSII monomers, and dimers were reduced and only trace amounts, if any, of the PSII supercomplexes accumulate in both mutants (Figure 6A). Although the amount of PSI was slightly reduced in ΔpsbN-F, no precomplexes could be detected. A small portion of the PSI antenna proteins LHCa2 and LHCa3 was not associated with PSI but accumulated as subcomplexes in both mutants, as revealed by immunological analysis and identification of appearing spots by mass spectrometry (Figure 6A). To provide evidence that this is caused by a secondary effect, we analyzed a previously described PSII mutant in tobacco, which lacks the PSII low molecular weight subunit Psbl. The Δpsbl line is much less affected in PSI function than ΔpsbN mutants since they exhibit an Fv/Fm ratio of ~0.74 and their PSII proteins are reduced by only 50% (Schwenkert et al., 2006). BN-PAGE analysis of Δpsbl plants showed a similar accumulation of LHCa2 and LHCa3 in smaller subcomplexes, affirming that this effect is independent of the absence of PsbN and appears when PSII activity is diminished in tobacco (Supplemental Figure 3). We suggest that these subcomplexes appear because LHCa2 and LHCa3 protein levels are not reduced compared with a slight reduction in PSI core protein levels. Thus, PsbN does not seem to be important for the assembly of PSI.

To further investigate the protein composition in the appearing complexes and to achieve a higher sensitivity for the detection of low abundant precomplexes, immunoblot analyses with antibodies directed against PsbN as well as PSII core (CP47, CP43, D2, PsbH, and Psbl) and PSI (PsaD) subunits were performed on replicate 2D BN/SDS-PAGE gels (Figures 6A and 6B). The data showed reduced levels of Psbl and PsbH in the mutants and that these proteins are predominant in PSII monomers. This finding also confirmed the lack of PSII dimers and supercomplexes in ΔpsbN-F and ΔpsbN-R. A moderate dissociation of the low molecular weight protein Psbl could be seen in both mutants but not in the wild type. The dissociated Psbl protein was found in a complex streaking in the low molecular weight range. Notably, part of PsbH was found as a distinct spot in the free fraction of membrane proteins in the wild type. No free PsbH could be found in either mutant. Instead, a smeared band was shifted toward higher molecular weight, indicating an impaired assembly of this subunit, which is stably associated with smaller
precomplexes. PsbN was absent in the mutants and could only be found in the free fraction of thylakoid proteins in the wild type, confirming previous databases on mass spectrometric analysis (Plöscher et al., 2009). This strongly indicates that PsbN is not or only loosely and/or transiently associated with other proteins or complexes. The immunological data using antisera for PSII proteins also confirmed the lack of PSII supercomplexes and dimers in ΔpsbN-F and ΔpsbN-R (Figure 6B). It appeared that the ratios of pre-CP43/PSII monomer and RC47/PSII monomer were more pronounced in the mutants than in the wild type. Importantly, pre-CP47 could be detected in both mutants but not in the wild type. The finding that pre-CP47 and pre-CP43 are detectable demonstrates that these precomplexes are stably assembled in the mutants. Based on the above data that several PSII precomplexes accumulate while higher order PSII complexes are lacking in ΔpsbN-F and ΔpsbN-R, we conclude that PsbN is essential primarily for efficient assembly and/or stability of PSII complexes.

**Time-Resolved Assembly Studies in ΔpsbN Mutants**

To investigate whether PSII complexes are unstable or unable to assemble properly in the ΔpsbN mutants, de novo-synthesized native complexes were studied by in vivo radiolabeling and subsequent BN-PAGE analysis (Figure 7A). Unlike detection of stationary levels of thylakoid megacomplexes, this method allows detection of smaller precomplexes in statu nascendi such as comigrating pre-D1 and pre-D2, as well as pre-CP43, pre-CP47, and PSII RC (D1/D2 heterodimers). This strongly indicates that precomplexes detected with radiolabeling represent assembly intermediates and not solubilized subcomplexes. In order to reduce the complexity of the appearing pattern of gel bands, we inhibited cytoplasmic translation with cycloheximide. Compared with the wild type, considerably less radioactive label was detectable in lower and higher order PSII complexes, such as PSII RC, RC47, PSII monomer and dimer, as well as PSII supercomplexes in the mutants (Figure 7A). Instead, significantly more labeling occurred in the smallest precomplexes pre-D1, pre-D2, pre-CP43, and pre-CP47 in the mutants. With the exception of pre-CP47 and pre-CP43, which both accumulated in the mutants (Figure 6B), pre-D2 and pre-D1 were not detectable at all when stationary levels of proteins were analyzed via immunoblotting (Figure 6B), demonstrating that they represent transient assembly intermediates, which do not efficiently assemble into PSII RC in the mutants. Therefore, we conclude that these precomplexes, which neither stably accumulate nor form higher order assemblies, are rather unstable in the mutants.

![Figure 7. Time-Resolved Assembly Studies of Thylakoid Membrane Proteins in Wild-Type and ΔpsbN Mutant Plants.](image-url)
In order to strengthen this finding, radiolabeled proteins were separated in the second dimension by SDS-PAGE without previous translation inhibition. To figure out at what point during assembly PsbN is required and to get a better resolution of the PSI assembly process, two different labeling times were chosen (Figures 7B and 7C). Substantial amounts of higher order PSI-LHCII supercomplexes as well as dimers, monomers, RC47, PSII RC, pre-CP43, and pre-CP47 were already labeled in the wild type within 15 min. Incorporation of label into larger PSII complexes was barely detectable if at all in the mutants (Figure 7B). Notably, incorporation of [35S]Met into pre-CP43 and pre-CP47 was as efficient as in the wild type, but only trace amounts of RC, RC47, PSII monomer, and PSI-LHCII supercomplexes were labeled in the mutants. Instead, pre-D1 and pre-D2 precursor complexes predominantly accumulate in the mutants, indicating that formation of the RC, the next assembly step, is mainly abolished (Figure 7B). After a longer incorporation period, pronounced labeling of all lower and higher order PSII complexes was detectable in the wild type (Figure 7C). By contrast, more labeling again occurred into the smaller precomplexes pre-D1 and pre-D2 in the mutants. Remarkably, pre-CP43 and pre-CP47 were much more strongly labeled after the longer incubation time, indicating that these complexes efficiently assemble but their association to higher order PSI complexes is abolished in the ΔpsbN mutants. Importantly, the RC is the first prominent PSI assembly product that showed less labeling, although pre-D1 and pre-D2 are formed efficiently, demonstrating that its formation is primarily impaired in the mutants (Figures 7A and 7C). Consequently, less RC47 and PSII monomers and no higher order PSII complexes were labeled in the mutants (Figure 7C). We therefore conclude that PsbN is particularly essential for efficient assembly during early PSI biogenesis rather than for stabilization of PSI precomplexes. Since pre-D1 and pre-D2 are rapidly produced in the mutants but do not form the PSII RC efficiently, we hypothesize that PsbN is primarily involved in the formation of this early heterodimeric PSI assembly intermediate.

Without the inhibition of translation, short pulse labeling does not provide meaningful information about PSI assembly due to poor labeling and noisy background signals in this molecular range. Therefore, we incubated leaves with cycloheximide and separated proteins labeled for 1 h in the second dimension. According to stationary levels, based on equal loading of chlorophyll, more labeling occurred into RbcL and CF1 (Figure 7D). The size and the shape of the chloroplast were comparable in the wild type and mutants. However, the thylakoid system mainly consisted of densely packed and severalfold enlarged grana stacks and less extended stroma lamellae in the mutants.

Low-Temperature Chlorophyll Fluorescence Analysis

The 77K fluorescence emission analysis is suitable for studying the effect of the mutation on the assembly of PSI and PSII as well as for inferring association of LHCII antenna proteins with the photosystems. A minor shift of the PSI signal at 735 nm by a maximum of 1 nm to shorter wavelengths was detected in the mutants (Figure 8A). This can be explained by accumulation of LHCa2 and LHCa3 subcomplexes, which are not associated with PSI due to their overrepresentation relative to the PSI core complex (Figure 6A). Importantly, the intensity of PSII signals in the range between wavelengths 685 and 695 nm was severely reduced in the mutants. Based on immunological and biochemical data, mutant plants possess more of the unassembled LHCII relative to PSII RC proteins compared with the wild type (Figures 5A, 6A, and 7C). Consequently, the emission shoulder of the LHCII at 680 nm is more pronounced in the mutants compared with the wild type (Figure 8B). In conclusion, 77K data again reinforce the notion that the primary deficiency in the ΔpsbN mutants is the failure to properly assemble PSI complexes.

The Thylakoid Ultrastructure Is Altered in the psbN Mutants

The effect of impaired assembly and reduced amount of PSII on the thylakoid ultrastructure was investigated in the mutants (Supplemental Figure 4). The size and the shape of the chloroplast were comparable in the wild type and mutants. However, the thylakoid system mainly consisted of densely packed and severalfold enlarged grana stacks and less extended stroma lamellae in the mutants.

Photosensitivity of psbN Mutants

An impaired PSII assembly presumably increased the probability of damage to D1 protein through chlorophyll triplet formation, free radical production, and photooxidation in ΔpsbN plants. Therefore, we studied the aspects of photosensitivity in the mutants. Since plants were acclimated to 10 µmol photons m⁻² s⁻¹, we chose a moderate light intensity of 50 µmol photons m⁻² s⁻¹ as photoinhibitory light and measured Fv/Fm over the time of exposure (Figure 9A). Wild-type leaves did not show a significant change within 12 h of treatment, but the Fv/Fm ratio decreased significantly to ~50% of its original value in the mutants. This decrease could be due to an elevated photosensitivity and/or a failure of the mutant plants to recover from photodestruction. To distinguish between these possibilities, leaves were treated with lincomycin to inhibit D1 turnover, which is necessary for the recovery process to occur (Figure 9A). Treatment of wild-type plants resulted in an increased photodestruction, which reflects loss of repair. By contrast, treated mutant plants showed an identical decrease in the Fv/Fm value compared with untreated leaves, indicating that the recovery process is severely affected in the mutants. Fv/Fm decreased only ~25% in treated wild-type leaves. To study the recovery process more precisely, we photoinhibited wild-type and mutant leaves until Fv/Fm reached ~50% of the initial level and compared the recovery process under growth light conditions. For a better comparison, Fv/Fm in the wild type was further reduced to
25% using high light to reach the same Fv/Fm as that of inhibited mutants (Figure 9B). Higher light intensities (500 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)) and prolonged exposure times were necessary to reduce Fv/Fm to 50 and 25% in the wild type compared with the mutants. The recovery rate was comparable in both treated wild-type samples and reached \( \approx 85 \) and 65%, respectively, within 14 h (Figure 9B). The Fv/Fm value did not increase at all within 14 h in both mutants, which is indicative of a very high light sensitivity even under moderate light intensity and of a failure to recover from photoinhibition during this time.

To assess whether PSII is more sensitive than PSI to light stress in \( \Delta \text{psbN} \)-F and \( \Delta \text{psbN} \)-R, immunoblot analysis was performed with the core subunits PsaA, CP43, and D1, 3 and 6 h after treatment with photoinhibitory light (Figure 9C). Wild-type plants only showed a slight decrease in all three subunits. Also, the amount of PsaA did not change in both mutants, while those of the PSII core proteins D1 and CP43 showed a substantial decrease after 3 and 6 h treatment, indicating that PSII is the primary target for photoinhibition.

**Phosphorylation of PSII Antennae Is Altered in psbN Mutants**

Phosphorylation of PSII antenna proteins is triggered by the redox state of the plastoquinol pool and depends critically on the light regime. The RC proteins D1, D2, and CP43 are mainly phosphorylated by STN8 kinase, whereas the outer antenna proteins LHCII and CP29 are substrates for the STN7 kinase (Rochaix et al., 2012). We chose three conditions, dark, red light, and far-red light, to follow the PSII phosphorylation pattern in the wild type and mutants (Figure 10A). Phosphorylation of outer antenna proteins increased under red light but decreased under far-red light compared with the dark sample in the wild type (Figure 10A). Taking into account a slight increase in outer antenna protein levels in the mutants by \( \approx 25\% \) based on equal loading of chlorophyll, phosphorylation of outer antenna proteins was comparable to the wild type in the dark but in contrast with the wild type decreased considerably under red light (Figures 10A and 10B). Phosphorylation of D1 and D2 proteins showed an increase in red light and that of CP43 did not change significantly under all three conditions in the wild type. Since levels of RC proteins are reduced to \( \approx 25\% \) in the mutants, we loaded 25% of wild-type samples to better compare the relative phosphorylation status in red light (Figure 10B). It appeared that phosphorylation of RC proteins was comparable in the wild type and mutants. This result can be explained by an increased activity of PSI compared with PSII, leading to oxidation of the plastoquinone pool even under light conditions that preferentially activate PSII in the mutants. This again supports the finding of a severely decreased ratio of PSI/PSII activity in the mutants. Phosphorylation of PSII antenna proteins was decreased under far-red light, indicating that redox regulation of phosphorylation is functional in both the wild type and mutants (Figure 10A).
Allotopic Complementation of \( \text{psbN} \) Mutants

In order to prove whether the observed phenotype of the mutants is independent of the \( \text{aadA} \) insertion in the \( \text{psbB} \) gene cluster, the transplastomic \( \Delta \text{psbN-R} \) mutant was complemented with the wild-type copy of PsbN. This was achieved by integration of the \( \text{PsbN} \) gene into the nuclear genome of \( \Delta \text{psbN-R} \) using \( \text{Agrobacterium tumefaciens} \)-mediated transformation. For this, the PsbS transit peptide was fused to the N terminus of \( \text{PsbN} \) to enable its plastid import. In order to follow expression and localization of PsbN, green fluorescent protein (GFP) was fused to its C terminus. We successfully generated and analyzed six independently complemented lines (\( \Delta \text{psbN-R}_{\text{com}} \)). The PsbN-GFP fusion was localized in the chloroplast of recovered lines, confirming its successful targeting (Supplemental Figure 5).

Expression of the chimeric fusion was also confirmed immunologically in \( \Delta \text{psbN-R}_{\text{com}} \) plants (Figure 5B). This allotopic expression caused the recovery of the chlorophyll \( a/b \) ratio, PSII-specific fluorescence signals, and all other photosynthetic parameters measured (Table 1, Figure 8; Supplemental Figure 2). Levels of PSII and other thylakoid proteins, which were reduced in the mutants, were completely restored and the assembly of PSII dimers and supercomplexes was recovered in \( \Delta \text{psbN-R}_{\text{com}} \) lines (Figures 5B and 6A). In summary, this provides proof that lack of PsbN is exclusively responsible for the mutant phenotype.

**DISCUSSION**

**PsbN Is Present in Stroma Lamellae and Is Not a Constituent Subunit of PSII**

The assembly of PSII in plants occurs via an ordered association of a series of cofactor-containing intermediate precomplexes to

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**Figure 9.** PSII Photosensitivity of \( \text{psbN} \) Mutants.

(A) Untreated and lincomycin-treated (+) wild-type and mutant leaves were exposed to photoinhibitory light for 12 h, and Fv/Fm was recorded at 2-h intervals. Error bars indicate SD (n = 12).

(B) Wild-type (a) and mutant leaves were first inhibited to 50% and wild type (b) in addition to 25% of the original Fv/Fm value and the PSII recovery was followed for 14 h. Error bars indicate SD (n = 12).

(C) Immunoblot analysis of the wild type, \( \Delta \text{psbN-F} \), and \( \Delta \text{psbN-R} \) with PsaA, CP43, and D1 antibodies before (0) and after photoinhibition for 3 and 6 h using 100 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) light intensity. Samples of 2.5 \( \mu \text{g of chlorophyll} \) were loaded in each lane, and loading was checked by Coomassie blue (CBB) staining of the proteins.

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Expression of the chimeric fusion was also confirmed immunologically in \( \Delta \text{psbN-R}_{\text{com}} \) plants (Figure 5B). This allotopic expression caused the recovery of the chlorophyll \( a/b \) ratio, PSII-specific fluorescence signals, and all other photosynthetic parameters measured (Table 1, Figure 8; Supplemental Figure 2). Levels of PSII and other thylakoid proteins, which were reduced in the mutants, were completely restored and the assembly of PSII dimers and supercomplexes was recovered in \( \Delta \text{psbN-R}_{\text{com}} \) lines (Figures 5B and 6A). In summary, this provides proof that lack of PsbN is exclusively responsible for the mutant phenotype.
a functional assembly together with the outer antenna system. Substantial progress has been made to identify factors involved in this sequential order of PSII assembly. With the exception of the plastid-encoded PsbN, all other factors described that are specifically involved in the accumulation of PSII, such as HCF136, LPA1, LPA2, LPA3, PAM68, HCF243, Psb27, Psb28, CYP38, and RBD1, are encoded by the nuclear genome (Calderon et al., 2013; Nickelsen and Rengstl, 2013). Remarkably, most of the factors, including their functions, are conserved from cyanobacteria to flowering plants, presumably owing to the highly conserved structure, dimension, and composition of the PSII complex. In order to decipher the function of PsbN, we knocked out this gene in tobacco. We also successfully raised antibodies against its short C-terminal part to show that ΔpsbN-F and ΔpsbN-R are indeed lacking this protein (Figure 5B). In most of the described mutants specifically affected in accumulation of PSII, de novo synthesis of one or more PSII core proteins was markedly reduced, indicating that the corresponding factors also interfere with translational events and that some steps in PSII assembly occur in a cotranslational manner. By contrast, translation of PSII core subunits D1, D2, CP43, and CP47 was not or was only marginally affected, although their levels were strongly reduced in ΔpsbN-R mutants. This finding provides evidence that the plastid-encoded PsbN protein is not a PSII subunit but is specifically required for efficient assembly of PSII. Thus, PsbN represents a plastid-encoded protein that shows the characteristics of an authentic PSII assembly factor. A single transcription factor, SIG3, has been shown to be primarily important for the expression of psbN (Zghidi et al., 2007). Most likely additional factors are required for fine-tuning and adjusting PsbN expression at the posttranscriptional level for the establishment of early PSII assembly in a concerted way.

PsbN resides as a bitopic membrane protein in the stroma lamellae with the conserved C terminus exposed to the stroma. The I-Tasser algorithm predicted a longer transmembrane helix of PsbN, representing the membrane anchor, and a shorter helix, which is exposed to the stroma. Both helices are separated by a flexible spacer, which might allow dynamic movement of the short stroma-exposed C terminus (Supplemental Figure 1B).

Complementation of the ΔpsbN Mutants by Ectopic Expression of PsbN in the Nuclear Genome

PsbN is expressed from the strand opposite the psbH gene in most cyanobacteria and in the psbB gene cluster in plastids of all plant lineages (Stoppel and Meurer, 2013). PsbN is one of the few plastid genes that has never been transferred successfully to the nucleus in photosynthetic eukaryotes during endosymbiosis (Race et al., 1999) presumably because its plastidic expression is coupled to early PSII assembly. Furthermore, it seems that PsbN expression has to be separated from the general photosynthetic regulatory expression network, which involves hundreds of coregulated nuclear and plastid genes, and is reflected during light activation and tight transcriptional clustering (Richly et al., 2003; Cho et al., 2009). Nevertheless, we were able to substitute for the loss of plastidic PsbN expression by the import of a nuclear-encoded version, equipped with a transit peptide, into the chloroplast of the transplastomic mutant. This ectopic expression has completely restored the wild-type phenotype under the chosen conditions, confirming that the observed mutant phenotypes in both ΔpsbN-F and ΔpsbN-R are solely due to the loss of PsbN.

Although the phenotype of ΔpsbN-F was apparently slightly stronger than that of ΔpsbN-R with respect to a lower Fv/Fm and ɸPSII and a higher nonphotochemical quenching, altogether the photosynthetic performances and molecular characteristics were comparable between the two lines (Table 1). The milder phenotype of ΔpsbN-R might have resulted from the insertion of an additional 16S promoter of the aadA cassette on the opposite strand to the psbB gene cluster in ΔpsbN-F.

PsbN Is Specifically Required for Assembly of the PSII RC

Several lines of evidence presented here show that the thylakoid-integral PsbN protein is particularly necessary for PSII activity in tobacco. The ΔpsbN mutants displayed drastically reduced PSII activities and abnormally low levels of PSII core subunits. We show that these phenotypes resulted from a failure to efficiently assemble the dimeric D1- and D2-containing RC of PSII, although the synthesis of PSII proteins and the assembly of the intermediate precomplexes pre-D1, pre-D2, pre-CP43, and pre-CP47 occur as efficiently as in the wild type (Figures 6B and 7A). This is accompanied by a transient enrichment of all precomplexes at the expense of all higher order PSII assemblies in the mutants. However, in contrast with the wild-type regime, the pre-CP47 stably accumulates but all other precomplexes formed are subjected to degradation after assembly in the mutants. This could indicate that D1 and/or D2 proteins are not properly folded and/or that the precomplexes are not correctly configured in the ΔpsbN mutants, which renders them highly unstable. Furthermore, association of low molecular weight subunits to PSII is also changed, as revealed by streaking PsbI and PsbH signals in the low molecular weight range on the native gels (Figure 6A). Although the lack of higher order complexes in the mutants might be ascribed, as a secondary effect, to the failure to assemble PSII RCs, an additional role of PsbN during later stages of PSII assembly and/or the repair cycle after photoinhibition cannot be excluded.

PsbN Shares Functional Characteristics with HCF136

Our finding that transplastomic ΔpsbN mutants are able to survive on soil only at very low light intensities is in contrast with corresponding ΔpsbN mutants in Synechocystis sp PCC 6803, which show no obvious additional effects of deleting psbN alongside psbH, and indicates that PsbN is not essential for PSII biogenesis and function in this organism (Mayers et al., 1993). However, due to the conserved C terminus, it is likely that the function of PsbN is also conserved, although the phenotype of knockout lines might differ substantially even in closely related species. A similar situation has been found in HCF136, which is essential for PSII biogenesis and, therefore, for photoautotrophic growth in Arabidopsis. Remarkably, the homologous protein in Synechococcus PCC 7002 seems not to be required for PSII biogenesis (Meurer et al., 1998; Shen et al., 2002). On the contrary, the corresponding knockout in Synechocystis sp PCC
6803 showed an obvious but much milder PSI phenotype (Komenda et al., 2008), reflecting a variable necessity of the protein for PSI biogenesis even in cyanobacteria. A similar functional divergence was reported for PAM68 in cyanobacteria and chloroplasts of land plants (Armbruster et al., 2010). Interestingly, HCF136 and PsbN also share several other characteristics. For instance, both proteins are conserved from cyanobacteria to vascular plants, reside in the stroma lamellae, and are required for the formation of the dimeric PSII RC. Loss of PsbN has a drastic effect on plant growth and photosynthetic performance. These effects are similar to those seen in hcf136 but much stronger than in most of the PSI assembly/repair mutants, emphasizing an important role of PsbN during early PSI assembly. Furthermore, hcf136 and ΔpsbN mutants exhibit a similar phenotype with respect to increased and dense grana stacking as well as photosynthetic performance (Supplemental Figure 4) (Meurer et al., 1998). Both precomplexes, pre-D1 and pre-D2, are assembled in hcf136 and ΔpsbN mutants at wild type–comparable rates but do not form the dimeric RC efficiently (Figure 7A) (Plücken et al., 2002). The increased efficiency of PSI assembly in the mutants could either be attributed to a direct role of PsbN in PSI assembly or it reflects a secondary effect due to the altered thylakoid ultrastructure (Supplemental Figure 4). Also, in this respect, the PSI-specific hcf136 mutant resembles the ΔpsbN phenotype. In contrast with hcf136, PsaA/PsaB-containing subcomplexes were more strongly labeled at the expense of PSI-LHCI complexes in the wild type (Plücken et al., 2002). Therefore, the effect on the assembly efficiency of PSI can be regarded as a secondary effect when PSI RC assembly is affected and in consequence the thylakoid structure is changed. Alternatively, both HCF136 and PsbN could also play a less important role in PSI assembly. However, neither PSI precomplex stably accumulates nor is assembly of PSI hindered in the mutants. Furthermore, fully assembled PSI-LHCI complexes accumulate at almost wild-type comparable levels, and in contrast with PSI proteins, the PSII RC protein PsaA is stable upon light stress in the mutants (Figure 10), indicating a minor role for PsbN in PSI accumulation or a secondary effect on PSI when PsbN is lacking.

The notably joint role of PsbN and HCF136 in early PSI biogenesis is also consistent with their similar expression patterns. In contrast with most photosynthetic proteins, which show a strong light-dependent expression, PsbN and HCF136 are already present in dark-grown seedlings (Meurer et al., 1998). The PsbN protein shows maximum levels between 8 and 24 h after illumination when the translation and assembly of photosynthetic proteins occur at higher rates. Therefore, it is likely that these two proteins work in a concerted manner toward the biogenesis of PSII RC assembly. The early expression pattern also explains why germinated mutant plants survive in soil only under extremely low light or state I favored condition, whereas older plants or plants transferred to soil from in vitro cultures were able to grow even under state II conditions induced by moderate light.

HCF136 was found to be associated with the stroma lamellae within the lumen with no evidence of membrane-spanning segments (Meurer et al., 1998), whereas the supposed functional C-terminal domain of PsbN is stroma exposed. Therefore, it seems unlikely that the two proteins interact with each other. This finding suggests that assembly factors assist in the formation of the heterodimeric PSII RC on both sides of the non-appressed thylakoid membrane without direct physical contact.

Unlike PsbN, all PSI assembly factors investigated, including HCF136, form stable complexes, indicating an intimate and firm interaction with other assembly factors and/or PSI subunits (Nickelsen and Rengstl, 2013). PsbN is found in an unassembled state even when using cross-linkers or lower detergent concentrations, indicating its transient and/or very loose interaction with other proteins (Figure 6B). This is presumably due to the small size of the conserved C-terminal part of Δ17 amino acids, which is assumed to form the functional domain of the peptide. Also based on the predicted structure of PsbN, we hypothesize that the less conserved membrane-spanning helix functions as a membrane anchor, which orients the flexible C terminus to the location where assembly of the PSI RC occurs (Supplemental Figure 1B). Apparently, PsbN may act as a low molecular weight chaperone either on its own or in transient association with other factors needed for efficient assembly of the PSI RC. Alternatively, PsbN could be vital for proper folding of D1 and/or D2, which is a prerequisite for the formation of the PSII RC. Our finding marks an important advancement in our understanding of the PSI assembly process.

ΔpsbN Mutants Are Extremely Light Sensitive

The fact that treatment with lincomycin had no effect on the light sensitivity of PSI and that D1 is susceptible to degradation in moderate light in the mutants confirms an important role of PsbN in the repair cycle of D1. This is also substantiated by a failure of the mutants to recover from photoinhibition. The PSII repair cycle takes place in stroma lamellae, where PsbN was found. Most likely, early PSI assembly and PSII repair cycle share identical steps involving PsbN. PsbN is assumed to exert a direct role of PsbN in PSI assembly or it re...
m$^{-2}$ g$^{-1}$ in this work. It has been shown in several studies that these still moderate light intensities are also deleterious to PSI when PSII assembly is primarily abolished (Meurer et al., 1998; Plücken et al., 2002; Swiatek et al., 2003). Furthermore, six hours of photooinhibition induced reduction only of PSII but not of PSI protein levels in the $\Delta$psbN mutants. However, due to the localization of PsbN in the stroma lamellae, a minor role in PSI assembly at higher light intensities cannot be excluded completely. A small secondary effect on PSI may also be a consequence of the increased grana/stroma lamellae ratio in the $\Delta$psbN mutants (Supplemental Figure 4). The same might be true for the PSI mutant hcf136, which also showed a severe PSI deficiency when grown at 40 µmol photons m$^{-2}$ s$^{-1}$ (Meurer et al., 1998; Plücken et al., 2002).

The accumulation of LHCA2/LHCA3 subcomplexes not associated with PSI in $\Delta$psbN mutants most likely reflects a secondary effect as revealed by comparison with $\Delta$psbI, which is primarily affected in PSII dimer stability, but shows a much milder reduction in PSII accumulation and activity compared with $\Delta$psbN mutants (Schwenkert et al., 2006) (Supplemental Figure 3).

**Establishment of a Semisterile Culture System**

$\Delta$psbN mutants need almost a year to set seeds when grown photoautotrophically (Krech et al., 2013). We have overcome this problem by developing a straightforward semisterile culture technique that allows rapid growth, genetic crossing experiments, and sufficient quantity of viable seeds (Figure 4). Most likely this technique is also applicable to many other non-photosynthetic mutants in vascular plants, which otherwise do not survive photoautotrophically.

**METHODS**

**Generation of psbN Knockouts in Tobacco**

Using a transplastomic approach in tobacco (Nicotiana tabacum cv Petit Havanna), the PsbN gene was dissected by the insertion of a terminator-less aadA cassette, conferring resistance to spectinomycin, into its S’ region. In order to recognize possible secondary effects of the insertion on the expression of upstream or downstream located genes within the psbN gene cluster, the aadA cassette was inserted in both directions to generate $\Delta$psbN-F and $\Delta$psbN-R plants (Figure 3A). The knockout constructs of psbN were generated following introduction of an artificial restriction site, Hpal. For this, two initial PCR reactions with primer combinations Hpal-for/psbH-rev and psbB-for/Hpal-rev were performed. The partially overlapping PCR products were reamplified using primers psbB-for and psbH-rev. The resulting fragment was cloned into a pDrive cloning vector (Qiagen) to produce plasmid pΔN (Figure 3A). A similar strategy has been used to generate knockouts of psbTc, also present in the psbB gene cluster (Umate et al., 2008). A Small-HindIII fragment of 916 bp containing the chimeric terminator-less aadA cassette was excised, blunted, and ligated to the Hpal site of pΔN. Recombinant clones with the orientation of the aadA cassette in both directions were selected. Tobacco chloroplasts were transformed as described (Svab et al., 1990). Wild-type control plants contained the aadA cassette at an insertion-neutral site in the tobacco plastome (Ohad et al., 2004). The homoplasmic state of the $\Delta$psbN-F and $\Delta$psbN-R was analyzed by PCR analysis with a mixture of primers psbH-rev, psbTc-for, and aadA-for, as well as RNA gel blot analysis (Figures 3A to 3C).

**Plant Growth Conditions**

Transformed wild-type and mutant plants were grown under continuous light at 25°C for 6 to 8 weeks on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 3% Suc, 0.8% agar, and 500 mg/L spectinomycin. Due to the extreme light sensitivity, plants were adapted to light intensity of 10 µmol photons m$^{-2}$ s$^{-1}$. Unless otherwise indicated, all analyses were performed with young leaves of 3-week-old plants grown in vitro. Growth under preferential state I condition was achieved by covering the light source (HE 21W/60; Osram) with a dark-red filter (527 Medium Red; Lee Filters). Before transferring the plants into glass jars, seeds were surface sterilized, incubated at 4°C in the dark for 2 d, and grown for 8 d at 25°C under 10 µmol photons m$^{-2}$ s$^{-1}$ continuous light in a Petri dish with Murashige and Skoog medium (Schwenkert et al., 2006).

**RNA Gel Blot Analysis**

RNA isolation and gel blot analysis was performed as described (Meurer et al., 2002) using end labeling of strand-specific 80mer oligonucleotides with T4 polynucleotide kinase (New England Biolabs).

**Chlorophyll a Fluorescence Induction and Light-Induced PSI Absorbance Changes**

Chlorophyll a fluorescence induction kinetics and PSI absorbance changes at 820 nm of mutant and wild-type leaves were measured using a Dual-PAM-100 system (Walz, 2004). $\Phi_{PSII}$ and $\Phi_{PSI}$ ND was expressed as described (Kljughammer and Schreiber, 1994).

**Fluorescence Imaging**

Fluorescence from the PsbN-GFP fusion in transgenic plants was visualized in leaves using a confocal laser scanning microscope at 20°C (Leica; TCS SP5; objective lens, HCX PL APO CS; magnification, ×63; numerical aperture, 1.3; imaging medium, glycerol; software, Leica Application Suite/Advanced Fluorescence).

**Protein Analysis**

Thylakoid membrane proteins were isolated, solubilized, and subjected to SDS-PAGE (15% acrylamide) as described (Schwenkert et al., 2008). PsbN antisera were generated against the synthetic peptide QPSQGYQLDPFEEHGD of the hydrophilic C terminus (Agrisera). CP43, PsbH, and PsaA antisera were obtained from Agrisera. Other antibodies used in this study were described elsewhere (Umate et al., 2008; Stoppel et al., 2011). Loading was estimated by staining with Coomassie Brilliant Blue prior to immunohybridization. In vivo labeling of tobacco leaves was performed using [35S]Met at a final concentration of 0.7 mCi/mL as described (Amann et al., 2004). For inhibition of cytosolic translation, leaf discs were incubated in labeling buffer with 20 µg/mL cycloheximide 30 min prior to infiltration. A model to predict the structure of PsbN was built using I-Tasser (http://zhanglab.ccmb.med.umich.edu/).

**Separation of Solubilized Thylakoid Membrane Complexes by BN-PAGE and in Vivo Labeling**

BN-PAGE analysis was performed as described (Schwenkert et al., 2006). Thylakoid membranes corresponding to 30 µg of chlorophyll were solubilized with 1% n-dodecyl-β-d-maltoside and separated on a 4 to 12% acrylamide gradient. Lanes of the first dimension were excised, denatured, and run in the second dimension of SDS-PAGE with 15% acrylamide and 4 M urea (Schwenkert et al., 2006). Gels were either stained with silver nitrate or colloidal Coomassie Brilliant Blue or used for immunoblot analysis.
Identification of Proteins by Mass Spectrometry

Mass spectrometric analysis was performed by the Proteomics Service, Department Biology I, Ludwig-Maximilians-University Munich, using a quaternary HPLC pump (Flux) together with an autosampler (CTC) coupled to a nanoLC LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific).

Analysis of PSII Photoinhibition and Recovery Rates

The sensitivity of PSII to oxidative stress, measured as changes in Fv/Fm as a function of exposure time, has been determined with leaves of mutant and wild-type plants grown at 10 μmol photons m⁻² s⁻¹ heterochromatic light (Schwenkert et al., 2006). Due to adaptation to low-light intensities, 50 μmol photons m⁻² s⁻¹ heterochromatic light was sufficient to induce significant photoinhibition in mutant plants. All incubations and measurements were conducted with leaf discs covered with moist lens paper. To estimate the contribution of the translation-dependent repair process, leaf disks were incubated with the translation inhibitor lincomycin (200 μg/L) for 20 min in darkness before measurements. As a control, leaf discs were incubated in water. Recovery from photoinhibition was determined under low light (5 μmol photons m⁻² s⁻¹) for 14 h following exposure of leaf disks to photoinhibitory light. To assess the target of photoinhibition, levels of photosynthetic proteins were determined after different time points by immunoblot analysis.

Localization and Topology of PsbN

Thylakoid membranes of the wild type were treated with different salt-containing buffers (2 M NaBr, 2 M NaSCN, 0.1 M Na₂CO₃, and 0.1 M NaOH) for 1 h at 0°C and subsequently centrifuged before subjecting the pellet and the supernatant to SDS-PAGE. Thylakoids were treated with the protease thermolysin (200 μg/mL) for 60 s at 20°C before immunoblot analysis. Thylakoid membranes were fractionated into grana, margins, and stroma lamellae. For this purpose, 5 mL of thylakoid suspension (0.8 mg chlorophyll/mL) was solubilized with 5 mL of 0.4% digitonin solution and incubated at room temperature for 2 min with gentle agitation. The reaction was terminated by the addition of 90 mL of ice-cold phosphate buffer (100 mM Suc, 10 mM sodium phosphate, pH 7.4, 5 mM NaCl, and 5 mM MgCl₂). The grana membranes were collected by centrifugation at 10,000g for 15 min at 4°C. The supernatant was centrifuged for 30 min at 40,000g and 4°C to sediment the margins, which were localized at the lower part of the tube. In a third centrifugation step of 60 min at 100,000g and 4°C, the stroma lamellae were recovered.

Electron Microscopy

The studies for the ultrastructure of the chloroplast membranes of the wild type and ΔpsbN mutants was performed as described (Amann et al., 2004).

Studies of Low-Temperature Fluorescence Induction

The 77K fluorescence emission spectra were performed with thylakoid membranes isolated from young mutant and wild-type leaves. Thylakoids corresponding to 10 μg chlorophyll/mL were transferred into a glass capillary (0.7-mm internal diameter) and immediately frozen in liquid nitrogen. Fluorescence was excited at 430 nm, and the emission was recorded between 670 and 770 nm. All spectra were recorded with a Jobin Yvon Spex Fluorolog spectrophuorometer (Hamamatsu R 374). The slits used were in the range of 1 to 2 nm.

Complementation of ΔpsbN-R by Nuclear Expression of PsbN

The psbN coding sequence was amplified by PCR using primers psbN-for and psbN-rev, and the resulting product was digested with HindIII. The transit peptide of Arabidopsis thaliana PsbS (AT1G44575) was amplified by PCR using primers PsbS-for and PsbS-rev, and the resulting product was also digested with HindIII. Both fragments were first ligated to produce a single product, which was then purified with spin columns (Qiagen) and subsequently cloned into pENTRY/D-TOPO followed by insertion into the binary Gateway vector pB7WG2 (Plant Systems Biology) in frame with the GFP coding region using LR Clonase II (Invitrogen). Transformation of leaf discs was performed essentially as described (Koncz and Schell, 1986). Complemented ΔpsbN-Rcom plants were regenerated on selection medium containing 2.5 μg/mL phosphinotricine.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: PsbN (P62114) and PsbS (AT1G44575).

Supplemental Data

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

Supplemental Figure 1. Sequence Conservation and Structure of PsbN.

Supplemental Figure 2. Chlorophyll Fluorescence Induction Analysis of 6-Week-Old Wild-Type, ΔpsbN Mutants, and ΔpsbN-Rcom Plants.

Supplemental Figure 3. Silver Staining of Thylakoid Membrane Proteins Separated by BN/SDS-PAGE in the Wild Type and ΔpsbI Mutants.

Supplemental Figure 4. Electron Micrographs of the Chloroplast Membrane System in the Wild Type and ΔpsbN-R.

Supplemental Figure 5. Imaging of Chlorophyll Fluorescence and GFP in Leaves of the Wild Type and Complemented ΔpsbN-Rcom Lines Using Confocal Laser Scanning Microscopy.

Supplemental Table 1. Oligonucleotides Used as PCR Primers and Probes for 5’ End Labeling for RNA Gel Blot Hybridization.

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AUTHOR CONTRIBUTIONS

S.T., P.U., N.M., M.P., L.K., H.B., G.W., W.S., and J.M. performed the research. S.T. and J.M. analyzed the data. S.T., R.G.H., and J.M. designed the work. S.T. and J.M. wrote the article.

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