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The PsbW protein stabilizes the supramolecular organization of photosystem II in higher plants

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SUMMARY

PsbW, a 6.1-kDa low-molecular-weight protein, is exclusive to photosynthetic eukaryotes, and associates with the photosystem II (PSII) protein complex. In vivo and in vitro comparison of Arabidopsis thaliana wild-type plants with T-DNA insertion knock-out mutants completely lacking the PsbW protein, or with antisense inhibition plants exhibiting decreased levels of PsbW, demonstrated that the loss of PsbW destabilizes the supramolecular organization of PSII. No PSII-LHCII supercomplexes could be detected or isolated in the absence of the PsbW protein. These changes in macro-organization were accompanied by a minor decrease in the chlorophyll fluorescence parameter \( F_v/F_m \), a strongly decreased PSII core protein phosphorylation and a modification of the redox state of the plastoquinone (PQ) pool in dark-adapted leaves. In addition, the absence of PsbW protein led to faster redox changes in the PQ pool, i.e. transitions from state 1 to state 2, as measured by changes in stationary fluorescence \( F_s \) kinetics, compared with the wild type. Despite these dramatic effects on macromolecular structure, the transgenic plants exhibited no significant phenotype under normal growth conditions. We suggest that the PsbW protein is located close to the minor antenna of the PSII complex, and is important for the contact and stability between several PSII-LHCII supercomplexes.

Keywords: Blue native polyacrylamide gel electrophoresis, circular dichroism, chloroplast, electron microscopy, phosphorylation, thylakoid membrane.

INTRODUCTION

The light-driven water-splitting reaction of oxygen-evolving photosynthesis occurs in a multisubunit complex called photosystem II (PSII), which is embedded in the thylakoid membranes of cyanobacteria and the chloroplast of photosynthetic eukaryotes. Crystallographic analyses (Zouni et al., 2001; Kamiya and Shen, 2003; Ferreira et al., 2004; Loll et al., 2005; Muh et al., 2008; Adachi et al., 2009 and Guskov et al., 2009) and biochemical data obtained from cyanobacteria (Kashino et al., 2002) have shown that roughly half of the protein composition of PSII consists of low-molecular-weight (LMW) proteins with molecular masses of <10 kDa (Shi and Schröder, 2004). Most of these peptides are integrated into the thylakoid membrane as single \( \alpha \)-helices. Data acquired in these studies, in addition to electron cryo-microscopic analyses (Rhee et al., 1998; Nield and Barber, 2006), have allowed the identification and localization of the major subunits within PSII complexes, as well as bona fide allocation for some of the LMW proteins (Guskov et al., 2009). Notably, higher plants contain three LMW proteins (PsbR, PsbTn, and PsbW) that are not present in cyanobacteria (Shi and Schröder, 2004), and consequently both their localization and functional properties remain unclear.
Although the cyanobacterial PSII reaction center is highly homologous with its higher plant counterpart in terms of intrinsic protein composition and co-factor binding, there are differences with regards to the composition of the extrinsic subunits (Boekema et al., 1995) and their peripheral antenna systems. Cyanobacteria contain large extrinsic light-harvesting phycobilisomes that presumably obstruct the formation of grana stacking in the thylakoid membranes (Sidler, 1994), whereas higher plants have integral membrane antenna complexes composed of proteins belonging to the family of light-harvesting complexes (LHCs) (Jansson, 1994). The peripheral antenna of PSII in higher plants is comprised of the major antenna LHCII, constituted by heterotrimeric complexes of Lhcb1 (Lhcb1), Lhcb2 (Lhcb2), and Lhcb3 (Lhcb3), and the minor antenna composed of CP24 (Lhcb4), CP26 (Lhcb5), and CP29 (Lhcb4). This peripheral, integral antenna permits the formation of stacked (grana) regions of the thylakoid membrane in which PSII is predominant. Grana formation in the thylakoid membrane provides PSII with an intricate macro-organization, in which PSII homodimers associate with the peripheral antenna to form so-called PSII-LHCII supercomplexes. Homogeneous preparations of various types of PSII-LHCII supercomplex from solubilized and fractionated grana membranes have been described previously (Caffarri et al., 2009). The PSII-LHCII supercomplex from Arabidopsis thaliana contains a homodimeric PSII core (C2), with two LHCII trimers tightly bound to the core antenna protein CP43 and the minor antenna protein CP26 (trimer S), and one or two LHCII trimers, less tightly bound and in contact with the minor antenna proteins CP29 and CP24 (trimer M). This supercomplex is known as C2S2M or C2S2M2, and can form semicrystalline arrays in the grana membrane (Dekker and Boekema, 2005). The functional relevance of this macro-organization is not fully understood, but it has been suggested to provide PSII with a large functional antenna that facilitates excitation energy transfer between different PSII complexes. Consequently, this structure enhances the efficiency of antenna excitation energy trapping in PSII, especially in photosynthetic species such as land plants that are exposed to ever-changing environmental conditions (Chow et al., 2005).

One of the most interesting LMW proteins associated with PSII is the PsbW subunit, a 6.1-kDa protein initially described as a PSII intrinsic component in spinach (Irrgang et al., 1995; Lorkovic et al., 1995). The protein is exclusively associated with PSII protein complexes (Shi and Schröder, 1997; Bishop et al., 2003), and a putative role in the stabilization of the PSII homodimer was proposed (Shi et al., 2000). However, this view has recently been challenged by the observation that PsbW is incorporated during the later steps of PSII assembly, concomitantly with Lhcb proteins (Rokka et al., 2005), and that its primary location is within PSII-LHCII supercomplexes (Thidholm et al., 2002; Granvogl et al., 2008).

In this study we have investigated A. thaliana T-DNA knock-out (koPsbW) and antisense (asPsbW) plants that completely lacked or exhibited decreased levels of the PsbW protein, respectively. We show that in the complete absence of the PsbW protein, the supermolecular organization of PSII-LHCII in the grana membranes is severely compromised. Without the PsbW protein the ordered rows of semicrystalline macromdomains of PSII-LHCII supercomplexes cannot be formed. This in turn leads to a decrease in the efficiency of energy transfer between PSII units, and to a slowed regulation response of PSII upon light stress.

RESULTS

PsbW knock-out and antisense transgenic lines of A. thaliana exhibit no significant phenotype

To investigate whether A. thaliana plants depleted of the LMW PsbW protein exhibit a significant phenotype, we compared T-DNA knock-out PsbW (koPsbW) and antisense PsbW (asPsbW) with wild-type A. thaliana ecotype Columbia plants. Seeds for koPsbW plants were obtained from the Syngenta Arabidopsis Insertion Library (SAIL) collection; asPsbW plants were described in Shi et al. (2000). The absence or reduction of PsbW was confirmed in isolated thylakoid membranes by immunodetection of PsbW (Figure 1, top lane). As expected, no PsbW protein was detected in the koPsbW plants, and the asPsbW plants displayed <25% of wild-type levels (Figure 1). Despite the absence or reduction of the PsbW protein in the transgenic plants, there were no significant differences in photoautotrophic growth or flowering under normal growth conditions (Figure S1). This is in agreement with our earlier observation using asPsbW grown under reduced light (90 μmol m⁻² sec⁻¹ photon) and normal light conditions (Shi et al., 2000). Furthermore, plant fitness in field studies of the koPsbW plants did not differ significantly from the wild type in terms of biomass, growth rate, flowering time, or herbivore attack (H. Johansson-Jänkänpää, W.P. Schröder and S. Jansson, unpublished data).

The abundance of several components of protein complexes from the thylakoid membrane was determined by immunodetection: these included subunits from PSII (reaction center proteins D1 and D2, and the LMW proteins PsbX and PsbH), the light-harvesting antenna subunits Lhcb1 and Lhcb2, photosystem I (PSI) Psao subunit, and the stromal large subunit of the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Figure 1). No significant differences in abundance of these proteins between the knock-out, antisense or wild-type plants were observed. Moreover, the ratio of PSII to PSI in the thylakoid membranes measured by electron paramagnetic resonance (EPR; Danielsson et al., 2004) showed no significant variation (Table 1). This is in agreement with comparable measurements of chlorophyll content and chlorophyll a/b ratio in the wild type and the
chlorophyll. \textit{in vivo} as shown by PsbW is important for the PSII macro-organization, in the thylakoid membranes. Different chlorophyll-containing protein complexes found essential for the organization and/or stoichiometry of the wild type. Thus, the PsbW protein seems not to be reduced or depleted levels of PsbW when compared with complexes resolved by deriphat gel from samples with was visible in any of the different pigmented protein supercomplex (CP29-CP24-LHCII), LHCII trimer, LHCII PSI-LHCI, PSII-core dimer, PSII-core monomer, antenna were described from highest to lowest molecular weight as seven green bands were separated in the deriphat gel, which was described from highest to lowest molecular weight as PSI-LHCI, PSII-core dimer, PSII-core monomer, antenna supercomplex (CP29-C24-LHCII), LHCII trimer, LHCII monomer and free pigment. Interestingly, no difference was visible in any of the different pigmented protein complexes resolved by deriphat gel from samples with reduced or depleted levels of PsbW when compared with the wild type. Thus, the PsbW protein seems not to be essential for the organization and/or stoichiometry of the different chlorophyll-containing protein complexes found in the thylakoid membranes.

PsbW is important for the PSII macro-organization, as shown by \textit{in vivo} circular dichroism spectroscopy

Wild-type and transgenic plants were characterized by \textit{in vivo} circular dichroism (CD) spectroscopy, a non-invasive method to monitor structural changes of pigment–protein complexes and their higher-order associations (Keller and Bustamante, 1986; Garab and van Amerongen, 2009) (Figure 2). The CD spectra of whole leaves from wild-type plants reveal typical features: the so-called psi-type CD bands seen around 690 and 505 nm (Keller and Bustamante, 1986) indicate the presence of chiral LHCII-containing macrodomains of the pigment–protein complexes in thylakoid grana membranes (Garab et al., 1988; Finzi et al., 1989). Strikingly, despite the lack of effect of PsbW levels on the organization and abundance of the pigmented protein complexes of the thylakoid membranes, whole leaves from mutant plants exhibited a CD spectrum with suppressed levels of psi-type CD bands in comparison with wild-type leaves (Figure 2). The amplitude of the psi-type band at 690 nm, measured as the difference in absorption between 690 and 675 nm, was decreased by 38% in koPsbW (27.1 ± 2.63 mdeg) compared with wild-type leaves (43.7 ± 3.9 mdeg), whereas leaves of asPsbW plants exhibited an intermediate decrease of 22% (33.9 ± 8.6 mdeg). Similarly, the blue psi-type CD band in the Soret region at 505 nm, decreased in plants lacking, or with reduced levels, of PsbW. The proportional decrease of both psi-type bands in the PsbW mutant leaves indicates a reduction in the extent of long-range order in the macro-organization of PSII-LHCII supercomplexes, rather than a modified structure.

Absence of the PsbW protein alters PSII macro-organization of the grana membranes, as measured \textit{in vitro}

Transmission electron microscopy (TEM) was used to assess whether the observed changes in macro-organization

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<th>Table 1 Photosynthetic parameters of Arabidopsis thaliana wild-type and PsbW knock-out plants</th>
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<tbody>
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<tr>
<td>-----------------------------------------------</td>
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<tr>
<td>Chlorophyll a/b ratio</td>
</tr>
<tr>
<td>PSI/PSII ratio\textsuperscript{b}</td>
</tr>
<tr>
<td>Oxygen evolution\textsuperscript{c}</td>
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<tr>
<td>PPBQ</td>
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<tr>
<td>DMBQ</td>
</tr>
<tr>
<td>Fluorescence (PAM)</td>
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<tr>
<td>Antenna size\textsuperscript{d}</td>
</tr>
<tr>
<td>Spectral half-time (fluor. rise time)</td>
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<td>Connectivity (J)</td>
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<td>State transitions\textsuperscript{e}</td>
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\textsuperscript{a}Errors reported are plus and minus one standard deviation (n = 5).
\textsuperscript{b}PSI/PSII ratio was measured by electron paramagnetic resonance (EPR) in isolated thylakoid membranes according to the method described by Danielsson et al. (2004).
\textsuperscript{c}Oxygen evolution rates were measured at 20°C with 0.25 mM phenyl-p-benzoquinone (PPBQ) and 1 mM 2,5-dimethyl-p-benzoquinone (DMBQ) at saturating light intensities.
\textsuperscript{d}Fluorescence transient measurements in the presence of 3-(3,4-dichloro-phenyl)-1,1-dimethylurea (DCMU).
\textsuperscript{e}State transitions were calculated as described in Haldrup et al. (2001).
of PSII-LHCII, as indicated by the altered CD spectra of the koPsbW plants compared with wild-type plants, influenced the chloroplast ultrastructure, particularly the grana stacking. No obvious differences were observed in overall chloroplast membrane organization when the plants were dark-adapted (Figure 3a), or exposed to normal or high light levels (data not shown), before sample fixation for TEM analysis. We extended our TEM analyses to study the PSII macrostructure of thylakoid grana membrane fragments by means of TEM combined with image analyses (Boekema et al., 2000). Negatively stained paired grana membranes from wild-type plants exhibited well-ordered rows of semicrystalline macrodomains of PSII supercomplexes (Figure 3b, left panel), similar to results described in Kovacs et al. (2006). However, in grana membrane fragments isolated from koPsbW plants, these ordered two-dimensional semicrystalline domains were replaced by seemingly random aggregates in the mutant (Figure 3b, right panel).

The alteration of PSII macrostructure in the thylakoid grana membranes of the koPsbW mutant resulted in an inability to isolate PSII-LHCII supercomplexes by either blue-native (BN)-PAGE (Figure 4a) or sucrose-density gradient ultracentrifugation (Figure 5a). For the BN-PAGE (Figure 4a), thylakoid membrane protein complexes were mildly solubilized in the presence of the detergent n-dodecyl-β-D-maltoside, and were electrophoretically separated in polyacrylamide gels under native conditions in the presence of Coomassie blue dye (R-250), which provides the necessary charges for separation of the protein complexes (Schägger and Von Jagow, 1991). Typically, BN-PAGE of wild-type thylakoid membranes resolved protein complexes, in which the higher molecular weight complexes correspond to PSII supercomplexes, followed by the PSI and PSII core dimers, PSII core monomers, PSII monomer without the CP43 subunit, LHClI trimers and LHClI monomers (Schwenkert et al., 2006) (Figure 4a, left lane). Two-dimensional analysis (BN-PAGE followed by denaturing SDS-PAGE) of wild-type thylakoid membranes followed by immunoblotting identified the PSII reaction center protein D2 in all bands corresponding to PSII. Of note, PsbW was immunostained mainly in the PSII supercomplexes (Figure 4b), but was not detected in PSII monomers or PSII subcomplexes. PSII supercomplexes were drastically reduced in the absence of PsbW, as visualized after native BN-PAGE (Figure 4a, koPsbW mutant), and asPsbW membranes demonstrated reduced levels of PSII-LHCII supercomplexes. Consequently, bands in koPsbW thylakoid extracts corresponding to LHClI components (CP29-CP24-LHClI-trimers), PSI core monomer and PSII core monomer without CP43 were increased (Figure 4a, middle lane). Hence, PsbW appears to facilitate PSII-LHCII supercomplex assembly.

Recently, Caffarri et al. (2009) reported a new method for isolating and characterizing stable PSII-LHCII supercomplexes by combining sucrose-density gradient ultracentrifugation in the presence of mild detergent (0.3% n-dodecyl-α-D-maltoside), followed by EM characterization. Several types of PSII supercomplexes were described, in which PSII core monomer (C) binds antenna trimers strongly (S) or moderately (M). The supercomplexes described are C2S2M2, C2S2M, C2SM, C2M, C2S and CS. With the aim of characterising the supercomplexes present in the koPsbW, we analysed PSII-enriched membrane fractions of wild-type and mutant plants. A total of 13 bands were resolved from the wild type and the corresponding absorption spectra...
were measured (Figure 5a,b). Of these bands, those numbered B3–B5 were found to correspond to monomeric LHCII, trimeric LHCII and LHCII complex, respectively. B7 corresponded to dimeric PSII core, and B8–B13 corresponded to different PSII-LHCII supercomplexes containing a dimeric core complex with various numbers of bound LHCII trimers.

Strikingly, only five bands were resolved from the PSII-enriched membrane fraction of koPsbW plants (Figure 5a, right side). From these, the absorption spectra were measured: bands B2* and B3* contained antenna proteins (LHCII monomer, trimer and LHCII assembly), and the two faint bands B4* and B5* corresponded to monomeric and dimeric PSII complexes, respectively. Surprisingly, all of the higher molecular weight bands (B8–B13) that corresponded to various PSII-LHCII supercomplexes in the wild type were absent in the koPsbW mutant. To rule out the possibility that different solubilization efficiencies resulted in the loss of PSII-LHCII supercomplexes in koPsbW, a higher detergent concentration of 0.45% n-dodecyl-β-D-maltoside (β-DM) at 0.3 and 0.45%, were tested. No supercomplexes were resolved from koPsbW membranes, regardless of the detergent or concentration tested (data not shown).

Photosynthetic performance of transgenic plants is slightly decreased

Despite the lack of significant changes in protein abundance of PSII subunits, the rates of oxygen evolution in isolated thylakoid membranes from koPsbW plants were 30 and 15% lower than wild-type rates when measured using the artificial electron acceptors phenyl-p-benzoquinone and 2,5-dimethyl-p-benzoquinone, respectively (Table 1). These results suggest that although PSII accumulates in the thylakoid membrane in similar levels as found in the wild type, PSII function is slightly impaired in the absence of PsbW.

We further investigated the photosynthetic performance of these transgenic plants by analysing the photosynthetic chlorophyll fluorescence parameters using pulse amplitude modulated (PAM) fluorometry. The chlorophyll fluorescence parameter $F_{v}/F_{m}$ indicates the maximum photochemical efficiency of PSII in the dark, and is around 0.83 in most higher plant species (Björkman and Demmig, 1987). In dark-adapted wild-type plants this value of 0.83 was confirmed (Table 1), whereas a slightly decreased value of 0.78 in koPsbW and 0.80 in asPsbW plants was measured ($n = 10$). The slight reduction of $F_{v}/F_{m}$ observed in koPsbW plants was caused by a reduced level of variable fluorescence. This result indicates that the light energy absorbed by the antenna chlorophyll molecules is dissipated further via non-photochemical quenching of PSII excitation energy. Also, a more detailed analysis of other fluorescence parameters (Table S1) and EPR analysis (Table S2) of the electron transport components of PSII did not reveal any dramatic effects on PSII. These results suggest that the primary PSII function is somewhat impaired in the absence of the PsbW protein.

The PSII antennae size is not changed, meanwhile the connectivity is reduced

Fast fluorescence transient measurements in the presence of 3-(3,4-dichloro-phenyl)-1,1-dimethylurea (DCMU) gives information on the antenna size of PSII and the cooperation between PSII subunits. The functional antenna size can be estimated by time ($t_{2/3}$) of the fluorescence induction (Table 1). KoPsbW exhibited a $t_{2/3}$ of 0.114 ± 0.013 msec ($P < 0.05$) compared with the wild type 0.115 ± 0.011 msec, indicating a similar antenna size in the studied plants, independent of the presence or absence of PsbW. The rise of fluorescence upon illumination follows a typical sigmoid kinetics, implicating cooperation between PSII complexes (Joliot and Joliot, 1964; Lavergne and Trissl, 1995). At the beginning of illumination, the excitation energy arriving at a closed PSII reaction center can be transferred to an open...
one, rather than being emitted as fluorescence, resulting in a fluorescence lag. The connectivity parameter (referred to as $J$) is a mathematical expression of the sigmoidicity of the fluorescence transient, and so measures the cooperativity of PSII units (Koblizek et al., 2001). A marked change in the shape of the fluorescence curves of the transgenic plants revealed a slight but significant ($P < 0.05$) difference in connectivity between the koPsbW plants (0.782 ± 0.114) compared with the wild type (0.975 ± 0.156) (Table 1). These measurements show that the antenna size is not changed in koPsbW; however, there is a disturbance of the macro-domain organisation of PSII subunits that lowers the probability of energy transfer between PSII-LHCII supercomplexes, resulting in a less efficient cooperation of reaction centres in sharing the captured light energy.

### The redox potential of the acceptor side of PSII is altered in koPsbW plants

It seems reasonable to assume that a change in PSII macro-organisation would influence the interaction of the PSII acceptor side with the plastoquinone pool. In photosynthetic material, thermoluminescence occurs because of the thermally activated recombination of electrons trapped on the acceptor side of PSII, with positive charges stored in the water splitting complex. The thermoluminescence signal of the dark-adapted sample, referred to as the B-band, originates from $Q_A$/$S_2$,$S_3$ charge recombination (Rutherford et al., 1982), whereas the signal obtained in the presence of DCMU (preventing $Q_A$ oxidation) is referred to as the Q-band, and arises from $Q_A$/$S_2$ charge recombination. In thylakoid membranes isolated from both wild-type and koPsbW plants the B-band was detected at about 25°C, but interestingly the intensity was significantly lower in the koPsbW mutant (Figure 6a). The Q-band was shifted towards higher temperatures in the mutant (16.6°C, compared with 12.5°C in wild-type), whereas the intensities were the same in the wild type and the mutant (Figure 6b). These results could be explained in terms of a redox shift in the Q-band ($Q_A$/$S_2$ pair) leading to a decrease in the redox potential gap between $Q_A$ and $Q_B$ sites, which leads to the increased stability of the charge separation in PSII. As the B-band, which also depends on $S_2$, was not shifted, the redox potential of $Q_A$ must have changed in the absence of PsbW. The unchanged amplitude of the Q-band demonstrates that the primary photochemistry (charge separation) of the PSII reaction center is not altered in the mutant. A possible explanation for the decreased B-band without the concomitant decrease of the Q-band would be that the non-irradiative competitive reaction is enhanced in the koPsbW plants at the expense of $Q_A$/$S_2$ charge recombination, most probably caused by a faster exchange between the $Q_B$ and the plastoquinone (PQ) pool in the koPsbW mutants. This conclusion is further supported by EPR analysis of the electron transport components of PSII, where the $Q_A$–Fe$^{2+}$ interaction signal was decreased, in agreement with the thermoluminescence analysis (Table S2).

### Altered PSII macro-organization does not influence state transitions

A well-studied phenomenon of short-term adaptation of plants are state transitions in which the level of light energy absorbed by the two photosystems is balanced in response...
half-time of about 40 sec (Figure 7a). This suggests that in the absence of PsbW, the PQ pool undergoes redox changes at a higher rate.

**Loss of PsbW decreases PSII core protein phosphorylation**

Despite clear changes in \( F_S \) in koPsbW compared with the wild type upon state transitions (Figure 7a), no differences were observed in the maximal fluorescence \( F_{m1}/F_{m2} \) ratio (Table 1). To further investigate this phenomenon, the extent of inducible phosphorylation of LHCII was analysed in detached leaves of wild-type and koPsbW plants that had been kept in darkness or pre-treated under state-1 or state-2 light conditions (see Damkjaer et al., 2009) for 25 min. Immediately following the light/dark treatment, thylakoid membranes were isolated, constituent proteins were resolved by SDS-PAGE and immunostained using phosphothreonine antibodies (Figure 7b), or stained with Coomassie Brilliant Blue to demonstrate equal loading (Figure 7c). The band migrating at around 25 kDa corresponds to LHCII.

In the wild type, a very low level of phosphorylated LHCII was detected after dark or state-1 illumination, whereas state-2 illumination strongly induced LHCII phosphorylation (Figure 7b, left lanes). In the koPsbW mutant, the LHCII phosphorylation levels after state-1 or state-2 illumination were comparable with those of the wild type. Notably, however, the extent of LHCII phosphorylation of dark-adapted leaves was elevated in koPsbW compared with the wild type.

The absence of the PsbW subunit strikingly reduced the extent of PSII core phosphorylation: D1 and D2, represented by the two bands around 30 kDa, were poorly phosphorylated in koPsbW (Figure 7b) compared with the wild type. The largest difference in phosphorylation level between the wild type and koPsbW LHCII was in the band above 37 kDa, corresponding to CP43. CP43 was heavily phosphorylated under all light conditions in the wild type, whereas in the koPsbW mutant, phospho-threonine immunostaining was weak. PSII core phosphorylation is performed in Arabidopsis mainly by the kinase STN8, whereas LHCII is phosphorylated by STN7 (Bellaﬁore et al., 2005; Bonardi et al., 2005; Vainonen et al., 2005). To exclude the possibility that PsbW is only indirectly involved in the observed changes in the phosphorylation pattern by affecting the level of kinases in the cells, we quantified levels of STN8 and STN7 protein kinases in the thylakoid membrane preparations using speciﬁc antisera (Figure S2). The level of each protein kinase was comparable between the wild type and koPsbW, indicating that the reduced PSII core phosphorylation observed in the mutant is not caused by a lack of protein kinases.

**Transgenic plants lacking PsbW show modest changes in photoinhibition and recovery**

Photosystem-II core phosphorylation is important in the turnover of the D1 reaction center protein, and the recycling
of photoinhibited PSII complexes in the grana membranes is dependent on their dephosphorylation (Rintamäki et al., 1996). As PsbW protein is important for PSII core phosphorylation and PSII-LHCII supercomplex stabilization in the grana membranes, it probably protects PSII from photodamage under high light levels, and/or aids PSII recovery under low light levels. To test this, leaves from 5-week-old wild-type and koPsbW plants were exposed to a continuous high light level (900 l mol photon m⁻² sec⁻¹) for 22 h, followed by a low light level (10 l mol photon m⁻² sec⁻¹) for another 22 h. Changes in Fv/FM fluorescence ratios and protein abundance in isolated thylakoid membranes were measured. Photodamage induced by high light levels was slightly enhanced in koPsbW plants, depicted by a decline in the Fv/FM ratio from 0.78 to 0.63, in contrast with the wild type, where the ratio only decreased from 0.82 to 0.71 (Figure 8a). These changes were corroborated by immunological analyses: levels of PSII core proteins D1, D2 and PsbO from wild-type leaves were lower after treatment with high light levels (HL22), compared with dark-adapted leaves (Figure 8b). Interestingly, in the koPsbW mutant, the D1 protein and, to a lesser extent, D2 were degraded to a greater degree than in wild-type leaves (Figure 8b). According to fluorescence measurements, treatment with low light levels was sufficient for recovery in wild-type plants, where the Fv/FM ratio returned to 0.80, whereas in the koPsbW mutant, a lower final Fv/FM value of 0.74 demonstrated that recovery was compromised (Figure 8a). This was accompanied by reduced levels of PSII core protein subunits, particularly for D1, which was present at levels below wild-type D1, and was decreased following treatment with low light levels compared with dark pre-treatment (Figure 8b). Interestingly, during recovery under low light levels, koPsbW samples exhibited degradation fragments that were immunostained by antibodies directed against the reaction center subunits D1 and D2, suggesting an imbalance in post-translational modification.

Lincomycin inhibits de novo synthesis of plastid-encoded repair proteins, and thus facilitates the study of photodamage in the absence of repair. As expected, following 3 h of treatment with high light levels, Fv/FM ratios fell to 0.13 in the wild type, and close to 0 in the mutant (Figure 8a). Levels of D1, D2 and PsbO were significantly lower in the presence of lincomycin, particularly for koPsbW (Figure 8b), indicating that PSII core protein degradation was markedly increased. Interestingly, exposure to lincomycin for 3 h had little effect on the turnover of the PsbW protein (Figure 8b, LINC), and only after treatment at a high light level for 22 h was the PsbW protein significantly degraded (Figure 8b, HL22), in accordance with previous observations (Hagman et al., 1997). Thus, the PsbW protein indeed affects the extent of PSII photodamage under high light levels and/or PSII recovery under low light levels.

DISCUSSION

PSII-LHCII supercomplexes are destabilized in plants lacking the PsbW protein

Earlier studies on the PsbW protein have been performed on antisense plants with variable residual levels of the PsbW protein. Here, we report analyses of Arabidopsis knock-out plants completely lacking the PsbW protein. Our results conclusively show that the PsbW protein is critical for the stabilization of the PSII-LHCII supercomplexes located in
the granal thylakoid membrane. All our efforts to isolate any type of PSII-LHCII supracomplex from koPsbW plants failed: neither mild solubilization followed by BN-PAGE (Figure 4) nor density gradient ultracentrifugation (Figure 5a) could resolve any PSII-LHCII supercomplexes from thylakoid membranes of plants lacking PsbW. Furthermore, TEM analyses showed that instead of the normal formation of semicrystalline arrays of PSII-LHCII supercomplexes found in wild-type samples, koPsbW plants display random aggregates (Figure 7b). Using non-invasive CD spectroscopy of intact leaves we detected changes in the long-range order of PSII macro-organization in koPsbW plants (Figure 2). Thus, using various biochemical and biophysical analyses on transgenic plants, we were able to conclusively show that PsbW must be present for PSII-LHCII supercomplex stabilization.

Antisense plants with strongly reduced levels of PsbW protein were also analysed. In comparison, antisense plants displayed an intermediate phenotype between wild-type and knock-out plants. The latter, strongly supports the observation that these effects are dependent on the reduction of the PsbW protein content in the plants, and are not a secondary effect resulting from random insertions of DNA. Shi et al. (2000) reported that the level of PSII reaction centre proteins was reduced by almost 40% in PsbW antisense plants. In our hands no reduction of PSII or PSI proteins was detected, and the PSI/PSII ratio was unchanged in both antisense and koPsbW plants. Instead, our data show that plants lacking the PsbW protein are somewhat more susceptible to photoinhibition (Figure 8), with the consequence that D1 and D2 degradation is increased. We therefore assume that the antisense plants used in the earlier work could have been somewhat stressed, which would have led to the reduction of the reaction centre protein content.

**PSII function and electron transport are slightly impaired in koPsbW transgenic plants**

Despite the changes in PSII-LHCII supercomplex organization in the koPsbW plants, chloroplast infrastructure resembled that of wild-type plants (Figure 7a). This indicates that the stabilization of PSII-LHCII supercomplexes and the formation of semicrystalline macrodomains of PSII supercomplexes is not a prerequisite for grana formation of the thylakoid membrane. In addition, no changes were measured in protein composition, pigment complex organization, PSII/PSI ratio (Table 1), chlorophyll content or chlorophyll a/b ratio. Despite these observations, steady-state measurements of oxygen evolution by PSII were decreased by 15–30% (Table 1), the S2 multiline signal was reduced by 50%, in which the formation of the Q_A Fe^2+ signal was reduced by 30% (Table S2), and the midpoint redox potential of the QA site was altered in comparison with the wild type (Figure 6). The latter indicates that PSII electron transport is affected in the absence of PsbW, despite the lack of an obvious phenotype in koPsbW plants and the antisense asPsbW mutants (Figure S1). These data support the conclusion that the primary PSII function and composition is slightly compromised when the PSII-LHCII supercomplex organization is modified in absence of the PsbW protein.

**How can the absence of one LMW protein affect PSII macro-organization?**

In order to have such a major effect on PSII-LHCII supercomplex formation, the LMW PsbW protein must occupy a highly specific position within the PSII complex. According
to the proposed model based on EM imaging analysis, the PSII-LHCII supercomplex (C2S2M2) of higher plants consists of a dimeric core (C2), two tightly bound LHCII trimers (S2) and two moderately bound trimers (M2) containing an unidentified small helix, located in close proximity to the S-trimer and CP26, which was assigned as an unknown ‘X’ protein (Dekker and Boekema, 2005). In addition, based on TEM projections, the structure of the PSII-LHCII supercomplex proposed by Caffarri et al. (2009) accommodates an additional small protein subunit in between CP47 of one of the dimeric PSII core complexes (C2) and CP24. We hypothesize that PsbW could be located in one of these regions, in close proximity to the minor antenna. In this position, PsbW could fulfill its important role in supercomplex stabilization, as well as participating in contacts between different PSII-LHCII supercomplexes in the grana membranes.

An exclusive, conserved motif in the amino acid sequence of PsbW has been recognized at the C terminus, facing the stromal side of the thylakoid membrane (Lirang et al., 1995). Of the 14 C-terminal stromal-exposed amino acids, five consecutive amino acids are negatively charged (EE-), a feature that has not been observed in any other LMW five consecutive amino acids are negatively charged (EE-)

Why do higher plants form ordered rows of semicrystalline macrodomains of PSII-LHCII supercomplexes?

The removal of the PsbW protein affects the formation of stable PSII-LHCII supercomplexes conforming to the typical well-ordered rows of semicrystalline macrodomains of PSII supercomplexes in the thylakoid grana. Thus, the koPsbW mutant enables us to address the role of such ordered domains in higher plant grana membranes. The data presented here point toward several important functions of these ordered domains.

(i) Optimization of energy transfer between different PSII complexes: the absorbed light energy reaching a closed ‘occupied’ PSII is not as efficiently transferred to the next photosystem in the koPsbW mutant, but is instead re-emitted as fluorescence. Basal chlorophyll fluorescence was increased in the koPsbW mutant (Figure 7; Table 1), and energy transfer between PSII units was reduced, although differences in antenna size were not observed (Figure 2). Thus, the absorption and utilization of light energy is less efficient in disordered PSII complexes.

(ii) Regulation of state transition of energy distribution: the lack of ordered rows of PSII supercomplexes did not influence state transitions. However, the transiently accumulated reduced Qa− pool seemed to relax more promptly during state-1 to state-2 transitions, reaching the steady-state higher fluorescence level (F2) faster in the mutant. The latter indicates that PQ pool undergoes redox changes at faster rates. These changes observed in F2 did not affect the extent of inducible LHCII phosphorylation in state 2, or the extent of transitions as measured by Fm1/Fm2. The latter indicates that the mobile LHCII experienced state transitions.

(iii) Optimization of the interaction between PSII and the PQ pool. The changes observed in the steady-state level of chlorophyll fluorescence may well be related to changes in the redox properties of the PQ pool of the mutant thylakoid membranes. The reduction of the PQ pool in dark-adapted koPsbW thylakoid membranes correlated well with the extent of LHCII phosphorylation. Furthermore, thermoluminescence measurements of dark-adapted samples indicated higher activation energy for the recombination of Qa−/S2 pairs in the mutant, and a lower pool of recombining Qa−/S2,3 pairs, which seem to lose electrons faster, presumably to the PQ pool.

(iv) Facilitation of PSII protein phosphorylation: a dramatic reduction in PSII core phosphorylation was observed in koPsbW plants. Altered and/or lower core phosphorylation has been reported to result from knock-out or antisense inhibition of other small PSII subunits in the chloroplast, e.g. PsbZ (Swiatek et al., 2001), Psbl (Schwenkert et al., 2006), Psbm (Umate et al., 2007), PsbTc (Umate et al., 2008) and PsbX (Garcia-Cerdan et al., 2008). Two plausible scenarios could explain the decreased PSII core protein phosphorylation: a reduction in the steady-state level of PSII core subunits (as observed, for instance, in tobacco Psbl knock-out plants or PsbX antisense mutants), or changes in the PSII macro-organization, as in tobacco PsbZ, PsbTc or PsbM knock-out plants. The latter proteins are known to be intrinsic subunits of PSII, conserved in both cyanobacteria and the chloroplast. The PsbZ protein influences the interaction of the minor antenna with PSII dimers, whereas the location of PsbTc and PsbM has been proposed to be at the interface of the PSII dimer, according to the crystal model (Henmi et al., 2008; Gusakov et al., 2009). Thus, a lack of these specific subunits may affect the stability or formation of PSII dimers and/or the association of the minor antenna, and consequently alter PSII macro-organization. In a similar manner, the PsbW protein may participate in the stabilization of PSII-LHCII supercomplexes in the grana stacks. A lack of ordered rows of PSII complexes in the absence

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of PsbW may affect specific site recognition by the protein kinase STN8 within the PSII core, leading to a lower phosphorylation rate.

(v) Reduction of photoinhibition for fast and optimal repair: in the absence of PsbW, plants are moderately photodamaged by high light levels, and recover more slowly in low light levels than wild-type plants (Figure 8A). Such effects have been reported for several plants with mutations in LMW subunits, e.g. PsbTc (Umate et al., 2008), PsbI (Schwenkert et al., 2006), PsbM (Umate et al., 2007) and PsbX (Garcia-Cerdan et al., 2008). Thus, macro-organizational changes of PSII may facilitate protease attack and repair, and recycling may be delayed by the destabilized PSII macrostructure. Interestingly, small fragments of the subunits D1 and D2 accumulated during the recovery phase in the mutant, indicating abnormal post-translational processing of the main PSII core subunits undergoing repair.

The present work shows that PsbW knock-out and antisense plants are capable of photo-autotrophic growth, suggesting a non-essential role for this subunit in photosynthetic eukaryotes. However, the evolutionary origin of the PsbW subunit in the higher plant chloroplasts could have taken place during the evolution of land plants, in conjunction with the presence of membrane-located light-harvesting antenna pigment proteins. This LMW protein may have enabled the fine-tuning of assembly and/or stabilization of PSII-LHCII supercomplexes, and thus the formation of ordered rows of semicrystalline macromodules of PSII-LHCII supracomplexes within the grana stack membranes, to optimize photosynthesis for the land-living eukaryotic plants facing large and fast changing environmental conditions with the need for effective regulation.

EXPERIMENTAL PROCEDURES

Plant material

Arabidopsis thaliana T-DNA insertion mutants of the psbW gene were generated from the Syngenta Arabidopsis Insertion Library collection (SAIL), mutant line SAIL_885_A03, in Columbia ecotype (Col-0) (Sessions et al., 2002), and obtained from the European stock centre (NASC) line N839813 (Alonso et al., 2003). Antisense plants were the same as those described in Shi et al. (2000). Arabidopsis seedlings were germinated and cultivated in soil and/or in MS agar plates for 3–7 weeks at a photon flux density of 80 mol m$^{-2}$ sec$^{-1}$, with a light/dark cycle of 8/16 h, respectively.

Isolation of thylakoid membranes and grana-enriched fractions

Thylakoid membranes were prepared using the method described by Noren et al. (1999), and their chlorophyll concentrations were measured following extraction in 80% acetone, using the method described by Porra et al. (1989). In addition, extraction buffers were supplemented with sodium fluoride (10 mM final concentration) for measuring the extent of phosphorylation. For the preparation of grana-enriched fractions, the procedure outlined in Caffarri et al. (2009) was followed.

Oxygen evolution measurements

The oxygen-evolving activity of thylakoid membrane preparations was measured in assay medium (25 mM Hepes, pH 7.6, 0.2 M sucrose, 10 mM NaCl, 5 mM CaCl2), supplemented with the artificial electron acceptors phenyl-p-benzoquinone or 2,6-dimethyl-p-benzoquinone at final concentrations of 0.25 and 1 mM, respectively. Oxygen evolution rates were measured at 20°C under saturating light using a Clark-type electrode (Hansatech Instruments, http://www.hansatech-instruments.com/).

Blue native polyacrylamide gel electrophoresis

Blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed as described in by Schägger and Von Jagow (1991), with modifications as described in Cline and Mori (2001). Solubilization of thylakoid membranes (concentrated to 1 mg ml$^{-1}$ chlorophyll) was performed with one volume of 2% n-dodecyl-β-D-maltoside. Solubilized samples were loaded onto a 1-mm-thick native gel (gradient of 5–12.5% acrylamide) and separated at 4°C.

Non-denaturing Deriphat-PAGE

Non-denaturing Deriphat-PAGE was carried out according to the method described by Peter et al. (1991), with minor modifications: isolated thylakoid membranes were solubilized with 1% n-dodecyl-β-D-maltoside at a chlorophyll to detergent ratio of 1:10 for 30 min at 4°C. After pelleting non-solubilized material, 10-μl aliquots of the samples were loaded onto a 1.5-mm-thick acrylamide gel (38:1 acrylamide/bisacrylamide) with a 3% stacking gel and a 4–15% acrylamide gradient resolving gel. The electrode buffer (12.4 mM Tris–HCl, 96 mM glycine, pH 8.3) was supplemented with 0.2% Deriphat-160 and 0.01% SDS (final concentrations). Electrophoresis was performed at a constant current of 10 mA at 4°C.

SDS-PAGE and immunoblot analyses

SDS-PAGE was performed on gels containing 12.5 or 15% acrylamide (the latter for LMW protein analyses) and 2 μM urea. For immune staining, the proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, http://www.bio-rad.com/). Polyclonal antisera directed against PsbW, PsbO, D1, D2, Lhcb1, Lhcb2, Rubisco and PsaO were purchased from AgriSera AB (http://www.agrisera.com). Antisera directed against STN7 and STN8 protein kinases were kindly provided by Professor D. Leister, Munich, Germany, and phosphothreonine antibodies were purchased from Cell Signaling Technology Inc. (http://www.cellsignal.com). Appropriate horseradish peroxidase-labeled secondary antibodies were used and immunodetection was visualized by enhanced chemiluminescence (GE Healthcare, http://www.healthcare.com) using an LAS-3000 cooled CCD camera (Fujiﬁlm, http://www.fujiﬁlm.com). Only samples that had been separated on the same gel were compared.

Sucrose density gradient ultracentrifugation

Photosystem II supercomplexes prepared from grana-enriched fractions were measured according to the method described in Caffarri et al. (2009). Grana membrane-enriched fractions were solubilized with n-dodecyl-α-D-maltoside with or without n-dodecyl-β-D-maltoside (0.3 or 0.45% final concentrations, respectively). Ultracentrifugation was performed using an LE-70 instrument (Beckman Coulter, http://www.beckmancoulter.com) at 160 000 g for 16 h in an SW41 rotor.
Chlorophyll fluorescence measurements

Chlorophyll a fluorescence was measured in intact leaves of 5-week-old wild-type plants. A PAM 101/103 pulse-amplitude modulated fluorometer (Heinz Walz, http://www.waltz.com) was used to evaluate the photochemical and non-photochemical quenching parameters, as described in Maxwell and Johnson (2000). To evaluate PSII activity under high level of light (900 µmol photons m$^{-2}$ sec$^{-1}$), and recovery under a low level of light (110 µmol photons m$^{-2}$ sec$^{-1}$), the typically used parameter $F_{0}$/$F_{M}$ was measured in detached leaves in a time-course experiment in the presence or absence of lincycin (1.25 µM final concentration; Sigma-Aldrich, http://www.sigmaaldrich.com) as follows: the leaves were immersed in water or lincycin solution in Petri dishes under a transparent water bath connected to a cooling system in a temperature-regulated room to maintain the leaves at 22°C. High-light treatments lasted for 22 h, and then leaves were allowed to recover in a low level of light for 22 h, and the maximum quantum yield of PSII ($F_{M}$/$F_{M}$) fluorescence was measured using a portable PAM 201 fluorometer (Heinz Walz). In addition, thylakoid membranes were isolated from wild-type and koPsbW leaves that were dark-adapted for 15 min, lincycin-treated for 3 h, and high-light treated for 22 h and low-light recovered for 22 h. Fast fluorescence induction curves were recorded for detached, dark-adapted leaves vacuum infiltrated with 100 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Fluorescence was measured with a DUAL DR head in a Dual PAM100 chlorophyll fluorescence photosynthesis analyzer (Heinz Walz) in fast-kinetics mode. To obtain the fast chlorophyll a fluorescence transient, 4000 µmol photons m$^{-2}$ sec$^{-1}$ actinic illumination was applied. The measured fluorescence induction curve was numerically fitted using the function $F(t, I, sPSII, J)$ based on a sigmoidal fluorescence induction model (Koblizek et al., 2001), where $J$ is the connectivity parameter that determines the shape of the curve, and $sPSII$ is the functional cross section of PSII.

State 1 $\rightarrow$ state 2 transition measurements

State transitions and state-1 or state-2 light pre-treatments were measured in intact leaves using a Dual-PAM 100 chlorophyll fluorescence analyzer (Heinz Walz), following the method described in Damkjær et al. (2008).

Circular dichroism spectroscopy for membrane structure analysis

Circular dichroism spectra were recorded on whole leaves or isolated thylakoid membranes between 400 and 750 nm at 25°C in a J810 dichrograph (Jasco, http://www.jasoinc.com) using a band-pass of 3 nm and a resolution of 1 nm. Detached leaves were quickly infiltrated with distilled water prior to measurement, and placed perpendicular to the optical path. In order to improve the signal-to-noise ratio, nine spectra were collected and averaged for each sample. Thylakoid membranes solubilized with n-dodecyl-β-D-maltoside were measured at a chlorophyll concentration of 20 µg ml$^{-1}$ in a glass cuvette with a 1-cm optical path length.

Thermoluminescence

Thermoluminescence measurements were carried out in a home-built apparatus, described elsewhere by Demeter et al. (1994). After 5 min of dark adaptation at 25°C, 200 µl of thylakoid membrane suspension (equivalent to 100 µg of chlorophyll) was loaded into the sample holder and excited at ~80°C in the presence or absence of 10 µM DCMU by a saturating single-turnover flash. Immediately after excitation, the emitted thermoluminescence was measured during heating of the sample in the dark at a heating rate of 20°C min$^{-1}$ using a photomultiplier (Hamamatsu Phototonics, http://www.hamamatsu.com).

Transmission electron micrographs and electron microscopy

Samples for chloroplast micrographs were prepared, embedded and sectioned according to the method described by Keskkula et al. (2005). For electron microscopy analyses, purified membranes were prepared as described in Kovacs et al. (2006), and were negatively stained with 2% uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120 electron microscope equipped with an LaB6 tip operating at 120 kV. Images were recorded with a Gatan 4000 SP 4K slow-scan CCD camera at 80 000× magnification at a pixel size (after binning the images) of 0.375 nm at the specimen level.

Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance (EPR) measurements were performed with a Bruker ELEXYS E500 spectrometer with a SuperX EPR049 microwave bridge and a SHQ4122 cavity, equipped with an ESR 900 liquid helium cryostat and ITC503 temperature controller (Oxford Instruments, http://www.oxninst.com). The S(2) state multilinie signal was induced by illumination at 200 K for 6 min, and oxidation of Cytb559 was induced by illumination at 77 K for 6 min, as described in Mamedov et al. (2008).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Photograph of wild-type Col-0 (WT), PsbW knock-out (koPsbW) and PsbW antisense (asPsbW) plants grown in MS media agar plates, showing that deleting or decreasing PsbW has no visible effect on the phenotype.

Figure S2. Immunodetection of protein kinases STN7 and STN8 in thylakoid membranes of dark-adapted leaves harvested from wild-type and koPsbW.

Figure S3. Characterization of the thylakoid membrane chlorophyll-containing protein complexes isolated from WT, asPsbW and koPsbW.

Table S1. The photosynthetic properties of koPsbW plants were examined in more detail by measuring other fluorescence parameters (Maxwell and Johnson, 2000), including the operational PSII quantum yield (ΦPSII), photochemical quenching (qP) and non-photochemical quenching (NPQ) (Table 1).

Table S2. Electron paramagnetic resonance (EPR) analysis of the electron transport components of photosystem II (PSII) showed that neither Tyr$_{D}$ nor the level of oxidized and reduced Cytb559 is changed in koPsbW plants.

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