The low molecular mass PsbW protein is involved in the stabilization of the dimeric Photosystem II complex in *Arabidopsis thaliana*

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**Running title:** Functional analysis of the PsbW protein in PSII

**Abbreviations:** PSII, photosystem II; LHCII, light harvesting complex II; Chl, chlorophyll; D1 and D2 proteins, products of the *psbA* and *psbD* genes, respectively; CP47 and CP43, chlorophyll-binding proteins encoded by the
*psb*B and *psb*C genes, respectively; \( Q_A \) and \( Q_B \) the first and second PSII plastoquinone electron acceptor;

**Summary**

*Arabidopsis thaliana* plants have been transformed with an antisense gene to the *psbW* of photosystem II (PSII). Eight transgenic lines containing low levels of *psbW* mRNA have been obtained. Transgenic seedlings with low contents of PsbW protein (more than 96% reduced) were selected by Western blotting and used for photosynthetic functional studies. There were no distinct differences in phenotype between the antisense and wild type plants during vegetative period under normal growth light intensities. However, a sucrose gradient separation of briefly solubilized thylakoid membranes revealed that no dimeric PSII supracomplex could be detected in the transgenic plants lacking the PsbW protein. Furthermore, analysis of isolated thylakoids demonstrated that the oxygen-evolving rate in antisense plants decreased by 50% compared to the wild type. This was found to be due to up to 40% of D1- and D2- reaction center proteins of PSII disappearing in the transgenic plants. The absence of the PsbW protein also altered the contents of other PSII proteins to differing extents. These results show that in the absence of the PsbW protein, the stability of the dimeric PSII is diminished and consequently the total number of PSII complexes is greatly reduced. Thus the nuclear encoded PsbW protein may play a crucial role in the biogenesis and regulation of the photosynthetic apparatus.
Introduction

Photosystem II (PSII) of higher plants catalyzes the light-driven oxidation of water to molecular oxygen and the reduction of plastoquinone to plastoquinol. The PSII supracomplex consists of almost 30 different subunits of which two, the D1 and D2 proteins, bind most, if not all, of the cofactors needed for primary and secondary electron transfer reactions. The D1, D2 and the inner antennae proteins, CP43 and CP47 bind chlorophyll $a$, and constitute together with the extrinsic proteins the PSII core. The PSII core in turn is surrounded by the outer antennae, light harvesting complex II (LHCII) which binds both chlorophyll $a$ and $b$ (1-5). Both biochemical studies (6-9) and single particle analysis of two-dimensional crystals (10-12) suggest that the PSII supracomplex forms a dimer in vivo. Recently, intact and highly active dimeric PSII-LHClII supracomplexes were isolated directly from spinach thylakoids (9) supporting the idea that the dimer is the natural state of PSII.

Both the monomeric and the dimeric forms of PSII have been found to contain several low molecular mass (<7 kDa) proteins (6, 8). One of these small proteins is the nuclear encoded PsbW (6.1 kDa) protein (13, 14) that is highly conserved in spinach, Arabidopsis and Chlamydomonas. Figure 1 shows the Arabidopsis PsbW protein sequence dealt with in this work. The PsbW protein was found to have only a single membrane span, with 14 and 20 amino acids stretching out to the stromal and lumenal sides of the membrane, respectively (Fig 1). The orientation of the PsbW protein in the thylakoid membrane is opposite to other transmembrane PSII reaction center proteins with its N-terminus at the lumenal side and the C-terminus at the stromal side (13-15). Localization studies showed that the PsbW protein is not present in PSI, but is instead tightly associated with the PSII reaction center (13, 15). This was further supported by the finding that the PsbW protein undergoes
degradation under photoinhibitory conditions. The extent and pattern of degradation was similar to that of the D1 protein except that it was not phosphorylated before degradation (16). The protein is expressed in dark-grown seedlings, i.e. it is synthesized before other PSII reaction center proteins, and the protein level increases upon illumination (14, 17). In order to obtain insights into the function of the PsbW protein in the photosynthetic process, we generated transgenic A. thaliana plants expressing an antisense construct of psbW. In this report we present the functional analysis of a nuclear-encoded low molecular mass protein in PSII from higher plants. The data demonstrate that the PsbW protein is involved in the stabilization of dimeric PSII complexes in Arabidopsis.

MATERIALS AND METHODS

Generation of A. thaliana - PsbW antisense plants. The genomic fragment encoding A. thaliana PsbW (18) was cloned in an antisense orientation into pBin19 downstream of the repeated CaMV 35S promoter. The construct was transferred into Agrobacterium tumefaciens strain LBA4404 by triparental mating (19). Arabidopsis plants were transformed by an inflorescence infiltration method (20). Transgenic plants (T1) were selected on kanamycin-containing Murashige and Skoog (21) plates, transferred into soil and allowed to self-pollinate to produce T2 seeds. The T1 plants were also verified by Southern and Northern blot analyses.

Growth of Arabidopsis thaliana - The wild type and the T2 Arabidopsis thaliana (Colombia) transgenic seeds were placed on wet filter paper and incubated at 4°C for three days. The cold treated seeds were sown in a mixture containing soil and vermiculite with the ratio of 1:1:2. The seedlings were growing under white light (90 or 180 µmol of photons m⁻² s⁻¹), and the light/dark cycle was 8/16 h. Plants were also grown hydroponically (22). For
biochemical studies, leaves were harvested before plant flowering.

Isolation of thylakoids and chlorophyll concentration measurement - Isolation of thylakoid membranes from *A. thaliana* was carried out according to Norén (22) with minor modifications. One gram of *A. thaliana* leaves was homogenized with 40 ml preparation medium (300 mM sorbitol, 20 mM Tricine, pH 8.4, 10 mM EDTA, 10 mM KCl 0.25% (w/v) BSA, 5 mM sodium ascorbate and 5 mM DTT). The slurry was filtered through 4 layers of cheesecloth and centrifuged at 1000 x g for 1 min. The pellet was resuspended in 20 ml of 5 mM MgCl₂ to lyse the chloroplasts. After 30 seconds the same volume of double concentrated resuspension medium (600 mM sorbitol, 40 mM Hepes, pH 7.6, 5 mM MgCl₂, 10 mM EDTA and 20 mM KCl) was added. The thylakoid membranes were reisolated at 1000 x g for 1 min, washed once with resuspension medium and resuspended in the same medium. For measurement of chlorophyll concentration, samples were diluted in 80% acetone, centrifuged at 10 000 x g for 10 min and measured spectroscopically (23).

Sucrose density gradient centrifugation - A continuous sucrose gradient containing 0.03% (w/v) *n*-dodecyl β-D-maltoside (DM) was prepared by the freeze and thaw method described by Eshaghi *et al* (9), except that the sucrose gradients were buffered with 25 mM Hepes, pH 7.6. The solubilization of thylakoid membranes by DM detergent and centrifugation were carried out exactly as in (9).

Western blotting and protein analysis - SDS-PAGE was carried out according to SchÄgger (24) with minor modifications. The polyacrylamide gel contained 6 M urea and the Tris-Tricine running buffer was used. The proteins on polyacrylamide gels were either transferred to PVDF membrane
or stained with silver (26). Immunoblotting was carried out using a semidry blotting system (Millipore). A polyclonal antiserum was raised in rabbit against the N-terminal 15-mer oligopeptide of PsbW protein and purified using protein A Sepharose chromatography (13). Immunodecorations were visualized using the alkaline phosphatase system with CDP-Star substrate (BioLabs). Quantification of immunoblots was performed by laser scanning densitometry.

Measurement of steady state Oxygen-evolution - Oxygen-evolution activity of PSII was measured using a Clark-type electrode in reaction medium (0.1 M sorbitol, 5 mM MgCl₂, 5 mM NaCl, 50 mM Hepes, pH 7.6) at 20°C under saturating light. Potassium ferricyanide (2 mM), phenyl-p-benzoquinone (PpBQ, 0.05 mM, 0.1 mM and 0.2 mM), 2,6-dichlorobenzoquinone (DCBQ, 1 mM) and 2,6-dichlorophenolindophenol (DCPIP, 0.1 mM) were supplied as electron acceptors.

Chlorophyll fluorescence and flash oxygen-evolving measurements - Chlorophyll fluorescence was measured directly on intact leaves after 15 min dark adaptation using a Walz PAM-200. Flash-induced oxygen oscillation patterns were measured with a modified Joliot-type electrode at 20°C (27). The flow medium contained 30 mM Mes, pH 6.5 and 10 mM KCl. The samples were dark adapted on the electrode for 3 min and the polarization voltage of 700 mV was switched on 30 s before a train of short (10 µs) Xenon flashes separated by 500 ms were given. The flash-induced oxygen yield (Yₙ) was measured and normalized to the average yield on flashes 3-6.

RESULTS

Generation of transgenic Arabidopsis thaliana - Eight independent lines of transgenic A. thaliana expressing the antisense psbW gene were produced
and analyzed. Northern blotting demonstrated that the antisense construct of \textit{psbW} was highly expressed in the transgenic plants and the level of \textit{psbW} mRNA was dramatically reduced (not shown) and consequently the translated PsbW protein was greatly reduced as well. In one of the different plant lines the level of PsbW protein was reduced to less than 4\% of wild type PsbW protein (Table 1). A 96\% reduction of the PsbW protein is very close to a total knock-out and hence provides an excellent system for further functional analyses. These low levels of PsbW protein were detected at different developmental stages of the plants indicating that the antisense gene was constitutively expressed, resulting in low levels of PsbW protein expression, through the whole life of the plant. The T2 plants were grown directly in soil, each individual plant was tested by Western blotting before any further analyses were performed. This showed that the transgene was segregated 3:1 (not shown), as expected for T2 plants and that both heterozygous and homozygous plants had decreased levels of PsbW protein.

\textbf{Phenotype} - In spite of the 96\% reduction in PsbW protein levels, no drastic change in phenotype of the antisense plants as compared to the wild type occurred (Fig. 2). In addition, growth of the plants under two different light regimes, 90 and 180 $\mu$mol photons m$^{-2}$s$^{-1}$ for at least 50 days and on two types of growth media (soil and hydroponic culture), did not result in phenotypic changes in the antisense plants (Fig. 2). However, the antisense plants flowered about two weeks earlier than the wild type, which indicated a certain kind of stress.

\textbf{Steady state oxygen evolution is affected} - When steady state oxygen evolving rates of isolated thylakoid membranes were measured in the presence of different electron acceptors, a dramatic effect was observed. Using PpBQ as an electron acceptor only 50\% of activity was present in thylakoids
from antisense plants (Table 2), and the oxygen-evolving activity supported by DCBQ was only 38% of the wild type activity. Also, oxygen-evolving activities supported by other electron acceptors, such as ferricyanide- and DCPIP- decreased significantly.

**Stability of the dimeric PSII complex is reduced** - We analyzed the structural conformation of PSII in the transgenic Arabidopsis plants. A new direct method was applied for the isolation of PSII-LHCII supercomplexes, i.e. PSII dimer complexes (9) from thylakoids of wild type and antisense Arabidopsis plants. In this method, the isolated thylakoids are briefly solubilized by n-dodecyl β-D-maltoside and then applied onto a sucrose gradient. By density gradient centrifugation the main complexes of the thylakoid membrane can be separated without affecting their intactness. In Fig. 3, the pattern of chlorophyll-containing bands from thylakoids of the wild type plant shows strong similarities to that from spinach thylakoids (9). The upper and middle bands contain LHCII and PSI, respectively, while the third, somewhat diffuse band contains the LHCII-PSII supercomplexes (PSII in dimeric form). When thylakoids from antisense plants were treated in the same way, the PSII dimer supracomplex could not be detected (Fig. 3). Even if the ratio of detergent to chlorophyll was decreased, no dimeric PSII band could be detected in the antisense plant (not shown). Instead, an increased chlorophyll $a/b$ ratio was detected in the lower part of the LHCII band, which is the location of the monomeric PSII (6). This experiment clearly shows that in the absence of the PsbW protein, no dimeric supracomplex of PSII can be isolated.

**Electron transport in PSII does not change significantly** - The electron transfer within the PSII complex of the *psbW* antisense Arabidopsis plants was analyzed by measuring the chlorophyll fluorescence and by flash oxygen measurements. The chlorophyll fluorescence measurements of both intact
leaves and isolated thylakoids (not shown) showed that F0 was slightly higher, and Fv/Fm was somewhat lower (Fig. 4) in the transgenic plant compared to the wild type plant. However, no dramatic effects were observed, showing that electron transfer in the PSII complexes lacking the PsbW protein was not seriously affected. Moreover, the measurement of flash oxygen evolution from isolated thylakoid membranes did not indicate any significant effects caused by the antisense gene (Fig. 5). This suggests that the lack of the PsbW protein does not alter energy transfer within the PSII complex and that the remaining PSII complex is functionally active.

The amount of functional PSII core complex was greatly reduced - If electron transport in PSII complexes is nearly normal, what causes the 50% decrease of the steady state oxygen evolution? Analyses of the chlorophyll content showed a small decrease of 0.2 in the chlorophyll a/b ratio in the transgenic plants indicated a loss of some chlorophyll a which lead us to assume that PSII core proteins must diminish. The decreased chlorophyll a/b ratio suggested a change of the chlorophyll a content, e.g., a reduced amount of PSII core proteins. To test this, immunoblotting using various antibodies raised against PSII proteins was performed. We found that the total levels of the different PSII proteins in thylakoid membrane preparations from PsbW antisense plants had changed (Table 3). The most affected proteins were the PSII reaction center proteins, D1 and D2, of which up to 40% disappeared. The amounts of oxygen-evolving enhancer proteins, PsbO and PsbP, were reduced by 20% and 40%, respectively. The inner antennae proteins CP43 and PsbS decreased by 30% and 40%, respectively. Two low molecular mass proteins, cytochrome b559 which is associated with the reaction center and PsbX located in PSII core, were less affected (75% and 90% remained respectively). In contrast to the proteins mentioned above, LHCII proteins were in fact slightly increased (8%). These results clearly demonstrated that
the amount of PSII core complexes decreased by about 40%, but the antennae complex remained intact. Since the PSII core contains mainly chlorophyll \( a \), this explains the decrease in the chlorophyll \( a/b \) ratio. It is consistent, too, with the fact that no bleaching occurred in the transgenic plants as the major part of chlorophyll pigment in plants is bound to the antennae complex, which was not drastically affected. Consequently, the decreased oxygen evolution rate was caused by a lower number of functional PSII centers.
DISCUSSION

Transgenic Arabidopsis plants with a 96% reduction in PsbW protein level did not show any drastic phenotype changes, which indicated that the PsbW protein is not directly involved in electron transfer within the PSII complex. However, when isolated thylakoids from these plants were analyzed with respect to steady state oxygen evolution, a reduction of PSII oxygen evolution of 50-60% (depending on the electron acceptor used), was observed. The remaining PSII complexes seemed to work normally as no drastic changes could be detected by flash oxygen evolution or chlorophyll fluorescence measurements when compared to thylakoids from wild type Arabidopsis.

The decreased oxygen evolution in the transgenic Arabidopsis thylakoids lacking PsbW protein was instead found to be due to the reduced amount of the PSII core proteins D1, D2 and CP43, which decreased by roughly 40%. Also the extrinsic proteins PsbO and PsbP proteins decreased, whereas the LHCII antennae was not affected. It is interesting to note that oxygen evolution seems to be somewhat more affected by the absence of the PsbW protein compared to the protein content of the PSII core complex. This could simply be due to variations using western blots for protein quantification, but it could also indicate an unidentified role of the PsbW protein in PSII. Further experiments are in progress to answer this question by using radiolabelled-DCMU for PSII quantification.

No dimeric PSII complexes could be isolated or detected in the PsbW antisense thylakoids, which suggests that the PsbW protein is essential for the stabilization of the dimeric PSII complex. The functional role of the dimeric organization of PSII is not yet fully understood. However, our results show that if the PSII dimeric form is not formed or is not stable enough, the amount of functional PSII is reduced. This suggests that the stability of the dimeric
form of PSII is higher than the monomeric form and thus the formation of
dimers could be a way of protecting the complex from being attacked by
proteases. On the other hand, when the complex is damaged by strong light
for instance, the complex monomerize, the D1 and PsbW protein are removed
and the degradation/repair can start. When the degradation process is
complete a newly synthesized PsbW protein will again combine the two
monomers to become a stable functional PSII dimer.

Our finding that the absence of the PsbW protein dramatically decreases the
amount of functional PSII dimers, and the fact that the PsbW protein is a
nuclear encoded protein in higher plants, allows for the interesting
speculation that this could be a way for the plant cell nucleus to control the
photosynthetic activity in the partly autonomous chloroplast.

How can a single α-helix transmembrane protein be crucial for the
dimerization of such large protein complexes? There are some reports
suggesting various factors that could indeed contribute to the dimerization of
PSII. In addition to D1, D2, CP43 and CP47, the PSII core contains the low
molecular weight proteins PsbE, PsbF, PsbH, PsbI, PsbK, PsbL, PsbTc and
PsbW (6, 8, 28). Recent crystallographic data on the oxygen-evolving core
PSII dimer suggested that the connector region between the two monomers
might be attributed to the small PSII subunits (12). The PsbL, PsbK and PsbH
were suggested to be involved in dimer stabilization (8, 12, 28). Genetic
dissection of PSII has shown that PsbL and PsbH are primarily required for
functioning of QA, the primary acceptor quinone in PSII (29, 31), and electron
transfer from QA to QB (31, 32), respectively. Requirement of PsbH for the
accumulation of PSII core proteins has also been reported (33), whereas the
PsbK seems to be entirely dispensable in Synechocystis (34) but not in
Chlamydomonas (35). Recent data has also suggested a function for
phosphatidylglycerol in the dimerization process of PSII (28).

The PsbH has a positively charged N-terminus at the stromal side of the thylakoid membrane and this could be the site of interaction with the negatively charged C-terminus of PsbW (Fig. 1). This interaction would then stabilize the PSII dimer. The exact mechanism by which the PsbW protein promotes PSII dimerization is not clear. However, as the PsbW protein is found in the monomeric PSII (6, 8), assembled dimeric PSII supracomplex (12), as well as in the reaction center pre-complexes in etioplasts (36), the protein seems to be involved both in guiding the assembly of monomeric PSII complexes and in stabilization of the dimeric PSII. Interconversion between the PSII dimer and monomers has been implicated in the D1 protein repair cycle (37) and this process could be controlled by reversible phosphorylation of PsbH at its N-terminus. In its phosphorylated form PsbH can not interact with PsbW and consequently the PSII dimer will monomerize. PsbW itself is not phosphorylated, but PSII damage under photoinhibitory conditions results in the degradation of D1 and PsbW proteins at a similar rate and extent (16).

A trEMBL database search revealed that roughly 10% of the total entries were proteins with a molecular mass below 7 kDa. Several of these are single α-helix transmembrane proteins lacking prosthetic groups, very similar to the PsbW protein. The results presented here for the PsbW protein give an incitement to search for low molecular mass proteins in other protein complexes and to analyze their possible involvement in complex oligomerization.
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**Table 1.**
The contents of PsbW protein in wild type and transgenic plants. Western blotting of thylakoid membranes was performed using antibody raised against the PsbW protein. The relative content was quantified by laser scanning densitometry.

|                      | WT | -PsbW |
|----------------------|----|-------|
| **Western Blot**     | ![Image] | ![Image] |
| **µg Chl loaded**    | 0.2 | 0.2   | 5.0 |
| **PsbW Content (%)** | 100 | 4     |
Table 2.
PpBQ-supported oxygen evolution. Thylakoid membranes were isolated and the stead-state oxygen evolution was measured with a Clark-type electrode at 20 °C and saturating light. PpBQ (0.1 mM) was used as electron acceptor. Values are means ± s.e. (n = 5).

|           | μmol O2/mgChl.h | Relative activity(%) |
|-----------|-----------------|----------------------|
| WT        | 128 ± 19        | 100                  |
| -PsbW     | 67 ± 8          | 51 ± 6               |
Table 3.
The contents of PSII proteins in wild type and transgenic plants. Western blotting of thylakoid membranes was performed using antibodies raised against D1, D2, CP43, PsbS, LHCII, PsbO, PsbP, cyt b559 and PsbX protein. The relative contents were quantified by laser scanning densitometry. Values are means ± s.e. (n = 5).

|                | WT -PsbW | WT (%) | -PsbW (%) |
|----------------|----------|--------|-----------|
| D1             | 100      | 61±5   |
| D2             | 100      | 59±7   |
| CP43           | 100      | 68±4   |
| PsbS           | 100      | 61±9   |
| LHCII          | 100      | 108±2  |
| PsbO           | 100      | 81±11  |
| PsbP           | 100      | 61±12  |
| Cyt b559       | 100      | 75±9   |
| PsbX           | 100      | 91±12  |
Figure legend

Figure 1

Schematic representation of the PsbW protein. The sequence was deduced from the Arabidopsis thaliana psbW gene sequence (X90769).

Figure 2

The phenotypes of wild type and PsbW antisense Arabidopsis plants. Arabidopsis thaliana (Colombia) plants were grown in soil for 50 days with white light and a light/dark cycle of 8/16 h. Light intensities were 180 (A) or 90 (B) µmol photons m\(^{-2}\) s\(^{-1}\).

Figure 3

Sucrose gradient separation of n-dodecyl β-D-maltoside solubilized thylakoid membranes from wild type (WT) and PsbW antisense (-PsbW) Arabidopsis plants. The major complexes are LHCII (light harvesting complex II), PSI (photosystem I), PSII\(_{D}\) (photosystem II dimer).

Figure 4

Chlorophyll fluorescence measurements of wild type and PsbW antisense Arabidopsis plants. Chlorophyll fluorescence was measured directly on intact leaf after 15 min dark adaptation using a Waltz PAM 200. Panel (A) wild type and panel (B) antisense plants.

Figure 5

Oxygen yield pattern measurements. Dark-adapted wild type (filled circle and solid line) and transgenic Arabidopsis (open circle and dashed line), detected with a Joliot-type electrode after illumination with a train of 15 flashes separated 700 ms, no electron donors or acceptors were added.
A

WT

-PsbW

180 μmol photons m\(^{-2}\) s\(^{-1}\)

B

WT

-PsbW

90 μmol photons m\(^{-2}\) s\(^{-1}\)
Fig. 4

A

Fluorescence (a.u.)

Time (s)

0.2

0.4

0.6

0.8

1.0

0

40

80

120

160

200

240

280

320

B

Fluorescence (a.u.)

Time (s)

30

60

90

120

150

180

210

240

270

300

330
Fig. 5

![Graph showing relative oxygen yield versus flash number with two lines representing wt and PsbW-antisens](image-url)
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