Comparison of the electron transport properties of the psbo1 and psbo2 mutants of Arabidopsis thaliana

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Genome sequence of Arabidopsis thaliana (Arabidopsis) revealed two psbo genes (At5g66570 and At3g50820) which encode two distinct PsbO isoforms: PsbO1 and PsbO2, respectively. To get insights into the function of the PsbO1 and PsbO2 isoforms in Arabidopsis we have performed systematic and comprehensive investigations of the whole photosynthetic electron transfer chain in the T-DNA insertion mutant lines, psbo1 and psbo2. The absence of the PsbO1 isoform and presence of only the PsbO2 isoform in the psbo1 mutant results in (i) malfunction of both the donor and acceptor sides of Photosystem (PS) II and (ii) high sensitivity of PSII centers to photodamage, thus implying the importance of the PsbO1 isoform for proper structure and function of PSII. The presence of only the PsbO2 isoform in the PSII centers has consequences not only to the function of PSII but also to the PSI/PSII ratio in thylakoids. These results in modification of the whole electron transfer chain with higher rate of cyclic electron transfer around PSI, faster induction of NPQ and a larger size of the PQ-pool compared to WT, being in line with apparently increased chlororespiration in the psbo1 mutant plants. The presence of only the PsbO1 isoform in the psbo2 mutant did not induce any significant differences in the performance of PSII under standard growth conditions as compared to WT. Nevertheless, under high light illumination, it seems that the presence of also the PsbO2 isoform becomes favourable for efficient repair of the PSII complex.

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1. Introduction

Photosystem II (PSII) is a multisubunit water–plastoquinone oxidoreductase embedded in the thylakoid membrane [12,3]. The unique water oxidizing complex (WOC) of PSII is located on the luminal side of the thylakoid. WOC consists of the Mn4Ca-cluster surrounded by three extrinsic proteins. PsbO protein of 33 kDa is one of the extrinsic WOC proteins and universal for all PSII complexes from higher plants to cyanobacteria [4]. The two other WOC proteins in higher plants are the PsbP and PsbQ proteins, and differ from the corresponding PsbU and PsbV proteins present in cyanobacterial WOC. The PsbO protein has the tightest interaction of all WOC proteins with the luminal loops of the PSII core subunits [5]. It is generally accepted that the PsbO protein plays an important role in stabilization of the Mn4Ca-cluster [6,7]. For this reason, PsbO is also known as ‘MSP’—the Manganese-Stabilizing-Protein, although none of its amino acid residues are likely ligands to the Mn4Ca-cluster. This protein undergoes pH dependent conformational changes, which regulate the binding of Ca2+ and Mn2+ [8]. Nevertheless, recent results suggest that the PsbO protein is additionally involved in several regulatory processes of PSII function [9,10].

Genome sequence of Arabidopsis thaliana (from here on referred as Arabidopsis) revealed two psbo genes (At5g66570 and At3g50820) which encode two distinct PsbO isoforms: PsbO1 and PsbO2, respectively. Consistently, the subsequent proteome analyses of Arabidopsis have identified the At5g66570 and At3g50820 gene products in the luminal compartment of thylakoids [11,12,13]. The psbo1 and psbo2 genes show 80.45% identity at the nucleotide level and 95.18% identity at the protein level [14]. PsbO1 isoform is the major isoform in Arabidopsis wild-type (WT) plants and is about 5-fold more abundant than PsbO2. However, in the absence of the PsbO1 protein, the steady-state level of the PsbO2 protein increases several fold in order to compensate the total amount of PsbO in thylakoids [15,9]. High-resolution X-ray data of cyanobacterial PSII complex have revealed
only one PsbO protein per PSII reaction center [16–18]. However, biochemical data provides evidence of two PsbO proteins per PSII monomer in higher plants [19–21]. The precise stoichiometry of PsbO protein in Arabidopsis PSII complexes is still unclear.

Already the first report on Arabidopsis mutant with a defect in the psbO1 gene but with intact psbO2, concluded that there is a functional difference between the PsbO1 and PsbO2 proteins due to the fact that the psbO1 mutant plants showed distinct growth retardation despite the presence of the PsbO2 isoform [22]. A subsequent report, however, suggested that the poor activity of PSII in the psbO1 mutant plants mainly was a result from a shortage of the total amount of the PsbO protein [15,9]. It was also proposed that the role of the PsbO1 isoform in Arabidopsis is mostly related to the support of PSII activity, whereas the PsbO2 protein mainly regulates the D1 protein turnover but can nevertheless substitute the PsbO1 protein in stabilizing WOC [9]. Yet another report [23,24] comes to a different conclusion and demonstrates that the PSII complex of the psbO1 mutant plants has significantly retarded electron transfer from QA to QB and long lifetimes for the S2- and S3-states of Mn4Ca-cluster. Moreover, it was reported that the PsbO2 isoform cannot efficiently use Ca2+ ions for proper function of the Mn4Ca-cluster and therefore cannot fully substitute PsbO1 in Arabidopsis [25]. Indeed, it is still controversial whether the two PsbO isoforms can substitute each other.

To get detailed insights into the function of the PsbO1 and PsbO2 proteins in Arabidopsis WOC, we have performed systematic and comprehensive investigations of the whole photosynthetic electron transfer chain in the T-DNA insertion mutant lines, psbO1 and psbO2 [9]. We also present the first data on a detailed study of the functional state of the PSII complex in the Arabidopsis mutant lacking the PsbO2 isoform by applying different biochemical and biophysical approaches.

2. Material and methods

2.1. Plant material

A. thaliana WT (ecotype Columbia) and mutant plants were grown on soil under standard growth chamber conditions (23 °C, 120 μmol photons m⁻²s⁻¹, a light/dark cycle 8/16 h) for 5 weeks. Mutants defective in the At5g66570 (psbO1) and At3g50820 (psbO2) genes were obtained from the Salk T-DNA collection and screened for homozygosity as discussed and characterized in [9].

2.2. Isolation of thylakoids and PSII-enriched membranes

Thylakoid membranes were isolated from mature WT and mutant plant leaves as previously described [26] and re-suspended in 50 mM Hepes/KOH (pH 7.5), 0.1 M sorbitol, 10 mM MgCl₂. PSII-enriched membranes (BBY-type) were prepared according to the procedure of Berthold et al. [27] with some modifications. Leaves were ground in ice-cold buffer containing 20 mM Tricine/NaOH (pH 8.4), 0.45 M sorbitol, 10 mM Na-EDTA, 5 mM NaCl, 5 mM MgCl₂ and freshly added 0.2% BSA and 0.2% NaAsc. The homogenate was filtered through Miracloth and centrifuged at 4200 × g for 10 min at +4 °C. The pellet was washed with 20 mM Tricine/NaOH (pH 7.6), 0.3 M sorbitol and re-suspended in buffer containing 20 mM Tricine/NaOH (pH 7.6), 5 mM MgCl₂. After centrifugation at 4200 × g, the pellet was re-suspended in 20 mM MES/NaOH (pH 6.3), 5 mM MgCl₂, 15 mM NaCl. Chlorophyll concentration was adjusted to 2.67 mg/ml, 1/3 volume of 20% Triton X-100 was added slowly and the sample was stirred for 30 min on ice in darkness. The sample was centrifuged at 9300 × g for 3 min and the supernatant again at 42,000 × g for 30 min. The pellet was re-suspended in the same buffer and again centrifuged at 42,000 × g for 30 min. Finally the pellet was suspended in buffer containing 20 mM Mes/NaOH (pH 6-3), 0.4 M sorbitol, 5 mM MgCl₂, 15 mM NaCl. This buffer also functioned as a measuring buffer for various experiments. In case of Ca-reactivation experiments 25 mM CaCl₂ and 50 μM Ca-ionophore (Calbiochem) were added to the measuring buffer and samples were incubated for 5 min at room temperature and 10 min on ice before conducting the measurements. The Chl concentration was measured according to Porra et al. [28].

2.3. SDS-PAGE and immunoblot analysis

Thylakoid membrane proteins were separated by 12% SDS-PAGE (6 M urea). For immunoblot analysis, the proteins were electro-transferred onto a PVDF membrane (Millipore, Watford, Herts, UK) and probed with the respective antibody and visualized with ECL (Western Blotting Detection Reagents from GE Healthcare).

2.4. Oxygen evolution measurements

The change in the redox state of P700 was measured from intact leaves by monitoring the absorbance at 820 nm and 860 nm using PAM 101/103 Fluorometer (Walz, Effeltrich, Germany) equipped with ED-700DW unit. Re-reduction of P700⁺ was recorded in darkness after 30 s illumination by far-red light applied from the LED (FR-102, Walz). The size of intersystem electron pool capable of donating electrons to oxidized P700⁺ was measured using single turnover and multiple turnover pulses under far-red background and estimated as previously described [29].

2.5. P700 oxido-reduction

The photochemical efficiency of the PSII complex (Fv/Fm) and the F₀ level were recorded by using Hansatech Plant Efficiency Analyzer (King's Lynn, UK) after 30 min dark incubation of leaves.

Flash-induced increase of the Chl fluorescence yield and its subsequent relaxation (FF-relaxation) in darkness were determined by the FL3300 dual-modulation fluorometer (Photon System Instruments, Brno, Czech Republic) as described in [30].

Thermoluminescence (TL) signal was measured with TL200/PMT Thermoluminescence System (P.S.I., Brno, Czech Republic). Excitation of thylakoid membranes was performed by a single turnover flash at −10 °C in the presence or in the absence of 20 μM DCMU.

Non-photochemical quenching (NPQ) of Chl fluorescence in WT and mutant plant leaves was determined using PAM-2000 Fluorometer (Walz, Effeltrich, Germany). Leaves were dark adapted for 30 min prior to measurements and then illuminated with actinic white light (800 μmol photons m⁻²s⁻¹) in order to induce NPQ, the quenching of excess excitation energy into heat. The NPQ parameter was calculated according to the equation: NPQ = (Fm' − Fm')/Fm' (where Fm' and Fm' represent the maximal fluorescence of dark adapted and illuminated leaves, respectively).

2.7. EPR spectroscopy

EPR measurements were performed from isolated thylakoid membranes and PSII-enriched membranes with a Bruker ELEXYS E500 spectrometer with a SuperX EPR049 microwave bridge and a SHQ4122 cavity. PSI/PSII ratio was determined from isolated thylakoids at room temperature in the flat cell on the basis of the intensity of the non-saturated EPR spectra from chemically oxidized P700⁺ and from the dark stable Yr redox (one spin per PSI and PSII, respectively) as described in [31,32]. P700⁺ was oxidized by 5 mM ferricyanide and...
Y₀ was oxidized by 2 min room light illumination. The spectral intensities of the Y₀ and P700⁺ spectra were determined by double integration of the EPR spectra. Y₀ stability was measured on the left hand peak after illumination with white light from an 800 W projector lamp filtered through saturated CuSO₄ solution. For measurements at liquid He temperatures the spectrometer was equipped with an ESR 900 liquid helium cryostat and ITCS03 temperature controller from Oxford Instruments, UK. The S₂-state multiline and g = 4.1 signals were induced by illumination at 200 K for 6 min as described in Mamedov et al. [32].

### 3. Results

#### 3.1. Phenotype of the mutant plants

The psbo1 mutant plants showed a distinct phenotype with pale green leaves, reduced rosette size and slower growth rate as compared to WT and psbo2 plants (data not shown) as described earlier [22,9]. On the other hand, the phenotype of the psbo2 plants closely resembled the phenotype of WT plants. This differs from the previously described phenotype of the psbo2 plants with slower growth than WT as described in [9]. This discrepancy might result from different growth conditions and the age of the plants, since the plants in Lundin et al. [9] were grown hydroponically and the distinct phenotype of psbo2 appeared only after 6–8 weeks of growth, whereas here the 5-week old plants were used for experiments.

#### 3.2. Comparative functional analysis of the PSII complex in psbo1 and psbo2 mutant plants

In order to clarify the functional consequences of the presence of either the PsbO1 or the PsbO2 isofrom in PSII, we monitored the function of the PSII complex by measuring the flash-induced increase in the fluorescence yield and its subsequent relaxation in darkness (FF-relaxation) in the presence and in the absence of DCMU in WT, psbo1 and psbo2 mutant plants of Arabidopsis.

In the absence of DCMU the FF-relaxation kinetics of WT thylakoids exhibited three distinct relaxation phases [33,34]. The fast and dominating phase of the FF-relaxation in the absence of DCMU originates from Qₓ⁻ to Qₓ electron transfer in PSII reaction centers, which had an occupied Qₓ⁻ pocket at the moment of firing the saturating single turnover flash. The middle phase, which arises from Qₓ⁻ to Qₓ electron transfer in PSII reaction centers, which had empty Qₓ⁻ pocket in darkness at the moment of the flash application and thus had to bind the PQ molecule from the pool. The slow phase originates from S₂Qₓ⁻ recombination, via Qₓ⁻ Qₓ → QₓQₓ⁻ equilibrium.

The FF-relaxation curves obtained from the psbo2 mutant thylakoids were almost overlapping with the WT curves (Fig. 1). On the contrary, the psbo1 mutant thylakoids demonstrated significantly modified shape of the FF-relaxation curve compared to the WT and psbo2 thylakoids. This is in line with earlier results obtained from intact leaves of the psbo1 plants [23].

Detailed kinetic analysis of FF-relaxation in the psbo1 thylakoids revealed a slower time constant for the fast phase and even more slower middle phase (0.78 and 59.5 ms, respectively) as compared to WT (0.51 and 17.8 ms, respectively) or to psbo2 (0.49 and 24.7 ms, respectively). These data demonstrate significantly retarded electron transfer from Qₓ⁻ to Qₓ in the psbo1 mutant plants, as compared to the psbo2 mutant plants or WT. Slower time constant and increased relative amplitude of the middle phase may also indicate a more reduced state of the PQ-pool in psbo1 thylakoids as compared to WT and the psbo2 plants.

The slow phase of the FF-relaxation curve of psbo1 thylakoids demonstrated rather similar time constant (7.9 s) when compared to the WT (7.8 s) or psbo2 thylakoids (7.6 s).

The FF-relaxation in the presence of DCMU mainly arises from recombination of Qₓ⁻ with the donor side components of the PSII complex and thus describes mainly the donor side status of the PSII complex. Again, the psbo2 mutant thylakoids exhibited very similar FF-relaxation kinetics as recorded for WT (Fig. 1, Table 1) whereas the psbo1 thylakoids demonstrated significant modifications. The relative amplitude and the time constant of the dominant slow phase of the FF-relaxation arising from S₂Qₓ⁻ relaxation were about 97% and 1.53 s, respectively in WT. Corresponding values in the psbo1 thylakoids were 88% and 1.98 s. In WT and psbo2 thylakoids the fast exponential component of the FF-relaxation curve was hardly detectable. Interestingly, the FF-relaxation kinetics of psbo1 thylakoids demonstrated the fast exponential component (28 ms), which constituted about 2% of the total amplitude of fluorescence. This fast component of the FF-relaxation kinetics in the presence of DCMU might originate from recombination of Qₓ⁻ with the less stable components of WOC than the S₂-state and can thus be an indicator of donor side modifications of the PSII complex in the psbo1 plants [30,35,36].

The amplitude of the flash-induced fluorescence yield in the presence of DCMU, which demonstrates the amount of PSII centers able to perform charge separation, was only slightly lower in psbo2 mutant thylakoids as compared to WT (Table 1) whereas the psbo1 mutant thylakoids showed about 30% lower fluorescence yield than WT and psbo2. The F₉/F₆ value, measured from intact leaves, was also similar in WT and the psbo2 plants (0.82 ± 0.01–0.81 ± 0.01), whereas in the psbo1 mutant plants it was only 0.47 ± 0.03 (Table 2, Fig. 5).

### Table 1

<table>
<thead>
<tr>
<th>Total Ampl</th>
<th>y₀ (%)</th>
<th>Fast phase τ (ms)/Ampl (%)</th>
<th>Middle phase τ (ms)/Ampl (%)</th>
<th>Slow phase τ (s)/Ampl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.4</td>
<td>0.51/60.7</td>
<td>17.8/16.6</td>
<td>7.81/18.3</td>
</tr>
<tr>
<td>psbo1</td>
<td>4.5</td>
<td>0.78/51.3</td>
<td>59.5/19.7</td>
<td>7.91/26.7</td>
</tr>
<tr>
<td>psbo2</td>
<td>5.6</td>
<td>0.49/59.4</td>
<td>24.7/18.5</td>
<td>7.60/16.6</td>
</tr>
<tr>
<td>WT + DCMU</td>
<td>100</td>
<td>2.9/0.005</td>
<td>–</td>
<td>1.53/97.4</td>
</tr>
<tr>
<td>psbo1</td>
<td>70</td>
<td>2.79/1.9</td>
<td>–</td>
<td>1.98/88.1</td>
</tr>
<tr>
<td>psbo2</td>
<td>90</td>
<td>4.05/0.08</td>
<td>–</td>
<td>1.59/95.5</td>
</tr>
</tbody>
</table>

Thylakoid membranes, at Chl concentration of 10 µg Chl ml⁻¹, were dark adapted for 5 min and Qₓ⁻ reoxidation in darkness after a single-turnover flash was recorded. Multicomponent deconvolution of the FF-relaxation curves was performed by using a fitting function with two exponential and one hyperbolic component as shown earlier [34]. The nonlinear correlation between the fluorescence yield and the redox state of Qₓ⁻ was corrected by using the Joliot model [52] with a value of 0.5 for the energy-transfer parameter between PSII units.

![Fig. 1. Characteristics of the flash-induced fluorescence yield and its subsequent relaxation in the darkness in the psbo mutant thylakoids.](image-url)

In order to facilitate comparison of the kinetics, F₀ and F₉₆ values were normalized to 0 and 1 values, respectively. WT is indicated as square, psbo1 as circle, psbo2 as triangle. DCMU curves are marked with open symbols.
These results are in line with steady-state oxygen evolution rates recorded from WT and mutant thylakoids in the presence of an artificial electron acceptor (Table 2), and with previous reports [9,17].

Next we used thermoluminescence technique to get further insights into the function of the PSII complex in WT and the mutants. The main TL band, the B-band, which originates from charge recombination, arises at about +38 °C in WT thylakoids (Fig. 2). In psbo1 mutant thylakoids the peak position of B-band was up-shifted to +40 °C, whereas in psbo2 thylakoids it was downshifted to +35 °C. The main TL band in the presence of DCMU, the Q-band, arises from S2QX charge recombination. In WT thylakoids the peak position of the Q-band was at +29 °C. TL measurements from the psbo2 thylakoids demonstrated no significant change in the peak position of the Q-band compared to WT. However, the psbo1 thylakoids again showed a slight upshift in the peak position (+31 °C) compared to WT. The up shift in the peak positions of the B- and Q-bands in the psbo1 mutant thylakoids, as compared to WT and psbo2, indicates the stabilization of the S2QX and S2QX charge pairs in the psbo1 thylakoids. Since the S2-state is a common component of both charge pairs, we propose that the stabilization in psbo1 mutant plants occurs mainly at the S2-state of WOC rather than at the acceptor side of PSII. This is in line with the previous results demonstrating that the S2- and S3-states of WOC in the psbo1 mutant plants exhibit significantly longer lifetimes compared to those in WT [23]. However, we cannot exclude the possibility of changes in the midpoint redox potential of Q and/or Qb in the psbo1 mutant plants.

In order to further investigate the electron transport properties of PSII and WOC in the psbo1 and psbo2 mutants, the induction of the S2-state EPR signals was measured in PSII-enriched membranes. Fig. 3 shows the light-minus-dark wide scan EPR spectra from PSII-enriched membranes of WT and both psbo mutants induced by illumination at 200 K. It is known that such a procedure induces one full S1- to S2-state turnover and a concomitant reduction of the acceptor side in PSII [37]. Indeed illumination of the WT PSII membranes induced the multiline and the g = 4.1 signals [38,39] both originating from the S2- state of the Mn4Ca-cluster. In addition to the S1- to S2-state transition, this illumination regime produced oxidation of the high potential form of Cytb559 and reduction of Qa, as the underlying QaFe2+ interaction signal [40,41] on the acceptor side of PSII (Fig. 3).

We clearly observed the induction of the S2-state EPR signals, induction of high potential Cytb559 and the reduction of Qa in both the psbo1 and psbo2 mutants (Fig. 3). However, the amplitudes of the induced signals from the S2-state (both multiline and g = 4.1) were in

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**Table 2**

Photosynthetic parameters of the psbo1 and psbo2 mutant plants.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>psbo1</th>
<th>psbo2</th>
</tr>
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<tbody>
<tr>
<td>( F_v/F_m )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GL</td>
<td>0.62±0.01</td>
<td>0.47±0.03</td>
<td>0.81±0.01</td>
</tr>
<tr>
<td>HL-grown (3+3)</td>
<td>0.72±0.05</td>
<td>0.70±0.03</td>
<td>0.68±0.04</td>
</tr>
<tr>
<td>( F_o )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GL</td>
<td>0.49±0.03</td>
<td>1.06±0.08</td>
<td>0.56±0.05</td>
</tr>
<tr>
<td>HL-grown (3+3)</td>
<td>0.84±0.12</td>
<td>0.69±0.07</td>
<td>0.89±0.11</td>
</tr>
<tr>
<td>( \Delta F )</td>
<td>333±25</td>
<td>193±10</td>
<td>346±24</td>
</tr>
<tr>
<td>( e^-/P700 )</td>
<td>18±5</td>
<td>87±9</td>
<td>18±5</td>
</tr>
<tr>
<td>PSI/PSII (GL)</td>
<td>0.91</td>
<td>1.54</td>
<td>0.91</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Thermoluminescence characteristics of psbo1 and psbo2 thylakoids. Thylakoids were excited by a single turnover flash at −10 °C in the presence or in the absence of 20 µM DCMU. Solid line—WT, dashed line—psbo1, dotted line—psbo2 samples.

**Fig. 3.** Light minus dark difference wide scan EPR spectra from PSII-enriched membranes isolated from WT and the psbo1 and psbo2 mutants. The figure shows the formation of the S2 state g = 4.1 signal (double bar) and the multiline signal (bars indicate multiline peaks used for quantification of the signal), as well as the oxidation of the high potential Cytb559 (g = 3.05, gz region) and the reduction of Qa, as the underlying QaFe2+ interaction signal [40,41] on the acceptor side of PSII (Fig. 3).
the psbo1 mutant only about half of that in WT and the psbo2 mutant. This correlated well with the reduced oxygen evolving activity of psbo1 mutant thylakoids (Table 2). Oxidation of Cytb559, on the other hand, was more pronounced in the mutant thylakoids (Table 2). Oxidation of Cytb559, on the other hand, was more pronounced in the

3.3. Susceptibility of the psbo1 and psbo2 plants to short-term and long-term high light

To analyze the susceptibility of the mutant plants to high light, the psbo1, psbo2 and WT plants grown under standard growth light (GL) conditions were transferred to high light (HL, 900 μmol photons m⁻²s⁻¹) for 3 h and the photochemical efficiency of PSII, the Fv/Fm value, was measured from intact leaves. Relative decrease in the Fv/Fm value as compared to respective values under GL conditions was at similar extent (68–72%) in WT and the psbo1 and psbo2 mutants upon the HL treatment (Fig. 5). The Fv value of Chl fluorescence, typically responding to HL stress, increased both in WT and the psbo2 leaves to 0.63 ± 0.06 and 0.75 ± 0.04, respectively. On the contrary, the psbo1 plants behaved differently and showed lower Fv level after the HL treatment (0.83 ± 0.09) as compared to that in GL (1.06 ± 0.08, also Suppl. Fig. 2). This suggests that the psbo1 mutant plants have reduced PQ-pool at GL conditions, whereas exposure to HL results in more oxidized PQ-pool.

To examine the effects of the psbo1 and psbo2 mutations on the efficiency of the PSII repair process, the recovery of Fv/Fm was monitored after the high light treatment. Transfer of the high light exposed plants back to growth light for the next 3 h resulted in nearly equal recovery of Fv/Fm with respect to the original values before the HL treatment in WT and both psbo mutants (Fig. 5).

To test the susceptibility of PSII to photodamage in the absence of concomitant repair, the psbo1, psbo2 and WT plants were exposed to illumination at 450 μmol photons m⁻²s⁻¹ for 2 h in the presence of lincomycin, an inhibitor of chloroplastic protein synthesis. Less severe illumination conditions were selected in these experiments not to induce too strong photoinhibition in the absence of PSII repair cycle, which could have masked the differences between the WT and mutants. The Fv/Fm value recorded from intact leaves after two hours illumination demonstrated about 80% loss in active PSII centers in the

![Fig. 4. EPR spectra on the stability of YD from thylakoid membranes isolated from WT and the psbo1 and psbo2 mutants. Arrow indicates the position where the light was switched off, i.e. the position of the maximally induced radical (100%). The inset shows the YD radical spectrum and the position where the kinetic spectra were recorded (bar at the left hand shoulder). Black traces are without and grey traces are with 25 mM CaCl₂. The spectra are normalized to the same Chl concentration. EPR conditions: microwave frequency 9.75 GHz, microwave power 8 mW, modulation amplitude 5 G, temperature 294 K.](image-url)
psbo1 plants, whereas the loss in WT and the psbo2 mutant plants was about 44% and 48%, respectively when compared to corresponding F_v/F_m values at GL (Fig. 5). This indicates that the PSI complexes of the psbo1 plants (i.e. in the presence of only the PsbO2 isoform in PSII centers) are more prone to PSI photodamage compared to WT and the psbo2 plants (Fig. 5).

To evaluate the long-term susceptibility of the psbo1 and psbo2 mutant plants to HL, three week old GL plants were transferred to HL (500 µmol photons m^{-2}s^{-1}) for the next 3 weeks (hereafter HL-grown). Interestingly, the F_v/F_m value recorded from HL-grown psbo1 plants (0.70) did not differ from those recorded for WT (0.72) and the psbo2 mutant (0.68) as drastically as under GL conditions (Table 2). Indeed, a distinctively lower F_0 level of Chl fluorescence in psbo1 after HL acclimation resulted in higher F_v/F_m value of PSI in the HL-grown psbo1 plants as compared to GL-grown psbo1. These results imply that the psbo1 plants have a capacity to cope and acclimate to HL.

3.4. Non-photochemical quenching (NPQ)

Induction of NPQ is an important phenomenon related to the high light response of photosynthetic organisms. In higher plants NPQ is induced under high light excitation and functions in dissipation of excess excitation energy in harmless form as heat [45,46,47]. It was therefore tested whether the psbo mutant plants deficient in one of the PsbO isomers are able to develop similar protective mechanisms to cope with excess light as the WT plants. Fig. 6 shows that upon exposure of dark adapted plants to high light illumination, the psbo1 plants were capable of developing NPQ faster than WT or psbo2, but the steady-state level of NPQ remained slightly lower in psbo1 than in WT or the psbo2 plants.

3.5. Size of the intersystem electron pool, chlororespiratory components and cyclic electron flow around PSI in the psbo mutants

The size of the intersystem electron chain in WT and the mutant plants was determined by following the redox changes of P700 upon applying single and multiple turnover saturating flashes on far-red background [29]. The psbo2 plants showed a similar pattern of P700 redox changes and thus also a similar status/size of the intersystem electron pool as WT (data not shown). On the contrary, the estimated intersystem electron pool size (e/P700) of the psbo1 plants was much bigger than that in WT and the psbo2 plants (Table 2). This prompted us to get insights into the possible inputs and outputs of electrons to and from the PQ-pool via chlororespiration and cyclic electron flow around PSI. Accordingly, the main components of chlororespiration were analysed using antibodies against PTOX and the NdhH subunit of the NDH complex in the thylakoid membrane. Interestingly, the psbo1 mutant thylakoids demonstrated significant upregulation in GL of both proteins associated with chlororespiration. Illumination at high light stimulated the upregulation of PTOX in WT and the psbo2 plants, whereas in the psbo1 plants no further upregulation was detected and the PTOX level even slightly decreased as compared to GL (Fig. 7A). As to the cyclic electron flow around PSI, the kinetics of P700+ re-reduction in darkness, a priori oxidized by far-red illumination, was analyzed. It was interesting to note that the psbo1 plants exhibited much faster P700+ re-reduction rate and thus higher cyclic electron transfer around the PSI complex than WT or the psbo2 plants (Fig. 7B). Again, no difference was observed in the kinetics of P700+ re-reduction between WT and the psbo2 plants.

3.6. PSI/PSII ratio in the psbo mutants

The relative amounts of PSII and PSI in the psbo1 and psbo2 mutants were estimated by EPR spectroscopy from the maximal size of the Y_s and P700+ EPR signals, respectively [31]. In WT and the psbo2 mutant plants the PSI/PSII ratio was 0.91 (Table 2), indicating approximately similar amounts of both photosystems in the thylakoid membrane. In contrast, the psbo1 mutant contained about 40% less of PSII centers estimated on the basis of the stable Y_s radical (1 spin per PSI reaction center), as compared to WT and the psbo2 mutant. Thus, in the psbo1 mutant the PSI/PSII ratio was 1.54 (Table 2). These results

![Fig. 6. Induction of NPQ in the psbo mutant plants. NPQ induction was recorded from dark adapted leaves by exposing them to the actinic light (800 µmol photons m^{-2}s^{-1}) for 15 min. The NPQ parameter was calculated according to the equation: NPQ = (F_m - F_{m'})/F_m' (where F_m and F_{m'} represent the maximal fluorescence of dark adapted and illuminated samples, respectively. WT (square), psbo1 (circle), psbo2 (triangle).](image-url)

![Fig. 7. Characterization of alternative electron transfer routes. Quantification of the PTOX and NdhH proteins and the re-reduction of P700+ in darkness. A. Immunoblots of the PTOX and NdhH proteins isolated from thylakoids of WT and the psbo1 (O1) and psbo2 (O2) mutant plants. The gels were loaded on Chl basis (2 µg Chl), which is in a linear range of immunoresponse with antibodies used for respective proteins. B. Re-reduction of F700 in darkness. Leaves were illuminated with far-red light for 30 s and the subsequent re-reduction of oxidized P700 was monitored in darkness. Leaves were dark adapted for 3 min prior to the measurement. P700 curves are normalized to the same amplitude in order to facilitate comparison of the kinetics.](image-url)
are not in accord with the report of Lundin et al. [9], where the calculation of the PSI/PSII ratio was based on immunoblot quantification, which is not as accurate as the EPR spectroscopy.

4. Discussion

Although the importance of the PsbO protein as a Manganese-Stabilizing-Protein (MSP) in the water splitting PSII complex is well known and accepted, the specific function of the two PsbO isoforms in Arabidopsis is still under discussion. The PsbO1 isoform is dominating in WT thylakoids and constitutes about 90% of the total amount of PsbO in WT. In the psbO1 mutant, the amount of the PsbO2 protein increases several fold, and some upregulation of PsbO1 takes place in the psbo2 mutant [9,15] leading to relatively constant amount of the PsbO protein in WT and both the psbO1 and psbo2 mutant plants. This makes it feasible to use the psbo1 and psbo2 mutants to compare the functional roles of the two PsbO isoforms in PSII centers.

The presence of only the PsbO1 isoform in the psbo2 mutant did not induce differences in the performance of PSII centers under standard growth conditions as compared to WT. On the contrary, the biophysical properties of PSII complex in the psbO1 plants, as reported here and, in line with the reports [23–25], clearly demonstrate that the PsbO2 isoform cannot fully substitute the PsbO1 protein as an efficient MSP in the PSII complex. Indeed, in the absence of the PsbO1 isoform, and consequently in the presence of only the PsbO2 isoform, the functional properties of the PSII complex are significantly different from those in WT and the psbo2 mutant plants, particularly under standard growth conditions. One distinguishing feature of the psbO1 mutant plants is the malfunction of the donor and acceptor sides of PSII, as demonstrated by the FF-fluorescence measurements in the presence and absence of DCMU.

Interestingly, despite the malfunction of PSII centers in the psbO1 mutant, the high light treatment of WT and the psbO1 and psbo2 plants did not result in any significant differences in the relative loss of PSII activity from that recorded at GL conditions (Fig. 5). The PSII centers of the WT and both the psbO1 and psbo2 mutant plants showed about 28–32% decrease in Fv/Fm upon short-term transfer from growth light to high light (3 h at 900 µmol photons m⁻²s⁻¹) illumination. However, when the concomitant PSII repair was inhibited by lincomycin during the high light treatment, the psbO1 mutant plants experienced a much more severe decline in Fv/Fm than WT and the psbo2 plants (Fig. 5).

Two important conclusions can be drawn from these experiments. Firstly, the presence of the PsbO2 isomer alone in PSII complexes renders the PSII centers extremely susceptible to photodamage. This result thus emphasises an important structural role for the PsbO1 isomer in maintaining the proper function of the PSII complex, particularly that of the Mn₄Ca-cluster. Secondly, the presence of only PsbO2 isoform in PSII centers, instead of the dominating PsbO1 isomer, induces an efficient PSII repair cycle even at GL conditions that more or less balances the photodamage to PSII. Indeed, upon HL illumination such an intensive repair cycle in the psbo2 mutant plants readily took care of the large number of photodamaged PSII centers induced under such conditions.

It is worth mentioning that also the psbo2 mutant, which has only the PsbO1 isoform in all PSII centers, showed slight difference in functional parameters of PSII and somewhat more sensitivity to high light as compared to WT (Table 2, Fig. 5). The absence of the PsbO2 isomer in the psbo2 mutant seems to lead to a slightly slower PSII repair cycle and thus to the accumulation of photodamaged PSII centers under HL, although the results obtained were not statistically significant.

Malfunction of PSII in the presence of the PsbO2 isoform may also be related to the fact that the psbO1 mutant has a higher amount of PSII monomer complexes as compared to WT or the psbo2 mutant [48]. In line with accumulation of PSII monomers in the psbO1 mutant plants, they have also been shown to exhibit a high ratio of PSI/PSII centers to PSII centers [23,24]. PSI/PSII centers include the newly restored PSII complexes in the stroma-exposed thylakoid membranes after repair of photodamaged PSI. Thus the accumulation of PSII/PSI centers in psbo1 relative to that of the PSII centers, which reside in stacked grana membranes as PSI supercomplexes, implies malfunction of the lateral migration of PSII complexes between the grana and stroma lamellae upon the photo-inhibition-repair cycle of PSI. Importantly, the lumen compartment, where the PsbO protein is located, is an extremely crowded place and exerts strong limitation to the migration of complexes in the thylakoid membrane [49]. It is thus conceivable that the migration of PSII is critically dependent on the PsbO isoform present in the PSII complex, the PsbO2 isoform being less efficient than the PsbO1 isoform in optimal migration of the PSII complexes and thereby leading to accumulation of PSII/PSII complexes in the psbo1 mutant. Interestingly, upon long-term high light acclimation of plants the difference in photochemical efficiency of PSII became less evident between the psbO1 and psbo2 mutants (Table 2). This might, at least partially, result from general destacking of the thylakoid network at high light, thus facilitating in the psbo1 mutant the migration and relocation of the PSII complexes under repair. It is conceivable that the PsbO1 isoform is crucial for optimal function of PSII in grana stacks, where the most efficient PSII complexes are located, and for the migration of the PSII complex between the grana and stroma lamellae. The PsbO2 isoform, in turn, might be favourable for optimal repair process when damaged D1 protein is replaced with the de novo synthesised new D1 copy in the stroma lamellae.

The presence of the PsbO2 isoform in PSII has consequences at normal GL conditions not only to the function of PSII but also to the whole electron transfer network. The higher cyclic electron transfer around PSI in the psbo1 mutant plants induces fast development of NPQ, which is known to protect PSII against photo-inhibition [50]. Higher PSI/PSII ratio and a larger size of the PQ-pool in psbo1 mutants than in WT are in line with apparently increased chlororespiration in the psbo1 mutant plants, as interpreted from the high amount of the Ndh and PTOX proteins, important players in nonphotochemical reduction and oxidation of the PQ-pool, respectively. In mature WT chloroplasts, the contribution of the chlororespiration pathway is minor when compared to the linear electron transport [51]. In psbo1 plants, however, the upregulation of chlororespiration might have an essential role in balancing the photosynthetic electron transport between the two photosystems. It remains to be shown whether these modifications in thylakoid electron transfer properties of the psbo1 mutant are due to signalling mechanisms induced by the malfunctioning PSII in the presence of the PsbO2 isoform, possibly by production of reactive oxygen species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.05.013.

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