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Insights into the function of PsbR protein in *Arabidopsis thaliana*

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Abstract

The functional state of the Photosystem (PS) II complex in Arabidopsis *psbR* T-DNA insertion mutant was studied. The ΔPsbR thylakoids showed about 34% less oxygen evolution than WT, which correlates with the amounts of PSII estimated from Y_D^{ox} radical EPR signal. The increased time constant of the slow phase of flash fluorescence (FF)-relaxation and upshift in the peak position of the main TL-bands, both in the presence and in the absence of DCMU, confirmed that the $S_2Q_A^-$ and $S_2Q_B^-$ charge recombinations were stabilized in ΔPsbR thylakoids. Furthermore, the higher amount of dark oxidized Cyt-b559 and the increased proportion of fluorescence, which did not decay during the 100s time span of the measurement thus indicating higher amount of $Y_D^+Q_A^-$ recombination, pointed to the donor side modifications in ΔPsbR. EPR measurements revealed that S_1-to-S_2-transition and S_2-state multiline signal were not affected by mutation. The fast phase of the FF-relaxation in the absence of DCMU was significantly slowed down with concomitant decrease in the relative amplitude of this phase, indicating a modification in Q_A to Q_B electron transfer in ΔPsbR thylakoids. It is concluded that the lack of the PsbR protein modifies both the donor and the acceptor side of the PSII complex.

Keywords: Arabidopsis; Photosynthesis; Photosystem II; PsbR; Thylakoid membrane

1. Introduction

Photosystem (PS) II is a multisubunit pigment protein complex embedded in the thylakoid membrane. In oxygen-evolving photosynthetic organisms the oxidation of water occurs at a tetramanganese-cluster, known as a water oxidizing complex (WOC). The WOC is bound to the reaction center of the PSII complex [1].

The mechanism of water oxidation is still unknown. Although many models have been suggested, the site of water binding and oxidation has not been unequivocally identified. There are a number of studies in the literature concerning the structure and assembly of the WOC, particularly the position and function of extrinsic WOC proteins [2]. In higher plants three extrinsic proteins (PsbO, PsbP, PsbQ) with the molecular masses of 33, 23 and 17 kDa are bound to the lumenal surface of the PSII complex. Modeling of WOC and its association with the reaction center of the PSII complex has demonstrated that the position of the PsbO protein is the same as in cyanobacteria, whereas PsbP and PsbQ are located in different positions as compared to the cyanobacterial PsbU and PsbV [3]. The PsbO protein is the most strongly bound extrinsic protein. Some experimental evidence suggests that the PsbP protein cannot bind efficiently to the PSII complex in the absence of PsbO and that the binding of PsbQ requires the presence of PsbP [4]. PsbP not only interacts with PsbO but also with the lumenal surface of the PSII core in the vicinity of CP43. There is some evidence that PsbP can bind weakly to the lumenal surface of the PSII complex even in the absence of PsbO [5].

Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorphenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; DMBQ, 2,6-dimethyl-*p*-benzoquinone; DPC, 2,2′-diphenylcarbonic dihydrazide; EPR, electron paramagnetic resonance; F_v, variable fluorescence yield; PSII, Photosystem II; Q_A and Q_B, primary and secondary quinone acceptors in PSII; Y_Z and $Y_Z^\bullet$, tyrosine 161 of the PSII D1 polypeptide and its radical; Y_D and $Y_D^\bullet$, tyrosine 161 of the PSII D2 polypeptide and its radical; TL, thermoluminescence

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While the structures of the extrinsic proteins have become solved, their function still remains obscure [6,7]. PsbO pretein is known as a Mn-stabilizing protein. In addition to the Mn ions, the ionic cofactors, especially Ca^{2+} and Cl^{-} are essential for the activity of WOC, although their specific functions still remain unknown [2]. The two extrinsic proteins—PsbP and PsbQ have been proposed to facilitate the binding of Ca^{2+} and Cl^{-} cofactors, to the WOC [8,9]. There is evidence that Ca^{2+} is required for the structure of the Mn cluster of the WOC [10] and modulates electron transfer between Y_z and $P680^{+}$ [11,12]. It has also been reported that Ca^{2+} influences the redox potential of Cyt-b559 [13] and Q_A [14], as well as the oxidation of Q_A^{-} [11,12]. In spite of the fact that the role of Ca^{2+} in water oxidation by PSII has been extensively studied over the years [2], a clear understanding of the involvement of Ca^{2+} ions in the function of the PSII complex is still missing. The importance of Ca^{2+} in oxygen evolution was realized when characterizing preparations, where the PsbP and PsbQ extrinsic proteins had been removed by 2 M NaCl treatment. After this treatment the electron transfer from the Mn cluster to $Y_z^{\bullet +}$ was inhibited, however addition of Ca^{2+} at millimolar concentration restored oxygen evolution to a large extent [10]. Later it was shown that the loss of oxygen evolving activity is due to the loss of Ca^{2+} rather than the PsbP and PsbQ proteins. These extrinsic proteins are thought to shield WOC and modulate the affinity of the Ca^{2+} binding site. Cl^{-} ions have been proposed to regulate the redox properties of WOC, which are readily interconvertible by addition or removal of Cl^{-}. Also the involvement of Cl^{-} in the process of PSII photoactivation has been suggested [15].

Recently PsbP has been suggested to play an important role in providing Mn during the process of photoactivation [16]. This protein also has structural features, which suggest that it might be a GTPase activating protein [6].

Besides PsbO, PsbP and PsbQ yet another PSII protein, PsbR, is thought to exert important structural stability to the WOC and is probably also required for proper function of the PSII complex [17,18].

In order to investigate the role of the PsbR protein in higher plant PSII, we have here functionally characterized the Arabidopsis T-DNA insertion mutant *psbR*.

2. Materials and methods

2.1. Plant material and isolation of thylakoid membranes

Arabidopsis thaliana L. ecotype Columbia (Col-0) plants were grown at 23 °C under 60-80 μE m^{-2} s^{-1} light intensity in 8-h light/16-dark photoperiod (for more details see [18]). The *psbR* T-DNA insertion line (SALK_114469) was purchased from the Salk Institute. PCR analysis of the plants confirmed that mutant was homozygous [18]. Fully-grown rosettes were used for the experiments. Leaves were homogenized in ice-cold buffer containing 40 mM HEPES/NaOH pH 7.6, 5 mM $MgCl_2$, 10 mM NaCl, 2 mM EDTA, 0.4 M sucrose, and 2 g/L BSA, 2 g/L NaAsc. The homogenate was filtered through Miracloth and centrifuged for 4 min at 6000×*g*. The resulting pellet was washed with 10 mM HEPES/NaOH pH 7.5, 5 mM $MgCl_2$, 10 mM NaCl and finally re-suspended in 40 mM Hepes/NaOH pH 7.0, 5 mM $MgCl_2$, 15 mM NaCl, and 0.4 M sucrose and stored at −80 °C until use. Unstacked thylakoids were isolated by the same procedure, however, $MgCl_2$ and NaCl were excluded from all buffers. PSII-enriched membranes were isolated as described by Berthold et al. [19].

Chlorophyll was extracted in 80% (v/v) buffered acetone and quantified as described [20].

2.2. Measurements of electron transport activity

Light-saturated steady-state rates of oxygen evolution from thylakoids were measured at 20 °C with Clark-type oxygen electrode (Hansatech, King's Lynn, UK) using 1 mM DMBQ as an artificial electron acceptor. Electron transfer from H_2O to DCIP and from H_2O+DPC to DCIP was measured as DCIP photoreduction at 560 nm in Shimadzu UV3000 spectrophotometer equipped with side illumination as described in [21].

2.3. Fluorescence measurements

Flash induced increase and subsequent relaxation of the chlorophyll fluorescence yield (FF-relaxation) were measured by a double-modulation fluorometer (Photon System Instruments, Brno, Czech Republic) in the 150 μs–100 s time range. Samples at 10 μg Chl mL^{-1} concentration were dark adapted for 5 min before fluorescence detection. Multicomponent deconvolution of the relaxation curves was performed by using a fitting function with two exponential and one hyperbolic component as shown earlier [22]. The nonlinear correlation between the fluorescence yield and the redox state of Q_A was corrected by using the Joliot model [23] with a value of 0.5 for the energy-transfer parameter between PSII units. For in vivo measurements DCMU (50 μM) was vacuum infiltrated into leaf discs. After 10 min dark adaptation FF-relaxation was measured as described above.

2.4. Thermoluminescence

Thermoluminescence (TL) was measured with home-build apparatus as described in [24]. Samples were excited with single turnover (2 μs, 1 Hz) Stroboslave Xenon flashes at −10 °C in the presence or absence 40 μM DCMU. TL curves were recorded from −40 to +80 °C at a heating rate 40 °C min^{-1}.

2.5. EPR spectroscopy

X-band low-temperature EPR spectra were recorded at 9.43 GHz microwave frequency with a Bruker ELEXSYS E500 spectrometer equipped with an Oxford-900 cryostat and ITC-503 temperature controller (Oxford Instruments Ltd, UK). Samples were pre-illuminated at room light for 1 min to fully oxidize Y_D and incubated in the dark for 5 min before freezing. The S_2-state multiline EPR signal was induced by illumination at 200 K for 6 min as described in [25]. Oxidation level of Cyt-b559 in the dark adapted samples was estimated from the amplitude of the EPR-signal in g_z-region ($g \sim 3.0$). Full oxidation of Cyt-b559 in PSII centers was achieved by illumination at 77 K for 6 min as in [25]. Analysis of EPR spectra was performed using Bruker Xepr 2.1 software.

3. Results

We have functionally characterized the T-DNA insertion mutant line SALK_114469, having an insert located in the third exon of the *psbR* gene (At1g79040). ΔPsbR mutant plants showed no specific phenotype under normal growth conditions when compared to WT plants (for more details see [18]).

3.1. Reduced amount and less activity of the oxygen evolving PSII complex in ΔPsbR mutant

Steady-state oxygen evolution measurements, the FF-relaxation, TL and EPR spectroscopy were applied to clarify the functional state of the PSII complex in an Arabidopsis *psbR* T-DNA insertion mutant. The ΔPsbR mutant thylakoids

showed about 34% less steady-state oxygen evolution activity measured in WT thylakoids at saturating light intensities. Under similar conditions, the steady-state yield of oxygen evolution in PSII-enriched membranes was 23% less than in WT, and on the basis of estimation of the size of the Y_D^{ox} radical EPR signal, we found that the ΔPsbR thylakoids have about 28% less of PSII complexes (on Chl basis) than in WT ([18], Table 1). The amount of non-oxygen evolving PSII complexes in ΔPsbR thylakoids were estimated in order to clarify whether the mutant PSII complex is more fragile and undergo more damage during the preparation of thylakoid membranes. When monitoring the electron transfer from H_2O to DCIP and from H_2O+DPC to DCIP it turned out that the amount of non-oxygen-evolving PSII centers was nearly the same in WT and ΔPsbR thylakoids (Table 1). Variable Chl fluorescence, the F_v values, obtained from the FF-relaxation curve of DCMU-treated intact leaves and isolated thylakoids were in ΔPsbR mutant 12% and 23% lower than in WT, respectively. The decrease in the F_v value, the sensitive indicator of the PSII activity, in both the intact leaves and in isolated thylakoids of ΔPsbR plants as compared to WT, also points to the lower content of the PSII complex in the mutant.

3.2. Flash induced increase and subsequent relaxation of the chlorophyll fluorescence yield

To investigate the functional status of the donor and acceptor sides of the PSII complex in the ΔPsbR mutant, the kinetics of the FF-relaxation was compared between WT and ΔPsbR thylakoids. Applying a single turnover saturating flash to dark adapted thylakoid samples induces a high fluorescence yield, which subsequently relaxes in the dark via forward and backward electron transfer reactions. Fig. 1A shows a typical FF-relaxation curve of the WT thylakoids, where three different phases can be detected. The kinetics of the FF-relaxation was dominated by the fast phase (0.86 ms, relative amplitude 49.5%), which arises from Q_A^- to Q_B/Q_B^- electron transfer. The contribution of the middle phase (21.3 ms) to the whole relaxation curve was about 18.6% (Table 2). This phase reflects the Q_A^- reoxidation in the PSII centers, which had an empty Q_B pocket at the moment of firing the flash and had to bind plastoquinone molecule from the pool. The relative amplitude of the slow phase (5.5 s), which arises from $S_2(Q_AQ_B)^-$ charge recombination, was about 29%. In ΔPsbR thylakoids the overall FF-relaxation kinetics was slowed down as compared to WT. The fast and the middle phases showed about two times slower time constants (1.78 ms and 42.9 ms, respectively) compared to WT. The relative amplitude of the fast phase of the ΔPsbR FF-relaxation decreased to 46%, whereas the middle and slow phases slightly increased to 20.1% and 30.8% respectively.

The FF-relaxation was measured also in the presence of DCMU, which blocks the reoxidation of Q_A^- by forward electron transfer and thus the FF-relaxation reflects the reoxidation of Q_A^- via recombination with the donor side components allowing us to analyze the functional status of the donor side of the PSII complex. The fast phase of the decay has a very small contribution to the total fluorescence amplitude and arises from the recombination of Q_A^- with Y_Z^+ in PSII centers in which the Mn-cluster is disturbed. The slow phase of the FF-relaxation arises from $S_2Q_A^-$ charge recombination. The overall FF-relaxation kinetics in ΔPsbR thylakoids in the presence of DCMU was slowed down compared to that of WT (Fig. 1A insert and Table 2). Analysis of the WT FF-relaxation curve obtained in the presence of DCMU resulted in a fast phase of 3.2 ms and 0.5% amplitude and in a slow phase of 1.74 s and 93.2% amplitude whereas in ΔPsbR thylakoids the corresponding values were 18.2 ms, 3% and 2.27 s, 76.7%. Interestingly, in ΔPsbR thylakoids in the presence of DCMU an increased proportion (20%) of fluorescence did not decay during the 100 s time span of the measurement.

It is known that the thylakoid isolation procedure may have specific effects on FF-relaxation kinetics. Therefore the experiments were also conducted with intact leaves of WT and ΔPsbR plants. These results showed a similar tendency as was obtained with isolated thylakoid membranes; the fast phase of FF-relaxation in the absence of DCMU was significantly slowed down in ΔPsbR mutant leaves, to 0.91 ms as compared to 0.61 ms in WT. Also in the presence of DCMU, the slow component of FF-relaxation detected from ΔPsbR leaves (1.04 s) is slower than that in WT (0.89 s), confirming the stabilization of $S_2Q_A^-$ charge recombination in mutant leaves (Fig. 1B).

3.3. Thermoluminescence

Upon illumination by a single flash at −10 °C, the WT thylakoid samples showed a TL band (B-band) peaking at 22 °C

Table 1
Characterization of the PSII complex in isolated thylakoid membranes and PSII particles from the WT and ΔPsbR plants

	O_2 evolution		O_2-evolving centers (% of total PSII centers)	F_v		PSII content (Y_D^{ox})	S_2-state multiline
	TM	PSII-particles		Leaf	TM		
WT	267 (100%)	326 (100%)	90%	100%	100%	100%	100%
ΔPsbR	176 (66%)	250 (77%)	88%	88%	77%	71%	70%

O_2 evolution is given as μmol O_2/mg Chl×h. TM—thylakoid membrane.
Number of O_2-evolving centers is given in % to the total PSII centers. The fraction of O_2-evolving PSII centers was estimated as the ratio between the electron transfer from H_2O to DCIP and the electron transfer to DCIP measured in the presence of DPC, an exogenous electron donor. See Materials and methods for more details.
F_v—value was obtained from the FF-relaxation curve by applying a single turnover flash to the dark adapted and DCMU treated leaves and thylakoids.
PSII content was estimated from the EPR measurements of Y_D^{ox} at 15 K (1 spin per PSII reaction center, [37]).
S_2-state EPR multiline signal was induced by saturating illumination at 200 K.

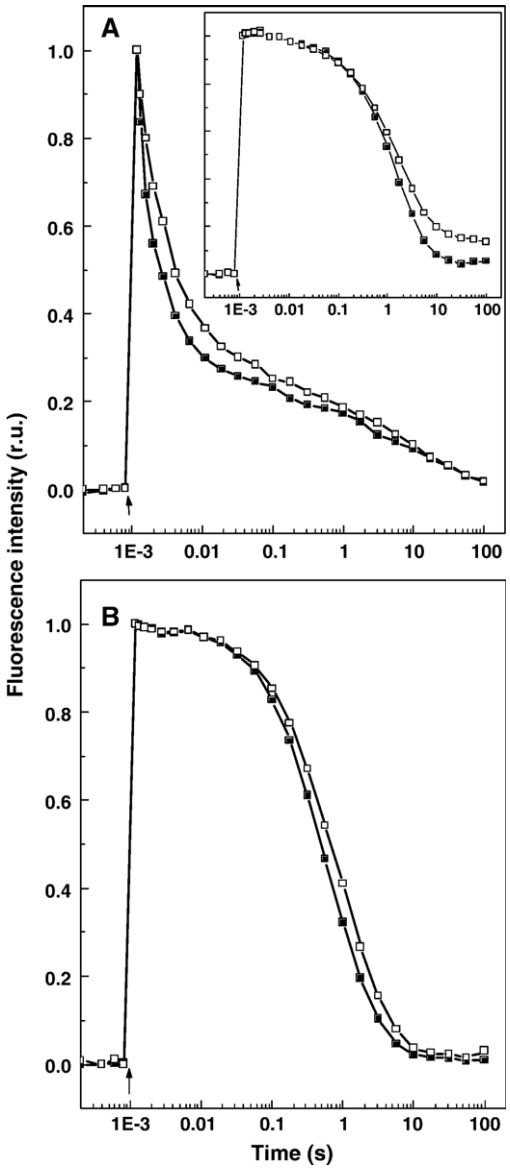

Fig. 1. Relaxation of flash-induced chlorophyll fluorescence yield in WT and ΔPsbR thylakoids (A) and leaves (B). The measurements were performed after a single flash excitation of WT (■) and ΔPsbR (□) thylakoids in the absence (A) and in the presence of 10 μM DCMU (A insert) and in intact leaves (B) in the presence of 50 μM DCMU. For the easy comparison, the fluorescence relaxation curves were normalized to the F_0 and F_m fluorescence level.

and arising from charge recombination between S_2 and Q_B^- (Fig. 2A). In ΔPsbR thylakoids the peak position of the B-band was slightly upshifted to 24 °C. The peak position of the main TL band (Q-band), obtained in the presence of DCMU and arising from $S_2Q_A^-$ charge recombination, was around 14 °C in WT. In ΔPsbR mutant thylakoids the Q-band was upshifted (17 °C) as compared to WT (Fig. 2B). Also the intensity of the main band of the TL glow curve in the mutant thylakoids was markedly lower than that in WT.

When excited with single turnover flashes, the amplitude of the B-band undergoes a period-four oscillation demonstrating the involvement of different oxidation states of the Mn-cluster of WOC [26]. Both the WT and mutant thylakoids showed typical oscillation with maxima at 2nd and 6th flashes and minima at 4th and 8th flashes, however, the ΔPsbR mutant thylakoids showed higher damping in the oscillation pattern (Fig. 3). After two and more flash excitations, the difference in the emission peak temperature of B-band between WT and ΔPsbR was nearly eliminated, which indicates that S_3 state of WOC was not affected in the mutant samples.

3.4. EPR spectroscopy

To gain more insights into the function of the donor side of the PSII complex in ΔPsbR thylakoids, we performed EPR measurements. Fig. 4A shows light minus dark EPR spectra from the WOC in the WT and ΔPsbR mutant. Illumination at 200 K efficiently accumulates all PSII centers in the S_2-state, which has a characteristic multiline EPR signal [25]. This signal has been reported to be very sensitive to small structural changes in the vicinity of the Mn-cluster [2,25,27]. However, lack of the PsbR protein did not affect the integrity of the Mn-cluster (Fig. 4). Estimation of the signal size in the base of Y_D^{ox} radical indicated that all PSII centers in the mutant produced the S_2-state multiline signal implying that S_1 to S_2 transition was not affected in the mutant (Table 1).

Cyt-b559 is an integral component of the PSII reaction center [28]. It has been proposed that Cyt-b559 is possibly involved in the protection of the PSII complex against donor- and acceptor-side photoinhibition as a 'molecular switch' by reversible conversion between high and low potential forms [29]. Here were examined how the lack of the PsbR protein affects the properties of Cyt-b559 in mutant thylakoid membrane using EPR spectroscopy. Usually, dark adapted membranes contain some oxidized Cyt-b559. Illumination at 77 K induces maximal oxidation of Cyt-b559, allowing estimation of the oxidized fraction in the dark adapted material [25,30]. In the dark adapted Arabidopsis WT thylakoids we observed a significant amount (55%) of oxidized Cyt-b559 and the dark adapted ΔPsbR

Table 2
Characteristic kinetic parameters of the FF-relaxation curve in the WT and ΔPsbR thylakoids

	y0	Fast phase τ (ms)/Amp (%)	Middle phase τ (ms)/Amp (%)	Slow phase τ (s)/Amp (%)
No addition				
WT	3.2±0.2	0.86±0.05/49.5±1.2	21.3±4.2/18.6±0.5	5.48±0.3/28.8±1.1
ΔPsbR	3.6±0.9	1.78±0.04/46.0±0.6	42.9±5.7/20.1±0.2	6.74±0.6/30.8±1.5
+ DCMU				
WT	6.5±0.8	3.2±0.9/0.5 ±0.02	–/0	1.74±0.08/93.2±1.4
ΔPsbR	20.3±2.4	18.2±1.5/3.0±0.6	–/0	2.27±0.04/76.7±0.9

Fluorescence curves measured as in Fig. 1, and analyzed as described in Materials and methods.

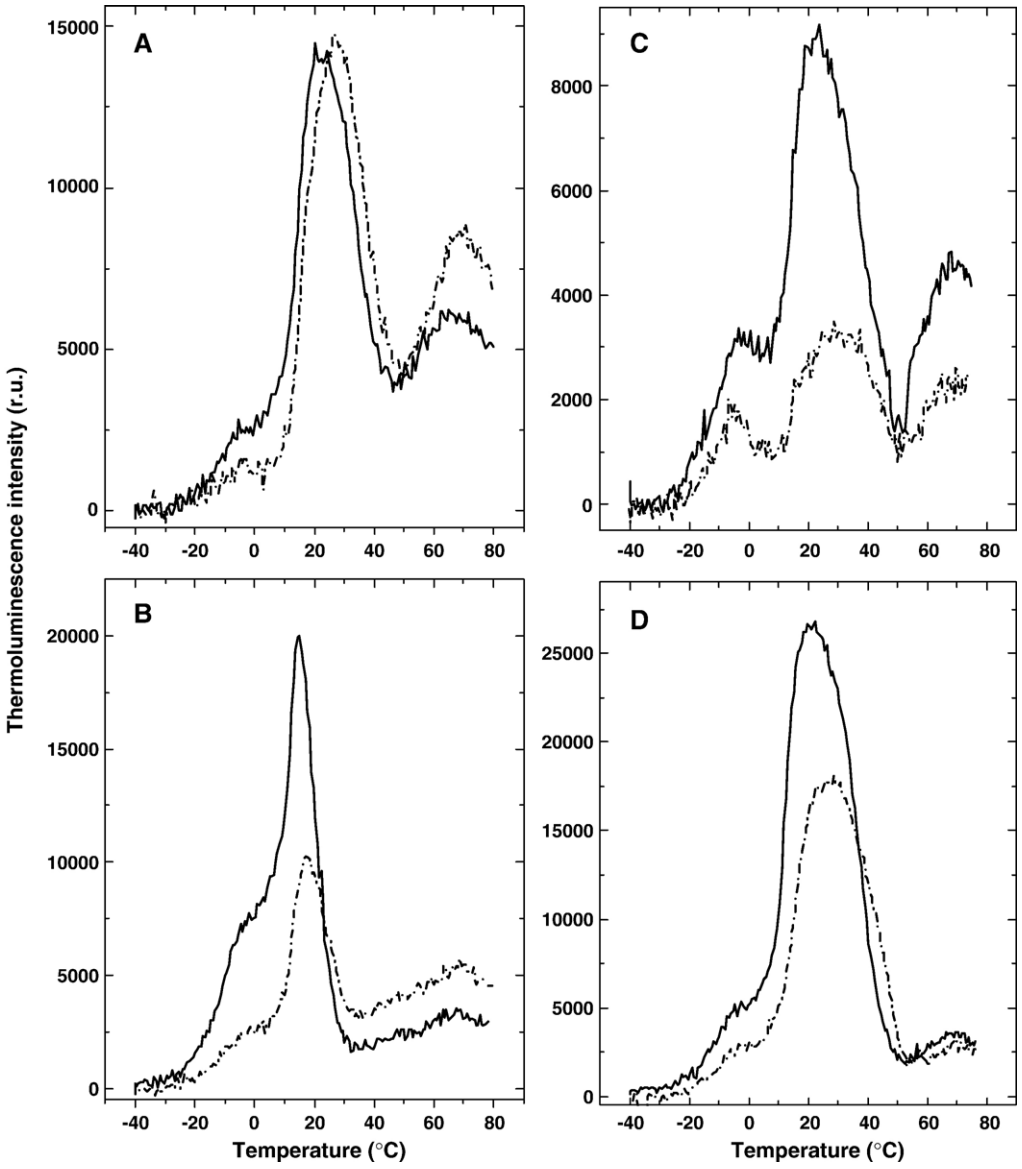

Fig. 2. Thermoluminescence characteristics of Arabidopsis WT and ΔPsbR thylakoids. The WT (solid line) and ΔPsbR (dotted line) thylakoids were excited with one flash at −10 °C in the absence (A, C) and in the presence of 40 μM DCMU (B, D). (A, B) The measurements performed in medium containing 40 mM Hepes/NaOH pH 7.0, 10 mM $MgCl_2$, 10 mM NaCl, and 0.4 M sucrose. (C, D) $MgCl_2$ and 10 mM NaCl were excluded from the isolation and measuring buffer.

thylakoids contained even higher amount of oxidized Cyt-b559 (72%, Fig. 4).

3.5. Ionic effect on the ΔPsbR thylakoids

Since the lack of the PsbR protein induces significant reduction of the lumenal PsbP and PsbQ proteins [18], which are known as Ca^{2+} and Cl^- binding proteins [8], we tested the effect of these ions on electron transfer properties ΔPsbR and WT thylakoids.

Table 3 shows the effects of various mono- and divalent cations on the steady-state oxygen evolution rate of WT and ΔPsbR thylakoids. As expected, in the absence of salts from the measuring buffer, the oxygen evolution activity of normal WT thylakoids was inhibited to some extent, and conversely, the activity was restored by addition of different salt combinations restored the activity. Addition of NaCl, NaCl+$CaCl_2$, and NaCl+$MgCl_2$ to the measuring buffer reactivated oxygen evolution of the WT thylakoids to 113%, 124% and 115%, respectively (Table 3). Interestingly, additions of salts had nearly the same reactivation effect on the oxygen evolution rate in ΔPsbR and in WT thylakoids (Table 3). However, even the maximal activity of ΔPsbR thylakoids measured in the presence of 5 mM NaCl and 5 mM $MgCl_2$ demonstrated only 66% of oxygen evolution activity of the WT thylakoids.

To evaluate the effect of added ions on oxygen evolution more precisely and to eliminate the background ions for some extent, we isolated thylakoid membranes in the absence of salts. (It should be noted, however, that there is a fixed background of [Na^+] from the NaOH used to adjust the pH of the basic medium). It is known that the medium containing no divalent cations and only low concentrations of monovalent cations

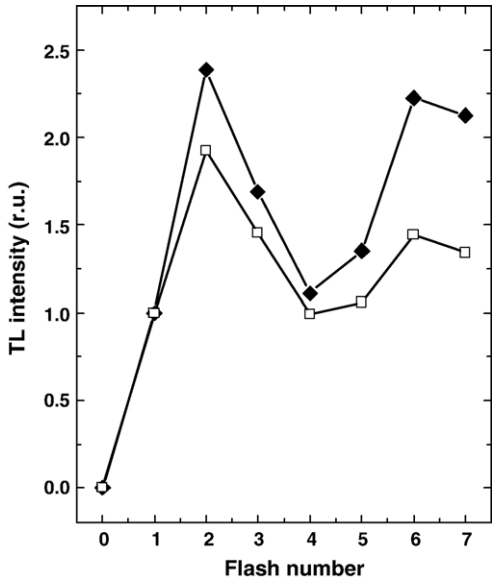

Fig. 3. Flash induced oscillation of the thermoluminescence B-band in Arabidopsis WT and ΔPsbR thylakoids. Samples were excited by a series of single saturating flashes at −10 °C and thermoluminescence B-band was measured from WT (■) and ΔPsbR (□) thylakoids.

induces an unstacking of the thylakoid membranes [31]. In the absence of salts, the initial oxygen evolution rate of the ΔPsbR thylakoids was only 40% of that observed in WT. Upon addition of NaCl, $MgCl_2$ and/or $CaCl_2$, a more prominent restoration of oxygen evolution occurred in ΔPsbR thylakoids than in similarly treated WT thylakoids. Addition of NaCl, $MgCl_2$ and $CaCl_2$ reactivated oxygen evolution in WT and ΔPsbR thylakoids to 123% and 189%, respectively. In the presence of NaCl and $MgCl_2$, WT unstacked thylakoids showed reactivation to 130%, whereas in the ΔPsbR thylakoids reactivation of oxygen evolution reached 186%.

In the media containing 20 mM NaCl and 10 mM $MgCl_2$, the WT thylakoids showed a typical sigmoidal shape of the FF-relaxation curve in the presence of DCMU, as described above (Fig. 1). Interestingly, the same WT thylakoids in the absence of salts showed two times smaller amplitude of variable fluorescence and a non-sigmoidal shape of the FF-relaxation curve (Fig. 5 and also Table 2). Addition of 20 mM NaCl slightly changed the shape of the FF-relaxation curve but the amplitude of the total fluorescence yield was not affected. On the contrary, addition of NaCl at high concentration (100 mM) leading to restacking of thylakoids, increased the relative amplitude of the fluorescence yield and also restored the sigmoidal shape of the FF-relaxation curve in the presence of DCMU. This indicates that prominent stacking of thylakoids is required for the sigmoidal shape of the FF-relaxation curve in the presence of DCMU. Including 10 mM $CaCl_2$ in the media further increased the total amplitude of fluorescence but 5 mM $MgCl_2$ was most effective in restoring the fluorescence parameters. It has been established that cation induced increase in chlorophyll fluorescence yield is related to changes in the degree of energy transfer between the light harvesting chlorophylls of PSI and PSII [32,33]. Investigation of the FF-relaxation in ΔPsbR thylakoids demonstrated similar effects of the studied ions on the fluorescence parameters as in WT. However, in ΔPsbR thylakoids the Mg^{2+} ion was slightly more effective in restoring the fluorescence yield.

Table 3
The effects of various combinations of salts on oxygen evolution in WT and ΔPsbR thylakoids

	Oxygen evolution (μmol O_2 per mg Chl × h)				
	HS	HSN	HSNC	HSNCM	HSNM
WT	233 (100%)	264 (113%)	288 (124%)	261 (112%)	267 (115%)
ΔPsbR	126 (100%)	150 (119%)	160 (128%)	148 (117%)	176 (140%)
R/WT	0.54	0.57	0.55	0.57	0.66
WT*	200 (100%)	–	–	247 (123%)	259 (130%)
ΔPsbR*	80 (100%)	–	–	151 (189%)	149 (186%)
R*/WT*	0.40	–	–	0.61	0.58

Measurement of oxygen evolution was performed in following media: HS—40 mM HEPES/NaOH pH 7.0, 0.4 M sucrose; HSN—HS + 5 mM NaCl; HSNM—HS + 5 mM NaCl + 5 mM $MgCl_2$; HSNC—HS + 5 mM NaCl + 25 mM $CaCl_2$; HSNCM—HS + 5 mM NaCl + 5 mM $MgCl_2$ + 25 mM $CaCl_2$. WT* and PsbR*, unstacked thylakoids isolated in the absence of salts.

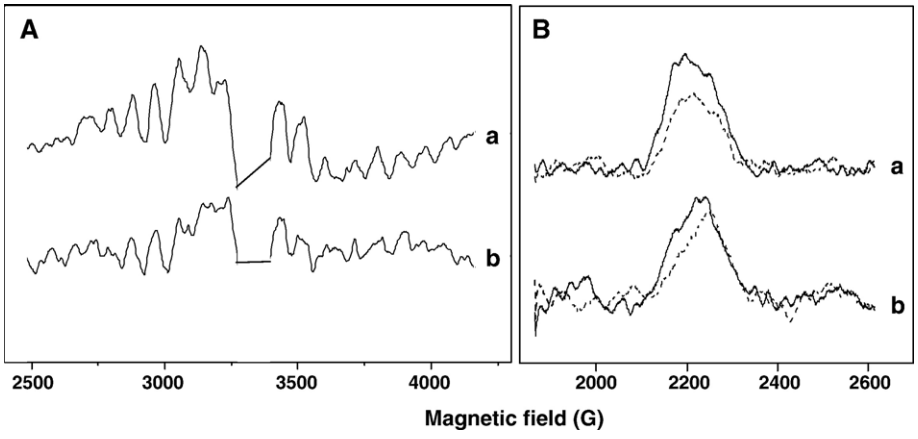

Fig. 4. Light minus dark EPR spectra of (A) the S2-state multiline signal and (B) of g_z region of oxidized Cyt-b559 (dark spectra—dashed line, after illumination at 77 K—solid line) from the WT (a) and ΔPsbR mutants (b). The middle part of the spectra in panel A with large absorption from Y_{Dox} was omitted. EPR conditions: microwave frequency 9.43 GHz, microwave power 10 mW (A) and 5 mW (B), modulation amplitude 15 G, temperature 7 K (A) and 15 K (B).

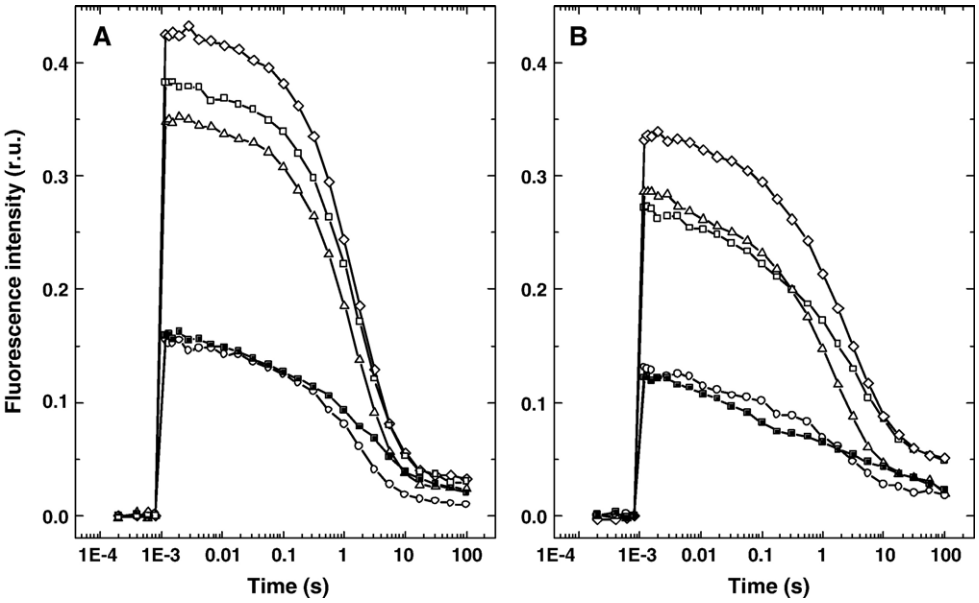

Fig. 5. Effect of various salts on the relaxation of flash-induced chlorophyll fluorescence yield in WT (A) and ΔPsbR (B) thylakoids. Measurements were performed in the presence of 10 μM DCMU in following media: (■) 40 mM HEPES/NaOH pH 7.0, 0.4 M sucrose (HS); (○) HS+20 mM NaCl; (Δ) HS+100 mM NaCl; (□) HS+10 mM $CaCl_2$; (◊) HS+5 mM $MgCl_2$.

In order to clarify the possible effect of salts on TL glow curves we next repeated TL measurements using isolated unstacked thylakoid membranes and omitting the salts from the measuring buffer. The TL B-band obtained from ΔPsbR and WT thylakoids, was arising at 29 °C and 24 °C, respectively (Fig. 2C). Also TL Q-band in ΔPsbR thylakoids (27 °C) was more significantly upshifted to higher temperature than that in WT (21 °C) (Fig. 2D). With the addition of 10 mM NaCl and 10 mM $MgCl_2$ to the measuring buffer the peak temperature of B-band was downshifted to 25 °C and 23 °C, whereas Q-band was downshifted to 20 °C and 15 °C in ΔPsbR and WT thylakoids, respectively (data not shown).

It is important to note that in all conditions the peak temperatures of the main TL bands of ΔPsbR thylakoids were higher than those of WT. One should notice also that the intensity of the TL bands largely increased with addition of salts to the measuring buffer.

4. Discussion

To gain insights into the role of the PsbR protein in the function of the PSII complex, we performed detailed investigation of the electron transport features of the ΔPsbR mutant plant. It is shown that the absence of the PsbR protein clearly modifies both the acceptor and the donor side of the PSII complex indicating that PsbR is essential for proper function of the PSII complex. Moreover, the fact that the amount of PSII complexes in the ΔPsbR mutant is clearly lower than in WT strongly suggests instability of the PSII complex in the absence of the PsbR protein. The lack of the ΔPsbR protein apparently leads to conformational changes in the PSII complex, thereby modifying both the donor and acceptor side properties of PSII.

A characteristic feature of the ΔPsbR mutant thylakoids is a slower electron transfer from Q_A to Q_B, which was revealed by slower time constants of the fast and middle phases of the FF-relaxation curve as compared to WT (Table 2, Fig. 1). In addition, a decrease in the relative amplitude of the fast relaxation phase and an increase in the relative amplitude of the middle phase in ΔPsbR thylakoids point to a lower amount of PSII centers which have an occupied Q_B site at the moment of giving the flash and also a limited PQ pool compared to WT.

The slow phase of the FF-relaxation curve measured in the absence of DCMU reflects the recombination of the S_2 state with Q_B^- via the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium. Increased time constant of this phase in ΔPsbR thylakoids compared to WT, points to the stabilized $S_2Q_B^-$ charge pair. Indeed, the peak position of TL B-band ($S_2Q_B^-$ recombination) also slightly shifted to higher temperature in ΔPsbR thylakoids compared to WT (Fig. 2).

In the presence of DCMU, the FF-relaxation curve is an indicator of the donor side status of the PSII complex. Overall slowdown of the FF-relaxation kinetics was typical to ΔPsbR thylakoids. The dominant phase of the FF-relaxation curve, which arises from $S_2Q_A^-$ recombination, showed slower time constant in ΔPsbR thylakoids, which indicates a stabilization of the $S_2Q_A^-$ charge pair in the ΔPsbR mutant thylakoids relative to WT. This result is also consistent with the TL measurements showing slight upshift in the peak position of the Q-band ($S_2Q_A^-$). One should also notice that the equilibrium constant for sharing an electron between Q_A and Q_B, calculated from the time constants of the slow phases of FF-relaxation curve in the absence and in the presence of DCMU, revealing ($S_2/(Q_AQ_B)^-$) and ($S_2Q_A^-$) charge recombination, respectively, decreased in ΔPsbR thylakoids.

The fast phase of the FF-relaxation curve, in the presence of DCMU, is expected to reflect charge recombination between Q_A^- and the electron donors, which are less stable than the S_2 state [22,34,35]. Such phase has in particular been assigned to

the $Y_z^{\cdot}Q_A^-$ recombination when the donor side is impaired by mutations [34,35,36] or by exposure to UV-B light [22]. In WT thylakoids the relative amplitude of this fast relaxation component (3.2 ms) is only about 0.5% of total fluorescence. The situation is clearly different in ΔPsbR thylakoids with fast relaxation phase being significantly slower (18.2 ms) and representing as much as 3% of the whole fluorescence relaxation (Table 1). This phenomenon is similar to the previously reported fluorescence relaxation times in several mutant and Tris-treated cells [36]. It is assumed that in ΔPsbR thylakoids the electron donation from the Mn-cluster to Y_z is slowed down and thus the $Y_zQ_A^-$ recombination competes with the photooxidation of Mn. Furthermore, the increased proportion of fluorescence that does not decay during the 100 s time span of the measurement indicates higher amount of $Y_D^+Q_A^-$ recombination and thus confirms the donor side modification in the ΔPsbR mutant. The TL B-band of ΔPsbR thylakoids showing a period four oscillation pattern with higher damping, points to the functional but slightly modified S-cycle of WOC (Fig. 3). The upward shift of the peak temperatures of the main TL bands after 1 flash occurred both in the absence and in the presence of DCMU indicating that in ΔPsbR thylakoids the S_2-state of WOC has different redox properties than in WT. Moreover, the lack of differences in the peak temperature of the B-band from ΔPsbR and WT thylakoids after 2 and more flashes, confirms our proposal that the S_2-state of WOC is stabilized and the S_3-state is not affected by mutation.

EPR measurements, however, revealed that the S_2-state multiline was induced in all centers and the shape of the signal was not affected in ΔPsbR thylakoids (Fig. 4). This implies that possible alteration of the donor side reactions in the PSII complex due to the lack of the PsbR protein, must take place in the late S-state transitions. Taking into account the fact that the PsbR protein acts in modulating the binding of peripheral subunits of WOC [18], we suggest that it can in principle also modulate the proton release from WOC. This would have no affect on the formation of the S_2 multiline EPR signal, since it does not require deprotonation.

The modifications of the donor side reactions, as discussed above, lead to a requirement of alternative electron donors in PSII [37]. Increased fraction of dark oxidized Cyt- b559 in ΔPsbR thylakoids indicates such requirement. This oxidized fraction most probably contains both low and high potential forms of Cyt-b559. We could not distinguish between these two forms at present stage since the *g*-values for the high potential and the low potential forms in Arabidopsis thylakoids are not known and therefore special investigations would have been required. However, it is highly likely that Cyt-b559 was mostly in the high potential form.

For explanation of the modified acceptor side electron transport kinetics, we speculate that the lack of the PsbR protein induces donor side modification in PSII, which is transmitted by conformational re-arrangement of the PSII complex to the acceptor side or vice versa.

It was recently proposed that PsbR is an important link in the PSII core complex for stable assembly of the WOC protein PsbP and also indirectly for PsbQ [18]. It is worth noting that the modifications revealed in the photosynthetic electron transport properties of the PSII complex in ΔPsbR mutant can result from limited amounts of PsbP and PsbQ proteins present in the mutant thylakoids. These two extrinsic proteins – PsbP and PsbQ – have been proposed to facilitate the binding of Ca^{2+} and Cl^- cofactors, in the WOC [8,9]. Indeed our experimental results show that addition of salts restores to a large extent the oxygen evolution activity of ΔPsbR thylakoids. However, the activity of the PSII complex remained lower in ΔPsbR thylakoids compared to WT even after restoration of the oxygen evolving activity by the addition of salts into the measuring media. Also, thylakoids isolated from high light grown plants, where the PsbP and PsbQ content of the thylakoids is higher than in our experimental conditions, still exhibited the same electron transport modifications (data not shown). We conclude that modification revealed in the ΔPsbR thylakoids are not due to the effect of a low content of PsbP and PsbQ proteins, but the PsbR protein itself plays a major structural role in the conformational changes of the PSII complex and thus modifies both sides of the PSII complex.

Taken together, we conclude that the PsbR protein has two particular distinct roles in the function of PSII complex. PsbR clearly stabilizes the PSII complex affecting the properties of both the acceptor and donor side electron transfer reactions. Moreover, PsbR is crucial in providing the additional ion concentration for the function of WOC, which is likely to occur by stabilizing the binding of the PsbP and PsbQ proteins in the PSII complex.

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