Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1767 (2007) 905–912
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbabio

Spermine and spermidine inhibition of photosystem II: Disassembly of the oxygen evolving complex and consequent perturbation in electron donation from Tyr_Z to $P680^+$ and the quinone acceptors Q_A^- to Q_B

Rémy Beauchemin, Alain Gauthier, Johanne Harnois, Steve Boisvert, Sridharan Govindachary, Robert Carpentier *

Groupe de recherche en Biologie Végétale, Université du Québec à Trois-Rivières, CP 500, Trois-Rivières (Québec), Canada G9A 5H7

Received 3 November 2006; received in revised form 30 March 2007; accepted 5 April 2007
Available online 12 April 2007

Abstract

Polyamines are implicated in plant growth and stress response. However, the polyamines spermine and spermidine were shown to elicit strong inhibitory effects in photosystem II (PSII) submembrane fractions. We have studied the mechanism of this inhibitory action in detail. The inhibition of electron transport in PSII submembrane fractions treated with millimolar concentrations of spermine or spermidine led to the decline of plastoquinone reduction, which was reversed by the artificial electron donor diphenylcarbazide. The above inhibition was due to the loss of the extrinsic polypeptides associated with the oxygen evolving complex. Thermoluminescence measurements revealed that charge recombination between the quinone acceptors of PSII, Q_A and Q_B, and the S_2 state of the Mn-cluster was abolished. Also, the dark decay of chlorophyll fluorescence after a single turn-over white flash was greatly retarded indicating a slower rate of Q_A^- reoxidation.

Keywords: Electron transport; Photosystem II; Oxygen evolution; Fluorescence; Extrinsic polypeptides; Polyamines

1. Introduction

Polyamines are organic cations widely distributed in bacteria, plants, and animals. They are considered to take part in the regulation of basic processes in plants such as cell division, morphogenesis, stability, as well as responses to environmental and other stress conditions [1]. Polyamines such as putrescine, spermine, and spermidine were shown to be of importance for chloroplast development [2]. They play a major role in protecting thylakoid membrane integrity during senescence and various environmental stress conditions such as chilling, osmotic, UV-B, and photoinhibitory stress [3–7]. Hence, the amount and/or ratio of various polyamines may strongly vary during stress adaptation. For example, an increase of up to 300–900% in spermine and spermidine levels was observed in tobacco plants exposed to UV-B irradiation [8]. Also, it was shown that overexpression of spermidine synthase in Arabidopsis mutants improves tolerance to several stress conditions [9].

Treatment of intact leaves or isolated photosynthetic membranes with exogenous polyamines is usually beneficial for membrane integrity. For example, spermine could prevent lipid peroxidation of thylakoid membranes thus retaining their structural integrity [10]. The release of the manganese stabilizing protein (33 kDa, extrinsic polypeptide) of PSII, plastocyanin, and cytochrome *f* from thylakoid membranes caused by treatments with palmitoleic acid seems to be prevented by spermidine [11].

Polyamines are protonated at physiological pH and thus contain charged amino groups [12]. Due to their delocalized positive charges, they can form electrostatic links with protein, phospholipids, and nucleic acids [13]. In chloroplasts, polyamines also bind covalently to proteins through an enzymatic process that involves a plastidic transglutaminase [14,15]. The chloroplastic transglutaminase is calcium- and light-dependent being almost

Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1, 1-dimethylurea; FI, fluorescence induction; F_0, basal level of chlorophyll fluorescence; F_m, maximal level of chlorophyll fluorescence; F_v, variable fluorescence; OEC, oxygen evolving complex; P680, primary electron donor of photosystem II; PS, photosystem; Q_A and Q_B, primary and secondary quinone acceptors of photosystem II; T_m, temperature of maximal thermoluminescence emission

* Corresponding author. Tel.: +1 819 376 5011; fax: +1 819 376 5057.
E-mail address: Robert.Carpentier@uqtr.ca (R. Carpentier).

0005-2728/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2007.04.001

non functional in the dark [5,14]. Apoproteins of the light-harvesting chlorophyll a/b complexes of PSII were proposed as substrates for the transglutaminase reaction [16]. Spermine is the most efficiently conjugated polyamine to isolated light harvesting complex proteins of PSII [14]. Native thylakoid membranes were also found to contain various concentrations of the three main polyamines, putrescine, spermine, and spermidine. Putrescine enrichment was observed in the antenna complexes of PSII, while spermine accumulates in the PSII reaction center complex [17].

Even though the influence of polyamines in plants and chloroplast was shown to be beneficial in nature, it was reported that interaction of spermine and spermidine with isolated PSII submembrane fractions elicited a strong inhibition of oxygen evolution [18]. However, the mechanism of inhibitory action of polyamines in PSII was not studied previously. Because the polyamine level in leaves can increase considerably under stress conditions, it is important to understand the different modes of action of these cations within the photosynthetic apparatus. In the present work, the interaction of spermine and spermidine with the oxygen evolving complex (OEC) of PSII is analyzed in detail. With polyamines, extrinsic polypeptides were released and the Mn_4CI complex was dysfunctional. Consequently, the electron transport on both donor and acceptor sides of PSII was strongly affected.

2. Materials and methods

2.1. Isolation of photosystem II submembrane fractions

PSII submembrane fractions were isolated from spinach (*Spinacea oleracea* L.) according to Berthold et al. [19] with the modification described elsewhere [20]. The PSII preparations were finally suspended in 400 mM sucrose and 20 mM MES-NaOH (pH 6.3) and were stored at −80 °C until use.

2.2. Oxygen evolution

Oxygen evolution was measured at 22 °C using Oxylab system (Hansatech Instruments, Norfolk, England). The assay medium contained 400 mM sucrose, 20 mM MES-NaOH (pH 6.3), 1 mM NaCl, 0.5 mM $MgCl_2$, 0.35 mM 2,6-dichlorobenzoquinone as PSII electron acceptor, 25 μg Chl mL^{-1} and the specified concentrations of polyamine. The control oxygen evolution rates were 700–800 μmol O_2 mg Chl^{-1} h^{-1}.

2.3. Fluorescence induction

Fluorescence induction (FI) measurements were performed at room temperature using Plant Efficiency Analyser (Hansatech, King' Lynn, Norfolk, UK). The assay medium contained 400 mM sucrose, 20 mM MES-NaOH (pH 6.3), 15 mM NaCl, 10 mM $MgCl_2$, 25 μg Chl mL^{-1} and the specified concentrations of polyamine. Dark-adapted samples were excited with saturating red actinic light (655 nm and an intensity of 3000 μmol m^{-2} s^{-1}) provided by light emitting diodes. As the fluorescence signal during the first 40 μs is ascribed for artifacts due to delay in response time of the instrument, these data were not included in analyses of FI traces. The signal at 40 μs is taken as F_0, the initial fluorescence. Variable fluorescence, F_v (the difference between F_0 and the maximal fluorescence, F_m in dark adapted samples), was used to calculate the F_v/F_m and F_v/F_0 ratios.

2.4. Thermoluminescence

Thermoluminescence measurements were carried out with a laboratory built equipment described in details elsewhere [21,22]. Photosystem II submembrane fractions (25 μg Chl mL^{-1}) were used in a medium containing 50 mM Tricine–NaOH (pH 7.8), 400 mM sorbitol, 10 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$ and the specified concentrations of polyamine. About 200 μL of the diluted suspension was added to the sample well (15 mm diameter) positioned just above a Peltier plate. All measurements were performed in the dark. Dark-adapted samples were incubated for 120 s at 20 °C. Following this step, temperature was brought down to 0 °C within 5–10 s and kept for 60 s. This incubation temperature was selected in order to avoid freezing induced damages to the OEC that usually gives rise to artifacts [21]. A probing single turn over saturating white flash of about 1-μs width (setting 10) from XE-ST Pump Flash unit (Walz, Effeltrich, Germany) was applied to initiate charge separation in PSII. During the last step of the measurements, linear warming of samples in total darkness activated the recombination of PSII charge pairs that can be detected by the appearance of emission bands with characteristic temperature optima. The data were analyzed using ThermoLite, a software developed in our laboratory for the purpose of working with Windows OS [22]. This software is an up-graded version of the original one [22,23].

2.5. Flash-induced fluorescence decay kinetics

In order to detect the reduction and oxidation kinetics of Q_A, Chl fluorescence rise and its relaxation in the dark were measured with a pulse amplitude double modulated fluorometer (PAM, Walz, Effeltrich, Germany) as described previously [24,25]. PSII submembrane fractions (Chl concentration of 25 μg/mL) were incubated for 3 min at room temperature in complete darkness without or with polyamines before initiating the fluorescence measurements. Reduction of Q_A was obtained using a single turn over saturating flash generated from the XST 103 flash lamp (Walz, Effeltrich, Germany). The flash reached its maximum intensity within 8 μs and decayed completely in 50 μs. Since the actual fluorescence signal stabilized 100 μs after the flash was applied, the data pertaining to this time-period were not used for the analyses of Q_A^- oxidation kinetics. The fluorescence signal was collected with the PDA 100 data acquisition system through WinControl software (Walz, Effeltrich, Germany) using a 20 μs/data point window with measuring light modulated at 100 kHz. The duration of the measuring time with the weak modulated light at a frequency of 1.6 kHz and the measuring light modulated at 100 kHz was less than 290 ms. The signal to noise ratio was improved by measuring the Chl fluorescence rise and its decay in the dark from nine independent samples of the same preparation. The traces were averaged with WinControl software to estimate the half-times and amplitudes of the fluorescence decay components using the following three-exponential function:

$$F(t) - F' = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + A_3 e^{-k_3 t}$$

where $F(t)$ is the fluorescence value at time t, k_n is the rate constant, A_n is the amplitude of the fluorescence relaxation phases, and F' is the stable minimal fluorescence at the end of the decay. The fluorescence decay curves from control samples could not be fit with a bi-exponential function.

2.6. Polyacrylamide gel electrophoresis

To determine the polypeptides released by the polyamines, the PSII preparations were incubated for 5 min at room temperature in the presence of spermine or spermidine and harvested immediately by a 5-min centrifugation (12,400 rpm) in an Eppendorf microcentrifuge. The pellets were washed twice in 20 mM MES-NaOH (pH 6.2) and used for polypeptide analysis. The first supernatants were further centrifuged (12,400 rpm for 5 min) to remove remaining membrane fragments. Tris-alkali extraction of the 17, 23 and 33 kDa polypeptides was carried out following the standard procedure of Nakatani [26]. The supernatants of the polyamine or Tris-alkali treated PSII submembranes were concentrated against sucrose using Spectra/Por molecularporous membranes (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) before analysis by polyacrylamide gel electrophoresis. The latter was performed at room temperature using miniature slab gels (Bio-Rad Laboratories, Hercules, California) containing 13% acrylamide and 6 M urea. The gels were stained with Coomassie brilliant blue and the polypeptide content was analyzed with the Gel-Doc 2000 system (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Manganese determination

The manganese content was determined in both pellet and supernatant fractions of spermidine and spermine-treated PSII using Analyst 100 Atomic Absorption Spectrophotometer (Perkin Elmer, Wellesley, Massachusetts, USA) as described previously [27].

3. Results

The polyamines spermine and spermidine added to isolated PSII submembrane fractions strongly inhibited oxygen evolution. Sub-millimolar concentrations above 0.5 mM of spermidine or spermine already caused measurable inhibition of electron transport with 2,6-dichlorobenzoquinone as an electron acceptor (Fig. 1). About 50% of the oxygen evolution was lost with 2 mM spermidine or spermine. This inhibition by polyamines was studied in detail using various techniques in order to establish their site of action as described below. A similar inhibition was observed in whole thylakoid membranes. However, we have used PSII submembrane fractions that represent a more simple and specific system without interference from other components of the thylakoid membrane.

The Chl fluorescence parameters F_m (the maximal fluorescence observed in dark-adapted samples exposed to saturating red-light illumination) and F_0 (the initial fluorescence level) were measured in PSII submembrane fractions treated with various polyamine concentrations. F_0 was not affected but F_m greatly declined (data not shown). This decline in F_m observed above 1 mM of spermidine corresponded with a decrease in F_v/F_0 (Fig. 2A), a parameter that accounts for the simultaneous variations in F_m and F_0 in determinations of the maximum quantum yields of PSII [28]. Importantly, a steep decrease in

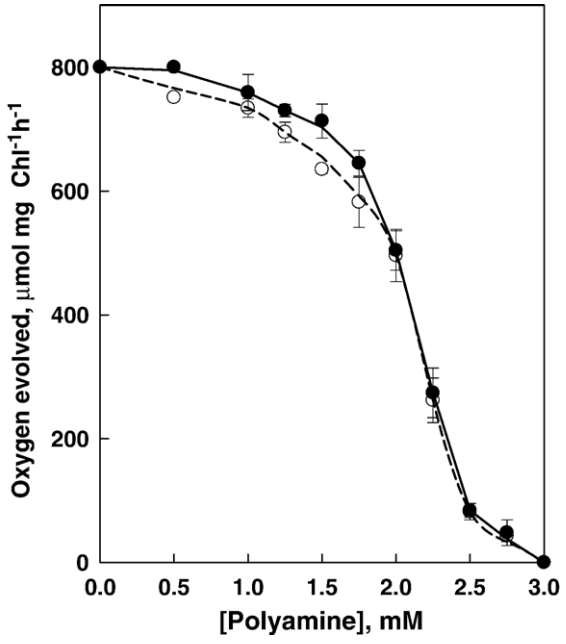

Fig. 1. Inhibition of oxygen evolution activity in PSII submembrane fractions after a 5-min incubation with various concentrations of spermine (open circles) or spermidine (closed circles). Other conditions are given in Materials and methods.

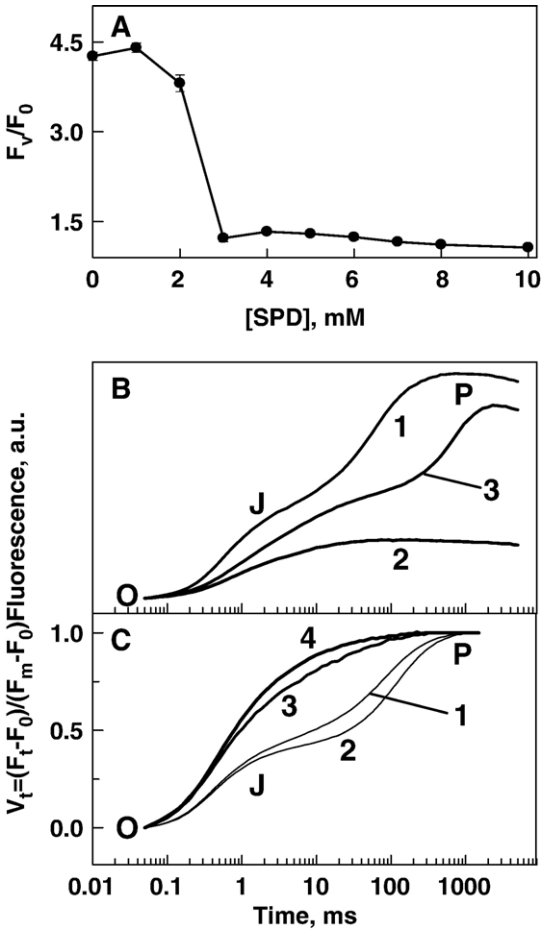

Fig. 2. (A) Effect of increasing spermidine (closed circles) on the F_v/F_0 ratio in PSII submembrane fractions. (B) Fluorescence induction traces with diphenylcarbazide: (1) control; (2) 3 mM spermidine; (3) 3 mM spermidine and 1 mM diphenylcarbazide. (C) Fluorescence induction traces of PSII membranes treated with various concentrations of spermidine: (1) control; (2) 2 mM; (3) 4 mM; (4) 7 mM. All traces were normalized at both initial and maximal intensities.

F_v/F_0 ratio observed with 1–3 mM spermidine correlated with the inhibition of oxygen evolution illustrated in Fig. 1.

The Chl fluorescence properties of PSII submembrane fractions in the presence of polyamines were further subjected to the comprehensive analysis of fluorescence induction (FI) kinetics (Fig. 2B and C). The FI traces illustrate the progressive reduction of the quinones located at the acceptor side of PSII with three main phases named O–J, J–I, and I–P [29,30]. The induction traces of PSII submembrane fractions contained only two well defined phases (Fig. 2B and C, trace 1) because the J–I phase is negligible in these preparations [31,32]. The first phase (O–J) reflects the reduction of the primary quinone acceptor of PSII, Q_A, and the second phase (I–P, here J–P) is ascribed for the reduction of the plastoquinone pool together with reduction of the secondary quinone acceptor Q_B. Upon treatment with 3 mM spermidine, the FI was greatly damped (Fig. 2B, trace 2), showing that the OEC failed to provide electrons for PSII to reduce the quinone acceptors.

The artificial electron donor diphenylcarbazide restored the kinetics of Chl fluorescence in polyamine-treated samples

(Fig. 2B, traces 2 and 3). This electron donor provided electrons to PSII, though at a slower rate compared to a native OEC. As a result, the reduction of quinones was partially restored as shown by the recovery of FI. Diphenylcarbazide can donate electrons only when the extrinsic polypeptides associated with the OEC are lost from the luminal side of PSII and the Mn_4Ca complex necessary for water splitting becomes dysfunctional [33]. The positive action of diphenylcarbazide thus indicates strong perturbations of the OEC in the presence of polyamines.

In Fig. 2C, the FI traces are normalized at both minimal and maximal values (V_t curves). A faster rise of fluorescence observed in the V_t curves of PSII submembrane fractions incubated with 4 or 7 mM spermidine (Fig. 2C, traces 3 and 4) might be explained by a delayed rate of Q_A^- re-oxidation by Q_B caused by polyamines.

To clarify the extent of the polyamine action on the OEC, polyacrylamide gels were run with PSII submembrane fractions treated with both polyamines. PSII submembrane fractions were incubated for 5 min with polyamines and then centrifuged to separate the submembrane fractions from the polypeptides that were released in the supernatants as described in Materials and methods. The polypeptide profiles are shown in Fig. 3 (upper panel) for spermidine. Similar data were obtained for PSII submembrane fractions incubated with spermine (results not shown). The polypeptides in the spermidine-treated PSII submembrane fractions are shown in lane 3. Notably, three polypeptides were lost at the position that corresponded to the well known extrinsic polypeptides of apparent molecular weight of 17, 23, and 33 kDa associated with the OEC [34] as shown in native PSII preparations. The polypeptide profile of the supernatant obtained from the spermidine-treated samples further confirms the release of these polypeptides (Fig. 3, lane 4). About 50% of 33 kDa polypeptide was lost when PSII membranes (100 μg/mL) were treated with 8 mM spermidine (Fig. 3, lower panel) that corresponds to 2 mM spermidine used for oxygen evolution measurements (25 μg Chl/mL). The above illustrates the correlation between the release of extrinsic proteins and the loss of oxygen evolution presented in Fig. 1. We also submitted the PSII submembrane fractions to Tris-alkali treatment that specifically releases these extrinsic polypeptides. Note that the Tris-alkali treated PSII supernatant fractions contained the polypeptides (Fig. 3, lane 5) of migrating distances coinciding with the ones shown in the supernatant fractions of PSII incubated with polyamines (Fig. 3, lane 4). Also, the bands representing these polypeptides were only faintly seen in the protein profile of the pellets of PSII membrane fractions subjected to Tris-alkali treatment (Fig. 3, lane 6).

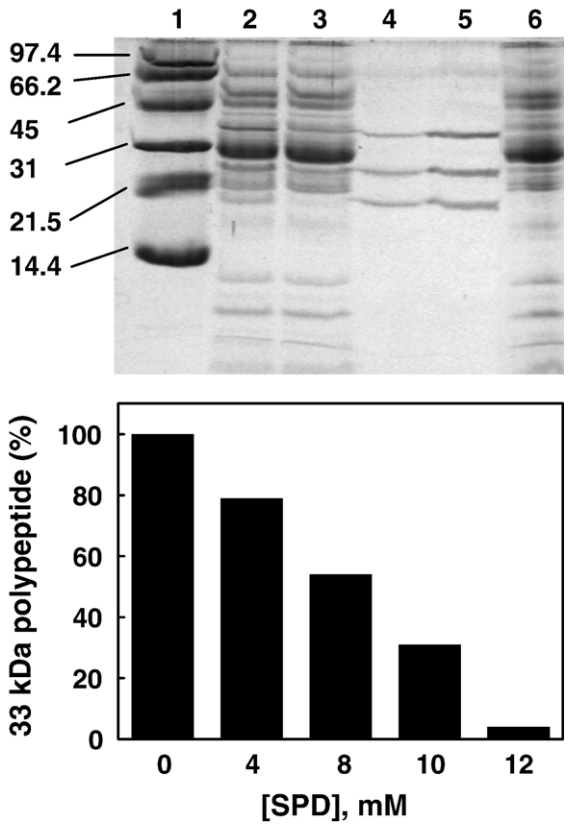

Fig. 3. Effects of a 5-min incubation of PSII submembrane fractions (100 μg Chl/mL) in the presence of spermidine on the polypeptide profile. Upper panel: Lane 1, molecular weight standards; lane 2, control PSII; lane 3, spermidine-treated PSII; lane 4, supernatant of spermidine-treated PSII; lane 5, supernatant of the Tris-alkali treated PSII; lane 6, Tris-alkali treated PSII. Numbers on the left side indicate apparent molecular masses (kDa) of the markers. Lower panel: 33 kDa polypeptide content of the PSII submembrane fractions as a function of spermidine concentration.

Thermoluminescence was used to characterize the charge recombination processes in the presence of polyamines. To initiate charge separation, PSII submembrane fractions were pre-illuminated at 0 °C with a 1-μs single turn-over white flash. The results obtained with spermine are presented in Fig. 4. Experiments with spermidine provided the same results (data not shown). With a linear increase in temperature at the rate of 0.5 °C/s, the amplitude of the thermoluminescence signal rapidly increased to attain its maximum (T_m) at 25 °C in control samples (Fig. 4A, trace 1). This major emission band corresponds to the B-band attributable to charge recombination between Q_B^- and the oxidized Mn-cluster predominantly in the S_2-state [35]. The T_m of the B-band seen in non-frozen control PSII samples is consistent with the peaking temperatures for the same preparations shown previously [36]. This band was progressively abolished as the concentration of spermine was raised (Fig. 4A, traces 2–5). Also, this was accompanied by a small shift of the T_m toward higher temperatures. With 10 mM spermine, the B-band was totally suppressed. The changes in the amplitude and T_m of the B-band could be related to changes in the hole properties of the S_n state of the Mn-cluster or to the inhibition of Q_B reduction in the presence of polyamines.

In order to segregate the effects of polyamines on the two-electron gate involving Q_B/Q_B^- couple, the inhibitor DCMU was used to prevent electron transfer between Q_A^- and Q_B. With DCMU, a major luminescent band with T_m of 18 °C was observed (Fig. 4B, trace 1) as shown previously [36]. This luminescent band is the Q-band that arises due to the back-flow of electrons from Q_A^- to the S_2-state. The peaking temperature of Q-band is somewhat higher at the concentration of Chl (25 μg Chl/mL) used. A T_m of 5–10 °C for Q-band in control PSII

samples could be seen only when the assay medium contained ~200 μg Chl/mL as indicated in several reports. The Q-band was shifted to higher temperatures in the presence of polyamines. A minor band, the C-band, also appeared at about 50 °C in the glow curves of the control samples (Fig. 4B, trace 1). This emission band originates from the recombination between Q_A^- and oxidized Tyr_D. The amplitude of these two bands also declined with polyamines. Also, the progressive loss of thermoluminescence intensity observed with increasing polyamine concentration was very similar for both B- and Q-bands (see inset of Fig. 4B). Thus, the strong inhibitory effect of polyamines on charge recombination was mainly due to the loss of the donor side partners for the above radiative recombination pathways.

The fluorescence induction traces indicated that the reoxidation of Q_A^- was retarded to some extent in the presence of polyamines (Fig. 2C). This possibility was verified using the fluorescence properties of PSII submembrane fractions submitted to a single turn over flash. Typical fluorescence decay kinetics is shown on a logarithmic scale in Fig. 5. The fluorescence rise induced by the flash is due to the reduction of Q_A and the decay ensured in the dark is related to the reoxidation of Q_A^-. The total amplitude of the fluorescence rise greatly declined as spermine concentration increased. Also, the fluorescence decay in the dark was slower as mentioned below.

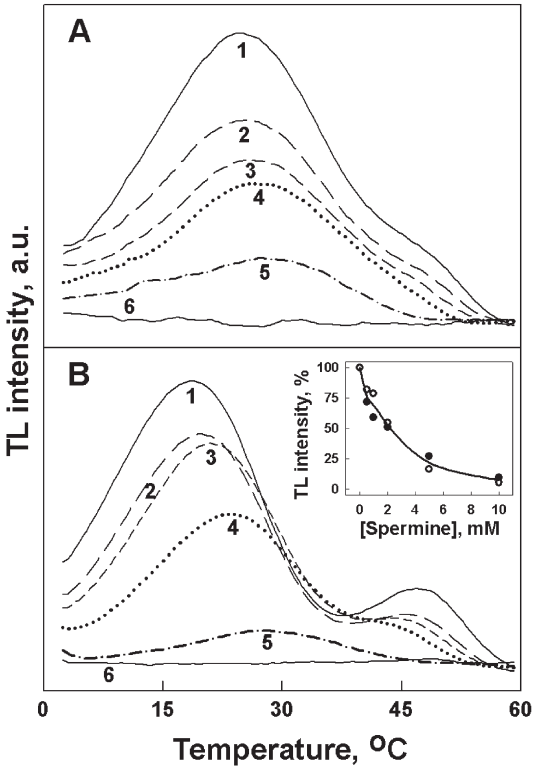

Fig. 4. Temperature profile of the thermoluminescence emission from PSII submembrane fractions in the absence (B-band) (A) or presence (Q- and C-bands) (B) of 5 μM DCMU with various spermine concentrations: 1, control; 2, 0.5 mM; 3, 1 mM; 4, 2 mM; 5, 5 mM; 6, 10 mM. Inset: Relative thermoluminescence intensity of the B-band (closed circles) and the Q-band (open circles) at various spermine concentrations.

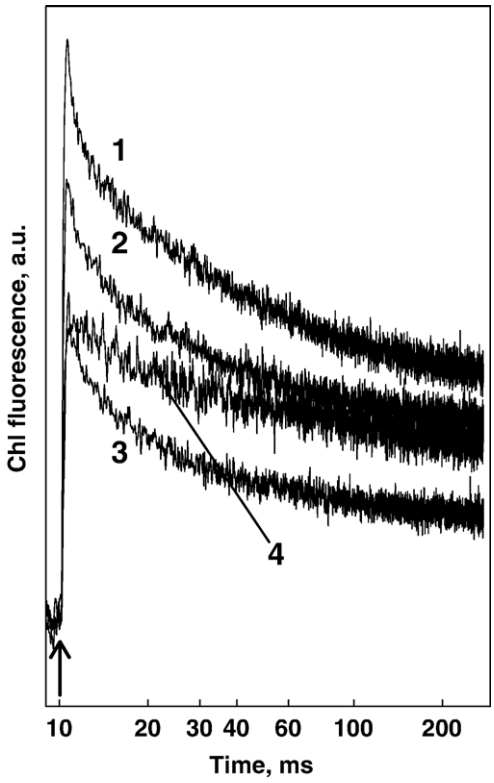

Fig. 5. Chl fluorescence decay kinetics of PSII submembrane fractions alone (trace 1) or treated with 1 (trace 2), 2 (trace 3) or 4 (trace 4) mM spermine. Upward arrow indicates the application of the single turn-over saturating white flash. Each trace is the average of nine independent measurements.

The dark decay of fluorescence in control samples was modeled with a three-exponential function. The amplitude and half-time of each component are shown in Table 1. The fast component with a half-time of ~0.66 ms is attributed to the reoxidation of Q_A^- by Q_B. The middle phase with a half-time of ~9.49 ms is ascribed for the reoxidation of Q_A^- in PSII centers with an empty Q_B pocket where the diffusion time of PQ to its binding site should be considered. The slow phase is attributed to the constant background fluorescence as a shorter time of ~290 ms was used for decay measurements. The half-times of the fast component greatly increased in PSII submembrane fractions incubated with 1 mM spermine. With higher spermine concentration, the fast component was completely lost (Table 1).

Table 1
Half-times ($t_{1/2}$, in ms) and amplitudes (A) of the three exponential components of the Chl fluorescence decay in PSII submembrane fractions (25 μg/mL) incubated for 3 min the dark at 20±2 °C in the absence or presence of spermine

Spermine	Fast phase		Middle phase		Slow phase[a]		A_{total}
	A (%)	$t_{1/2}$ (ms)	A (%)	$t_{1/2}$ (ms)	A (%)	$t_{1/2}$ (ms)	(%)
No addition	33.03	0.66	39.27	9.49	27.70	68.1	100
1 mM	30.25	1.78	38.53	11.52	31.22	66.1	100
2 mM	–	–	63.82	4.21	36.18	40.7	100
4 mM	–	–	50.45	5.96	49.55	87.9	100

The amplitudes and $t_{1/2}$ of each component were calculated from the averaged trace of nine independent measurements of the PSII sub-membrane fractions.
[a] This slow phase is considered as the constant background fluorescence.

The above confirms that Q_A^- reoxidation was much slower in polyamine-treated samples as the electron transfer between Q_A^- and Q_B was highly retarded.

4. Discussion

The data presented here clearly demonstrate a strong inhibition of electron transport in isolated PSII submembrane fractions by millimolar range of the polyamines, spermine and spermidine. The polyamine putrescine, a dication, was shown to enhance PSII electron transport in a manner similar to Mg^{2+} in low salt chloroplast membranes due to its coulombic effect [37]. However, this type of cationic effect was avoided by the use of physiological cation concentrations in our experiments [37]. The polyamines strongly interacted with the luminal side of the photosystem causing the release of the three extrinsic polypeptides of 17, 23 and 33 kDa associated with the OEC (Fig. 3). At physiological pH, these polycations are protonated with 4 positive charges for spermine and 3 positive charges for spermidine. Studies using Fourier transform infrared difference spectroscopy have shown that their binding to PSII proteins causes significant alteration of the protein secondary structure [18]. Such changes could have produced the release of the extrinsic polypeptides. Several divalent cations such as Cu^{2+}, Cd^{2+}, Hg^{2+}, Zn^{2+}, and Pb^{2+}, were also shown to release the extrinsic polypeptides of PSII to various extents [38–41] and therefore polyamines may act similarly in that respect. The inhibitory action of polyamines was also observed in whole thylakoid membranes (results not shown) demonstrating that the polyamines could diffuse into the thylakoid lumen as previously discussed for putrescine [37].

The two extrinsic polypeptides of 17 and 23 kDa modulate the affinity of the Ca^{2+} binding site [42,43]. Hence, Ca^{2+} is lost when these two polypeptides forming a barrier between the lumen and the calcium binding site [44] are released from the OEC by the action of polyamines. The other extrinsic polypeptide of apparent molecular mass of 33 kDa, also known as the manganese-stabilizing protein, is important for the integrity and function of the Mn_4Ca complex involved in water oxidation [45]. In its absence, two of the four Mn ions are often released and the oxygen evolving activity is lost unless high concentration (100 mM) of Cl^- is added. In the present case, however, it was verified by atomic absorption spectroscopy that no Mn was released from the PSII submembrane fractions even at high spermine or spermidine concentrations (data not shown). Also the inhibition could not be overcome by $CaCl_2$ (data not shown) as it is usually the case with toxic divalent cations such as heavy metals [38–41]. The inhibition by polyamines is thus different.

The release of the three extrinsic polypeptides in the presence of polyamines and thus the disassembly of the OEC are expected to modify electron transport within PSII. Indeed, we observed a lack of electron supply for reduction of the quinone acceptors of PSII as shown by FI measurements (Fig. 2B and C). Alteration of the OEC was demonstrated by the partial recovery of the O–J and J–P phases with diphenylcarbazide (Fig. 2B). This electron donor can gain access to its photo-oxidation site at Tyr_Z^+ only when the OEC is dysfunctional [33]. Thus, even though Mn was not lost, the Mn-cluster was disorganized by polyamine action. This is demonstrated by the impairment of quinone reoxidation through charge recombination as revealed by the polyamine-dependent loss of B and Q thermoluminescence bands (Fig. 4). The above indicates that the S_2 state of the Mn-cluster needed for recombination was lost with the perturbation of the OEC. An upshift in the T_m of thermoluminescence bands in PSII submembrane fractions was previously associated with an abnormal ligand environment of the Mn_4Ca complex [46]. Also, a large increase of the thermoluminescence temperature of the Q-band was reported in PSII submembrane fractions depleted in Cl^- and 33 kDa polypeptide due to a stronger stabilization of S_2 [47]. Hence, the shift of the T_m of thermoluminescence towards higher temperatures observed for both B- and Q-bands (Fig. 4) may originate from a population of PSII centers where the Mn_4Ca complex is stabilized in S_2 state by polyamines as this could represent an intermediate step in the disorganization of the OEC.

The data presented in Fig. 2C reveal that the FI was relatively fast at high polyamine concentration reflecting a reduced rate of Q_A^- reoxidation. Further, flash induced fluorescence decay measurements have shown that the half-times for reoxidation of Q_A^- increased with high concentrations of polyamine (Table 1). This clearly demonstrates that electron transfer from Q_A^- to Q_B was delayed. The total amplitude of the flash induced fluorescence rise was also decreased by polyamines (Fig. 5). This apparent loss of the amplitude of fluorescence indicates a fast decline of fluorescence that occurs much before the start of the decay measurement after 100 μs. We propose that this decline in the flash-induced fluorescence yield is due to recombination between Q_A^- and $P680^+$ in a fraction of the PSII centers. PSII centers with $P680^+$ are known to have a fluorescence yield close to F_0 [48]. The accumulation of $P680^+$ after a single flash illumination is only observed when electron transfer between the primary electron donor Tyr_Z and $P680^+$ is inhibited or delayed [49]. The decline in the rate of electron transfer from Tyr_Z to $P680^+$ in PSII centers that have lost the integrity of the OEC is consistent with the structural data indicating Tyr_Z is localized in close proximity with the Mn_4Ca complex [50,51]. Therefore, the specific orientation of Tyr_Z or its deprotonation that is needed for electron transfer to $P680^+$ may be hindered [52]. Alternatively, a direct interaction between polyamines and Tyr_Z can be postulated.

As can be expected, the restoration of quinone photo-reduction in polyamine-treated PSII submembrane fractions by diphenylcarbazide does not overcome the slower electron transfer from Tyr_Z to $P680^+$ or from Q_A^- to Q_B. Though the FI is partially recovered with the electron donor, it is clear from Fig. 2B that the induction in the ms time scale is greatly retarded owing to the slower electron transport reactions and also due to the weaker electron donation by diphenylcarbazide to Tyr_Z compared to a native OEC.

A binding site for polyamines near the quinones on the stromal side of PSII can be proposed to explain the retarded electron transfer between Q_A^- and Q_B. However, it was reported that Ca^{2+} release from the OEC changes the midpoint redox potential of Q_A and affect Q_A^- to Q_B electron transfer [53,54].

Increased half-times of Q_A^- reoxidation were observed in PSII submembrane fractions with a disorganized OEC such as in NaCl-treated PSII that are depleted of the two extrinsic polypeptides of 17 and 23 kDa and Ca^{2+} [24]. This was explained by a transmembrane conformational change that modifies the acceptor side of PSII reflecting a long-range allosteric coupling with the OEC. Similar alteration of the stromal side of PSII as a consequence of a transmembrane effect in polyamine-treated PSII is plausible as the polycations greatly affected the integrity of the OEC. The recent structural information about the stromal side of cyanobacterial PSII indicates that the Q_B pocket is formed by several transmembranous polypeptides including the polypeptide D1, cytochrome b_{559}, PsbL, and PsbJ [51]. Cytochrome b_{559} was proposed as an alternative electron acceptor preventing the over-reduction of the PSII acceptor side and as an auxiliary electron donor for oxidized chlorophylls of the reaction center [55,56]. The cytochrome is mostly in a high potential form in PSII complexes with an intact OEC but transformed into a low potential form when the OEC is damaged [57]. PsbL and PsbJ were shown to regulate electron flow to the plastoquinone pool with PsbJ specifically regulating the oxidation of Q_A^- [58]. These polypeptides are also involved in the assembly of the OEC [59] and, together with D1 and cytochrome b_{559}, they can therefore create a direct link between the functions of PSII on the luminal and stromal sides of the photosystem.

In conclusion, the action of polyamines on the OEC is more profound than Cl^- or Ca^{2+} depletion as $CaCl_2$ could not restore the activity. Also, even if Mn is not released, the loss of thermoluminescence shows that the S_2 state of the Mn cluster is not formed at relatively high polyamine concentrations and that the Mn_4Ca complex is disorganized.

Acknowledgment

This work was funded by the Natural Science and Engineering Research Council of Canada.

References

[1] P.T. Evans, R.L. Malmberg, Do polyamines have roles in plant development, Annu. Rev. Plant Physiol. Plant Mol. Biol. 40 (1989) 235–269.
[2] K. Kotzabasis, A role for chloroplast-associated polyamines, Bot. Acta 109 (1996) 5–7.
[3] K. Kotzabasis, D. Dornemann, Differential changes in the photosynthetic pigments and polyamine content during photoadaptation and photoinhibition in the unicellular green alga *Scenedesmus obliquus*, Z. Naturforsch C 53 (1998) 833–840.
[4] R.T. Besford, C.M. Richardson, J.L. Campos, A.F. Tiburcio, Effect of Polyamines on stabilization of molecular complexes in thylakoid membranes of osmotically stressed oat leaves, Planta 189 (1993) 201–206.
[5] J. Legocka, I. Zajchert, Role of spermidine in the stabilization of the apoprotein of the light-harvesting chlorophyll a/b-protein complex of photosystem II during leaf senescence process, Acta Physiol. Plant. 21 (1999) 127–132.
[6] L. Sfichi, N. Ioannidis, K. Kotzabasis, Thylakoid-associated polyamines adjust the UV-B sensitivity of the photosynthetic apparatus by means of light-harvesting complex II changes, Photochem. Photobiol. B80 (2004) 499–506.
[7] G. Szalai, T. Janda, T. Bartok, E. Paldi, Role of light in changes in free amino acid and polyamine contents at chilling temperature in maize (*Zea mays*), Physiol. Plant. 101 (1997) 434–438.
[8] C. Lütz, E. Navakoudis, H.K. Seidlitz, K. Kotzabasis, Simulated solar irradiation with enhanced UV-B adjust plastid- and thylakoid-associated polyamine changes for UV-B protection, Biochim. Biophys. Acta 1710 (2005) 24–33.
[9] Y. Kasukabe, L.X. He, K. Nada, S. Misawa, I. Ihara, S. Tachibana, Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress regulated genes in transgenic *Arabidopsis thaliana*, Plant Cell Physiol. 45 (2004) 712–722.
[10] E.Z. Stoynova, E.N. Karanov, V. Alexieva, Subcellular aspects of the protective effect of spermine against atrazine in pea plants, Plant Growth Regul. 29 (1999) 175–180.
[11] J.S. Peters, C.K. Chin, Inhibition of photosynthetic electron transport by palmitoleic acid is partially correlated to loss of thylakoid membrane proteins, Plant Physiol. Biochem. 41 (2003) 117–124.
[12] R.D. Slocum, R. Kaur-Sawhney, A.W. Galston, The physiology and biochemistry of polyamines in plants, Arch. Biochem. Biophys. 235 (1984) 283–303.
[13] F. Schuber, Influence of polyamines on membrane function. Biochem. J. 260 (1989) 1–10.
[14] M. la Mea, A. Di Sandro, L. Dondini, S. Del Duca, F. Vantini, C. Bergamini, R. Bassi, D. Serafini-Fracassini, A *Zea mays* 39-kDa thylakoid transglutaminase catalyses the modification by polyamines of light-harvesting complex II in a light-dependent way, Planta 219 (2004) 754–764.
[15] S. Del Duca, S. Beninati, D. Serafini-Fracassini, Polyamines in chloroplasts: identification of their glutamyl and acetyl derivatives, Biochem. J. 305 (1995) 233–237.
[16] S. Del Duca, V. Tidu, R. Bassi, C. Esposito, D. Serafini-Fracassini, Identification of chlorophyll-a/b proteins as substrates of transglutaminase activity in isolated chloroplast of *Helianthus tuberosus*, Planta 193 (1994) 283–289.
[17] K. Kotzabasis, C. Fotinou, K.A. Roubelakisangelakis, D. Ghanotakis, Polyamines in the photosynthetic apparatus. Photosystem II highly resolved subcomplexes are enriched in spermine, Photosynth. Res. 38 (1993) 83–88.
[18] A. Bograh, Y. Gingras, H.A. TajmirRiahi, R. Carpentier, The effects of spermine and spermidine on the structure of photosystem II proteins in relation to inhibition of electron transport, FEBS Lett. 402 (1997) 41–44.
[19] D.A. Berthold, G.T. Babcock, C.F. Yocum, A highly resolved oxygen evolving photosystem II preparation from spinach thylakoid membranes. EPR and electron transport properties, FEBS Lett. 134 (1981) 231–234.
[20] A. Rashid, M. Bernier, L. Pazdernick, R. Carpentier, Interaction of Zn^{2+} with the donor side of photosystem II, Photosynth. Res. 30 (1991) 123–130.
[21] J.M. Ducruet, Chlorophyll thermoluminescence of leaf discs: simple instruments and progress in signal interpretation open the way to new ecophysiological indicators, J. Exp. Bot. 54 (2003) 2419–2430.
[22] A. Gauthier, S. Govindachary, J. Harnois, R. Carpentier, Interaction of *N,N,N′,N′*-tetramethyl-p-phenylenediamine with photosystem II as revealed by thermoluminescence: reduction of the higher oxidation states of the Mn cluster and displacement of plastoquinone from the Q_B niche, Biochim. Biophys. Acta 1757 (2006) 1547–1556.
[23] J.M. Ducruet, T. Miranda, Graphical and numerical analysis of thermoluminescence and fluorescence F_0 emission in photosynthetic material, Photosynth. Res. 33 (1992) 15–27.
[24] I.I. Putrenko, S. Vasil'ev, D. Bruce, Modulation of flash-induced photosystem II fluorescence by events occurring at the water oxidizing complex, Biochemistry 38 (1999) 10632–10641.
[25] T.A. Ono, T. Noguchi, Y. Nakajima, Characteristic changes of function and structure of photosystem II during strong light photoinhibition under aerobic conditions, Biochim. Biophys. Acta 1229 (1995) 239–248.
[26] H.Y. Nakatani, Photosynthetic oxygen evolution does not require the participation of polypeptides of 16 and 24 kilodaltons, Biochem. Biophys. Res. Commun. 120 (1984) 299–304.

[27] S. Boisvert, D. Joly, S. Leclerc, S. Govindachary, J. Harnois, R. Carpentier, (In Press). Inhibition of the oxygen evolving complex of photosystem II and depletion of extrinsic polypeptides by nickel, BioMetals.
[28] F. Babani, H.K. Lichtenthaler, Light-induced and age-dependent development of chloroplasts in etiolated barley leaves as visualized by determination of photosynthetic pigments, CO_2 assimilation rates and different kinds of chlorophyll fluorescence ratios, J. Plant Physiol. 148 (1996) 555–566.
[29] D. Lazár, The polyphasic chlorophyll a fluorescence rise measured under high intensity of exciting light, Funct. Plant Biol. 33 (2006) 9–30.
[30] X.G. Zhu, N.R. Govindjee, E. Baker, D.R. deSturler, S.P. Ort, Chlorophyll a fluorescence induction kinetics in leaves predicted from a model describing each discrete step of excitation energy and electron transfer associated with photosystem II, Planta 223 (2005) 114–133.
[31] P. Pospišil, H. Dau, Valinomycin sensitivity proves that light-induced thylakoid voltages result in millisecond phase of chlorophyll fluorescence transients, Biochim. Biophys. Acta 1554 (2002) 94–100.
[32] P. Pospišil, H. Dau, Chlorophyll fluorescence transients of Photosystem II membrane particles as a tool for studying photosynthetic oxygen evolution, Photosynth. Res. 65 (2000) 41–52.
[33] M.L. Ghirardi, T.W. Lutton, M. Seibert, Interactions between diphenylcarbazide, zinc, cobalt, and manganese on the oxidizing side of photosystem II, Biochemistry 35 (1996) 1820–1828.
[34] A. Seidler, The extrinsic polypeptides of photosystem II, Biochim. Biophys. Acta 1277 (1996) 35–60.
[35] P.V. Sane, Thermoluminescence: a Technique for Probing Photosystem II, in: R. Carpentier (Ed.), Photosynthesis Research Protocols, Humana Press, Totova, NJ, USA, 2004, pp. 229–248.
[36] P.H. Homann, Reliability of photosystem II thermoluminescence measurements after sample freezing: few artifacts with photosystem II membranes but gross distortions with certain leaves, Photosynth. Res. 62 (1999) 219–229.
[37] N.E. Ioannidis, L. Sfichi, K. Kotzabasis, Putrescine stimulates chemiosmotic ATP synthesis, Biochim. Biophys. Acta 1757 (2006) 821–828.
[38] A. Rashid, E.L. Camm, A.K.M. Ekramoddoullah, Molecular mechanism of action of Pb^{2+} and Zn^{2+} on water oxidizing complex of photosystem II, FEBS Lett. 350 (1994) 296–298.
[39] M. Bernier, R. Carpentier, The action of mercury on the binding of the extrinsic polypeptides associated with the water oxidizing complex of photosystem II, FEBS Lett. 360 (1995) 251–254.
[40] C. Jegerschöld, J.B. Arellano, W.P. Schroder, P.J.M. Vankan, M. Baron, S. Styring, Copper(II) inhibition of electron transfer through photosystem II studied by EPR spectroscopy, Biochemistry 34 (1995) 12747–12754.
[41] I. Yruela, M. Alfonso, M. Baron, R. Picorel, Copper effect on the protein composition of photosystem II, Physiol. Plant. 110 (2000) 551–557.
[42] D.F. Ghanotakis, J.N. Topper, G.T. Babcock, C.F. Yocum, Water-soluble 17-kDa and 23-kDa polypeptides restore oxygen evolution activity by creating a high-affinity binding-site for Ca^{2+} on the oxidizing side of photosystem II, FEBS Lett. 170 (1984) 169–173.
[43] P. Adelroth, K. Lindberg, L.E. Andreasson, Studies of Ca^{2+} binding in spinach photosystem II using $^{45}Ca^{(2+)}$, Biochemistry 34 (1995) 9021–9027.
[44] C.F. Yocum, Calcium activation of photosynthetic water oxidation, Biochim. Biophys. Acta 1059 (1991) 1–15.
[45] T.M. Bricker, L.K. Frankel, The structure and function of the 33 kDa extrinsic protein of Photosystem II: a critical assessment, Photosynth. Res. 56 (1998) 157–173.
[46] P.H. Homann, L.V. Madabusi, Modification of the thermoluminescence properties of Ca^{2+} depleted photosystem II membranes by the 23 kDa extrinsic polypeptide and by oligocarboxylic acids, Photosynth. Res. 35 (1993) 29–39.
[47] I. Vass, T. Ono, Y. Inoue, Stability and oscillation properties of thermoluminescent charge pairs in the O_2-evolving system depleted of Cl^- or the 33 kDa extrinsic protein, Biochim. Biophys. Acta 892 (1987) 224–235.
[48] J. Deprez, A. Dobek, N.E. Geacintov, G. Paillotin, J. Breton, Probing fluorescence induction in chloroplasts on a nanosecond time scale utilizing picosecond laser pulse pairs, Biochim. Biophys. Acta 725 (1983) 444–454.
[49] R.D. Britt, Oxygen Evolution, in: D.R. Ort, C. Yocum (Eds.), Oxygenic photosynthesis: the light reactions, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1996, pp. 137–164.
[50] S. Iwata, J. Barber, Structure of photosystem II and molecular architecture of the oxygen-evolving centre, Curr. Opin. Struck. Biol. 14 (2004) 447–453.
[51] B. Loll, J. Kern, W. Saenger, A. Zouni, J. Biesiadka, Towards complete cofactor arrangement in the 3.0 angstrom resolution structure of photosystem II, Nature 438 (2005) 1040–1044.
[52] G. Christen, G. Renger, The role of hydrogen bonds for the multiphasic $P680^{+\bullet}$ reduction by Y_z in photosystem II with intact oxygen evolution capacity. Analysis of kinetic H/D isotope exchange effects, Biochemistry 38 (1999) 2068–2077.
[53] A. Krieger, E. Weis, S. Demeter, Low pH-induced Ca^{2+} ion release in the water-splitting system is accompanied by a shift in the midpoint redox potential of the primary quinone acceptor Q_A, Biochim. Biophys. Acta 1144 (1993) 411–418.
[54] P. Pospišil, E. Tyystjärvi, Molecular mechanism of high-temperature-induced inhibition of acceptor side of Photosystem II, Photosynth. Res. 62 (1999) 55–66.
[55] M. Poulson, G. Samson, J. Whitmarsh, Evidence that cytochrome b559 protects photosystem II against photoinhibition, Biochemistry 34 (1995) 10932–10938.
[56] P. Faller, A. Pascal, A.W. Rutherford, Beta-carotene redox reactions in photosystem II: electron transfer pathway, Biochemistry 40 (2001) 6431–6440.
[57] M. Roncel, J.M. Ortega, M. Losada, Factors determining the special redox properties of photosynthetic cytochrome b559, Eur. J. Biochem. 268 (2001) 4961–4968.
[58] I. Ohad, C. Dal Bosco, R.G. Herrmann, J. Meurer, Photosystem II proteins PsbL and PsbJ regulate electron flow to the plastoquinone pool, Biochemistry 43 (2004) 2297–2308.
[59] M. Suorsa, R.E. Regel, V. Paakkarinen, N. Battchikova, R.G. Herrmann, E.M. Aro, Protein assembly of photosystem II and accumulation of subcomplexes in the absence of low molecular mass subunits PsbL and PsbJ, Eur. J. Biochem. 271 (2004) 96–107.