Biochimica et Biophysica Acta 1797 (2010) 566–575

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Role of FtsH2 in the repair of Photosystem II in mutants of the cyanobacterium *Synechocystis* PCC 6803 with impaired assembly or stability of the $CaMn_4$ cluster

Josef Komenda [a,b,*], Jana Knoppová [a,b], Vendula Krynická [a,b], Peter J. Nixon [c], Martin Tichý [a,b]

[a] *Institute of Microbiology, Academy of Sciences, Opatovický mlýn, 37981 Třeboň, Czech Republic*
[b] *Institute of Physical Biology, University of South Bohemia, Zámek 136, 37333 Nové Hrady, Czech Republic*
[c] *Wolfson Biochemistry Building, Department of Life Sciences, Imperial College London, S. Kensington Campus, London, SW7 2AZ, UK*

ARTICLE INFO

Article history:
Received 23 November 2009
Received in revised form 25 January 2010
Accepted 4 February 2010
Available online 11 February 2010

Keywords:
CtpA protease
D1 degradation and maturation
FtsH protease
Photosystem II
psbA gene
psbO gene
psbV gene
Synechocystis PCC 6803

ABSTRACT

The FtsH2 protease, encoded by the *slr0228* gene, plays a key role in the selective degradation of photodamaged D1 protein during the repair of Photosystem II (PSII) in the cyanobacterium *Synechocystis* sp. PCC 6803. To test whether additional proteases might be involved in D1 degradation during high rates of photodamage, we have studied the synthesis and degradation of the D1 protein in ΔPsbO and ΔPsbV mutants, in which the $CaMn_4$ cluster catalyzing oxygen evolution is less stable, and in the D1 processing mutants, D1-S345P and ΔCtpA, which are unable to assemble a functional cluster. All four mutants exhibited a dramatically increased rate of D1 degradation in high light compared to the wild-type. Additional inactivation of the *ftsH2* gene slowed the rate of D1 degradation dramatically and increased the level of PSII complexes. We conclude that FtsH2 plays a major role in the degradation of both precursor and mature forms of D1 following donor-side photoinhibition. However, this conclusion concerned only D1 assembled into larger complexes containing at least D2 and CP47. In the Δ*psbEFLJ* deletion mutant blocked at an early stage in PSII assembly, unassembled D1 protein was efficiently degraded in the absence of FtsH2 pointing to the involvement of other protease(s). Significantly, the ΔPsbO mutant displayed unusually low levels of cellular chlorophyll at extremely low-light intensities. The possibilities that PSII repair may limit the availability of chlorophyll for the biogenesis of other chlorophyll-binding proteins and that PsbO might have a regulatory role in PSII repair are discussed.

1. Introduction

Energy and electron transport processes of oxygenic photosynthesis are accompanied by frequent damage to the photosynthetic apparatus by oxidants, such as reactive oxygen species (ROS), that are generated during illumination of plant chloroplasts or cyanobacterial cells [1,2]. Cellular proteases that control the quality of photosynthetic proteins and remove their inactive, possibly even harmful versions play a key role in maintaining the functionality of the photosynthetic apparatus [3]. The complex most sensitive to light-induced damage is Photosystem two (PSII) [4–6], which consists of a number of membrane as well as peripheral proteins, that form a molecular machine responsible for the oxidation of water and the reduction of plastoquinone [7]. The primary target for light-induced damage in PSII is the D1 subunit which together with the homologous D2 protein binds most of the cofactors and prosthetic groups participating in electron transfer through the complex [8]. As a response to its frequent damage, the D1 subunit undergoes an unusually high rate of synthesis and degradation (or turnover) in the light that represents a crucial part of the so-called PSII repair cycle. This cycle maintains the PSII complex in a photochemically active state (for review see [9]).

In the cyanobacterium *Synechocystis* sp. PCC 6803 (referred to hereafter as *Synechocystis* 6803), D1 is synthesized as a precursor (pD1) with a carboxyl-terminal extension consisting of 16 amino-acid residues [10]. The precursor is subsequently cleaved by a specific processing endoprotease, CtpA [11], in two steps [12]. The primary cleavage occurs after Ala352 resulting in formation of a processing intermediate termed iD1 [13] or pre-D1-2 [14], which in *Synechocystis* 6803 is mainly associated with reaction center (RC) complexes lacking the chlorophyll-binding proteins CP47 and CP43 [15]. Maturation is completed by cutting on the carboxyl side of residue Ala344 [10]. Removal of the extension is required for the assembly of the $CaMn_4$ oxygen-evolving center of PSII and is therefore essential for water oxidation [16]. The exact function of the D1 extension is not yet clear, although it is known that it improves the viability of the *Synechocystis* wild-type cells under high irradiance in comparison

Abbreviations: $CaMn_4$, calcium/manganese metal cluster involved in water oxidation; Chl, chlorophyll; cyt, cytochrome; DM, dodecylmaltoside; OEC, oxygen-evolving complex; PSI, Photosystem I; PSII, Photosystem II; RC, reaction center; ROS, reactive oxygen species

* Corresponding author. Institute of Microbiology, Academy of Sciences, Opatovický mlýn, 37981 Třeboň, Czech Republic. Fax: +420 384340415.
E-mail address: komenda@alga.cz (J. Komenda).

0005-2728/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2010.02.006

with strains lacking the extension [17], most probably by making the wild-type cells more resistant to photoinhibition [18]. One possible role for the extension could be an increased resistance of the D1 protein to proteolysis preventing premature degradation of the unassembled protein before its insertion into the PSII complex.

The proper function of the oxygen-evolving complex (OEC) is enabled by a set of extrinsic proteins that stabilize the $CaMn_4$ cluster and separate it from the bulk lumenal space making an optimal ionic environment for the reactions of water oxidation [7]. The isolated cyanobacterial PSII complex contains three such subunits: PsbO, PsbV (also known as cytochrome *c*-550 or cyt *c*-550) and PsbU [19]. In addition, recent studies suggest that cyanobacterial homologues of the PsbP and PsbQ extrinsic subunits found on the lumenal side of chloroplast PSII might also bind to the cyanobacterial PSII complex *in vivo*, most probably to optimize oxygen evolution (for recent review see [20]).

PsbU seems to primarily regulate S-state transitions in the OEC, and its binding to PSII is dependent on the presence of PsbO and PsbV. Deletion of the *psbU* gene does not significantly affect autotrophic growth nor the content of PSII complexes [21]. In contrast, the PsbO and PsbV proteins are essential for dark stability of the $CaMn_4$ cluster and their binding to PSII is largely independent of each other [22–24]. Mutants lacking PsbO grow autotrophically, but very slowly, and oxygen-evolving activity is decreased to about 70% of that in wild-type despite a similar content of PSII complexes [22,25,26]. The PsbV-less mutant similarly exhibits slow autotrophic growth and displays even lower oxygen evolution than the PsbO deletion strain [24]. The cellular content of PSII reaches maximally 50% of the wild-type level suggesting an additional influence of the PsbV protein on the PSII assembly process and/or turnover of PSII protein subunits, especially D1. However, details on these processes in the mutant have not been studied.

Members of the FtsH protease family play an important role in the degradation of D1 during PSII repair [27]. Of the four FtsH homologues identified in the cyanobacterium *Synechocystis* 6803 [28], FtsH2, encoded by *slr0228*, has been shown to participate in the early stages of the selective D1 degradation following damage by high light or heat stress [29,30]. This protease also plays a key role in the quality control of other PSII proteins by removing mutated or unassembled subunits [31]. Recent evidence suggests that D1 degradation in *Synechocystis* is mainly initiated from the N-terminus and continues progressively to the end of the molecule without formation of distinct degradation products [32]. The mechanism that triggers the degradation process remains unclear.

In *Synechocystis* 6803 we have previously shown that the absence of the PsbO protein leads to strong acceleration of D1 degradation which is related to an enhanced rate of light-induced donor-side damage to PSII [33]. While acceptor-side damage (occurring for example in the *Synechocystis* PsbH-less mutant [33]) is related to the light-induced over-reduction of the PSII quinone electron acceptors, modification of the quinone-binding pockets and subsequent formation of singlet oxygen, donor-side damage is typical for mutants with inefficient donation of electrons to the reaction center, which leads to the oxidative damage mediated by reactive cations like $P680^+$ [5,6]. With the exception of the ΔPsbO strain, D1 turnover has not been rigorously studied in the other donor-side mutants and it is not known whether the donor-side induced turnover involves action of the same FtsH protease (encoded by the *slr0228* gene) as the "normal" D1 turnover detected in the wild-type strain [29].

In the present paper we have analyzed the synthesis, assembly and degradation of PSII proteins in mutants of *Synechocystis* 6803 either lacking the important extrinsic oxygen-evolving enhancer proteins PsbO and PsbV, or unable to completely process the precursor of the D1 protein. These defects caused an accelerated turnover of the D1 protein and also a decreased accumulation of PSII complexes in the mutant cells. Inactivation of the *slr0228* gene coding for the FtsH2 protease led in all these mutants to the inhibition of D1 turnover and to an increase in accumulation of PSII core complexes showing that donor-side defects are effectively recognized by FtsH2. Moreover, analysis of chlorophyll content and its distribution between PSII and PSI in the mutants suggested a key importance of the D1 synthesis and degradation for the cellular content of both photosystems.

2. Materials and methods

2.1. Strains and culture conditions

The following previously described mutants of the glucose-tolerant strain of *Synechocystis* sp. PCC 6803, referred to here as wild-type (WT) [34], were used in the study: (i) the PsbO-less strain, ΔPsbO, with *psbO* gene inactivated by a spectinomycin ($spec^R$) resistance cassette [22]; (ii) the PsbV-less strain, ΔPsbV, with *psbV* gene inactivated by an erythromycin (ery^R) resistance cassette [24]; (iii) the site-directed mutant, D1-Ser345Pro, having the *psbA1* and *psbA2* genes inactivated by cm^R and kan^R cassettes, respectively, and the codon for residue Ser345 in the *psbA3* gene replaced by a codon for Pro [10]; (iv) the CtpA-less strain, ΔCtpA, with the *ctpA* gene inactivated by an spectinomycin ($spec^R$) resistance cassette [13] and (v) *psbEFLJ* deletion mutant ΔCYT lacking both subunits of cytochrome *b*-559 (cyt *b*-559) and an additional two PSII subunits PsbL and PsbJ [35]. The newly constructed FtsH2-less mutants were obtained by transformation of the described strains as in [34] using genomic DNA isolated from a strain with an inactivated *slr0228* gene [31]. Genotypes of the resulting multiple mutants were confirmed by PCR.

The strains were grown in BG-11 medium containing 5 mM glucose, solid media contained in addition 10 mM TES/NaOH, pH 8.2, 1.5% agar and 0.3% sodium thiosulphate [35]. 50–100 ml liquid cultures were shaken in 250 ml conical flasks at 29 °C with a surface irradiance of 5 μmol photons m^{-2} s^{-1} of white light due to light sensitivity of *slr0228* deletion strains. Cultures were analyzed in the exponential phase (OD_{750nm} in the range 0.6–0.8). For high-light treatment, the cultures of ΔPsbO and ΔPsbV (OD_{750nm} = 0.8) grown at irradiance of 15 μmol photons m^{-2} s^{-1} in the absence of glucose were placed in 150 ml cuvettes (optical path 20 mm) and exposed to irradiance of 500 μmol photons m^{-2} s^{-1} for 90 min at 29 °C with constant air bubbling, during exposure the oxygen-evolving activity of the cultures was measured by Clarke electrode in the presence of benzoquinone.

2.2. Radioactive labeling of the cells

For 2D analysis of protein complexes, cells were radiolabeled using a mixture of L-[^{35}S]-methionine and L-[^{35}S]-cysteine (>1000 Ci $mmol^{-1}$, 400 μCi ml^{-1} final activity, Trans-label, MP Biomedicals) [36]. For the pulse-chase experiments, cells were resuspended to 75 μg ml^{-1} chlorophyll, and exposed to 200 μmol photons m^{-2} s^{-1} at 29 °C in the presence of radioactive methionine and cysteine (100 μCi ml^{-1} final activity). After 30 min, cells were washed twice with BG11, resuspended in BG11 supplemented with 4 mM non-radiolabelled methionine and 1 mM non-radiolabeled cysteine, and exposed to 500 μmol photons m^{-2} s^{-1} for time indicated.

2.3. Thylakoid preparation and protein analyses

Cyanobacterial membranes were prepared by breaking the cells using glass beads [32]. For analysis of protein complexes, isolated membranes were solubilized in 1% (w/w) dodecyl-β-D-maltoside (DM) and analyzed by blue-native electrophoresis at 4 °C in 5–14% polyacrylamide gel according to [37]. Samples with the same Chl content (6 μg for gel staining and 1 μg for Western blot) were loaded onto the gel.

Protein composition of complexes was assessed by electrophoresis in a denaturing 12–20% linear gradient polyacrylamide gel containing 7 M urea [36]. The whole lanes from the native gel were excised, incubated for 30 min in 25 mM Tris/HCl pH 7.5 containing 1% SDS and 1% dithiothreitol (w/v), and placed on the top of the denaturing gel; two lanes were analyzed in a single denaturing gel. One-dimensional SDS-PAGE for analysis of pulse-chase labeled proteins was carried out in the same 12–20% polyacrylamide gel containing 7 M urea. The gels were either used for immunoblotting or they were stained by Coomassie Blue, dried and exposed to Phosphorimager plate (GE Healthcare) overnight. The intensity of the radioactively labeled bands was quantified by ImageQuant TL software (GE Healthcare). For immunodetection, proteins were electroblotted from gel onto PVDF membrane (Sigma) and detected using (i) D1-specific antibodies raised against amino-acid residues 59–76 of the *Synechocystis* D1; (ii) D2-specific antibodies raised against amino-acid residues 341–352 of the *Synechocystis* D2; (iii) CP47-specific antibodies raised against amino-acid residues 380–394 of barley CP47; (iv) CP43-specific antibodies raised against whole isolated CP43 from *Synechocystis*; and (v) PsaD-specific antibodies raised against whole isolated PsaD from *Synechocystis*.

2.4. RNA isolation, reverse transcription and quantitative PCR

Total RNA was isolated from frozen cells following the hot Trizol protocol as described in [32], purified by an Rneasy MinElute Cleanup Kit (Qiagen) and treated with TURBO Dnase (Ambion). 20 ng of purified RNA was used for cDNA synthesis using random primers and SuperScript II Reverse Transcriptase (Invitrogen). Real time-quantitative PCR reactions were performed on the Rotor-Gene 3000 using the iQ SYBR Green Supermix (BioRad). Each quantitative PCR experiment was performed on two replicates from two independent RNA isolations from the same culture. *rnpB* (encoding the B subunit of the ribonuclease) was used as a reference gene. Its level was found to be proportional to total RNA (measured spectrophotometrically) in all strains. The ΔΔCt method was used to calculate *psbA* gene expression normalized against *rnpB*. The ΔCt values were reproducible to within 0.5 cycle.

2.5. Spectroscopic methods

77 K fluorescence spectra were obtained using an Aminco Bowman Series 2 luminescence spectrometer (Spectronic Unicam). The same number of cells for each strain was excited at 435 nm (bandwidth 4 nm), spectra were recorded in the range 550–800 nm, subsequently corrected for the sensitivity of the photomultiplier and normalized to the 570 nm fluorescence maximum of rhodamine, which was used as an internal standard.

Absorption spectra of cells *in vivo* were measured using a Shimadzu UV3000 spectrophotometer in a compartment close to the photomultiplier with a slit width of 2 nm. The suspensions with the same OD_{750nm} were always used for measurement of each strain.

The concentration of chlorophyll was calculated from the absorbance values of the extract at 666 nm and 720 nm using a Shimadzu UV3000 spectrophotometer [38].

3. Results

3.1. The FtsH2 protease participates in fast D1 replacement in the psbO deletion strain

The strain lacking the PsbO oxygen-evolving enhancer protein grows autotrophically, exhibits PSII oxygen evolution but it is much more sensitive to photoinhibition than the wild-type strain [22,33]. This sensitivity was attributed to the donor-side damage of PSII induced in illuminated mutant cells [33]. Surprisingly, the measurement of Chl content and 77 K chlorophyll fluorescence spectra of the mutant cells grown under low irradiance (5 μmol photons m^{-2} s^{-1}) in the presence of glucose revealed an unusually low cellular content of Chl (Table 1) related to a low level of both photosystems (Fig. 1A) and no oxygen-evolving activity (Table 1). The strain grew much slower than WT (Fig. S1A) and also the level of phycobiliproteins was much lower in the mutant in comparison with WT (Fig. S2). After additional inactivation of the *slr0228* gene encoding FtsH2 [28] the chlorophyll content in the cells of the double mutant ΔPsbO/ΔSlr0228 increased due to a higher level of both PSII and PSI as determined from fluorescence spectra (Fig. 1A, Table 1) and immunoblotting (Fig. S1B). The cellular content of phycobiliproteins was also higher in ΔPsbO/ΔSlr0228 than in the single mutant ΔPsbO (Fig. S2). On the other hand, measurement of oxygen evolution in the mutant cells did not reveal any activity increase after inactivation of the *slr0228* gene showing that the PSII complexes accumulating in cells of the double mutant ΔPsbO/ΔSlr0228 were inactive in oxygen evolution (Table 1).

Assessment of PSII complexes in the ΔPsbO strain by means of 2D-Blue-Native (BN)/SDS-PAGE revealed the absence of the dimeric form of the PSII core complex, RCC(2), whereas level of the monomeric core complex, RCC(1), was higher than in WT (Fig. 2A, panels a and c). The ΔPsbO/ΔSlr0228 double mutant contained a comparable amount of PSII complexes as the single mutant ΔPsbO; however, the relative contents of RC47, the core complex lacking the inner antenna CP43, and the unassembled inner PSII antenna, CP47, increased (Fig. 2A, panel e, dotted squares), as previously shown for the single *slr0228* deletion strain [31]. The correct assignment of the large PSII proteins on 2D gel was confirmed in immunoblots using specific antibodies (Fig. S3).

Since 2D-BN/SDS-PAGE was performed with membranes isolated from radioactively labeled cells, the degree of radiolabeling of PSII subunits in a particular complex on autoradiograms gave us information on the relative contributions of *de novo* synthesis and selective replacement to the overall accumulation of a subunit in the complex. In comparison with the stained gel, the same PSII complexes were also recognized on the autoradiograms. In WT and ΔPsbO, the radiolabeling of the D1 protein in RCC(1) and RC47 was much higher than that of D2 and CP43 due to ongoing selective D1 replacement (Fig. 2A, panels b and d, Table 1). Fast resynthesis of the D1 protein was also accompanied by about 70% increase in the level of the *psbA* transcript estimated by RT-PCR (Table 1). In contrast, the level of radioactive labeling of D1 in RCC(1) of the strain lacking both PsbO and FtsH2 was lowered to that of D2 and CP43 (Fig. 2A, panel f), despite the maintained high level of the *psbA* transcript (Table 1). This result suggested that the complex was assembled predominantly from newly synthesized D1, D2 and CP43 proteins without significant contribution of the label from selective D1 turnover. Another typical feature of Slr0228-less strains is the presence of newly synthesized, unassembled D1 protein, especially its non-mature forms pD1 and iD1 [31], and this was indeed observed in the ΔPsbO/ΔSlr0228 double mutant (Fig. 2A, panel f, arrows) but not in the ΔPsbO single mutant.

Frequent damage to PSII in the ΔPsbO strain leads to the accelerated turnover of the D1 protein [33]. This was confirmed in the present experiments where the D1 half life was estimated to be about 30 min in the ΔPsbO strain (Fig. 2B, middle panel) compared to 200 min in WT (Fig. 2B, left panel; see also [35]). Fast degradation of D1 was accompanied by a massive resynthesis of D1 as indicated by intensive D1 radiolabeling. In the ΔPsbO/ΔSlr0228 double mutant, preferential labeling of mature D1 was abolished (Fig. 2B), and instead, weakly labeled incompletely processed forms of the D1 protein, termed pD1 and iD1, were observed that were absent in cells of WT or the single ΔPsbO mutant. During the chase, the labeled mature form of D1 was rather stable and its labeling intensity decreased by 40% after 120 min of the label chase. A similar slow decrease in the labeling intensity was also observed for the D2 subunit further confirming the absence of selective D1 turnover in the ΔPsbO/

Table 1
Characteristics of the *Synechocystis* sp. PCC 6803 PSII donor-side mutants and their FtsH2-less variants grown under low irradiance (5 μmol photons m^{-2} s^{-1}) in the presence of glucose.

	WT	ΔPsbO	ΔPsbO/ΔSlr0228	ΔPsbV	ΔPsbV/ΔSlr0228	ΔCtpA	ΔCtpA/ΔSlr0228	D1-S345P	D1-S345P/ΔSlr0228
Chl content[a]	2.6	1.13	1.51	3.25	2.30	1.72	1.1	3.60	1.36
F695/F571[b]	1.5	0.9	1.5	0.6	1.6	0.6	1.9	0.5	2.2
F728/F571[b]	11.1	6.0	8.0	13.1	10.4	9.1	8.4	13.2	10.5
F728/F695[b]	7.5	7.0	5.3	23.6	6.6	16.2	4.4	26.7	4.8
Oxygen evolution[c]	830 ± 40	<20	<20	45 ± 10	65 ± 10	<20	<20	<20	<20
D1/D2 label[d]	2.6	6.8	1.6	0.9	1.3	3.0	0.8	0.7	1.0
psbA transcript[e]	100	170 ± 40	210 ± 50	120 ± 20	60 ± 20	250 ± 60	50 ± 10	25 ± 10	90 ± 20

[a] Cellular content of Chl (μg Chl per OD_{750} unit) in cultures at the exponential growth phase ($OD_{750\ nm}$ 0.6–0.8); means of 3 measurements, SD did not exceed 12%.
[b] Ratio of the fluorescence maximum heights of PSII at 695 nm (F695) or PSI at 728 nm (F728) and the height of the maximum of rhodamine at 571 nm (F571) used as an internal standard; the values were taken from chlorophyll fluorescence spectra at 77 K shown in Fig. 1 that were measured using blue light excitation at 435 nm.
[c] Light-saturated rate of oxygen evolution in μmol O_2 per mg Chl and h in WT and mutant cells measured in the presence of 1 mM p-benzoquinone and 5 mM potassium ferricyanide; means of 3–6 measurements ± SD.
[d] Ratio of the intensities of the radioactively labeled bands of the D1 and D2 proteins determined from the pulse-chase labeling experiments at the time zero obtained by quantification of the signals from the Phosphoimager plate.
[e] Level of the *psbA* transcript assessed by RT-PCR and expressed as % of the level detected in wild-type strain; means of 3 measurements ± SD.

Δslr0228 double mutant. Taken together the experimental data provide clear evidence for a role of FtsH2 in the selective replacement of D1 in the PsbO-less mutant of *Synechocystis* 6803.

3.2. Low cellular content of PSII in the PsbV-less strain lacking cytochrome c-550 is increased after inactivation of FtsH2

Previous mutagenesis experiments have revealed the importance of cyt *c*-550 (the *psbV* gene product) for maintaining the stability and function of the oxygen-evolving $CaMn_4$ cluster [23]. In comparison to WT, the *psbV* null mutant exhibits about 40% capacity of PSII-dependent electron transfer and a corresponding decrease in PSII content [23]. In our low-light culture, the oxygen-evolving activity of the cells was much lower than this earlier value, determined in cells grown at a higher irradiance [23], reaching only about 5% of the WT value (Table 1). Unlike the ΔPsbO strain, the ΔPsbV strain grew quickly in the presence of glucose (Fig. S1A), contained normal level of phycobiliproteins (Fig. S2) and assessment of its photosystem content

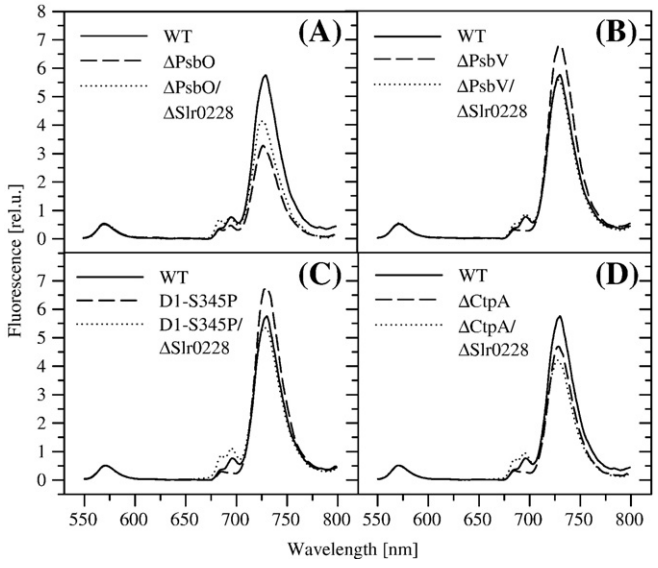

Fig. 1. 77 K chlorophyll fluorescence emission spectra of wild-type (solid lines), PSII donor-side mutants (dashed lines) and their FtsH2-less variants (dotted lines). A: ΔPsbO and ΔPsbO/ΔSlr0228; (B) ΔPsbV and ΔPsbV/ΔSlr0228; (C) D1-S345P and D1-S345P/ΔSlr0228; and (D) ΔCtpA and ΔCtpA/ΔSlr0228. The spectra were obtained using Aminco Bowman Series 2 luminescence spectrometer. The suspensions with the same number of cells were excited at 435 nm, spectra were corrected for the sensitivity of photomultiplier and normalized to the fluorescence maximum of rhodamine (570 nm) which was used as an internal standard.

showed a high level of PSI on a cell basis, which even exceeded that of WT. On the other hand the level of PSII was rather low as documented by the low fluorescence peak at 697 nm (Fig. 1B) and Western blot (Fig. S1B). Additional inactivation of the *slr0228* gene resulted in an increase in PSII content which was accompanied by a slight decrease in the PSI level in the resulting double mutant (Fig. 1B, Fig. S1B and Table 1).

2D analysis of membrane protein complexes confirmed the low level of PSII complexes on Chl basis in the ΔPsbV strain (Fig. 3A, panel a), in line with a previous report [23]. RCC(1) and a small amount of RC47 were distinguishable on the Coomassie-stained gel, while the dimeric PSII core complex (RCC(2)) was virtually absent, as in the ΔPsbO strain. Autoradiography also revealed the presence of PSII assembly intermediates in the ΔPsbV strain, namely two RC complexes, RC* and RCa, lacking both inner antennae CP47 and CP43 (Fig. 3A, panel b). While RCa was shown to contain D2, iD1 (Fig. 3A, panel b, box), D1, PsbE, PsbF, PsbI and the assembly factor YCF48, the larger RC* most probably contains in addition to the RCa subunits one or more unidentified protein factors [39]. The RC complexes, which were not detected in ΔPsbO, are usually typical for strains lacking the CP47 antenna [15]. However, in the ΔPsbV strain unassembled CP47 was apparent on both the stained gel and the autoradiogram and, therefore, deficiency in CP47 could not be the reason for the occurrence of RC complexes in ΔPsbV. Unlike ΔPsbO, the ΔPsbV strain also accumulated an increased level of pD1, iD1 and D1 in the region of unassembled proteins (Fig. 3A, panels b and d, arrows) indicating lower efficiency of incorporating D1 into PSII complexes. The *psbA* transcript level in the mutant was similar to wild-type (Table 1).

Inactivation of the *slr0228* gene in the ΔPsbV strain markedly increased the accumulation RCC(1) and RC47 complexes but still there was no accumulation of RCC(2) (Fig. 3A, panel c). An increase in the level of PSII complexes (and decrease in the PSI content) was also documented in the 77 K emission fluorescence spectrum (Fig. 1B) and western blot (Fig. S1B) but the negligible oxygen-evolution activity of the double mutant showed that the accumulated PSII complexes fluorescing at 697 nm do not have a functional OEC (Table 1). The absence of FtsH2 was again typically manifested by an equal labeling of D1, D2 and CP43 in RCC1 on a 2D gel (Fig. 3A, panel d) confirming the absence of the selective turnover of D1. In addition, despite the higher level of RC47 in the double mutant, (Fig. 3A, panel c, dashed box) the D1 and D2 proteins in this complex were radiolabeled only minimally indicating that this complex originated from the disassembly of older RCC complexes. The level of the *psbA* transcript in the double mutant reached only about 60% of that in WT (Table 1).

Compared to ΔPsbO, pulse-labeling of the D1 protein in the ΔPsbV strain was weaker and similar to the labeling of D2. Surprisingly, both

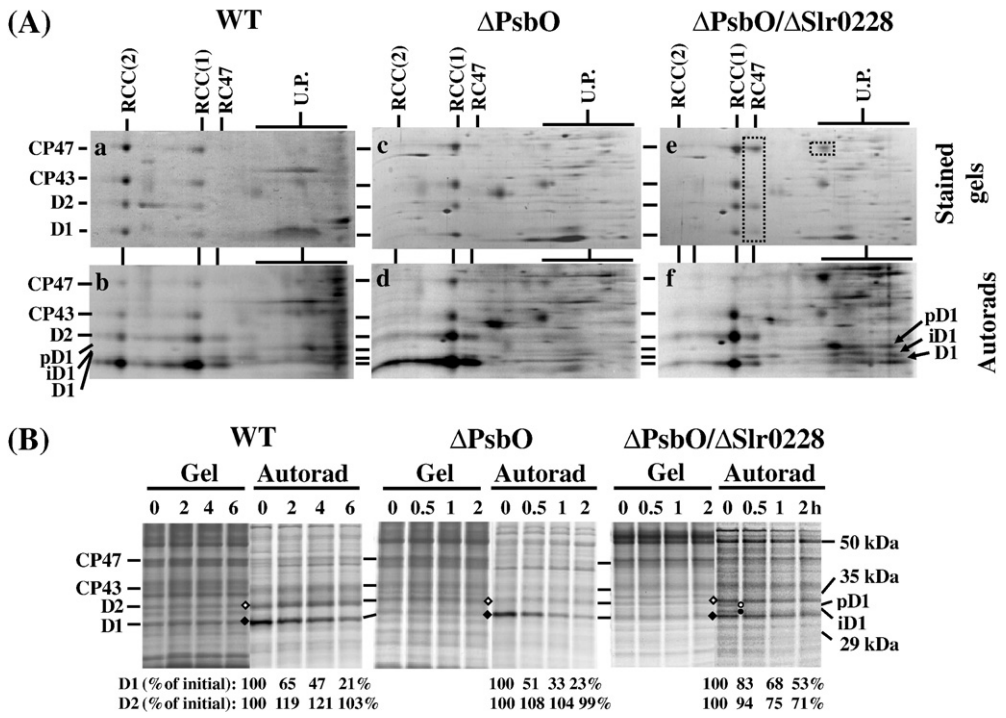

Fig. 2. Two dimensional analysis of thylakoid membrane proteins (A) and effect of high irradiance on turnover of D1 and D2 proteins (B) in cells of WT and the mutants ΔPsbO and ΔPsbO/ΔSlr0228. A: Radiolabeled thylakoid membrane proteins of WT (a and b) and the mutants ΔPsbO (c and d) and ΔPsbO/ΔSlr0228(e and f) were separated by a combination of BN-PAGE and SDS-PAGE as described in Materials and methods. Gels were stained (Stained gels; panels a, c and e), dried and exposed to a Phosphoimager plate (Autorads; panels b, d and f). Loaded thylakoids corresponded to 6 μg of Chl. Designation of complexes: RCC(1) and RCC(2): monomeric and dimeric PSII reaction center core; RC47: PSII reaction center core lacking CP43; U.P.: unassembled proteins. Overaccumulated RC47 and unassembled CP47 in ΔPsbO/ΔSlr0228 (panel e) are marked by dotted rectangles, unassembled D1 forms in autoradiogram of the same strain (panel f) are marked by arrows. B: Cells of WT (left panel), ΔPsbO (middle panel) and ΔPsbO/ΔSlr0228 (right panel) were subjected to 250 μmol photons m^{-2} s^{-1} of white light for 20 min in the presence of radioactively labeled mixture of Met and Cys (0 h). Then the cells were washed and cold methionine and cysteine were added and the cells were subjected to 500 μmol photons m^{-2} s^{-1} of white light for designated time. Thylakoids were isolated, their proteins separated by SDS-PAGE, gels were stained by Coomassie (Gel) and the radioactive labeling of the proteins was visualized using a PhosphorImager (Autorad). Quantification of radioactivity in bands of D1 and D2 was performed by ImageQuant TL software with all samples equally loaded on chlorophyll basis (6 μg of Chl, for equal loading see stained Gel). The radioactivity incorporated into D1 and D2 after the pulse period (0 h) was taken as 100%; numbers show the means of three measurements, SD did not exceed 9%. The D1 and D2 proteins are marked by closed and empty diamonds, respectively, the unprocessed D1 forms iD1 and pD1 are marked by closed and empty circles, respectively.

proteins were degraded in parallel with a half-time similar to ΔPsbO (about 30 min, Fig. 3B, left panel). Additional inactivation of the *slr0228* gene in the ΔPsbV strain led to the stabilization of both D1 and D2 and the proteins were not degraded during the 120 min of the chase period (Fig. 3B, right panel).

3.3. FtsH2 is also needed for efficient degradation of incompletely processed forms of the D1 protein

To examine whether FtsH2 is also involved in the removal of the D1 protein containing either part or all of the C-terminal cleavable extension, we investigated the effect of removing the *slr0228* gene on the degradation rate and quantity of PSII proteins in two strains unable to complete the maturation of the D1 precursor. The first one designated D1-S345P cannot cleave the extension at position 344 due to the replacement of the D1 residue Ser345 by Pro but can cleave after D1-Ala352 [13], while the second one, designated ΔCtpA, lacks the CtpA processing protease and accumulates pD1 [11].

The D1-S345P strain is not able to grow autotrophically due to an inability to assemble active oxygen-evolving complexes [10] and accumulates a limited amount of PSII core complexes containing only the partially processed form iD1 [13]. We found that this strain also contained significantly higher levels of PSI than WT as judged from the increased cellular chlorophyll content and the cell-normalized chlorophyll fluorescence spectra (Table 1, Fig. 1C). On the other hand, the reduced intensity of the fluorescence band at 695 nm pointed to a low PSII content (Fig. 1C). The PSI/PSII ratio decreased in cells of D1-S345P after the additional inactivation of the *slr0228* gene due to both the increase in PSII content and a decrease in PSI (Fig. 1C, Table 1). 2D protein analysis with gels loaded on an equal chlorophyll basis confirmed the high PSI/PSII ratio, and showed only a small amount of the monomeric PSII core complex RCC(1) while the dimeric PSII core was absent similar to the other PSII donor-side mutants (Fig. 4A, panel a). This strain also contained a rather high level of unassembled CP47 and CP43. Autoradiography further showed a quite high level of newly synthesized but unassembled D2, CP43 and CP47 (Fig. 4A, panel b). Their accumulation is likely to be a consequence of the genetic background of the strain: the site-directed mutation was created in the *psbA3* gene with a downstream tetracycline-resistance cassette while the remaining two *psbA* copies were deleted [10]. Insertion of the selection marker cassette close to the 3′ terminus of *psbA3* was previously shown to drastically suppress the level of *psbA3* transcript and reduce the synthesis of the D1 protein [40]. Consistent with this, RT-PCR showed that the level of the *psbA* transcript in D1-S345P was only 25% of that of WT. Therefore it is likely that accumulation of free D2, CP43 and CP47 in D1-S345P was due to low availability of newly synthesized D1.

2D gel analysis of the D1-S345P/ΔSlr0228 double mutant showed an at least twofold increase in the PSII content and this was documented by the level of RCC(1), RC47 as well as free CP47 (Fig. 4A, panel c). Interestingly, the labeling of the D2 protein in comparison with D1 and CP43 was distinguished from previous mutants by its low extent in RCC(1) and high extent in the free unassembled fraction. On the other hand, a quite low labeling of CP47 in the PSII complexes was a common feature of the all tested FtsH2-less strains. The result suggests that the new core complex in this strain was largely assembled from a long-lived pool of D2 and CP47 while newly synthesized CP43 and D1 were quickly incorporated into

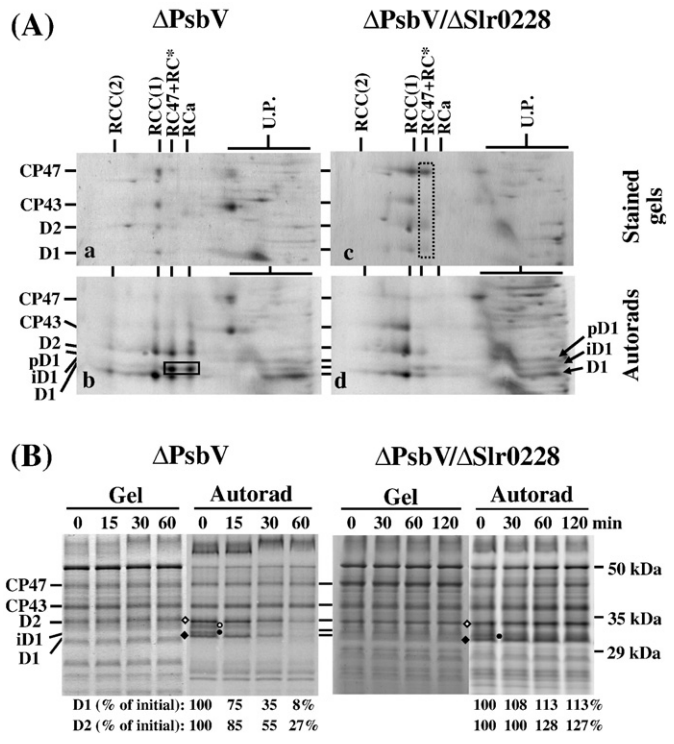

Fig. 3. Two dimensional analysis of thylakoid membrane proteins (A) and effect of high irradiance on turnover of D1 and D2 proteins (B) in cells of the ΔPsbV and ΔPsbV/ΔSlr0228 mutants. A: Radiolabeled thylakoid membrane proteins of the mutants ΔPsbV (a and b) and ΔPsbV/ΔSlr0228(c and d) were separated by a combination of BN-PAGE and SDS-PAGE as described in Materials and methods. Gels were stained (Stained gels; panels a and c), dried and exposed to a Phosphoimager plate (Autorads; panels b and d). Loaded thylakoids corresponded to 6 μg of Chl. Designation of complexes and bands as in the legend of Fig. 2A, RC* and RCa, complexes containing iD or D1 together with D2, cytochrome *b*-559 and PsbI but lacking CP47 and CP43. Overaccumulated RC47 in ΔPsbV/ΔSlr0228 (panel c) is marked by a dotted rectangle, labeled unassembled D1 forms in autoradiogram of the same strain (panel d) are marked by arrows and the labeled iD1 assembled in RCa and RC* of ΔPsbV (panel b) is marked by the non-dotted rectangle. B: Cells of ΔPsbV (left panel) and ΔPsbV/ΔSlr0228 (right panel) were labeled and analysis was performed as described in the legend to Fig. 2B. The D1, iD1, pD1 and D2 proteins are marked as in Fig. 2B.

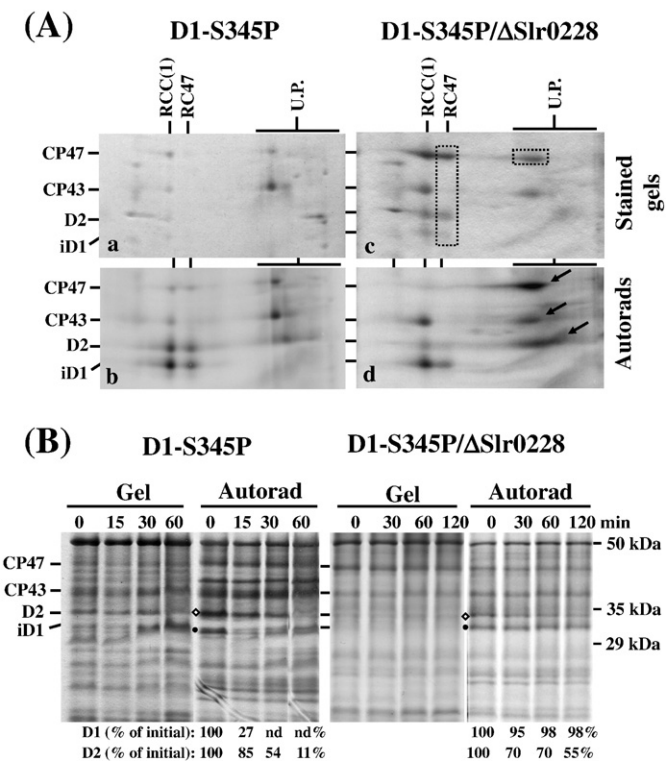

Fig. 4. Two dimensional analysis of thylakoid membrane proteins (A) and effect of high irradiance on turnover of D1 and D2 proteins (B) in cells of the mutants D1-S345P and D1-S345P/ΔSlr0228. A: Radiolabeled thylakoid membrane proteins of the mutants D1-S345P (a and b) and D1-S345P/ΔSlr0228 (c and d) were separated by a combination of BN-PAGE and SDS-PAGE as described in Materials and methods. Gels were stained (Stained gels; panels a and c), dried and exposed to a Phosphoimager plate (Autorads; panels b and d). Loaded thylakoids corresponded to 6 μg of Chl. Designation of complexes as in the legend of Fig. 2A. Overaccumulated RC47 and CP47 in D1-S345P/ΔSlr0228 (panel c) are marked by dotted rectangles and labeled unassembled CP47, CP43 and D2 in autoradiogram of the same strain (panel d) are marked by arrows. B: Cells of D1-S345P (left panel) and D1-S345P/ΔSlr0228 (right panel) were labeled and analysis was performed as described in the legend to Fig. 2B. The iD1 and D2 proteins are marked as in Fig. 2B.

the RCC(1) complex. Interestingly, inactivation of the slr0228 in the D1-S345P mutant led to restoration of the wild-type level of the *psbA* transcript in the D1-S345P/ΔSlr0228 double mutant (Table 1).

Pulse-chase measurement of the protein degradation in D1-S345P revealed very quick degradation of the iD1 protein with a half-time of less than 15 min (Fig. 4B, left panel). Inactivation of the *slr0228* gene resulted in an at least ten times prolongation of the half-time of iD1, demonstrating participation of FtsH2 in this extremely fast degradation process. Such an inhibition of the iD1 degradation was most probably the reason for the high content of PSII detected in the double mutant although the contribution of the high *psbA* transcript level could be also important (Figs. 1 and 4A).

Like the D1-S345P mutant, the ΔCtpA strain, which lacks the D1 processing protease, is also unable to evolve oxygen [11]. The ΔCtpA strain also exhibited a significantly lower cellular content of Chl in comparison with WT, and, in agreement with 2D gel data, both PSI and, especially, PSII levels were lowered (Fig. 1D, Table 1). ΔCtpA contained only the D1 precursor, termed pD1 [11,13], which accumulated in the RCC(1) complex and also in the abundant RC47 complex (Fig. 5A, panel a). As in the case of the other donor-side mutants, the dimeric PSII core complex, RCC(2), was almost undetectable in ΔCtpA. The 2D autoradiogram further revealed that pD1 was labeled selectively and was efficiently inserted into PSII complexes with little newly synthesized, unassembled pD1 being detected (Fig. 5A, panel b). In contrast, a lot of newly synthesized pD1 was seen in the ΔCtpA/ΔSlr0228 strain in the region of unassembled proteins (Fig. 5A, panel d, arrow). The double mutant also accumulated about twice the level of RCC(1) and RC47 compared to the single mutant when loaded on the same Chl basis (Fig. 5A, panel c). This agreed well with the fluorescence spectra which showed a much higher PSII/PSI ratio in ΔCtpA/ΔSlr0228 compared to ΔCtpA. Despite the absence of the CtpA protease, in both CtpA-less mutants the PSII complexes on 2D gels contained not only pD1 but also iD1 and mature D1 (Fig. 5A, panels b and d) due to the additional CtpA- and CtpB-unrelated proteolytic activity observed during 2D, but not 1D, gel electrophoresis [13]. In agreement with the enhanced pD1 synthesis, the *psbA* transcript level in ΔCtpA was almost three times higher than in WT, but the inactivation of the *slr0228* gene led to its subsequent fivefold decrease (Table 1).

The pulse-chase experiment confirmed a massive synthesis of pD1 and its subsequent rapid and FtsH2-dependent degradation in the ΔCtpA mutant (Fig. 5B). Nevertheless, the rate of D1 degradation in ΔCtpA was not as fast as in D1-S345P, and in the double mutant ΔCtpA/ΔSlr0228, some pD1 was quickly degraded during the initial 30 min of the chase, while iD1 in the D1-S345P/ΔSlr0228 strain was stable. 2D analysis of the pulse labeled cells and cells after 30 min of the chase (not shown) confirmed that the pD1 that was most quickly degraded in ΔCtpA/ΔSlr0228 represented a sub-population of pD1, absent in D1-S345P/ΔSlr0228, which was present in an unassembled state.

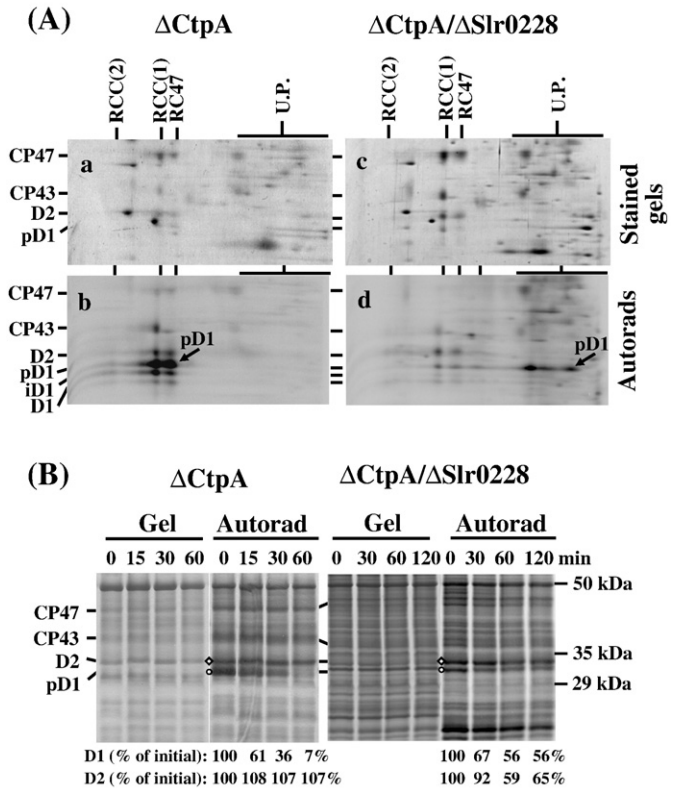

Fig. 5. Two dimensional analysis of thylakoid membrane proteins (A) and effect of high irradiance on turnover of D1 and D2 proteins (B) in cells of the mutants the ΔCtpA and ΔCtpA/ΔSlr0228. A: Radiolabeled thylakoid membrane proteins of the mutants ΔCtpA (a and b) and ΔCtpA/ΔSlr0228 (c and d) were separated by a combination of BN-PAGE and SDS-PAGE as described in Materials and methods. Gels were stained (Stained gels; panels a and c), dried and exposed to a Phosphoimager plate (Autorads; panels c and d). Loaded thylakoids corresponded to 6 μg of Chl. Designation of complexes as in the legend of Fig. 2A. The labeled pD1 assembled in RCC(1) and RC47 of ΔCtpA (panel b) and the labeled unassembled pD1 in ΔCtpA/ΔSlr0228 (panel d) are marked by arrows. B: Cells of ΔCtpA (left panel) and ΔCtpA/ΔSlr0228 (right panel) were labeled and analysis was performed as described in the legend to Fig. 2B.

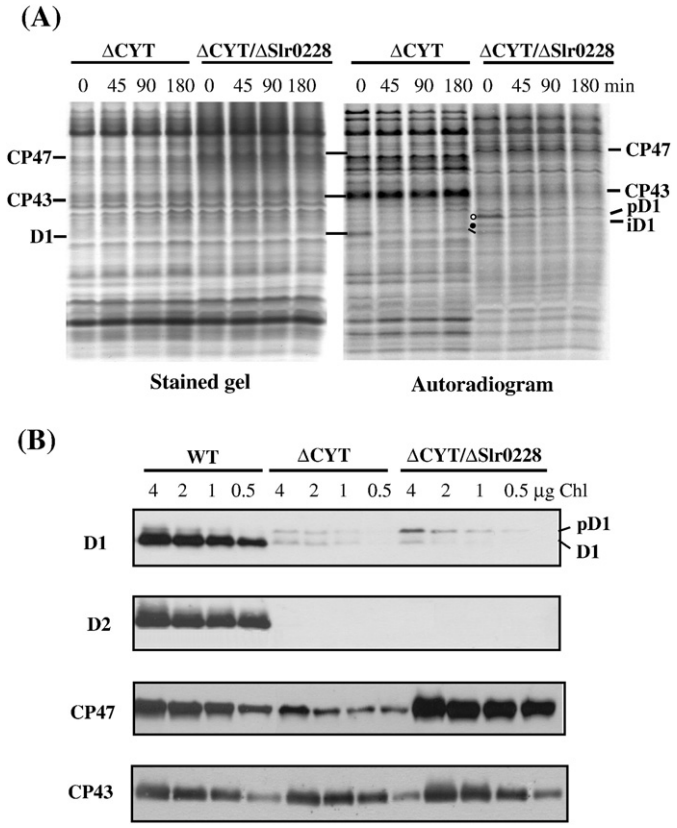

Fig. 6. Turnover (A) and steady-state levels of PSII proteins (B) in cells of the *Synechocystis* psbEFLJ deletion mutant ΔCYT, and the double mutant ΔCYT/ΔSlr0228. A: Cells were labeled and analysis was performed as described in the legend to Fig. 2B. B: Thylakoids of each strain containing 4, 2, 1 and 0.5 μg of chlorophyll were immunoblotted using antibodies specific for D1, D2, CP47 and CP43. For comparison, amount of proteins in WT corresponding to 4, 2, 1 and 0.5 μg of chlorophyll is also shown on the left side of the membrane. Bands of CP43 were used as the internal standard documenting the equal loading of the lanes.

To test further the idea that unassembled pD1 is effectively degraded even in the absence of FtsH2, we investigated the accumulation and degradation of precursor and mature forms of D1 in the ΔCYT strain lacking the *psbEFLJ* operon. This strain is unable to accumulate D2 protein and contains detectable level of all three forms of D1 in the unassembled state [15,35]. Pulse-chase measurements indeed showed efficient degradation of D1 in the ΔCYT strain. However, the same effective degradation of D1 was also detected in the ΔCYT/ΔSlr0228 strain, the FtsH2-less variant of the *psbEFLJ* deletion mutant (Fig. 6A). In agreement with this result, the steady-state level of the various D1 forms was not markedly increased in the ΔCYT/ΔSlr0228 strain in comparison to ΔCYT (Fig. 6B), and the content of D2 was not elevated in the absence of FtsH2 either. On the other hand, the levels of unassembled CP47 increased in the double mutant, in line with previous results on accumulation of CP47 in a mutant lacking the D1 protein [31]. In summary, these results showed that FtsH2 participates in the selective degradation of all forms of D1 when assembled in PSII complexes but it is not required for removal of unassembled precursor and mature forms of D1.

4. Discussion

4.1. Comparison of the phenotypes of the ΔPsbO and ΔPsbV mutants

The recent crystallographic models of PSII have suggested a possible physiological role for PsbO in stabilizing the dimeric form of the PSII core complex via the interaction of PsbO of one monomer with a CP47 subunit in the second monomer [7,19]. The importance of this structural feature for dimerisation is supported here by results of the 2D protein analysis which showed the absence of the dimeric form of PSII in the ΔPsbO mutant. A similar destabilization of the PSII dimeric structure was also exhibited by the ΔPsbV strain. Although PsbV does not reach the contact site between the two monomers, its absence may lead to a general perturbation in the arrangement of the lumenally exposed part of PSII and, consequently, the binding between monomers within the dimer might be indirectly weakened. The same explanation may be used for the observed absence of the dimer in the D1-S345P and ΔCtpA mutants containing incompletely processed forms of D1. Alternatively, the high rate of PSII damage together with rapid repair cycle could also contribute to the dominance of the monomer in the various donor-side mutants. The lack of PSII dimer, which occurred regardless of the detergent concentration used for solubilization in all mutants studied here, argues against recent suggestions that dimeric PSII is a detergent-induced artefact [41,42].

The absence of the PsbO has been previously shown to increase the turnover of the D1 protein due to light-induced donor-side damage [33]. The increase in the rate of D1 synthesis is also accompanied by efficient integration of D1 into PSII without detectable accumulation of unassembled forms of D1 (Fig. 2A). The fast synthesis of the D1 protein corresponds well with the high level of *psbA* transcript

detected here (Table 1) and previously [43] in the *psbO*-less strain. After additional inactivation of the FtsH2 protease, the cellular level of PSII complexes was increased, the degradation of the D1 protein was inhibited and a significant amount of the newly synthesized D1, especially pD1, accumulated as unassembled protein. We can conclude that FtsH2 is essential for fast degradation of the D1 protein induced by the light-mediated donor-side damage occurring in the PsbO-less strain as well as in the other donor-side mutants studied here. This conclusion is in accordance with recent results showing a similar FtsH2-dependency of the D1 degradation in cells of *Synechocystis* 6803 with OEC impaired either by ammonia [44] or by UV-B [45].

The effect of deleting *psbV* on the accumulation and stability of PSII RC proteins D1 and D2 was surprisingly different to that of the *psbO* deletion strain, despite the fact that both proteins are located in close proximity on the lumenal side of PSII and their absence causes destabilization of OEC and fast light-induced donor-side damage [23,33]. By comparison with ΔPsbO, there is a higher PSI/PSII ratio in the ΔPsbV strain and consequently a lower level of PSII on a Chl basis. 2D gel analysis and pulse-chase labeling also showed a lower rate of D1 synthesis as reflected by the low level of D1 radiolabeling which was similar to the labeling of D2. The ΔPsbV strain also showed similar rates of D1 and D2 degradation (Fig. 3B) what means that both proteins must be synthesized *de novo* in order to get new PSII complexes. The detection of increased levels of the PSII RC assembly complexes, RCa and RC*, in the ΔPsbV strain is consistent with this conclusion.

The reason why the selective replacement of the D1 protein in ΔPsbV is inefficient remains unclear. The rate of light-induced damage was similar in both ΔPsbO and ΔPsbV strains (oxygen-evolving activity in cells of both ΔPsbO and ΔPsbV strains grown autotrophically at 15 μmol photons m^{-2} s^{-1} and subsequently exposed to irradiance of 500 μmol photons m^{-2} s^{-1} declined very quickly with half-time 15–20 min, Fig. S1C). RT-PCR indicated a slightly higher level of the *psbA* transcript in ΔPsbV than in WT which would rule out a significant effect at the transcriptional level (Table 1). On the other hand, the presence of unassembled D1 suggests that the repair-related insertion of the protein into the PSII complex is affected when PsbV is absent. One possibility for the inhibition of selective D1 replacement could be the parallel damage to both D1 and D2 which is often accompanied by the formation of D1–D2 or D1-cytochrome *b*-559 cross-linked products [46]. However, neither cross-linked products, nor fragments were identified in cells of ΔPsbV or ΔPsbO exposed to high irradiance of 500 μmol photons m^{-2} s^{-1} for 90 min (not shown). As the selectivity of D1 replacement was restored in the ΔPsbV/ΔPsbO double mutant (not shown), the presence of the PsbO protein seems to be of crucial importance for this effect. It is possible that absence of PsbV in the ΔPsbV mutant perturbs PsbO binding so that D1 degradation is triggered less efficiently despite the same extent of the light-induced damage. In conclusion, PsbO and PsbV seem to play an important regulatory role in the selective replacement of D1 during PSII repair.

4.2. FtsH-mediated degradation of D1

Recent studies on the PSII photoinactivation mechanism *in vivo* indicate that the initial damaging events might actually occur at the $CaMn_4$ cluster on the lumenal side of the complex [47–49]. Thus the mechanism of D1 degradation seen for the donor-side mutants might actually represent the process that occurs in WT. The ability of the oxygen-evolving machinery to signal the need for replacement of damaged D1 protein has the added advantage of preventing even more extensive irreversible damage to the PSII RC with the possible necessity to replace additional PSII subunits besides D1. It is also important to note that PSII mutations impairing the PSII acceptor side (for instance, deletion of the *psbH* gene [33] or D1 mutants with mutations close to the Q_B pocket [50]) often exhibit an inhibitory effect on the D1 replacement process. This would suggest a lower efficiency of acceptor-side impairment in triggering D1 degradation in comparison to donor-side impairment.

FtsH2-mediated D1 degradation is likely to occur when D1 is in a destabilized state [27]. This would be promoted by damage to the $CaMn_4$ cluster and the D1 subunit, detachment of the extrinsic subunits and partial disassembly of the PSII complex. The question arises as to how FtsH2 might recognize structural changes to PSII on the lumenal side of the membrane following donor-side photoinhibition. The 627 amino-acid FtsH2 subunit is predicted by CyanoBase to contain two transmembrane regions (residues 5–26 and 117–138) in the N-terminal region. The large C-terminal soluble fragment containing the ATP-ase module and the Zn^{2+}-protease domain is predicted to be located in the cytoplasm whereas the 91 amino-acid region linking the two transmembrane regions would be in the lumen. Consequently FtsH might engage with damaged PSII through interactions on the lumenal side of the membrane, as well as via transmembrane helices in the membrane, prior to initiation of degradation of damaged D1 from the N-terminus on the opposite side of the membrane. Accordingly the binding of PsbO and possibly the other extrinsic proteins to PSII might regulate this interaction and control D1 degradation. There is some experimental support for this idea from studies in *Arabidopsis* where there are two *psbO* genes termed *psbO1* and *psbO2*. The latter is predominantly expressed under high-light conditions and its presence underlies higher resistance of wild-type to photoinhibition in comparison to a mutant expressing just PsbO1 [51]. This stimulatory action of PsbO2 seems to be related to the increased GTPase activity of the protein that is a specific feature of the plant protein [51].

4.3. Existence of an FtsH2-independent pathway for degradation of unassembled D1

Previous results [29,31,32] have led to the clear conclusion that the FtsH2 protease plays a key role not only in the degradation of assembled D1 protein but also in the degradation of assembled and unassembled D2, CP43 and CP47. However, it is important to note that there is still residual degradation of assembled D1 and D2 in the absence of FtsH2, especially in strains with fast turnover of D1 like ΔPsbO and ΔCtpA.

Study of the turnover of pD1 in the strain lacking the pD1 processing protease, CtpA, and more clearly in the ΔCYT strain (Fig. 6), unexpectedly revealed the efficient FtsH2-independent degradation of unassembled D1 protein. It is possible that the unassembled forms of the D1 protein detected in ΔCtpA/ΔFtsH and in ΔCYT are located in a specific, FtsH2-free membrane compartment in which different proteases are located. This compartment could be cytoplasmic membrane or connecting membrane regions between thylakoid and cytoplasmic membranes which were previously proposed as the site of the D1 synthesis based on results with the *pratA*-deficient mutant [52]. The three remaining FtsH proteases encoded by *Synechocystis* 6803 are obvious candidates for the proteolysis of unassembled pD1, iD1 and D1 and the residual proteolysis of assembled D1 and D2, but other proteases must be also considered.

In all FtsH2-less strains autoradiography suggested that the newly synthesized D1, D2 and CP43 subunits are inserted into the PSII core complexes with very similar efficiency, while incorporation of the newly synthesized CP47 is negligible. However, in the strains with a large fraction of unassembled D2 typical of the low availability of D1 (for instance D1-S345P/ΔSlr0228) the incorporation of the newly synthesized D2 into PSII complexes is also quite low. This suggests that in the presence of a larger pool of unassembled proteins like CP47 and D2 in D1-S345P/ΔSlr0228, the newly synthesized molecules are not immediately inserted into PSII complexes. On the other hand radiolabeled D1 and CP43 quickly appear in the PSII complex

indicating that there is no significant pool of these unassembled molecules and, consequently, that newly synthesized subunits are immediately inserted into the PSII complex.

4.4. Does PSII repair limit chlorophyll availability?

Comparison of the relative rates of D1 synthesis and cellular content of Chl among the studied mutants showed that strains with highest rate of D1 synthesis (D1/D2 ratio, Table 1) and highest amount of the *psbA* transcript (ΔPsbO and ΔCtpA) contain a decreased level of Chl. As the majority of Chl in *Synechocystis* 6803 is bound to PSI, a low Chl level also means a low level of PSI as confirmed by the cell-normalized 77 K chlorophyll fluorescence spectra (Fig. 1). In contrast, the ΔPsbV and D1-S345P strains, in which D1 synthesis is slower than in wild-type, exhibited an elevated content of PSI. Similar results were obtained also for other strains deficient in D1 synthesis, for instance ΔYCF48 [39] (data not shown). In summary, these results raise the possibility that there is a high consumption of Chl during D1 turnover which seems to limit Chl availability for other Chl-binding proteins, especially PSI.

In all the donor-side mutants studied here, deletion of the *slr0228* gene resulted in the increase of the PSII content while the level of PSI is mostly decreased leading to general lowering of the cellular Chl content. The increase in PSII content is directly related to the inhibition of PSII protein degradation, while the decrease in the PSI content previously described for the single mutant ΔSlr0228 [28] could be an indirect effect reflecting a higher utilization of Chl for more accumulated PSII proteins. However, a more direct effect of FtsH2 on biogenesis of PSI cannot be excluded. In this context it is understandable that the ΔPsbV and D1-S345P strains with the highest PSI content also show the most extensive decrease in PSI after inactivation of the *slr0228* gene.

4.5. Novel role for PsbO at low irradiances

Our study has showed a previously unrecognized phenotypic feature of the ΔPsbO strain: its inability to grow under very low-light conditions. The mutant grew very slowly in the presence of 5 mM glucose at an irradiance of 5 μmol photons m^{-2} s^{-1} (Fig. S1A) but could not grow below this light threshold regardless of the presence of glucose. Growth under low-light conditions led to the strong suppression of Chl as well as phycobiliprotein content in the mutant cells (Fig. S2). The effect seems to be directly related to the function and quality control of PSII complex as the addition of the PSII inhibitor diuron, and the inactivation of the FtsH2 protease improved the rate of photoheterotrophic growth and even restored the ability of the ΔPsbO mutant to grow at irradiance below 5 μmol photons m^{-2} s^{-1}. Given the FtsH2 dependency of the mutant phenotype we speculate that dark destabilization of the $CaMn_4$ cluster in ΔPsbO cells [22] induces a permanent D1 replacement process that consumes most of the tetrapyrroles produced in the low-light grown cells. This explanation would indicate that the impairment of the $CaMn_4$ cluster and related modification of its neighborhood, including a change in PsbO binding, could play a key role in the signal that triggers D1 replacement. In summary, regardless of the exact mechanism, the low Chl and phycobiliprotein level in low-light grown ΔPsbO cells suggests that PsbO is not only an important PSII-specific factor in stabilizing the OEC, but it is required for a more general process ensuring a balanced pigment distribution among various thylakoid proteins under conditions of intensive D1 turnover. In line with this proposed role in the biogenesis of pigment-proteins, the PsbO protein has been detected not only in thylakoids but also in the cytoplasmic membrane [53] where some of the early steps in the biogenesis of the photosystems are proposed to occur [54].

Acknowledgements

The authors are grateful to Drs. Dexter A. Chisholm and Bruce Diner for the ΔPsbO and ΔCtpA strains, Profs. Wim Vermaas and Himadri Pakrasi for ΔPsbV and ΔCYT strains, and Remco De Vries and Stana Kuviková for construction of the ΔPsbO/ΔSlr0228 and D1-S345P/ΔSlr0228 mutants. The work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (project no. MSM6007665808), Czech Academy of Sciences (research concept no. AV0Z50200510) and its Grant Agency (project no. IAA400200801) and BBSRC.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.bbabio.2010.02.006.

References

[1] Ch. Foyer, M. Lelandais, K.J. Kuvert, Photooxidative stress in plants, Physiol. Plant. 92 (1994) 696–717.
[2] K. Asada, The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons, Annu. Rev. Plant. Physiol. Plant Mol. Biol. 50 (1999) 601–639.
[3] Z. Adam, A.K. Clarke, Cutting edge of chloroplast proteolysis, Trends Plant Sci. 7 (2002) 451–456.
[4] S.B. Powles, Photoinhibition of photosynthesis induced by visible light, Annu. Rev. Plant Physiol. 35 (1984) 14–44.
[5] O. Prasil, N. Adir, I. Ohad, Dynamics of photosystem II: mechanism of photoinhibition and recovery processes, in: J. Barber (Ed.), The Photosystems: Structure, Function and Molecular Biology, Elsevier Science Publishers, Amsterdam, 1992, pp. 295–348.
[6] E.-M. Aro, I. Virgin, B. Anderson, Photoinhibition of photosystem II. Inactivation, protein damage and turnover, Biochim. Biophys. Acta 1143 (1993) 113–134.
[7] K.N. Ferreira, T.N. Iverson, K. Maglaoui, J. Barber, S. Iwata, Architecture of the photosynthetic oxygen-evolving center, Science 303 (2004) 1831–1838.
[8] B. Loll, J. Kern, W. Saenger, A. Zouni, J. Biesiadka, Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II, Nature 438 (2005) 1040–1044.
[9] K. Yokthongwattana, A. Melis, Photoinhibition and recovery in oxygenic photosynthesis: mechanism of a photosystem II damage and repair, in: B. Demmig-Adams, W.W. Adams, A. Matoo (Eds.), Advances in Photosynthesis and Respiration, Photoprotection, Photoinhibition, Gene Regulation, and Environment, Springer, Dordrecht, 2006, pp. 175–191.
[10] P.J. Nixon, J.T. Trost, B.A. Diner, Role of the carboxy terminus of polypeptide-D1 in the assembly of a functional water-oxidizing manganese cluster in Photosystem-II of the cyanobacterium *Synechocystis* sp. PCC 6803 — assembly requires a free carboxyl group at C-terminal position 344, Biochem. 31 (1992) 10859–10871.
[11] P.R. Anbudurai, T.S. Mor, I. Ohad, S.V. Shestakov, H.B. Pakrasi, The CtpA gene encodes the C-terminal processing protease for the D1 protein of the photosystem-II reaction-center complex, Proc. Natl. Acad. Sci. USA 91 (1994) 8082–8086.
[12] N. Inagaki, Y. Yamamoto, K. Satoh, A sequential two-step proteolytic process in the carboxyl-terminal truncation of precursor D1 protein in *Synechocystis* sp. PCC 6803, FEBS Lett. 509 (2001) 197–201.
[13] J. Komenda, S. Kuviková, B. Granvogl, L.A. Eichacker, B.A. Diner, P.J. Nixon, Cleavage after residue Ala352 in the C-terminal extension is an early step in the maturation of the D1 subunit of photosystem II in *Synechocystis* PCC 6803, Biochim. Biophys. Acta 1767 (2007) 829–837.
[14] S.I. Allakhverdiev, N. Tsvetkovaa, P. Mohanty, B. Szalontai, B.Y. Moon, M. Debreczenya, N. Murata, Irreversible photoinhibition of photosystem II is caused by exposure of *Synechocystis* cells to strong light for a prolonged period, Biochim. Biophys. Acta 1708 (2005) 342–351.
[15] J. Komenda, V. Reisinger, B.Ch. Müller, M. Dobáková, B. Granvogl, L.A. Eichacker, Accumulation of the D2 protein is a key regulatory step for assembly of the photosystem II reaction center complex in *Synechocystis* PCC 6803, J. Biol. Chem. 279 (2004) 48620–48629.
[16] J.L. Roose, H.B. Pakrasi, Evidence that D1 processing is required for manganese binding and extrinsic protein assembly into photosystem II, J. Biol. Chem. 279 (2004) 45417–45422.
[17] N.B. Ivleva, S.V. Shestakov, H.B. Pakrasi, The carboxyl-terminal extension of the precursor D1 protein of photosystem II is required for optimal photosynthetic performance of the cyanobacterium *Synechocystis* sp. PCC 6803, Plant Physiol. 124 (2000) 1403–1411.
[18] S. Kuviková, M. Tichý, J. Komenda, A role of the C-terminal extension of the photosystem II D1 protein in sensitivity of the cyanobacterium *Synechocystis* PCC 6803 to photoinhibition, Photochem. Photobiol. Sci. 4 (2005) 1044–1048.
[19] A. Guskov, J. Kern, A. Gabdulkhakov, M. Broker, A. Zouni, W. Saenger, Cyanobacterial photosystem II at 2.9-Å resolution and the role of quinones, lipids, channels and chloride, Nat. Struct. Mol. Biol. 16 (2009) 334–342.

[20] J.L. Roose, K.M. Wegener, H.B. Pakrasi, Extrinsic proteins of photosystem II, Photosynth. Res. 92 (2007) 369–387.
[21] J.-R. Shen, M. Ikeuchi, Y. Inoue, Analysis of the psbU gene encoding the 12-kDa extrinsic protein of photosystem II and studies on its role by deletion mutagenesis in *Synechocystis* sp. PCC 6803, J. Biol. Chem. 272 (1997) 17821–17826.
[22] R. Burnap, L.A. Sherman, Deletion mutagenesis in *Synechocystis* sp. PCC 6803 indicates that the Mn-stabilizing protein of photosystem II is not essential for O_2 evolution, Biochemistry 30 (1991) 440–446.
[23] J.R. Shen, Y. Inoue, Binding and functional properties of 2 new extrinsic components, cytochrome *c*-550 and a 12 kDa protein, in cyanobacterial photosystem II, Biochemistry 32 (1993) 1825–1832.
[24] J.R. Shen, W.F. Vermaas, Y. Inoue, The role of cytochrome *c*-550 as studied through reverse genetics and mutant characterization in *Synechocystis* sp. PCC 6803, Biochemistry 270 (1995) 6901–6907.
[25] J.B. Philbrick, B.A. Diner, B.A. Zilinskas, Construction and characterization of cyanobacterial mutants lacking the manganese stabilizing polypeptide of photosystem II, J. Biol. Chem. 266 (1991) 13370–13376.
[26] S.R. Mayes, K.M. Cook, S.J. Self, Z. Zhang, J. Barber, Deletion of the gene encoding the photosystem II 33 kDa protein from *Synechocystis* sp. PCC6803 does not inactivate water-splitting but increases vulnerability to photoinhibition, Biochim. Biophys. Acta 1060 (1991) 1–12.
[27] P.J. Nixon, M. Barker, M. Boehm, R. de Vries, J. Komenda, FtsH mediated repair of the photosystem II complex in response to light stress, J. Exp. Bot. 56 (2005) 357–365.
[28] N. Mann, N. Novac, C. Mullineaux, J. Newman, S. Bailey, C. Robinson, Involvement of an FtsH homologue in the assembly of functional photosystem I in the cyanobacterium *Synechocystis* sp. PCC 6803, FEBS Lett. 479 (2000) 72–77.
[29] P. Silva, E. Thompson, S. Bailey, O. Kruse, C.W. Mullineaux, C. Robinson, N.H. Mann, P.J. Nixon, FtsH is involved in the early stages of repair of photosystem two in *Synechocystis* sp PCC 6803, Plant Cell 15 (2003) 2152–2164.
[30] M. Yoshioka, S. Uchida, H. Mori, K. Komayama, S. Ohira, N. Morita, T. Nakanishi, Y. Yamamoto, Quality control of photosystem II: cleavage of reaction center D1 protein in spinach thylakoids by FtsH protease under moderate heat stress, J. Biol. Chem. 281 (2006) 21660–21669.
[31] J. Komenda, M. Barker, S. Kuviková, R. de Vries, C.W. Mullineaux, M. Tichý, P.J. Nixon, The FtsH protease slr0228 is important for quality control of photosystem II in the thylakoid membrane of *Synechocystis* sp. PCC 6803, J. Biol. Chem. 281 (2006) 1145–1151.
[32] J. Komenda, M. Tichý, O. Prášil, J. Knoppová, S. Kuviková, R. de Vries, P.J. Nixon, The exposed N-terminal tail of the D1 subunit is required for rapid D1 degradation during photosystem II repair in *Synechocystis* sp. PCC 6803, Plant Cell 19 (2007) 2389–2854.
[33] J. Komenda, J. Barber, Comparison of psbO and psbH deletion mutants of *Synechocystis* PCC 6803 indicate that degradation of D1 protein is regulated by the QB site and is dependent on protein synthesis, Biochemistry 34 (1995) 9625–9631.
[34] J.G.K. Williams, Construction of specific mutations in PSII photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803, Methods Enzymol. 167 (1998) 766–778.
[35] H.B. Pakrasi, J.G.K. Williams, C.J. Arntzen, Targeted mutagenesis of the *psbE* and *psbF* blocks photosynthetic electron transport: evidence for a functional role of cytochrome *b*-559 in photosystem II, EMBO J. 7 (1988) 325–332.
[36] M. Dobáková, M. Tichý, J. Komenda, Role of the PsbI protein in photosystem II assembly and repair in the cyanobacterium *Synechocystis* sp. PCC 6803, Plant Physiol. 145 (2007) 1681–1691.
[37] H. Schägger, G. von Jagow, Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form, Anal. Biochem. 199 (1991) 223–231.
[38] A.R. Wellburn, K. Lichtenthaler, Formulae and programs to determine total carotenoids and chlorophyll a and b of leaf extracts in different solvents, in: C. Sybesma (Ed.), Advances in Photosynthesis Research, Martinus Nijjhoff, Dordrecht, 1984, pp. 10–12.
[39] J. Komenda, J. Nickelsen, L.A. Eichacker, M. Tichý, O. Prášil, P.J. Nixon, The cyanobacterial homologue of hcf136/ycf48 is a component of an early photosystem II assembly complex and is important for both the efficient assembly and repair of photosystem II in *Synechocystis* sp. PCC6803, J. Biol. Chem. 283 (2008) 22390–22399.
[40] J. Komenda, H.A. Hassan, B.A. Diner, R.J. Debus, J. Barber, P.J. Nixon, Degradation of the photosystem II D1 and D2 proteins in different strains of the cyanobacterium *Synechocystis* PCC 6803 varying with respect to the type and level of *psbA* transcript, Plant Mol. Biol. 42 (2000) 635–645.
[41] T. Takahashi, N. Inoue-Kashino, S. Ozawa, Y. Takahashi, Y. Kashino, K. Satoh, Photosystem II complex in vivo is a monomer, J. Biol. Chem. 284 (2009) 15598–15606.
[42] M. Watanabe, M. Iwai, R. Narikawa, M. Ikeuchi, Is the photosystem II complex a monomer or a dimer? Plant Cell Physiol. 50 (2009) 1674–1680.
[43] G. Friso, J. Barber, Comparison of the level of psbA, psbD and psbB transcripts in psbO-less, psbH-less mutants and wild-type of *Synechocystis* sp. PCC 6803, in: P. Mathis (Ed.), Photosynthesis: From Light to Biosphere, Kluwer Academic Publishers, Netherlands, 1995, pp. 517–520, Vol. III.
[44] M. Drath, N. Kloft, A. Batschauer, K. Marin, J. Novak, K. Forchhammer, Ammonia triggers photodamage of photosystem II in the cyanobacterium *Synechocystis* sp. strain PCC 6803, Plant Physiol. 147 (2008) 206–215.
[45] O. Cheregi, C. Sicora, P.B. Kós, M. Barker, P.J. Nixon, I. Vass, The role of the FtsH and Deg proteases in the repair of UV-B radiation-damaged photosystem II in the cyanobacterium *Synechocystis* PCC 6803, Biochim. Biopys. Acta 1767 (2007) 820–828.
[46] J. Komenda, L. Lupinková, J. Kopecký, Absence of the psbH gene product destabilizes the photosystem II complex and bicarbonate binding on its acceptor side in *Synechocystis* PCC 6803, Eur. J. Biochem. 269 (2002) 610–619.
[47] N. Ohnishi, S.I. Allakhverdiev, S. Takahashi, S. Higashi, M. Watanabe, Y. Nishiyama, N. Murata, Two-step mechanism of photodamage to photosystem II: step 1 occurs at the oxygen-evolving complex and step 2 occurs at the photochemical reaction center, Biochemistry 44 (2005) 8494–8499.
[48] P. Sarvikas, M. Hakala, E. Pätsikkä, T. Tyystjärvi, E. Tyystjärvi, Action spectrum of photoinhibition in leaves of wild type and npq1–2 and npq4–1 mutants of *Arabidopsis thaliana*, Plant Cell Physiol. 47 (2006) 391–400.
[49] N. Murata, S. Takahashi, Y. Nishiyama, S.I. Allakhverdiev, Photoinhibition of photosystem II under environmental stress, Biochim. Biophys. Acta 1767 (2007) 414–421.
[50] M. Dalla Chiesa, G. Friso, Z. Deak, I. Vass, J. Barber, P.J. Nixon, Reduced turnover of the D1 polypeptide and photoactivation of electron transfer in novel herbicide resistant mutants of *Synechocystis* sp. PCC 6803, Eur. J. Biochem. 248 (1997) 731–740.
[51] B. Lundin, M. Nurmi, M. Rojas-Stuetz, E.-M. Aro, I. Adamska, C. Spetea, Towards understanding the functional difference between the two PsbO isoforms in *Arabidopsis thaliana* — insights from phenotypic analyses of psbO knockout mutants, Photosynth. Res. 98 (2008) 405–414.
[52] M. Schottkowski, S. Gkalympoudis, N. Tzekova, Ch. Stelljes, D. Schuenemann, E. Angele, J. Nickelsen, Interaction of the periplasmic PratA factor and the PsbA (D1) protein during biogenesis of photosystem II in *Synechocystis* sp. PCC 6803, J. Biol. Chem. 284 (2009) 1813–1819.
[53] D. Smith, C.J. Howe, The distribution of photosystem I and photosystem II polypeptides between the cytoplasmic and thylakoid membranes of cyanobacteria, FEMS Microbiol. Lett. 110 (1993) 341–348.
[54] E. Zak, B. Norling, R. Maitra, F. Huang, B. Andersson, H.B. Pakrasi, The initial steps of biogenesis of cyanobacterial photosystems occur in plasma membranes, Proc. Natl. Acad. Sci. USA 98 (2001) 13443–13448.