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Cyanobacterial *psbA* families in *Anabaena* and *Synechocystis* encode trace, constitutive and UVB-induced D1 isoforms

Cosmin I. Sicora [a], Sarah E. Appleton [a], Christopher M. Brown [b], Jonathon Chung [a], Jillian Chandler [a], Amanda M. Cockshutt [b], Imre Vass [c], Douglas A. Campbell [a,*]

[a] *Department of Biology, Mount Allison University, Sackville, NB, Canada E4L1G7*
[b] *Environmental Proteomics Inc., Canada*
[c] *Institute of Plant Biology, Biological Research Center, Szeged, Hungary*

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Abstract

Cyanobacteria cope with UVB induced photoinhibition of Photosystem II by regulating multiple *psbA* genes to boost the expression of D1 protein (in *Synechocystis* sp. PCC6803), or to exchange the constitutive D1:1 protein to an alternate D1:2 isoform (in *Synechococcus* sp. PCC7942). To define more general patterns of cyanobacterial *psbA* expression, we applied moderately photoinhibitory UVB to *Anabaena* sp. PCC7120 and tracked the expression of its five *psbA* genes. *psbAI*, encoding a D1:1 protein isoform characterized by a Gln130, represented the majority of the *psbA* transcript pool under control conditions. *psbAI* transcripts decreased upon UVB treatment but the total *psbA* transcript pool increased 3.5 fold within 90 min as a result of sharply increased *psbAII*, *psbAIV* and *psbAIII* transcripts encoding an alternate D1:2 protein isoform characterized by Glu130, similar to that of *Synechococcus*. Upon UVB treatment the relaxation of flash induced chlorophyll fluorescence showed a characteristic acceleration of a decay phase likely associated with the exchange from the D1:1 protein isoform encoded by *psbAI* to the alternate D1:2 isoform encoded by *psbAIV*, *psbAII* and *psbAIII*. Throughout the UVB treatment the divergent *psbA0* made only a trace contribution to the total *psbA* transcript pool. This suggests a similarity to the divergent *psbAI* gene from *Synechocystis*, whose natural expression we demonstrate for the first time at a trace level similar to *psbA0* in *Anabaena*. These trace-expressed *psbA* genes in two different cyanobacteria raise questions concerning the functions of these divergent genes.

Keywords: *Anabaena*; D1; Photoinhibition; Photosystem II; *psbA*; *Synechocystis*; UVB

1. Introduction

One of the main sites of damage by UVB radiation in photosynthetic organisms is the pigment–protein complex of photosystem II (PSII) (for a review, see [1,2]), in particular the D1 protein subunit [3]. Photo-damaged D1 proteins are replaced with newly synthesized ones in a D1 protein repair cycle. If the turn-over rate of the D1 protein or reassembly of PSII centers is slower than the rate of damage, the overall oxygen evolving capacity of the cells decreases due to net inhibition of the pool of PSII centers. The critical D1 protein is encoded by the *psbA* gene present as a single chloroplastic gene in eukaryotic photoautotrophs. In contrast, in known cyanobacteria the D1 protein is encoded by a small gene family with one to five members (http://www.kazusa.or.jp/cyano/, http://genome.jgi-psf.org/mic_home.html).

To date, UVB influences on expression patterns of the *psbA* genes have been studied in *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC7942 which both have three *psbA* genes, but which exhibit different responses to environmental stresses. In *Synechococcus* PCC 7942 the three *psbA* genes encode two distinct D1 protein isoforms [4–9]. Under environmental stress conditions such as high light [5,6,10,11], blue light [12], low temperature [13,14], UVB [15], or oxygen depletion [16], this cyanobacterium alters *psbA* expression to selectively exchange the D1:1 isoform encoded by *psbAI* with

Abbreviations: Ci, inorganic carbon; DCMU, 3-(3′, 4′-dichlorphenyl)-1,1-dimethylurea; DMBQ, 2,5 dimethyll, *p*-benzoquinone; PSII, photosystem II; UVB, ultraviolet B radiation

* Corresponding author. Tel.: +1 506 364 2521; fax: +1 506 364 2505.
E-mail address: dcampbell@mta.ca (D.A. Campbell).

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the D1:2 isoform, encoded by *psbAII* and *psbAIII*. The overall regulation of *Synechococcus* D1 content involves environmentally dependent changes in form-specific transcription, translation and D1 turnover [17–20]. Mutant strains of *Synechococcus* [4] in which the exchange of D1:1 to D1:2 is blocked suffer enhanced inhibition under UVB [21], showing that the two isoforms are functionally distinct. Furthermore Tichy et al., [9] used *Synechocystis* mutant strains in which all three native genes were inactivated to show that a transgenic strain expressing only the D1:2 isoform from *Synechococcus* showed a faster decay of variable fluorescence in the presence of DCMU, reflecting faster recombination of the reduced Q_A with positive charges from the donor side of PSII [22], compared to a *Synechocystis* mutant expressing only the D1:1 form from *Synechococcus* [9]. Thermoluminescence [8] and fluorescence life time data [7] from *Synechococcus* cells containing only D1:1 or D1:2 also support enhanced charge recombination in PSII centers containing D1:2. This functional difference is associated with the presence of a glutamate residue instead of a glutamine at position 130 in the D1 protein sequence, which interacts with a key phaeophytin co-factor [23,24].

In *Synechocystis* sp. PCC6803 only one type of D1 protein, encoded by both the *psbAII* and *psbAIII* genes, has been detected under normal growth conditions as well as under stress [25]. The transcript from *psbAII* accounts for 90% of the total *psbA* transcript pool under normal growth conditions [26]. A study using a highly selective S1 nuclease protection assay showed that UVB exposure of the cells induces an increase in the total *psbA* transcript pool primarily through increased accumulation of *psbAIII* transcripts [25]. Other studies using micro-array [27] or non-specific probes and hybridization blotting [28] showed general induction of the *psbAII* and/or *psbAIII* genes in *Synechocystis* under UVB. This *Synechocystis* pattern of supplemental expression of an identical protein isoform under excitation stress is therefore distinct from the D1 isoform exchange found in *Synechococcus*.

In *Synechocystis*, the *psbAI* gene encodes a divergent D1 protein called D1′, expression of which has not been detected to date in wild type cells [26,29,30]. Artificially induced expression of this *Synechocystis psbAI* does result in a functional D1 protein with somewhat distinct redox properties [30–32]. The presence of *psbAI* in the genome of *Synechocystis* has been enigmatic because, if not exposed to selective pressure through expression, the gene is sufficiently divergent [29] to be statistically predicted to have accumulated multiple inactivating mutations.

The different transcriptional response patterns of the three *psbA* genes in the two characterized cyanobacteria raises the question of what, if any, general principles guide the expression patterns of *psbA* families in cyanobacteria in response to environmental stresses. In particular, we sought to understand transcriptional patterns under UVB in cyanobacterial species with more than three genes in the *psbA* gene family. Among known cyanobacterial genomes the heterocystous, filamentous cyanobacterium *Anabaena* PCC 7120 shares the largest known five-member *psbA* gene family with the closely related *Nostoc punctiforme* ATCC 29133 (http://genome.jgi-psf.org/mic_home.html) and more surprisingly with the deeply divergent cyanobacterium *Gloeobacter violaceus* PCC7421 (Cyanobase: http://www.kazusa.or.jp/cyano [33,34]). *Anabaena* was initially characterized with four *psbA* genes named *psbAI*, *psbAII*, *psbAIII* and *psbAIV* [35]. More recently, a divergent fifth gene alr3742, which we herein term *psbA0*, was identified once the complete *Anabaena* genome sequence was published (Cyanobase: http://www.kazusa.or.jp/cyano, [36,37]). We therefore used gene-specific RT-RT-Q-PCR to analyze *psbA* transcript patterns in *Anabaena* PCC 7120 exposed to moderately inhibitory UVB. In parallel we tracked changes in the decay profiles of flash-induced fluorescence which show characteristic changes in response to changes in the D1 isoform composition [9] of the PSII reaction centers.

2. Materials and methods

2.1. Strain, growth and treatment conditions

Anabaena sp. PCC 7120 cells were obtained from the Pasteur Culture Collection and cultured in BG-11 growth medium [38] at 30 °C under fluorescent white growing light of 55 μmols photons m^{-2} s^{-1}. Most results are presented for cultures growing under high inorganic carbon (2–4 mM total dissolved inorganic carbon) achieved by bubbling the media with 5% CO_2. Similar experiments were also conducted with cultures grown under low inorganic carbon achieved by bubbling with ambient air. The cultures were inoculated into rectangular polycarbonate tissue culture flasks (BD Biosciences, Two Oak Park, Bedfort, MA, 01730, USA) to 0.125 μg chl ml^{-1} and grown for 5 generations until they reached 4 μg chl ml^{-1}, within 6 days. Cells were then, in addition to the growth light, exposed to 1.3 W m^{-2} UVB light, provided from Philips TL 20W/12 RS fluorescent sun lamps with an emission spectrum between 275 and 380 nm, peak, 310–315 nm (Philips Lighting, Stockholm, Sweden), and measured by a Skye Instruments UVB light sensor (no. SKU430) and a Spectrosensmeter (no. RS232; Skye Instruments Ltd., Llandrindod Wells, Wales, UK). We used cellulose-acetate filters to filter out the UVC component of the lamp spectrum below 280 nm. This UVB light treatment caused an approximate 50% drop in oxygen evolution and fluorescence induction parameters within 90 min, with subsequent functional recovery tracked over 60 min after the end of the UVB treatment. The concentration of chlorophyll was assayed with 100% methanol extracts from cell samples [39].

2.2. Oxygen evolution and chlorophyll fluorescence parameters

Oxygen evolution of culture samples was measured in the presence of the Photosystem II electron acceptor compound 2,5-dimethyl-*p*-benzoquinone at 0.5 mM (DMBQ) [40] at 30 °C using a temperature regulated Clark-type electrode and cuvette (DW2, Hansatech, Kings Lynn, England) (Fig. 1). Oxygen consumption or evolution was recorded in the dark, at the growing light of 55 μmol photons m^{-2} s^{-1} which drove oxygen evolution at ca. 75% of the saturated value and at 150 μmol photons m^{-2} s^{-1}, determined to be saturating by light response curves (data not shown). The measurements under growth light were to track the actual running performance of PSII under growth conditions while the saturating light was used to determine changes in the maximum capacity for PSII activity. A pulse amplitude modulated fluorometer (Xe-PAM, Walz, Effeltrich, Germany) was used to monitor the chlorophyll a fluorescence parameters Fv′/Fm′ and ΦPSII [21].

2.3. D1 protein determinations

PsbA, the D1 core subunit of PSII, and PsbB, the CP47 stable pigment protein subunit of PSII, were quantified from subsamples of culture taken simultaneously with culture samples used for biophysical measurements and transcript analyses. 1.8 ml subsamples of culture were centrifuged at 12,000 g for 5 min immediately after withdrawal from the culture. Cell pellets were stored

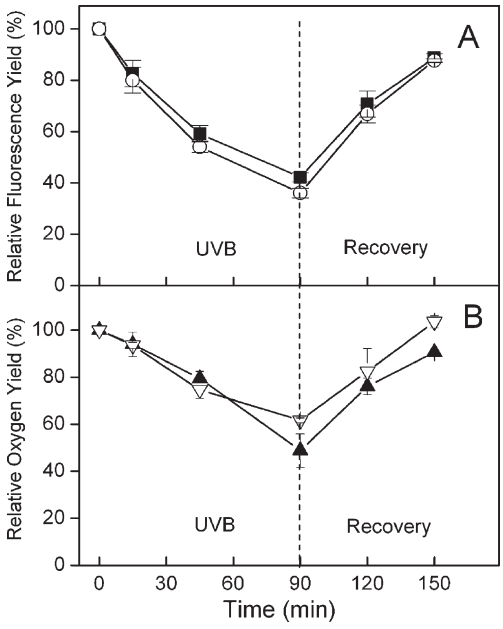

Fig. 1. UVB induced damage and recovery of the photosynthetic parameters in *Anabaena* sp. PCC 7120 intact cells. Samples were illuminated with 1.3 mW m^{-2} UVB light under continuous 55 μmol photons m^{-2} s^{-1} visible light and fluorescence parameters Fv'/Fm', (solid squares) and ϕPSII (open circles) were recorded (A) as well as oxygen evolution (B) in the presence of DMBQ as electron acceptor using saturating (open down triangles) and growing (solid up triangles) light intensity. *N*=3, ±S.E.M.

at −20 °C. For the extraction of total protein, we resuspended pellets in 200 μl LDS solubilization buffer (140 mM Tris base, 105 mM Tris–HCl, 0.5 mM EDTA, 2% LDS, and 10% glycerol), and froze them in liquid nitrogen. We sonicated frozen samples with a Branson Ultrasonics model 450 fitted with a microtip attachment. Once samples were completely thawed, tubes were immediately refrozen in liquid nitrogen. Following 3 rounds of sonication and flash freezing, samples were centrifuged for 3 min and chlorophyll *a* contents were determined by withdrawing 50 μl of cell lysate, extracting chlorophyll with 950 μl 90% $MgCO_3$ saturated acetone, and measuring absorbance at 663 nm and 646 nm [41].

Samples were supplemented with dithiothreitol (DTT) to 50 mmol l^{-1} final concentration with a 1/10 volume addition of 0.5 mol l^{-1} DTT. Extracts were heated to 70 °C for 5 min, and recombinant PsbA or PsbB protein standards (www.environmentalproteomics.ca) were heated to 95 °C for 5 min.

Proteins were separated on NuPAGE Bis–Tris gels (Invitrogen, Burlington, ON, Canada) in MES 1× SDS running buffer (Invitrogen) in an XCell Sure-Lock Tank (Invitrogen), with 3 lanes bearing a concentration range of quantified PsbA/D1 or PsbB protein (www.environmentalproteomics.ca) run as standards on each gel. Gels were electrophoresed at 200 V for 40–50 min. Following electrophoresis the proteins were transferred to polyvinylidene difluoride (PVDF) membranes pre-wetted in methanol and equilibrated in 1× transfer buffer (Invitrogen) using the XCell blot module (Invitrogen) for 60 to 80 min at 30 V.

Blots were blocked immediately following transfer in 2% ECL Advance (Amersham Biosciences, Piscataway, NJ, USA) blocking reagent in Tris buffered saline 0.1% Tween-20 (TBS-T, 20 mmol l^{-1} Tris, 137 mmol l^{-1} sodium chloride pH 7.6 with 0.1% (v/v) Tween-20) for 1 h at room temperature with agitation. Primary and secondary antibodies were used at a dilution of 1 in 50,000. Blots were incubated in primary anti-PsbA or anti-PsbB antibody (www.agrisera.se) solution for 1 h at room temperature with agitation. The antibody solution was decanted and the blot was rinsed briefly twice, then washed once for 15 min and 3 times for 5 min in TBS-T at room temperature with agitation. Blots were incubated in secondary antibody (rabbit anti-chicken horse radish peroxidase conjugate, AbCAM, www.abcam.com) diluted to 1:50,000 in 2% ECL Advance blocking solution for 1 h at room temperature with agitation. The blots were washed as above and developed for 5 min with ECL Advance detection reagent according the manufacturer's instructions (3 ml reagent per 68×81 mm blot). Images of the blots (Fig. 2A) were obtained using a FluorSMax CCD imager (Bio-Rad Laboratories Mississauga, Ontario, Canada) and Quantity One software (Bio-Rad).

Protein levels from samples and protein standards on immunoblots were quantitated using Quantity One software (Bio-Rad). The volume contour tool was used to define the band to be quantitated (standards and samples). Adjusted volume values (in counts×mm^2) were obtained with the volume analysis and report function. Standard curves were plotted and used to interpolate the amounts of protein in the experimental samples (Fig. 2B). The standard curve fell within the ca. 10-fold pseudo-linear region of the sigmoidal signal response curves for the immunoblotting.

2.4. Fluorescence relaxation kinetics

Flash induced increase and subsequent decay of fluorescence was measured using a double-modulation fluorometer (PSI Instruments, Brno, CZ) [22]. Both actinic (20 μs) and measuring (2.5 μs) flashes were given by red LEDs. The measurements were done in the time interval of 150 μs to 100s with the measuring flashes applied in a logarithmic succession and in the presence of the Photosystem II inhibitor 3-(3′, 4′-dichlorphenyl)-1,1-dimethylurea (DCMU) at 10 μM final concentration in order to block the electron transfer between Q_A and Q_B. We adjusted the intensity of the measuring flashes low enough to avoid any actinic effect to minimize distortion of the relaxation kinetics. *Anabaena* cells at 4 μg chl ml^{-1} were dark adapted for 10 min prior to measurement. Analysis of the

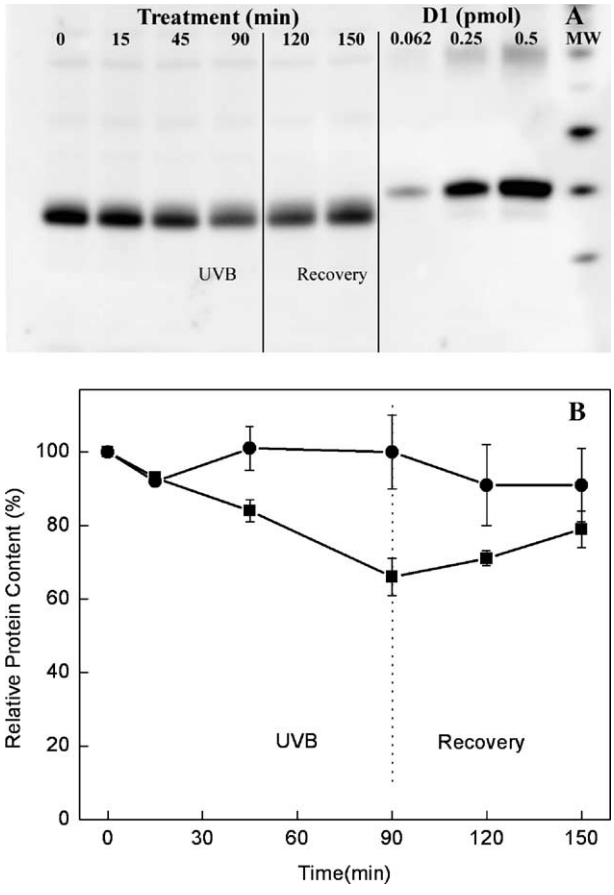

Fig. 2. UVB induced loss and recovery of the total D1 content in *Anabaena* sp. PCC 7120. (A) shows a representative detection of total D1 protein using a PsbA Global antibody (www.agrisera.se) binding a peptide target conserved in all the D1 isoforms. Three lanes of D1 molar quantitation standard (www.environmentalproteomics.ca) and a MW marker are also shown. (B) shows quantitation of the D1 protein (squares) and CP47 (circles) using the quantitation standards and BioRad Quantity One software. *N*=4, ±S.E.M.

decrease of the fluorescence was based on the model of the two electron gate as described earlier [22]. In the presence of DCMU the relaxation of the fluorescence depends on Q_A^- recombination with positive charges on the donor side of PSII. UVB exposure provokes two characteristic modifications in the relaxation kinetics [9,22]. First is a UVB generated fast phase of the initial decay caused by the impairment of the water oxidation complex. Second is an acceleration of the late decay component of the curve caused by changes in the acceptor side redox environment, apparently as a consequence of the change from glutamine at position 130 of D1:1 to glutamate at position 130 of D1:2 [7–9]. By deconvoluting the curves, we were able to calculate the half-life time of the slow phase and thereby detect the presence of functional PSII centres containing D1:2 in the population. The deconvolution of the curves was done using an equation with two exponential and one hyperbolic components that is usually used for deconvoluting curves in the absence of DCMU. To adapt this equation to the presence of DCMU the first exponential component was set to zero.

$$F(t) - F_0 = A_1\exp(-t/T_1) + A_2\exp(-t/T_2) + A_3/(1 + t/T_3) \quad (1)$$

where $F(t)$ is the variable fluorescence yield; F_0 is the fluorescence level before the flash; A_1–A_3 are the amplitudes; T_1–T_3 are the time constants from which the half-lives can be calculated as $t_{1/2}$=ln 2 T for the exponential component and T for the hyperbolic component.

2.5. Transcript determinations

Total RNA was isolated from cells using the Trizol (Invitrogen) phenol–guanidine–isothiocyanate–chloroform extraction protocol as described in McGinn et al. [42] and dissolved in nuclease free water (Invitrogen DEPC treated water). A treatment with 1U DNA-ase (Ambion Turbo DN-ase, 2130 Woodward, Austin, TX, USA) per sample was applied in order to remove traces of genomic DNA present in our isolate. The DNA-ase reaction was stopped by a double phenol extraction. The concentration of our RNA solution was calculated from the absorbance measurement at 260 nm assuming one unit absorbance in water to be equivalent with 40 μg RNA/ml.

First strand cDNA synthesis from 1 μg of purified RNA was performed using the BioRad iScript cDNA Synthesis kit (Bio Rad Laboratories Inc). We used a common reverse primer (Table 1) directed against a fully conserved region found about 600 bp within all the *psbA* genes, in order to create a common initial reverse transcriptase reaction covering for all *psbA* transcripts. This lowered the potential for variation among the genes at the reverse transcriptase step. In order to ensure gene specificity for each transcript detection, we designed the forward primers against the divergent transcribed non-translated 5′ regions of the *psbA* genes (Table 1). The primer pairs were thus designed to generate amplicons of similar length (600–650 bases) from each *psbA* transcript pool, which nonetheless varied sufficiently to be differentiated by a melting curve. Although the resulting amplicons are near the long end for recommended lengths for RT-RT-Q-PCR we obtained high and uniform amplification efficiencies (data not shown).

A specific primer pair was designed to amplify transcripts from the gene encoding the constitutive, moderate abundance transcript encoding phospho-enolpyruvate carboxylase, *pepC*, to use as a reference gene to verify the equivalence of the initial RNA levels. The constitutive expression of this gene has been found earlier in other cyanobacterial strains (P. McGinn and D. Campbell unpublished data) and was confirmed during our experiments (Fig. 3). The expression level of *pepC* is in the range of *psbA* gene expression in control cells (Fig. 3).

Table 1
Primer sequences

Primer name	Primer sequence 5′–3′
psbA0 forward	CCTTGTCGGTTGATATTTCACCATC
psbAI forward	GCGTCTGAATTAATCTGCACAAATCC
psbAII forward	TTGTTACAGGCAAAAAACAACTGCC
psbAIII forward	CCGCCTGATTCATAAATAATTAATCGC
psbAIV forward	TGTAACAGGTGGAAACGCCTACAA
psbA common reverse	GGAACCGTGCATTGCAGAGAA

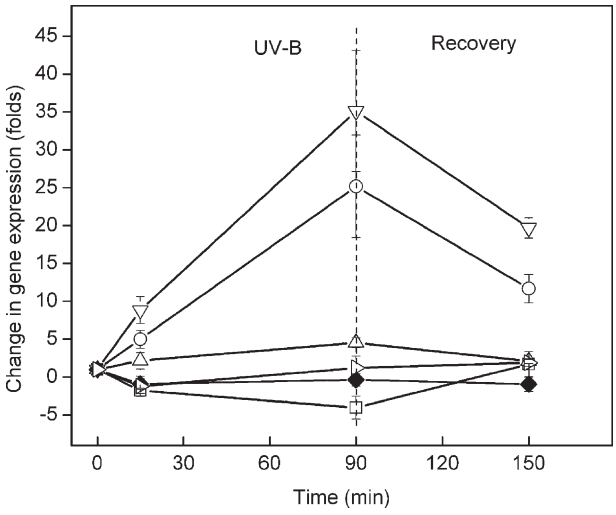

Fig. 3. Changes in *psbA* transcripts relative to control level of the respective transcript during the UVB treatment and recovery in *Anabaena* sp. PCC 7120. *psbA0* (open forward triangle), *psbAI* (open square), *psbAII* (open circle), *psbAIII* (open up triangle) and *psbAIV* (open down triangle). We also present *pepC*, transcripts (solid diamond) that we used when we calculated *psbA* transcripts relative to reference (Fig. 4). N=4, ±S.E.M.

The primer pairs were tested in silico using the Amplify software (Bill Engels, 2004, University of Wisconsin) and were compared using BLAST against the *Anabaena* genome (http://www.kazusa.or.jp/cyano) in order to ensure their gene specificity. The *psbA* primer pairs were tested as well by PCR using *Anabaena* genomic DNA as a template and the expected specific amplicon fragments were verified through agarose gel electrophoresis (data not shown).

Cell samples taken at time points during the treatment were used to measure the transcripts from the five *psbA* genes plus the *pepC* reference gene using Real Time Quantitative PCR on a BioRad iCycler using BioRad iTaq Polymerase (Bio Rad Laboratories Inc) and SyBR Green as a fluorescent dye to detect accumulation of double-stranded amplicons. For each RNA sample we performed triplicate RT-RT-Q-PCR determinations for each transcript. At the conclusion of the PCR cycling, we performed a melt curve analysis to confirm the presence and specificity of the expected amplicon product. The temperature was decreased to 60 °C and then increased in steps to 99 °C. The fluorescence yield of the double-stranded PCR product was monitored relative to temperature ($-dF/dt$) and a single melt peak indicating the presence of a single product with a gene-specific melting temperature was demonstrated in each case. The efficiency of the PCR reaction for each triplicate was estimated using the LineReg program [43] and an average of the three was made and used in the subsequent estimation of the expression levels.

We started with the same amount of RNA for each cDNA preparation, and then we spectrophotometrically verified the equivalent efficiency of each reverse transcription reaction (data not shown). Furthermore, we verified that each *psbA* gene gave an equal accumulation of amplicon when starting from an equal concentration of template, by performing quantitative PCR reactions using a concentration calibration range of *Anabaena* genomic DNA, which contains one copy of each *psbA* gene per genome (data not shown). The change in the transcripts for each gene during the treatment and recovery was then quantified relative to the transcripts for the respective gene in control samples:

$$\text{Target transcript relative to Time Zero control} = eff^{(\text{CT target at control} - \text{CT target at specific time point})} \quad (2)$$

where: CT is the cycle value where the amplicon fluorescence emission reached the detection threshold, and *eff* is amplification efficiency. Values for target transcripts were plotted as fold change relative to the control level at time zero (Fig. 3). Fig. 3 therefore shows gene-by-gene changes in the levels of each *psbA* transcript relative to the pre-UVB treatment control levels for the respective gene.

We found the pool of *pepC* transcripts did not vary with UVB treatment and was highly comparable across replicates (Fig. 3). We therefore used the steady

pool of *pepC* transcripts as a reference to estimate the changing pool sizes of the *psbA* transcripts for each time point under UVB exposure and recovery:

$$\text{Target transcript relative to reference} = eff^{(\text{CT reference gene}-\text{CT target gene})}, \quad (3)$$

where *eff* is again amplification efficiency.

This allowed us to use the expression of *pepC* as a base unit to express the amounts of transcripts from each *psbA* gene. We then estimated the total *psbA* transcript pool as the sum of each target transcript calculated relative to the reference gene (Eq. (3)), after verifying that each *psbA* gene generates an equal level of amplicon when starting from an equal level of template (data not shown). For presentation, we then expressed each *psbA* transcript pool and the sum of *psbA* transcript pools at each treatment time point on a scale relative to the *pepC* transcript pool (Fig. 4). Fig. 4 therefore shows the changes in the total *psbA* pool under UVB treatment and recovery, as well as the changing proportional contributions of each *psbA* transcript to the total pool.

2.6. Data presentation

All the graphs and statistical calculations were done using Origin 7.5 data analysis software (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. UVB induced inactivation of PSII function in Anabaena PCC 7120 cells

To assess changes in *psbA* transcript pools as a result of UVB inhibition we exposed the cells to UVB for a period of 90 min with continued visible growth light at 55 μmol photons m^{-2} s^{-1}. The Photosystem II fluorescence parameters F*v*′/F*m*′ and ΦPSII measured under growth light decreased by 60% relative to control values within 90 min of UVB treatment (Fig. 1A). The oxygen evolution capacity of the cells relative to control decreased by 55–60% when measured under growth light and by 45–50% when measured under saturating light (Fig. 1B).

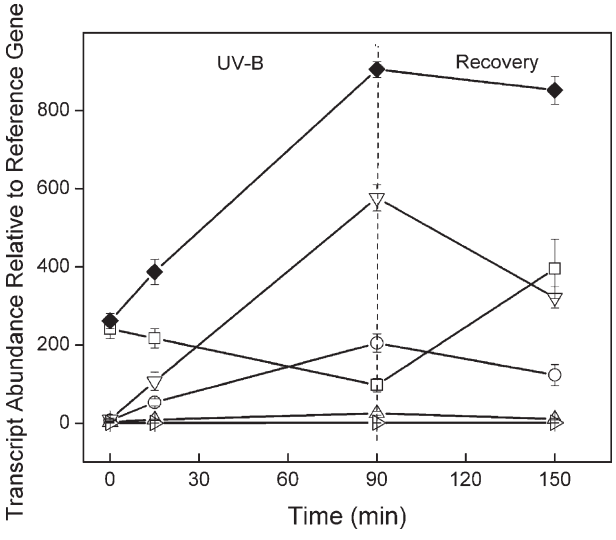

Fig. 4. Changes in the *psbA* transcripts normalized to the expression level of *pepC* gene during UVB exposure and subsequent recovery of the *Anabaena* sp. PCC 7120 cells. For each time point the sum of *psbA0* (open forward triangle), *psbAI* (open square), *psbAII* (open circle), *psbAIII* (open up triangle) and *psbAIV* (open down triangle) transcript was plotted (solid diamond) showing the change in the total *psbA* transcript pool. *N*=4, ±S.E.M.

After the UVB treatment, the cells were maintained under growth light and the recovery of their PSII function was assessed. F*v*′/F*m*′ and ΦPSII parameters recovered to 80% of their control values (Fig. 1A) within 60 min, while oxygen evolution recovered to control values when saturating light was used and to about 85% of the control values when growth light was used for the measurement (Fig. 1B).

3.2. Loss of D1 content during UVB treatment in Anabaena PCC 7120 cells

During the UVB treatment the total content of D1 protein was assessed using a quantitative immunodetection technique (Fig. 2). The UVB treatment decreased the total D1 content to about 65% of control levels, while during the recovery the D1 content recovered to 80% of control values. PsbB/CP47 protein content did not change significantly over the course of the UVB treatment or recovery (Fig. 2).

3.3. UVB induced changes in psbA transcript pools in Anabaena PCC 7120

Using gene specific primers we assessed the transcript pools of each member of the *psbA* gene family as well as the transcripts of *pepC*, used in our experiments as a constitutively expressed reference gene. Relative to the pre-treatment controls, UVB induced consistent and significant changes in levels of the transcripts of four of the five *psbA* genes. Within 90 min UVB treatment induced the expression of *psbAII* (25 fold), *psbAIII* (4.5 fold) and *psbAIV* (35 fold), while *psbAI* transcripts dropped (4 fold) from their control expression values. (Fig. 3). During the subsequent 60 min recovery period, the *psbAII*, *psbAIII* and *psbAIV* transcripts declined and *psbAI* transcripts re-accumulated to control levels (Fig. 3). The *psbA0* transcripts remained at low but detectable levels throughout UVB treatment and recovery. UVB, as expected, did not change the *pepC* transcript levels (Fig. 3).

The abundance of the transcript from each *psbA* gene was, therefore, also quantified with reference to the *pepC* transcripts in the same sample. The total *psbA* gene family transcript pool was then calculated as the sum of the transcripts from each of the five genes at the respective time points, calculated relative to the constitutive *pepC* transcript pool as a base of comparison. Under control conditions, the transcript from *psbAI* accounted for 92% of the *psbA* transcript pool (Fig. 4). Once the UVB treatment started, an increase in the total *psbA* transcript pool resulted from a sharp increase in transcripts from *psbAII*, *psbAIV* and to a lesser extent *psbAIII* (Fig. 4). These UVB induced transcript accumulations more than offset the concomitant decrease in *psbAI* transcripts under UVB. After 90 min of UVB, the total *psbA* transcript pool was about 3.5 fold higher than the control level, with a majority contribution from *psbAIV* (63%) and a significant contribution from *psbAII* (23%) (Fig. 4). The *psbAI* transcript decreased steadily during the UVB treatment to about 10% of the total transcript pool by 90 min (Fig. 4). During the 60 min of recovery the total *psbA* transcript pool slightly decreased following the decrease of the transcripts from *psbAIV*, *psbAII* and *psbAIII*, which were partially compensated by the sharp re-

accumulation of the *psbAI* transcripts (Fig. 4). *psbA0* remained at a trace, constitutive contribution of about 0.2% of the total transcript pool throughout the treatment and recovery (Fig. 4).

psbAIII was only weakly induced by UVB in the cultures growing under high inorganic carbon (Ci). We conducted parallel UVB treatments on cells grown under low inorganic carbon, achieved by bubbling the growth media with air instead of 5% CO_2. In these low Ci cultures, *psbAIII* transcripts were strongly induced by UVB to an extent comparable to *psbAIV*. The other *psbA* transcript pools showed similar responses to UVB under both high and low Ci growth conditions (data not shown). Therefore, *psbAIII* responds specifically and synergistically to the combination of UVB and low Ci.

3.4. UVB induced changes of the flash fluorescence decay indicate D1 isoform exchange

In measurements of the decay of the flash induced fluorescence in the presence of the PSII inhibitor DCMU, the amplitude of the fluorescence signal is directly proportional to the number of Photosystem II centers capable of Q_A reduction, while the shape of the decay curve reflects the recombination of Q_A^- with positive charges from the donor side of PSII [22]. Small changes in the structure of the D1 protein that modify the redox potential of PSII therefore cause detectable changes in the kinetics of the fluorescence decay trace. Tichy et al. [9] showed that cells containing the D1:2 isoform of *Synechococcus* 7942 display a characteristic acceleration of the slow decay phase of flash fluorescence when compared to cells containing the D1:1 isoform of *Synechococcus* 7942. In untreated *Anabaena* cells, the flash fluorescence decay is dominated by a slow hyperbolic phase with a half lifetime of 1.5 s, which reflects the recombination of Q_A^- with the S_2 state of the water-oxidizing complex. During UVB exposure, the amplitude of the flash-induced fluorescence signal decreased to 50% of its initial value (Fig. 5B), consistent with the previously measured inhibition of oxygen and fluorescence measurements (Fig. 1). Upon recovery from UVB exposure the flash-induced fluorescence signal recovered. UVB exposure also caused qualitative changes in the fluorescence decay, firstly by generating an additional fast exponential phase in the fluorescence decay curve (Fig. 5A) which reflects damage to the water oxidation complex, thereby forcing Q_A^- into recombination with other positively charged components like the redox-active tyrosine Z residue [9,22]. By deconvoluting the decay curve with an equation accounting for both a fast exponential and a slower hyperbolic component (Eq. (1)), we calculated the half life time ($T_{1/2}$) of the slow hyperbolic phase of fluorescence decay. After 15 min of UVB exposure $T_{1/2}$ steadily decreased from 1.5 s to about 0.85 s and then stabilized during the recovery period (Fig. 5C), even though the amplitude of the flash fluorescence was increasing. This change in the life time of the slow phase is consistent with an exchange between the two D1 isoforms [9] and is directly associated with changes in the redox potential of the acceptor side of PSII caused by the glutamine at position 130 of D1:1 compared to a glutamate at position 130 of D1:2 [7,23,24].

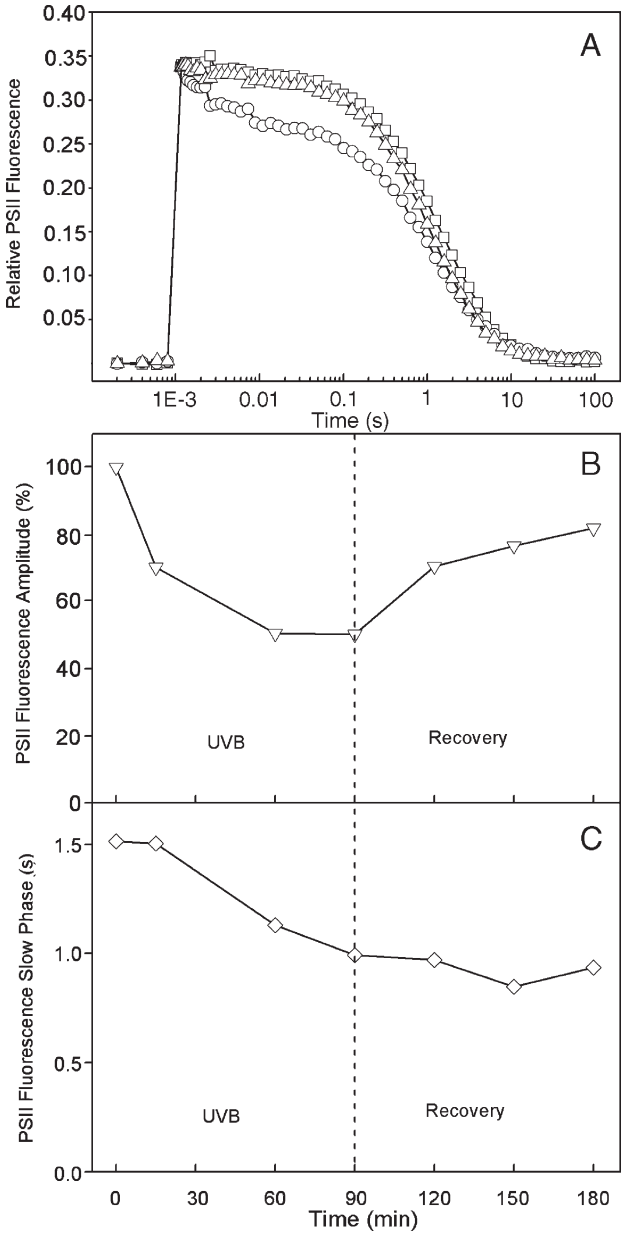

Fig. 5. Effects of UVB on the relaxation of flash-induced fluorescence in the presence of DCMU in *Anabaena* sp. PCC 7120. Cells were exposed to UVB in the presence of growth visible light for 90 min and then allowed to recover in visible light for a further 90 min. The control (open squares), 90 min UVB treated (open circles) and 90 min recovery (open up triangles) curves were plotted normalized to the control maximum (A). The change of the maximum fluorescence amplitude (open down triangles) during the UV treatment and recovery period (B) is proportional to the number of PSII centers capable of forming Q_A^-. (C) shows the change in the value of the time constant of the slow phase of fluorescence decay (open diamonds) that arises from the recombination of Q_A^- with the S_2 state of the water-oxidizing complex ($T_{1/2}$).

3.5. Trace expression of the psbA0 gene in Anabaena 7120 and the psbAI gene in Synechocystis 6803

In the case of the trace-expressed *psbA0* gene (alr3742) we used a negative control (−RT) that was processed in the same way as the other samples but had no reverse transcriptase added, to eliminate the possibility of a false positive resulting from

amplification of traces of genomic DNA. Even after 45 cycles of amplification, there was no *psbA0* amplicon fluorescence signal from the −RT sample, whereas amplicon fluorescence signal accumulated for the *psbA0*+RT tubes within 32 cycles of amplification (Fig. 6A). The amplicon from *psbA0* also showed a single denaturation melting peak, close to but distinct from the melting peaks of the other *psbA* amplicons (data not shown). This shows that the *psbA0* specific primers did indeed generate a specific product for *psbA0* transcripts distinct from the other *psbA* transcript amplicons. To determine whether the *psbA0* gene in *Anabaena*, with its low but detectable expression is functionally analogous to *psbAI* in *Synechocystis*, we used a gene-specific primer pair to check the expression of the *Synechocystis psbAI* gene against a −RT control. We consistently detected a low but measurable level of mRNA produced from *Synechocystis psbAI* (Fig. 6B), at levels similar to the divergent *psbA0* gene transcripts from *Anabaena*.

4. Discussion

4.1. Effect of UVB on PSII function and content in Anabaena

Low to moderately inhibitory UVB light is sufficient to induce changes in *psbA* gene expression in *Synechococcus* [15,44], depending on the growth light history of the culture. In *Anabaena* we first applied a very mild UVB treatment of 0.14 W m^{-2} that did not lead to a drop in oxygen evolution nor in the fluorescence parameters (data not shown). This mild treatment did not drive consistently detectable changes in the *psbA* transcript pools (data not shown). Therefore, to ensure an effect on the *psbA* gene expression and to make the treatment conditions reproducible we increased the UVB light to a level where we lost approximately 50% of the oxygen evolution and fluorescence induction parameters (Fig. 1) within 90 min. The cells were still able to recover to near control levels within 60 min (Fig. 1). The total D1 protein pool followed the loss of the functional PSII centers, declining to approximately 65% of the control values (Fig. 2), while the PsbB/CP47 PSII subunit remained steady across the treatment.

4.2. Effect of UVB on the expression of psbA genes in Anabaena

The two best-characterized cyanobacterial model species *Synechocystis* and *Synechococcus* show different strategies of *psbA* expression in response to UVB. Furthermore, cyanobacterial genomic sequences (e.g., [33,36]) reveal a diversity of *psbA* gene families. We therefore studied the expression of the 5-membered *psbA* gene family of *Anabaena* under UVB stress, in order to discern more general patterns for *psbA* and D1 isoform expression and their roles in the PSII repair cycle across cyanobacterial taxa.

In *Anabaena* under control, acclimated growth the large majority of the *psbA* transcripts comes from the *psbAI* gene (Fig. 4) and in this respect *Anabaena* corresponds with *Synechocystis* [26,29] and *Synechococcus* [4,13,15,45] where one of the *psbA* genes produces most *psbA* transcript under acclimated conditions. Based on the amino acid sequence (Fig. 7) showing the key Gln130 residue and on the fluorescence decay characteristics (Fig. 5) the *Anabaena psbAI* encodes a

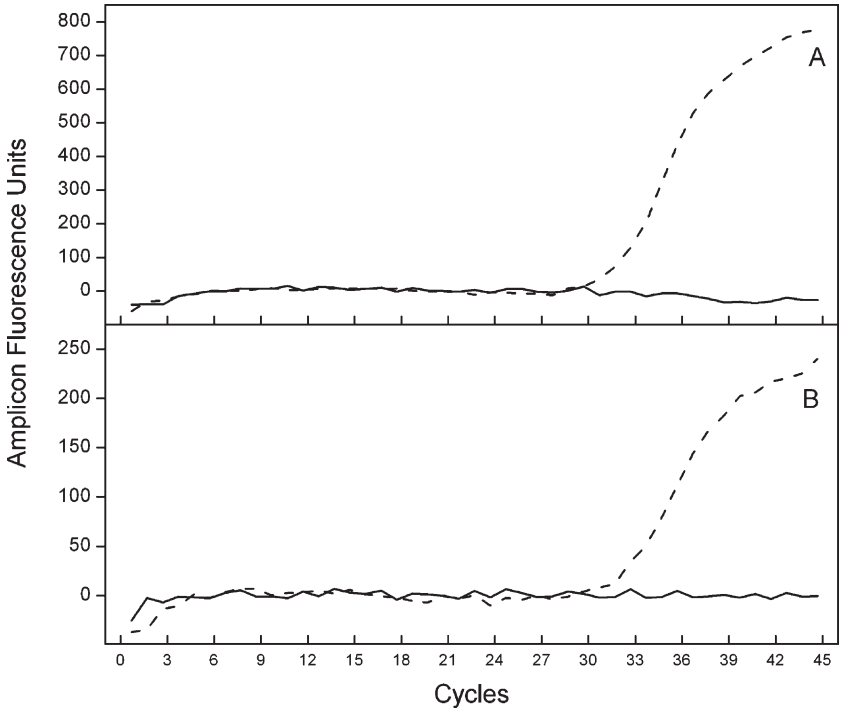

Fig. 6. Amplification of the *psbA0* transcript from *Anabaena* sp. PCC 7120 (A) and *psbAI* transcript from *Synechocystis* sp. PCC 6803 (B). RT-RT-Q-PCR was performed using *psbA0* and *psbAI* gene specific primers on cDNA samples (dashed lines) and in −RT samples (solid lines) processed in the same way but without reverse transcriptase enzyme.

```
      1                                                                                                                120
  psbA0  MTTLLEQRSS ANLWHRFGNW ITSTENRMYV GWFGVLLIPT ALTAAIVFIL AFIAAPPVDV DGIREPVSGS LLYGNNIITA TVVPTSAAIG LHLYPIWEAA SLDEWLYNGG PYQMIVLHFL
  psbAI  MTTTLQQRSS ANVWERFCTW ITSTENRIYV GWFGVLMIPT LLAATVCFII AFVAAPPVDI DGIREPVAGS LIYGNNIISG AVVPSSNAIG LHFYPIWEAA SLDEWLYNGG PYQLVIFHFL
 psbAII  MTATLQQRKS ANVWEQFCEW ITSTNNRLYI GWFGVLMIPT LLAATTCFII AFIAAPPVDI DGIREPVAGS LIYGNNIISG AVVPSSNAIG LHFYPIWEAA SLDEWLYNGG PYQLVIFHFL
psbAIII  MTATLQQRKS ANVWEQFCEW ITSTNNRLYI GWFGVLMIPT LLAATTCFII AFIAAPPVDI DGIREPVAGS LIYGNNIISG AVVPSSNAIG LHFYPIWEAA SLDEWLYNGG PYQLVIFHFL
 psbAIV  MTATLQQRKS ANVWEQFCEW ITSTNNRLYI GWFGVLMIPT LLAATTCFII AFIAAPPVDI DGIREPVAGS LIYGNNIISG AVVPSSNAIG LHFYPIWEAA SLDEWLYNGG PYQLVIFHFL

      121     130                                                                                                240
  psbA0  IAIYAYMGRQ WELSYRLGMR PWIPVAFSAP VAAATAVLLI YPIGQGSFSD GMMLGISGTF NFMIVFSPEH NILMHPFHMI GVAGVFGGAL FSAMHGSLVT STLVRETSEV ESANTGYKFG
  psbAI  IGCACYLGRQ WELSYRLGMR PWICVAYSAP LASATAVFLI YPIGQGSFSD GMPLGISGTF NFMIVFQAEH NILMHPFHML GVAGVFGGSL FSAMHGSLVT SSLVRETTEI ESQNYGYKFG
 psbAII  TGVFCYLGRE WELSYRLGMR PWICLAFSAP VAAATAVFLI YPIGQGSFSD GMPLGISGTF NFMIVFQAEH NILMHPFHML GVAGVFGGSL FSAMHGSLVT SSLVRETTEN ESQNYGYKFG
psbAIII  TGVFCYLGRE WELSYRLGMR PWICLAFSAP VAAATAVFLI YPIGQGSFSD GMPLGISGTF NFMIVFQAEH NILMHPFHML GVAGVFGGSL FSAMHGSLVT SSLVRETTEN ESQNYGYKFG
 psbAIV  TGVFCYLGRE WELSYRLGMR PWICLAFSAP VAAATAVFLI YPIGQGSFSD GMPLGISGTF NFMIVFQAEH NILMHPFHML GVAGVFGGSL FSAMHGSLVT SSLVRETTEN ESQNYGYKFG

      241                                                                                                        360
  psbA0  QEEETYNIVA AHGYFGRLIF QYASFNNSRS LHFFLAAWPV IGIWFAALGI STMSFNLNGF NFNNSILDHQ GRTIDTWADL LNRANLGIEV MHERNAHNFP LDLASGEVQP IALTAPAIAS
  psbAI  QEEETYNIVA AHGYFGRLIF QYASFNNSRQ LHFFLAAWPV IGIWFTALGV STMAFNLNGF NFNQSIIDSQ GRVINTWADI INRANLGMEV MHERNAHNFP LDLAAGEVAP VALTAPAING
 psbAII  QEEETYNIVA AHGYFGRLIF QYASFNNSRQ LHFFLAAWPV IGIWFTALGV STMAFNLNGF NFNQSIIDSQ GRVINTWADI INRANLGMEV MHERNAHNFP LDLAAGEVAP VAISAPAING
psbAIII  QEEETYNIVA AHGYFGRLIF QYASFNNSRQ LHFFLAAWPV IGIWFTALGV STMAFNLNGF NFNQSIIDSQ GRVINTWADI INRANLGMEV MHERNAHNFP LDLAAGEVAP VAISAPAING
 psbAIV  QEEETYNIVA AHGYFGRLIF QYASFNNSRQ LHFFLAAWPV IGIWFTALGV STMAFNLNGF NFNQSIIDSQ GRVINTWADI INRANLGMEV MHERNAHNFP LDLAAGEVAP VAISAPAING
```

Fig. 7. Amino acid sequence alignment of the five PsbA polypeptides encoded by the *psbA* genes from *Anabaena* sp. PCC 7120. Note that at position 130 the *psbA0* and *psbAI* sequences have a glutamine, while *psbAII*, *psbAIII* and *psbAIV* have a glutamate at 130.

D1:1 isoform functionally similar to the D1:1 isoform from *Synechococcus* [9] and we therefore propose the D1:1 nomenclature for the *Anabaena* protein also.

When UVB was applied, we saw the induction of transcripts from *psbAII, psbAIII* and *psbAIV* (Fig. 3) that all encode an alternate D1:2 isoform with 17 amino acid differences including a Glu130 residue (Fig. 7), similar to the D1:2 isoform from *Synechococcus*. The transcript induction was detectable within 15 min from the beginning of the treatment (Fig. 3). The three genes were induced to different extents, and their relative induction might depend on the prior growth conditions of the cells and/or the type of environmental stress. In particular, UVB induction of *psbAIII* transcripts was much stronger in cells growing under low inorganic carbon than in cells growing under high inorganic carbon (data not shown). It is possible that other combinations of environmental conditions would result in alternate patterns of induction among the three *Anabaena* genes encoding D1:2. Over the duration of our treatment the composition of the *psbA* transcript pool changed in favour of the transcripts encoding the D1:2 isoform but the *psbAI* transcript encoding D1:1 was still present in significant amounts (Fig. 4), similar to the pattern found in *Synechococcus* under comparable UVB treatments [15]. Nevertheless, the change in gene expression apparently drove a significant change in the composition the D1 protein subunits of the functional PSII pool, as indicated by a characteristic acceleration in the hyperbolic slow fluorescence decay phase (Fig. 5), reflecting an increased propensity towards fast charge recombination in PSII centers containing the D1:2 protein [7–9,23].

During the recovery phase, we detected a decrease in the abundance of the transcripts encoding D1:2 and a reaccumulation of *psbAI* transcripts encoding D1:1 (Fig. 4) suggesting the return of the cells towards their original pre-stress status. This process was gradual and after 60 min of recovery the transcripts encoding the two forms were in approximately equal amounts (Fig. 4). The almost complete recovery of PSII function (Fig. 1) coupled with the maintenance of an accelerated hyperbolic slow fluorescence decay phase (Fig. 5) suggests that the recovery of PSII function is accomplished initially with the D1:2 isoform remaining in the majority of the photosystems, presumably followed later by re-exchange of the protein isoforms.

For *Synechococcus* sp. PCC 7942, D1 isoform specific antibodies were developed [18] against non-conserved regions of the proteins and used to characterize the D1 exchange process (e.g., [5,6,18]). Developing form specific antibodies is not, however, practical for characterizing the D1 isoform exchange in multiple species since prohibitively expensive separate antibody sets would need to be developed for variant protein sequence regions for each cyanobacterial species of interest. The functionally critical Gln130/Glu130 residue change distinguishing D1:1 from D1:2 is not in itself sufficient to reliably generate form-specific antibodies (D. Campbell, unpublished). We therefore propose flash fluorescence as a more reproducible and reliable alternative to track the exchange of D1 isoforms in the pool of functional PSII [9].

4.3. Expression of the psbA0 gene in Anabaena and psbAI in Synechocystis

The publication of the *Anabaena* genome [36,37] showed the presence of the divergent *psbA0* (alr3742), whereas previously this cyanobacteria was believed to carry just four *psbA* genes [35]. The *Anabaena psbA*0 (alr3742) gene is expressed at a low constitutive level and is not responsive to photo-oxidative UVB or light stress, nitrogen stress (data not shown) nor changes in inorganic carbon levels (data not shown). This suggested a possible similarity with *Synechocystis*, where hybridization blotting techniques did not detect the expression of the *psbAI* gene [26,29,30] under standard growth conditions nor under a range of stressors including UVB, light and temperature.

We therefore decided to measure the expression of *psbAI* in *Synechocystis* using gene specific primers and RT-RT-Q-PCR, a method far more sensitive than the hybridization blots originally employed to track *psbA* expression [26,29,30]. We tested *Synechocystis* cells from normal growth conditions and indeed detected the presence of *psbAI* transcript at a level much higher than the negative control (−RT), showing that the gene is indeed expressed, albeit at quantitatively trace levels compared to the total *psbA* transcript pool (Fig. 6).

Like *Anabaena* and *Synechocystis*, the cyanobacteria *Nostoc* (http://genome.jgi-psf.org/mic_home.html) and *Gloeobacter*

(http://www.kazusa.or.jp/cyano/) also contain a divergent *psbA* gene. *psbA0* provides only quantitatively insignificant contributions to the total *Anabaena psbA* transcript pool under our tested conditions. We therefore exclude the necessity of its presence for the quantitative function of the PSII repair cycle of replacement of damaged D1 under UVB stress or basal growth conditions. Nevertheless, the *psbA0*, like *psbAI* in *Synechocystis* must be under selective pressure to be maintained intact, functional and expressed, since the *psbA0* sequence is sufficiently divergent to have accumulated multiple inactivating stop codons if the sequences were diverging randomly. Furthermore, in *Synechocystis* artificial over-expression of *psbAI* [30] generates a D1 protein capable of creating a functional, albeit aberrant, PSII [32].

Recently, intact and potentially functional *psbA* genes have been detected in the genomes of roughly 50% of phages infecting marine strains of cyanobacteria [46]. The sequences of these phage encoded *psbA* are divergent from the host *psbA* sequences, hinting at possible similarities to the divergent *psbA* genes in cyanobacterial genomes.

In summary, the five-member *psbA* gene family of *Anabaena* encodes a divergent but trace expressed *psbA* gene as in *Synechocystis*, but also three genes encoding an alternate D1:2 protein isoform expressed under environmental stress as in *Synechococcus.* Our characterization of the *Anabaena psbA* family thus demonstrates an integration of the features of the previous model species, and suggests a general pattern of three D1 isoforms in cyanobacteria, a divergent but intact and trace expressed D1′ of unknown function in some strains, a constitutive D1:1 with Gln130 expressed under acclimated growth with supplemental induction in some strains, and an alternate D1:2 with Glu130 induced under excitation stress in many strains.

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