ORIGINAL ARTICLE

Function and evolution of the *psbA* gene family in marine *Synechococcus*: *Synechococcus* sp. WH7803 as a case study

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In cyanobacteria, the D1 protein of photosystem II (PSII) is encoded by the *psbA* multigene family. In most freshwater strains, a D1:1 isoform of this protein is exchanged for a D1:2 isoform in response to various stresses, thereby altering PSII photochemistry. To investigate PSII responses to stress in marine *Synechococcus*, we acclimated cultures of the WH7803 strain to different growth irradiances and then exposed them to high light (HL) or ultraviolet (UV) radiation. Measurement of PSII quantum yield and quantitation of the D1 protein pool showed that HL-acclimated cells were more resistant to UV light than were low light- (LL) or medium light- (ML) acclimated cells. Both UV and HL induced the expression of *psbA* genes encoding D1:2 and the repression of the *psbA* gene encoding D1:1. Although three *psbA* genes encode identical D1:2 isoforms in *Synechococcus* sp. WH7803, only one was strongly stress responsive in our treatment conditions. Examination of 11 marine *Synechococcus* genomic sequences identified up to six *psbA* copies per genome, with always a single gene encoding D1:1. In phylogenetic analyses, marine *Synechococcus* genes encoding D1:1 clustered together, while the genes encoding D1:2 grouped by genome into subclusters. Moreover, examination of the genomic environment of *psbA* genes suggests that the D1:2 genes are hotspots for DNA recombination. Collectively, our observations suggest that while all *psbA* genes follow a concerted evolution within each genome, D1:2 coding genes are subject to intragenome homogenization most probably mediated by gene conversion.

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Introduction

Although light is essential for photosynthesis, it also provokes photoinactivation of photosystem II (PSII). The D1 polypeptide is a key protein of the core of the PSII, and is the primary target for photoinactivation (reviewed in Nishiyama *et al.*, 2006), which must be countered by a repair cycle to replace photodamaged D1 proteins (Aro *et al.*, 1993). When irradiance and/or ultraviolet (UV) levels increase, photoinactivation may outrun the rate of repair, leading to net photoinhibition of PSII (Aro *et al.*,

2005) and a decrease of photosynthetic capacity. Hence, the D1 protein shows a high rate of synthesis and degradation, so that photodamaged D1 proteins are usually rapidly replaced with newly synthesized ones (Schaefer and Golden, 1989; Bustos *et al.*, 1990; Aro *et al.*, 1993; Clarke *et al.*, 1993a; Nishiyama *et al.*, 2006). While in eukaryotes D1 is usually encoded by a single chloroplastic *psbA* gene, a multigene *psbA* family is present in most cyanobacterial genomes encoding multiple isoforms of D1 protein in many strains (Schaefer and Golden, 1989; Vrba and Curtis, 1990; Mate *et al.*, 1998; Sicora *et al.*, 2006).

Synechococcus sp. PCC 7942 and *Thermosynechococcus elongatus* BP-1 contain three *psbA* copies, with *psbA1* encoding the primary D1:1 isoform constitutively present in PSII centres (Schaefer and Golden, 1989). Excess irradiance, low temperature or other stresses leading to a

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decrease in electron transport induce expression of the *psbAII* and *psbAIII* genes encoding the alternate isoform D1:2, which then progressively replaces the D1:1 isoform through the ongoing action of the PSII repair cycle (Bustos *et al.*, 1990; Clarke *et al.*, 1993a,b, 1995; Campbell *et al.*, 1995, 1998, 1999; Sippola *et al.*, 1998; Kos *et al.*, 2008). In *Synechococcus* sp. PCC 7942, the presence of D1:2 in PSII centres decreases the cellular susceptibility to PSII photoinactivation (Clarke *et al.*, 1993a; Campbell *et al.*, 1998). Amino-acid position 130, a glutamine in D1:1 and a glutamate in D1:2, is essential in the differential function of these proteins in PSII (Clarke *et al.*, 1993a; Campbell *et al.*, 1996; Giorgi *et al.*, 1996b; Sane *et al.*, 2002; Tichy *et al.*, 2003; Kos *et al.*, 2008). *Synechocystis* sp. PCC 6803 is quite different, with three *psbA* copies where both *psbAII* and *psbAIII* encode a D1:1 isoform. Under stress conditions, only *psbAIII* expression is enhanced to provide supplemental transcripts for D1 repair (Mate *et al.*, 1998). *psbAI* has a divergent sequence encoding a D1 form termed D1', which is expressed at only constitutive trace levels (Sicora *et al.*, 2006). In *Anabaena* sp. PCC 7120 five *psbA* genes, *psbA0–IV*, are present and expressed (Sicora *et al.*, 2006). The *psbAI* copy encodes a D1:1 constituting the major D1 protein under moderate excitation conditions. The *psbAII–IV* genes encode D1:2 isoforms and are induced upon UVB or high irradiance exposure. Similarly, to the *psbAI* gene of *Synechocystis* sp. PCC 6803 and *psbA2* in *Thermosynechococcus elongatus* BP-1 (Kos *et al.*, 2008), the *psbA0* gene of *Anabaena* sp. PCC 7120 is phylogenetically divergent and transcribed at only trace levels. The deeply divergent cyanobacteria *Gloeobacter violaceus* also contains five *psbA* genes, but two encode divergent D1' forms expressed at only constitutive trace levels, while the remaining three *psbA* genes all encode a D1:2-type isoform, with differential induction of the transcripts under excitation stress (Sicora *et al.*, 2008).

Marine *Synechococcus* are ubiquitous picoplanktonic cyanobacteria, which contribute significantly to primary production, food webs, biogeochemical cycles and climate feedbacks (Waterbury *et al.*, 1986; Olson *et al.*, 1990; Partensky *et al.*, 1999). These cyanobacteria constitute a well-defined phylogenetic group but exhibit a large phenotypic diversity across a large number of phylogenetic clades as defined by 16S RNA analyses (Fuller *et al.*, 2003) and 16S–23S internal spacer sequence analyses (Ahlgren and Rocap, 2006). Although many studies have described the distribution of marine *Synechococcus*, the relation between their photophysiology and their phylogeny and ecology is not well understood (Six *et al.*, 2007a). In particular, whether the functional diversity of *psbA* genes in marine *Synechococcus* is similar to that of freshwater cyanobacteria has not been investigated yet.

In this paper, we have used the recently available genomes of 11 strains of marine *Synechococcus* to draw the first overview of the numbers and

distributions of *psbA* genes in this cyanobacterial group. Through genomic context analyses, phylogeny and recombination tests, we provide new insights into the evolutionary processes that maintain the unusual properties of this important gene family. In parallel, the WH7803 strain, which is so far the physiologically best-characterized marine *Synechococcus* strain, was used to investigate the PSII response to high light (HL) and UV stresses and to determine the function of the different *psbA* genes in a representative marine *Synechococcus*.

Materials and methods

Culture conditions and experimental procedures

Synechococcus sp. WH7803 was grown at 25°C in 0.2 µM filtered PCR-S11 medium (Rippka *et al.*, 2000) supplemented with 5 mM NaNO₃. Three cultures were acclimated for several weeks to continuous low light (LL, 15 µmol photons m⁻² s⁻¹), medium light (ML, 75 µmol photons m⁻² s⁻¹) and HL (350 µmol photons m⁻² s⁻¹; Sylvania Daylight 58W/154 fluorescent bulbs). For each growth irradiance, 9 l of exponentially growing culture (about 10⁷ cell ml⁻¹) were split in 800 ml aliquots in 1 l glass or quartz Erlenmeyer flasks (Ellipse, La Chapelle la Rennes, France) and left under their initial growth irradiance for 1 h. One of the 800 ml glass flasks was kept in the initial conditions and used as a control. For the LL- and the ML-acclimated cultures, four glass flasks were placed under 350 µmol photons m⁻² s⁻¹ PAR (photosynthetically active radiation, that is, HL), while four quartz flasks were kept under the initial growth irradiance supplemented with UV (4.7 W m⁻² UVA, 0.8 W m⁻² UVB) for 5 h. Four aliquots of the HL-acclimated culture were subjected to the latter treatment, while four others were shifted to 75 µmol photons m⁻² s⁻¹ (that is, ML). One Erlenmeyer flask for each treatment (light shift, UV shift) was withdrawn after 15 min, 1, 2.5 and 5 h to study the kinetics of the responses to light and UV stress. The PAR was measured in the 400–700 nm range using a QSL-2100 quantameter (Biospherical, La Jolla, CA, USA). UVA and UVB radiations, generated from UVA-351 and UVB-313 fluorescent bulbs (Q-Panel Lab products, Cleveland, OH, USA), were measured between 280–320 and 320–400 nm, respectively using a USB2000 spectroradiometer (Ocean Optics, Ew Duiven, The Netherlands).

Synechococcus sp. WH7803 growth was monitored by cytometry (Marie *et al.*, 1999, 2000). Immediately after sampling, culture aliquots were fixed with 0.25% glutaraldehyde grade II (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 4 °C, flash was frozen in liquid nitrogen and stored at –80 °C until analysis. Cell concentration in the cultures was determined using a FacSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with laser emission set at 488 nm. The flow rate was about 70 µl min⁻¹ and the sheath fluid was distilled water.

Fluorescence measurements

Photosystem II fluorescence was studied using a pulse amplitude-modulated fluorimeter (PhytoPAM, Walz, Effeltrich, Germany). This instrument includes modules providing excitation light at four distinct wavelengths (470, 520, 645 and 665 nm), red actinic light peaking at 655 nm filtered through a short-pass filter (<695 nm), as well as a photomultiplier equipped with a long-pass filter set (>710 nm). A volume of 2 ml culture was dark acclimated for 10 min in a quartz cell displaying a mirrored facet facing the photomultiplier to enhance the signal. The excitation-modulated light (13 Hz) was then applied to measure the basal fluorescence level F_0 with excitation at 520 nm. The maximal fluorescence F_M with excitation at 520 nm was determined in the presence of 50 μM 3-(3',4-dichlorophenyl)-1,1-dimethylurea under 1400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of red actinic light. The PSII quantum yield was calculated as:

$$F_V/F_M = (F_M - F_0)/F_M$$

D1 protein quantitation by immunoblotting

A volume of 400 ml culture was centrifuged and the pellet was drained, flash frozen in liquid nitrogen and stored at -80°C until analysis. Cell pellets were resuspended in extraction buffer (140 mM Tris base, 105 mM Tris-HCl, 0.5 mM ethylenediaminetetraacetic acid, 2% lithium dodecyl sulphate, 10% glycerol and 0.1 mg ml $^{-1}$ Pefabloc SC (AEBSF) protease inhibitor (Roche, Basel, Switzerland) and the total protein concentration was determined using the modified Lowry DC protein assay kit (Bio-Rad, Hercules, CA, USA). The samples were then brought up to 50 μM dithiothreitol, and then heated for 5 min at 70°C . A volume containing 2.5 μg of total protein was loaded on a 4%–12% acrylamide precast NuPAGE Bis-Tris mini-gel (Invitrogen, Carlsbad, CA, USA) along with recombinant protein standards of D1 (Agrisera, Vännäs, Sweden) to establish a standard curve. Gels were electrophoresed for 40 min at 200 V and the proteins were transferred onto a methanol prehydrated polyvinylidene fluoride (PVDF) membrane for 80 min at 30 V. Membranes were immediately immersed in Tris Buffer Saline-Tween (TBS-T) buffer pH 7.6 (0.1% Tween 20, 350 mM sodium chloride, 20 mM Trizma base) containing 2% (w:vol) ECL Advance blocking agent (Amersham Biosciences, Piscataway, NJ, USA) for 1 h. Aliquots of primary antibodies against D1 or RbcL (that is, the large subunit of RuBisCO) proteins (Agrisera) were diluted at 1:50 000 in TBS-T in the presence of 2% blocking agent and membranes were soaked in this solution for 1 h with agitation. The membranes were then extensively washed in TBS-T buffer. Antichicken secondary antibodies were applied with the same procedure as for primary antibodies. Membranes were developed by chemoluminescence using the ECL Advance reagent kit (Amersham Biosciences) with a CCD imager

(FluorSMax, Bio-Rad). Signals were quantified using the Quantity One software (Bio-Rad, Hercules, CA, USA) and the protein standard curve was generated. Protein amounts were determined by fitting the signal of the samples on the standard curve, which spanned the range of protein target abundance in the samples.

RNA extraction and cDNA synthesis

Immediately after sampling, 200 ml of culture were harvested by centrifugation at 18 500 g for 9 min at 4°C (Avanti J25 centrifuge, Beckman, Fullerton, CA, USA), followed by a second centrifugation in microtubes (3 min, 4°C at 20 800 g in an Eppendorff 5417R centrifuge). Cell pellets were then resuspended in 300 μl Trizol (Invitrogen), frozen in liquid nitrogen and stored at -80°C until RNA extraction. For RNA extraction, cells resuspended in Trizol were thawed by 15 min incubation at 65°C with regular vortexing. After phenol chloroform extraction, RNA was purified on Qiagen RNAeasy columns (Qiagen, Valencia, CA, USA) as recommended by the manufacturer. A DNase treatment was performed on the columns using the Qiagen RNase-free DNase Set (Qiagen). RNA was then eluted from the column in 30 μl DEPC-treated water, precisely quantified using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA) and stored at -80°C .

Real time PCR

Reverse transcription was carried out on 100 ng RNA using SuperScriptII reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA) as described previously (Six *et al.*, 2007b). Real-time PCR was performed using the GeneAmp 5700 sequence detection system and SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) on 1:100th diluted cDNA (but 1:1000th for *rnpB*) in the presence of 200 nM primers. The PCR reaction programme consisted of a sequence of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C . Occurrence of a single amplicon was systematically checked by performing a melting curve.

Gene-specific primers for both reverse transcription and real-time PCR were designed using Primer-Express software (V2.0) from Applied Biosystems (Table 1). To amplify specifically each of the four *psbA* copies present in *Synechococcus* sp. WH7803, primers were selected in the 5'-UTR of the genes. Primer specificity was checked on plasmid DNA (PCR2.1; Invitrogen) containing the upstream sequences of the four *psbA* copies. Furthermore, these plasmids, after linearization, were used to perform a standard curve for absolute quantification of each *psbA* transcript in LL-, ML- and HL-acclimated cultures.

The relative expression of each *psbA* gene in response to HL and UV stresses were calculated

Table 1 Primers used for real-time PCR reactions for *Synechococcus* sp. WH7803. All primers are specific for the targeted gene except primers designed to amplify the four *psbA* copies present in *Synechococcus* sp. WH7803

Open reading frame (ORF)	Forward primer (5' → 3')	Reverse primer (5' → 3')
<i>SynWH7803_0366</i>	CCATGGCTGGACGCTAACA	GTCGTTGTGAAGAAGCGTTGAG
<i>SynWH7803_0784</i>	AATCCAGGCTTTCTCTGAACGT	GCGAATTGCGGTAGCCATAA
<i>SynWH7803_0790</i>	TTTCTTTACCGCTGAAGGGTTT	GCTGAATGGTGGTGGTCATG
<i>SynWH7803_2084</i>	TCTTAGCGGCCGTCGAT	CGGAAGTTATCCAGAGCTTTATGAA
All ORFs	TGGCTGCCACGGTTACT	TGCGGCTGTTGTTGAAGGA

using the $2^{-\Delta\Delta C_t}$ method as described in the Applied Biosystems user bulletin no. (http://dna-9.int-ed.uiowa.edu/RealtimePCRdocs/Compar_Anal_Bulletin2.pdf) with the sample taken just before the shift to stress conditions (T_0) as a reference and *rnpB* as an internal standard to normalize the relative transcript levels (Mary and Vaultot, 2003). These induction values, together with the absolute quantification of each *psbA* transcript at T_0 , allowed measuring the proportion of each transcript within the *psbA* pool throughout the light treatments. To ensure a similar amplification efficiency of the reference and target genes (difference < 5%) on both plasmids and cDNA template, standard curves were generated for each set of primers.

Phylogenetic and genomic analyses

Annotation files were downloaded from the Genome section of the NCBI databases for the following *Synechococcus* and *Prochlorococcus* genomes: *Synechococcus* spp. WH7803 (NC_009481), RCC307 (NC_009482), WH8102 (NC_005070), CC9902 (NC_007513), CC9605 (NC_007516), CC9311 (NC_008319), RS9916 (NZ_AAU000000000), RS9917 (NZ_AANP000000000), BL107 (NZ_AATZ000000000), WH7805 (NZ_AAO000000000) and WH5701 (NZ_AANO000000000) and *Prochlorococcus* spp. MED4 (NC_005072), SS120 (NC_005042), MIT9313 (NC_005071) and NATL2A (NC_007335). Nucleotide and protein sequences of the *psbA* genes were extracted from these files for *Synechococcus* and *Prochlorococcus* and retrieved from the CYORF database (<http://cyano.genome.jp/>) for the freshwater cyanobacteria. Because of the extreme conservation of the *psbA* genes at the protein level, nucleotide sequences were aligned using protal2DNA (<http://bioweb.pasteur.fr/seqanal/interfaces/protal2dna.html>) according to a protein alignment generated using the CLUSTALW (Thompson *et al.*, 1994) as implemented in the BIOEDIT freeware (Hall, 1999). To avoid biases due to large GC% variability, phylogenetic analyses were performed using the first and second codon position. Maximum likelihood tree was inferred using PHYML V2.4.4 (Guindon and Gascuel, 2003) with the K2P substitution model and the estimation of the gamma distribution parameter of the substitution rates among sites and of the proportion of invariables sites. Bootstrap analyses including 1000 replicate data sets were

used to estimate the relative confidence of internal nodes using both neighbor-joining and maximum parsimony methods. Phylogenetic trees were edited using the program MEGA3 V3.1 (Kumar *et al.*, 2004). To complement these phylogenetic analyses and highlight potential recombination events, an evolutionary network was developed with SplitsTree v4.2 (Huson and Bryant, 2006) using an alignment of the nucleotide sequences of all marine *Synechococcus psbA* genes including the variable third codon position. Computing of the network was made using the K2P model for the distances and the Neighbor-Net method for distance transformation. The Phi test was used to detect the occurrence of recombination events between *psbA* sequences (Bruen *et al.*, 2006). Moreover, the Genetic Algorithm Recombination Detection (GARD) (Kosakovsky Pond *et al.*, 2006) was used to identify recombination breakpoints in the alignments of *psbA* genes coding either D1:1 only, D1:2 only or both isoforms. Analysis was performed using the HKY85 model for the substitution bias, the beta-gamma model with three rate classes for the site-to-site variation of substitution rates and the GARD option for the detection method.

To examine the conservation of the gene order around the *psbA* genes, orthologous relationships among *psbA* flanking genes were investigated. Families of orthologous genes were delineated using the TribeMCL algorithm after Basic Local Alignment Search Tool (BLAST) all-against-all comparisons of every D1 protein sequence from the 11 *Synechococcus* genomes (Altschul *et al.*, 1997; Enright *et al.*, 2002). An e-value cut-off of 10^{-12} was chosen as threshold for the BLAST comparisons. Non-modelled genes, which were missed by the open reading frame (ORF) finding softwares during genome annotations were manually added to the data set.

Results

Growth of *Synechococcus* sp. WH7803

Cultures of *Synechococcus* sp. WH7803 acclimated to 15 (LL), 75 (ML) and $350 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (HL) exhibited different growth rates of 0.46 ± 0.06 , 1.04 ± 0.14 and $1.15 \pm 0.19 \text{ d}^{-1}$, respectively. These values are typical for marine *Synechococcus* (Kana and Glibert, 1987; Moore *et al.*, 1995; Six *et al.*, 2004). UV inhibited cell growth in the three culture

conditions (Figure 1). We did not observe significant growth recovery within 24 h after UV exposure in any culture condition. When transferred from LL or ML to HL conditions, only a slight decrease of growth rate was observed relative to control cultures and both light-acclimated cultures (LL and ML) had recovered about 80% of their growth rate after 24 h (Figures 1a and b). HL cultures shifted to ML responded by a slight growth decrease (Figure 1c). Although some variability in the results was observed, the HL cultures were capable of almost regaining their initial growth rate when shifted back under HL (not shown).

Figure 1 Growth curves of *Synechococcus* sp. WH7803 acclimated to (a) LL, (b) ML and (c) HL and transiently submitted to UV (black symbols), HL (white symbols except diamonds) or ML (white diamonds) for 5 h followed by return to the original growth conditions. The cell concentration is expressed as percentage of the initial condition. Control cultures are indicated by grey symbols and the treatment period is delineated by dotted lines. These data are based on three independent experiments. HL, high light; LL, low light; ML, medium light; UV, ultraviolet.

PSII photoinactivation in *Synechococcus* sp. WH7803
We used pulse amplitude-modulated fluorimetry to assess PSII activity through the different light treatments applied to LL, ML and HL cultures of *Synechococcus* sp. WH7803 (Figure 2). UV treatments induced a strong PSII photoinhibition for cells acclimated to all three PAR conditions, shown by a drastic drop of F_v/F_m at 5 h. The cells were capable of a slow but significant recovery of the function of their PSII after 24 h, demonstrating that the applied UV dose was not lethal. It should be noted that the control cultures showed some decrease in F_v/F_m due to culture aging, especially, in the fast growing HL cultures.

The rate of UV photoinhibition was interestingly quite different depending on the cell light history, with LL cultures suffering net photoinhibition under UV about twice as fast as the HL culture ones (Figure 2d). LL and ML cells exhibited different patterns of PSII quantum-yield variation upon HL exposure (Figures 2a and b). In the LL cultures, this parameter dropped from 0.6 at the beginning of the experiment to 0.3 after 5 h of HL, while ML cultures stabilized at 0.4 after 1 h HL exposure, which corresponds to the initial value of HL cultures. When shifted back to initial light conditions, the ML-acclimated cells recovered faster than LL-acclimated cells and reached their initial PSII quantum-yield value within 8 h. When the HL cultures were transferred under ML, F_v/F_m monitoring indicated a rapid increase in PSII quantum yield, which almost reached the initial value of ML cultures (Figure 2c). When shifted back to HL, this HL culture returned to its initial F_v/F_m value within 1 h.

D1 protein content in *Synechococcus* sp. WH7803

Cultures from all the growth light conditions responded to UV with a drop in D1 content (Figure 3a). However, the LL cultures showed a more rapid and pronounced decrease compared to ML and HL cultures. In response to HL stress, the D1 content of LL-acclimated cultures rapidly and strongly increased with a transient doubling after 2.5 h exposure (Figure 3b), followed by a decline back towards the initial D1 levels. In contrast, D1 cell contents of ML cultures were not affected by an increase of irradiance. Likewise, no variation in the size of the cellular D1 protein pool was observed on the immunoblots of the HL cultures upon ML exposure.

Expression of *psbA* genes in *Synechococcus* sp. WH7803

The total pool of *psbA* transcripts, monitored using primers common to all four *psbA* genes in *Synechococcus* sp. WH7803, showed significant changes upon UV and HL exposure (Figure 4). In cultures from all the LL and ML light-acclimation states, UV provoked a sharp 8–12-fold increase in the *psbA*

Figure 2 Variations of PSII maximal photochemical yield (F_v/F_m) in *Synechococcus* sp. WH7803 acclimated to (a) LL, (b) ML and (c) HL and submitted to UV (filled triangles), HL (open squares) or ML (open diamonds) for 5 h. Control cultures are indicated by grey circles and dashed lines. The end of the stress and beginning of the recovery period is indicated by the dotted line. (d) PSII photoinhibition rates in response to UV treatment. These data are based on at least three independent experiments. HL, high light; LL, low light; ML, medium light; UV, ultraviolet; PSII, photosystem II.

transcript pool within 1 h, which then partly fell back towards control levels. In HL-acclimated cultures, however, UV exposure provoked only a small induction of *psbA* transcripts above the initial levels. In the LL-acclimated culture, HL treatment led to an increase of the global *psbA* transcript pool.

Using gene-specific primers, it is revealed that *SynWH7803_0784* and *SynWH7803_0790* transcripts constituted most of the *psbA* mRNA pool. While *SynWH7803_0784*, encoding D1:1, was the dominant form in LL-acclimated cells (90%), the proportion of *SynWH7803_0790*, encoding D1:2, strongly increased in cultures acclimated to higher light intensities (ML and HL) reaching up to 85% of total *psbA* mRNA in HL-acclimated cells. Thus, it seems that the PSII of LL cultures contained mostly D1:1 proteins, whereas the PSII of HL cells contained more D1:2 proteins as a result of photoacclimation.

Furthermore, the proportion of different *psbA* transcripts was rapidly altered by HL or UV exposure (Figure 4). Although in our experimental conditions, this alteration was faster in response to UV than to HL stress and in LL-compared to HL-acclimated cells, the *SynWH7803_0790* transcript, encoding D1:2, made up at least 80% of the total *psbA* mRNA pool after 1 h of stress in all stress conditions. Measurement of the fold change of each transcript relative to T_0 showed that these modifications of *psbA* mRNA distribution are due to both repression of *SynWH7803_0784* expression and activation of *SynWH7803_0790* expression. A slight induction of the two other *psbA* gene copies encoding D1:2, *SynWH7803_0366* and *SynWH7803_2084*, also occurred although both transcripts constituted only a small proportion of the *psbA* mRNA pool even under stress conditions. In contrast, the *psbA* genes were inversely regulated in HL cultures shifted to ML, leading to the rapid accumulation of the *SynWH7803_0784* mRNA encoding D1:1 to 56% after 15 min. Altogether, these data show that by exerting a strong influence on the relative contribution of *psbA* transcripts in the initial pool, light history of the cells is the key to responses to high light and UV stresses.

psbA gene diversity in marine cyanobacteria

Table 2 summarizes the number and the isoform type of *psbA* genes present in all marine *Synechococcus* sequenced to date as well as in six freshwater cyanobacteria and four *Prochlorococcus* spp. strains shown for comparison. As a working definition, we define D1:1 and D1:2 protein isoforms on the basis of the occurrence of amino acid Gln130 in D1:1 and Glu130 in D1:2 (Giorgi et al., 1996a). While *Prochlorococcus* spp. possess 1–3 *psbA* copies encoding only the D1:1 isoform, all other studied cyanobacteria except *Synechocystis* sp. PCC 6803 (Mate et al., 1998) and *Gloeobacter* (Sicora et al., 2008) possess both D1 isoforms. Three (CC9605) to

Figure 3 Variations of PsbA protein pool determined by immunoblotting with polyclonal antibody on *Synechococcus* sp. WH7803 grown under LL (circles), ML (triangles) or HL (squares or diamonds) and submitted to (a) UV (filled symbols), (b) HL or ML (open symbols). The plots present quantifications of biological triplicates of the representative blots on the left. These data are based on four independent experiments. HL, high light; LL, low light; ML, medium light; UV, ultraviolet.

six (WH7805) *psbA* genes have been identified per *Synechococcus* genome, with most of the strains possessing four *psbA* gene copies (Table 2). In each strain, only one *psbA* gene encodes the D1:1 isoform, which shares a high percentage of nucleotide identity (90–94%) with the D1:2 coding genes as in freshwater cyanobacteria. Furthermore, in both marine and freshwater cyanobacteria, D1:2 isoform encoding genes show an extremely low level of divergence within genomes. Indeed, these D1:2 isoform genes are nearly identical within each marine *Synechococcus* strain at both the protein (99.94%–100% identity; Supplementary Table 1) and nucleotide level (99.86%–100% identity; Supplementary Table 2). These D1:2 protein sequences are even identical between the two clade IV *Synechococcus* strains, BL107 and CC9902, and the same applies to *Synechococcus* spp. WH7803 and WH7805. This clearly shows that the D1:2 isoform encoding genes have undergone concerted evolution in all cyanobacterial genomes examined in this study. *Synechococcus* sp. RS9917 possesses one *psbA* sequence very distant from all marine *Synechococcus psbA* genes. Such a divergent *psbA* gene copy has also been identified in *Synechocystis* sp. PCC 6803 (Mate *et al.*, 1998; Sicora *et al.*, 2006), *Anabaena* sp. PCC 7120 (Sicora *et al.*, 2006)

and *Gloeobacter violaceus* (Sicora *et al.*, 2008). Lastly, two fragments encoding partial C-terminal regions of the D1 protein were found in WH7803 (SynWH7803_2082; 42 amino acids) and in RS9916 (RS9916_39526; 112 amino acids).

Genomic environment of *psbA* genes in marine *Synechococcus*

The investigation of the genomic context of each *psbA* copy in all marine *Synechococcus* strains revealed that most *psbA* genes are located into three main genomic regions (Figure 5). The two first regions contain only D1:2-encoding genes, while the third one carries the D1:1-encoding gene as well as 0–2 genes encoding the D1:2 isoform. Four *Synechococcus* genomes possess an additional *psbA* gene (SYNW0983, RS9917_12975, WH5701_10719 and RCC307_2009), which is present in none of the three main genomic regions. Notably, groups of orthologues located upstream from the *psbA* gene in the three main regions have a conserved gene order, which has been modified mainly by strain-specific gene deletion or insertion of unique genes. In contrast, orthologues found downstream of *psbA* genes are characterized by a large number of translocations as shown by the occurrence of

Figure 4 Relative contributions of the four *psbA* transcripts present in *Synechococcus* sp. WH7803 throughout light treatments after normalizing each transcript to the sum of the transcript pool, expressed as 100% (left scale): SynWH7803_0784 (encoding D1:1 isoform, light grey), SynWH7803_0790 (encoding D1:2 isoform, dark grey), SynWH7803_0366 (encoding D1:2 isoform, black) and SynWH7803_2084 (encoding D1:2 isoform, white). The relative size of the total *psbA* transcript pool normalized to the expression level of the *mnpB* gene was plotted as black circles (right scale). The cultures were grown under LL, ML or HL and submitted to UV (left panel), HL or ML (right panel). Each real-time PCR was performed in triplicate. HL, high light; LL, low light; ML, medium light; UV, ultraviolet.

strain-specific associations of the same genes in a different order. These observations strongly suggest that *psbA* genes constitute hotspots for intragenomic homologous recombination.

Phylogeny and evolution of *psbA* genes

Phylogenetic analyses performed using the first and second codon position showed that all *psbA* gene sequences of marine cyanobacteria, except the divergent *psbA* sequence of the halotolerant *Synechococcus* sp. RS9917, form a monophyletic group within cyanobacteria (Figure 6). *psbA* genes of marine *Synechococcus* are dispersed within two main clusters of sequences corresponding to the two D1 isoforms (Figure 6). The first cluster gathers the D1:1-encoding genes of each of the marine *Synechococcus* genomes. The second cluster contains the D1:2-encoding genes. Within the D1:2 cluster,

the genes always group together according to the genome to which they belong. A much larger genetic diversity exists among the freshwater cyanobacteria sequences used in this study, leading to a more complex pattern of clustering of the *psbA* genes in the phylogenetic tree (Figure 6). As in marine *Synechococcus*, D1:2 *psbA* genes tend to group according to the genome to which they belong. However, contrary to what is observed in marine cyanobacteria, D1:2 genes also cluster with D1:1 genes from the same genome. *Thermosynechococcus elongatus* appears to be an exception, since one D1:2 *psbA* gene seems to be closer to the D1:1 gene than to the second D1:2 copy. The divergent *psbA* genes either group with their D1:2 counterparts as in *Gloeobacter violaceus* or form a separate cluster as in the *Anabaena* strains and in *Synechocystis* sp. PCC 6803. Interestingly, the genetic distances among the organisms seem to affect the clustering of

Table 2 *psbA* gene copies in some freshwater and marine cyanobacteria. Note that D1:1 and D1:2 isoforms were distinguished based on the amino-acid 130 residue

Cyanobacteria strains	Divergent <i>psbA</i>	D1:1 isoform coding gene	D1:2 isoform coding gene	Light response ^a	References
<i>Synechococcus</i> sp. PCC 7942		<i>Synpcc7942_0424</i>	<i>Synpcc7942_1389</i> , <i>Synpcc7942_0893</i>	HL, UV,	(Bustos <i>et al.</i> , 1990; Clarke <i>et al.</i> , 1993b; Campbell <i>et al.</i> , 1998)
<i>Thermosynechococcus elongatus</i> BP-1		<i>tlr1843</i>	<i>tlr1477</i> , <i>tlr1844</i> ,	HL	(Sander <i>et al.</i> , 2007)
<i>Synechococcus</i> sp. PCC 6301		<i>syc1093</i>	<i>syc0647</i> , <i>syc0166</i>		
<i>Synechocystis</i> sp. PCC 6803	<i>slr1181</i>	<i>slr1311</i> , <i>sll1867</i>		HL,UV	(Mate <i>et al.</i> , 1998; Vass <i>et al.</i> , 2000; Tichy <i>et al.</i> , 2003; Allakhverdiev and Murata, 2004)
<i>Anabaena</i> sp. PCC 7120	<i>alr3742</i>	<i>alr4866</i>	<i>all3572</i> , <i>alr3727</i> , <i>alr4592</i>	HL, UV	(Sicora <i>et al.</i> , 2006)
<i>Anabaena variabilis</i>	<i>Ava4121</i> , <i>Ava1583</i>	<i>Ava2138</i>	<i>Ava2460</i> , <i>Ava1597</i> , <i>Ava3553</i>		
<i>Gloeobacter violaceus</i> sp. PCC 7421	<i>glr1706</i> , <i>glr2656</i>		<i>glr2322</i> <i>glr0779</i> , <i>gll3144</i>	HL, UV	(Sicora <i>et al.</i> , 2008)
<i>Prochlorococcus</i> sp. MED4 (HL clade)		<i>PMM0223</i>		HL	(Garcia-Fernandez <i>et al.</i> , 1998)
<i>Prochlorococcus</i> sp. SS120 (LL clade)		<i>Pro0252</i>		HL	(Garcia-Fernandez <i>et al.</i> , 1998)
<i>Prochlorococcus</i> sp. MIT9313 (LL clade)		<i>PMT0419</i> , <i>PMT1532</i>			
<i>Prochlorococcus</i> sp. Natl2a (LL clade)		<i>NT06PM0673</i> , <i>NT06PM0907</i> , <i>NT06PM1877</i>			
<i>Synechococcus</i> sp. CC9311 (clade I)		<i>sync_1858</i>	<i>sync_1856</i> , <i>sync_0368</i> , <i>sync_2384</i>		
<i>Synechococcus</i> sp. CC9605 (clade II)		<i>Syncc9605_1042</i>	<i>Syncc9605_0307</i> , <i>Syncc9605_0519</i>		
<i>Synechococcus</i> sp. WH8102 (clade III)		<i>SynW1470</i>	<i>SynW0983</i> , <i>SynW1919</i> , <i>SynW2151</i>		
<i>Synechococcus</i> sp. BL107 (clade IV)		<i>BL107_14260</i>	<i>BL107_08169</i> , <i>BL107_08179</i> , <i>BL107_09336</i> , <i>BL107_09351</i>		
<i>Synechococcus</i> sp. CC9902 (clade IV)		<i>Syncc9902_0943</i>	<i>Syncc9902_1814</i> , <i>Syncc9902_1817</i> , <i>Syncc9902_2036</i>		
<i>Synechococcus</i> sp. WH7803 (clade V)		<i>SynWH7803_0784</i>	<i>SynWH7803_0790</i> , <i>SynWH7803_0366</i> , <i>SynWH7803_2084</i>	HL, UV	This study
<i>Synechococcus</i> sp. WH7805 (clade VI)		<i>WH7805_00300</i>	<i>WH7805_00315</i> , <i>WH7805_00325</i> , <i>WH7805_07341</i> , <i>WH7805_12128</i> , <i>WH7805_12138</i>		
<i>Synechococcus</i> sp. RS9917 (clade VIII)	<i>RS9917_12975</i>	<i>RS9917_10626</i>	<i>RS9917_03498</i> , <i>RS9917_08025</i>		
<i>Synechococcus</i> sp. RS9916 (clade IX)		<i>RS9916_27899</i>	<i>RS9916_34917</i> , <i>RS9916_39506</i> , <i>RS9916_39561</i>		
<i>Synechococcus</i> sp. RCC307 (clade X)		<i>SynRCC307_1441</i>	<i>SynRCC307_1440</i> , <i>SynRCC307_2009</i> , <i>SynRCC307_2183</i>		
<i>Synechococcus</i> sp. WH5701 (subcluster 5.2)		<i>WH5701_12653</i>	<i>WH5701_01120</i> , <i>WH5701_10719</i> , <i>WH5701_15526</i>		

^aChange in *psbA* expression in response to high light or ultraviolet stress measured in previous studies.

Figure 5 Genomic rearrangements in the vicinity of *psbA* genes. Marine *Synechococcus psbA* genes (in red) surrounded by 10 upstream and downstream genes are shown according to the strain (horizontally) and to the genomic area they belong to (vertically). The first three columns represent the three main genomic regions (GR) surrounding the *psbA* genes. The fourth column groups the *psbA* genes whose upstream regions are not conserved in the other genomes. *psbA* genes are labelled with the numerical part of their gene names, while prefixes of gene names are mentioned in brackets on the left. The same colour has been given to the open reading frame number of identical D1:2-coding genes. Genes coding for the D1:1 isoform (GR 3) are labelled in bold, while fragments of *psbA* genes are labelled in italic. Identical colours have been used for orthologues forming colinear units in the different genomes. Grey genes have orthologues in at least one other *Synechococcus* genome but which are not located near a *psbA* gene. Strain-specific genes are shown in white. Genomic rearrangements upstream and downstream of *psbA* genes are indicated by coloured bars linking single orthologues or groups of orthologues. When several strains show the same recombination event as *Synechococcus* sp. WH7803, rearrangements are indicated only in this strain. When *Synechococcus* sp. WH7803 is not concerned by a recombination event, the next strain affected by this event with the highest position in the figure will be used as reference.

the *psbA* genes. Indeed, *psbA* genes from closely related cyanobacteria group together as well as according to the isoform they are coding for, such as for *Synechococcus* PCC 6301 and PCC 7942, and in the two *Anabaena* strains. Altogether, this suggests that the concerted intragenomic evolution observed between D1:2 copies also occurred among the D1:2 and the D1:1 genes over longer time scales.

A similar situation is found in marine *Synechococcus* when all three codon positions are taken into account. Indeed, phylogenetic trees (not shown) as well as evolutionary network (Supplementary Figure 1) showed that D1:1 and D1:2 isoforms encoding genes cluster together in several strains such as CC9902, BL107, CC9311 and WH5701. This

strongly suggests the occurrence of intragenomic recombination events between both isoform-coding genes. Statistical tests (Phi test) indeed allowed to detect a significant recombination signal in the alignment of all *psbA* genes of marine *Synechococcus* (Table 3). Furthermore, recombination analyses using the GARD genetic algorithm allowed identification of three recombination breakpoints, dividing the *psbA* genes alignment into four segments, each of them leading to phylogenetic trees with discordant topologies (Figure 7, Table 3). Only the tree inferred from the first 151 nucleotides showed a clear separation of the D1:1- and D1:2-encoding genes as in the tree based on the first two codon positions (Figure 6). In contrast, all three phylogenetic trees based on the other segments,

Figure 6 Maximum likelihood tree of *psbA* gene sequences from freshwater and marine cyanobacteria. Numbers at the nodes correspond to bootstrap values for 1000 replicate trees obtained with the maximum likelihood, neighbor-joining and maximum parsimony methods (ML/NJ/MP). Only bootstrap values higher than 65 are shown. Details on sequences used in this analysis are listed in Table 2. For readability, marine *Synechococcus* clusters containing several closely related D1:2-encoding genes have been compressed. The number of gene copies within each *Synechococcus* genome is indicated in brackets and the encoded isoform under square brackets.

although different, showed the clustering of D1:1- and D1:2-coding genes from the same genome, indicating intragenomic recombination events

affecting these *psbA* fragments. Likewise, recombination breakpoints have been identified in the alignment gathering either the D1:1- or the

Table 3 Statistical (Phi test) and recombination Genetic Algorithm Recombination Detection (GARD) tests performed on three *Synechococcus psbA* alignments

Alignment	Number of unique sequences	Phi test P-value	Number and positions of recombination breakpoints
All <i>psbA</i> genes	35	1.83×10^{-22}	3 (151, 522, 858)
D1:1-coding genes	11	6.58×10^{-08}	6 (186, 291, 480, 642, 774, 971)
D1:2-coding genes	23	5.70×10^{-03}	1 (660)

100% identical nucleotide sequences have been excluded from the alignments.

Figure 7 Recombination analysis of marine *Synechococcus psbA* genes using the Genetic Algorithm Recombination Detection (GARD). The identified *psbA* segments are represented as contiguous shaded boxes with the breakpoint positions indicated above. Phylogenetic trees corresponding to each segment of D1:1 and D1:2 coding genes are shown, rooted with the RS9917 divergent *psbA* gene (RS9917_12975). Clusters suggesting putative recombination events between the two isoforms of *psbA* genes are framed in black (see online version for colour figure).

D1:2-coding genes, providing segments (Table 3) with distinct phylogenetic trees (not shown). The Phi test also confirmed that the recombination events occurred within each D1 isoform-coding genes (Table 3). Taken together, all these analyses show that recombinations have affected the evolution of *psbA* genes coding for both isoforms.

Discussion

The photophysiology of cyanobacterial PSII has been primarily studied in freshwater model organisms such as *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942 and *Anabaena* sp. PCC 7120. To understand the stress response of PSII in marine *Synechococcus*, and more specifically the function of the *psbA* gene family, we submitted the

WH7803 strain acclimated to different growth irradiances to UV and PAR stresses.

Ultraviolet treatments induced a strong decrease in PSII quantum yield whatever be the initial growth irradiance (Figures 1 and 2). Cells acclimated to HL, however, appeared to be better prepared to withstand subsequent UV stress, since the photoinhibition rate was lower for the HL cultures than for the other cultures (Figure 2d). The smaller decrease of D1 content in HL cultures than in ML and LL cultures suggests that the D1 turnover is faster in HL cells than in the other cultures (Figure 3). The influence of light history of cultures was also quite visible at the transcriptomic level, as the relative contribution of the different *psbA* transcripts differed significantly depending on growth irradiance (Figure 4). Similarly, to most freshwater cyanobacteria, we show that in *Synechococcus*

Figure 8 Model of evolution of marine *Synechococcus psbA* genes. After duplication of a *psbA* gene precursor (black box), leading to the emergence of the D1:1 (light grey box) and D1:2 (white box) isoform-coding genes, the triplication of the latter gene probably occurred in an ancestral cyanobacterium. Although there is a low degree of divergence between *psbA* sequences of marine *Synechococcus* strains, all *psbA* gene copies have followed a concerted evolution driven by intragenomic recombinations and lateral transfers from other strains and possibly viruses. D1:2-encoding genes also seem to have undergone an additional homogenization by gene conversion. Through this process, a mutation (indicated by a star) occurring in a D1:2-encoding gene, would be either eliminated or transmitted to other D1:2-encoding genes of the same strain (see online version for colour figure).

sp. WH7803, UV stress led to the induction of expression of *SynWH7803_0790* encoding the D1:2 isoform, whereas the gene encoding D1:1, *SynWH7803_0784*, was repressed. These modifications were particularly striking in LL-acclimated cultures for which *psbA* gene encoding D1:1 isoform initially made up most of the *psbA* pool transcripts. In contrast, HL cultures already possess a larger initial pool of *psbA* transcripts encoding the D1:2 isoform, likely allowing better immediate resistance to UV stress. These observations are consistent with those of MacDonald *et al.* (2003) who reported that acclimation to higher PAR potentiated *Synechococcus* sp. PCC 7942 to tolerate a higher UVB level. Similarly, field studies have shown that phytoplankton sensitivity to surface UVB exposure was influenced by their previous light history (Bouchard *et al.*, 2005).

Several mechanisms have been proposed to explain PSII photoinhibition by UV, all of them involving reactive oxygen species. It is thought that UV generates oxidative stress either by excessive reduction of the primary electron acceptor of PSII Q_A (Vass *et al.*, 1992) or by provoking charge recombination between PSII acceptor and donor sides (Keren *et al.*, 1997). Reactive oxygen species have been suggested to be involved in the direct inactivation of PSII reaction centre through D1 protein cleavage (Vass *et al.*, 1992), and also inhibits PSII repair by blocking the elongation step of protein translation, preventing D1 protein *de novo* synthesis (Nishiyama *et al.*, 2001, 2006). Indeed, we have

observed in the LL (and some of the ML) cultures exposed to UV the formation of cross-linked products of the large subunit of the RuBisCO (RbcL; data not shown), a phenomenon previously correlated with transient changes in cellular levels of reactive oxygen species (Wilson *et al.*, 1995; Yosef *et al.*, 2004). This indicates that these LL and ML cultures underwent a strong oxidative stress under UV exposure. Since no cross-linked products of the RuBisCO subunit were detected in any of the cultures exposed to HL, a difference in reactive oxygen species accumulation between the two types of treatments could partially explain the differential susceptibility of *Synechococcus* to UV and HL stresses.

LL and ML cells also exhibited different responses throughout HL exposure. When exposed to HL, the PSII quantum yield of LL cultures dropped sharply (Figure 2), while D1 protein content transiently doubled (Figure 3). Hence, most of these D1 proteins were not participating in functional PSII centres. These may be *de novo* synthesized products still not incorporated in the PSII cores, and/or D1 proteins that have not been cleared from photoinactivated PSII. In contrast to LL cultures, ML cultures rapidly acclimated to HL conditions with damaged D1 clearance coordinated with PSII photoinactivation. Indeed, although the PSII quantum yield of ML cultures decreased somewhat during the first hour of HL exposure, no fluctuations in D1 protein abundance was observed (Figures 2b and 3d). In fact, PSII quantum yield rapidly stabilized at the initial value of HL cultures, indicating a nearly full acclimation to the new light conditions. The complementary acclimation was also observed in the reverse experiment, when the HL cultures were transferred to ML, as the D1 content did not vary during these experiments and the PSII quantum yield quickly increased and stabilized at a level close to that of ML cultures. In both cases, the change observed in PSII quantum yield may not reflect insufficient turnover of the D1 protein but rather a modification of the photosynthetic activity of the PSII complex. It is likely that the D1:1 isoform confers a higher PSII activity than D1:2 (Campbell *et al.*, 1996), but the latter form provides a lower quantum yield but a higher PSII resistance to photoinhibition (Krupa *et al.*, 1991; Clarke *et al.*, 1993a,b; Campbell *et al.*, 1995, 1998; Tichy *et al.*, 2003), by increasing the probability of excitation dissipation in PSII centres (Sane *et al.*, 2002). This hypothesis is consistent with the fact that HL-acclimated cells, which express mostly the D1:2 isoform genes (Figure 4), exhibit a lower PSII quantum yield. The switch between D1 isoforms, indicated by transcript (Figure 4), could thus be seen as a trade off between PSII activity and PSII photoprotection.

It is likely that the mechanism described here for *Synechococcus* sp. WH7803 occurs in most marine *Synechococcus* and constitutes a fundamental

photophysiological trait of these ubiquitous organisms that inhabit diverse light environments. Indeed, examination of all marine *Synechococcus* genomes available to date shows a range for the *psbA* copy number, with all having a single D1:1 but several, up to five, D1:2-encoding genes per genome. The extremely low level of sequence divergence among the D1:2 coding genes and their independent clustering within all marine *Synechococcus* indicates a concerted evolution of these genes within a genome. This strongly suggests a homogenization of these sequences by gene conversion, defined as a non-reciprocal transfer of genetic information between highly homologous sequences (Santoyo and Romero, 2005). Through this process, paralogues within a species stay highly similar and yet continue to diverge among species. This mechanism is associated here with the occurrence of numerous translocations of orthologues adjacent to D1:2-encoding genes (Figure 5), indicating recurrent recombination events between these genes (Hurles, 2004). Noteworthy, the two *psbA* genes responding most sharply to the exposure to UV and HL, *SynWH7803_0790* and *SynWH7803_0784*, are located in the same genomic region (genomic region 3) in which more genomic rearrangements seem to have occurred when there is a D1:2 copy located downstream the D1:1 gene. This situation suggests that some strains could have carried a D1:2 gene copy in the genomic region 3, which would have been secondarily lost during one of the recombination events. The evolution of the D1:2-coding sequences thus appears to be the result of two counteracting processes: mutation and gene conversion. While mutation creates diversity between the D1:2 *psbA* copies, gene conversion tends to homogenize all copies within the genome in favour of the mutated or the original one and thus to decrease intragenomic divergence.

This concerted evolution of D1:2-encoding genes seems to be common to most cyanobacteria. Examination of freshwater cyanobacteria such as the two *Anabaena* or the two *Synechococcus* strains indeed shows that each strain possesses a set of nearly identical D1:2-encoding genes, as in marine *Synechococcus* (Figure 6). Furthermore, considering more distantly related strains, both isoforms cluster according to their genome. This observation clearly shows that, on a larger time scale, a certain degree of concerted evolution by recombination also occurred between both isoforms in freshwater cyanobacteria. In marine *Synechococcus* strains, the existence of a significant level of intragenomic recombination between D1:1- and D1:2-coding genes was confirmed by evolutionary network as well as recombination and statistical tests (Table 3). Recombination events have also been identified between marine *Synechococcus* strains, suggesting lateral transfers of *psbA* fragments between strains co-occurring in a same niche. Lastly, previous studies have shown that most *Synechococcus* cyanophage genomes

contain a *psbA* gene closely related to the ones of their host and that genetic exchange of *psbA* fragments may have occurred from phage to host through direct environmental DNA uptake or through viral intermediates (Zeidner *et al.*, 2005; Sullivan *et al.*, 2006). As suggested by Sullivan *et al.* (2006), these lateral transfers could be one of the reasons explaining the incongruent phylogeny of *Synechococcus psbA* genes compared to the one obtained with concatenated ribosomal protein sequences (Six *et al.*, 2007c) or other phylogenetic markers (Palenik, 2001; Rocap *et al.*, 2002; Fuller *et al.*, 2003; Lindell *et al.*, 2005).

Such a mechanism of evolution, involving recombinations across all *psbA* genes, can also be related to the function and structure of the D1 protein. This protein indeed belongs to a core set of proteins that are extremely conserved due to protein–protein, protein–cofactor, and protein–substrate interactions that are critical to energy transduction (Shi *et al.*, 2005). A striking example concerns the essential amino acid at position 130: although localized in a *psbA* segment affected by recombination between both isoform genes (Figure 7), the Gln and Glu residues are maintained in D1:1 and D1:2, respectively. Therefore, D1 proteins undergo a strong purifying selection and most amino acid changes are likely selected against. The coevolution of both D1 isoforms, while maintaining their respective functional specificity, would then allow maintaining the essential function of these proteins and their interactions with other protein members in the core photosynthetic machinery.

Several general trends in the evolutionary process of this protein family can be inferred from our study (Figure 8). The occurrence of both D1 isoforms in most cyanobacteria indicates that the common ancestor of these organisms probably possessed one D1:1- and two or three D1:2-encoding genes. Noticeable exceptions are the green oxyphotobacteria, *Prochloron*, *Prochlorothrix* and *Prochlorococcus*, which only carry one to three *psbA* gene copies encoding only the D1:1 isoform (Hess *et al.*, 1995; Partensky and Garczarek, 2003). However, these organisms are atypical cyanobacteria in that they differ in their light harvesting system, which is an integral membrane Chl *b*-binding antenna instead of the extrinsic phycobilisome found in most cyanobacteria (LaRoche *et al.*, 1996; Garczarek *et al.*, 2000). Considering the strong interactions among PSII apoproteins and antenna proteins, the acquisition of the green oxyphotobacteria-specific antenna most likely co-occurred with the loss of the D1:2-encoding genes. In contrast, all marine *Synechococcus* still possess D1:1- and D1:2-encoding genes. As in most cyanobacteria, both genes encoding D1 isoforms seem to be submitted to two distinct evolutionary processes, both of them leading to concerted evolution (Figure 8). While all *psbA* gene copies follow a concerted evolution through intragenomic recombinations and lateral

transfers from other strains and possibly viruses, D1:2-encoding genes seem to undergo an additional homogenization through gene conversion, which would either eliminate or transmit mutation(s) to other D1:2-encoding genes of the same strain. In contrast, we hypothesize that the need to maintain the functional specificity of the D1:1 isoform prevents gene conversion between D1:1 and D1:2 genes. In any case, functional and structural constraints of D1 protein isoforms appear to be the driving force of the coevolution of *psbA* genes. Such numerous recombination events probably also affect the regulation of these genes at the transcriptional level in *Synechococcus* strains.

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