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The D1 and D2 proteins of dinoflagellates: unusually accumulated mutations which influence on PS II photoreaction

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2Tables, 4 Figures

Abstract

Plastid encoded genes of the dinoflagellates are rapidly evolving and most divergent. The importance of unusually accumulated mutations on structure of PS II core protein and photosynthetic function was examined in the dinoflagellates, *Symbiodinium* sp. and *Alexandrium tamarense*. Full-length cDNA sequences of *psbA* (D1 protein) and *psbD* (D2 protein) were obtained and compared with the other oxygen-evolving photoautotrophs. 23 amino acid positions (7%) for the D1 protein and 34 positions (10%) for the D2 were mutated in the dinoflagellates although amino acid residues at these positions were conserved in cyanobacteria, the other algae and plant. Many mutations likely to distribute in the N-terminus and the D-E interhelical loop of the D1 protein and helix B of D2 protein, while the remaining regions were well-conserved. The different structural properties in these mutated regions were supported by hydropathy profiles. The chlorophyll fluorescence kinetics of the dinoflagellates was compared with *Synechocystis* sp. PCC6803 in relation to the altered protein structure.

Keywords *Alexandrium*, Chlorophyll fluorescence, Hydropathy plot, *psbA/D*, *Symbiodinium*, *Synechocystis* sp. PCC6803

Abbreviations

a. a.	Amino acid
cDNA	DNA complementary to RNA
Chl	chlorophyll
C-terminus	carboxyl terminus
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
F_V	variable fluorescence
F_M	maximum fluorescence
N-terminus	amino terminus
PSII	photosystem II
Rubisco	ribulose 1,5-bisphosphate carboxylase/oxygenase

Introduction

Photosystem II (PSII) functions as the water:plastoquinone oxidoreductase in oxygenic photosynthesis (Satoh et al. 2005). The important light-induced electron transfer reactions occur within a heterodimer composed of the D1 and the D2 reaction center proteins encoded by *psbA* and *psbD* genes, respectively (Nixon et al. 2005). The D1 protein is synthesized as the precursor protein with extension on its carboxyl terminus (C-terminus), which typically consists of nine amino acid residues in green algae and plants (Chlorophyta), and 16 residues in cyanobacteria and the other eukaryotic algae. The processing of C-terminal extension of D1 precursor protein is catalyzed by a specific processing endoprotease, CtpA (Satoh and Yamamoto 2007).

Dinoflagellates (Dinophyta) contain important primary producers in both marine and freshwater environment (Taylor 1987). The typical dinoflagellate plastid has Chl *a*, Chl *c*₂ and peridinin (Prézelin 1987), and is surrounded by three envelopes (Dodge 1987). The peridinin-containing dinoflagellate is suggested to have acquired its plastid from the red algae (Rhodophyta) through the secondary symbiosis (Palmer 2003). Many remarkable modifications occurred in the dinoflagellate plastid, such as the presence of form II Rubisco (a form known only in proteobacteria and the dinoflagellates), instead of the usual plastid-coded form I Rubisco, encoded in the nucleus, marked reductions of their plastid genomes, the presence of extensive RNA editing and specific polyuridylation in plastid gene transcripts (Green 2004; Howe et al. 2008). In contrast to plastid genomes of 120-200 kbp (in which more than 100 genes were encoded) sequenced from many plants and algae, one or limited number of genes was shown to be located on separate plasmid-like molecules of 2-10 kbp (minicircles) in the dinoflagellates plastid. To date, only 17 genes have been found on independent minicircles (six for PSII including *psbA* and *psbD*, two for PSI, two for cytochrome *b₆f* complex, two for ATP synthase, and five for ribosomal and transfer RNA).

The D1 protein appears to restrict amino acid exchanges and has been extensively conserved during evolutionary process that transformed the ancestral cyanobacteria into a contemporary plastid of various algae and plants, probably due to functional constraints on this structural component of the PSII complex (Golden 1994). However, comparison with *psbA*, together with chloroplast encoded 23S ribosomal RNA, of other oxygenic organisms shows that dinoflagellate plastid genes are rapidly evolving of all and the most divergent (Zhang et al. 2000). A markedly accelerated rate of evolution was also confirmed when dinoflagellate plastid genes (*psbA*, *psbD* and eight other photosynthetic genes) were compared with those of *Emiliania huxleyi* (Haptophyta), one of the other organisms with secondary plastid of red algal origin like the dinoflagellate (Bachvaroff et al. 2006). Although such exceptionally rapid evolution of plastid genes in the dinoflagellate

increases chances to fix mutations involved in structure and function of the D1 and the D2 proteins, the influence of mutations on organization and function of PSII has not been considered except for unordinary deletion of the C-terminal extension (9 or 16 a. a. residues) in precursor D1 protein (Takishita and Uchida 1999; Satoh and Yamamoto 2007; cf. Fig. 1a).

The C-terminus domain of the predicted mature D1 protein is strictly conserved among diverse oxygen-evolving photoautotrophs (-Glu-Val-Met-His-Glu-Arg-Asn-Ala-His-Asn-Phe-Pro-Leu-Asp-Leu-Ala³⁴⁴ in sequence) (cf. Satoh 1998; Satoh and Yamamoto 2007), while it has been predicted to terminate exceptionally as -Leu-Asp-Leu-Gly-Leu based on the genomic sequence of *psbA* in the dinoflagellate *Alexandrium tamarense* (AB025589, Takishita and Uchida 1999). Since the carboxyl group of terminal residue Ala³⁴⁴ of D1 protein is supposed to be an essential ligand for manganese atom in the Mn₄Ca-cluster (Nixon et al. 1992; Debus 2005; Kimura et al. 2005; Zouni 2008), the presence of such unusual but functional D1 protein may provide invaluable opportunities to examine the organization and function of PSII. For this purpose, the examination of DNA complementary to RNA (cDNA) rather than genomic DNA is prerequisite to infer protein primary structure in the dinoflagellate plastid since the presence of translationally defective pseudogenes and extensive RNA editing are frequently reported in the dinoflagellate (Zauner et al. 2004; Wang and Morse 2006; Howe et al. 2008).

This paper presents the structure of D1 and D2 proteins of the two dinoflagellates, *Symbiodinium* sp. and *Alexandrium tamarense*, based on full-length cDNA sequences and protein secondary structure prediction, and examined whether the dinoflagellate accumulated unusual mutations. In addition, Chl fluorescence kinetics of dinoflagellates with altered structure of PSII were compared with *Synechocystis* sp. PCC 6803. Our results showed the extensive mutations with amino acid substitutions were accumulated in particular regions of D1 and D2 proteins and some mutations were related to alteration in protein structure and PS II function in the dinoflagellates.

Materials and Methods

Cell Culture

Two peridinin-containing dinoflagellates, *Symbiodinium* sp. strain SGCH-03 (Nomaru 2005) and *Alexandrium tamarense* strain OFAT0105-8 (Kobiyama et al. 2006), were used in the present study. *Symbiodinium* sp. was cultured in Daigo's IMK medium (Nihon Pharmaceutical, Tokyo, Japan) at 20°C and 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ on a 12-h light/12-h dark cycle. *Alexandrium tamarense* was cultured in SWII medium (Iwasaki 1961) at 15°C and

100 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ on a 15-h light/9-h dark cycle. *Synechocystis* sp. PCC 6803 was cultured in aerated BG-11 at 20°C and 50 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$. The fluorescent lamp (FL20SSW/18 National, Osaka, Japan) was used for all cultures.

RNA isolation, cDNA synthesis and sequencing

Cells at the mid-exponential growth phase of *Symbiodinium* were harvested and centrifuged (4,000 g, 10 min, 15°C). *Alexandrium* were harvested in the same way (5,000 g, 10 min, 4°C) after filtration through a nylon net (20- μm mesh). Cells were immersed in RNAlater (Ambion, Austin, USA) immediately after cell harvesting according to the manufacturer's instructions and stored at -20°C until analysis. RNA was isolated and purified using an RNeasy Mini Kit and RNase-Free DNase (Qiagen, Hilden, Germany).

cDNAs were synthesized using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, USA). Plastid gene specific first-strand cDNAs were synthesized using the primer 5'-GACTAGATCTCCATGGCCTAGGAAAAAAAAAAAAAAAAA-3' (oligo dA17), which polyA sequence was complementary to polyuridine tract at 3' termini of the dinoflagellate plastid gene transcript (Wang and Morse 2006). Target cDNA was amplified using a gene specific primer and its sequence was determined directly. Following gene specific primers were designed based on the sequences of the initial PCR products using degenerate primers: *Symbiodinium psbA* (5'-GGCCCGAATATTGATCAGCTGAC-3'), *Symbiodinium psbD* (5'-CTTCAAGATTGAAATTAGGTGCAATTCG-3'), *Alexandrium psbA* (5'-CAGCATGAAGCTTAAACAATAGGGAC-3') and *Alexandrium psbD* (5'-CTTTGGGCATTTGTGAC ATCAC-3'). Templates for sequencing analyses were prepared using a BigDye Terminator v3.1 (Applied Biosystems, Foster City, USA). Sequencing was carried out using an ABI PRISM 3100 or 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Sequence alignment and protein structure analyses

Predicted amino acid sequence of D1 and D2 proteins of *Symbiodinium* and *Alexandrium* were aligned and compared with phylogenetically diverse organisms listed in Table 1 using MEGA version 3.1 (Kumar et al. 2004). In the dinoflagellates, many genomic sequence data for *psbA* and *psbD* were registered in open database but the genomic sequence might be different from information of cDNA sequence due to mis-inclusion of pseudogenes (Howe et al. 2008) and RNA editing (Zauner et al. 2004; Wang and Morse 2006). Therefore, only published cDNA data for two other dinoflagellates were used (Table 1). To compare amino acid sequences from

different organisms, the divergence (the proportion of different amino acids between two sequences, Nei and Kumar 2000) was calculated. Kyte-Doolittle hydropathy plot (Kyte and Doolittle 1982) was drawn using a program ProtScale with window size 15, which are available through a web server of Expasy (<http://www.expasy.ch/tools/protscale-ref.html>, Gasteiger et al. 2005).

Chlorophyll fluorescence measurements

Fluorescence induction and decay kinetics of Chl *a* were monitored with a FL3000 dual modulation kinetic fluorometer (Photon System Instruments, Brno, Czech Republic) at room temperature. For all the fluorescence experiments, the cells were dark incubated for 5 min before initiation of the measurements. In the fluorescence induction measurements, data were collected in a logarithmic time series between 200 μ s and 1 s after the onset of saturated actinic light. In the fluorescence decay experiments, flash-induced increase and the subsequent decay of fluorescence were measured in the 200 μ s to 10 s time ranges. *Alexandrium* cells were continuously stirred to prevent from cell sedimentation during the measurements.

Results

Examined sequence of *Symbiodinium* sp. cDNA contained 344 codons for *psbA* (AB456560) and 357 codons for *psbD* (AB456561) excluding the termination (Table 1). And that of *Alexandrium tamarense* contained 346 codons for *psbA* (AB433582) and 355 codons for *psbD* (AB433583) (Table 1). Alignments of predicted amino acid sequence of the D1 and the D2 proteins with other oxygen-evolving photoautotrophs were successfully carried out in the region excluding amino-terminus (N-terminus) region in each protein (Fig. 1). The N-terminus region of the dinoflagellates D1 (ca. 16 a. a. residues) and D2 protein (ca. 10 a. a. residues) differ from the all other oxygen-evolving photoautotrophs in length and in amino acid sequence. In *Alexandrium*, a codon other than ATG was identified in expected site of translation initiation (TTG in *psbA* and CTT in *psbD*). We determined the C-terminus sequence of *Alexandrium* D1 protein was –Leu-Asp-Leu-Ala which was identical to consensus of mature D1 protein sequence among all oxygen-evolving photoautotrophs (from cyanobacteria to green plants). As reported in the previous studies of both genomic and cDNA sequences of the other peridinin-containing dinoflagellate species (e.g. Takishita and Uchida 1999; Wang and Morse 2006), the C-terminal extension of precursor D1 protein is equally deleted in *Symbiodinium* and *Alexandrium* (Table 1).

Table 2 shows amino acid sequence divergence of the D1 and the D2 proteins between phylogenetically diverse organisms listed in Table 1. Generally 13-15 % of total amino acids in the D1 protein and 11-16 % in the D2 protein were different from *Synechocystis* sp. PCC6803 sequence in each oxygen-evolving photoautotrophs (lane 1 from lower or upper diagonal in Table 2), but divergence from the dinoflagellates was almost doubled (24-26 % in D1 and 19-23% in D2). Similar to *Synechocystis*, high divergence values were detected between the dinoflagellates and the rest of the other organisms (21-27 % in D1 and 16-26 % in D2, Table 2). The extent of divergence was also higher than those within primary endosymbiotic lineages of plastid (Chlorophyta, Rhodophyta and Glaucophyta) (5-16 % in D1 and 6-14 % in D2, Table 2). Organisms of Chromophyta, Euglenophyta and Chlorarachniophyta have secondary origin of plastid as the dinoflagellate, but the divergence values were similar to those of primary lineages and significantly lower than the dinoflagellate ones (7-16 % in D1 and 5-16 % in D2, Table 2). Similarity within the dinoflagellates (*Symbiodinium*, *Alexandrium*, *Gonyaulax* and *Ceratium*) was observed in both D1 and D2 proteins (Table 2).

Figure 2 shows amino acid mutation sites of the dinoflagellate D1 and D2 protein sequences. Among 328 amino acid residues compared among D1 proteins of phylogenetically diverse organisms excluding N-terminus region, 23 amino acid positions were mutated in all (12 positions) or some (11 positions) dinoflagellate species. A total of 342 amino acid residues were compared in D2 protein and 34 positions were specific to all (10 positions) or some (24 positions) dinoflagellate species. Although the mutation sites did not include key residues predicted from sequence comparison to be involved in binding various cofactors within PSII, some were distributed near possible ligands to the functional Mn_4Ca -cluster (D1-His337), tyrosine electron donor (D2-Tyr160), and Q_A and Q_B binding pockets supported by the D-E interhelical loops of D1 and D2 proteins (Fig. 2) (Kern et al. 2005; Nixon et al. 2005; Petrouleas and Crofts 2005).

To examine whether these mutations of dinoflagellate affect the conformation of proteins, Kyte-Doolittle hydropathy plot was drawn and the protein structural features of the dinoflagellate *Symbiodinium* and *Alexandrium* were compared with those of other organisms. Five transmembrane helices (A, B, C, D and E) and two surface (CD and DE) helices suggested in the folding model of cyanobacteria, *Thermosynechococcus* (Loll et al. 2005, Fig. 1), were assigned in the hydropathy profiles of both D1 and D2 proteins (Fig. 3). Hydropathy profiles were conserved among all oxygen-evolving photoautotrophs, however extensive increase in hydrophobicity was found in N-terminus region (1-12 a. a. position) and the D-E interhelical loop (223-247 a. a. position) of D1 and helix B (110-136 a. a. position) of D2 protein of the both dinoflagellates (Fig. 1, 3). Considerable modification in hydrophobicity was also observed in helix B of *Alexandrium* (111-137 a. a. position) and helix C of *Symbiodinium* D1 (148-165 a. a. position) protein, respectively.

To assess the influence of mutations around the D-E interhelical loop of D1 protein, a light-induced redox reaction of Q_A/Q_B was examined by Chl *a* fluorescence. The fluorescence induction (Kautsky curves) and fluorescence decay (re-oxidation rates of Q_A) were compared with *Synechocystis* sp. PCC6803 (Fig. 4). In the fluorescence induction experiments, fluorescence curve of *Synechocystis* had two maximum at times 2 ms and 0.3 s (Fig. 4a). In both dinoflagellate strains, two fluorescence maxima (10 – 15 ms, 1.3 s) were obscured and delayed compared with those of *Synechocystis* (Fig. 4a). Slower fluorescence induction during initial phase was especially marked in the dinoflagellates. In the presence of 30 μ M DCMU, however, difference between the dinoflagellate and *Synechocystis* was almost disappeared.

In flash-induced fluorescence experiment, rapid fluorescence decay was observed in *Synechocystis* after a single saturating flash (Fig. 4b, Allahverdiyeva et al. 2004). Fluorescence decay of the dinoflagellate was very similar to that of *Synechocystis* in the absence of DCMU indicating electron transfer from Q_A to Q_B was not modified in dinoflagellate PSII. In the presence of 30 μ M DCMU, fluorescence decay of the dinoflagellate was slightly faster than that of *Synechocystis* (Fig. 4b).

Discussion

Dinoflagellates are important primary producers in the aquatic environment, especially in tropical coral reef (repr. *Symbiodinium* sp.), while occasional blooms cause harmful red tides (repr. *Alexandrium* sp.) (Taylor 1987). Detailed characterization of structure and function of PSII in the dinoflagellate will provide good opportunity to understand coral bleaching (Warner et al. 1999; Jones 2005; Takahashi et al. 2008) and complex blooming of red tide (Taylor 1987). In this study, we found the dinoflagellate D1 and the D2 proteins accumulated amino acid mutations in the region conserved among the other oxygen-evolving photoautotrophs including red algae whose plastid was suggested to be direct ancestry of the dinoflagellate one (Palmer 2003) (Table 2, Fig. 1, 2). As some mutations changed the hydropathy profile of the D1 and the D2 proteins extensively, they might affect the local conformation of PSII complex. Among all, marked increase in hydrophobicity in the D-E interhelical loop may have influence the Q_A and Q_B binding pockets consisted from D and E helices (Fig. 3). However, only slight modification of these binding pockets in the dinoflagellate was indicated by the flash-induced fluorescence decay curves, while significant difference in the fluorescence induction curves were observed between the dinoflagellate and *Synechocystis* sp. PCC6803 (Fig. 4). Detailed experimental study on organization and reactivity of PSII complex is necessary to understand its unique structure of the dinoflagellate D1 and D2 proteins.

The C-terminus of the mature form of the D1 protein provides a ligand to the Mn_4Ca -cluster for water oxidation in PSII (Nixon et al. 1992; Debus 2005; Kimura et al. 2005; Zouni 2008). The C-terminus of the predicted mature D1 protein is highly conserved (-Leu-Asp-Leu-Ala in sequence) in all oxygen-evolving photoautotrophs (Satoh and Yamamoto 2007) with the only exception of one dinoflagellate species, *Alexandrium tamarense* (-Gly-Leu-Asp-Leu; Takishita and Uchida 1999). In this study, cDNA from *A. tamarense* was examined to assess the presence of ordinary D1 protein with conserved -Leu-Asp-Leu-Ala sequence in the C-terminus (Fig. 1). Our result determined the C-terminus of the mature D1 protein and critical function of the C-terminus were conserved even in *A. tamarense*. The dinoflagellate plastid genome has a peculiar minicircular structure which is unique among the oxygenic photoautotrophs (Green 2004; Howe et al. 2008). Since various pseudogenes were present in the dinoflagellate minicircular plastid genome (Howe et al. 2008), *psbA* pseudogene in *A. tamarense* has been mistaken as normal one in the previous genomic DNA analysis (Takishita and Uchida 1999).

The D1 protein was identified as the rapidly turning over protein (Adir et al. 2003). The turnover (damage and repair) of this protein is related to the inactivation and the recovery of PSII activity induced by high light stress (photoinhibition) (Adir et al. 2003). The damaged D1 protein is suggested to be cleaved primary in the stromal exposed domain either the D-E interhelical loop (Greenberg et al. 1987; Adir et al. 2003; Lindahl et al. 2000) or the N-terminus (Komenda et al. 2007). The hydropathy profiles of D1 proteins were conserved adequately among *Synechocystis* (cyanobacteria), *Spinacia* (plant) and *Porphyra* (red algae) including the N-terminus and the D-E interhelical loop (Fig. 3). The well-conserved hydropathy profiles were also indicated in a divergent and silent copy of cyanobacteria *psbA* gene family (e.g. *Synechocystis* sp. PCC6803-slr1181) (data not shown). However, in the dinoflagellate D1 proteins, both stromal exposed domains (D-E interhelical loop and N-terminus) accumulated mutations (Fig. 1, 2) and their hydrophobicity increased markedly (Fig. 3). FtsH protease found in cyanobacteria and chloroplasts is involved in the degradation of the D1 protein and supposed to initiate proteolysis from stromally exposed regions of D1 protein (Adam et al. 2005; Komenda et al. 2007). Modified dinoflagellate D1 and D2 protein must be examined from the view point of D1 turnover and substrate recognition of FtsH protease. Future study must also focus on the other modification unique to the dinoflagellates such as in the basal part of helix B of the D2 protein (Fig. 1-3).

The mature form of D1 protein is produced by enzymatic cleavage of the C-terminal extension of the precursor protein by the D1 processing protease, CtpA (Satoh and Yamamoto 2007). The presence of the C-terminal extension was suggested to contribute to systematic assembly and precise organization of PSII complex in genetically engineered mutants of *Synechocystis* sp. PCC6803 (e.g. Ivelva et al. 2000; Diner 2001). Although

almost universal presence of the C-terminal extension (9-16 a. a.) was shown by *psbA* genes encoding the D1 protein registered in the open database, the C-terminal extension was occasionally lost in several derived lineages of secondary symbiosis (Euglenophyta and Chlorarachniophyta with plastid of green algal origin and Dinophyta with plastid of red algal origin)(Sato and Yamamoto 2007). In this study, we confirmed this evolutionary trend in the dinoflagellate by showing loss of the C-terminal extension in *Symbiodinium* sp. and *Alexandrium tamarense* D1 sequences. However, not all dinoflagellate species lost the C-terminal extension (Sato and Yamamoto 2007). One dinoflagellate species, *Karenia mikimotoi* (the former *Gymnodinium mikimotoi*), whose plastid contains fucoxanthin and fucoxanthin derivatives instead of peridinin, has the C-terminal extension in the precursor D1 protein (Takishita et al. 1999). Comparative study of heterogeneous dinoflagellates and the examination of evolutionary requirement which allow the organisms to delete the C-terminal extension may provide important insights in understanding unelucidated function of the C-terminal processing of the D1 protein.

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Table 1 D1 and D2 proteins of phylogenetically diverse oxygen-evolving photoautotrophs used in this study

Organism		Amino acid no.		Acc. no.
		Mature protein	C-terminal extension	
D1 protein				
Cyanophyta	<i>Synechocystis</i> sp. PCC6803	344	16	NC_000911 ^e
	<i>Thermosynechococcus elongatus</i>	344	16	NC_004113 ^f
Chlorophyta	<i>Spinacia oleracea</i>	344	9	NC_002202
	<i>Chlamydomonas reinhardtii</i>	344	8	NC_005353
Rhodophyta	<i>Porphyra purpurea</i> Avonport	344	16	NC_000925
	<i>Cyanidioschyzon merolae</i> 10D	344	16	NC_004799
Glaucophyta	<i>Cyanophora paradoxa</i>	344	16	NP_043238
Chromophyta	<i>Phaeodactylum tricornutum</i>	344	16	NC_008588
	<i>Fucus vesiculosus</i>	344	16	DQ307679
Euglenophyta	<i>Euglena gracilis</i> Z	345	0	NC_001603
Chlorarachniophyta	<i>Bigelowiella natans</i>	344	0	DQ851108
Dinophyta	<i>Gonyaulax polyedra</i>	345	0	DQ264844
	<i>Symbiodinium</i> sp. SGCH-03	344	0	AB456560
	<i>Alexandrium tamarense</i> OFAT0105-8	346 ^a	0	AB433582
D2 protein				
Cyanophyta	<i>Synechocystis</i> sp. PCC6803	352	- ^d	NC_000911 ^e
	<i>Thermosynechococcus elongatus</i>	352	-	NC_004113 ^f
Chlorophyta	<i>Spinacia oleracea</i>	353	-	NC_002202
	<i>Chlamydomonas reinhardtii</i>	352	-	NC_005353
Rhodophyta	<i>Porphyra purpurea</i> Avonport	351	-	NC_000925
	<i>Cyanidioschyzon merolae</i> 10D	351	-	NC_004799
Glaucophyta	<i>Cyanophora paradoxa</i>	352	-	NP_043238
Chromophyta	<i>Phaeodactylum tricornutum</i>	351	-	NC_008588
Euglenophyta	<i>Euglena gracilis</i> Z	(342) ^b	-	NC_001603
Chlorarachniophyta	<i>Bigelowiella natans</i>	352	-	DQ851108
Dinophyta	<i>Gonyaulax polyedra</i>	355 ^c	-	DQ264847
	<i>Ceratium horridum</i>	355 ^c	-	DQ318028
	<i>Symbiodinium</i> sp. SGCH-03	357	-	AB456561
	<i>Alexandrium tamarense</i> OFAT0105-8	355 ^a	-	AB433583

Sequences of *Alexandrium* and *Symbiodinium* were reported in this study. cDNA was examined in dinoflagellate strains

^aA codon representing leucine was identified at expected translation initiation site of both D1 and D2 proteins

^bThe sequence has been reported partially

^cThe initiation site was estimated based on *Alexandrium tamarense* data

^dD2 protein have no carboxyl terminal extension

^eThe sequences were deduced from *psbA2* and *psbA3* in D1, and *psbD1* and *psbD2* in D2 proteins

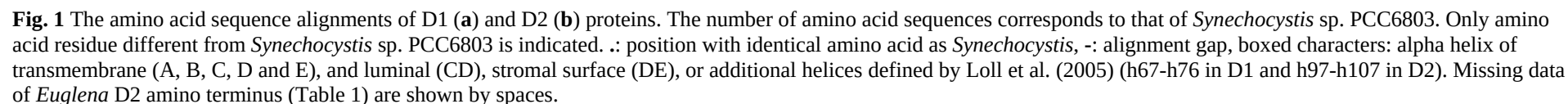
^fThe sequence was deduced from *psbA1* in D1, and *psbD1* and *psbD2* in D2 proteins. Each has identical amino acid sequence to that of crystal structure (PDB accession number: 2axt, Loll et al. 2005)

Table 2 Divergence in amino acid sequences of D1 (lower diagonal) and D2 (upper diagonal) proteins among phylogenetically diverse oxygen-evolving photoautotrophs listed in Table 1

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 <i>Synechocystis</i>		11.4	13.7	14.3	12.0	10.2	14.3	11.4	-	15.8	13.5	19.3	22.2	22.8	20.5
2 <i>Thermosynechococcus</i>	12.9		9.9	9.4	10.8	11.4	9.9	9.9	-	13.7	11.1	17.8	20.5	20.8	17.8
3 <i>Spinacia</i>	13.5	12.3		5.9	11.7	12.9	11.4	11.4	-	12.6	7.6	20.8	23.1	23.1	20.8
4 <i>Chlamydomonas</i>	13.8	12.3	5.0		12.0	13.2	10.8	11.7	-	12.3	8.5	21.4	24.6	23.7	21.1
5 <i>Porphyra</i>	13.5	14.4	9.1	10.3		7.3	11.7	5.3	-	14.6	12.6	16.4	19.9	19.3	17.0
6 <i>Cyanidioschyzon</i>	13.8	13.5	9.7	10.9	10.0		10.5	6.7	-	15.2	13.7	17.3	19.3	19.9	16.4
7 <i>Cyanophora</i>	14.4	16.1	8.8	10.0	10.0	12.0		10.5	-	15.5	12.6	21.1	22.5	23.1	21.1
8 <i>Phaeodactylum</i>	14.4	15.0	9.4	9.7	8.5	10.0	10.0		-	14.0	12.0	16.1	19.3	18.4	16.4
9 <i>Fucus</i>	15.2	17.0	12.0	12.6	11.1	13.5	14.4	8.2		-	-	-	-	-	-
10 <i>Euglena</i>	15.5	16.1	12.9	11.7	16.1	14.4	15.2	15.0	17.3		12.3	21.9	26.0	24.9	22.8
11 <i>Bigeloviella</i>	13.5	15.2	7.3	7.9	14.1	11.4	12.6	12.0	15.8	12.0		21.4	24.3	24.9	21.9
12 <i>Gonyaulax</i>	24.6	26.1	24.3	23.8	24.6	25.2	22.9	22.3	22.6	24.3	24.0		12.6	10.5	6.4
13 <i>Ceratium</i>	-	-	-	-	-	-	-	-	-	-	-	-		14.3	12.0
14 <i>Symbiodinium</i>	24.3	26.1	23.2	22.9	23.8	24.0	21.1	20.8	22.6	25.5	24.3	12.3	-		9.7
15 <i>Alexandrium</i>	26.1	27.0	25.2	25.2	25.5	26.1	23.5	24.6	24.6	27.0	24.9	10.0	-	15.0	

The divergence is indicated by the proportion (%) of different amino acids between two sequences (Nei and Kumar 2000). The length mutations were treated as missing data and excluded from the analysis. The values on dinoflagellate are highlighted with colored background (bold type: between the dinoflagellate and the others, normal type: between two dinoflagellates). -: no data

Cyanophyta



b. D2 protein

			(h97)	A (h98)		
Cyanophyta	<i>Synechocystis</i>	(Syn)	MTIAVGRAP- ¹⁰ VERGWFDVLD ²⁰ DLKRD ³⁰ RFVF ⁴⁰ IGWSGLLLFPCAFMALGGWL ⁵⁰ TGTTFTVTSWYTHGLASSYLEGANFLT ⁶⁰ VA ⁷⁰			
	<i>Thermosynechococcus</i>	(The)	... I. ... -A. ... I. ... V. ... I. ... YL. ... C. ...			
Chlorophyta	<i>Spinacia</i>	(Spi)	... KFTKD. KDL. SM. R. V. ... YF. ... F. ... C. ... A. ...			
	<i>Chlamydomonas</i>	(Chl)	... I. TYQ-EK. T. DA. RQ. V. ... YF. ... T. ... C. ... A. ...			
Rhodophyta	<i>Porphyra</i>	(Por)	... I. QEK--T. G. LV. ... V. ... YL. V. ... C. ... A. ...			
	<i>Cyanidioschyzon</i>	(Sch)	... I. EQ-- ... LM. ... SYL. A. F. ... C. ... A. ...			
Glaucophyta	<i>Cyanophora</i>	(Cya)	... V. I. SD-S. ... L. ... L. ... YL. V. A. F. ... C. ... A. ...			
Chromophyta	<i>Phaeodactylum</i>	(Pha)	... I. QNQ-- ... I. LI. ... K. V. ... T. YL. V. M. ... C. ... A. ...			
Euglenophyta	<i>Euglena</i>	(Eug)	... KN. A. K. I. ... SYL. ... I. ... F. C. A. A. ...			
Chlorarachniophyta	<i>Bigelowiella</i>	(Big)	... I. K. E-E. V. II. R. ... YL. ... F. ... C. ... A. ...			
Dinophyta	<i>Gonyaulax</i>	(Gon)	LIYSTKFIENKGYLP. SVTL. ... S. YLS. F. ... T. ... C. ... A. ...			
	<i>Ceratium</i>	(Cer)	LLYSTKYMATQGYLP. A. TL. ... Y. IF. N. YLS. A. FS. LF. ... C. I. AS. ...			
	<i>Symbiodinium</i>	(Sym)	MKVIYSTRFmaskayLP. SLTL. ... T. YL. A. F. A. FF. ... T. AC. A. A. ...			
	<i>Alexandrium</i>	(Ale)	LIYSTQFLATKGYLP. SITL. ... L. YLS. A. F. S. F. ... T. C. A. ...			

			(h99)	B (h100)	C (h101)	CD (h102)	D (h103)
Syn	SSPADAFGHSL ⁸⁰ LLFLWGPEAQGN ⁹⁰ LTRWFQ ¹⁰⁰ IGGLWPFVALHGAFGL ¹¹⁰ IGFMLRQFE ¹²⁰ ISRLVGIRPYNA ¹³⁰ IAFSGP ¹⁴⁰ IAVFVSVFLMYPLGQSSWFFAP ¹⁵⁰ SFGVAG ¹⁶⁰ IFRFILFLQGFHNWTLNPFHMMGVAG ¹⁷⁰ ILGGALLCAIHG ²¹⁰						
The	... T. NSM. ... L. ... DF. ... C. L. ... T. I. ... A. ... V. ... I. ... A. ... L. F. ... V. ...						
Spi	... T. NSLA. ... L. ... DF. ... C. L. ... A. ... A. ... LA. S. QL. ... A. ... I. ... G. ... A. ... F. ... V. A. ...						
Chl	... T. NSMA. ... V. ... DF. ... C. L. ... A. ... A. S. NL. ... A. ... I. ... G. ... A. ... F. ... V. A. ...						
Por	... T. NSM. ... DF. ... C. A. I. ... S. ... C. ... A. ... L. ... A. ... L. ... A. ... L. ... V. A. ...						
Sch	... T. NSM. ... DF. ... C. T. T. ... C. ... A. ... L. ... L. ... A. ... A. ... L. F. ... V. A. ...						
Cya	... T. NSM. ... I. V. ... DF. ... C. T. T. ... L. ... T. ... A. I. L. ... L. ... A. ... L. F. ... V. A. ...						
Pha	... T. NSM. ... L. ... DF. ... C. ... T. ... S. ... C. ... A. ... L. ... L. ... A. ... L. ... V. A. ...						
Eug	... T. PNSM. ... L. L. S. ... WDF. ... L. ... I. ... AKA. Q. ... A. S. ... I. ... G. ... A. ... F. ... V. A. ...						
Big	... T. SN. V. ... L. ... F. ... C. L. ... S. A. ... AQS. RL. ... S. ... I. ... G. ... A. ... F. ... V. A. ...						
Gon	... T. NCM. ... L. ... YF. VLF. ... T. IV. ... L. VVA. C. ... A. N. ... L. ... V. YS. ... I. ... A. ... L. ... S. ...						
Cer	... T. NCM. ... L. ... S. SM. ... L. ... S. L. I. LLS. A. S. Y. A. ... S. ... Y. II. ... Y. G. ... A. ... L. ... S. ...						
Sym	... T. NSMA. ... L. ... HLG. A. LC. ... T. I. F. LI. ... A. S. ... A. L. ... L. ... Y. T. ... I. AI. ... A. ... L. ... S. ...						
Ale	... T. NSM. ... L. ... V. ... YF. VLF. ... T. I. F. L. V. A. S. ... A. N. ... L. ... Y. T. ... I. ... A. ... L. ... S. ...						

			DE (h104)	E (h105)	(h106)	(h107)
Syn	ATVENTLFEDGEDSN ²²⁰ TFRAFEPTQAEETYSMTANRFWSQ ²³⁰ IFGI ²⁴⁰ AFSNKRWLHFFMLFVPVTGLWMSSVG ²⁵⁰ IVGLALNLRAYDFVSGELRAAEDPEFETFTYKN ²⁶⁰ ILLNEGMR ²⁷⁰ AWMAPQDQPHENF ²⁸⁰ IFPEEVLPRGNAL ³⁵⁰					
The	... Q. ... GAS. ... N. ... V. ... AI. V. ... S. ... I. ... I. ... L. ... I. ... V. ...					
Spi	... DGA. ... N. ... V. ... AL. V. ... I. ... I. ... A. ... L. ...					
Chl	... DGA. ... N. ... V. ... AL. V. ... I. ... I. ... A. ... RLV. ...					
Por	... Q. ... DAAD. ... T. S. ... V. ... T. AF. ... I. S. ... A. ...					
Sch	... Q. ... A. D. ... T. S. ... V. ... L. ... A. ... A. ... I. ... A. ... V. ...					
Cya	... DG. ... P. N. ... V. ... AI. V. ... GV. ... I. ... L. ... I. ... V. ... S. ...					
Pha	... DAA. ... T. S. ... V. ... T. AI. ... I. ... A. ... V. ...					
Eug	... I. ... DSP. ... N. L. S. ... V. ... V. ... R. ... AL. ... I. ... A. ... Q. ...					
Big	... DGA. ... N. Q. ... V. ... AL. V. ... I. ... I. ... VA. ... KIV. ...					
Gon	... V. IYQ. VAYT. ... S. P. ... V. ... L. M. T. ... I. F. ... I. K. ... I. L. V. ... Q. ... S. ...					
Cer	... V. YQ. GAYT. ... S. P. ... V. ... Y. ... LA. M. T. ... I. LI. F. ... I. K. ... I. L. V. ... LQ. ... S. ...					
Sym	... V. YQ. ARAYT. ... S. P. ... L. ... V. ... LG. M. T. ... I. LI. F. ... I. K. ... I. L. V. ... YQ. ... S. ...					
Ale	... V. YQ. GAYS. ... T. S. P. ... V. V. ... LA. M. T. ... I. LI. F. ... I. K. ... I. L. V. ... LQ. ... S. ...					

Fig. 1. Continued

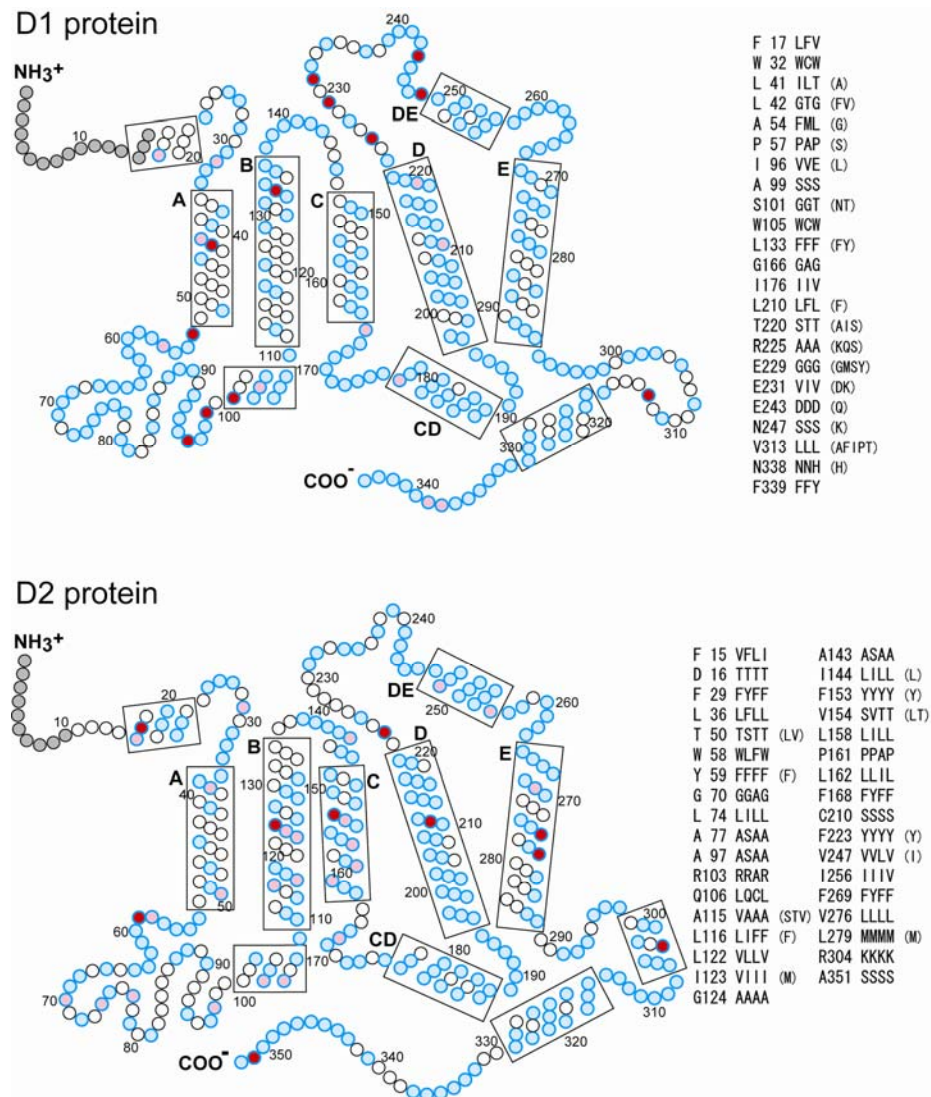


Fig. 2 Amino acid changes in D1 and D2 proteins of the dinoflagellates. The dinoflagellate plastids include amino acid positions uniquely mutated in all (red colored circles) or several dinoflagellate species (light red colored circles) when compared with *Synechocystis* sp. PCC6803, *Thermosynechococcus elongatus*, plant and the other eukaryotic algae (see Table 2 for list of species). Amino acid residues conserved among these species and the dinoflagellates are also indicated (blue colored circles). Cyanobacterial sequence with highly variable and often multicomponent of *psbA/D* families (registered in Cyanobase website <http://bacteria.kazusa.or.jp/cyanobase/>) basically has the same amino acid residue as *Synechocystis* and *Thermosynechococcus* at these conserved amino acid positions. Open circles show amino acid positions relatively variable among diverse organisms and gray colored circles show the extremely mutated N-terminus region difficult to align. The column on the right shows substituted patterns of the dinoflagellates (*Gonyaulax*, *Symbiodinium* and *Alexandrium* in D1 protein; *Gonyaulax*, *Ceratium*, *Symbiodinium* and *Alexandrium* in D2 protein) indicated after conserved amino acid residue and mutated position. Amino acid change observed in a few cyanobacterial sequences of a highly divergent *psbA* copy (e.g. *Synechocystis* sp. PCC6803-slrl181) or of species with unusual photosynthetic system (e.g. *Gloeobacter violaceus*, *Prochlorococcus marinus*, *Acaryochloris marina*) is also shown in the brackets. The primary structure of D1 and D2 proteins was superimposed on a five transmembrane (A, B, C, D and E) and two surface (CD and DE) helix model (Loll et al. 2005).

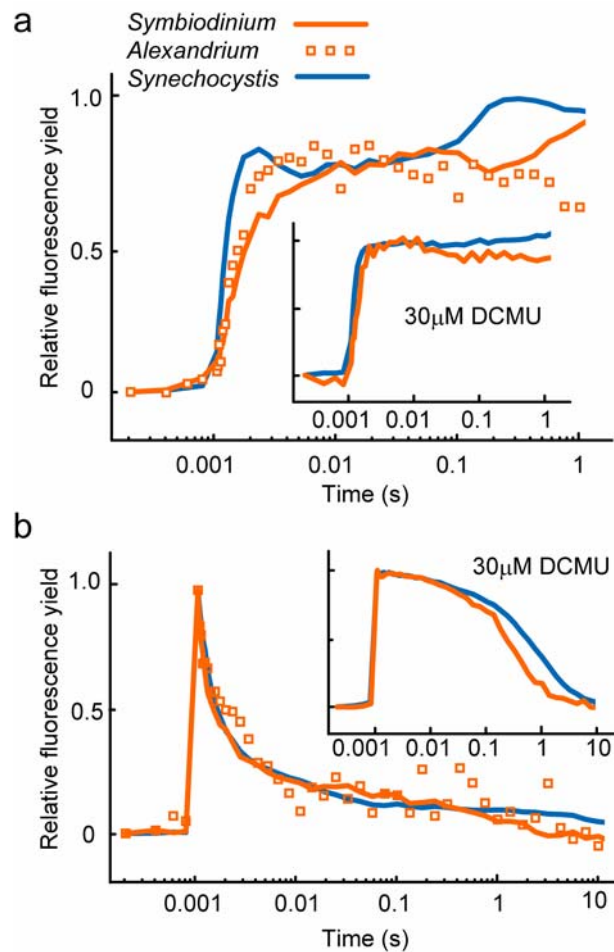


Fig. 4 Chlorophyll fluorescence of the dinoflagellates in comparison to *Synechocystis* sp. PCC 6803. **(a)** The standard fluorescence induction rise following continuous illumination (Kautsky curves). **(b)** The fluorescence decay kinetics following a single saturating flash. F_v/F_M value is 0.51 for *Symbiodinium* and 0.39 for *Synechocystis* in the absence of DCMU. The measurement with 30 μM DCMU are shown in the inset, respectively. The averaged value of four measurements was used for *Symbiodinium* sp. and *Synechocystis* sp. PCC 6803 data. In *Alexandrium*, a single series of data without DCMU was plotted ($n = 3$ or 4). The curves are normalized to the maximum yield. The Chl *a* concentration of each sample was 1-3 μg/mL.