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SHORT COMMUNICATION

An update on carotenoid biosynthesis in algae: phylogenetic evidence for the existence of two classes of phytoene synthase

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Abstract Carotenoids play crucial roles in structure and function of the photosynthetic apparatus of bacteria, algae, and higher plants. The entry-step reaction to carotenoid biosynthesis is catalyzed by the phytoene synthase (PSY), which is structurally and functionally related in all organisms. A comparative genomic analysis regarding the PSY revealed that the green algae *Ostreococcus* and *Micromonas* possess two orthologous copies of the PSY genes, indicating an ancient gene duplication event that produced two classes of PSY in algae. However, some other green algae (*Chlamydomonas reinhardtii*, *Chlorella vulgaris*, and *Volvox carteri*), red algae (*Cyanidioschyzon merolae*), diatoms (*Thalassiosira pseudonana* and *Phaeodactylum tricornutum*), and higher plants retained only one class of the PSY gene whereas the other gene copy was lost in these species. Further, similar to the situation in higher plants recent gene duplications of PSY have occurred for example in the green alga *Dunaliella salina/bardawil*. As members of the PSY gene families in some higher plants are differentially regulated during development or stress, the discovery of two classes of PSY gene families in some algae suggests that carotenoid biosynthesis in these algae is differentially regulated in response to development and environmental stress as well.

Keywords Algae · Carotenoid biosynthesis · Phytoene synthase

Abbreviations
PSY Phytoene synthase

Introduction

Carotenoids are isoprenoids which derived from the precursor molecule isopentenyl pyrophosphate and its isomer dimethylallyl diphosphate. All photosynthetic organisms including higher plants and algae synthesize carotenoids (Armstrong and Hearst 1996; Cunningham and Gantt 1998; DellaPenna and Pogson 2006; Fraser and Bramley 2004; Goodwin 1980; Hirschberg et al. 1997; Lichtenthaler 1999) which are structurally constituted in the thylakoid membrane and function as accessory molecules for light harvesting and for prevention from photo-damage and as antioxidants under stress conditions (Goodwin 1980; Hirschberg et al. 1997; McCarthy et al. 2004; DellaPenna and Pogson 2006; Lichtenthaler 2007).

The phytoene synthase (PSY) is considered as the enzyme performing the rate-limiting entry reaction into the carotenoid biosynthesis pathway in photosynthetic organisms (Chen et al. 2007; Lindgren et al. 2003; Salvini et al. 2005; Yan et al. 2005; Li et al. 2008b). Multiple paralogous PSY genes were discovered in higher plants such as corn (Li et al. 2008a, b) and tomato (Bartley and Scolnik 1993) in which chromoplasts of specialized tissue cells over-accumulate carotenoids. When paralogous genes exist in higher plants they are often differentially up-regulated depending

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on environmental conditions or developmental stages of various tissues (Bartley and Scolnik 1993; Buckner et al. 1996; Li et al. 2008b). In the past biosynthesis of carotenoids of bacteria and higher plants has been studied extensively, whereas research on carotenoid biosynthesis in algae is still in its infancy. For unicellular green algae, PSY was previously investigated for example in *Chlamydomonas reinhardtii* (Bohne and Linden 2002; McCarthy et al. 2004; Lohr et al. 2005) and *Haematococcus pluvialis/lacustris* (Steinbrenner and Linden 2001). The genome of *C. reinhardtii* contains only one functional *psy* gene (McCarthy et al. 2004; Lohr et al. 2005). For *Haematococcus* the number of *psy* copies per genome is unknown, but *psy* was shown to be up-regulated under stress conditions of high light and low nutrient availability (Steinbrenner and Linden 2001, 2003). Further, *psy* genes were also cloned from different species of the unicellular green alga *Dunaliella* (Yan et al. 2005), but so far only one gene was reported for each species.

Recently, the genome sequences for a number of different microalgae became available from the DOE Joint Genome Institute (http://www.jgi.doe.gov). An examination of these algae genomes with representatives from very different groups such as green algae, red algae, diatoms, and haptophytes was performed to identify their *psy* genes. The results presented here demonstrate that some algae contain only a single gene coding for *psy* whereas other algae contain either multiple paralogous or orthologous copies of the *psy*. From the discovery of small *psy* gene families in algae it can be expected that analogous to the diversity of *psy* genes and their differential expression in higher plants (Li et al. 2008a, b) algae also differentially regulate expression of their paralogous *psy* gene copies. Similarly, it may be hypothesized that orthologous *psy* gene copies identified in some algae could also be differentially regulated.

Materials and methods

Phytoene synthase cDNA and protein sequences of *Aureococcus anophagefferens*, *Chlamydomonas reinhardtii*, *Chlorella sp. NC64A*, *Volvox carteri*, *Micromonas pusilla*, *Micromonas sp. RCC299*, *Ostreococcus lucimarinus*, *Ostreococcus tauri*, *Ostreococcus* RCC809, *Emiliania huxleyi*, *Thalassiosira pseudonana*, and *Phaeodactylum tricornutum* were obtained from the website of the DOE Joint Genome Institute (Walnut Creek, CA, USA; http://www.jgi.doe.gov). The cDNA and protein sequence of the PSY for the red alga *Cyanidioschyzon merolae* were obtained from the *Cyanidioschyzon merolae* Genome Project (http://merolae.biol.s.u-tokyo.ac.jp). Genomes from these algae were examined for presence of *psy* genes. In a first step analysis of the number of *psy* genes present in the algae genomes, we followed JGI's or the *Cyanidioschyzon merolae* Genome Project's annotation. In a following step, the cDNA and the protein sequence of the PSY of *C. reinhardtii* were each used independently as query sequences to perform BLAST searches in each algal genome to identitfy *psy* homologues.

Phytoene synthase protein sequences for the algae *Dunaliella salina* (Accession no. AY601075), *Dunaliella bardawil* (Accession no. DBU91900), *Dunaliella spec.* 366 (Accession no. DQ463305), and *Haematococcus pluvialis* (Accession no. DQ057355) were obtained from the NCBI GenBank database. The protein sequences for PSY of the higher plants *Zea mays* (Accession no. AY773475, AY773476, DQ356430), *Arabidopsis thaliana* (Accession no. P37271), and *Solanum lycopersicum* (Accession no. P08196, ABU40771) were also obtained from the NCBI GenBank. PSY protein sequences for *Synechocystis sp.* PCC6803 and *Anabaena sp.* PCC7120 were obtained from the Kazusa Cyanobase (http://bacteria.kazusa.or.jp/cyanobase/).

Based on a PSY cDNA sequence of *Dunaliella bardawil* that was available from NCBI (NCBI no. U91900) various forward and reverse primers (Table 1) were designed to clone the corresponding full length genomic PSY gene by PCR. In addition, two other different partial genomic *psy* sequences were obtained by PCR. Genomic DNA was isolated from cells using the DNeasy Plant Mini Kit (Qiagen Cat. no. 69104) and PCR was performed with the following standard conditions: 1 cycle of 95°C, 5 min; 32 cycles of 95°C, 1 min; 59°C, 1–3 min depending on the length of the products; 72°C, 1 min; and holding the sample at 4°C. The PCR products were gel-purified by agarose gel electrophoresis and following gel extraction (Qiagen Cat no. 28704) and cloned into the vector pSA-C using Strataclone PCR cloning kit (Cat. no. 240205-5) before being sent out for sequence determination at MWG Biotech Inc. (High Point, NC, USA). Resulting sequences were submitted to NCBI (PSY1A Accession no. DQ057342; PSY1B Accession no. FJ262988; PSY2 Accession no. FJ262989).

All protein sequences from our PSY dataset were multiply aligned using ClustalW, version 1.83 (Thompson et al. 1994). A primary PSY phylogenetic tree was constructed in MrBayes, version 3.12 (Huelsenbeck and Ronquist 2001), under 100,000 runs, using the Jones amino acid substitution matrix with a fixed rate among sites. A second PSY phylogenetic tree was constructed using the Seqboot, Neighbor, and Consense programs in the Phylip package, version 3.66 (Felsenstein 1989). Bootstrap support values were derived from 100 randomized, replicate datasets.

Results

In contrast to higher plants, for genomes of unicellular green algae such as *C. reinhardtii* and *V. carteri* only one

Table 1 List of primer sequences used for PCR amplification to clone one full and two partial genomic *psy* in *Dunaliella bawdawil* UTEX LB2538

No	Primers	Forward/ reverse	Sequence 5′→3′	Tm
1	DBPSF	F	GAC CCT GTC TAT GCT GGA C	60
2	DBPSP3	R	AAG AGG AGG CTG ATC GC	54
3	PSF1a	F	TCA GCC TCC TCT TCC TCC	58
4	PSR1a	R	GTG AGT GCT GCA TCC AGC	58
5	PSF2a	F	CAG GGA CAT GAT TGA GGG C	60
6	PSR2a	R	CTC CTC TTC AGT CAT GCC A	58
7	PSF5	F	GGC ATG ACT GAA GAG GAG	56
8	DBPSR3	R	TTA CTT GTT CTG GTT CTG GG	58
9	PSYSTART2	F	ACC GAA TTC ATG ACC CTG TCT ATG CTG GAC G	94
10	PSYSTOP2	R	GGC CTC GAG TTA GTG GTG ATG GTG ATG ATG CTT GTT CTG GTT CTG GGG CAC C	62
11	PSYF1a	F	ATG GGG TGG ACT GCT TGG ACG T	60
12	PSYR1b	R	CAT AGT CAT TCT TCT CAA TTG AA	60
13	PSYF2b	F	TAT GGG GTG GAC TGT ATG GAC CC	62
14	PSYR2b	R	CAT AGT CAT TCT TTT CGA TGG CG	66

psy gene was known to exist. However, recently the genomes of a variety of microalgae were sequenced by the DOE Joint Genome Institute (http://genome.jgi-psf.org/) and by the *Cyanidioschyzon merolae* Genome Project. These available microalgae genomes were analyzed for presence of *psy* genes and results were summarized in Table 2. The translated PSY proteins were then used to perform a phylogenetic analysis.

Figure 1 displays a Bayesian phylogeny of our PSY dataset, rooted using the outgroup PSY proteins of the cyanobacteria *Synechocystis* and *Anabaena*. The tree topology matches our neighbor-joining tree (not shown), and the high clade support values coincide with high neighbor joining bootstrap values, suggesting the PSY phylogeny is robust to different tree reconstruction methods. The tree exhibits one major ancestral duplication event at the root node, which gave rise to two distinct PSY classes. To illustrate similarities and differences between the two PSY classes, Fig. 2 shows an exemplary alignment with selected class I and class II PSY protein sequences. The alignment indicates that both PSY classes share the essential characteristics of PSY including predicted substrate-Mg^{2+}-binding sites (Aspartate-rich regions) and catalytic residues. Major differences between the two PSY classes appear to exist only in regions not essential to the enzymatic function.

Figure 1 also shows that gene loss is present for both classes of PSY, and higher plants appear to have retained only class I PSY. Similarly, within the green algae members of the *Chlorophyceae* as well as the group of the *Trebouxiophyceae* seemed to have also only retained class I PSY. In contrast, loss of class I PSY occurred in the *Rhodophyta* with the example of *Cyanidioschyzon*, in the *Haptophyta* with the example *E. huxlei,* and in the *Heterokontophyta*, which appeared to have retained only the class II PSY as shown in Fig. 1 for *P. tricornutum* and *T. pseudonana* (*Bacillariophyceae*) as well as for *Aureococcus* (*Pelagophyceae*). It seems that only green algae within members of the *Prasinophyceae* represented by the species *O. tauri*, *O. lucimarinus*, *Ostreococcus* strain RCC809, *Micromonas* strain RCC299, and *M. pusilla* retained both ancestral PSY copies.

A special case appears to be represented by the two *psy* genes both coding for class II PSY identified in the alga *A. anophagefferens* which belongs to the *Pelagophyceae* within the phylum *Heterokontophyta*.

In addition to an ancient gene duplication leading to two PSY classes, more recent *psy* gene duplications appeared to have occurred independently in higher plants and some microalgae. For example, multiple paralogous *psy* genes were not only found in some higher plants such as corn and tomato, but were also present in the haploid genome of the chlorophyte *D. bardawil*. For *D. bardawil* one full genomic *psy* sequence and two more partial genomic *psy* sequences were cloned by PCR indicating that also this alga has multiple paralogous *psy* genes (class I PSY duplications) in its haploid genome. To demonstrate the existence of multiple class I *psy* genes from *D. bardawil*, Fig. 3 shows the selectively PCR-amplified parts of two paralogous *psy* genes by use of specific primers. In contrast to *D. bardawil* which is a haplont, the two copies of class I PSY found in the haptophyte *E. huxlyei* are diploid alleles.

Discussion

Previously, it was known that some higher plants contained small *psy* gene families consisting of multiple

Table 2 Phytoene synthase genes identified in algae genomes

Species	Phytoene synthase gene locations
Chlamydomonas reinhardtii	Chlre3/scaffold_6: 413352-418669
Volvox carteri	Volcal/scaffold_65: 260712-263699
Chlorella NC64A	ChlnNC64A-1/ scaff_26: 434930-437268
Micromonas RCC299	MicpuN2/Chr10:704575-705633
	MicPuN2/Chr13:563686-564741
Micromonas pusilla CCMP1545	MicpuC2/scaffold 3: 823140-825717
	MicpuC2/scaffold 15:310666-312229
Ostreococcus tauri	Ostta4/chr_13.0001: 529538-530422
	Ostta4/chr_05.0001: 571812-572741
Ostreococcus lucimarinus	Ost9901_3/chr_5: 565049-565870
	Ost9901_3/chr_10: 12385-13290
Ostreococcus RCC809	OstRCC809_1/scaffold_5:571411-572479
	OstRCC809_1/scaffold_2:380345-381226
Thalassiosira pseudonana	Thaps3/chr_5: 1330264-1331582
Phaeodactylum triconutum	Phatr2/chr_5: 447594 – 449378
Aureococcus anophagefferens	Auran1/scaffold19: 181540-182658
	Auran1/scaffold_8:1304824-1305510
Emiliania huxleyi	Emihu1/scaffold_89:100348-101202
	Emihu1/scaffold_369:76183-77730 (diploid allels)
Cyanidioschyzon merolae	C17f0001 274022 < 275140

Locations of genes are indicated by positions on either chromosomes or scaffolds

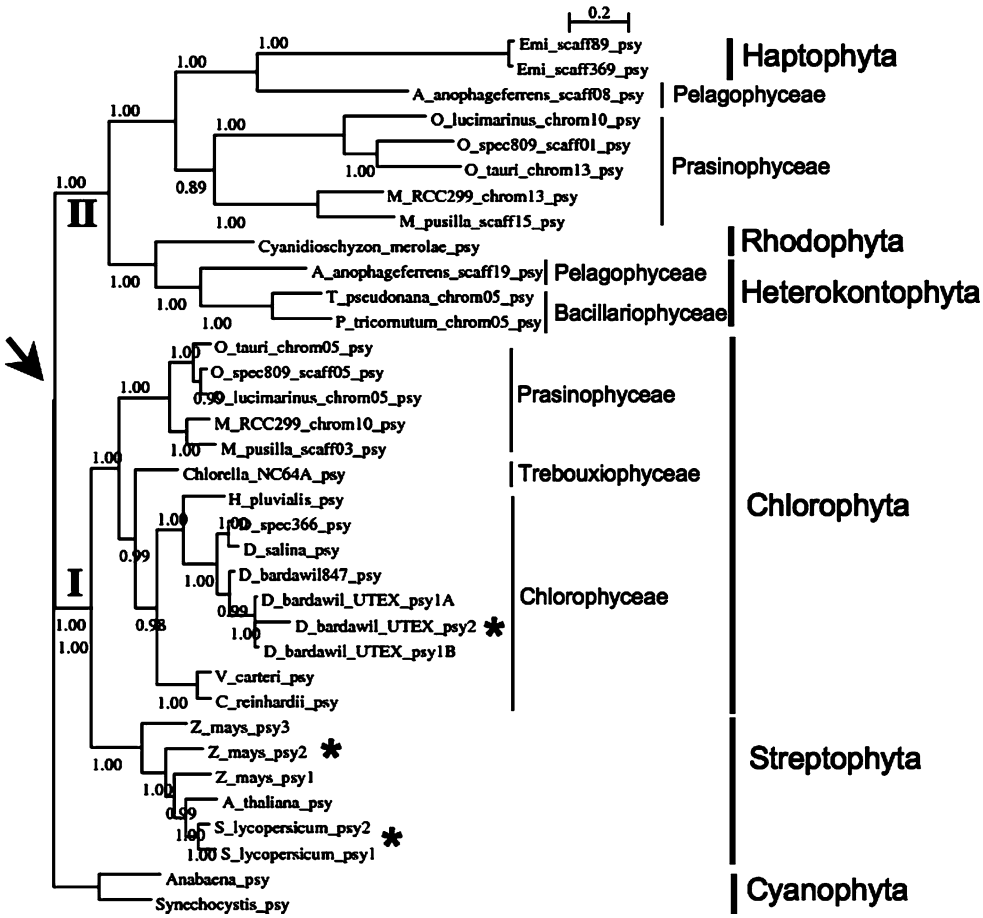

Fig. 1 Shown is a phylogenetic tree for the phytoene synthase from various organisms including cyanobacteria, algae, and higher plants. The *arrow* indicates an ancient gene duplication event creating a class I PSY (I) and a class II PSY (II). *Stars* indicate where later gene duplications led to creation of paralogous genes found within one species. Major groups of organisms are labeled to allow comparison between the phylogeny of PSY and algae evolution. Note that the overall phylogeny of PSY follows the currently accepted system of classification of algae

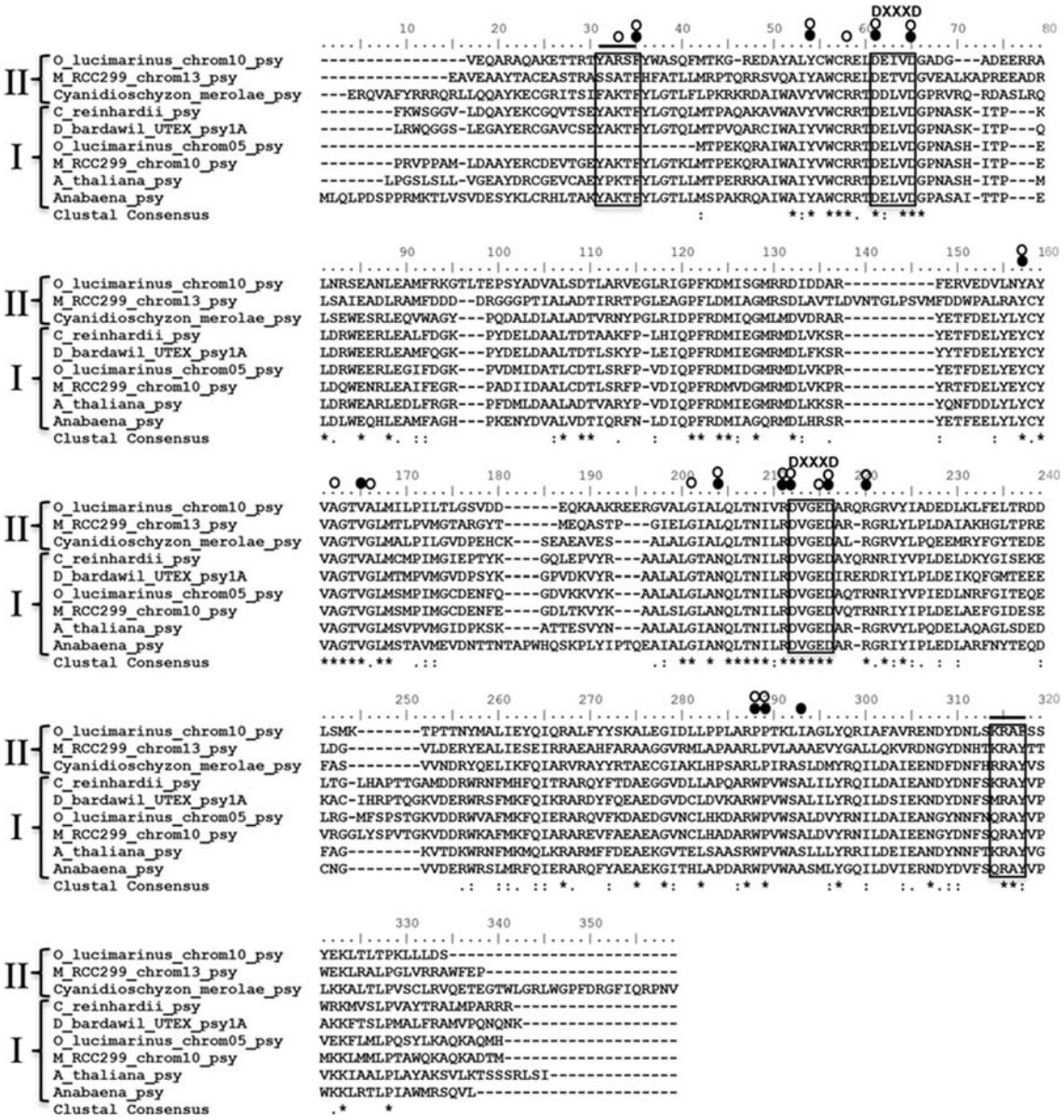

Fig. 2 Alignment of the selective PSY protein sequences from different algae produced with the BioEdit program by using Clustal W. The putative transit peptides were removed for this analysis. The alignment indicates selective sequences of PSY class I and class II, aspartate rich and regions/substrate-Mg^{2+}-binding sites (DXXXD), residues of the substrate binding pocket (*open circle*), catalytic residues (*dark filled circle*), and active site lid residues (*straight line*)

paralogous genes (Bartley et al. 1992; Bartley and Scolnik 1993; Gallagher et al. 2004; Li et al. 2008a). In higher plants containing small paralogous *psy* gene families, the different *psy* genes are differentially regulated during development (Bartley et al. 1992; Bartley and Scolnik 1993; Buckner et al. 1996) and/or in response to environmental stress (Li et al. 2008a, b). In contrast to the situation in higher plants, the number of *psy* present in microalgae genomes was largely unknown. However, recently genomes from different classes of microalgae became available. The comparative analysis of various algal genomes for PSY in combination with a phylogenetic analysis shown in Fig. 1 suggested an ancient gene duplication creating two classes of PSY. Both classes of PSY appeared to have only been retained in members of the *Prasinophyceae,* which belong to the *Chlorophyta*, whereas all other investigated species belonging to the *Chlorophyta* as well as the related higher plants (*Streptophyta*) seemed to have lost the class II PSY. In contrast, members of the algal classes *Rhodophyta*, *Heterokontophyta*, and *Haptophyta* investigated here lost the class I PSY. This unbalanced distribution of PSY genes within different algal classes may have been driven by neutral processes or by adaptive pressure.

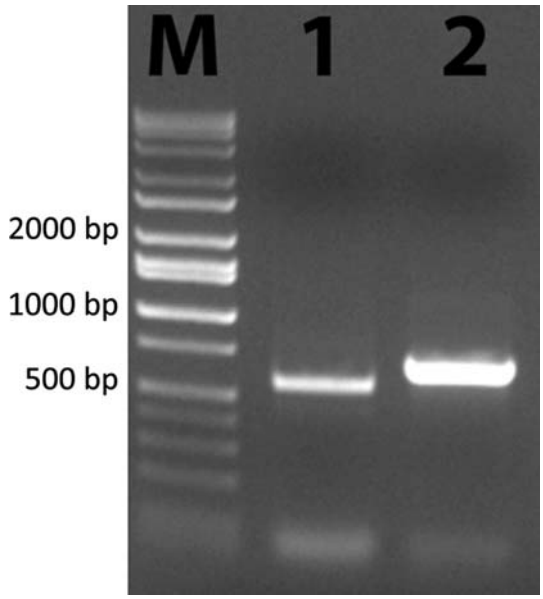

Fig. 3 Shown is the photo of a polyacrylamide gel. *Lane M* molecular weight marker, *lane 1* PCR product obtained by using the primer pair PSYF1A and PSYR1B to amplify the partial *psy1A* from genomic DNA of *D. bardawil*, *lane 2* PCR product obtained by using the primer pair PSYF2B and PSYR2B to amplify a partial *psy2* from genomic DNA of *D. bardawil*

The persistence of gene duplicates in only a subset of algal groups may be due to the acquisition of a novel function in one copy (neo-functionalization), or the degeneration of both copies facilitating their joint requirement (sub-functionalization) (Force and Lynch 1999). Evidence for such adaptive neofunctionalization can be found in a mutation rate variation between the two PSY classes. It appears that there is a relaxed selective constraint in class II, which may be indicative of rapid evolution of new function. This variation exists in those species containing both ancient PSY copies, and therefore, is not attributed to species-specific mutation rates. Taken together, the persistence of multiple PSY copies, and the relaxed selective constraint in class II PSY suggested that a functional novelty may have played a role in the maintenance of the two PSY gene classes. However, analysis of the conserved residues of class I and class II PSY showed that they shared the essential substrate binding and catalytic site residues. Such conservation of the critical catalytic residues suggested that in the case of PSY not the catalytic function was affected in evolution of the class I and II enzymes, but rather it is proposed that there may be regions of the enzyme impacted by evolution that are critical for regulation of the function. A structural analysis is necessary to further delineate the role of the more variable regions of the class II PSY proteins, but such an analysis is beyond the scope of this manuscript.

Phylogenetic analysis of PSY showed also that more recent gene duplication events creating small paralogous gene families did not only occur in some higher plants, but independently in microalgae such as the chlorophyte *D. bardawil*. Possibly, existence of multiple paralogous *psy* copies resulted in their differential regulation in response to developmental or environmental cues (Li et al. 2008b). This hypothesis remains to be tested for the alga *D. bardawil,* which over-accumulates carotenoids in response to abiotic stress.

It is generally accepted that chloroplasts of the algae in the phylum *Heterokontophyta* were acquired by secondary endosymbiosis involving a red alga (Bhattacharya and Medlin 1998; Braun and Phillips 2008; Boore 2008). Therefore, it may be hypothesized that the two different copies of *psy* coding for class II PSY found in *A. anophagefferens* may have originated from a host cell and from a secondary endosymbiosis event rather than from a more ancient gene duplication. Indirect evidence for this hypothesis comes from the fact that the genome of the red alga *C. merolae* contained only one gene coding for a class II PSY (Fig. 1). Analysis of further genomes of algae in the phylum is necessary to delineate between these two possibilities of *psy* origin in *A. anophagefferens*.

In summary, phylogenetic analysis of PSY from a variety of photosynthetic organisms revealed an ancient gene duplication resulting in two PSY classes. Further, it was shown that more recent gene duplications occurred which led to existence of small paralogous *psy* gene families in some algae and some higher pants. This finding raises new questions regarding the function of multiple PSY copies within some of the unicellular algae. It is postulated that similar to the situation in higher plants, in algae containing multiple copies of *psy* these genes are differentially regulated in response to developmental and/or environmental cues to fine-tune metabolic flux into carotenoid biosynthesis.

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