Phylogenetic analysis and cloning of *psaB* gene of *Dunaliella salina*

LU Zhao-Ming, LIU Hong-Tao, ZANG Wei-Dong, XUE Le-Xun*

(*Laboratory for Cell Biology, Zhengzhou University*, Zhengzhou 450052, China)

Abstract: One pair of degenerate primers was designed according to conserved motifs of the *psaB* (A2 subunit of photosystem I) of *Chlamydomonas reinhardtii*, *Chlamydomonas moewusii*, *Chlorella vulgaris*, and a total RNA of *Dunaliella salina* was extracted with TRIzol reagent. A cDNA fragment, about 1.8 kb in length, from green algal *D. salina* was obtained through RT-PCR method. The resulting PCR product was cloned into T-vector and screened to determine its sequence. Homologous analysis of the deduced amino acid sequence was performed by BLAST and subsequently compared to GenBank data. The obtained cDNA sequence was 1815 bp long, which encoded 605 amino acids (GenBank accession number: AY820754). The sequence shared high homologue with the following *psaB*: 92% for *Chlamydomonas reinhardtii*, 91% for *Chlamydomonas moewusii*, 86% for *Chlorella vulgaris*, 85% for *Mesostigma viride*, 85% for *Physcomitrella patens* subsp. Patens and 84% for *Nephroselmis olivacea*. It can be concluded that the cloned sequence is *psaB* cDNA fragment from *D. salina*. In addition, codon bias analysis shows that the content of A and T (35.7% and 39.17%, respectively) is used significantly more frequently in the third position composition than that of G and C (7.27% and 17.85%, respectively), that is to say, the codons of *psaB* of *D. salina* are mainly composed of NNA and NNT. At the same time, through phylogenetic analysis of the Chlorophyta with *psaB* gene, it is shown that *D. salina* is the nearest to the most species of Haematococcaceae in the phylogenetic relationship, which is contrary to previous opinion that *D. salina* is the nearest to the most species of Chlamydomonadaceae in the phylogenetic relationship. These studies will help clarify the genetic background of *D. salina*.

Key words: *Dunaliella salina*; A2 subunit; *psaB*; cDNA; degenerate primer

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Genetic engineering of microalgae has been greatly developed. At present, various kinds of microalgae, including prokaryotic microalgae cyanobacterium, and eukaryotic microalgae *Chlamydomonas reinhardtii*, chlorella, etc, have been used to pruduce a large number of bioactive substances. *Dunaliella salina*, an unicellular green alga, which was one of the most halotolerant eukaryotes, originally described by Dunal in 1938 (Avron *et al.*, 1992), has a thin cellular membrane without a rigid cell wall, and a single, large cup-shaped chloroplast with its photosynthetic thylakoid membranes, pyreniod, and starch, and abundanceβ-carotene globules. Studies on the genetics of *Dunaliella* have been conducted for decades at a few laboratories (Geng *et al.*, 2003; Li *et al.*, 2003; Chai *et al.*, 2004; Wang *et al.*, 2005).

Photosystem I(PSI) complex is an iron-sulfur type of reaction center (RC) which consists of 11～13 subunits and catalyzes the photoinduced oxidation of plastocyanin (Pc)/cytochrome (cyt) c6 and reduction of ferredoxin/£ avodoxin (Golbeck *et al.*, 1991; Brettel, 1997). The PSI reaction center contains two major core proteins *psaA* and *psaB* (Fish *et al.*, 1985), The following cofactors are bound to the *psaA*/*psaB* heterodimeric polypeptides (82～83 kDa): P700 (a pair of chlorophyll mole-

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作者简介: 鲁照明(1966-), 男, 河南原阳人, 博士, 从事分子生物学研究, (E-mail) lzm310@zzu.edu.cn。

* 通讯作者(Author for correspondence, E-mail: xuelx@371.net)

cules), A0 (monomeric chlorophyll), A1 (phylloquinone) and FX (4Fe-4S center). The terminal acceptors FA and FB (4Fe-4S centers) are located in the *psaC* subunit (8.9 kDa). In addition, *psaA*, *psaB*, *psb*D, *rpo*C2, and *rbc*L are good sources of phylogenetic information at a deep level (Nishiyama *et al.*, 1999). The plastid genes *psaA*, *psaB* and *rps*14, encoding the photosystem I reaction center chlorophyll proteins and ribosomal protein CS14, are organized into an operon on the circular plastid genome (Wu *et al.*, 1999).

In this study, one pair of degenerate primer was designed according to conserved motifs of the *psaB* (A2 subunit of photosystem I) of *Chlamydomonas reinhardtii*, *Chlamydomonas moewusii*, *Chlorella vulgaris* and *Mesostigma viride*, and a total RNA of *Dunaliella salina* (*D. salina*) was extracted with TRIzol reagent. A cDNA fragment from green alga *D. salina* was obtained through RT-PCR method. Cloning of the *psaB* cDNA can further easily obtain the upstream strong promoter which provide theoretic basis for the construction of transgene *D. salina* bioreactor and promote the studies of the phylogenetic relationships among *D. salina*, other algae and high plants. These studies are expected to provide a new insight into molecular mechanisms of electron transfer in PSI.

1 Materials and Methods

1.1 Algal and bacterial strains

Dunaliella salina (UTEX 1644 Teod) was purchased from the University of Texas, USA, E. coli JM109 was kept in our laboratory.

1.2 Culture of *Dunaliella salina*

Dunaliella salina was grown in batch cultures in liquid PKS medium at 27 ℃ under continuous irradiance of 4 500 Lux, cycle ratio of light and dark is 12 h:12 h.

1.3 Primers

According to conserved motifs of the *psaB* (A2 subunit of photosystem I) of *Chlamydomonas reinhardtii*, *Chlamydomonas moewusii*, and *Chlorella vulgaris*, two pieces of highly conservative regions (AWQGNFE and RGYWQE) were found. A pair of degenerate primers was designed as follows: P1: 5-GGNTGGCARG-GNAAYTTYGAR-3; P2: 5-YTCYTGCCARTANCC-NCX-3. Where N was random base pairs, Y R and X stand for C/T, G/A and G/T, respectively.

1.4 Total RNA extraction

After cultured for 4 days, cells of *D. salina* about 1×10^6/mL were harvested by centrifugation under 4 000 g for RNA extraction, the total RNA was isolated with TRIzol (Invitrogen) and measured by electrophoresis and UV analysis.

1.5 RT-PCR amplication of *psaB* cDNA

Two microgram of total RNA was reverse-transcribed into cDNA with Reverse Transcriptase (AMV First strand cDNA synthesis Kit, Shanghai Sangon Co. Ltd) following the protocol recommended by manufacturer. The product was then subjected to PCR-amplification with the degenerate primers above-mentioned. PCR amplification was performed using Biometra Thermal Cycler. PCR reactions contained 10×PCR Buffer 5 μL, P1 1 μL (50 μmol/μL), P2 1μL (50 μmol/μL), Taq polymerase 0.5 μL (5 U/μL), dNTP 4μL (10 mol/μL), template DNA 1 μL and H_2O 37.5 μL, with a final volume of 50 uL. PCR program was described as follows: 95 ℃ predenatrue 1min, then 94 ℃ 30 S, 50 ℃ 30S, 72 ℃ 90 S, 30 cycles, 72 ℃ 10min. The RT-PCR products were examined on electrophoresis in the 1% agarose gels with ethidium bromide stain.

1.6 Purification and cloning of PCR product

The PCR products were separated by 1% agarose gel ecectrophoresis and then the band of interest was purified according to manufactruer's instruction of the DNA gel extraction Kit (Hangzhou Vitagene Biochemical Technique Co. Ltd, China), and inserted into the pMD18T-vector. Competent cells of E. coli JM109 were transformed with the ligation product, then grown on LB-agar plates containing 100 μg/mL ampicillin, 80 μg/mL X-gal and 80 μg/mL IPTG. White colonies were cultured in a 3ml LB liquid medium containing 100 μg/mL ampicillin. Plasmid DNA mini-preparation, purification and enzyme digestion were carried out according to Molecular Cloning: A Laboratory Manual (Sambrook *et al.*, 1989).

1.7 Sequencing and homolog analysis of the *psaB* gene

The positive recombinant plasmids DNAs were se-

quenced by the Shanghai Sangon Co. Ltd. The amino acid sequences were deduced from the cDNA data with DNAMAN software. Sequence analysis was performed using blast of NCBI(http://www.ncbi.nlm.nih.gov/BLAST/).

Table 1 Results of ultraviolet absorbency of total RNA

OD_{230}	OD_{260}	OD_{280}	OD_{260}/OD_{230}	OD_{260}/OD_{280}
0.601	1.287	0.657	2.141	1.959

1.8 Phylogenetic analysis of the *pasB* gene

In all analyses taxa belonging to families of the *psaB* gene of Chlorophyta were given in Table 4. The nucleotide sequences alignments from *psaB* gene were constructed using CLUSTAL software. All phylogenetic analyses were carried out using TreeView software.

2 Results

2.1 Identification of RNA quality

Formaldehyde denatured electrophoresis on agarose gel showed the total RNA quality met the need of RT-PCR (Fig. 1). Two clear bands of 28S rRNA and 18S rRNA isolated with TRIzol reagent were shown in Fig. 1. The brightness of 28S rRNA was nearly 2 times that of 18S rRNA, showing that total RNA isolated is consistent with ultraviolet analysis of the yield and purity of total RNA.

Fig. 1 Electrophoresis of the *D. salina* total RNA

2.2 RT-PCR and cloning of the *psaB*

The agarose gel electrophoresis results of the RT-PCR from cpDNAs of *D. salina* were shown in Fig. 2. A fragment of interest was around 1800bp long, which was purified and subcloned into pMD18-T vector. The recombinant plasmids (pMD18-T-*psaB*) were identified with EcoRI and SalI, which were included in the multiple cloning sites (MCS) of pMD18-T vector, and a correct fragment was obtained (Fig. 3).

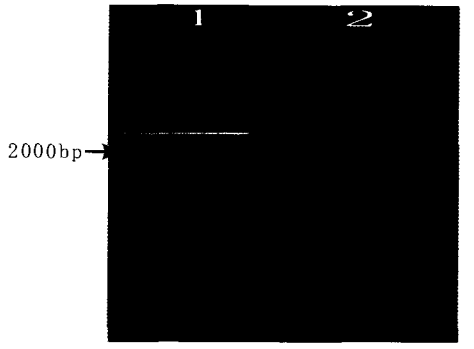

Fig. 2 Agarose gel electrophoresis analysis of the PCR products

1: Molecular size marker: Sangon GeneRuler™ DNA Ladder mix; 2: 1.8 kb of PCR product.

2.3 The deduced amino acids sequences and its homolog of the *psaB*

The multiple alignments of the deduced 605 amino acid polypeptides of *D. salina psaB* cDNA showed that the cloned *psaB* cDNA shared more high identities with other species in amino acid(Fig. 4): 92% for *Chlamydomonas reinhardtii*, 91% for *Chlamydomonas moewusii*, 86% for *Chlorella vulgaris*, 85% for *Mesostigma viride*, 85% for *Physcomitrella patens* subsp. Patens and 84% for *Nephroselmis olivacea*.

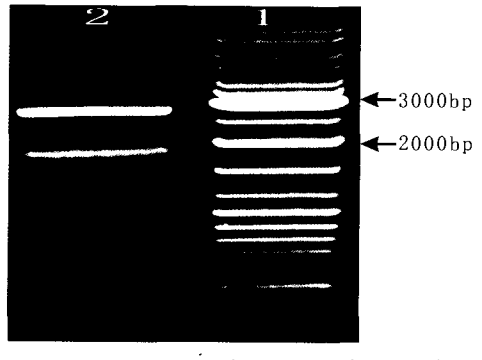

Fig. 3 Identification of the recombinant plasmid containing the *psaB* fragment

1: Molecular size marker: Sangon GeneRuler™ DNA Ladder mix; 2: pMD18-T-*psaB*/EcoR I + Sal I

2.4 Analysis of codon bias

For each amino acid, the table lists the number of times a given codon occurs in the *D. salina psaB* gene.

The codon usage in the *D. salina psaB* gene is apparently biased (GC% 39.4, AT% 60.6) (Table 2). It can be seen that A and T (35.7% and 39.17%, respectively) are used significantly more frequently in the third position composition than G and C (7.27% and 17.85%, respectively), that is to say, the codons of *psaB* of *D. salina*

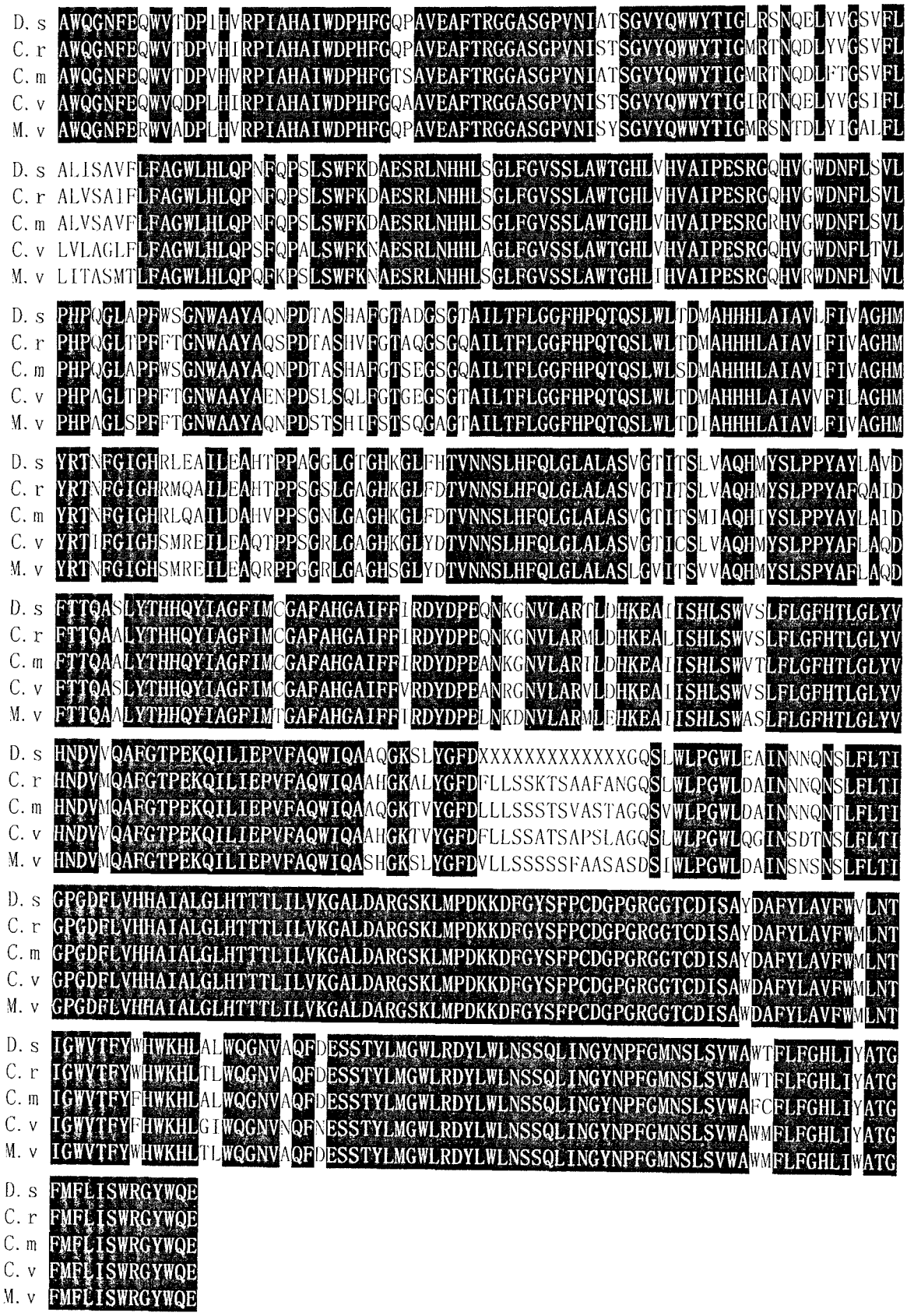

Fig. 4　Alignment of the deduced amino acid sequences of the *psaB*

D. s: *Dunaliella salina*; **C. r**: *Chlamydomonas reinhardtii*; **C. m**: *Chlamydomonas moewusii*; **C. v**: *Chlorella vulgaris*; **M. v**: *Mesostigma viride*.

Table 2 Codon usage in the *Dunaliella salina psaB* gene

TTT-Phe 12	TCT-Ser 13	TAT-Tyr 9	TGT-Cys 3
TTC-Phe 29	TCC-Ser 0	TAC-Tyr 14	TGC-Cys 0
TTA-Leu 62	TCA-Ser 24	TAA 0	TGA 0
TTG-Leu 0	TCG-Ser 0	TAG 0	TGG-Trp 27
CTT-Leu 8	CCT-Pro 7	CAT-His 8	CGT-Arg 12
CTC-Leu 0	CCC-Pro 0	CAC-His 27	CGC-Arg 0
CTA-Leu 1	CCA-Pro 17	CAA-Gln 26	CGA-Arg 1
CTG-Leu 0	CCG-Pro 2	CAG-Gln 2	CGG-Arg 0
ATT-Ile 27	ACT-Thr 20	AAT-Asn 3	AGT-Ser 7
ATC-Ile 4	ACC-Thr 0	AAC-Asn 21	AGC-Ser 0
ATA-Ile 0	ACA-Thr 13	AAA-Lys 11	AGA-Arg 0
ATG-Met 8	ACG-Thr 0	AAG-Lys 0	AGG-Arg 0
GTT-Val 12	GCT-Ala 33	GAT-Asp 14	GGT-Gly 49
GTC-Val 0	GCC-Ala 2	GAC-Asp 8	GGC-Gly 3
GTA-Val 18	GCA-Ala 23	GAA-Glu 12	GGA-Gly 8
GTG-Val 2	GCG-Ala 1	GAG-Glu 2	GGG-Gly 0

are mainly composed of NNA and NNT.

2.5 Phylogenetic analysis of the pasB gene

All the species belonging to Chlorophyta were given in Table 3. In order to clarify the phylogenetic relationships between *D. salina* and other species of the Chlorophyta (Fig. 5), alignments of the amino acid encoded by *psaB* gene were constructed using CLUSTAL software; phylogenetic trees were constructed using TreeView software. As shown in Fig. 5, *D. salina* is the nearest to the most species of Haematococcaceae in the phylogenetic relationship.

3 Discussions

Unicellular green alga, *Dunaliella salina*, is one of the most halotolerant eukaryotes. To date, there is a little information about genetic background of *D. salina*. Degenerate primer is a compound constituting of a great deal of oligonucleotide, of these compounds, one or more differences can be found. PCR with degenerate primer help detect new genes (Fietto *et al.*, 2002). In the present study, degenerate primers were designed according to conserved amino acid motifs of known species, and congeneric genes can be screened by PCR, which is a simple and feasible method (Jiang *et al.*, 2003).

According to conserved motifs of the *psaB* (A2 subunit of photosystem I) of *Chlamydomonas reinhardtii*, *Chlamydomonas moewusii*, *Chlorella vulgaris* and *Mesostigma viride*, two pieces of highly conservative regions (AWQGNFE and RGYWQE) were found. A pair of degenerate primers was designed. A cDNA frag-

Table 3 All the species belonging to Chlorophyta

Organism	Accession numbers	Length
Dunaliella salina	AAV67778	605 aa
D. parva	BAC20461	464 aa
Chlamydomonas reinhardtii	P09144	735 aa
Ch. moewusii	S41481	735 aa
Gonium multicoccum	BAC06438	498 aa
Nephroselmis olivacea	Q9TKW1	734 aa
Pandorina morum	BAB18378	498 aa
Hafniomonas montana	BAC87680	464 aa
Chlamydomonas chlorococcoides	BAC87678	464 aa
Ch. noctigama	BAC87677	464 aa
Volvox tertius	BAC77264	498 aa
Characiochloris sasae	BAC20462	464 aa
Phacotus lenticularis	BAC20460	457 aa
Chlorogonium fusiforme	BAC20456	464 aa
Ch. euchlorum	BAC20455	464 aa
Ch. elongatum	BAC20454	464 aa
Ch. kasakii	BAC20453	464 aa
Ch. neglectum	BAC20452	464 aa
Haematococcus lacustris	BAC20451	464 aa
Pseudocarteria mucosa	BAC20450	464 aa
Carteria radiosa	BAC20446	464 aa
C. cerasiformis	BAC20445	466 aa
C. crucifera	BAC20444	466 aa
Chlamydomonas tetragama	BAC20443	464 aa
Ch. kuwadae	BAC20442	464 aa
Chloromonas serbinowi	BAC20440	464 aa
Ch. palmelloides	BAC20435	464 aa
Ch. insignis	BAC20434	464 aa
Chlamydomonas mutabilis	BAC20430	464 aa
Ch. macrostellata	BAC20429	464 aa
Pediastrum duplex	BAC20426	464 aa
Scenedesmus quadricauda	BAC20425	464 aa
Vitreochlamys aulata	BAC06403	498 aa
V. pinguis	BAC06401	498 aa
Gonium viridistellatum	BAC06400	498 aa
Volvox africanus	BAC06398	498 aa
V. obversus	BAC06397	498 aa
V. gigas	BAC06396	498 aa
V. aureus	BAC06392	498 aa
Paulschulzia pseudovolvox	BAB18399	468 aa
Lobomonas monstruosa	BAB18398	498 aa
Vitreochlamys ordinata	BAB18397	498 aa
Chlamydomonas debaryana	BAB18395	498 aa
Tetrabaena socialis	BAB18392	498 aa
Gonium quadratum	BAB18390	498 aa
G. octonarium	BAB18388	498 aa
Volvulina boldii	BAB18377	498 aa
V. steinii	BAB18376	498 aa
V. pringsheimii	BAB18373	498 aa
V. compacta	BAB18372	498 aa

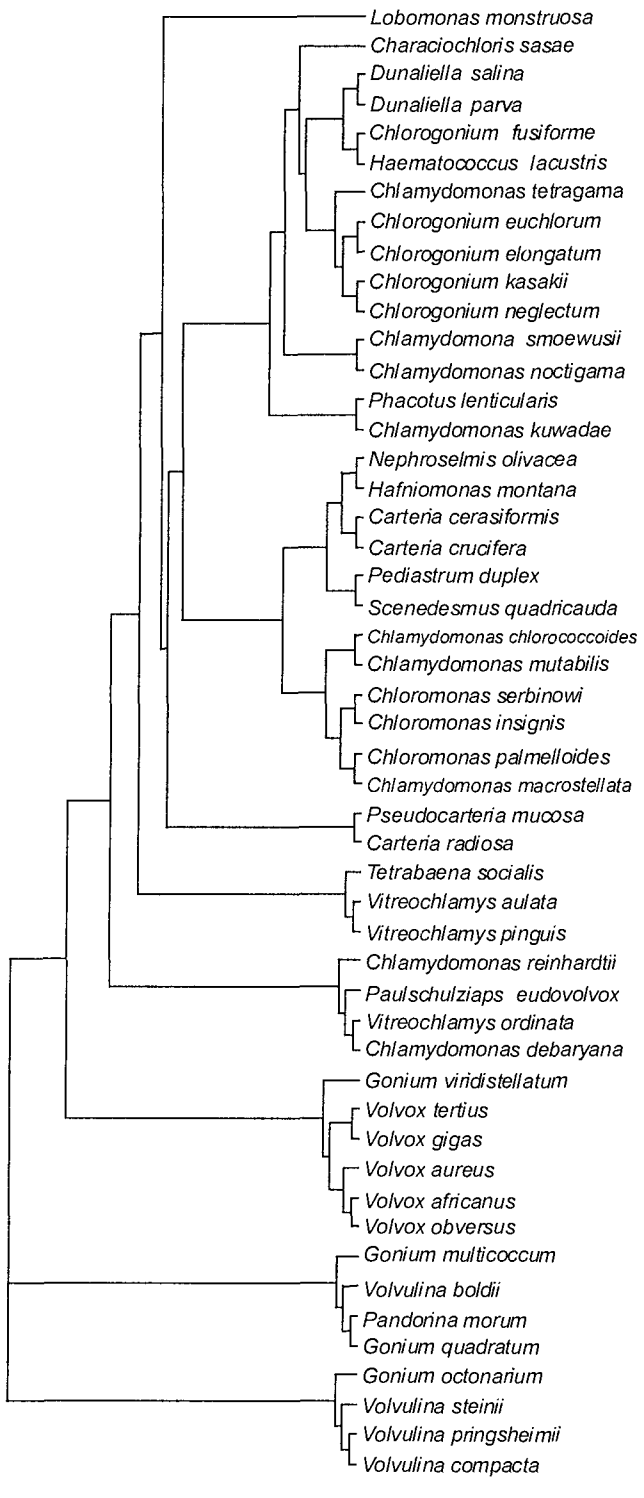

Fig. 5　Phylogenetic chart of *psaB* in the Chlorophyta

ment from green algal *D. salina* was obtained through RT-PCR method. Homologous analysis of the deduced amino acid sequences was performed by BLAST and subsequently compared to GenBank data. The obtained cDNA sequence was 1815 bp in length, which encoded 605 amino acids (GenBank accession number: AY820754). The sequences shared high homologue with the following *psaB*: 92% for *Chlamydomonas reinhardtii*, 91% for *Chlamydomonas moewusii*, 86% for *Chlorella vulgaris*, 85% for *Mesostigma viride*, 85% for *Physcomitrella patens* subsp. Patens and 84% for *Nephroselmis olivacea*. The findings indicate that the cloned sequence is a *psaB* cDNA fragment from *D. salina*.

In higher plants, the studies of the *psaB* gene mainly focus on special operon of *psaA-psaB-rps*14 (Wu *et al.*, 1999). The plastid genes *psaA*, *psaB* and *rps*14, encoding the photosystem I reaction center chlorophyll proteins and ribosomal protein CS14, respectively, are organized into an operon on the circular plastid genome. In the upstream, the activity of promoter is very strong. So the author can obtain the full *psaB*, *psaA* and upstream promoter sequences by 5'-RACE and genome walking (data not shown). These studies are expected to provide a new strategy into bioreactor of transgene in *D. salina*.

The chloroplast genome is an interesting system for studies of codon bias. Several complete chloroplast genome sequences are available from plants as well as from red, brown, and green algae. The chloroplast genome codes for a limited set of proteins involved in protein synthesis and photosynthesis (Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986; Hiratsuka *et al.*, 1989). Chloroplast genes of *D. salina* have a codon usage that reflects the genome compositional bias of a high A+T content with the translated *psaB* gene encoding the photosystem I A2 protein (Tab. 2). The codon usage of algal *psaB* corresponds more closely to the limited tRNA population of the chloroplast and is very similar to the codon use observed in the chloroplast genes of the green alga *Chlamydomonas reinhardtii* (Morton BR, 1996).

Opinions on the basal relationship of *Chlorophyta* vary considerably and no phylogenetic tree with significant statistical support has been obtained. Here, we report phylogenetic analyses using *psaB* gene sequences of 50 representative algal species of Chlorophyta. Analyses at the nucleotide level could not resolve the basal relationship with statistical confidence (Nishiyama *et al.*,

2004). Furthermore, Bryophyte monophyly inferred using amino acid sequences has a good statistical foundation and is not rejected statistically by other data sets (Nishiyama *et al.*, 2004). In the study, the analyses, using translated amino acid sequences, indicate that *D. salina* is the nearest to the most species of Haematococcaceae in the phylogenetic relationship, which is contrary to previous opinion that *D. salina* is the nearest to the most species of Chlamydomonadaceae in the phylogenetic relationship (Hou *et al.*, 2004; Hou *et al.*, 2006). So we can further know the genetic background of *D. salina* through various species of Haematococcaceae and find various new functional genes in *D. salina* under the condition of the research backgrounds.

杜氏盐藻 *psaB* 基因 cDNA 克隆及系统进化分析

鲁照明，刘红涛，臧卫东，薛乐勋*

（郑州大学 细胞生物学研究室，郑州 450052）

摘 要：根据真核生物莱茵衣藻（*Chlamydomonas reinhardtii*）、*Chlamydomonas moewusii* 及 *Chlorella vulgaris* 等光系统Ⅰ反应中心蛋白 *psaB* 基因的氨基酸高度保守序列，设计一对简并引物，利用 TRIzol 试剂提取杜氏盐藻（*Dunaliella salina*）细胞的总 RNA，通过 RT-PCR，得到的一段长为 1.8 kb 左右的 cDNA 片段。PCR 产物经 T-A 克隆并测序以及测序结果推导成氨基酸序列进行同源性比较，表明所克隆的 1815bp 序列为杜氏盐藻光系统Ⅰ反应中心 *psaB* 基因的 cDNA 片段，GenBank 收录号为 AY820754。根据已经得到的 *psa*B 的核苷酸序列推导成氨基酸序列与一些已知物种的 *psa*B 氨基酸序列相比较，同源性分别为 *Chlamydomonas reinhardtii* 92%，*Chlamydomonas moewusii* 91%，*Chlorella vulgaris* 86%，*Mesostigma viride* 85%，*Physcomitrella patens* subsp. Patens 85%，*Nephroselmis olivacea* 84%。此外，psaB 密码子偏爱性分析表明：杜氏盐藻 *psaB* 基因第三位密码子 A 和 T 的组成分别为 35.7%和 39.17%，而 G 和 C 分别为 7.27%和 17.85%，即杜氏盐藻 *psaB* 基因密码子的组成大多为 NNA 和 NNT。根据 *psaB* 基因的特征，作者对绿藻门的 50 个物种的 *psaB* 基因作了进化分析，结果表明：杜氏盐藻与 Haematococcaceae 中的大多数种类进化地位最为接近，这为更进一步弄清杜氏盐藻的遗传背景提供了理论依据。

关键词：杜氏盐藻；A2 亚基；*psa*B；cDNA；简并引物

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