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Isolation and Characterization of Phytoene Desaturase cDNA Involved in the β-Carotene Biosynthetic Pathway in *Dunaliella salina*

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The green alga *Dunaliella salina* is one of the best and most important biological sources of β-carotene; however, to date the molecular basis of the β-carotene biosynthesis process in *D. salina* is still unresolved. The dehydrogenation of phytoene is the second step in the carotenoids biosynthetic pathway, and the phytoene-related desaturases are the key enzymes in the β-carotene biosynthetic pathway. A phytoene desaturase (Pds) cDNA with a 1752 bp open reading frame was cloned by RT-PCR and RACE-PCR methods on the basis of a modified switching mechanism at 5′ end of the RNA transcript (SMART) technology from *D. salina*. The predicted protein sequence displays a high identity (up to 65%) with phytoene desaturases of higher plants and cyanobacteria. The highest amino acid sequence identity (91%) is shared with the phytoene desaturase sequence of *Dunaliella bardawil*, and a dinucleotide-binding motif lies in the N-terminal. The phylogenetic analysis shows that *D. salina* Pds is closer to higher plants and cyanobacteria than bacterial and fungi. These results together demonstrated the cloned Pds cDNA of *D. salina* is a *Pds*-type gene, and it is postulated that in *D. salina* the first two dehydrogenations, by which phytoene is converted into ζ-carotene, are carried out by this putative phytoene desaturase.

KEYWORDS: ***Dunaliella salina*; β-carotene biosynthesis; phytoene desaturase cDNA; SMART technology**

INTRODUCTION

Carotenoids are synthesized by all photosynthetic organisms including green alga, as well by some nonphotosynthetic fungi and bacteria. Carotenoids function in light-harvesting, photoprotection in photosynthetic organisms. Dietary carotenoids are essential precursors to vitamin A, and their possible properties as antioxidants and free radical scavengers contribute to their potential in reducing the occurrence of certain cancers and cardiovascular diseases (*1*). Carotenoids also appear to play a structural role in assembly and stabilization of some pigment−protein complexes in plants and purple bacteria (*2*). In a recent review of Sharoni et al. (*3*), it was indicated that carotenoids modulate transcription of regulatory proteins involved in cell growth, transformation, and differentiation.

C_{40} carotenoids are derived from terpenoid precursor. The initial C_{40} biosynthetic reaction is the condensation of two molecules of GGPP by phytoene synthase to form phytoene, followed by stepwise desaturations to convert *cis*-phytoene into all-*trans*-lycopene via probable intermediates, such as phytofluene, ζ-carotene, and neurosporene. The linear lycopene was catalyzed to acyclic carotenoids including β-carotene. These early steps of carotenoid metabolic pathway have been extensively studied, and many corresponding genes have been isolated from yeast, bacteria, algae, and plants (*4*).

The naturally occurring carotenoids are of significant commercial interest as food coloring, animal feeding, and agents in pharmaceuticals, neutraceuticals, and cosmetics (*5*), and many of them can be synthesized chemically or extracted from biological sources (*6*). Some carotenogenic microbial strains were commercially employed to produce important carotenoids, for instance, astaxanthin from the red basidiomycetous yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) (*7*). In some green algae such as *Dunaliella salina* and *Haematococcus pluvialis*, accumulation of carotenoids can be induced by various environmental stress factors including high light and unfavorable conditions. These green microalgae are important and attractive for commercial exploitation because they require minimal nutrients for growth, and they grow very fast attributed to a 1 day cycle of doubling the biomass. They are also exploited particularly in some countries including Australian, China, and Israel to produce high-value pigments, such as astaxanthin from *H. pluvialis*, β-carotene from *D. salina*, and lutein from *Muriellipsis* sp. (*8*). Due to the high accumulation of β-carotene, even up to 10% of the dry weight, the green alga *D. salina* may be one of the best and most important biological sources

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Table 1. Primers Used in This Paper (5′–3′)

RT-PCR	PDS-F1 CTTTGGTGCTTACCCCAACA
	PDS-F2 GAGCAGGATGAGCTGAC
	PDS-F3 TGGCGAAGGCCCTGAACT
	PDS-R1 GCAGGAAACGGTTCAGTG
	PDS-R2 CTTCATGATGTCCACTGGCA
	PDS-R3 TCACCCGCCAGGTAGAAG
3′RACE	Dspds3-u1 ATGAACGCACGCATCAAGCAAATCG
	Dspds3-u2 TGGAGGACAACAGCGTCAAGCACT
	Modified oligo (dT) CGCGTCGACGAGCCAGAGCTCTGGGAA $(dT)_{18}$VN
5′RACE	SMART oligo GGTCTAGAGAATTCGGATCCAGGGG
	Dspds5-d1 GGGCTGGGATGTCTGGGAACTCAA
RT-PCR to amplify the ORF	Pds ORF-F GGATCCCCGAGAAGGATGCAGGTTATG
	Pds ORF-R AAGCTTCTACAGGAGCACAGAGCAACGG

of β-carotene. The preference of consumers for natural products and the health-promoting role of carotenoids (*9*) have also resulted in considerable commercial interest in developing *D. salina* as competitive biological sources of β-carotene.

Despite many publications on the accumulation of carotenoids in *D. salina* in response to high light and unfavorable culture stress factors, little research has been carried out on the molecular process of β-carotene biosynthesis. An approximate scheme, based on a zeaxanthin-accumulating mutant, including the β-carotene biosynthesis in *D. salina* was proposed by Jin et al. (*10*). However, the molecular basis of the β-carotene production in *D. salina* is still unresolved. It is hypothesized that there is a specific secondary metabolic pathway to massively synthesize β-carotene in *D. salina*, possibly due to a relatively simple reaction sequence or special primary structure of the involved enzymes, including lycopene cyclases (Lyc), phytoene synthase (Psy), and phytoene desaturases.

In previous work, the *D. salina* phytoene synthase gene and its cDNA, which may be rate limiting for the synthesis of β-carotene, were isolated and analyzed (*11*). In this paper, the isolation and characterization of another key enzyme, phytoene desaturase cDNA from *D. salina*, which is involved in the dehydrogenation reactions of phytoene to form lycopene, is described.

MATERIALS AND METHODS

Strains and Cultivation Conditions. *D. salina* cells were grown in defined medium (*12*) containing 2 mol/L NaCl at 26 °C under a 14/10 h dark/light cycle and were collected at the log phase or late log phase. *Escherichia coli* DH5was used as the host for the multiplication of plasmids.

Cloning of the Phytoene Desaturase Coding Gene from *D. salina*. The total RNA was prepared from 10 mL of *D. salina* cells grown at the late log phase with Trizol reagent (Life Technology) according to the manufacture's instruction. The RT reaction, based on the switching mechanism At 5′ end of the RNA transcript (SMART) technique, was performed using a modified oligo (dT) primer, 5′ SMART oligo sequence and the Powerscript reverse transcriptase (BD Clontech). The PCR procedure to amplify the Pds cDNA fragment is as the following: 1 cycle of 94 °C, 2 min; 30 cycles of 94 °C, 30 s, 47 °C, 30 s, and 72 °C, 1 min; used primers listed in **Table 1**.

On the basis of the obtained partial cDNA fragment sequence, several gene specific primers were designed to carried out the 3′ and 5′ RACE-PCR, employing the same RT method, and the following gradient PCR program was used: initial denaturalization at 94 °C for 5 min, followed by 5 cycles of 94 °C, 30 s, 70 °C, 30 s, and 72 °C, 1 min; another 5 cycles of 94 °C, 30 s, 68 °C, 30 s, and 72 °C, 1 min; a final 20 cycles of 94 °C, 30 s, 66 °C, 30 s, and 72 °C, 1 min. All of the amplified fragments were cloned to pGEM-T easy (Promega) and sequenced before the further experiments.

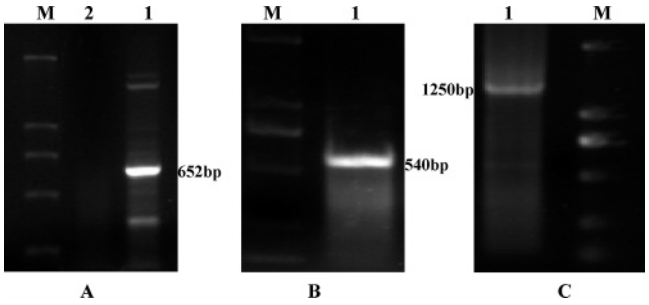

Figure 1. Isolation of the full length Pds cDNA from *D. salina*: (**A**) fragment of the Pds cDNA amplified by a combination pair of primers F1/R2; (**B**) 5′-end of the Pds cDNA isolated by 5′RACE; (**C**) 3′-end of the Pds cDNA isolated by 3′RACE. Key: M, DNA ladder (2000, 1000, 750, 500, 250, and 100 bp); 1, PCR products; 2, negative control.

Plasmid preparations, transformations, and other standard molecular biology techniques were carried out as described (*13*).

DNA Sequencing and Computer Analysis of DNA and Deduced Protein Sequences. The nucleotide sequence of the *D. salina* Pds cDNA was determined for both strands using the Abi Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). The DNAStar software package (Lasergene), Clustal X1.83 (NCBI, Bethesda, MD), and Treeview1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/ treeview.html) program were applied for the analysis of nucleotide and derived amino acid sequences as well as multiple alignments. Phylogenetic analyses were carried out using PHYLIP 3.63 phylogeny package.

Nucleotide Sequence Accession Number. The NCBI GenBank accession number for the phytoene desaturase cDNA reported in this paper is AY954517.

RESULTS AND DISCUSSION

Isolation of a cDNA Coding for *Dunaliella salina* Phytoene Desaturase. The reverse transcription was performed by using a SMART technology, based on the intrinsic terminal transferase activity of the Powerscript reverse transcriptase (MMLV, RNase H minus, point mutant, BD Clontech). By the SMART technology, a specialized SMART primer can be added to the 5′ end of the newly synthesized cDNA and used as universal primer. Second-strand cDNA synthesis is then accomplished using PCR with the universal sequences (a SMART primer, a modified oligo (dT) primer, and gene specific primers).

A series of specific primers were designed to obtain Pds cDNA fragment from *D. salina* on the basis of the Pds cDNA sequences of two green algae, *Dunaliella bardawil* (accession No. Y14807) and *H. pluvialis* (accession No. X86783), and also referring to several conserved regions of the Pds amino acid sequences of several plants and cyanobacteria. When using total RNA from different growth stage *D. salina* cells as RT substrate S, an expected 652 bp fragment amplified with the combination of primer PDS-F1/R2 was in existence consistently, and then it was cloned and sequenced (**Figure 1A**).

A complete homologous search in the GenBank database by using the BLAST programs (NCBI) demonstrates that the nucleotide and putative protein sequence of this fragment has a high up to 87% and 91% identity with that of *D. bardawil*. On the basis of this cDNA sequence, a gene specific primer Dspds3-u1 and an inner primer Dspds3-u2 were designed and 3′RACE was performed together with the modified oligo (dT) primer to obtain C-terminus of Pds cDNA; 5′RACE was carried out using primer pair Dspds5-d1/SMART oligo to obtained the N-terminus. Two corresponding fragments, 540 and 1250 bp in length, respectively, were amplified (**Figure 1B,C**). They were subcloned and sequenced as above. Lapping regions were found

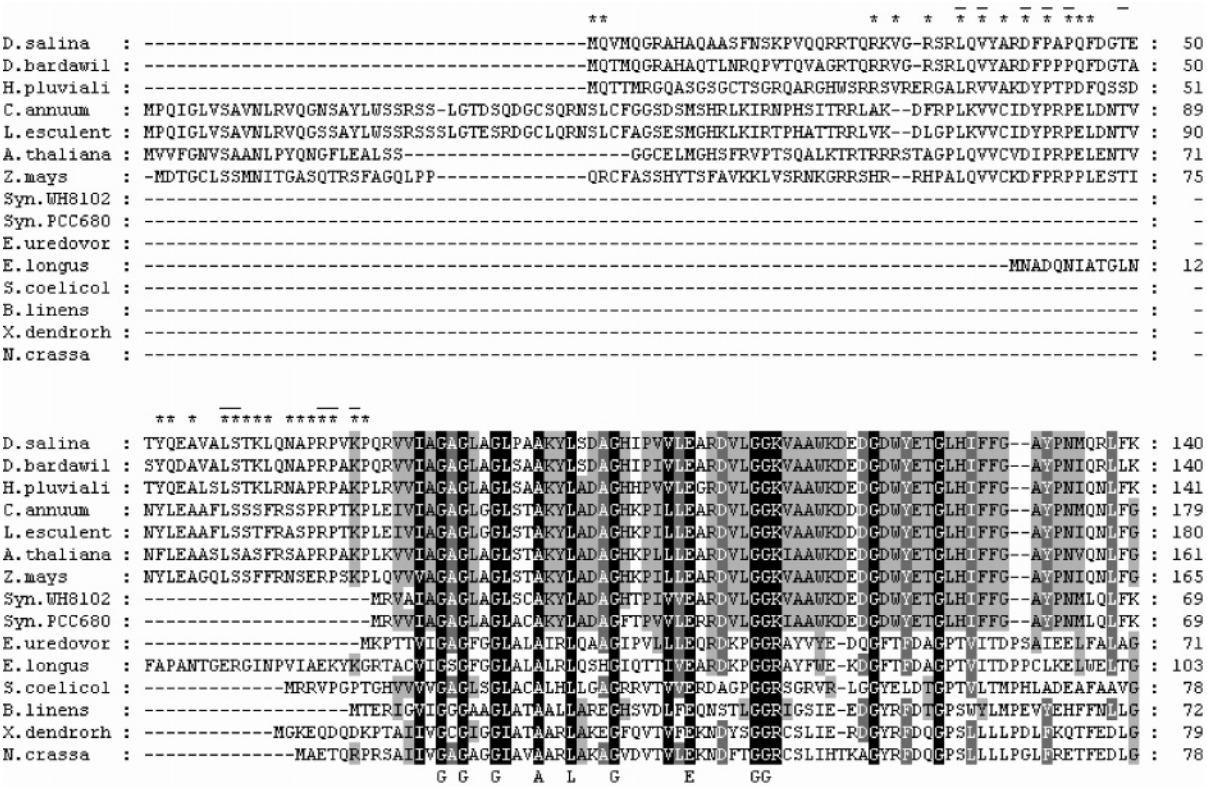

Figure 2. Alignment of sequences in the N-termini of phytoene desaturase sequences from bacterial, fungi, cyanobacteria, green algae, and plant. Consensus motif for the binding site of NADP (H) is given below the sequences. Residues in the putative transit sequences conserved between the plants and *D. salina* Pds sequences are overlined, and the residues conserved between three algaes are marked with asterisks. The number on the right is corresponding to the amino acid position of each Pds sequence.

between the initial 652 bp and each of the RACE-PCR fragments; homologous searches through the BLAST programs demonstrated that they are the expected 3′and 5′ ends of the *D. salina* Pds cDNA. The sequence of full length Pds cDNA was assembled according to the three fragments above and is 2198 bp in length. Two primers Pds ORF-F/R containing the *BamH* I and *Hind* III restriction site, respectively, were used to amplify a 2061 bp long sequence (data not shown), which was subjected to the verification by restriction analysis and sequencing and then cloned into the corresponding sites of expression vector for further expression.

Nucleotide Sequence of the Phytoene Desaturase cDNA of *D. salina*. The cloned 2061 bp cDNA fragment includes a 1752 bp putative open reading frame according to the Blast programs search results, which is equivalent to the length of the Pds cDNA ORF of *D. bardawil*. A short 5′ untranslated region of 10 bp precedes the first ATG start codon for the putative open reading frame, and a 3′ untranslated region of 370 bp follows the putative stop codon and precedes the poly (A) tail. A polyadenylation signal (the sequence AATAA at base pairs 2115−2119) occurs 62 bp before the poly (A) tail. The nucleotide sequence has a highest overall sequence similarity with the Pds counterpart of *D. bardawil* (87% identity over 1464 bp). For the amino acid sequence deduced from this cDNA fragment, a significant overall similarity was observed with other Pds sequences from other green algae and higher plants; it shares the highest identity with the *D. bardawil* Pds in the same way (91% identity). Due to the high sequence similarity, it was concluded that the 2061 bp cDNA fragment contains an ORF coding for phytoene desaturase. The *D. salina* phytoene desaturase was found to have 583 amino acids residues with a calculated mass of 64.9 kDa.

The deduced amino acid sequence of phytoene desaturase from *D. salina* was aligned with those from higher plants, another two green algae, cyanobacteria, fungi, and bacteria (**Figure 2**). Phytoene desaturase sequences and GenBank accession numbers are as the following: *Capsicum annuum*, X68058; *Lycopersicon esculentum*, X59948; *Arabidopsis thaliana*, L16237; *Zea mays*, U37285; *Synechococcus* sp. WH8102, BX548020; *Synechocystis* sp. PCC 6803, NC_000911; *Erythrobacter longus*, D83514; *Erwinia uredovora*, D90087; *Streptomyces coelicolor* A3(2), NC_003888; *Brevibacterium linens*, AF139916; *Xanthophyllomyces dendrorhous*, AY177424; *Neurospora crassa*, XM_324731.

In a comparison of phytoene desaturase protein sequences of different species, a lower homology of *D. salina* Pds was found for the prokaryote phytoene desaturases (14−18% identities) with the exception of the cyanobacterial ones (about 65% identities). The overall Pds sequence identity between the amino acid sequences of green algae, cyanobacteria, and higher plants is rather high (about 65% identities); it also revealed many conservative changes.

However, the *D. salina* Pds revealed an N-terminal extension when compared to the cyanobacterial, fungal, and bacterial phytoene desaturases, which has also been observed in the higher plant enzymes. The sequence similarities between the green algal and plant phytoene desaturases extend for about 20−40 amino acid residues toward the N-terminus, indicating an involvement of this region in the enzymatic activity (*14*). It can be postulated that this region serves as a transit sequence for the transport of Pds into plastids, as the phytoene desaturase gene located in the nucleus and the mature functional Pds in eukaryotic organisms would have the same size as its counterpart in cyanobacterial organisms. It requires proof to support the idea

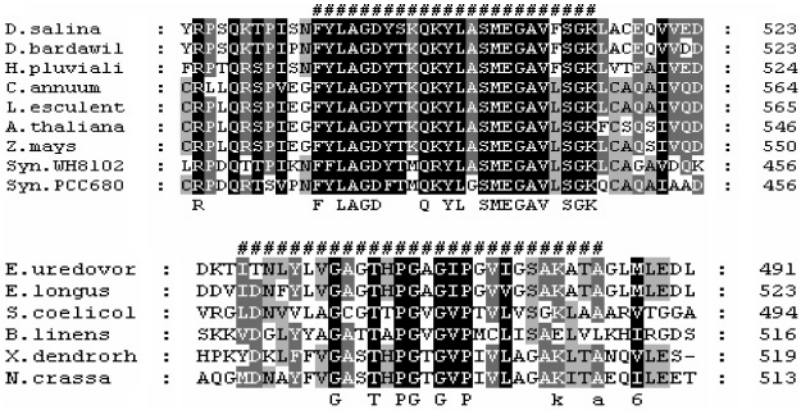

Figure 3. Postulated carotenoid-binding domain in the C-termini of phytoene desaturase sequences from bacterial, fungi, cyanobacteria, green algae, and plant. The predicted domain residues are marked with "#" over the sequences. The number on the right is corresponding to the amino acid position of each Pds sequence.

for *D. salina* Pds that a part of this N-terminus region is necessary for a fully functional enzyme.

The highest homology between phytoene desaturase proteins was found in the region forming dinucleotides (NAD (H)/NADP (H) or FAD (H))-binding domain in the amino termini that have been described (*15*). This is also present in the *D. salina* protein, and it is consistent with the fact that all of the known carotene desaturases and isomerases contain a conserved, N-terminal dinucleotide-binding motif: $GXGX_2GX_3AX_2LX_3G\ X_6EX_5GG$. This motif is characteristic of all known carotenoid desaturases and cis−trans isomerases (*16*−*18*). It is not possible to determine which kind of dinucleotide (NAD, NADP, or FAD) will specifically solely depend on the protein sequence. The comparison of all the dinucleotide-binding motif sequences from various species seems to reveal a composite sequence that is nubilous in its specificity for either NAD or NADP.

In despite of the low similarity of overall protein sequences, the predicted hydropathy structures of all phytoene desaturases C-termini are similar (data not shown). This may be the carotenoid-binding domain including a conserved carotenogenic box (*14*), as shown in **Figure 3**. The amino acid sequences of the corresponding domain in phytoene desaturases of *D. salina*, cyanobacteria, and plants are different from those of bacteria and fungi.

Phylogenetic Analysis and Possible Function in the β-Carotene Biosynthetic Pathway of the Cloned cDNA. The phylogenetic tree (**Figure 4**) indicates that, within phylogenetic clusters formed by green algae, plants, cyanobacteria, bacteria, and fungi, a clear homology is found. Green algal phytoene desaturases are more related to those enzymes of the higher plants and cyanobacteria than to the phytoene desaturase proteins within the clusters formed by plants and cyanobacteria. The two *Dunaliella* Pds proteins form a cluster, and they are phylogenetically closer to plants than to cyanobacteria. These results agree with those conclusions derived from the alignment analysis of the phytoene desaturase sequences.

In fact, it is well-known that, for conversion of phytoene into lycopene, it needs two desaturases, phytoene desaturase (called *crtP* in cyanobacteria) and ζ-carotene desaturase (crtQ) in organisms with oxygenic photosynthesis, while in organisms that lack oxygenic photosynthesis dehydrogenation is carried out by an enzyme completely unrelated to the former one, which carries out four dehydrogenations and is encoded by the *crtI* genes (*19*, *20*). In cyanobacteria, algae, and higher plants, the first phytoene desaturase (*Pds*-type) converts phytoene into ζ-carotene via phytofluene by two dehydrogenation reactions.

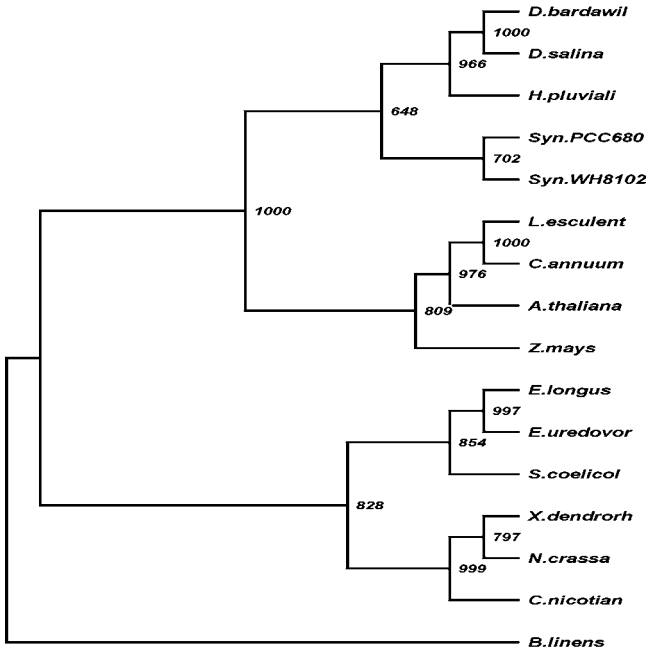

Figure 4. Phylogenic tree of Pds sequences from various species. (Numbers associated with the branches are bootstrap values; BOOTSTRAP was analyzed by using 1000 replicates.) The maximum likelihood (distance) method was used.

This intermediate is further dehydrogenated into lycopene via neurosporene by a *Zds* (*crtQ*) enzyme. Because of the significant deduced protein sequence identity and close phylogenetic relationship with the phytoene desaturase of plants and cyanobacteria, it is deduced that the *D. salina* cDNA in this paper codes the first phytoene desaturase (Pds), by which phytoene is dehydrogenated into ζ-carotene, and another distinct ζ-carotene desaturase (Zds) is required to finish the subsequent two dehydrogenation reactions.

In higher plants phytoene exists predominantly as the 15-cis isomer, while the predominant geometric isomer of lycopene is all-trans, suggesting isomerization must occur at some stage. Carotene cis−trans isomerase involved in the biosynthesis of all-*trans*-lycopene from *tangerine* tomato (CRTISO) (*21*) has been isolated. Simultaneously the *Arabidopsis* Crtiso homologue termed *Ccr*-2 was found (*22*). In the cyanobacterium Synechocystis sp. PCC6803, an ortholog of CrtISO was also

reported (*23*, *24*). In *D. salina* a homologous carotene isomerase is predicted to be existent and participate in the formation of all-*trans*-lycopene after finishing the four dehydrogenations (*25*).

In summary, a cDNA coding phytoene desaturase was isolated from the green algae *D. salina*, which is an important β-carotene biological source. The deduced protein is significantly homologous to phytoene desaturases of other green algae, higher plants, and cyanobacteria, and the closer phylogenetic relationship between them also demonstrates it is a *Pds*-type gene. On the basis of these data, it is postulated that to produce β-carotene in *D. salina* cells, the Pds needs to combine with another two gene products, ζ-carotene desaturase (Zds) and isomerase (CRTISO), to form the direct substrate for cyclization, all-*trans*-lycopene. Presently, the effects of deregulation and overexpression of the *Pds* on β-carotene biosynthesis in *D. salina* and the enzyme assay including the test of substrate specificity of the cDNA expression product, cofactor requirements are being tested.

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