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Analysis of an Essential Carotenogenic Enzyme: ζ-Carotene Desaturase from Unicellular Alga *Dunaliella salina*

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The green alga *Dunaliella* has become a valuable model organism for understanding the interesting mechanism of massive carotenoid accumulation. Previously, DNA sequences of several carotenogenic enzymes were obtained from *Dunaliella*. In this study, the cDNA of *zds* was isolated from *Dunaliella salina* using a polymerase chain reaction approach. The full-length cDNA sequence was 2178 base pairs (bp) containing a 1731 bp putative open reading frame which coded a 576 amino acid deduced polypeptide whose molecular weight was 63.9 kDa computationally. A complete homologous search displayed that the nucleotide and putative protein sequence have sequence identities of 69% and 66% with those of green alga *Chlamydomonas reinhardtii*, respectively. It was predicted that this ζ-carotene desaturase (Zds) may be located in the chloroplast of *D. salina*. Phylogenetic analysis demonstrated that the *D. salina* Zds had a closer relationship with the Zds of algae and higher plants than with those of other species.

KEYWORDS: *Dunaliella salina*; algae; carotenoids; carotenogenic pathway; ζ-carotene desaturase

INTRODUCTION

Widely distributed in nature, carotenoids are a structurally diverse class of isoprenoids which are synthesized by all photosynthetic organisms and many nonphotosynthetic organisms. So far, at least 700 types of carotenoids have been detected from natural sources (*1*). For their extensively commercial and industrial purposes, carotenoids are in high demand around the world with an expectation of the worldwide market value of more than one billion dollars by 2009 (*2*).

As a result of the capacity to accumulate a large quantity of highly bioavailable β-carotene (*3*), *Dunaliella salina* and *Dunaliella bardawil*, belonging to the genus *Dunaliella*, have been commercially exploited for the production of natural β-carotene and β-carotene-rich biomass all over the world since the 1980s (*4*). Though the biochemistry and physiology of *Dunaliella* have been sufficiently investigated, molecular elucidation of the carotenogenic pathway of *Dunaliella* has been conducted in recent years, and only several nucleotide sequences of carotenogenic enzymes have been isolated (*5*−*8*). The carotenogenic pathway of *Dunaliella* is in line with the one in higher plants, and the abbreviated pathway of carotenogenesis from geranylgeranyl pyrophosphate (GGPP) to β-carotene in *Dunaliella* is displayed in **Figure 1**. In the process of carotenogenesis, to generate the pink colorant pigment lycopene, phytoene experiences four desaturations catalyzed by carotene desaturase via the intermediates of phytofluene, ζ-carotene and neurosporene, sequentially. In bacteria and fungi, these four desaturations are independently accomplished by a single carotenoid enzyme coded by *crtI*, whereas in cyanobacteria and higher plants, two carotene desaturases, phytoene desaturase (Pds) and ζ-carotene desaturase (Zds), participate in this process together (*9*).

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Zds is a key enzyme in carotenogenesis severing downstream of ζ-carotene in the carotenogenic pathway. In the heterologous expression of carotenoids, the expression of *zds* in the host cell *Escherichia coli* implemented the formation of lycopene (*10*). Researchers demonstrated that the mutation of the *zds* gene led to impaired carotenogenesis and subsequent spontaneous cell death due to the increased content of superoxide generated from photooxidation (*11*). Analysis of phylogenesis indicated that carotenogenic enzymes of *Dunaliella* had a closer relationship with those of higher plants than with those of other species (*5*−*8*). In addition, the *Dunaliella pds* cannot solely replace *crtI* of *Erwinia uredovora* in achieving the conversion from phytoene to lycopene in heterologous expression (*12*). Therefore, we suggested that Zds may be present in *Dunaliella* and participate in the massive generation of carotenoids.

The exhaustive exhibition of the carotenogenic pathway of *Dunaliella* is essential in commerce and industry for the remarkable property of massive β-carotene accumulation of this alga and the high demand of globally natural carotenoids. Although the molecular elucidation of the carotenogenic pathway of *Dunaliella* has been conducted for nearly a decade, the molecular mechanism of the massive β-carotene accumulation in *Dunaliella* is still unclear (*2*). So far, little investigation on the *zds* of eukaryotic algae has been published owing to the limited data sets of the carotenogenic pathway of these species. In order to further elucidate the carotenogenic pathway of *Dunaliella*, we attempted to clone the cDNA (cDNA) of *zds* from *D. salina* and to subsequently characterize this essential enzyme with a series of bioinformatics tools.

MATERIALS AND METHODS

Strains and Cultural Conditions. *D. salina* strain UTEX LB 200 was purchased from the Institute of Hydrobiology (Wuhan, China) and cultured in a medium (pH 7.5) containing 1.50 mol/L NaCl, 4.94 mmol/L $NaNO_3$,

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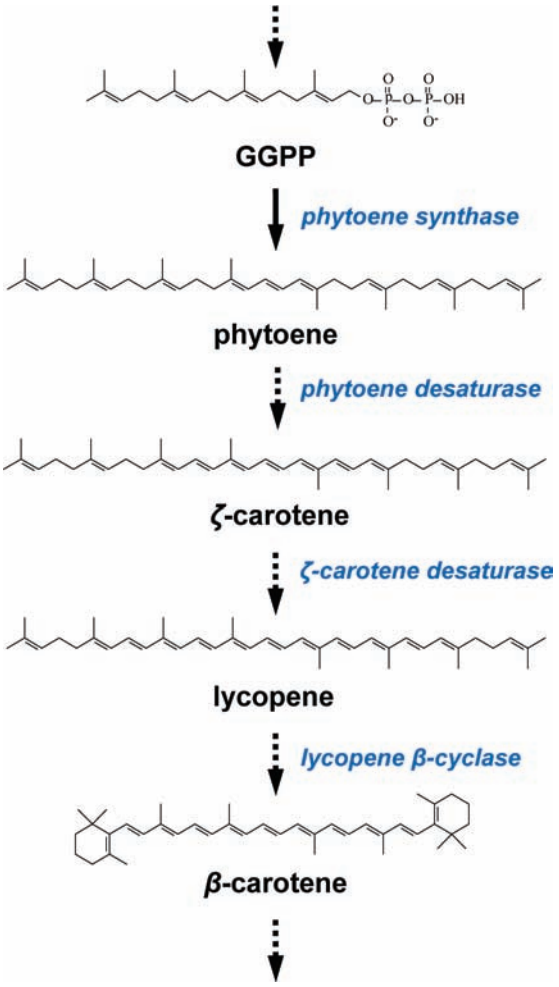

Figure 1. Abbreviated biosynthetic pathway of carotenoids from GGPP to β-carotene in *Dunaliella*. Commonly, the carotenogenic pathway is made up of three main parts: GGPP biosynthesis, lycopene generation, and the formation of carotenoids with cyclohexene and their derivatives. Metabolites are shown in boldface type, whereas enzymes for relative conversions are displayed in italics. Dotted arrows represent one or more intermediates being generated under the catalysis of related enzymes.

0.10 mmol/L $NaH_2PO_4 \cdot 2H_2O$, 5.00 mmol/L $MgSO_4 \cdot 7H_2O$, 0.99 mmol/L KCl, 10.00 mmol/L $NaHCO_3$, 0.30 mmol/L $CaCl_2 \cdot 2H_2O$, 1.00 mL/L trace elements solution, and 0.50 mL/L Fe-salting liquid. The trace element solution was composed of 46.13 mmol/L H_3BO_3, 9.14 mmol/L $MnCl_2 \cdot 4H_2O$, 0.77 mmol/L $ZnSO_4 \cdot 7H_2O$, 0.32 mmol/L $CuSO_4 \cdot 5H_2O$, and 0.03 mmol/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, whereas the Fe-salting liquid contained 0.42 mmol/L $Na_2EDTA \cdot 2H_2O$ and 0.90 mmol/L $FeCl_3 \cdot 6H_2O$. Three hundred milliliter *D. salina* cultures were grown in 1 L flasks under a 14/10 h light (6000 lx white fluorescent lamps)/dark cycle at 26 °C with continuous shaking at 100 rpm in an ATL-032LR incubator (Chem-star, Shanghai, China). Cells were collected at the log phase for the experiments in the present study (*7*).

Isolation and Purification of Total Ribonucleic Acid (RNA). *D. salina* was harvested via centrifugation at 15,000*g* for 10 min at 4 °C. Subsequently, cells were suspended in 5 mL of 0.1% diethyl pyrocarbonate (DEPC) solution (Takara, Dalian, China) and recollected as above. The total RNA was extracted from *D. salina* using Trireagent (Ambion, Austin, USA) following the manufacturer's protocols. Finally, the total RNA was treated by DNase I (RNase Free) (Takara, Dalian, China) and was dissolved in 0.1% DEPC solution.

Cloning of the 3′-End of the *zds* cDNA. In order to clone the 3′-end of the *D. salina zds*, a degenerated primer was designed on the basis of the *zds* cDNA of 7 species (*Anabaena*, Y15115; *Arabidopsis thaliana*, U38550; *Capsicum annuum*, X89897; *Chlamydomonas reinhardtii*, XM_001700734; *Citrus unshiu*, DQ309869; *Lycopersicon esculentum*, AF195507; and *Zea mays*, AF047490) and conserved amino acid regions from the Zds protein sequences of 11 species (*Anabaena variabilis*, ABA19826; *A. thaliana*, NP_187138; *C. annuum*, AAB35386; *C. reinhardtii*, EDP07040; *Citrus sinensis*, CAC85667; *Cyanothece* sp., EAZ90456; *Daucus carota*, ABB52070; *Solanum lycopersicum*, AAF13698; *Synechococcus elongatus*, BAD80687; *Thermosynechococcus elongatus*, BAC07889; and *Trichodesmium erythraeum*, ABG52981). The 3′-end of *zds* cDNA was isolated using a 3′ rapid amplication of cDNA ends (RACE) polymerase chain reaction (PCR) approach (*7*). The first strand cDNA was synthesized from 200 ng of total RNA using RNA PCR Kit (AMV) version 3.0 (Takara, Dalian, China) in a total volume of 10 μL containing 1 μL of 10× reverse transcription (RT) buffer, 5 mmol/L $MgCl_2$, 10 U RNase inhibitor, 2.5 U AMV reverse transcriptase, and 125 nmol/L Oligo dT-adaptor primer according to the manufacturer's instructions. Negative control was performed using nuclease free water as the template. Reverse transcription (RT) reaction was performed with the parameters set as 42 °C for 60 min followed by 95 °C for 5 min. PCR mediated amplifying reaction for the 3′-end of *zds* cDNA was performed with 2 μL of the RT product described above, 10 pmol forward degenerated primer, 10 pmol M13M4 primer (5′-GTCGTGACTGGGAAAAC-3′), 4 μL of 2.5 mmol/L dNTP mixture, 5 μL of 10× Ex Taq buffer (Mg^{2+} plus), and 1.25 U Ex Taq (Takara, Dalian, China), with ultrapure water added to a final volume of 50 μL. Negative control was performed using the negative control RT product generated above. PCR was performed with the touchdown program with the parameters set as follows: 95 °C for 2 min; 5 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min; 25 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 3 min; 72 °C for 7 min. In this study, all of the target sequences were cloned into pMD20-T vector (Takara, Dalian, China) and transformed in *E. coli* GT116 for multiplication and deoxyribonucleic acid (DNA) sequencing.

Amplification of the 5′-End of *zds* cDNA. In the process of amplifying the 5′-end of *zds* cDNA, the 5′ RACE PCR approach was employed with 5′-full RACE kit (Takara, Dalian, China) (*7*). (1) The total RNA of *D. salina* was treated by alkaline phosphatase (calf intestine) and tobacco acid pyrophosphatase successively. (2) The 5′ RACE adaptor was linked into the 5′-end of mRNA by T4 RNA ligase, and the mRNA template for the cloning of 5′-end of *zds* cDNA was obtained. (3) The first strand cDNA was synthesized using random 9 primers in a total reactive system volume of 10 μL by reverse transcriptase M-MLV (RNase H^-) according to the manufacturer's instructions. The parameters were set as 30 °C for 10 min, 42 °C for 60 min, and 70 °C for 15 min. (4) The first PCR for the amplification of the 5′-end of *zds* cDNA was performed with 2 μL of RT product, 20 pmol of 5′ RACE outer primer, and 20 pmol of a gene specific primer which was designed according to the cDNA 3′-end obtained above, using LA Taq (Takara, Dalian, China) following the manufacturer's protocols. The parameters were set as 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.5 min; 72 °C for 10 min. Negative control was performed according to the supplier's instructions. A second PCR was carried out with 1 μL of the product of the first PCR, 20 pmol of 5′ RACE inner primer, and 20 pmol of gene specific primer. The parameters were set as for the first PCR above.

Isolation of an Open Reading Frame (ORF) of *zds*. In the process of ORF isolation, the High Fidelity PrimeScript RT-PCR Kit (Takara, Dalian, China) was used following the manufacturer's protocols. The parameters were set as 94 °C for 3 min; 30 cycles of 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 2 min; 72 °C for 10 min.

Sequencing, Characterization, and Phylogenic Analysis. The nucleotide sequences of cDNA were determined for both strands using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA) by ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, USA). In this study, DNAstar software package program 7.1.0 (Lasergene), ProtParam tool, Compute pI/M_w, SignalP 3.0 Server, ProtScale, TMHMM server version 2.0, and WoLF PSORT were employed for the analysis of physical and chemical characteristics of the *D. salina* Zds (*13*−*15*). The program's basic local alignment search tool (BLAST) was employed with default parameters for the homologous search of nucleotide and putative protein sequences (*16*). In addition, the Conserved Domain Database (CDD) was used to analyze converse domains of the *D. salina* Zds (*17*). The three-dimensional structure of *D. salina* Zds was automatically predicted by 3D-JIGSAW Protein Comparative Modeling Server (version 2.0) and

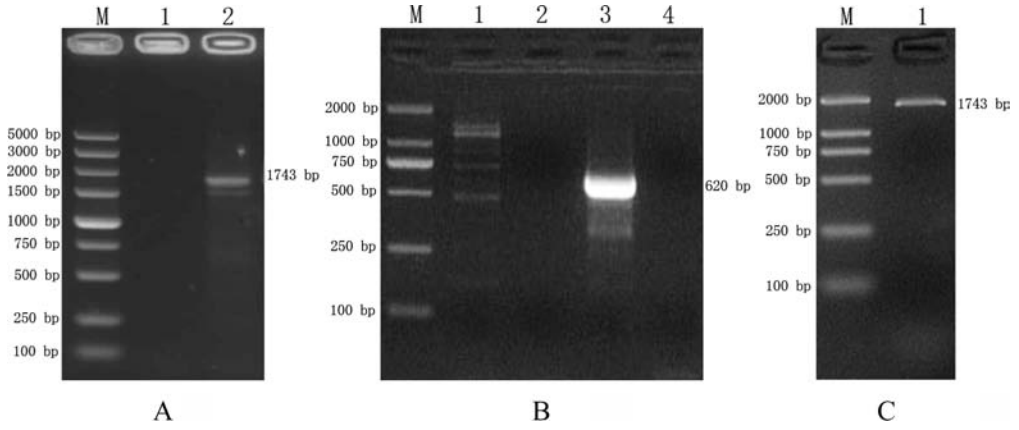

Figure 2. Isolation of the cDNA of *D. salina zds*. (**A**) Fragment of the 3′-end of *zds* cDNA. M, DNA marker; lane 1, negative control; lane 2, PCR product. (**B**) Fragment of the 5′-end of *zds* cDNA. M, DNA marker; lane 1, first PCR product; lane 2, first negative control; lane 3, second PCR product; lane 4, second negative control. (**C**) ORF of the *zds* cDNA. M, DNA marker; lane 1, PCR product.

CPHmodels 3.0 Server on the basis of the homologues of known structures (*18*, *19*). Results were visualized by RasMol software 2.7.2.1.1 (*20*). Finally, phylogenic analysis was performed using MEGA 4.0.2 software based on the neighbor-joining method with 45 Zds protein sequences of other species (*21*).

RESULTS

Cloning and Characterization of *zds* cDNA. A 1743 base pair (bp) long partial cDNA was amplified from *D. salina* during the cloning of the 3′-end of the *zds* cDNA (**Figure 2A**). The nucleotide and putative protein sequence had, respectively, sequence identities of 68% and 66% with those of the alga *C. reinhardtii zds* undergoing a homologous search by BLAST. Because of the high degree of sequence homology to the *C. reinhardtii zds*, a gene specific primer was designed on the basis of the obtained nucleotide sequence for the 5′ RACE PCR approach. During the process of cloning the 5′-end of *zds* cDNA, we obtained a 620 bp long sequence which shared a 140 bp identical nucleotide fragment with the 3′-end of *zds* cDNA (**Figure 2B**). On the basis of the results above, a 1743 bp long ORF of the *D. salina zds* was amplified using two gene specific primers (**Figure 2C**). As a result, the full-length cDNA of the *D. salina zds* was 2178 bp long and has been deposited in GenBank under the accession number HM754265.

Characteristics of the *D. salina Zds*. On the basis of the analysis of the nucleotide sequence, we demonstrate that the *D. salina zds* full-length cDNA contained 2178 bp nucleotides with a 1731 bp long putative ORF flanked by a 69 bp long 5′ untranslated region upstream of the start codon and a 378 bp long 3′ untranslated region after the stop codon. This 1731 bp long ORF encoded a deduced protein sequence of 576 amino acids long peptide which contained 63 strongly basic amino acids (Lys and Arg), 59 strongly acidic amino acids (Asp and Glu), 197 hydrophobic amino acids (Ala, Ile, Leu, Phe, Trp, and Val), and 138 polar amino acids (Asn, Cys, Gln, Ser, Thr, and Tyr). A complete homologous search displayed that the nucleotide and putative protein sequence had sequence identities of 69% and 66% with those of *C. reinhardtii zds*, respectively. Analysis of ProtParam tool and Compute pI/M_w revealed that the chemical formula of this peptide was $C_{2863}H_{4448}N_{784}O_{825}S_{26}$, the instability index was 45.38 (unstable), the aliphatic index was 77.38, the grand average of hydropathicity was −0.286, the estimated molecular mass of this deduced peptide was 63.9 kDa, and the isoelectric point was 8.44. SignalP 3.0 Server analysis with neural networks and hidden Markov models displayed that there was no signal peptide in this polypeptide. As a result, the *D. salina* Zds was a nonsecretory protein. Hydropathy analysis

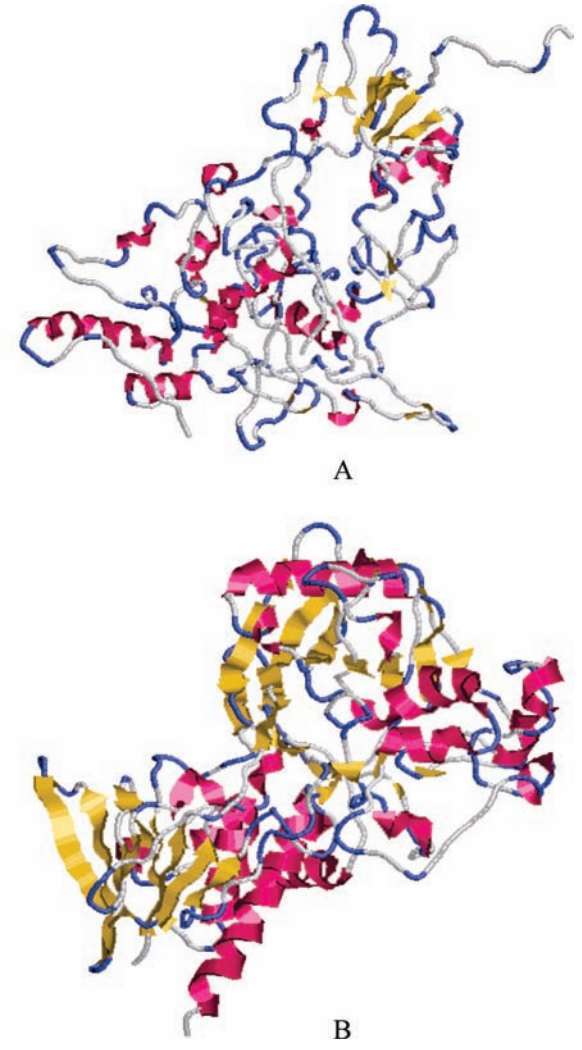

Figure 3. Schematic representation of three-dimensional models of the *D. salina* Zds. (**A**) Comparative modeling was performed using 3D-JIGSAW. (**B**) Comparative modeling was performed using CPHmodels. The α-helix and β-sheet regions of the putative protein are indicated with ribbons and arrows, respectively. The loop regions are also designated in the schematics.

by ProtScale indicated that several hydrophilic and hydrophobic regions exist in this polypeptide. Prediction of transmembrane region was performed using the TMHMM server, version 2.0.

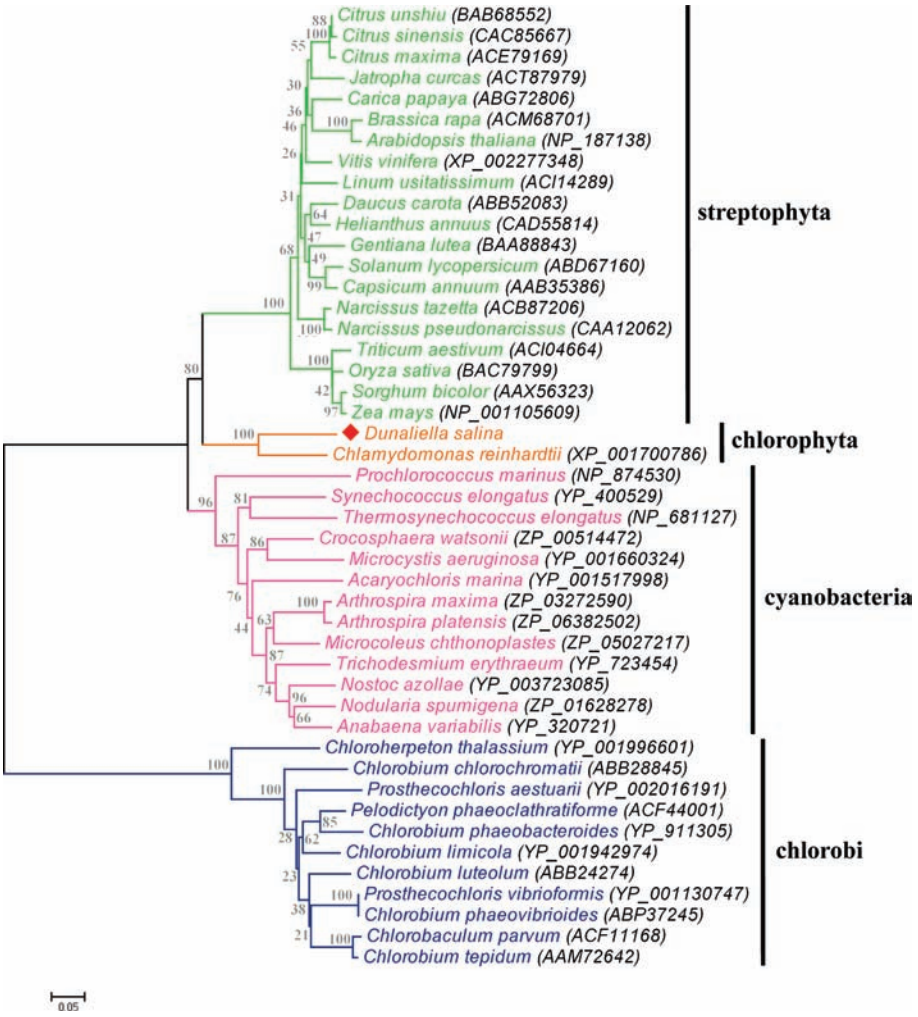

Figure 4. Phylogenetic tree (neighbor-joining) for Zds of 46 species. As shown, these protein sequences of Zds of 46 species can be divided into four groups as streptophyta (green), chlorophyta (orange), cyanobacteria (pink), and chlorobi (blue). The red diamond shows the position of the *D. salina* Zds on the phylogenetic tree. GenBank accession numbers of reference protein sequences are shown next to the name of each species. Numbers associated with the branches are the neighbor-joining bootstrap values (n = 1000). The length of the branch expresses evolutionary distance with its scale being 0.05.

Results demonstrated that the *D. salina* Zds was not a potential membrane protein. Analysis of WoLF PSORT exhibited that the *D. salina* Zds may be situated in the chloroplast.

Protein Structure of the *D. salina* Zds. It was indicated that the putative polypeptide contained a NAD(P)-binding Rossmann-fold domain cluster (superfamily) and a carotene 7,8-desaturase domain model (multidomain) based on the analysis of CDD. Consequently, the results predicted that this deduced protein was a carotenoid desaturase Zds (EC: 1.14.99.30). 3D-JIGSAW and CPHmodel comparative modeling programs were used for the automatic prediction of the three-dimensional structure of the *D. salina* Zds based on the homologues of known structures. The α-helix regions, β-sheet regions, and the loop regions of these three-dimensional models are displayed in **Figure 3**.

Phylogenetic Analysis. Phylogenetic analysis of 46 Zds protein sequences demonstrated that four defined clusters of Zds were displayed on the phylogenetic tree as follows: streptophyta, chlorophyta, cyanobacteria, and chlorobi (**Figure 4**). As shown, the *D. salina* Zds has a closer relationship with the Zds of the chlorophyta and streptophyta than with those of cyanobacteria and chlorobi.

DISCUSSION

The biosynthetic pathway of the diverse carotenoids is a typical system for understanding the evolution of secondary metabolism (*22*). Heterologous expression indicated that the desaturation of phytoene in Archaea, nonphotosynthetic prokaryotes, and fungi was significantly different from the one in cyanobacteria and eukaryotic photosynthetic organisms (*9*). In the former, the desaturation of carotenoids from phytoene to lycopene is catalyzed by a signal enzyme crtI, whereas the conversion is accomplished by Pds and Zds together in the latter. Zds is an essential carotenogenic enzyme responding to the conversion from ζ-carotene to lycopene. To date, mitochondrial and plastid genome sequencing of three eukaryotic algae, including *C. reinhardtii*, *Volvox carteri*, and *D. salina*, have been completed, but the characteristics of *zds* in these eukaryotic algae has not yet been released by the authors, and only the *zds* sequence of *C. reinhardtii* could be searched on the GenBank database currently (*23*−*25*). As a result of the insufficiently molecular investigation of carotenogenesis of eukaryotic algae, the carotenogenic enzymes of *Dunaliella* have not been isolated and characterized thoroughly, and the carotenogenic pathway of *Dunaliella* is still unclear.

Because of its interesting property of the massive accumulation of β-carotene, *Dunaliella* has become one of the most significantly industrial microalgae (*4*). As a result, interests have been focused on the molecular clarification of the distinctive carotenogenic

pathway of *Dunaliella* for the past few years. Several DNA sequences of carotenogenic enzymes of *Dunaliella* have been isolated owing to the hard work of previous researchers (*5*−*8*). As the reported carotenogenic enzymes of *Dunaliella* have a high degree of sequence homology to those of plants, we speculated that Zds existed in *Dunaliella* and was involved in carotenogenesis. In the present study, the cDNA of *zds* was isolated from *D. salina* employing the RACE PCR approach. The full-length cDNA shared, respectively, nucleotide and putative protein sequence identities of 69% and 66% with the *C. reinhardtii zds*. Analysis of CDD indicated that this putative polypeptide was a carotene 7,8-desaturase (EC: 1.14.99.30), namely, Zds. Microscopic analysis displayed that carotenoids were massively accumulated in the interthylakoid spaces of chloroplast in *Dunaliella* under stress conditions (*26*). The *D. salina* Zds may be situated in the chloroplast according to analysis of the protein localization predictor WoLF PSORT. As a result, the massive carotenoid accumulation may be triggered in the chloroplast of *Dunaliella*. In addition, the three-dimensional structure of the *D. salina* Zds was automatically modeled by 3D-JIGSAW and CPHmodels based on homologues of known structures. Because of the nonuniform computing methods, the three-dimensional structures predicted by different modeling servers were not quite similar (**Figure 3**).

The phylogenetic tree of Zds is shown in **Figure 4**. As displayed, the phylogenetic tree could be divided into four groups as streptophyta, chlorophyta, cyanobacteria, and chlorobi. The *D. salina* Zds had a closer relationship with the Zds of algae (chlorophyta) and higher plants (streptophyta) than that with those of other species. It is not hard to find that archaea, nonphotosynthetic prokaryotes, and fungi are absent from this phylogenetic tree owing to the presence of crtI instead of *pds* and *zds* in these species. This is consistent with the previous conclusion from the phylogenetic analysis that *zds* had a high homology to the *pds* and that both *zds* and *pds* are distantly related to *crtI* (*9*, *22*). Previously, investigators have suggested that genes for the isoprenoid biosynthetic pathway of eukaryote origin are from an endosymbiotic cyanobacterial ancestry (*8*, *27*). Consistent with this suggestion, the homologousness of streptophyta, chlorophyta, and cyanobacteria in the phylogeny here implied that eukaryotic algae and plants *zds* may shared a common cyanobacterial origin.

Currently, metabolically engineered microorganisms and transgenic plants are widely used in carotenoid production to meet the high global demand (*28*, *29*). As a result, a large carotenogenic genes pool should be provided first for the carotenoid production by engineered microorganisms (*2*, *30*). *Dunaliella* can accumulate a large quantity of highly bioavailable β-carotene, the 9-cis stereoisomer, under appropriate growth conditions (*3*). This remarkable property may result from the efficient catalytic function of carotenogenic enzymes in *Dunaliella*. Consequently, molecular identification on carotenogenesis in *Dunaliella* may promote the massive accumulation of highly bioavailable carotenoids in heterologous expression systems. However, the carotenogenic pathway of *Dunaliella* is still unclear due to the insufficient elucidation of the carotenogenesis of eukaryotic algae, especially the procedure of carotene desaturation. As a result, the rare analysis of the *zds* of eukaryotic algae has not been reported until now. Presently, the cDNA of *zds* was isolated from *D. salina* via the RACE PCR approach. The presence of *zds* in *Dunaliella* is favorable toward promoting the elucidation of the carotenogenic pathway of eukaryotic algae as well as the evolution of secondary metabolism. Moreover, the isolation of the cDNA of *D. salina zds* may play a crucial role in heterologous expression for the production of highly bioavailable carotenoids. Currently, the DNA sequences of carotenogenic enzymes were elucidated on the basis of previous studies. In future, investigations can now be focused on the carotenoid isomerases and the regulatory mechanisms of carotenogenic enzymes.

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