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Cloning, Expression, and Functional Characterization of the *Dunaliella salina* 5-enolpyruvylshikimate-3-phosphate Synthase Gene in *Escherichia coli*

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5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EC 2.5.1.19) is the sixth enzyme in the shikimate pathway which is essential for the synthesis of aromatic amino acids and many secondary metabolites. The enzyme is widely involved in glyphosate tolerant transgenic plants because it is the primary target of the nonselective herbicide glyphosate. In this study, the *Dunaliella salina* EPSP synthase gene was cloned by RT-PCR approach. It contains an open reading frame encoding a protein of 514 amino acids with a calculated molecular weight of 54.6 KDa. The derived amino acid sequence showed high homology with other EPSP synthases. The *Dunaliella salina* EPSP synthase gene was expressed in *Escherichia coli* and the recombinant EPSP synthase were identified by functional complementation assay.

Keywords: *Dunaliella salina*, EPSP synthase, glyphosate tolerant

5-enolpyruvylshikimate-3-phosphate (EPSP) synthase is a critical enzyme in the shikimate pathway for the biosynthesis of aromatic amino acids (Phe, Tyr, and Trp) and other essential aromatic compounds in bacteria, fungi, and plants (Schonbrunn *et al.*, 2001). It catalyzes the formation of EPSP and the inorganic phosphate from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) (Oliveira *et al.*, 2001).

The enzyme is widely researched because it has been identified as the primary target of the nonselective herbicide glyphosate (Priestman *et al.*, 2005). Glyphosate shows competitive inhibition for PEP but no inhibition for S3P. It binds to the EPSP synthase in place of PEP and forms a stable enzyme-S3P-glyphosate ternary complex (McDowell *et al.*, 2004). Therefore, glyphosate can block the synthesis of aromatic amino acids and result in the death of organism. Expression of a foreign glyphosate-tolerant EPSP synthase by gene engineering or amplification of EPSP synthase has been demonstrated to confer tolerance to the glyphosate (Stallings *et al.*, 1991). Many mutated sites which can generate resistance to glyphosate have been reported, such as Pro101 (Stalker *et al.*, 1985) of *Salmonella typhimurium* EPSP synthase, Gly96 (Padgette *et al.*, 1991) and Thr42 (He *et al.*, 2003) of *Escherichia coli* EPSP synthase as well as Lys23 (Huynh *et al.*, 1988) of mature *Petunia hybrida* EPSP synthase.

Furthermore, the shikimate pathway is completely absent in mammals, fish, birds, reptiles, and insects. Therefore, it is an attractive target for novel antibiotics (Alibhai *et al.*, 2001). It was reported that bacterial mutants lacking EPSP synthase can not survive and the interaction with other enzymes in shikimate pathway can also induce the antibacterial effect (Du *et al.*, 2000).

Two classes of EPSP synthase have been identified (Sun *et al.*, 2006). Class I enzymes are the research object of most studies about EPSP synthase and they are naturally sensitive to glyphosate. They have been found in plants, bacteria, and fungi, such as *Arabidopsis thaliana*, *Brassica napus*, *Oryza sativa*, *E. coli*, and *Mycobacterium tuberculosis*. Class II EPSP synthases have been identified from *Bacillus subtilis*, *Agrobacterium tumefaciens* CP4, and *Pseudomonas* sp. strain PG2982. In contrast to Class I EPSP synthase, they show natural tolerance to glyphosate.

Although EPSP synthase have been found in many species, it has never been cloned from algae. In this study, the gene encoding *D. salina* EPSP synthase, named *DsaroA*, was cloned and expressed in *E. coli* for the first time. The recombinant EPSP synthase were identified by functional complementation assay.

Materials and Methods

Reagents, bacterial strain, and enzymes

Trizol reagent was purchased from Invitrogen (USA). 5' RACE kit (SMART™ RACE cDNA Amplification kit) was purchased from Clontech (USA). 3' RACE kit (3'-Full RACE Core Set), pMD18-T Vector and all the ligases and endonucleases were purchased from Takara (Japan). Glyphosate was purchased from Sigma (USA). *E. coli* strain ER2799 (with the EPSP synthase gene deleted from its genome) was a kind gift from Professor Thomas C. Evans, Jr. (New England Biolabs, USA).

Plant material and growth conditions

Dunaliella salina strain 435 was obtained from Institute of Hydrobiology, Chinese Academy of Sciences. The algae grew

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with 16 h light and 8 h darkness at 25°C. The medium contained 1.5 M NaCl, 5.0 mM $NaNO_3$, 5.0 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $NaH_2PO_4 \cdot 2H_2O$, 1.0 mM KCl, 10.0 mM $NaHCO_3$, 0.3 mM $CaCl_2 \cdot 2H_2O$ and a mixture of micronutrients (Pick *et al.*, 1986). Cells in exponential phase were used for experiments.

Isolation of EPSP synthase expression sequence tag (EST) from D. salina

The cDNA library of *D. salina* has been constructed in our previous work (data not shown). Parts of the clones were sequenced randomly, then these sequenced clones were submitted to BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) for alignment. As a result, a cDNA sequence of 466 bp with high homology with other EPSP synthases was found, but it did not contain the entire open reading frame (ORF).

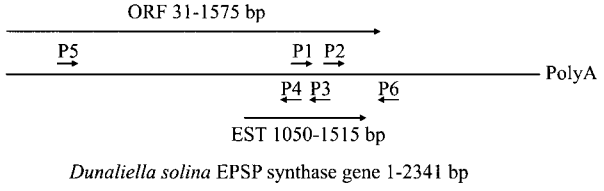

Fig. 1. Primer design and the cloned *D. salina* EPSP synthase gene. Primer5 and primer6 were designed to amplify the fragment encoding the mature *D. salina* EPSP synthase (without transit peptide).

Amplification of the cDNA ends

Total RNA was extracted using trizol reagent. 3' RACE was performed using the 3' RACE kit. 5' RACE was performed using the 5' RACE kit. According to the EST sequence, two primers, primer1; 5'-CATTGTCAACGAGACCCGTAAG-3' and primer2; 5'-GGACTACTGTGTCATCACCCCT-3' were designed for 3' RACE. Primer2 was used for nested amplification. In the same way, primer3; 5'-TTGACAATGGCGAC CATGCGTTCCGT-3' and primer4; 5'-TGCAGCGGCTTGC CAAAGGCTGAAG-3' were designed for 5' RACE (Fig. 1). Other manipulations followed the manufacturer's instructions. Fragments amplified by 3' and 5' RACE were purified by gel extraction, then cloned into pMD18-T Vector and sequenced. Sequencing and primer synthesis were performed at Sangon (Shanghai Sangon Biological Engineering Technology & Service Co., Ltd, China). Sequence data in this article have been de-

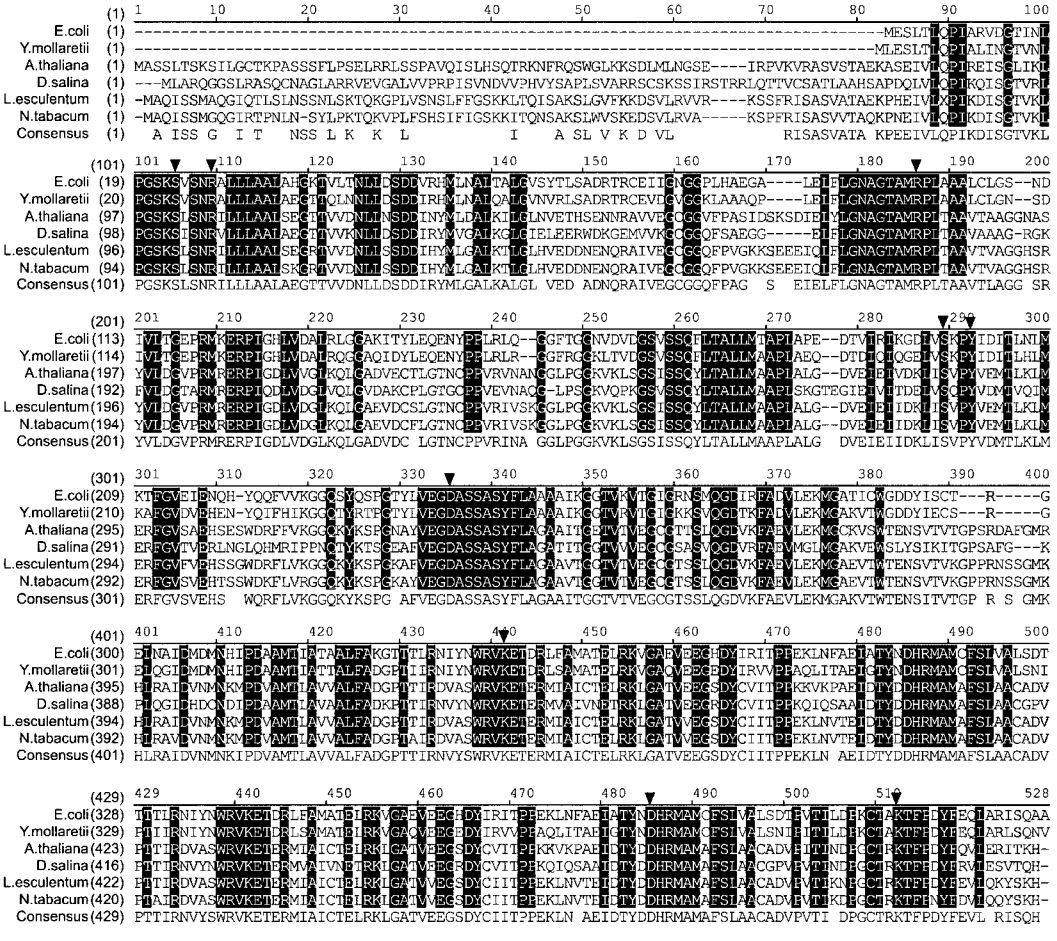

Fig. 2. Alignment of the amino acid sequence of the *D. salina* EPSP synthase with *Arabidopsis thaliana* EPSP synthase (GenBank accession number AAK64123), *Lycopersicon esculentum* EPSP synthase (P10748), *Nicotiana tabacum* EPSP synthase (P23981), *Escherichia coli* EPSP synthase (NP_415428) and *Yersinia mollaretii* EPSP synthase (ZP_00826904). The identical amino acids are shown as white letters on a black background. The conserved sites involved in enzyme activity are marked with triangles.

posited at GenBank under accession number EF051488.

Construction of the DsaroA gene contained vector

Primer5; 5'-CGGAATTCTCTGCTACTTTGGCGGCTCACA G-3', forward and primer6; 5'-CCCTCGAGAAGGACCCCA GGACTCAGAAGTCC-3', backward were designed to amplify *DsaroA* gene. An *Eco*RI and an *Xho*I site were added in the forward and backward primers, respectively (underlined). PCR was performed using the following procedure: initial denaturation, 94°C for 3 min; 30 cycles with 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min; final extension at 72°C for 5 min. PCR product was digested with *Eco*RI and *Xho*I. The digested fragments were purified by gel extraction, then ligated to the pGEX-4T-1 vector to generate the recombinant expression vector, named pGEX*DsaroA*. The ligated sequence was confirmed by sequencing.

Expression of the DsaroA gene in E. coli

E. coli JM109 cells were transformed with the pGEX*DsaroA*. Then the strain was inoculated into LB medium containing 50 mg/L ampicillin and grown at 37°C. When OD_{600} reached 0.6, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to final concentration of 0.1 mM and incubation was continued for 4 h. Protein expression was detected by SDS-PAGE.

Functional complementation and growth curves

E. coli ER2799 cells were transformed with the pGEX*DsaroA*. Then the strain was inoculated into M9 minimal medium [6.8 g/L Na_2HPO_4, 3 g/L K_2HPO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 0.12 g/L $MgSO_4$, 0.4% (w/v) glucose] containing 50 mg/L ampicillin and 0.1 mM IPTG. Cells were grown with shaking at 37°C. *E. coli* ER2799 and *E. coli* ER2799 transformed with pGEX-4T-1 were used as control. The cell growth was observed after 72 h incubation.

E. coli JM109 transformed with pGEX-4T-1 and pGEX*DsaroA* was inoculated into LB medium containing 50 mg/L ampicillin and 0.1 mM IPTG. When OD_{600} reached 0.2, glyphosate at various concentrations were added. Cells were grown at 37°C and the densities were measured at OD_{600}.

Results and Discussion

Cloning of DsaroA gene

DNA fragments about 900 and 1200 bp were cloned by 3' RACE and 5' RACE respectively. Finally, a cDNA sequence of 2341 bp was obtained. It contained an ORF of 1545 bp flanked by 30 and 766 bp of 5' UTR and 3' UTR regions respectively (Fig. 1). The 3' UTR contained the polyA which showed the cloned cDNA had reached the 3' end of the *DsaroA* gene.

Analysis of the cloned gene

The cloned cDNA sequence was submitted to the BLAST server for alignment. The result showed that the cloned cDNA belonged to EPSP synthase. The ORF encoded a protein of 514 amino acids with a calculated molecular weight of 54.6 kDa. The multiple alignments of the deduced amino acid sequence with other EPSP synthases were performed by Vector NTI ver. 9.0. As shown in Fig. 2, the *Dunaliella salina* EPSP synthase showed a high degree of sequence homology to the *Arabidopsis thaliana* EPSP synthase (56.3% sequence identity), the *Lycopersicon esculentum* EPSP synthase (55.7%), the *Nicotiana tabacum* EPSP synthase (55.3%), the *Escherichia coli* EPSP synthase (44.4%), and the *Yersinia mollaretii* EPSP synthase (46.5%). Several amino acid residues, Ser23, Arg27, Ser197, and Tyr200 (Stauffer *et al.*, 2001) of *E. coli* EPSP synthase which involved in S3P binding are well conserved in all enzymes. Moreover, Lys340 (Huynh, 1990), Asp242, Asp384, and Lys411 (Shuttleworth *et al.*, 1999), identified as the active sites, and the putative PEP binding site, Arg100 (Padgette *et al.*, 1991), are also conserved.

In plants, EPSP synthases are localized primarily in chloroplasts and other plastids (Forlani *et al.*, 1994). They need a transit peptide for translocation of the enzyme to the plastids where the transit peptide is cleaved to form the mature protein. The potential N-terminal chloroplast transit peptide (cTP) of *D. salina* EPSP synthase were determined with the ChloroP software provided online (http://www.cbs.dtu.dk/services/ChloroP/) (Emanuelsson *et al.*, 1999). The result showed that the EPSP synthase of *D. salina* had a potential cTP of 70 amino acids (data not shown). It is also shown in Fig. 2 that all the plant EPSP synthases, including *D. salina*, have an excess peptide about 75 amino acids compared to the bacteria at N-terminal. It is exactly the transit peptide that is absent in the bacteria EPSP synthase. The length of the predicted cTP agrees with the cTP of petunia and tomato EPSP synthases which are 72 and 76 amino acids respectively (Gasser *et al.*, 1988).

Expression of the DsaroA gene in E. coli

DNA fragment encoding the mature protein of *D. salina* EPSP synthase (without transit peptide) was amplified by PCR using primer5 and primer6. The 1391 bp PCR product was inserted into the pGEX-4T-1 vector and transformed into *E. coli* JM109 to express the recombinant protein (Fig. 3). The recombinant protein was expressed efficiently after 1 h induction of 0.1 mM IPTG. Expression became more efficient with increasing induction time. The molecular weight of the recombinant protein is about 73 kDa, it agrees with the expected molecular weight (26 kDa GST tag protein added with the 47 kDa mature *D. salina* EPSP synthase).

Functional complementation

To determine the activity of the recombinant *D. salina* EPSP synthase *in vivo*, pGEX*DsaroA* was transformed into the EPSP synthase gene deleted *E. coli* strain ER2799. The EPSP synthase is required for aromatic amino acid synthesis so that *E. coli* ER2799 will not grow on M9 minimal medium unless complemented with an active EPSP synthase (Chen *et al.*, 2001). As shown in Fig. 4, neither *E. coli* ER2799 nor *E. coli* ER2799 transformed with pGEX-4T-1 could grow on M9 minimal medium even after 72 h incubation. In contrast, when pGEX*DsaroA* was transformed into *E. coli* ER2799, the strain could grow on M9 minimal media. This result indicated that the recombinant *D. salina* EPSP synthase can catalyze the formation of EPSP in *E. coli* and sustain the synthesis of aromatic amino acid. In addition, the recombinant protein did not contain the chloroplast

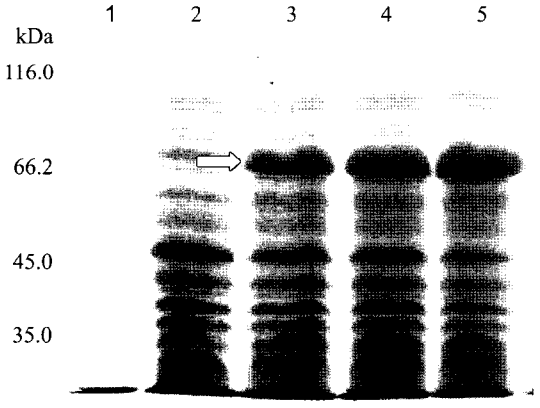

Fig. 3. SDS-PAGE analysis of the expression of the recombinant *D. salina* EPSP synthase. *E. coli* JM109 transformed with pGEX*DsaroA* was induced with 0.1mM IPTG. Proteins were separated by 15% SDS-PAGE. Lane 1, marker; lane 2, 0 h induction; lane 3, 1 h induction; lane 4, 2 h induction; lane 5, 4 h induction. The arrow indicates the expected protein products.

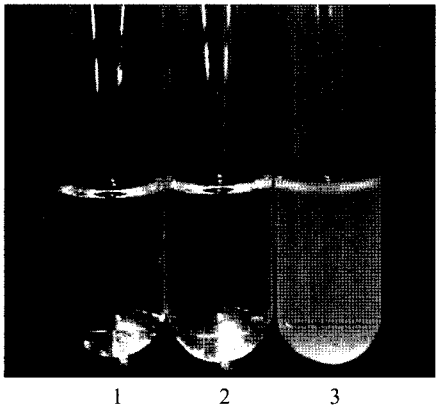

Fig. 4. *In vivo* complementation of *E. coli* ER2799 by the constructs of the *DsaroA*. The growth was observed after 72 h incubation. 1, *E. coli* ER2799; 2, *E. coli* ER2799 transformed with pGEX-4T-1; 3, *E. coli* ER2799 transformed with pGEX*DsaroA*.

transit peptide. It showed that the transit peptide made no contribution to the enzyme activity. It was only needed for translocation of the enzyme to the chloroplast.

Cells carrying DsaroA gene are tolerant to glyphosate

It was reported that amplification of EPSP synthase could confer tolerance to the glyphosate (Stallings *et al.*, 1991). To determine whether the over expression of the recombinant *D. salina* EPSP synthase can bring glyphosate tolerance to *E. coli*, the growth curves of *E. coli* JM109 harboring pGEX-4T-1 and pGEX*DsaroA* at various concentration of glyphosate was tested. As shown in Fig. 5, *E. coli* JM109 transformed with pGEX-4T-1 and pGEX*DsaroA* had the same growth rate when no glyphosate added to the culture. The growth rate of *E. coli* JM109 with pGEX-4T-1 was inhibited approximately 50% by 5 mM glyphosate (Fig. 5A). Inhibition became more severe with increasing concentration of glyphosate. At 50 mM, growth was barely detectable.

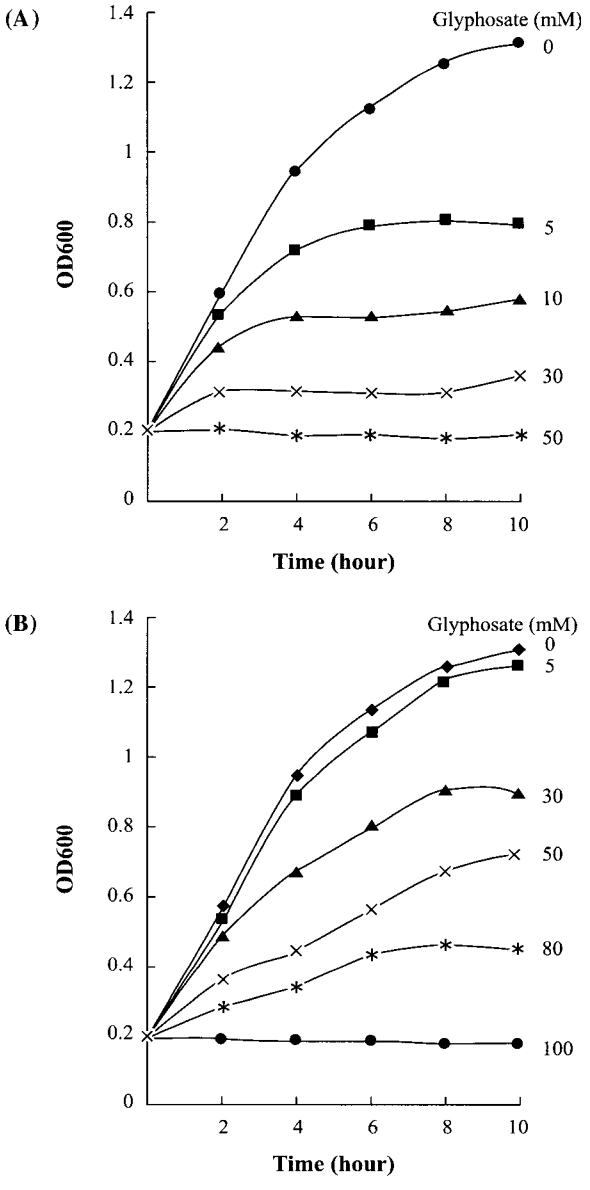

Fig. 5. Growth curves of *E. coli* JM109 harboring pGEX-4T-1 and pGEX*DsaroA* in the various concentration of glyphosate. (A) *E. coli* JM109 carrying pGEX-4T-1; (B) *E. coli* JM109 carrying pGEX*DsaroA*.

The results for growth of *E. coli* JM109 with pGEX*DsaroA* were different (Fig. 5B). During 10 h of treatment, the growth rate was minimally affected by 5 mM glyphosate and reduced by 50% in 50 mM glyphosate. Cells could grow slowly in 80 mM glyphosate and no growth could be detected in 100 mM glyphosate. When growth rates over 10 h are compared, cells with *DsaroA* gene show the same growth rate in 50 mM glyphosate as control cells in 5 mM glyphosate. This indicates a 10-fold increase in glyphosate tolerance.

In conclusion, we cloned the EPSP synthase gene from *D. salina* and expressed it in *E. coli* for the first time. The in vivo experiments showed that the activity of *E. coli* EPSP synthase can be reconstituted by recombinant *Dunaliella salina* EPSP synthase and over expression of *Dunaliella salina*

EPSP synthase can bring the glyphosate tolerance to *E. coli*.

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