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Short Communication

Single Amino Acid Changes in the 5-Enolpyruvylshikimate-3-phosphate Synthase from *Agrobacterium* sp. Strain CP4 and *Synechocystis* sp. PCC6803 Alter Enzyme Activity and Glyphosate Sensitivity

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The enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) catalyzes the sixth step in the shikimate pathway, which is essential for the biosynthesis of aromatic amino acids and aromatic compounds in algae, higher plants, bacteria, and fungi. However, the enzyme is absent from animals.[1,2] EPSP synthase (EPSPS) converts shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to EPSP and inorganic phosphate. It is inhibited by glyphosate [*N*-(phosphonomethyl)glycine], an active ingredient of the broad-spectrum herbicide, Round-up.[3] The inhibitor, glyphosate, is competitive with PEP, while uncompetitive with S3P.[4,5] Glyphosate (MW = 169.1), as an analog of PEP (MW = 167.0), appears to occupy the same binding site of PEP, mimicking an intermediate state of the ternary enzyme-substrates complex.[6]

The glycine at the 96th amino acid of *Escherichia coli* EPSPS, which is almost completely conserved in various EPSPSs, is an important site for the binding of PEP and glyphosate. The mutation of the 96th glycine to alanine (G96A) confers glyphosate insensitivity to EPSPS from *E. coli* and *Klebsiella pneumoniae*.[7,8] The same amino acid change (Gly to Ala) also resulted in a greatly reduced affinity for glyphosate in higher plants, such as petunia, tomato, soybean, maize, and *Arabidopsis*.[9] In addition, plastid-expressed, G100A-mutated EPSPS from the *Achromobacter* strain LBAA conferred a very high level of glyphosate resistance in higher plants.[10] All these results are consistent with the notion that the glycine residue is a part of the conserved EPSPS active site.[9]

Multiple sequence alignment in this study indicates that the unique 100th alanine of EPSPS from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) and the 109th glycine of EPSPS from *Synechocystis* sp. PCC6803 correspond to the 96th glycine of *E. coli* EPSPS (Table 1). CP4 EPSPS has a strong glyphosate tolerance as well as high affinity to PEP due to its unique amino acid composition at the active site. For this reason, CP4 EPSPS has been used to develop glyphosate-resistant crops for commercial purpose.[11] Mutations of the 100th alanine to glycine, serine, and valine in CP4 EPSPS were introduced in this study in order to figure out the effect of the R-group size of the 100th amino acid on enzyme activity and glyphosate sensitivity. *E. coli* BL21(DE3) transformants expressing parental and point-mutated CP4 EPSPSs were produced using pET-30a(+) expression system (Novagen, USA), and their growth rates were measured in the absence or presence of glyphosate. Overall, parental and point mutated CP4 EPSPSs were expressed in soluble fraction and their expression levels were similar (Fig. 1A). The growth rates of all five transformants were almost the same as in M9 minimal medium[12] supplemented with 30 $\mu g \cdot ml^{-1}$ of kanamycin as a selective chemical (Fig. 1B). *E. coli* BL21(DE3) has its own EPSPS. However, its resistance to glyphosate is very weak, therefore, the effect caused by its own EPSPS could be minimal and ignored. In fact, the transformant containing the vacant pET-30a(+) did not grow at all in the presence of glyphosate. Hence, cell growth rates in the presence of glyphosate were swayed by the transformed CP4 EPSPSs, and these could be the barometer for the differences in the enzyme activities and glyphosate sensitivities. The transformant expressing parental CP4 EPSPS showed the fastest growth in the presence of glyphosate (Fig. 1C & 1D), and OD_{600} reached 1.5 after 15 hours of culture at 37°C. The transformant expressing A100G CP4 EPSPS (A100G transformant) showed a similar growth rate with the parental transformant in the presence of 10 mM glyphosate, while showing some growth retardation in the

Table 1. Sequence alignment of the conserved region of several EPSPSs around the 96th glycine of *E. coli* EPSPS.

Source	Sequences
Staphylococcus aureus	HQVLYTGNSG[94]TTTRLLAG
Bacillus subtilis 1A2	ESLLDVGNSG[91]TTIRLMLG
Achromobacter sp. strain LBAA	EAALDFGNAG[100]TGARLTMG
Pseudomonas sp. strain PG2982	EAALDFGNAG[100]TGARLTMG
Agrobacterium sp. strain CP4	EAPLDFGNAA[100]TGCRLTMG
Synechocystis sp. PCC6803	STVLDAGNSG[109]TTMRLMLG
Samonella typhimurium	ALELFLGNAG[96]TAMRPLAA
Escherichia coli	ALELFLGNAG[96]TAMRPLAA
Consensus key	* **:. * * .

Consensus key means as follows: '*', single fully conserved residue; ':', highly conserved residue '.', weakly conserved residue ' ', no consensus.

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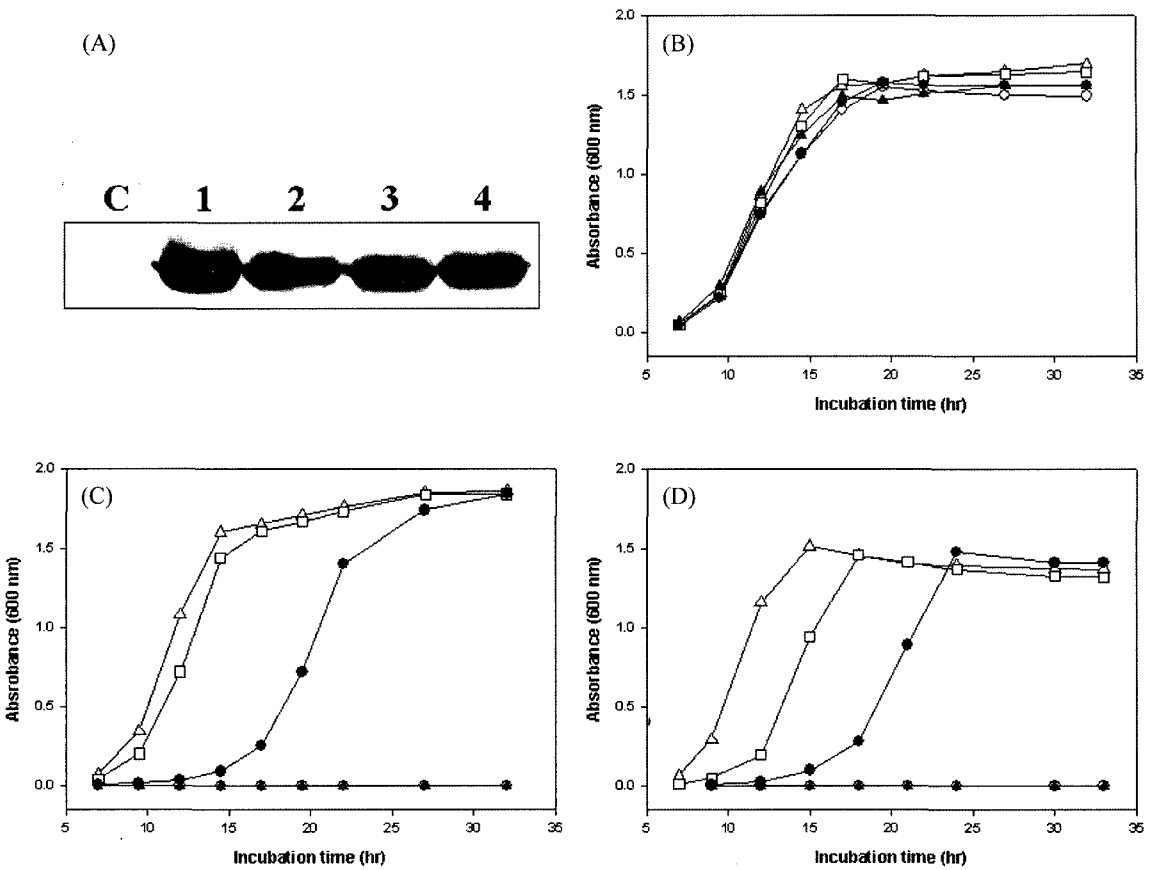

Fig. 1. CP4 EPSPS expression levels and growth rates of *E. coli* BL21(DE3) transformants expressing parental, A100G, A100S and A100V CP4 EPSPS. (A) Immunoblot analysis of the expressed CP4 EPSPS using anti-CP4 EPSPS polyclonal antibody. Total soluble proteins were prepared from *E. coli* BL21(DE3) transformants cultured in M9 minimal medium supplemented with 30 $\mu g \cdot ml^{-1}$ of kanamycin and 0.01 mM of IPTG. Lane C, *E. coli* BL21(DE3) transformant harboring the vacant pET-30a(+) as a negative control; lane 1, parental type; lane 2, A100G; lane 3, A100S; lane 4, A100V. Growth rates of *E. coli* BL21(DE3) transformants were measured using M9 minimal medium supplemented with (B) 30 $\mu g \cdot ml^{-1}$ of kanamycin as a inoculation control, (C) 30 $\mu g \cdot ml^{-1}$ of kanamycin, 0.01 mM of IPTG, and 10 mM of glyphosate, and (D) 30 $\mu g \cdot ml^{-1}$ of kanamycin, 0.01 mM of IPTG, and 20 mM of glyphosate, respectively. -○-, *E. coli* BL21(DE3) transformant harboring the vacant pET-30a(+) as a negative control; -△-, parental type; -□-, A100G; -●-, A100S; -▲-, A100V.

presence of 20 mM glyphosate. Severe growth retardation was observed in the case of the A100S transformant; OD_{600} reached 1.5 after approximately 25 hours of the culture. Furthermore, the A100V transformant could not grow at all in the presence of glyphosate (Fig. 1C & 1D). If alanine (R-group; -CH3) substitutes for glycine (R-group; -H) at the 96th amino acid in *E. coli* EPSPS, the methyl group substantially reduces the accessible cavity for the binding of glyphosate. In this case, glyphosate tolerance is often paralleled by a decrease in the affinity of EPSPS for PEP, because glyphosate and PEP occupy the same binding site.[7] However, the 100th alanine of CP4 EPSPS turned out to be the best fitting amino acid, whose R group size is most suitable for the binding of PEP and resistance to glyphosate. When this alanine was replaced by other amino acid which has a larger R-group, such as serine or valine, the enzyme activity was decreased or even completely lost, probably because PEP along with glyphosate, could not bind to the enzyme due to the reduced accessibility of the substrate toward the active site, thereby resulting in severe growth retardation or no growth of the transformed bacteria in the presence of glyphosate. In this context, it can be inferred that the growth of the A100V transformant in the absence of glyphosate originated from the EPSPS activity of the host bacteria.

The entire nucleotide sequence of EPSPS isolated from photosynthetic cyanobacteria *Synechocystis* sp. PCC6803, which has a naturally weak tolerance to glyphosate, has been reported.[13,14] *E. coli* transformants containing the plasmid pET-30a(+) and expressing parental and G109A EPSPS from *Synechocystis* sp. PCC6803, were shown to have similar growth rates when they were cultured in M9 medium without glyphosate (Fig. 2A). The parental and G109A EPSPS from *Synechocystis* sp. PCC6803 were expressed mostly in insoluble fraction and could not cross-react with anti-CP4 EPSPS polyclonal antibody (data not shown). The transformant expressing parental EPSPS from *Synechocystis* sp. PCC6803, did not grow at all in the presence of glyphosate. On the other hand, the transformant expressing G109A EPSPS from

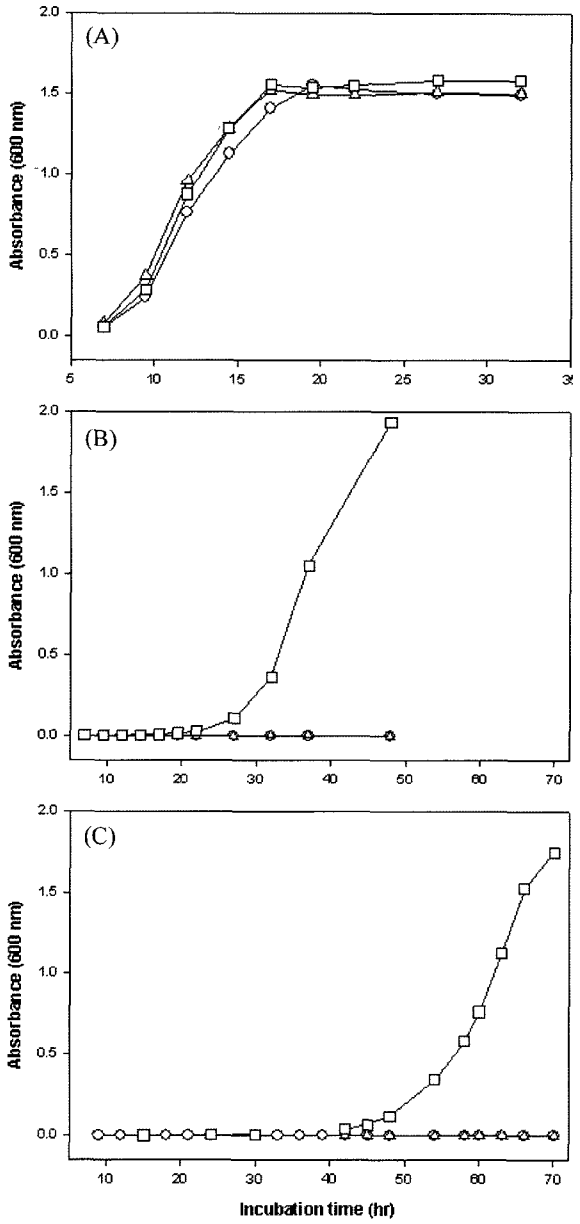

Fig. 2. Growth rates of *E. coli* BL21(DE3) transformants harboring parental and G109A EPSPS from *Synechocystis* sp. PCC6803. Growth rates of *E. coli* BL21(DE3) transformants were measured using M9 minimal medium supplemented with (A) 30 $\mu g \cdot ml^{-1}$ of kanamycin as a inoculation control, (B) 30 $\mu g \cdot ml^{-1}$ of kanamycin, 0.01 mM of IPTG, and 10 mM of glyphosate, and (C) 30 $\mu g \cdot ml^{-1}$ of kanamycin, 0.01 mM of IPTG, and 20 mM of glyphosate, respectively. -○-, *E. coli* BL21(DE3) transformant harboring the vacant pET-30a(+) as a negative control; -△-, parental type; -□-, G109A.

Synechocystis sp. PCC6803 could grow, although its OD_{600} could reach 1.5 after about 40 hours of incubation in the presence of 10 mM glyphosate (Fig. 2B), and after about 65 hours in the presence of 20 mM glyphosate (Fig. 2C). The growth rate of the transformant expressing G109A EPSPS from *Synechocystis* sp. PCC6803 was relatively lower than those of the transformants harboring various CP4 EPSPS (except for A100V mutant) probably due to the low level of the soluble G109A EPSPS enzyme in host bacteria (data not shown). In conclusion, the mutation of 109th glycine to alanine confers glyphosate insensitivity to the photosynthetic bacteria, *Synechocystis* sp. PCC6803. Therefore, the G109A-point mutated *epsps* gene from *Synechocystis* sp. PCC6803 could be exploited as a selection maker in the transformation of bacteria and plastids of higher plants.[15]

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