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SHORT COMMUNICATION

The three proline residues (P25, P242, and P434) of *Agrobacterium* CP4 5-enolpyruvylshikimate-3-phosphate synthase are crucial for the enzyme activity

Kyungsu Kang · Yong-Mei Jin · Hyesung Jeon · Sang-Ryoung Park · Dae-geun Song · Joo Young Lee · Cheol-Ho Pan · Minkyun Kim

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Abstract Multiple sequence alignments showed that the prolines at the 25th, 129th, 153rd, 242nd, 322nd, and 434th amino acids in 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Agrobacterium* sp. strain CP4 are strongly conserved in various prokaryotic EPSPS proteins. Single point mutations of the conserved prolines to alanine (P25A, P153A, P242A, P322A, and P434A) were introduced in the CP4 EPSPS in order to investigate the importance of the conserved prolines for the enzyme properties. The point mutations caused decreases in substrate binding affinity and catalytic efficiency as well as the glyphosate resistance, in general. Especially, the 25th and 242nd prolines located in the polypeptide hinges connecting top and bottom domains of CP4 EPSPS as well as the 434th proline at the C-terminus of the enzyme turned out to be crucial for the enzyme activity.

Keywords *Agrobacterium* sp. strain CP4 · 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) · Glyphosate · Herbicide resistance · Proline residue

K. Kang · Y.-M. Jin · H. Jeon · S.-R. Park · M. Kim (✉)
Department of Agricultural Biotechnology,
Center for Agricultural Biomaterials,
Research Institute for Agriculture and Life Sciences,
Seoul National University, Seoul 151-921,
Republic of Korea
e-mail: mkkim3@snu.ac.kr

K. Kang · D. Song · J. Y. Lee · C.-H. Pan
Natural Products Research Center,
Korea Institute of Science and Technology Gangneung Institute,
Gangwon-do 210-340, Republic of Korea

Introduction

5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) is an enzyme that catalyzes the biosynthesis of aromatic amino acids in bacteria, fungi, algae, and higher plants (Herrmann 1995; Kishore and Shah 1988). The enzyme is competitively inhibited by glyphosate, a widely used nonselective herbicide (Boocock and Coggins 1983; Steinrucken and Amerhein 1984; Malik et al. 1989; Duke and Powles 2008). However, EPSPS from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) has been known to have the resistance to glyphosate. Therefore, the gene encoding CP4 EPSPS has been used to develop genetically modified herbicide resistant crops (Padgette et al. 1996; Kang and Kim 2006a).

Previously, we reported that the 100th alanine of CP4 EPSPS is crucial for the enzyme activity and glyphosate sensitivity based on the growth rates of the *Escherichia coli* BL21 (DE3) transformants that expressed the point-mutated CP4 EPSPS in the presence of glyphosate (Kang and Kim 2006b). It was found that the growth rate of the bacterial transformant in the presence of glyphosate was governed only by the transformed CP4 EPSPS due to the complete inhibition of the endogenous EPSPS of *E. coli* host strain by glyphosate. Therefore, the growth rate of the *E. coli* transformant in the presence of glyphosate served as a barometer for the enzyme activity or glyphosate sensitivity of the point-mutated CP4 EPSPS. In this study, we employed the same experimental strategy and also the in vitro biochemical assays to investigate the importance of the proline residues of the CP4 EPSPS conserved among the various prokaryotic organisms for the enzyme activity and glyphosate sensitivity.

Materials and methods

Point mutations of CP4 EPSPS

Point mutations of *cp4-epsps* gene were generated by using a PCR-based overlap extension technique (Ho et al. 1989). The plasmid containing the parental *cp4-epsps* gene, pET-wild-CP4 EPSPS (Kang and Kim 2006b), was used as a template for the PCR-based point mutations. The point-mutated *cp4-epsps* genes were cloned into the pET-30a(+) vector (Novagen, USA), and then *E. coli* BL21(DE3) transformants expressing point-mutated CP4 EPSPSs were obtained.

Measurements of the growth rates of bacterial transformants

The growth rates of *E. coli* BL21(DE3) transformants expressing parental and point-mutated CP4 EPSPSs were measured by spectrophotometry as described previously (Kang and Kim 2006b).

Immunoblot analyses

The expression levels of the point-mutated CP4 EPSPS in *E. coli* were determined through western blot analyses by using antiserum raised against CP4 EPSPS, as described previously (Kang and Kim 2006b). Total soluble proteins were prepared from *E. coli* BL21 (DE3) transformants cultured in M9 minimal medium supplemented with 30 μg/mL kanamycin and 0.01 mM IPTG by using Bugbuster reagent (Novagen). Ten micrograms of the proteins were then separated by electrophoresis on 10% SDS-PAGE gels and transferred to nitrocellulose membrane. The membranes were incubated with anti-CP4 EPSPS antibody, and subsequently with alkaline phosphatase-conjugated secondary antibody. BCIP and NBT were used as substrates for alkaline phosphatase assays (Invitrogen, USA).

Purification of the recombinant CP4 EPSPS enzymes and their kinetic analyses

The parental and point-mutated recombinant CP4 EPSPS proteins were purified from the total soluble proteins of the *E. coli* BL21 (DE3) transformants via two-step purification with Ni–NTA resin (Novagen) and ÄKTA FPLC system with Tricon™ Superdex200 10/300 column (Amersham biosciences, Sweden). Protein concentration was determined by using the Bio-Rad Protein Assay Kit (USA). The enzyme assay was performed according to the previous method (Eschenburg et al. 2002) with a minor modification. The activities of the CP4 EPSPSs were analyzed using a 96-well plate in 10 μL of 50 mM HEPES, pH 7.5, 200 mM KCl, 2 mM DTT at 25°C by determining the amount of inorganic phosphate produced in the reaction (Lanzetta et al. 1979). The reaction was started by addition of the enzyme. Eighty microliters of Lanzetta reagent (freshly prepared mixture: 300 mL of 0.035% malachite green-HCl, 100 mL of 4.2% ammonium molybdate in 4 N HCl, and 0.8 g of CHAPS) was added after 3 min of enzyme reaction. Then, color development was stopped after 3 min by addition of 10 μL of 34% (w/v) sodium citrate. Absorbance at 660 nm was measured by using Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, USA), and the amount of inorganic phosphate was calculated using a phosphate standard curve. Potassium dihydrogen phosphate (KH_2PO_3) was used as the standard for inorganic phosphate. Enzyme activity was expressed as μmol phosphate produced per min reaction time per mg enzyme (U/mg). The final concentration of enzyme used for the assay was 100 nM.

Results and discussion

Proline has a unique cyclic structure of the side chain that lacks a primary amine group ($–NH_2$). It confers an exceptional conformational rigidity on proline, making proline important for protein conformation (Balbach and Schmid 2000). Multiple sequence alignments showed that the prolines at the 25th, 129th, 153rd, 242nd, 322nd, and 434th amino acids in CP4 EPSPS are strongly conserved in various prokaryotic EPSPSs (Fig. 1a). In this study, mutations of the conserved prolines of CP4 EPSPS to alanine (P25A, P153A, P242A, P322A, and P434A) were introduced, in order to investigate the effect of the single point mutations at the conserved proline residues on the enzyme activity and glyphosate sensitivity that are closely linked with the structure of the enzyme. Alanine and proline residues have similar hydrophobicity (Bull and Breese 1974; Eisenberg and McLachlan 1986), and alanine substitution has been known as one of the most frequent changes for the proline residue in related proteins from homologous organisms, which often play critical roles to change the enzymatic properties (Schulz and Schirmer 1979; Kelly and Richards 1987; Garcia-Effron et al. 2008). The mutation of the 129th proline was excluded for the study because it is adjacent to the active site (128th arginine) of the enzyme (Fig. 1a). Recently, the three-dimensional structure of CP4 EPSPS was known to have the same overall folding pattern as the EPSPSs from *E. coli* and *Streptococcus pneumoniae* (Schönbrunn et al. 2001; Park et al. 2003; Funke et al. 2006). EPSPS can be divided into the top and bottom domains, which are connected by two strings of polypeptide chains. Especially, the 25th and 242nd prolines are located at each connection hinge of CP4

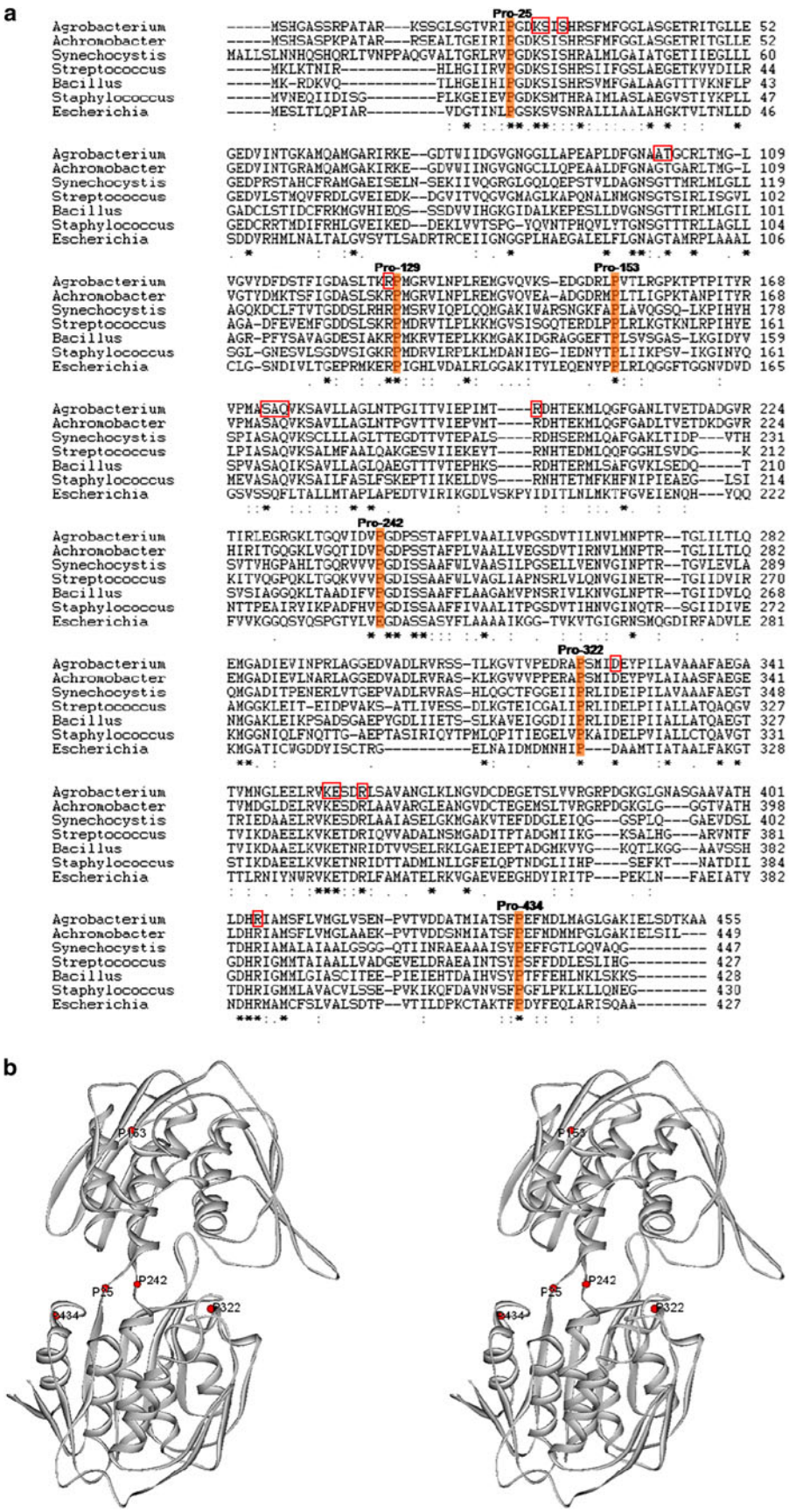

Fig. 1 Sequence alignment of the amino acid sequences of the various EPSPSs, and three-dimensional structure of CP4 EPSPS. **a** The amino acid sequences shown are from *Agrobacterium* sp. strain CP4 (Q9R4E4), *Achromobacter* sp. strain LBAA (P0A2Y5), *Synechocystis* sp. PCC6803 (Q59975), *Streptococcus pneumoniae* (1RF6A), *Bacillus subtilis* (P20691), *Staphylococcus aureus* (Q05615), and *Escherichia coli* (P0A6D3). Alignment was performed using CLUSTALW (1.82) program. Consensus key definitions as follows: *asterisks* identical in all sequences, *colon* conserved substitution, *dot* semi-conserved substitution. Strongly conserved proline residues are *shaded*. The important amino acids for ligand binding of CP4 EPSPS are indicated in *rectangular box* (Funke et al. 2006). **b** Stereo view of CP4 EPSPS structure. *Spheres* and their respective *numbers* indicate the positions of the conserved proline residues of CP4 EPSPS. Three-dimensional data were adopted from Funke et al. (2006) (PDB ID number 2gg4) and drawn by Discovery Studio Visualizer version 2.0 program (Accelrys Software, USA)

EPSPS (Fig. 1b) (Funke et al. 2006), implying their potential importance for the enzyme structure and function.

Immunoblot analyses showed that the single point mutations at the conserved prolines slightly decreased the level of the soluble mutant enzymes compared to the parental one in bacterial transformants: especially, P242A mutation caused approximately 2-fold decrease in the level of the soluble enzyme (Fig. 2a). The growth rates of all seven transformants were almost the same in M9 minimal medium in the absence of glyphosate (Fig. 2b). The parental transformant showed the fastest growth in the presence of 10 mM glyphosate, reaching 1.0 of the optical density at 600 nm (OD_{600}) approximately after 11.5 h of culture (Fig. 2c). The P153A and P322A transformants showed slightly lower growth rates compared to the parental one in the presence of the glyphosate. However, in the case of the P25A transformant, it showed significant growth retardation in the presence of the glyphosate, reaching 1.0 of OD_{600} approximately after 13 h of culture. This result indicates the importance of the 25th proline that is located at the connection hinge for the enzyme activity or glyphosate sensitivity. In addition, strong growth retardation was observed in the cases of the P242A and P434A transformants in the presence of the glyphosate, reaching 1.0 of OD_{600} approximately after 16 or 15 h of culture, respectively (Fig. 2c). The P242A transformant showed the slowest growth, and even slower growth rate was observed in the P242S transformant that has the amino acid substitution with bulkier serine residue compared to alanine substitution (data not shown). These results indicate the important roles of the 242nd proline located at the second connection hinge of CP4 EPSPS and the 434th proline at the C-terminus of the enzyme for the enzyme activity or glyphosate sensitivity.

The significant growth retardations of the P25A, P242A, and P434A transformants in the presence of glyphosate were likely due to the effects of the mutation of the conserved proline residues on either or both the enzyme activity and glyphosate sensitivity. Since bacterial growth rates shown in Fig. 2 are rather indirect clues for the enzyme activity or glyphosate sensitivity, we further performed in vitro biochemical studies to investigate the importance of the conserved proline residues for the enzyme kinetic properties as well as the glyphosate resistance.

In vitro biochemical studies were accomplished by using the purified parental or point-mutated recombinant CP4 EPSPS enzymes and various concentrations of the substrates (S3P and PEP). Michaelis–Menten curves were obtained in order to determine the kinetic values for the enzymes (Fig. 3a, b).

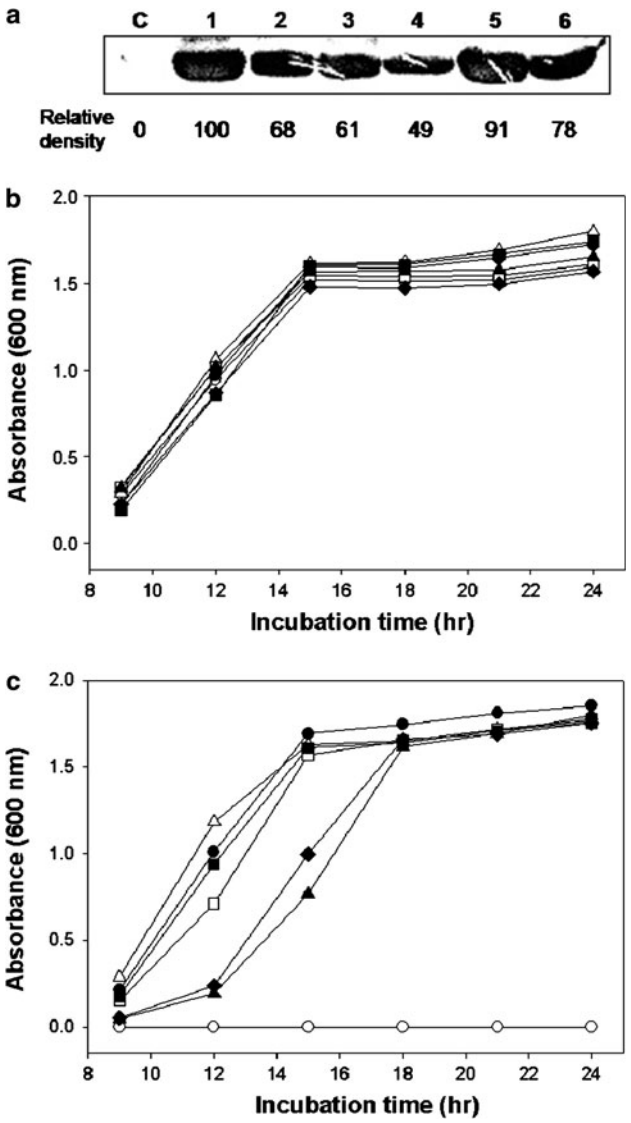

Fig. 2 The levels of the soluble CP4 EPSPS and its point-mutated enzymes in *E. coli* transformants and growth rates of the bacterial transformants. **a** Western blot analysis of the expressed CP4 EPSPS and its point-mutated enzymes. Total soluble proteins were prepared from *E. coli* BL21 (DE3) transformants cultured in M9 minimal medium supplemented with 30 μg/mL kanamycin and 0.01 mM IPTG. *Lane C E. coli* transformant harboring only the vacant pET-30a(+), *lane 1* parental CP4 EPSPS, *lane 2* P25A mutant, *lane 3* P153A mutant, *lane 4* P242A mutant, *lane 5* P322A mutant, *lane 6* P434A mutant. **b**,**c** Measurements of the growth rates of *E. coli* transformants via culture in M9 minimal medium supplemented with either 30 μg/mL kanamycin only as an inoculation control or 30 μg/mL kanamycin, 0.01 mM IPTG, and 10 mM glyphosate. *Open circles* *E. coli* transformant harboring the vacant pET-30a(+), *open triangles* parental type, *open squares* P25A, *filled circles* P153A, *filled triangles* P242A, *filled squares* P322A, *filled diamonds* P434A

The kinetic data were fit to the following equations using the SigmaPlot program (Systat Software, USA). First, the K_m values for S3P and PEP were determined by fitting the measured data to

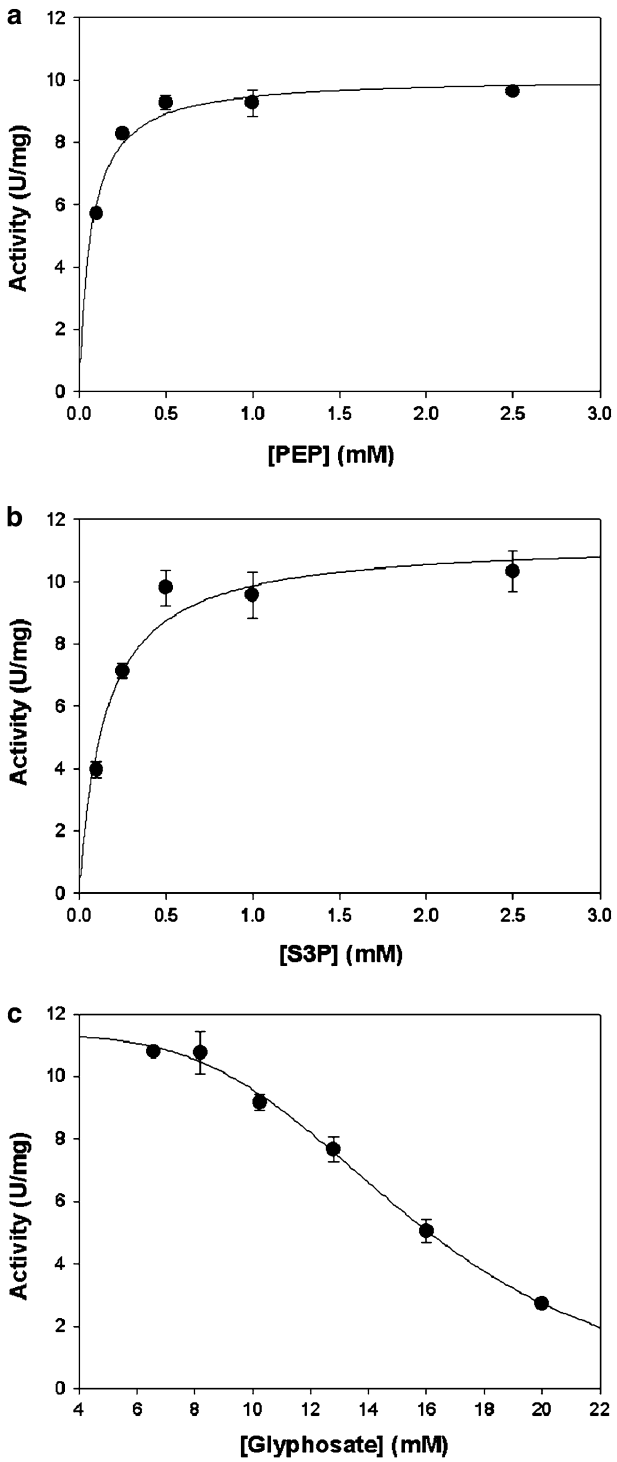

Fig. 3 Enzyme kinetics of the parental CP4 EPSPS. **a**, **b** Steady-state Michaelis–Menten plots for the parental CP4 EPSPS obtained with either 1 mM of S3P and increasing concentration of PEP ($n = 4$) or 1 mM of PEP and increasing concentration of S3P ($n = 4$). The measured data were fit to Eq. 1 (see text) and the calculated K_m values for PEP and S3P are shown in Table 1. **c** Inhibition of the parental CP4 EPSPS by glyphosate. Enzyme activities were assayed with 1 mM of PEP and S3P, respectively, and increasing concentration of glyphosate ($n = 2$). The measured data were fit to Eq. 2 (see text) and the calculated IC_{50} values are shown in Table 1

$$v = \frac{V_{\max} \times [S]}{K_m + [S]} \quad (1)$$

where v is the velocity of the reaction (expressed in U/mg), $V_{\max}$ is the maximum velocity, S is the concentration of S3P or PEP, and K_m is the Michaelis constant. Then, the IC_{50} value for the enzyme inhibition by glyphosate was determined by fitting data to

$$v = V_{\min} + \frac{V_{\max} - V_{\min}}{1 + \left(\frac{[I]}{IC_{50}}\right)^H} \quad (2)$$

where I and H are the concentration of glyphosate and Hillslope, respectively.

The results shown in Table 1 indicate that single point mutations in the conserved prolines of CP4 EPSPS caused the deterioration of the kinetic properties of the parental CP4 EPSPS (Fig. 3), such as substrate binding affinity and catalytic efficiency, in general. In other words, most of the K_m values for both substrates (S3P and PEP) were higher in the case of the mutant types of CP4 EPSPS compared to the parental enzyme. Consequently, the k_{cat}/K_m values for both substrates became lower in the mutant types of CP4 EPSPS compared to the parental one. In addition, the IC_{50} values for glyphosate were lower in the mutant types compared to the parental one, indicating that the point mutations also decreased the glyphosate resistance of the parental CP4 EPSPS (Fig. 3). Therefore, the growth retardations of the bacteria harboring the CP4 EPSPS mutants in the presence of glyphosate (Fig. 2) were most likely to be caused by the decreased enzyme activities and also the increased glyphosate sensitivity.

It was noteworthy that the point mutations in the conserved proline residues had greater negative effect on the kinetic efficiency for PEP than for S3P: in other words, the k_{cat}/K_m (PEP) values for the mutant types were 25–48% lower than the parental one, while k_{cat}/K_m (S3P) values for the mutant types were only 3–14% lower or even 6% higher than the parental one. Interestingly, however, P25A, P242A, and P434A transformants showed much lower k_{cat}/K_m values for S3P compared to P153A and P322A. These kinetic data, along with the relatively higher growth retardation of the corresponding transformants in the presence of glyphosate (Fig. 2), further support the idea that the 25th and 242nd prolines located at the connection hinges of CP4 EPSPS as well as the 434th proline at the C-terminus of the enzyme are crucial for the enzyme kinetic efficiencies for both PEP and S3P substrates.

In conclusion, the conserved prolines of the EPSPS of *Agrobacterium* sp. strain CP4 are important for the enzyme activity and glyphosate resistance. Especially, the 25th and 242nd prolines located at the connection hinges of CP4 EPSPS as well as the 434th proline turned out to be crucial

Table 1 K_m (PEP), k_{cat}/K_m (PEP), K_m (S3P), k_{cat}/K_m (S3P), and IC_{50} (glyphosate) for parental and mutant types of CP4 EPSPS

	K_m (PEP) (mM)[a]	k_{cat}/K_m (PEP) ($M^{-1}s^{-1}$)[a]	K_m (S3P) (mM)[b]	k_{cat}/K_m (S3P) ($M^{-1}s^{-1}$)[b]	IC_{50} (glyphosate) (mM)[c]
Parental	0.067 ± 0.011	1.3×10^5	0.14 ± 0.047	6.4×10^4	15.3 ± 2.01
P25A	0.14 ± 0.019	7.7×10^4 (41%↓)	0.18 ± 0.030	5.5×10^4 (14%↓)	12.8 ± 0.78 (16%↓)
P153A	0.16 ± 0.021	7.7×10^4 (41%↓)	0.21 ± 0.052	6.2×10^4 (3%↓)	11.6 ± 0.33 (24%↓)
P242A	0.10 ± 0.019	8.7×10^4 (33%↓)	0.23 ± 0.064	5.6×10^4 (13%↓)	13.3 ± 0.47 (13%↓)
P322A	0.080 ± 0.014	9.7×10^4 (25%↓)	0.11 ± 0.018	6.8×10^4 (6%↑)	12.8 ± 0.30 (16%↓)
P434A	0.10 ± 0.016	6.8×10^4 (48%↓)	0.14 ± 0.028	5.9×10^4 (8%↓)	13.0 ± 0.64 (15%↓)

The K_m and IC_{50} values, represented as mean ± standard error, were obtained by fitting the measured data to Eqs. 1 and 2, respectively (see text). Proportional changes (%) of the kinetic parameters (k_{cat}/K_m values for PEP and S3P, respectively) and IC_{50} (glyphosate) for mutant types of CP4 EPSPS as compared to the parental ones are indicated in parentheses

[a] Assayed with 1 mM of S3P and increasing concentration of PEP ($n = 4$)

[b] Assayed with 1 mM of PEP and increasing concentration of S3P ($n = 4$)

[c] Assayed with 1 mM of PEP and S3P, respectively, and increasing concentration of glyphosate ($n = 2$)

amino acids for catalytic efficiencies for both PEP and S3P substrates. The results obtained in this study provide valuable information to understand the biochemical properties of CP4 EPSPS, which would be useful for enzyme engineering to develop the new glyphosate-resistant crops in the future.

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