Genetics and Molecular Research

Cloning and characterization of 5-enopyruvylshikimate-3-phosphate synthase from *Pantoea* sp

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ABSTRACT. The shikimate pathway enzyme 5-enopyruvylshikimate-3-phosphate synthase (EPSPS) is the target of the broad spectrum herbicide glyphosate. A novel *aroA* gene encoding an EPSPS from *Pantoea* sp was identified and subcloned into the pET-28a vector to construct the recombinant pET-AroA$_{Pantoea\ sp}$ plasmid. Amino acid sequence analysis indicated that AroA$_{Pantoea\ sp}$ is a class I AroA enzyme. When expressed in *Escherichia coli*, it conveyed high tolerance to glyphosate. AroA$_{Pantoea\ sp}$ may be used to generate transgenic glyphosate-tolerant plants.

Key words: 5-Enopyruvylshikimate-3-phosphate synthase; *Pantoea* sp; Clone; *Escherichia coli*

INTRODUCTION

The widely used herbicide glyphosate, also known as *N*-(phosphonomethyl) glycine and under the brand name Roundup, inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS or AroA), thus blocking biosynthesis of aromatic amino acids in the penultimate step of the shikimate pathway (Dill et al., 2008). In this pathway, EPSPS catalyzes the formation of EPSP and inorganic phosphate from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP; Maeda and Dudareva, 2012). The shikimate pathway is essential to plants, microorganisms, and fungi, but is absent in mammals (Haslam, 1974). Glyphosate is an analog of PEP and inhibits the action of EPSPS. As a herbicide, glyphosate controls weeds but also kills food crops, therefore much attention is focused on the discovery of glyphosate tolerance genes for use in transgenic plants.

Recently, AroA proteins have been divided into Class I and Class II enzymes (Tian et al., 2012; Yi et al., 2015). The Class I enzyme found in all plants and bacteria such as *Escherichia coli* (Yu et al., 2015) and *Salmonella enterica* Typhimurium (Garg et al., 2014) is sensitive to glyphosate at low concentrations. The Class II enzyme present in bacterial species such as *Pseudomonas* sp strain PG2982 (Zhang et al., 2014), *Agrobacterium tumefaciens* strain CP4 (Heck et al., 2005), *Streptococcus pneumoniae* (Du et al., 2000), and *Staphylococcus aureus* (Priestman et al., 2005), however, can tolerate glyphosate even at high concentrations. Class I and II enzymes share less than 30% amino acid similarity (Ye et al., 2001). Genes encoding class I glyphosate-tolerant enzymes have been isolated from bacteria having been subjected to natural selection in contaminated environments (Chen et al., 1999; He et al., 2003; Gong et al., 2006). Thus, with a view to generating transgenic plants, novel *aroA* genes crucial for glyphosate tolerance may be isolated and cloned from microorganisms found in many natural or polluted environments (Tian et al., 2013).

In this paper, we identify an *aroA* gene from a *Pantoea* sp collected from heavily contaminated soil. Its isolation, cloning, and characterization are described. Our results demonstrate that $AroA_{Pantoea\ sp}$ is naturally insensitive to glyphosate.

MATERIAL AND METHODS

Bacterial strains and chemicals

Roundup, containing 41% *N*-(phosphonomethyl) glycine, was purchased from Monsanto (St. Louis, MO, USA). All other chemicals, culture media, and antibiotics were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). *E. coli* strain DH5α and the pET-28a vector were sourced from our laboratory, while *E. coli* strain ER2799, which carries a deletion of *aroA*, was provided by Dr. Li Juan Qiu (Chinese Academy of Agricultural Sciences). *Taq* DNA polymerase and the pMD-18 vector were purchased from Takara Co., Ltd. (Dalian, China). All primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

Isolation and identification of *Pantoea* sp

The glyphosate-tolerant strain was isolated from contaminated soil. Soil samples were washed with 0.9% NaCl (w/v) solution before being incubated on M9 medium plates with 60 mM glyphosate. After 2 days at 28°C, colonies were then isolated and incubated on M9 plates with 200 mM glyphosate. A glyphosate-tolerant colony was selected for further study.

Chromosomal DNA was extracted from this strain and purified according to a previously reported method (Ausubel et al., 1992). Amplification of 16S rDNA was carried out using the primers 16SR (5'-AGA GTT TGA TCC TGG CTC AG-3') and 16SF (5'-ACG GCT ACC TTG TTA CGA CTT C-3'), and the resulting polymerase chain reaction (PCR) product was sequenced.

Cloning and sequence analysis of the *aroA* gene

Genomic DNA from *Pantoea* sp was used as a template to amplify the putative *aroA* gene. Forward (5'-ATG CAG GAC TCC CTG AC-3') and reverse (5'-TCA GGC GCT CTG GCT GAT TT-3') primers were designed based on a sequence conserved across other *Pantoea aroA* genes, including those from strains YR343 (GI495377688), At-9b (GI317047556), GM01 (GI495162675), Sc1 (GI496382122), AS-PWVM4 (GI544755733), and A4 (GI515918973). The PCR product was subsequently inserted into the pMD-18 vector and sequenced.

Sequence analysis of $AroA_{Pantoea\ sp}$

Sequence alignment was performed using the National Center for Biotechnology Information database and GenBank (http://www.ncbi.nlm.nih.gov), while amino acid sequence analysis was carried out using DNAMAN version 6.0 (Lynnon Biosoft, Vaudreuil, Quebec, Canada). A phylogenetic tree was constructed with MEGA version 2.1 (Kumar et al., 2001) using Clustal W (Larkin et al., 2007) alignments.

Evaluation of glyphosate tolerance in *E. coli*

The DNA fragment encoding the mature $AroA_{Pantoea\ sp}$ protein was amplified by PCR using the following primers (introduced restriction enzyme sites are underlined): forward, 5'-CGG GAT CCA TGC AGG ACT CCC TGA CTT TAC AG-3'; and reverse, 5'-CCG AGC TCG GCG CTC TGG CTG ATT TTT GCC A-3'. The amplified *aroA* gene fragment was then digested with *Bam*HI and *Sac*I restriction enzymes before being inserted into a pET-28a vector to construct the recombinant pET-$AroA_{Pantoea\ sp}$ plasmid. Forward (5'-GGA ATT CCA TAT GAT GGA ATC CCT GAC GTT ACA A-3') and reverse (5'-CGG AAT TCC GTC GGC TGC CTG GCT AAT C-3') primers were used to amplify the *E. coli aroA* gene. The plasmid containing this gene, pET-$AroA_{E.\ coli}$, was then made using *Nde*I and *Eco*RI endonucleases. The ER2799 strain expressing either pET-$AroA_{Pantoea\ sp}$ or pET-$AroA_{E.\ coli}$ was grown by shaking at 37°C in liquid M9 minimal medium, to which concentrations of glyphosate ranging from 0 to 150 mM had been added. Cell densities were then measured by spectrophotometry at 660 nm.

RESULTS

Isolation and identification of the glyphosate-tolerant strain

After selection on high-concentration glyphosate, one strain was isolated from the polluted soil samples. This strain grew well on M9 plates containing 200 mM glyphosate. Analysis of 16S rDNA identified it as belonging to the genus *Pantoea*.

Cloning and sequence analysis of AroA$_{Pantoea\ sp}$

Genomic DNA was extracted from *Pantoea* sp and used as a template to amplify the *aroA* gene using specific primers. A 1287-bp fragment was obtained and inserted into the pMD-18 plasmid by means of the T/A cloning method. Subsequently, plasmids were extracted from four separate colonies and sequenced. Remarkably, the same 1287-bp fragment of the gene encoding AroA$_{Pantoea\ sp}$ was present in all four independent plasmids according to the sequencing results. Amino acid sequence analysis revealed that AroA$_{Pantoea\ sp}$ shares more than 80% homology with AroA$_{E.\ coli}$ (UniProt accession No. P07638), but less than 25% identity with AroA$_{A.\ tumefaciens}$ CP4 (Q9R4E4). In addition, residues in PEP and S3P binding domains in AroA$_{Pantoea\ sp}$ and AroA$_{E.\ coli}$ were highly conserved. A phylogenetic analysis indicated that AroA$_{Pantoea\ sp}$ belongs to the Class I EPSPS group (Figure 1).

To determine whether expression of an open reading frame of aroA from *Pantoea* sp (ORF427) was sufficient to convey resistance to glyphosate, the PCR fragment was inserted into the expression vector pET-AroA$_{Pantoea\ sp}$ and transformed into the *aroA* mutant *E. coli* strain ER2799 (Figure 2). These constructs rescued the *E. coli* AroA deficiency when cells were grown on M9 medium containing 50 mM glyphosate (Figure 3).

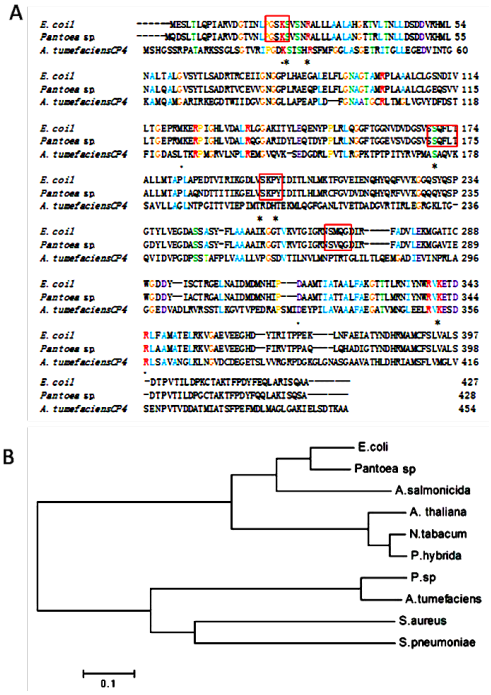

Figure 1. A. Amino acid sequence alignment of AroA$_{Pantoea\ sp.}$ AroA$_{E.\ coli}$ and AroA$_{A.\ tumefaciens\ CP4}$. Asterisks and circles represent residues important for shikimate-3-phosphate and phosphoenolpyruvate (PEP) binding in AroA$_{E.\ coli}$, respectively. Domains important for glyphosate tolerance and maintenance of productive PEP binding in class II AroA enzymes are boxed. **B.** Phylogenetic analysis of AroA$_{Pantoea\ sp.}$ using MEGA. Class I AroA proteins shown in this figure are from *Escherichia coli* (UniProt accession No. P07638), *Aeromonas salmonicida* (Q03321), *Arabidopsis thaliana* (P05466), *Nicotiana tabacum* (P23981), and *Petunia hybrida* (P11043); class II AroA proteins are from *Pseudomonas* sp strain PG2982 (P56952), *Agrobacterium tumefaciens* CP4 (Q9R4E4), *Staphylococcus aureus* (Q05615), and *Streptococcus pneumoniae* (Q9S400).

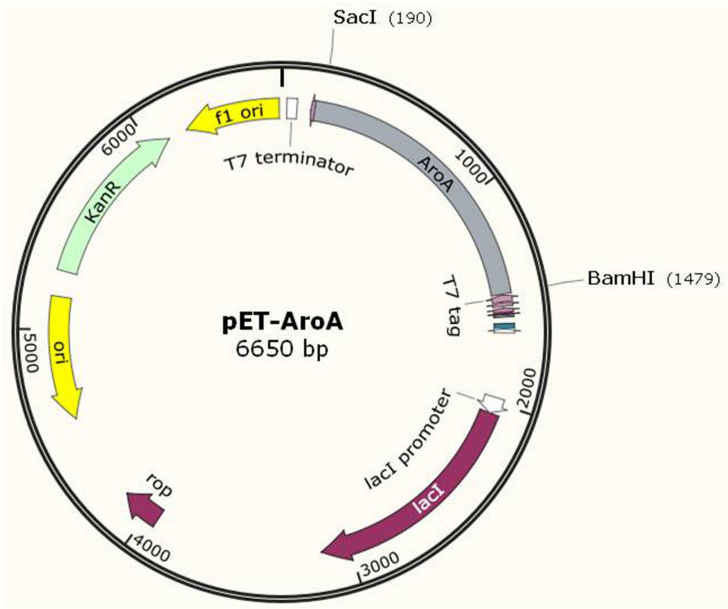

Figure 2. Diagrammatic representation of pET-AroA$_{Pantoea\ sp}$.

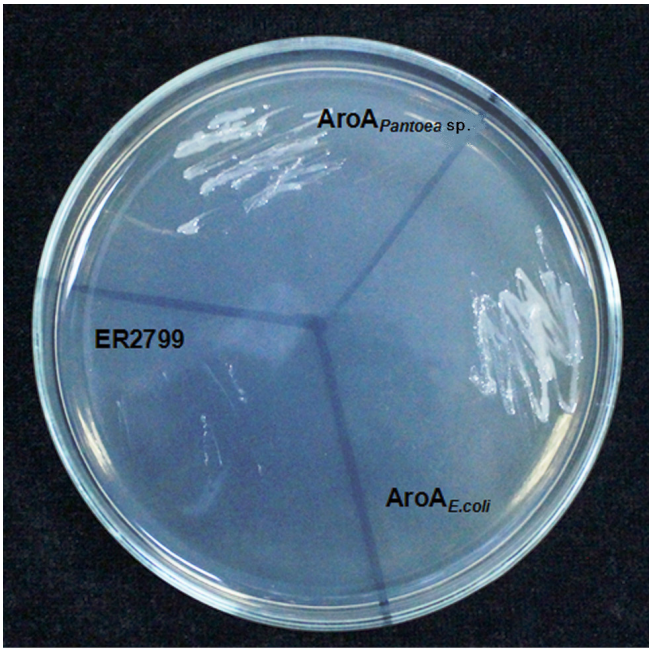

Figure 3. Functional analysis of the *Pantoea* sp AroA gene. *In vivo* rescue of the *E. coli* AroA mutant strain ER2799 by constructs containing *aroA* sequences. The growth of cells expressing pET-AroA$_{Pantoea\ sp}$ or pET-AroA$_{E.\ coli}$ was tested on M9 minimal medium agar plates with 50 mM glyphosate.

Structure of AroA$_{Pantoea\ sp}$

The structure of AroA$_{Pantoea\ sp}$ was modeled using SWISS-MODEL and drawn with Swiss-PdbViewer (Schwede et al., 2003). Its structure was found to be very similar to those of other known AroA proteins (Stallings et al., 1991; Park et al., 2004). The protein folds into two similar, globular, inside-out α-β barrel domains (Figure 4). AroA$_{Pantoea\ sp}$ shares 84.78% amino acid identity with AroA$_{E.\ coli}$ (Figure 1) and their two structures are much alike (Figure 4). These results also confirmed that AroA$_{Pantoea\ sp}$ is a class I EPSPS.

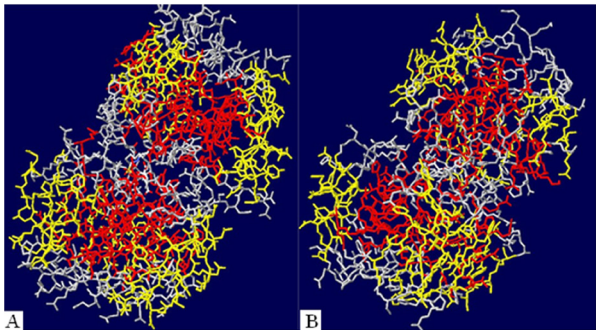

Figure 4. Backbone trace models of AroA proteins from (**A**) *Pantoea* sp and (**B**) *E. coli*. Red and yellow represent α-helices and β-sheets, respectively.

Identification of a conserved region within the *Pantoea* sp *aroA* gene

As shown in Table 1, the region to which glyphosate binds is conserved across many EPSPS sequences. This conserved region, XLGNAG TAXRX, has been reported in other species, including *E. coli*, *Salmonella enterica* Typhimurium, *Petunia hybrida*, *Glycine max*, *Arabidopsis thaliana*, *Brassica napus*, *Zea mays*, *Saccharomyces cerevisiae*, and *Aspergillus nidulans*. Within this region, the same sequence of amino acids is found in each of the abovementioned species, with the exception of *Aspergillus nidulans* and *Saccharomyces cerevisiae*. These observations show that the *Pantoea* sp EPSPS shares greater similarity with class I AroA enzymes than with those from fungi (Table 1). The tolerance of AroA$_{Pantoea\ sp}$ to glyphosate may therefore be increased by mutation of amino acids in the active site of this conserved region.

Table 1. Sequence comparison of the conserved region of several AroA proteins in *Pantoea* sp and other organisms.

EPSPS	Sequence
Escherichia coli	L^{90}FLGNAG^{96}TAMRP$^{101}L^{102}$
Salmonella enterica Typhimurium	L^{90}FLGNAG^{96}TAMRP$^{101}L^{102}$
Petunia hybrida	L^{95}FLGNAG^{101}TAMRP$^{106}L^{107}$
Glycine max	L^{98}FLGNAG^{104}TAMRP$^{109}L^{110}$
Arabidopsis thaliana	L^{95}FLGNAG^{101}TAMRP$^{106}L^{107}$
Brassica napus	L^{95}FLGNAG^{101}TAMRP$^{106}L^{107}$
Zea mays	L^{95}FLGNAG^{101}TAMRP$^{106}L^{107}$
Saccharomyces cerevisiae	L YLGNAG TASRF L
Aspergillus nidulans	L YLGNAG TASRF L
Pantoea sp	L FLGNAG TAMRP L
Consensus sequence	L XLGNAG TAXRX L

EPSPS = 5-enopyruvylshikimate-3-phosphate synthase.

Growth of cells in the presence of glyphosate

To ascertain whether $AroA_{Pantoea\ sp}$ was naturally glyphosate-tolerant, the growth of two *E. coli* ER2799 lines expressing either $AroA_{Pantoea\ sp}$ or $AroA_{E.\ coli}$ was compared over a period of 30 h in M9 media containing different concentrations of glyphosate. Growth curves for these lines and untransformed ER2799 cells are shown in Figure 5. All cells were observed to grow easily in medium lacking glyphosate. However, untransformed ER2799 cells and those harboring pET-$AroA_{E.\ coli}$ were strongly inhibited in medium containing 50 and 100 mM glyphosate. In contrast, cells expressing pET-$AroA_{Pantoea\ sp}$ grew well at these concentrations. These results indicate that $AroA_{Pantoea\ sp}$ can functionally rescue AroA deficiency in *E. coli* strain ER2799 and indeed, grant a high level of glyphosate-tolerance.

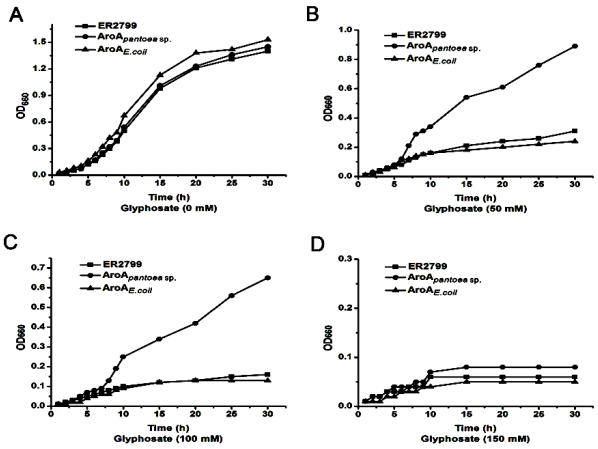

Figure 5. Growth curves of untransformed *E. coli* ER2799 cells, and those expressing $AroA_{Pantoea\ sp}$ or $AroA_{E.\ coli}$ in liquid M9 minimal medium supplemented with 0 to 150 mM glyphosate. OD_{660} = optical density measured at 660 nm wavelength.

DISCUSSION

In this study, we successfully isolated a glyphosate-resistant bacterial strain from contaminated soil and established it as belonging to the genus *Pantoea* by phylogenetic analysis of 16S rDNA sequences. A novel *aroA* gene from this species was identified, cloned, and expressed in high levels of glyphosate. Glyphosate has been used extensively over the past 40 years as a commercial herbicide (Amrhein et al., 1980), however, it also affects food crops (Zhou et al., 2012). Thus, we aimed to find a gene conferring glyphosate-tolerance for use in transgenic plants.

Two types of AroA have been cloned, identified, and examined for glyphosate tolerance. Although class I enzymes have been used to generate glyphosate-resistant crops, virtually all commercial crops with this trait, such as soybeans, corn, cotton, and canola, express the *Agrobacterium tumefaciens* strain CP4 class II protein (Dill, 2005). In this study, sequence analysis and structure modeling demonstrated that $AroA_{Pantoea\ sp}$ most closely resembles a class I EPSPS and possesses high glyphosate tolerance. Despite the prevalence of *Pantoea* spp. in soil, there have as yet been no reports concerning glyphosate resistance in these organisms. $AroA_{Pantoea\ sp}$ constitutes a candidate glyphosate-tolerance gene for use in transgenic crops.

The isolation and cloning of this *aroA* gene was the first step, with the focus of the current study being to examine the glyphosate tolerance that it confers. The next step will be its use in the generation of a glyphosate-resistant crop.

Conflicts of interest

The authors declare no conflict of interest.

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