Functional Characterization of aroA from Rhizobium leguminosarum with Significant Glyphosate Tolerance in Transgenic Arabidopsis

Jing Han, Yong-Sheng Tian, Jing Xu, Li-Juan Wang, Bo Wang, Ri-He Peng*, and Quan-Hong Yao*

Department of Shanghai Key Laboratory of Agricultural Genetics and Breeding, Biotechnology Research Institute of Shanghai Academy of Agricultural Sciences, Shanghai 201106, P. R. China

Glyphosate is the active component of the top-selling herbicide, the phytotoxicity of which is due to its inhibition of the shikimic acid pathway. 5-Enolpyruvyshikimate-3-phosphate synthase (EPSPS) is a key enzyme in the shikimic acid pathway. Glyphosate tolerance in plants can be achieved by the expression of a glyphosate-insensitive aroA gene (EPSPS). In this study, we used a PCR-based two-step DNA synthesis method to synthesize a new aroA gene (aroA<sub>R. leguminosarum</sub>) from Rhizobium leguminosarum. In vitro glyphosate sensitivity assays showed that aroA<sub>R. leguminosarum</sub> is glyphosate tolerant. The new gene was then expressed in E. coli and key kinetic values of the purified enzyme were determined. Furthermore, we transformed the aroA gene into Arabidopsis thaliana by the floral dip method. Transgenic Arabidopsis with the aroA<sub>R. leguminosarum</sub> gene was obtained to prove its potential use in developing glyphosate-resistant crops.

**Keywords:** 5-Enolpyruvyshikimate-3-phosphate synthase, Rhizobium leguminosarum, glyphosate tolerant, transgenic Arabidopsis

**Introduction**

Glyphosate (N-phosphonomethyl-glycine) is one of the most widely used herbicides in the world, having minimal human and environmental toxicity [30]. Glyphosate exhibits broad-spectrum herbicidal activity, as it inhibits a metabolic step in the biosynthesis of aromatic compounds [5]. The enzyme 5-enolpyruvyshikimate-3-phosphate synthase (EPSPS) encoded by the gene aroA (3-phosphoshikimate 1-carboxyvinyltransferase; E.C. 2.5.1.19) is the sixth enzyme in the shikimate pathway [1]. EPSPS catalyzes the conversion of phosphoenol-pyruvate (PEP) and shikimate-3-phosphate (S3P) into 5-enolpyruvyshikimate 3-phosphate (EPSP) and inorganic phosphate [26]. As an analog of PEP, glyphosate is a competitive inhibitor of EPSP synthase. Development of glyphosate-tolerant crops was a major breakthrough in agricultural biotechnology. Recent advances have been made in identifying, cloning, and testing the glyphosate-insensitive form of EPSPS enzymes [3, 8, 22, 23]. In these reports, EPSP synthases have been divided into two classes, sharing less than 30% amino acid identity. Class I EPSP synthases identified from plants and bacteria are generally sensitive to glyphosate, whereas Class II EPSP synthases usually have tolerance to glyphosate [9]. However, to date, only two aroA genes derived from the A. tumefaciens strain CP4 and Zea mays have been used in developing glyphosate-tolerant crops [7]. Exploration of new aroA genes could provide assistance in the engineering of glyphosate-tolerant crops.

*Rhizobium leguminosarum* is an alpha proteobacterial N<sub>2</sub>-fixing symbiont of legumes, and its genes for the symbiotic interaction with plants are well studied [10, 14, 34]. *R. leguminosarum* is also known for its essential contribution to agricultural production due to its excellent efficiency in fixing nitrogen [24]. It is a common object of study in biological nitrogen fixation and bacterial classification [15], but there are no published reports regarding EPSPS for glyphosate tolerance from *R. leguminosarum*. Functional characterization of aroA<sub>R. leguminosarum</sub> could provide new avenues of research.
In this study, we chemically synthesized a novel \textit{aroA} gene from \textit{R. leguminosarum} by a PCR-based two-step DNA synthesis (PTDS) method according to the \textit{aroA}_{\textit{R. leguminosarum}} gene sequence (GenBank: NC:Q1MN56) [32]. \textit{In vitro} glyphosate sensitivity assays of the \textit{E. coli} mutant (ER2799) harboring plasmid p251-\textit{aroA}_{\textit{R. leguminosarum}} were used to determine the level of glyphosate resistance [4]. Then, the gene was overexpressed and the enzyme was purified for kinetic characterization. Furthermore, an expression vector was constructed to obtain transgenic \textit{Arabidopsis} plants. Assay of the glyphosate resistance in different stages of transgenic plants showed outstanding resistance when compared with the wild type.

**Materials and Methods**

**Medium and Chemicals**

S3P, glyphosate (free acid form), PEP, and Ni^{2+}-NTA agarose affinity columns were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). All other chemicals were of analytical grade. \textit{E. coli} strain ER2799 was provided by Dr. Thomas C. Evans, Jr. (New England Biolabs, USA). \textit{A. tumefaciens} GV3101 and \textit{A. thaliana} plants (Columbia ecotype) were stored by our laboratory. The plants were grown on Murashige and Skoog medium with 1% agar [16], or in pots filled with a mixture of vermiculite/peat moss/perlite (9:3:1) in a controlled environment (22°C under 16h:8h day/night cycles).

**Chemical Synthesis of \textit{aroA}_{\textit{R. leguminosarum}}**

The sequence of \textit{aroA}_{\textit{R. leguminosarum}} was artificially synthesized using a PTDS strategy. All the codons in \textit{aroA}_{\textit{R. leguminosarum}} were optimized and preferentially designed for plants [17]. The amplified fragment was cloned into TA cloning vector Simple pMD-18 and sequenced. Errors in the synthetic gene were corrected by the overlap extension PCR method [21]. The sequence analysis was conducted through a database search using the BLAST program (National Center for Biotechnology Services; http://www.ncbi.nlm.nih.gov). Amino acid sequence analysis and construction of the phylogenetic tree were done using DNAMAN ver. 6.0.

**In Vitro Glyphosate Sensitivity Assays**

Plasmid p251-\textit{aroA}_{\textit{R. leguminosarum}} was obtained by inserting \textit{aroA}_{\textit{R. leguminosarum}} into pYPX251 [31]. \textit{In vitro} glyphosate sensitivity assays of the clone were performed with the \textit{aroA}-deleted \textit{E. coli} mutant (ER2799), ER2799 harboring plasmid p251-\textit{aroA}_{\textit{R. leguminosarum}} and ER2799 harboring plasmid pYPX251. The cell strains were grown with shaking at 37°C in liquid M9 minimal medium supplemented with glyphosate at various concentrations ranging from 0 to 100 mM. Cell densities were determined by measuring the absorbance at 600 nm (OD_{600}).

**Protein Purification and Enzyme Assay**

Synthetic \textit{aroA}_{\textit{R. leguminosarum}} was inserted into the expression vector pYM4087 [33], and then transformed into competent expression host \textit{E. coli} BL21. Cells were harvested and disrupted by sonication, and the soluble fraction was loaded onto a Ni^{2+}-NTA agarose affinity column at 4°C for purification. The protein was tested by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the concentration was measured by the Bradford assay. EPSPS activity was determined at 28°C by measuring the amount of inorganic phosphate produced in the reaction using the malachite green dye assay method [11]. Reaction mixtures without S3P served as controls. \textit{K_{m}} values for PEP, \textit{K_{i}} values for glyphosate, and IC_{50} values (the concentration of glyphosate inhibiting enzyme activity by 50%) were measured as described by Tian et al. [26].

**Construction of Plant Expression Vector and Plant Transformation**

EPSPS in plants is located in the chloroplast [6]; thus, the plant expression vector was modified by adding a chloroplast transit peptide of \textit{Arabidopsis} (TSP) to ensure that the transgene would be targeted to the chloroplast. The construction of the vector was performed as in Xu et al. [33]. Then, the vector was introduced into \textit{A. tumefaciens} GV3101 by electroporation. The plant transformation was carried out by a floral dip method into \textit{A. thaliana} (ecotype Columbia) [35].

**Transgenic Plant Selection and Assay of Glyphosate Resistance**

Transgenic plants were selected from plantlets containing 30 mg/l hygromycin. The T3 sterilized \textit{A. thaliana} seeds were vertically grown on MS medium containing 5 mM glyphosate to analyze root development. Several transgenic lines chosen in the selection were used to test the level of gene transcription by reverse transcription (RT)-PCR. The \textit{A. thaliana} actin gene (GenBank: U41998) served as an internal standard to normalize the amount of cDNA. Primers RIZ (5'-tgccagaaagtctggtc-3') and RIF (5'-ccacagaggactgctacctg-3') with a specific sequence for \textit{aroA}_{\textit{R. leguminosarum}} were used in RT-PCR with the same amount of cDNA. PCR products were separated on 1.5% agarose gels and quantified using a Model Gel Doc 1000 (Bio-Rad, USA). Then, the germination of transgenic lines was carried out on MS medium containing 8 mM glyphosate. Transgenic lines for the glyphosate spray treatment were grown in pots (nine seedlings per pot) in a controlled-environment chamber. After 4 weeks, plants were sprayed with 10 mM glyphosate.

**Results**

**Sequence Analysis of \textit{aroA}_{\textit{R. leguminosarum}}**

\textit{aroA}_{\textit{R. leguminosarum}} was synthesized by the PTDS method. The length of the gene was 1,389 bp with a protein of 452 amino acid residues (sequence accession number in GenBank: Q1MN56). The deduced molecular mass was
51.1 kDa, which was confirmed by SDS gel electrophoresis. The amino acid sequence is exhibited in Fig. 1. A BLAST search showed that the synthesized aroA<sub>R. leguminosarum</sub> gene shares 85.4% amino acid identity with aroA<sub>CP4</sub> (UniProt: Q9R4E4), and 23.2% amino acid identity with aroA<sub>E. coli</sub> (UniProt: P07638). Phylogenetic analysis also indicated that aroA<sub>R. leguminosarum</sub> is a Class II EPSPS.

**In Vitro Glyphosate Sensitivity Assay**

Growth curves of the E. coli aroA-deleted mutant ER2799
harboring p251-aroAR__leguminosarum are shown in Fig. 2. After 12 h of incubation, the growth of cells harboring plasmid pYPX251 was strongly inhibited by 40 mM glyphosate. The cultures harboring p251-aroAR__leguminosarum grew well compared with controls. At 80 mM glyphosate, cultures harboring p251-aroAR__leguminosarum still attained ~40% OD values of the blank, but growth was completely inhibited at 100 mM glyphosate.

Kinetic Properties of aroAR__leguminosarum

The aroAR__leguminosarum protein was overexpressed in E. coli, purified, and assayed for kinetic activity. As shown in Table 1, the V_max value of the enzyme was 23.4 U/mg, the K_m value of the enzyme for PEP was 0.267 mM, aroAR__leguminosarum had an IC_{50} value of 1.025 mM, and the K_i (glyphosate) value was 291.6 µM. The K_i/K_m value (PEP) was taken as a measure of the selectivity for PEP over glyphosate binding; aroAR__leguminosarum had a K_i/K_m value of 1.09. Compared with aroAR__E.coli [26], the IC_{50} and K_i/K_m values were significantly higher, indicating that the enzyme has a high glyphosate tolerance and a high level of affinity for PEP.

Transgenic Plant Selection

Twelve transgenic plants with aroAR__leguminosarum were obtained. Then, three transgenic lines with good root development (named AR1, AR2, and AR3) were further analyzed for gene expression using RT-PCR. For the steady transmission of the target gene, the scaffold attachment

Table 1. Kinetic properties of aroAR__E.coli and aroAR__leguminosarum.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (U/mg)</th>
<th>K_m [PEP] (µM)</th>
<th>K_m [S3P] (mM)</th>
<th>K_i [Glyphosate] (µM)</th>
<th>IC_{50} [Glyphosate] (mM)</th>
<th>K_i/K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>aroAR__E.coli</td>
<td>7.9</td>
<td>97</td>
<td>0.140</td>
<td>1.4</td>
<td>0.055</td>
<td>0.014</td>
</tr>
<tr>
<td>aroAR__leguminosarum</td>
<td>23.4</td>
<td>267</td>
<td>0.223</td>
<td>291.6</td>
<td>1.025</td>
<td>1.09</td>
</tr>
</tbody>
</table>

One unit (U) of EPSPS activity is defined as the amount of enzyme that catalyzes the liberation of 1 µmol inorganic phosphate per minute of reaction time.
region (SAR) was fused upstream of the double cauliflower mosaic virus 35S (D35S) promoter. The \( aroA_{R. leguminosarum} \) expression vector used in \( A. thaliana \) transformation is displayed in Fig. 3. We can see that the level of the \( A. thaliana \) actin gene from the transgenic lines (AR1, AR2, and AR3) and the wild-type control was average. The specific DNA fragments of \( aroA_{R. leguminosarum} \) were then amplified, and the agarose gel electrophoresis in Fig. 3 shows that the gene was transcribed actively in transgenic plants. Furthermore, the level of transcription in the three different transgenic lines was equal.

**Assay of Glyphosate Resistance**

For the assay of glyphosate resistance, the seedling stage and adult stage of transgenic plants were studied. Seeds and roots are usually poorly developed under glyphosate stress [27, 29], so the assays of transgenic \( A. thaliana \) were carried out on MS medium in a petri dish. After 2 weeks, the photos were taken as shown in Fig. 4. In each petri dish, we seeded six transgenic lines, arranged with the wild type as control. It can be seen that \( aroA_{R. leguminosarum} \) transgenic plants grew normally on MS medium. Three transgenic lines (named AR1, AR2, and AR3) were selected from 12 transgenic lines on MS medium containing 5 mM glyphosate. Furthermore, germination on MS medium with 8 mM glyphosate was also very good. We can see that the transgenic plants showed significant resistance to glyphosate in the seedling stage. To analyze the tolerance to glyphosate in the adult stage, transgenic lines grown in pots were sprayed with 10 mM glyphosate. As shown in Fig. 5, the
transgenic plants were still green after 7 days. The results show that the transgenic plants of \( aroA_{R.\ leguminosarum} \) had glyphosate resistance.

**Discussion**

In this study, a novel \( aroA \) gene from \( R.\ leguminosarum \) was synthesized. The PTDS method provided us with the \( aroA \) gene without using template DNA. Chemical synthesis was used as a powerful tool for characterizing gene structure, expression, and function. Sequence analysis showed that \( aroA_{R.\ leguminosarum} \) is most closely related to the EPSPS of \( A.\ tumefaciens \) strain CP4 and belongs to Class II \( aroA \). Class II EPSP synthases usually have a natural tolerance to glyphosate, and are found in bacteria such as \( S.\ pneumoniae \) [19] and \( P.\ aeruginosa \) [28]. However, the synthases reported often exhibit a low \( K_m \) for PEP. The characterization of enzyme \( aroA_{R.\ leguminosarum} \) showed a lower tolerance to glyphosate but a higher \( K_m \) for PEP compared with \( aroA_{CP4} \).

Amino acid sequence comparison of \( aroA_{R.\ leguminosarum} \), \( aroA_{CP4} \), and \( aroA_{E.\ coli} \) showed various residues located at different parts of the protein. The residues’ effects on glyphosate/PEP binding are marked in Fig. 1; we found many similarities between \( aroA_{R.\ leguminosarum} \) and \( aroA_{CP4} \). As is known to us, many active sites of EPSP synthase have been identified and studied [2]. Alterations at Gly100Ala and Pro105Ser in the EPSPS enzymes result in a significant change in glyphosate tolerance [18]. In contrast to the single residue in the active site ( Ala-100) of \( aroA_{CP4} \), causing insensitivity to glyphosate, this position in \( aroA_{R.\ leguminosarum} \) has a highly conserved Gly residue as in other known natural plant and bacterial EPSPSs. We also found a conserved Ile423, whereas the residue in \( aroA_{CP4} \) is Val. These differences in residues impart distinct kinetic properties to the enzyme. A single-site mutant for further research was later produced.

\( R.\ leguminosarum \) is an \( \alpha \)-proteobacterial \( N_2 \)-fixing symbiont of legumes in soil. The bacterium can infect legume roots and induce symbiotic root nodules, with the rhizobia fixing atmospheric nitrogen for the host plant [13]. In recent years, reports on genes for the symbiotic interaction with plants were more than a thousand, while a few were about glyphosate and the influence of the environment. Liu et al. [12] tested several strains of the Rhizobiaceae family for their ability to degrade glyphosate. Transgenic plants of \( aroA_{R.\ leguminosarum} \), obtained in our lab provide more materials for gene transfer in GMO and soil bacteria. The symbiotic interaction made the risk assessment of transgenic plants more dependable.

*In vitro* glyphosate sensitivity was examined by functional complementation of \( E.\ coli \) mutant ER2799. The result showed that \( aroA_{R.\ leguminosarum} \) was glyphosate tolerant at 80 mM (Fig. 2). Compared with other EPSPs [20, 25], \( aroA_{R.\ leguminosarum} \) has similar performance regarding glyphosate tolerance. However, transgenic plants with \( aroA_{R.\ leguminosarum} \) showed excellent glyphosate resistance. It is known that glyphosate can affect seed germination and root development. Through glyphosate selection, we obtained three transgenic lines with a high level of transcription. As shown in Fig. 4, the seedling stage of transgenic plants could survive in 8 mM glyphosate, which indicates that \( aroA_{R.\ leguminosarum} \)
encodes a highly glyphosate-tolerant EPSPS. Thus, gene aroA<sub>leguminosarum</sub> could be applied to the development of GM crops with glyphosate tolerance in the future. Glyphosate-tolerant crops offer farmers a powerful tool in fighting weeds, and make it possible to use no-till or conservation-till systems. Meanwhile, reduced glyphosate spraying helps farmers to control the total input of herbicides, which also has consequent benefits to soil structure and organisms.

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