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Error-prone PCR mutation of *Ls-EPSPS* gene from *Liriope spicata* conferring to its enhanced glyphosate-resistance

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ABSTRACT

Liriope spicata (Thunb.) Lour has a unique LsEPSPS structure contributing to the highest-ever-recognized natural glyphosate tolerance. The transformed Ls*EPSPS* confers increased glyphosate resistance to *E. coli* and *A. thaliana*. However, the increased glyphosate-resistance level is not high enough to be of commercial value. Therefore, Ls*EPSPS* was subjected to error-prone PCR to screen mutant *EPSPS* genes capable of endowing higher resistance levels. A mutant designated as ELs-*EPSPS* having five mutated amino acids (37Val, 67Asn, 277Ser, 351Gly and 422Gly) was selected for its ability to confer improved resistance to glyphosate. Expression of ELs-*EPSPS* in recombinant *E. coli* BL21 (DE3) strains enhanced resistance to glyphosate in comparison to both the Ls*EPSPS*-transformed and -untransformed controls. Furthermore, transgenic EL*s-EPSPS A. thaliana* was about 5.4 fold and 2-fold resistance to glyphosate compared with the wild-type and the Ls-*EPSPS*-transgenic plants, respectively. Therefore, the mutated ELs-*EPSPS* gene has potential value for has potential for the development of glyphosate-resistant crops.

1. Introduction

Glyphosate is the most widely used nonselective herbicide in the world [1]. It inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) in the shikimic acid pathway, an enzyme that is essential for the biosynthesis of the aromatic amino acids, tryptophan, phenylalanine and tyrosine in plants [2]. Being non selective, glyphosate cannot be directly applied to conventional crops to control weeds. Hence, many crops have been transgenically modified to withstand glyphosate [3–8]. Herbicide-resistance (primarily glyphosate resistance) traits have been transformed into canola (*Brassica napus* L.), corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), soybean [*Glycine* max (L.) Merr.], and sugar beet (*Beta vulgaris* L.). Glyphosate-resistant (GR) crops have been planted worldwide on an increasing area of more than 180 million ha [9].

The development of glyphosate-resistant crops mainly depends on glyphosate-resistance gene resources. A glyphosate-resistance *EPSPS* gene from *Agrobacterium tumefaciens* strain CP4 has been successfully used in commercial transgenic crops [10]. EPSPS of glyphosate-resistant *Eleusine indica* was recently patented [11]. Another mutant EPSPS from *Z. mays* (event GA21 carrying two mutations) has been utilized to produce the first commercial varieties of glyphosate-resistant maize in 1990s [12–15]. An EPSPS of *Vitis vinifera* modified by DNA shuffling conferred high resistance to glyphosate in transgenic *A. thaliana* and rice [16]. However, there are still limited EPSPS gene sources available to develop glyphosate-resistant commercial varieties. Therefore, identification and cloning of additional plant-derived glyphosate-resistance *EPSPS* genes will provide alternative options for developing new glyphosate-resistant crops and increasing the diversity of transgenic glyphosate-resistance technology.

Directed evolution based on error-prone polymerase chain reaction (PCR) and DNA shuffling has become a promising new method in protein engineering. Since initially proposed by Leung in 1988 [17], error-prone PCR has been a useful tool because of its simple operation and remarkable results. Zhou et al. (2006) produced a glyphosate-resistant P106L mutant of a rice (*Oryza sativa*) EPSPS using this technique [18]. Affinity of the P106L mutant for glyphosate and phosphoenolpyruvate decreased about 70-fold and 4.6-fold, respectively, compared to wild-type EPSPS [18]. We recently documented that *Liriope spicata* (Thunb.) Lour has a unique EPSPS structure contributing to the highest-ever-recognized natural glyphosate tolerance [19]. However, the increased glyphosate-tolerance level in transformed-plants was not enough to be suitable for commercialization. In this study, we employed the error-prone PCR technique to develop a high glyphosate-resistance *EPSPS* gene from Ls-*EPSPS* of *L. spicata*.

2. Materials and methods

2.1. Error-prone PCR

Based on the previous obtained *EPSPS* sequence of *Liriope spicata* (GenBank sequence accession: KP143747), two specific primers (EPSPS-

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F1: 5′-ATGGTGCCGGAGATCGTGCTGCAGCC-3′ and EPSPS-R1: 5′-TTAGTGCGTTGTGAACCTCTGCAATAC-3′) were designed and used for error-prone PCR. The reactions were carried out in 0.2 mL tubes (25 μL per tube) in a reaction buffer containing Taq polymerase, dNTPs, dCTP, dTTP, Mg^{2+}, Mn^{2+}, buffer, 0.2 μM primers and template DNA. PCR was performed in a thermal cycler as follows: 5 min at 94 °C (1 cycle); 30 s at 94 °C, 30 s at 50 °C, 2 min at 72 °C (30 cycles); and 10 min at 72 °C (1 cycle). The obtained fragments were cloned into the PMD-19T vector (Takara Code No. 6013).

2.2. Screening and identification of EPSPS genes conferring high glyphosate-resistance

Fragments of about 1.3 kb obtained by error-prone PCR were purified and inserted into the PMD-19T vector. The resulting ligation mixture was then transformed into *E. coli* strain DH5α (Takara Code No. 9057) and screened on LB agar plates supplemented with 100 mg L^{-1} ampicillin and 100 mM glyphosate. Clones surviving 100 mM glyphosate were inoculated in LB liquid medium for the extraction of plasmid DNA. To identify if the obtained *EPSPS* genes coded for high glyphosate resistance, the plasmids of *EPSPS* were transformed into DH5α again. *EPSPS* genes conferring high glyphosate-resistance were further selected, sequenced and analyzed.

2.3. Overexpression and identification of glyphosate resistance

DNA fragment encoding the mature protein of EPSPS was amplified by PCR using LSEYH-F (5′-CCATGGTGCCGGAGATCGTGCTG-3′) and LSEYH-R (5′-GAATTCTTAGTGCGTTGTGAACCTCTGCAAT-3′). The PCR product was digested with NcoI and EcoRI, cloned into the corresponding restriction sites of pET-28a (Novagen, Inc.), and confirmed by DNA sequencing. The plasmid was then transformed to *E. coli* BL21 (DE3) (Novagen, Inc.). The expressed protein was detected by discontinuous vertical SDS-PAGE electrophoresis.

The transgenic bacteria (with PET-28a, plasmid Ls-EPSP-PET-28a or ELs-EPSPS-PET-28a) were inoculated into liquid LB medium containing 1.0 mmol L^{-1} IPTG and glyphosate at increasing concentrations (0, 3000, 4500, 6000, 7500, 9000, 10,500, 12,000 and 20,000 mg ae L^{-1}) and shaken at 37 °C for 12 h. A strain only transformed with plasmid PET-28a was used as a negative control. Cell concentrations were calculated by optical density (OD_{600}) spectrophotometric measurements.

2.4. Construction of the plant expression vector and plant transformation

Construction of the plant expression vector and plant transformation was performed as previously described [20]. The PCR primers were EPSPS9 (5′-GCTCTAGAATGGAGCAAGCGATCATGGCTAAG-3′) and EPSPS6 (5′-GCTCTAGAGTGCGTTGTGAACCTCTGCAATAC-3′). The PCR product was cloned into the corresponding restriction sites of pBI121 (Novagen, Inc.), digested with XbaI. Then the final constructs, NOS: NPTII: NOS: 35S: Ls-*EPSPS*: β-Glu: NOS were introduced into *A. tumefaciens* EHA105 (provided by National Key Laboratory of Nanjing Agriculture University) by the freeze-thaw method [21], and subsequently transformed into *A. thaliana* (ecotype Columbia) by a previously described floral dip method [20] to generate transgenic plants.

2.5. Transgenic plant selection

The seed produced by infected plants was surface sterilized and planted in MS medium containing 50 mg L^{-1} kanamycin. A week later, surviving seedlings were transferred into pots filled with growth medium composed of vermiculite/ peat moss/ perlite (9:3:1). Leaves from three-week old plantlets were used for transgenic molecular identification. The transgenic *A. thaliana* plants were screened until homozygous. The transgenic nature of the *A. thaliana* plants was confirmed by PCR analysis of genomic DNA using the specific primers 35S1 (5′-ATCCGGAAACCTCCTCGGATTCCATTGC-3′) and EPSP 6(5′-GCTCTAGAGTGCGTTGTGAACCTCTGCAATAC-3′). DNA extraction was according to the manufacturer's protocol (Tiangen Code: DP305). PCR products were separated on 1% (w/v) agarose gels and quantified using a Model Gel Doc 1000 system (Bio-Rad, USA).

2.6. Glyphosate response assay

Non-transgenic and transgenic plants were grown in pots filled with growth medium as before in a controlled environmental chamber at 22 °C kept on a 16/8 h day/night cycle at a light intensity of 120 μmol photons m^{-2} s^{-1}. Four-week-old seedlings were sprayed with glyphosate (Roundup, Monsanto) at rates of 0, 164, 492, 984, 1640, 2460 and 3280 mg L^{-1} using a compressed-air tower sprayer (PT-1, Nanjing Agricultural University) calibrated to deliver 234 L ha^{-1} at 0.2 MPa. Symptom development was assessed visually one week after treatment. Percent injury was estimated based on discrete herbicide injury severity (HIS) values according to Song et al., 2011:

$$\text{Injury}\ (\%) = [\Sigma\ (\text{HIS} \times \text{plant number})/(\text{all plants} \times 5)] \times 100$$

The experiments were repeated three times. Injury data was subjected to ANOVA (SPSS 17.0, SPSS Institute Inc.) test and fitted to a log-logistic regression model, $Y = C + (D - C) / \{1 + \exp[b \times \ln(X / ED_{50})]\}$, where Y represents the HIS index of herbicide injury severity, X is the herbicide rate. To estimate the parameters of the log-logistic response curve, a non-linear regression routine was used with the Origin software (Origin 8.0, Origin Lab Company). The ED_{50} value was calculated with the above regression equation. The relative resistance level was determined by calculating the R/S ratio (ED_{50} for the test populations divided by ED_{50} for the susceptible population).

3. Results

3.1. Screening and identification of EPSPS genes conferring high glyphosate-resistance

E. coli DH5α strain with PMD-19T or Ls-*EPSPS*-PMD-19T grew well on LB plates without glyphosate (Fig. 1A–B), but neither could survive on 100 mM glyphosate LB (Fig. 1C–D). By error-prone PCR, the 1.3 kb products of the *EPSPS* gene were amplified and sub cloned into the pMD™19-T vector (Fig. 1E). After screening with 100 mM glyphosate, three surviving clones were obtained (Fig. 1F) and identified by PCR using primers for Ls-*EPSPS* (Fig. 1G).

The sequence and analysis of the targeted fragment determined that it had a complete open reading frame of 1332 bp with a 49% GC content encoding a protein of 443 amino acid residues that was named ELs-EPSPS. It had an ATG start codon; its deduced molecular mass was 47.04 kDa with an isoelectric point of 5.83. Nucleotide sequence analysis found five mutated amino acids in ELs-EPSPS: Glu37Val, Asp67Asn, Thr277Ser, Asp351Gly, and Arg422Gly (numbered according to *Amaranthus tuberculatus* EPSPS) compared to Ls-EPSPS (GenBank sequence accession number KP143747) (Fig. 2A).

3.2. 3D structure analysis of ELs-EPSPS

To predict the effect of the mutated sites of ELs-EPSPS on the unique enzyme on glyphosate resistance, the ELs-EPSPS sequence was submitted to Swiss Model (http://swissmodel.expasy.org/) under the automatic modeling mode. The output was analyzed with Swiss-PdbViewer 4.01 and PyMOL 1.5.0.3 softwares. The pdb of the x-ray structure used was DOI:10.2210/pdb2aay/pdb. The spatial positions of the five mutant amino acids, 37Val, 67Asn, 277Ser, 351Gly, and 422Gly, are shown in Fig. 2B.

Residue 37Glu is at the end loop of the 28Arg helix, which directly interacts with the substrate S3P (Fig. 3A). Both Glu and Arg are polar