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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

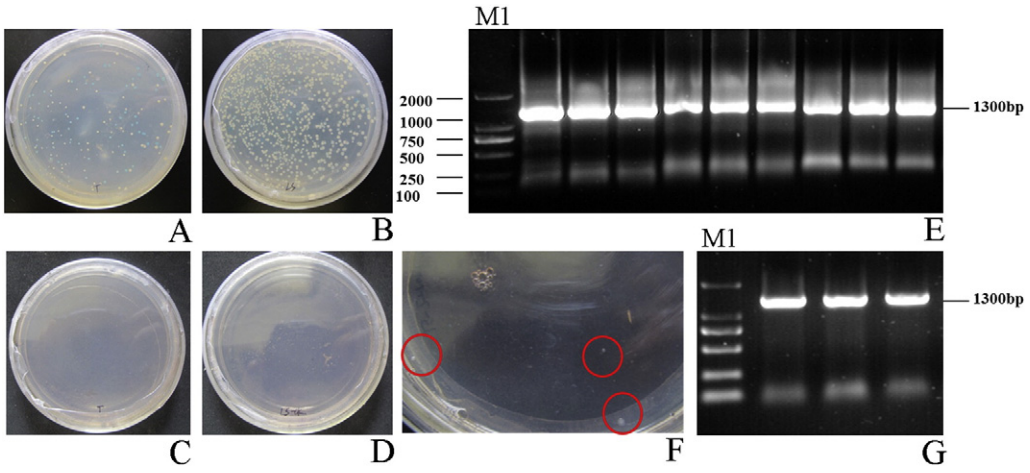


Fig. 1. Screening and molecular identification of glyphosate resistant strains. (A) PMD-19T without glyphosate; (B) Ls-*EPSPS*-PMD-19T without glyphosate; (C) PMD-19T with100mM glyphosate; (D) Ls-*EPSPS*-PMD-19T with 100 mM glyphosate; (E) Error-prone PCR on Ls-*EPSPS*; (F) Mutant Ls-*EPSPS* grown with 100 mM glyphosate; (G) Molecular identification of mutant Ls-*EPSPS*. Note: M1, DL2000 DNA Marker (Takara, D501A).

amino acids and there is dipole-dipole attraction between them. However, Val is a nonpolar amino acid. After mutation, the orientation force between Val and Arg weakened, so that the hydrogen bond distance between S3P and 28Arg was shortened to 2.59 Å in ELs-EPSPS from 2.83 Å in Ls-EPSPS (Fig. 3A).

Residue 422Arg is actually next to the 429Lys helix (Fig. 3B). The 429Lys H-bond stabilizes the glyphosate phosphonate or the PEP phosphate. Therefore, this substitution Arg422Gly might be a way to slightly shift the H-bond toward the phosphate because Gly is smaller (Fig. 3B). The model calculated that the hydrogen bond distance between

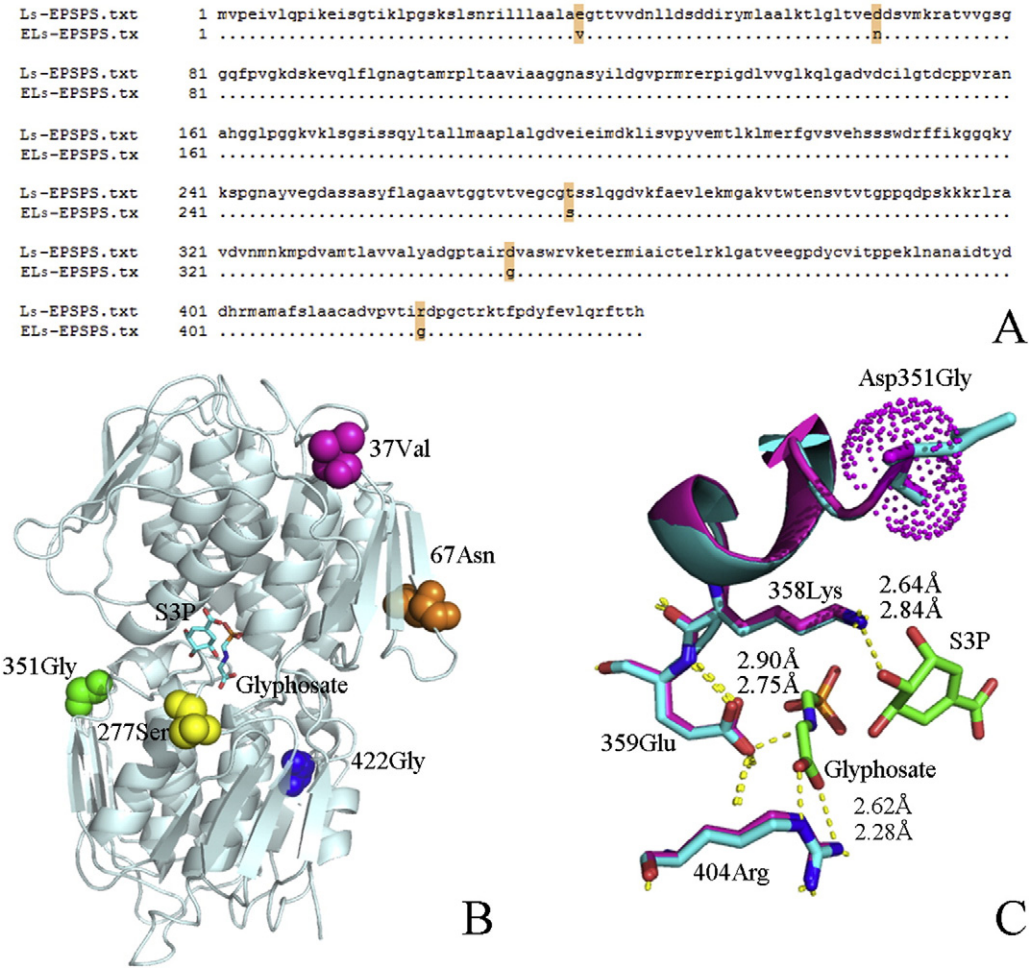


Fig. 2. 3D analyses of ELs EPSP synthase. (A) Alignment of LS and ELs-EPSPS; (B) The 3D structure of ELs-EPSPS displaying the five mutated amino acids (magenta: 37Val; orange: 67Asn; yellow: 277Ser; green: 351Gly; blue: 422Gly); (C) The location of Asp351Gly substitution in ELs-EPSPS (blue: A. Ls-EPSPS; magenta: ELs-EPSPS).

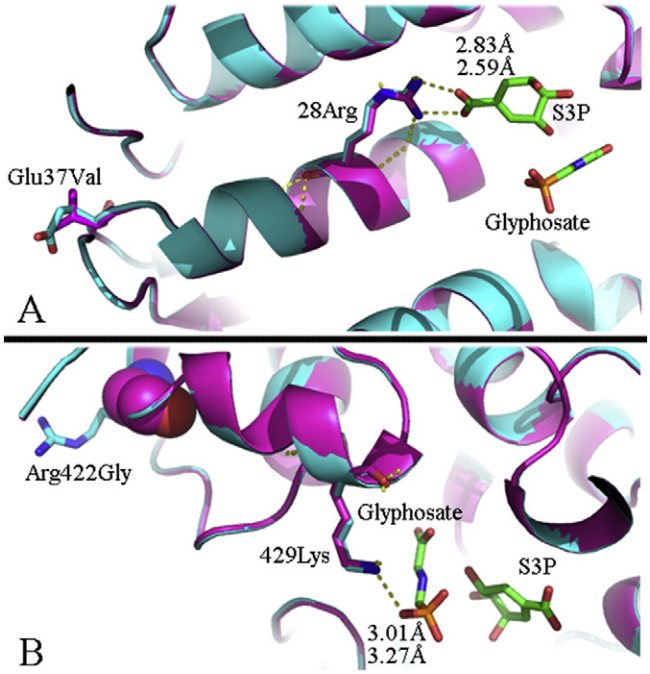


Fig. 3. Analysis of the three-dimensional structure of ELs-EPSP synthase by PyMOL. (A) Location of Glu37Val substitution of ELs-EPSPS. (B) Location of Arg422Gly substitution of ELs-EPSPS (blue: Ls-EPSPS; magenta: ELs-EPSPS).

glyphosate and 429Lys was lengthened from 3.01 Å in Ls-EPSPS to 3.27 Å in ELs-EPSPS (Fig. 3B).

351Asp is on the surface along with 358Lys. After substituted by Gly, the H-bond between S3P and 358Lys was extended by 0.2 Å due to the much smaller 351Gly just like in the Arg422Gly substitution (Fig. 2C). 359Glu is next to 358Lys and 404Arg is on the helix adjacent to 358Lys and 359Glu. Thus the displacement of 358 Lys might be a way to shift 359Glu and 404Arg toward glyphosate (Fig. 2C). The H bonds between glyphosate and 359Glu and 404Arg were shortened by 0.15 Å and 0.34 Å, respectively (Fig. 2C).

Finally, 67Asp is on the turning of the second and third helix in domain 3 and 277Thr is on the turning of the seventh and eighth helixes in domain 2. Both substitutions, Asp67Asn and Thr277Ser, are well away from the active sites, with which they do not interact given its surface exposure (Fig. 2B).

3.3. Overexpression and identification of glyphosate resistance

The construction of overexpression vectors with ELs-*EPSPS* genes is shown in Fig. 4. The recombinant plasmids ELs-EPSPS-PET-28a effectively expressed in *E. coli* BL 21 (DE3). The protein was about 47kD as determined by discontinuous vertical SDS-PAGE electrophoresis (Fig. 4E). The recombinant strains (with ELs-EPSP-PET-28a, Ls-EPSP-PET-28a or only PET-28a plasmid) were cultured in presence of glyphosate at increasing concentrations and their response assessed based on OD_{600} growth values then subjected to a logistic analysis. The calculated ED_{50} values for the trans-ELs-*EPSPS* strains were 15,207 mg ae L^{-1}, much higher than that of the wild and trans-Ls-*EPSPS* types that were 6657 and 8851 mg ae L^{-1}, respectively (Fig. 4F). Thus the ELs-*EPSPS* gene conferred transformed *E. coli* the ability to withstand glyphosate at higher concentrations than the other two types of strains.

3.4. Glyphosate response assay

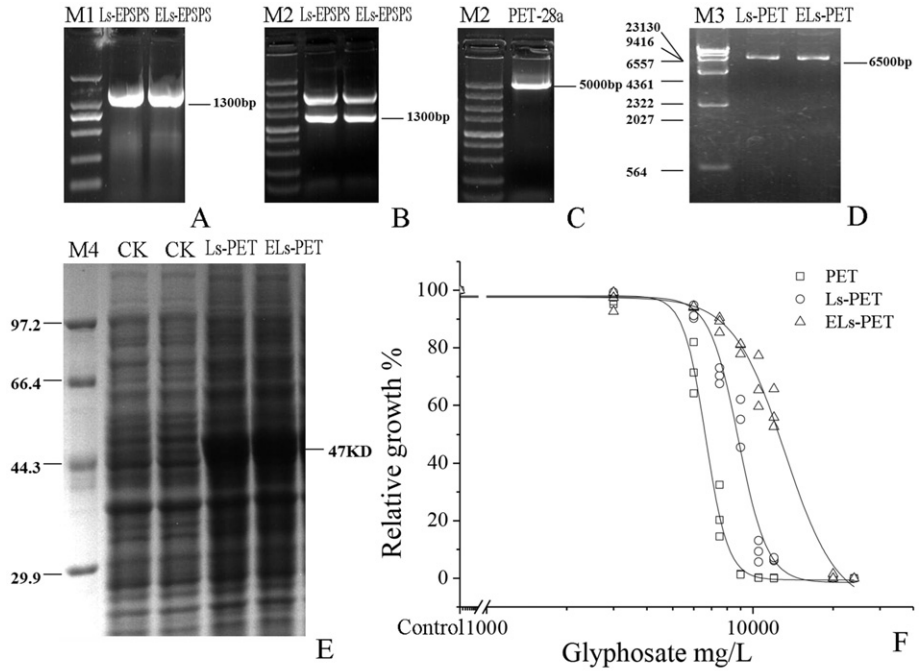
In absence of glyphosate, growth of transgenic and wild-type *A. thaliana* was similar. Glyphosate at 1640 mg ae L^{-1} strongly inhibited leaf growth of both the wild-type and Ls-*EPSPS*-transgenic *A. thaliana* (Fig. 5A–B). In contrast, leaves of ELs-*EPSPS*-transgenic plants only developed very slight phytotoxicity symptoms at this concentration (Fig. 5C). The logistic model provided a good description of the relationship between injury level and glyphosate dose (Fig. 5D). The calculated ED_{50} value of ELs-*EPSPS*-transgenic *A. thaliana* was 1844 mg L^{-1}, about 5.5 and 2.6 fold that of the wild-type and Ls-*EPSPS*-transgenic plants, respectively. Therefore, ELs-*EPSPS*-transgenic plants were more resistant to glyphosate than both Ls-*EPSPS*-transgenic and control plants.

4. Discussion

We performed error-prone PCR on Ls-*EPSPS* gene and obtained one highly glyphosate-resistant mutant with an EPSPS carrying five mutated amino acids. These mutations enhanced the mutant's glyphosate-resistance (GR) level likely by affecting the molecular interaction between glyphosate and its binding site in the EPSPS enzyme. Substrate-binding sites of EPSPS determine its affinity for glyphosate [15,22]. Amino acids interacting with glyphosate in the cavity of the EPSPS active site have been extensively studied and identified [23]. Thus, the mutation of these amino acids in active site can significantly change the plant's response to glyphosate. Amino acid mutations away from the active site can also alter glyphosate response, such as those in the 'hinge' region between two EPSPS globular domains and the helix region (second and third) in the N-terminal domain [24,25].

Three new mutations of ELs-EPSPS, 37Val, 351Gly, and 422Gly, affecting its active site cavity have been identified in our study. To better understand the function of these amino acid substitutions, the three mutations in this mutant were located on a structure model of ELs-EPSPS based on the crystal structure of *E. coli* EPSPS (Fig. 2B). In susceptible EPSPS, Pro106 adjusts the position of the 101Gly H-bond [23]; it is likely that the Arg422Gly substitution may also allow for a similar adjustment for 429Lys at the end of the helix due to the much smaller size of 422Gly (Fig. 3B). In addition, 359Glu and 404Arg that stabilize glyphosate could well be shifted "toward" the phosphonate due to the smaller size of 351Gly in the adjacent helix (Fig. 2C). Thus it is possible for residues distantly located from the active site to exert indirect effects on glyphosate/PEP binding leading to improved glyphosate resistance. Besides the molecular size of an amino acid, its molecular polarity can also affect the binding between glyphosate and EPSPS. The EPSPS of *C. arvensis* (CaEPSPS) has a Phe96Ser (numbered according to *A. tuberculatus* EPSPS but reported in the original paper as Phe101Ser based on *E. coli* numbering) substitution. Phe is a nonpolar amino acid whereas Ser is polar. This substitution could contribute to glyphosate tolerance in *C. arvensis* [26]. In our study, Glu37Val substitution had opposite change of polarity but could still impact the affinity between the substrate and EPSPS due to the weakened orientation force (Fig. 3A). Ultimately, experimental data on kinetic properties of EPSPS is required to further characterize the glyphosate resistance conferred by ELs-EPSPS.

The development of transgenic glyphosate-tolerant crops has revolutionized chemical weed control. The combination of glyphosate and a GR crop generally provides better, simpler, cheaper and more flexible weed management than the conventional alternatives [27,28]. The *EPSPS* gene of *A. tumefaciens* CP4 is the most widely used source for commercially-grown transgenic GR crops [10]. An *EPSPS* gene originally isolated from *Zea mays* (event GA21 carrying two mutations) was recently used successfully to produce the first commercial glyphosate-resistant maize cultivar [15]. Interestingly, an *EPSPS* gene from *Eleusine indica* populations that evolved field resistance to glyphosate carries the same mutations and was patented as a possible source for the development of transgenic crops [11,29]. The *EPSPS* gene from *Malus domestica* also has been added to those with potential for commercialization [30]. A number of promising enzymes were identified through selective evolution, site-directed mutagenesis, and microbial screens [30–34]. However, an increased tolerance for glyphosate in EPSPS is often

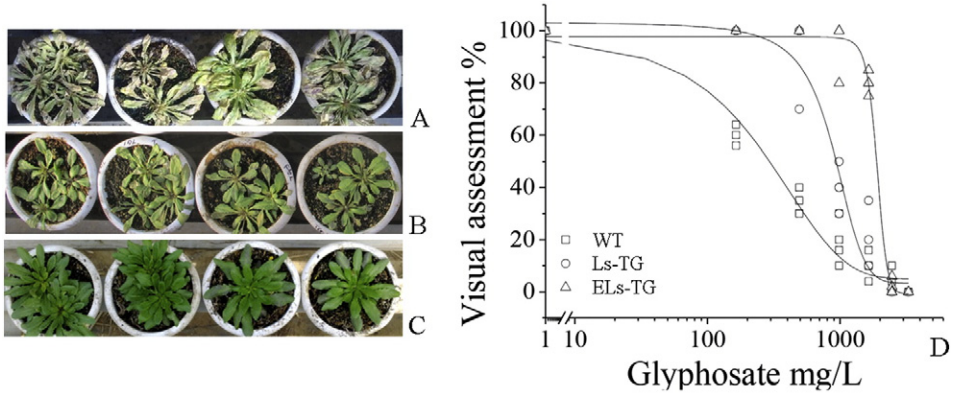


Biotype	D	b	ED_{50}	ED_{50} significance*	Resistance Index
ELs-PET	98.82 ± 1.72	3.46 ± 0.59	15207.24 ± 1777.38	a	2.28
Ls-PET	97.82± 2.46	7.85 ± 1.01	8851.44 ± 175.87	b	1.33
PET	98.09 ± 1.76	10.32 ± 0.91	6656.61 ± 75.63	c	1

Fig. 4. Overexpression of ELs-*EPSPS* gene. (A) Amplification of Ls-*EPSPS* and ELs-*EPSPS* by PCR; (B) Double-enzyme digestion of LS-*EPSPS*-PMD-19T and ELs-*EPSPS*-PMD-19T; (C) Double-enzyme digestion of PET-28a; (D) Recombinant vectors Ls-*EPSPS*-PET-28a and ELs-*EPSPS*-PET-28a; (E) SDS-PAGE analysis of Ls-*EPSPS*-PET-28a and ELs-*EPSPS*-PET-28a (BL21 (DE3)) induced by IPTG for 12 h; (F) Dose-response curve of transgenic BL21 (DE3) with PET-28a, Ls-*EPSPS*-PET-28a or ELs-*EPSPS*-PET-28a to increasing glyphosate doses 12 h after treatment with parameters of the curve provided in the inserted table. Note: M1, DL2000 DNA Marker (Takara, D501A). M2, DL5000 DNA marker (Takara, 3428Q). M3, λ-Hind III digest (Takara, 3403). M4, Premixed Protein Marker (Low) (Takara, 3595Q). CK, control check.

accompanied by a concomitant decrease in the enzyme's affinity for PEP, resulting in decreased catalytic efficiency. Additional mutations such as those in GA21/*Eleusine* restore the catalytic activity of the enzyme to suitable levels for commercialization.

The ELs-*EPSPS* gene bestowed *E. coli* strain with 100 mM glyphosate resistance. The calculated ED_{50} values for the trans-ELs-*EPSPS* strains were significantly higher than those of the wild and trans-Ls-*EPSPS* types (Fig. 4F). Furthermore, we evaluated the potential of the ELs-



Biotype	D	b	ED_{50}	ED_{50} significance*	Resistance Index
ELs-TG	97.51 ± 1.42	12.93 ± 4.51	1844.43 ± 81.67	a	5.45
Ls-TG	101.09± 3.23	3.37 ± 0.75	899.12 ± 62.54	b	2.66
WT	100.93 ± 2.69	0.83 ± 0.16	338.14 ± 89.04	c	1

Fig. 5. Injury response of transgenic and wild type *A. thaliana* to increasing glyphosate concentrations. Wild type (A), Ls-*EPSPS*-transgenic (B) and ELs-*EPSPS*-transgenic (C) *A. thaliana* plants sprayed with 1640 mg ae L^{-1} glyphosate at one week after application; (D) Dose-response curve of transformed and wild type *A. thaliana* based on a logistic function whose parameters are given in the embedded table.

EPSPS gene by transforming it into *A. thaliana* via a simple floral dip method. The EL*s-EPSPS* in transgenic *A. thaliana* was stable and heritable, as confirmed by the glyphosate response bioassay. EL*s-EPSPS* transgenic *A. thaliana* withstood glyphosate at substantially higher doses (ED_{50} of 1844 mg ae L^{-1} glyphosate) than L*s-EPSPS* transgenic *A. thaliana* (ED_{50} of 899 mg ae L^{-1} glyphosate) and, of course, the susceptible, wild type whose ED_{50} values was 338 mg ae L^{-1} glyphosate (Fig. 5D). Even when sprayed with 1640 mg L^{-1} glyphosate, the transgenic newly-mutated EL*s-EPSPS A. thaliana* plants grew almost normally (Fig. 5C). Plant-sourced *EPSPS* of *Z. mays* and *E. indica* conferred transgenic receptors the ability to withstand about 1900 mg ae L^{-1} glyphosate [12,29]. Glyphosate resistance level of transgenic rapeseed is about 1700 mg ae L^{-1} [35]. Therefore glyphosate resistance in EL*s-EPSPS A. thaliana* plants is equivalent to others deemed as of commercial value indicating that this novel gene sourced from *L. spicata* is a potential new choice for crop transformation.

Competing interests

The authors have declared that no competing interests exist.

Acknowledgments

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