

Cloning and characterization of 5-enolpyruvylshikimate-3-phosphate synthase from *Phragmites australis*

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Key words: Paepsps, Glyphosate, Tobacco

Abstract: Glyphosate is a non-selective broad-spectrum herbicide that blocks plant growth by inhibiting 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme of the shikimate pathway in microorganisms and plants. The full-length *epsps* cDNA sequence (*paepsps*, Genbank: KY860582.1) was cloned and characterized for the first time from *Phragmites australis*. The full-length cDNA of *paepsps* was 1308 bp encoding a polypeptide of 435 amino acids. The bioinformatic analyses showed that PaEPSPS has highly homologous with EPSPS from other plants. RT-PCR analysis of *paepsps* expression indicated that the gene expressed in leaves, stems, and roots, with higher expression in leaves. The expression of the *paepsps* gene increased with glyphosate application. In addition, the transgenic tobacco containing the *paepsps* gene showed glyphosate resistance in comparison with control. The novel *paepsps* is a good candidate gene in transgenic crops with glyphosate tolerance in the future.

Introduction

EPSPS is a critical enzyme in aromatic amino acids' biosynthesis that inhibits glyphosate (Herrmann, 1955). Glyphosate is an efficient herbicide mainly used to kill most types of broadleaf weeds and grasses (Baleanu *et al.*, 2019; Zobiolo *et al.*, 2012). Shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) can be converted into 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate by EPSPS (Pollegioni *et al.*, 2011; Raza *et al.*, 2019), respectively. Glyphosate inhibits EPSPS reaction and causes plant death (Pedotti *et al.*, 2010; Tian *et al.*, 2010). Glyphosate has been widely used as a commercial herbicide over the last three decades (Cao *et al.*, 2012; Tian *et al.*, 2013). Due to glyphosate's non-selective feature, it also kills food crops (Tian *et al.*, 2014). Today, there is a great interest in finding resistance genes in glyphosate-resistant plants. Since the *epsps* gene plays an important role in increasing herbicide resistance, many studies have been carried out to understand glyphosate resistance of EPSPS (Tian *et al.*, 2012; Sammons and Gaines, 2014; Guo *et al.*, 2015). Until now, the *epsps* gene has been cloned from eukaryotes and prokaryotes (Liu and Cao, 2015; Huang *et al.*, 2014).

Although the *epsps* gene has been isolated in many plant species, it has never been cloned from *Phragmites australis*. In this experiment, the open reading frame encoding the enzyme EPSPS was cloned, and the sequence was identified for the first time. The potential glyphosate resistance mechanism of *paepsps* was also investigated.

Materials and Methods

Seed source

P. australis seeds were collected from Heilongjiang Bayi Agricultural University, Heilongjiang, China. Young leaves of the *P. australis* were used RNA isolation (TaKaRa, Japan).

Cloning and sequence analysis *paepsps* gene

cDNA from *P. australis* as template amplified putative *paepsps* gene. Primers (F: 5'-ATGGGGGAGATCTCCGGCACCCTG-3', and R: 5'-TCAGTTC TTGACGAAAGTGCTCAG-3') was designed based on the conserved sequence of among all other EPSPS genes identified from other plants. The PCR products were linked to the pMD-18 vector and sequenced. The sequence comparison was observed through the NCBI database. MEGA 5.0 software was used to construct the phylogenetic tree.

RT-PCR and qRT-PCR

Transcriptional level expression of *epsps* in different tissues of *P. australis*, including leaves, stems, and roots. The cDNA

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Received: 11 March 2020; Accepted: 26 June 2020



synthesis was conducted by Invitrogen SuperScript IV (TaKaRa, Japan). cDNA was used as a template for RT-PCR. PCR products are separated and identified by agarose gel electrophoresis.

According to a previous report, the *paepsps* gene expression used to be observed by qRT-PCR analysis (Liu *et al.*, 2015; Yi *et al.*, 2016). Primers of *paepsps* (5'-TGGGGAATGCTGGAAGTCAATGCG-3' and 5'-CTCAAGTACTGACTGCTGATGG ATCCAG-3') were used to amplify of DNA. An actin gene as used as the housekeeping gene. At the six-leaf stage, 10 mM glyphosate was sprayed on reed plants, and the leaves were harvested at different times (12, 24, 36, and 48 h). The expression level of the *paepsps* gene was calculated by the $2^{-\Delta\Delta C_t}$ method.

In vitro glyphosate sensitivity assays

cDNA fragment encoding the protein of PaEPSPS was amplified by PCR via Primers (F5'-CCCA TATGATGGGGGAGATCTCCGGCACCGTG-3', and R 5'-CCGAGCTCTCAGTTCTTGACGAAAGTGCTCAG-3'). The amplified fragment of the *paepsps* gene was digestion by using BamHI and SacI and inserted into pET-28a prokaryotic vector to establish recombinant plasmids pET-EPSPS. ER2799 containing either pET-EPSPS or pET-28a was grown by shaking in liquid M9 minimal medium at 37°C. Different concentration of glyphosate ranging from 0–100 mM were added to the medium. The cell growth densities were tested by spectrophotometry at 600 nm.

Construction of the plant expression vector with *paepsps*

The method of plant vector construction followed the previous report (Tian *et al.*, 2012). In order to locate in the chloroplast, the DNA fragment encoding the chloroplast transit peptide of *Arabidopsis* (TSP) was connected with the front of the *epsps* gene. The recombinant plasmid was introduced into *A. tumefaciens* EH105 by electroporation.

Tobacco transformation

The tobacco genetic transformation method was followed, according to previous reports. 50 mg/L hygromycin, as a screening marker, was used for genetic transformation. The obtained tobacco plants were confirmed by PCR. The PCR products were expanded by primers (5'-TGGGGAATGCTGGAAGTCAATGCG-3; 5'-GGTCTCCT TCACT CTCCAGGAAGC-3); the PCR product was 791 bp (*paepsps*).

Analysis of Southern blot experiment

The extraction of whole plant genomic DNA was performed by using the CTAB method. Genomic DNA was entirely digested using *EcoR* I restriction enzyme. The digestion products were separated by electrophoresis and transferred to a Hybond N⁺ nylon membrane. The DIG-labeled probe amplified a 525 bp fragment from the *paepsps* gene by PCR.

The hybridization test was followed, according to the DNA Labeling and Detection Starter Kit II (Roche, USA).

Verification of glyphosate resistance in transgenic tobacco

The tobacco transformants were grown in sterile culture and then transferred to soil in the greenhouse. Tobacco plants were sprayed with 10 mM glyphosate at the five-to-six leaf stage. The spray tests were observed after two weeks.

Results

Cloning and sequence analysis of *epsps*

A 1308 bp sequence was obtained by PCR. Sequence analysis showed that the *paepsps* gene contained an ORF of 1308 bp (Genebank: KY860582.1), which encodes a polypeptide of 435 amino acids. PaEPSPS protein had one EPSPS conserved sequence (Fig. 1).

The amino acid homology analysis indicated that PaEPSPS was highly homologous to other plant species (Fig. 2a). The evolution tree indicated that PaEPSPS is most similar to *Oryza sativa* (Fig. 2b).

Expression pattern analysis of the *paepsps* gene

The expression of different tissues was also detected at the transcription level. It was found that the expression level of the *paepsps* gene was highest in the leaves, while the expression level in the stems and roots was relatively low (Fig. 3).

The expression of the target gene in the leaves of *P. australis* was analyzed by qRT-PCR during different time periods after spraying glyphosate treatment. The expression of the *paepsps* gene increased at the level of transcription after treatment, and the expression of the *paepsps* had risen three-fold after 36 h (Fig. 4).

Prokaryotic resistance of experiment

The growth curves of the ER2799 cells are shown in Fig. 5. After 36 h of incubation, the results indicated that all cells were well without glyphosate, while under the condition of 75 mM glyphosate, the growth of cells and cells containing pET-28a were severely limited. However, the ER2799 cells containing pET-EPSPS could grow well, but the cells containing pET-EPSPS were entirely suppressed in 100 mM glyphosate.

Obtaining transgenic tobacco with *paepsps* gene

As shown in Fig. 6, the transgenic *paepsps* tobacco plants were obtained by *Agrobacterium*-mediated transformation. First, 50-mg/L hygromycin tobacco in callus culture medium was obtained. Seven tobacco plants containing hygromycin resistance tested positive by PCR (Fig. 7). In addition, the leaves of four hygromycin-resistant plants were positive by RT-PCR (Fig. 8).

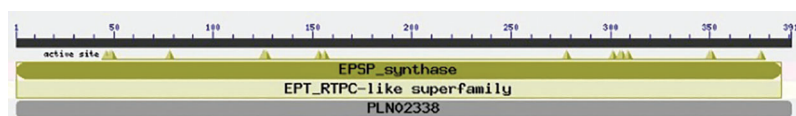


FIGURE 1. The conserved domain of the protein of PaEPSPS.

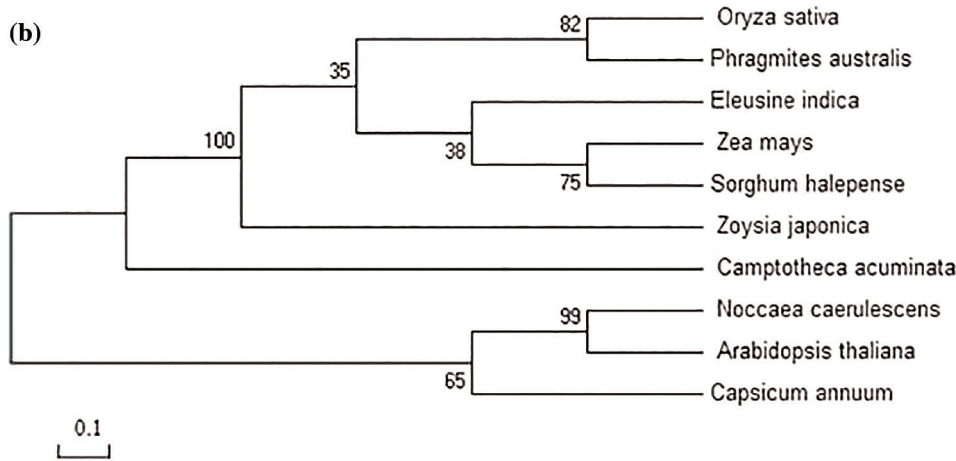
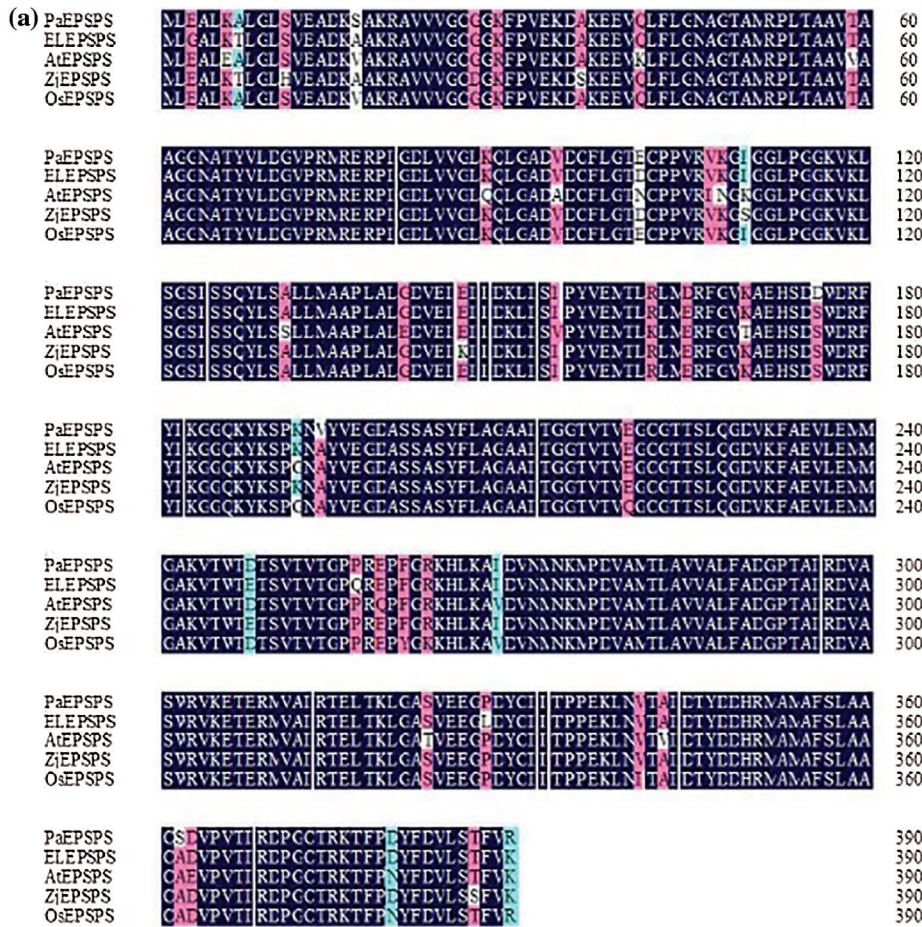


FIGURE 2. (a) Multiple alignments of the deduced amino acid sequence of PaEPSPS with other known plant EPSPS genes. Five EPSPS proteins were *Aegilops tauschii* (AtEPSPS, EMT02861.1), *Oryza sativa* (OsEPSPS, BAA32276.1), *Zoysia japonica* (ZjEPSPS, ADB85252.1), *Eleusine indica* (ELEPSPS, AEB80423.2), and *Phragmites australis* (PaEPSPS, KY860582.1). (b) Phylogenetic analysis of PaEPSPS protein orthologs. Sources of the orthologous proteins were indicated in parenthesis. The numbers on the branches represent bootstrap support for 1,000 replicates.

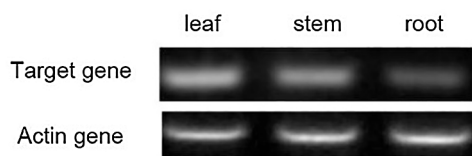


FIGURE 3. The expression of *paepsps* in different tissues.

Southern blot analysis and glyphosate tolerance in transgenic tobacco

Two transgenic tobacco plants were identified from these transgenic lines through Southern blot hybridization. As shown in Fig. 9, the results confirmed the stable integration

of *paepsps* into the tobacco plant genome. In the transgenic line, the copy number of the *paepsps* was a single copy.

The leaves of the wild-type tobacco appeared yellow and eventually died with spray 10 mM glyphosate after two weeks, while the transgenic tobacco continued to grow well (Fig. 10). The results also indicated that the transgenic tobacco with overexpression of *paepsps* had higher glyphosate resistance than the wild type.

Discussion

Although *P. australis* is widespread in the natural environment, there have been no previous reports of glyphosate resistance in

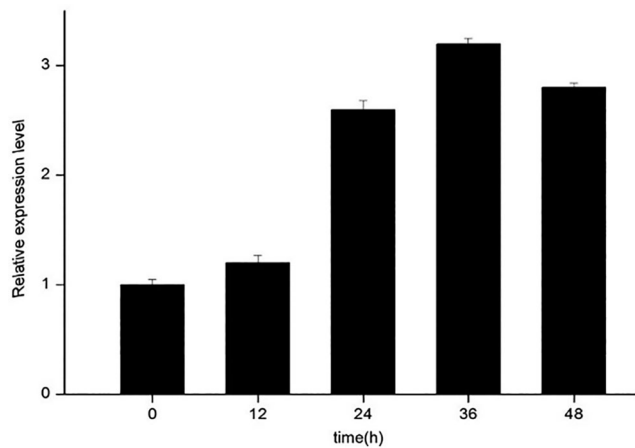


FIGURE 4. mRNA level of the *paepsps* after glyphosate treatment. The quantitative real-time polymerase chain reaction (qRT-PCR) was used for detecting expression level.

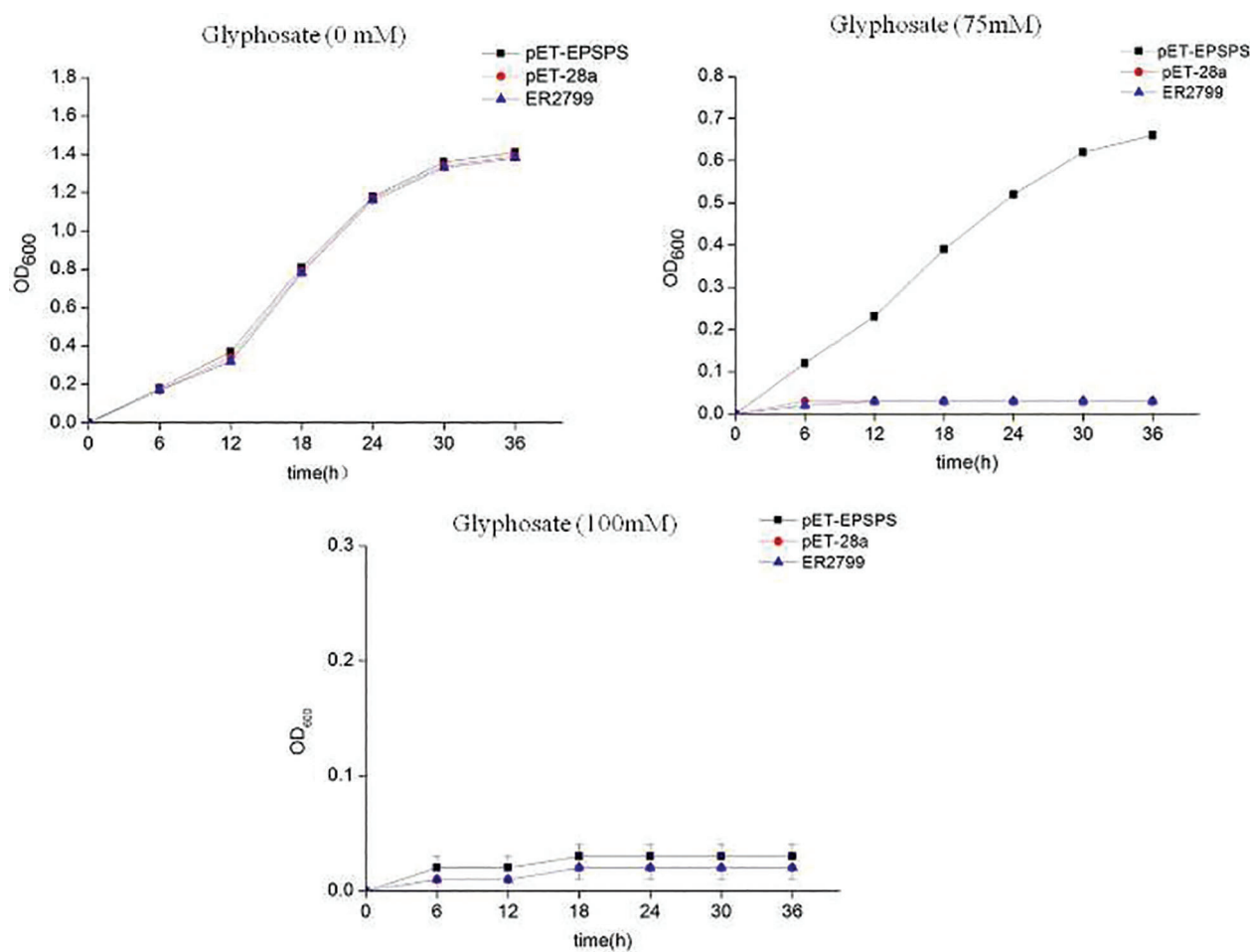


FIGURE 5. Growth of the *E. coli* *epsps* mutant ER2799 harboring either PET-28a, pET-EPSPS in a liquid M9 minimal medium supplemented with various concentrations of glyphosate.

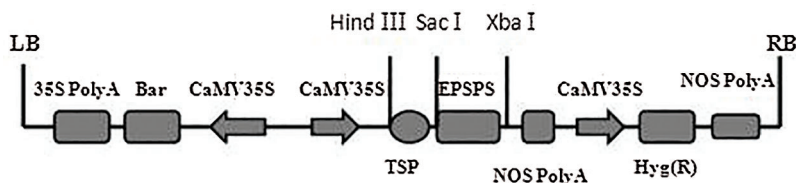


FIGURE 6. The *epsps* expression vector for tobacco transformation.

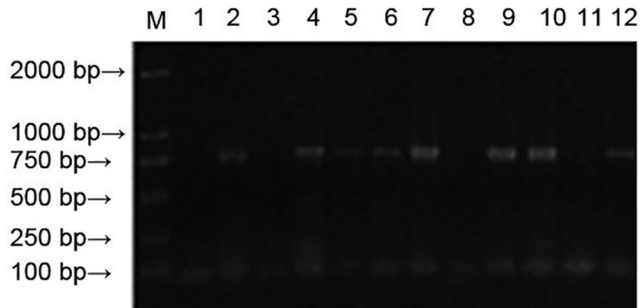


FIGURE 7. The glyphosate-tolerant transgenic plants were confirmed by PCR analysis.

M: 2000 bp DNA marker; 1: non-transgenic tobacco as the negative control; 2: PHB-TSP-EPSPS plasmid as the positive control; 3-12: putative transgenic plants.

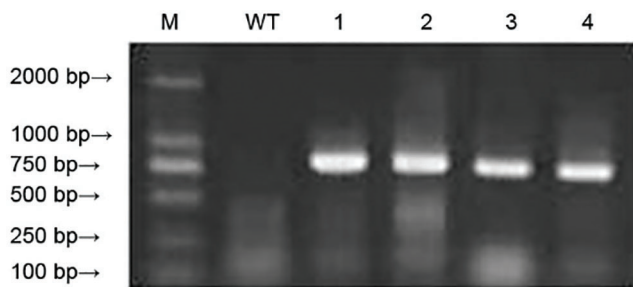


FIGURE 8. Expression analysis of four transgenic lines by RT-PCR. WT: non-transgenic plant; 1-4: four putative transgenic lines.



FIGURE 9. Southern blot analysis of T0 transgenic tobacco. P: The PHB-EPSPS plasmid as the positive control; WT: non-transgenic plant as the negative control; Lanes 1-2, transgenic events W1 and W2, respectively.

this species. Protein sequence analysis showed that PaEPSPS contained one EPSPS conserved sequence. To our knowledge, this is the first report on the cloning and identification of *epsps* from *P. australis*.

Glyphosate efficiently restrains EPSPS by preventing the synthesis of aromatic amino acids, leading to plant death (Shaner *et al.*, 2012; Liu and Cao, 2018a). The *epsps* gene has different transcription levels in plant tissues (Frescura *et al.*, 2013; Han *et al.*, 2014). The transcription level

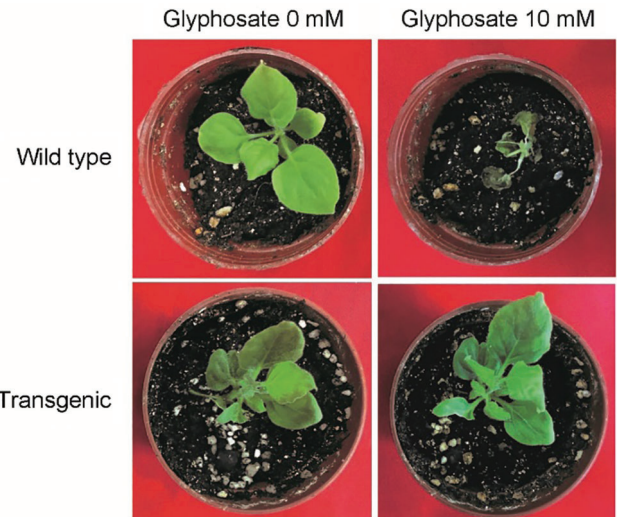


FIGURE 10. Photographs of tobacco plants sprayed with 10 mM glyphosate.

The picture in the left was taken before the spraying treatment, and the picture in the right was taken two weeks after the spraying treatment.

indicated that it is extensive for the expression of the *paepsps* gene, which is expressed in roots, stems, and leaves, and the leaf is the most highly expressed tissue. This transcription level pattern is in the same as *Field bindweed* and *Camptothecin* (Xiao *et al.*, 2012; Huang *et al.*, 2014). Spraying 10 mM glyphosate after 36 h, the transcription expression level of *paepsps* is higher than the control group. The result is the same in field bindweed (Huang *et al.*, 2014). These results illustrate that the *epsps* played a key role in the reaction to glyphosate.

According to a report, the amplification of the *epsps* gene could enhance plant resistance to glyphosate (Zhang *et al.*, 2013; Liu and Cao, 2018b). The petunia cells enhanced glyphosate to tolerance by overexpression of the wild-type *epsps* gene. In our study, the transgenic tobacco plants with the *paepsps* gene survived on 10 mM of glyphosate. These results indicated that the *paepsps* gene could improve the tolerance of transgenic plants to glyphosate. In the previous studies, the transgenic tobacco plants were tolerant to 5 mM glyphosate, while transgenic *paepsps* tobacco were tolerant to 10 mM glyphosate (Peng *et al.*, 2012). In brief, the cloning of the *paepsps* gene will help to further understand the function of *epsps* at the molecular level. In addition, *paepsps* may improve resistance to glyphosate by genetic engineering techniques. The novel *paepsps* is a good candidate gene in transgenic crops with glyphosate tolerance in the future.

Availability of Data and Materials: All data generated or analysed during this study are included in this published article.

Funding Statement: Heilongjiang Bayi Agricultural University Support Program for San Heng San Zong (ZRCQC201909).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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