Vol.64: e21200316, 2021
https://doi.org/10.1590/1678-4324-2021200316
ISSN 1678-4324 Online Edition

Article - Agriculture, Agribusiness and Biotechnology

Cloning and Characterization of 5-enopy ruvylshikimate-3-phosphate Synthase from *Fragaria vesca*

Feng Liu[1]
https://orcid.org/0000-0002-0522-6303

Yingying Ruan[2]*
https://orcid.org/0000-0002-8521-693X

[1]Ningbo City College of Vocational Technology, Department of Horticultural Technology, Ningbo City, Zhejiang Province, China;[2] Ningbo University, Department of Biotechnology, Ningbo City, Zhejiang Province, China

Editor-in-Chief: Paulo Vitor Farago
Associate Editor: Adriel Ferreira da Fonseca

Received: 2020.05.24; Accepted: 2020.11.13.

*Correspondence: coy24@163.com; Tel.: +86-13661552145 (Y.R.)

HIGHLIGHTS

- The *FvEPSPS* encodes a polypeptide of 520 amino acids.
- *FvEPSPS* was constitutively expressed in stems, leaves and roots, with lower expression in roots.
- *FvEPSPS* expression level could increase significantly with glyphosate treatment.
- Transgenic *Arabidopsis Thaliana* with *FvEPSPS* gene exhibited 10 mM glyphosate to resistance.

Abstract: To discover and isolate a glyphosate-resistant gene from *Fragaria vesca* through gene mining. An open reading frame (ORF) of 1563 bp encoding *EPSPS*was amplified from *Fragaria vesca* (*FvEPSPS*). *FvEPSPS* (Genebank: XP004306932.1) encodes a polypeptide of 520 amino acids and it has hightly homologous with EPSPS from other plants. qRT-PCR analysis showed that the *FvEPSPS* was expressed extensively in all tissues including leaves, roots and stems, with higher expression in leaves. Furthermore, transgenic *Arabidopsis Thaliana* exhibited 10 mM glyphosate to resistance. Therefore, this research offers a new glyphosate-resistant gene for development of transgenic crops.

Keywords: *Fragaria vesca*; *FvEPSPS*; glyphosate; transformation; *Arabidopsis Thaliana*.

INTRODUCTION

5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) is a key enzyme in aromatic amino acid synthesis pathway [1]. Because the target enzyme of glyphosate inhibition is EPSPS, this enzyme is widely studied in glyphosate resistant plants. Herbicide glyphosate inhibits EPSPS reaction and causes plant death, so it also kills food crops by its non-selective feature [2,3]. The 80% of commercial glyphosate-resistant GM crops are related to EPSPS.

The application of *EPSPS* gene in the transgenic glyphosate resistant crops is mainly used through overproduction of the wild-type *EPSPS* or expression of mutant *EPSPS* with glyphosate resistance [4-7]. Since the *EPSPS* gene plays an important role in increasing herbicide resistance, much studies have been

carried out to understand glyphosate resistance of *EPSPS* [8-10]. Until now, *EPSPS* gene has been cloned from from bacteria, fungi, herb plants and wood plants [11-13]. Today, there is a great interest in finding glyphosate tolerant genes for GM crops.

Although *EPSPS* gene has been isolated in many plant species, it has never been cloned from *Fragaria vesca*. A naturally glyphosate-resistant *Fragaria vesca* was found in Shanghai, China, whereas its mechanism of resistance to glyphosate is still unknown. In this experiment, the open reading frame encoding the enzyme FvEPSPS was cloned and the sequence was identified for the first time. The potential glyphosate resistance mechanism of *FvEPSPS* was also investigated.

MATERIAL AND METHODS

Seed source

The *Fragaria vesca* seeds were collected from Shanghai Jiaotong University, Shanghai, China. Young leaves were used as the starting material for RNA isolation.

Cloning and sequence analysis *FvEPSPS* gene

cDNA from *Fragaria vesca* as template amplified putative *FvEPSPS* gene. The PCR production that inserted into pMD-18 vector was sequenced. The PCR products linked to pMD-18 vector and sequenced. The sequence comparison was observed through NCBI database. MEGA 5.0 software was used to built the phylogenetic tree. The primers are listed in Table 1.

Quantitative real-time RT-PCR

According to a previous report, the *FvEPSPS* gene expression used to be observed by qRT-PCR analysis. Transcriptional level expression of *FvEPSPS* were used in different tissues of *Fragaria vesca*, including leaves, stems and roots at 30-day-old seedlings, respectively. A 18SrRNA gene was used as housekeeping gene. 10 mM glyphosate was sprayed on *Fragaria vesca*, and the leaves were harvested at different hours (12, 24, 36 and 48 h). The expression level of *FvEPSPS* gene was calculated by $2^{-\Delta\Delta t}$ method. The qRT-PCR primers are listed in Table 1.

Table 1. Primers used in study

Primer name	Sequence (5'– 3')	Purpose of primers
FvEPSPS-1	ATGGCCCAAGTGAGCAAAATCTGC	Amplify the *FvEPSPS*
FvEPSPS-2	TTAATGTTTTGTAAACTTCCCAAGG	
FvEPSPS-3	CCCATATGATGGCCCAAGTGAGCAAAAT	Amplify the *FvEPSPS* for prokaryotic expression vector
FvEPSPS-4	CCTCGAGTTAATGTTTTGTAAACTTCCCA	
TSP-1	CCCAAGCTTATGGCTCAAGTTAGCAGAATCTGC	Amplify the signal peptide
TSP-2	GAGTACTCATGACCTTAAGAGGACGAAGC	
FvEPSPS-5	GAGTACTATGGCCCAAGTGAGCAAAATC	Amplify the *FvEPSPS* for eukaryotic expression vector
FvEPSPS-6	GCTCAGATTAATGTTTTGTAAACTTCCC	
18SrRNA-f	AGAAACGGCTACCACATC	qRT-PCR analysis of *18SrRNA*
18SrRNA-r	CCATCCCAAAGTCCAAC	
qFvEPSPS-f	GCCGTTGACTGCTGCAGTAACTG	qRT-PCR analysis of *FvEPSPS*
qFvEPSPS-r	TTCAACATCTCCCAGAGCCAAAG	

In vitro glyphosate sensitivity assays

cDNA fragment encoding the protein of FvEPSPS was amplified by PCR. The amplified fragment of *FvEPSPS* gene was digestion by using NdeI and XhoI and inserted into pET-28a prokaryotic vector to establish recombinant plasmids pET-FvEPSPS. ER2799 containing either pET-FvEPSPS or pET-28a was grown by shaking in liquid M9 minimal medium at 37°C. The medium add to different concentration of glyphosate ranging from 0-100 mM. The cell growth densities were tested by spectrophotometry at 600 nm. The primers are listed in Table 1.

Construction of the plant expression vector with *FvEPSPS*

The method of plant vector construction follows the previous report [13]. In order to located in chloroplast, the DNA fragment encoding the chloroplast transit peptide of Arabidopsis (TSP) was connection with the front of the *FvEPSPS*. The recombinant plasmid was introduced into *A. tumefaciens* EH105 by electroporation. The method for genetic transformation of *Arabidopsis thaliana* was by *Agrobacterium*-mediated transformation. The primers are listed in Table1.

Arabidopsis Thaliana transformation

The floral-dipping method of *Arabidopsis Thaliana* follows according to previous reports. T_0 generation seeds of the transgenic *Arabidopsis Thaliana* were selected on 1/2 MS solid medium with 40 mg L^{-1} hygromycin. T_2 generation plants of hygromycin resistance confirmed by RT-PCR and harvested for further research.

Analysis of glyphosate resistance in transgenic *A. thaliana*

To test root length of *A. thaliana*, the T_2 Sterilized *A. thaliana* seeds were grown on 1/2MS solid medium containing glyphosate (0, 300, 500, and 800 μM). After grown for ten days, the root length was surveyed. The *A. thaliana* transformants were grown in sterile culture and then transferred to soil in the greenhouse. Spraying with 10 mM glyphosate on plants at 40-day-old. The spray tests were observed after six days.

RESULTS

Cloning and sequence analysis of *FvEPSPS*

A 1563 bp sequence (named *FvEPSPS*) was obtained by PCR. Sequence analysis showed that the *FvEPSPS* gene contained an ORF of 1563 bp (Genebank: XP004306932.1), which encodes a polypeptide of 520 amino acids. FvEPSPS protein had one EPSPS conserved sequence. The amino acid homology analysis indicated that FvEPSPS belongs to Class I EPSPS (Figure 1a). The evolution tree indicated that FvEPSPS is most similar to *Rosa Chinensis* (Figure 1b).

a

```
FvEPSPS        MAQVSKICSNGAQSFNLLPNVSKPQIPRSLNSISLKSQFLGSSNSLSLKRESGFVGRVRAGGLRVSASVVTAEKPSTVPE    80
E.coli-EPSPS   ..............................................................................     0
CP4-EPSPS      ..............................................................................     0

FvEPSPS        IVLQPIKEISGTITLPGSKSLSNRILLIAALSEGTTVVDNLLDSDDINYMLGALKTLGLNVEDDRANKRAVVEGCGGQFP   160
E.coli-EPSPS   ..........................................................TRCEIIGNGG..P    11
CP4-EPSPS      ..........................................................RKEGDTWIIDGVGNGGL    17

FvEPSPS        LGNSGEEVQLFLGNAGTAMRPLTAAVIAAGGHSRYVLDGVPRMRERPIGDLVTGLKQLGADVDCFLGTDCPPVRVIGKG   240
E.coli-EPSPS   LHAEG..ALELFLGNAGTAMRPIAAALCLGS..NDIVLTGEPRMKERPIGHLVIALRLGCAKITYLEQENYPPLRLQG..    85
CP4-EPSPS      LAPLA....PLDFGNAATGCRLTMGLVGVYD..FDSTFIGLASLTKRPMGRVLNPLREMGVQVKSEDGDRLP.VTLRG..    88

FvEPSPS        GLPGGKVKLSGSISSQYLTSLLMATPLALGDVEVEIIDKLISIPYVEMTLKLMERFGVTVEHSDSWDRFLVRGGQKYKSP   320
E.coli-EPSPS   GFTGGNVDVDGSVSSQFLIALLMTAPLAPEDTVIRIKGDLVSKPYIDITLNLMKTFGVEIEN.QHYQQFVVKGGQSYQSP   164
CP4-EPSPS      PKTPTPITYRVPMASAQVKSAVLLAGLNTPG.ITTVIEPIMTRDHTEKMLQGFGAN.LTVETIADGVRTIRLEGRGKLTG   166

FvEPSPS        GKAPVEGDASSASY.FLAGAAVTGGTVTVEGCGTSSLQGDVKFAEVLEKMGAEVTWTENSVTVTG.PQRHSSGRKHLKAV   398
E.coli-EPSPS   GTYIVEGDASSASY.FLAAAAIKGGTVKVTGIGRNSMQGDIRFADVLEKMGATICWGDDYISCT.........RGELNAI   234
CP4-EPSPS      QVIDVEGDPSSTAFPLVAALLVPGSDVTILNVLMNPTR..TGLILTLQEMGADIEVINPRLAGGEDVADLRVRSSTLKGV   244

FvEPSPS        DVNMNKMPD...DVAMTLAVVALFADGPTAIRDVASWRVKETERMIAICTELRKLGATVEEGPDYCIITP.PE.....KLN   469
E.coli-EPSPS   DMDMNHIPD...DAAMTIATAALFAKGTTTLRNIYNWRVKETDRLFAMATELRKVGAEVEEGHDYIRITP.PE.....KLN   305
CP4-EPSPS      TVPEDRAPSMICEYPILAVAAAFAEGATVMNGLEELRVKESDRLSAVANGLKLNGVDCDEGETSLVVRGRPDGKGLGNAS   324

FvEPSPS        VIAIDTYDDHRMAMAFSLAACG.DVPVTIKDPGCTRKTFPNYFEVLGKFTKH........                       520
E.coli-EPSPS   FAEIATYNDHRVAMCFSLVALS.DTPVTILDPKCTAKTFPDYFEQLARISQAA.......                       357
CP4-EPSPS      GAAVATHLDHRIAMSFLVMGLVSENPVTVDLATMIATSFPEFMDLMAGLGAKIELSDTKA                         384
```

b

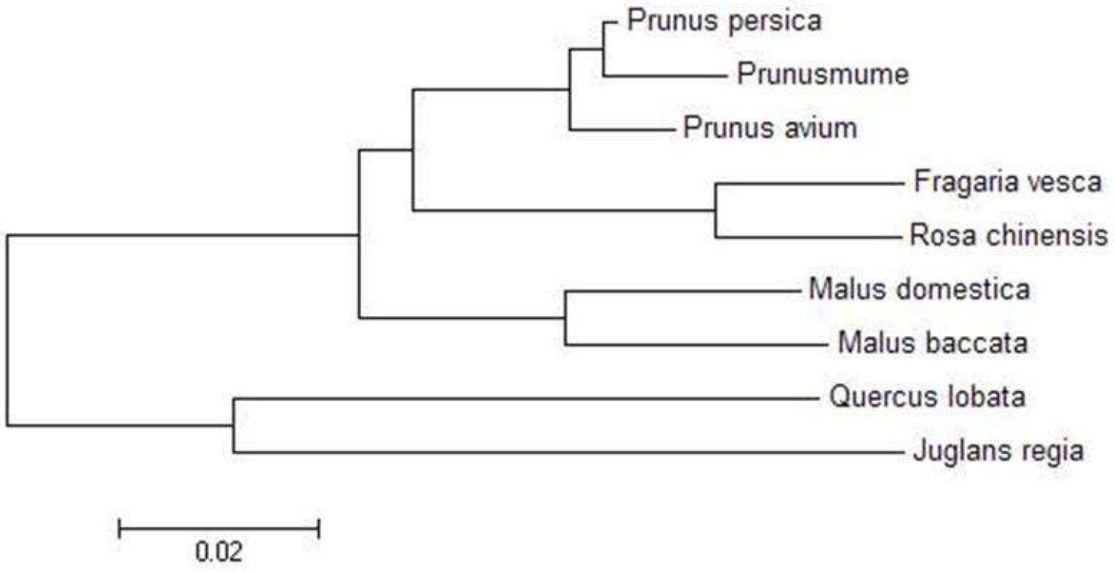

Figure 1. (a) Multiple alignments of deduced amino acid sequence of FvEPSPS with other known plant EPSPS genes. Three EPSPS proteins were *Fragaria vesca* (FvEPSPS, XP004306932.1), *Escherichia coli* (E.coli-EPSPS, P07638) and *Agrobacterium tumefaciens CP4* (CP-EPSPS, Q9R4E4). **(b)** Phylogenetic analysis of FvEPSPS protein orthologs. Sources of the orthologous proteins were indicated in parenthesis. The numbers on the branches represent bootstrap support for 1,000 replicates.

Expression pattern analysis of *FvEPSPS* gene

The expression of different tissues was also detected at the transcription level. It was found that the expression level of *FvEPSPS* gene was highest in the leaves, while the expression level in the stems and roots was relatively low (Figure 2). The expression of target gene in the leaves of *Fragaria vesca* was analyzed by qRT-PCR during different time periods after spraying glyphosate treatment. The expression of

the *FvEPSPS* gene increased at the level of transcription after treatment, and the expression of the *FvEPSPS* had risen more three-fold after 24h (Figure 3).

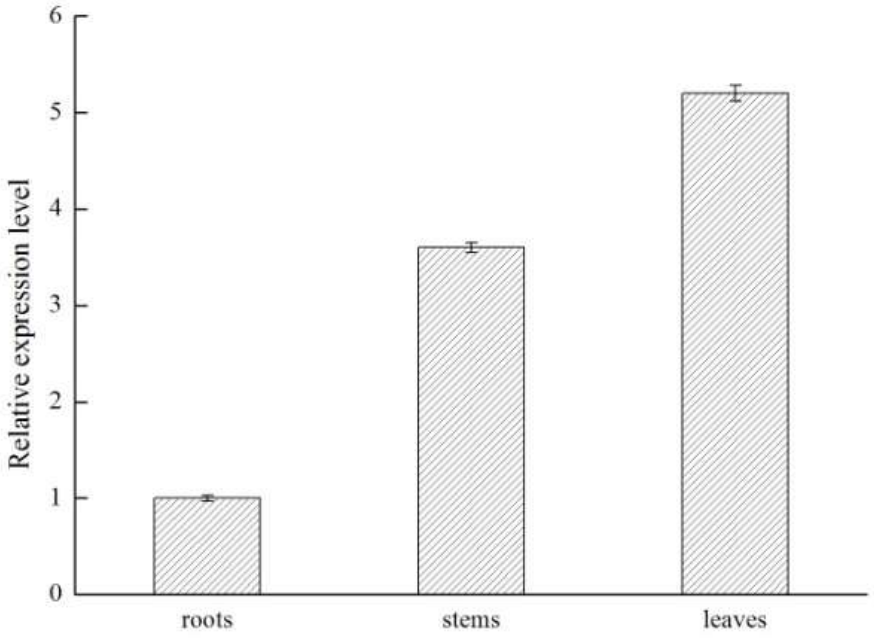

Figure 2. The expression of *FvEPSPS* in different tissues.

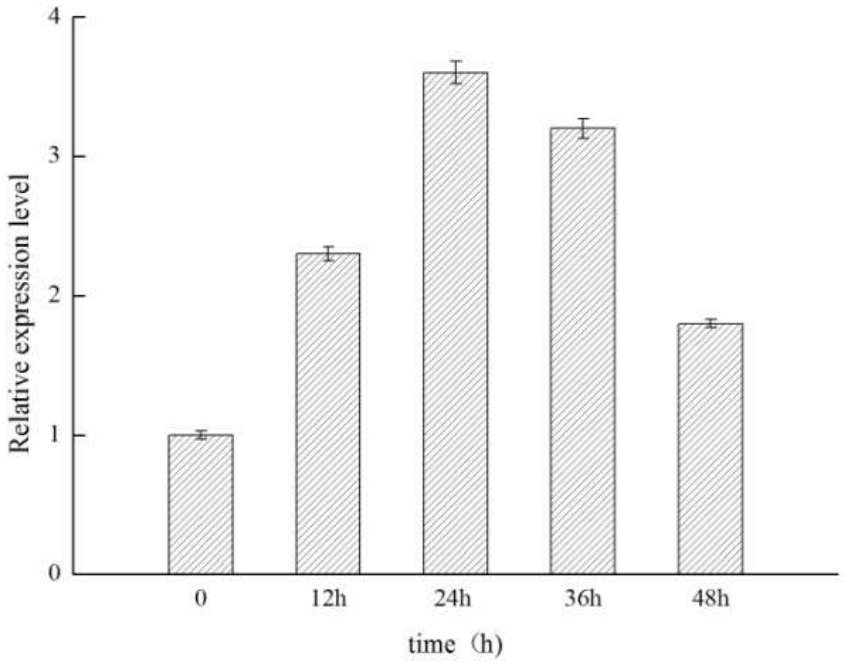

Figure 3. mRNA level of the *FvEPSPS* after glyphosate treatment.

Growth of cells in the presence of glyphosate

The growth curves of the ER2799 cells are shown in Figure 4. 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 grew well, but the cells containing pET-FvEPSPS was entirely suppressed in 100 mM glyphosate.

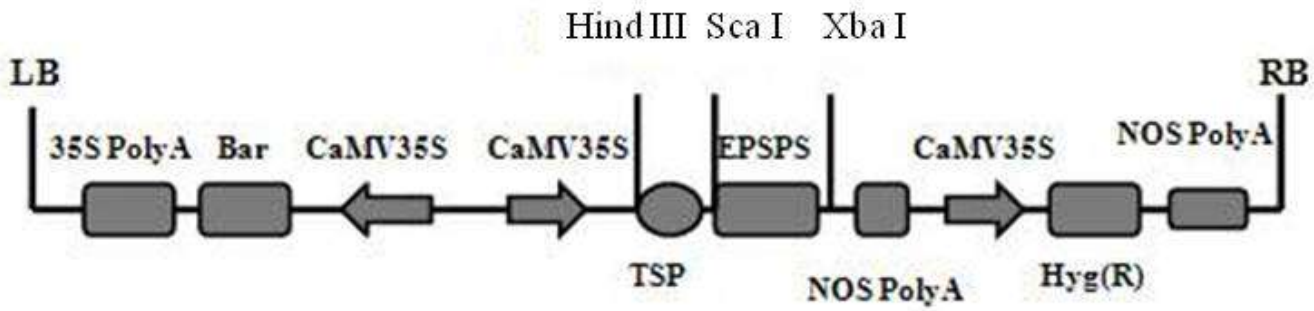

Figure 4. Growth of the *E. coli* EPSPS mutant ER2799 harboring either PET-28a, pET-FvEPSPS in a liquid M9 minimal medium supplemented with various concentrations of glyphosate**.**

Glyphosate tolerance in transgenic *Arabidopsis thaliana*

Construction of the plant expression vector with *FvEPSPS* was used to infect *Arabidopsis* (Figure 5). RT-PCR analysis showed that *FvEPSPS* expressed in three transgenic T_2 lines (Figure 6). One line was selected to analyze resistance to glyphosate with wild type as controls. Glyphosate affects plant root growth. Under the action of glyphosate, the root system grew weakly. As shown in Figure 7, the results showed that transgenic plants overexpressing *FvEPSPS* gene were able to grow with 800 μM glyphosate, whereas wild-type plants were strongly inhibited with 300 μM glyphosate. In addition, all transgenic lines showed more tolerance to glyphosate (Figure 8). The leaves of the wild-type appeared yellow and eventually died with spray 10 mM glyphosate after six days, while the transgenic plants continued to grow well. The results also indicated that the transgenic *Arabidopsis thaliana* with overexpression of *FvEPSPS* had higher glyphosate resistance than the wild type.

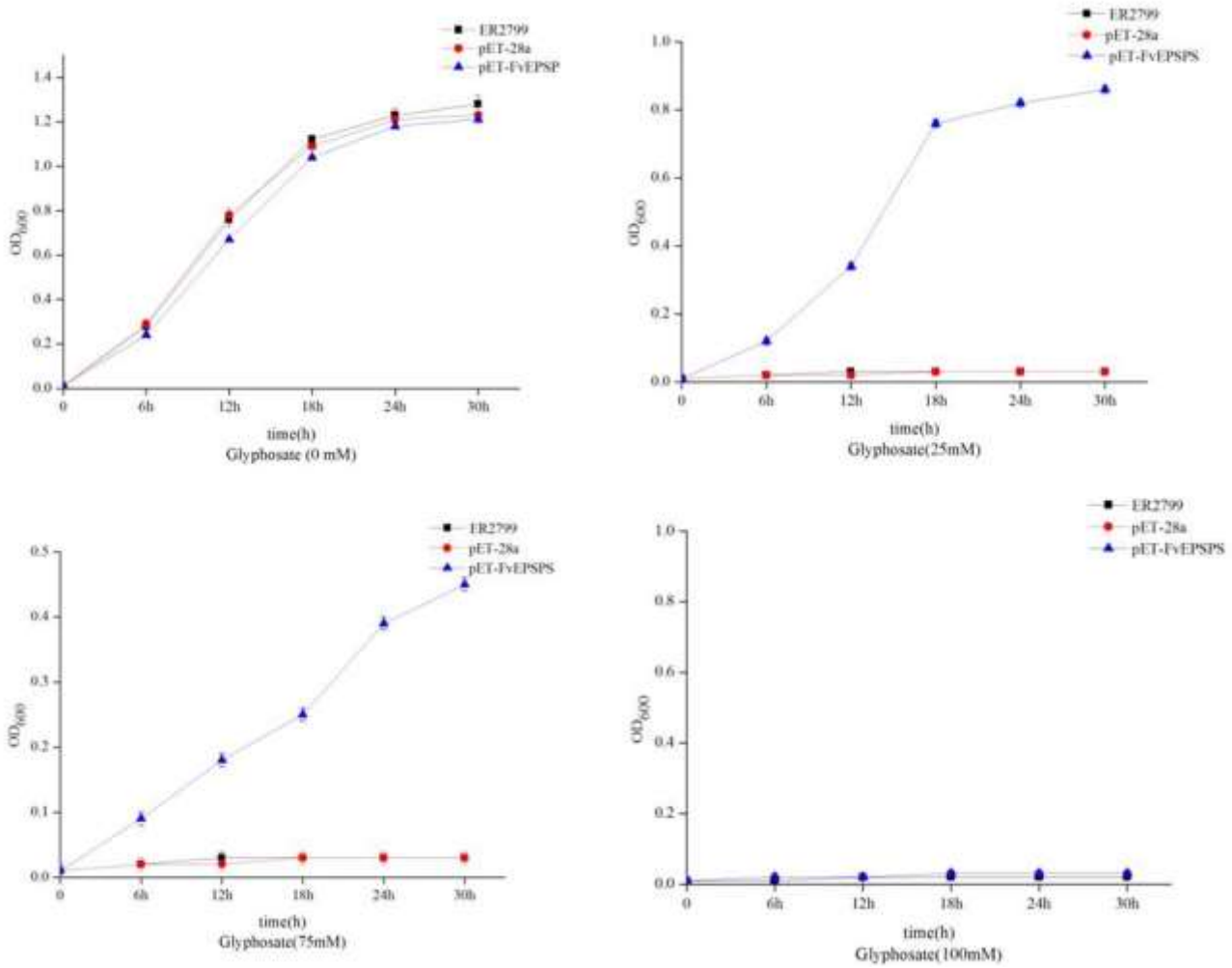

Figure 5. The plant expression vector with FvEPSPS.

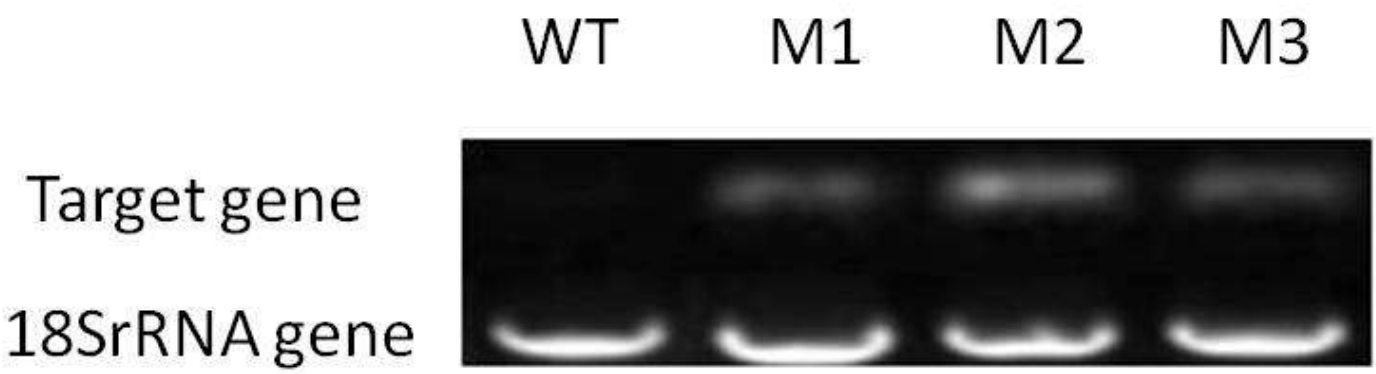

Figure 6. Expression analysis of three transgenic lines by RT-PCR. WT: non-transgenic plant; M1-M3: three putative transgenic lines.

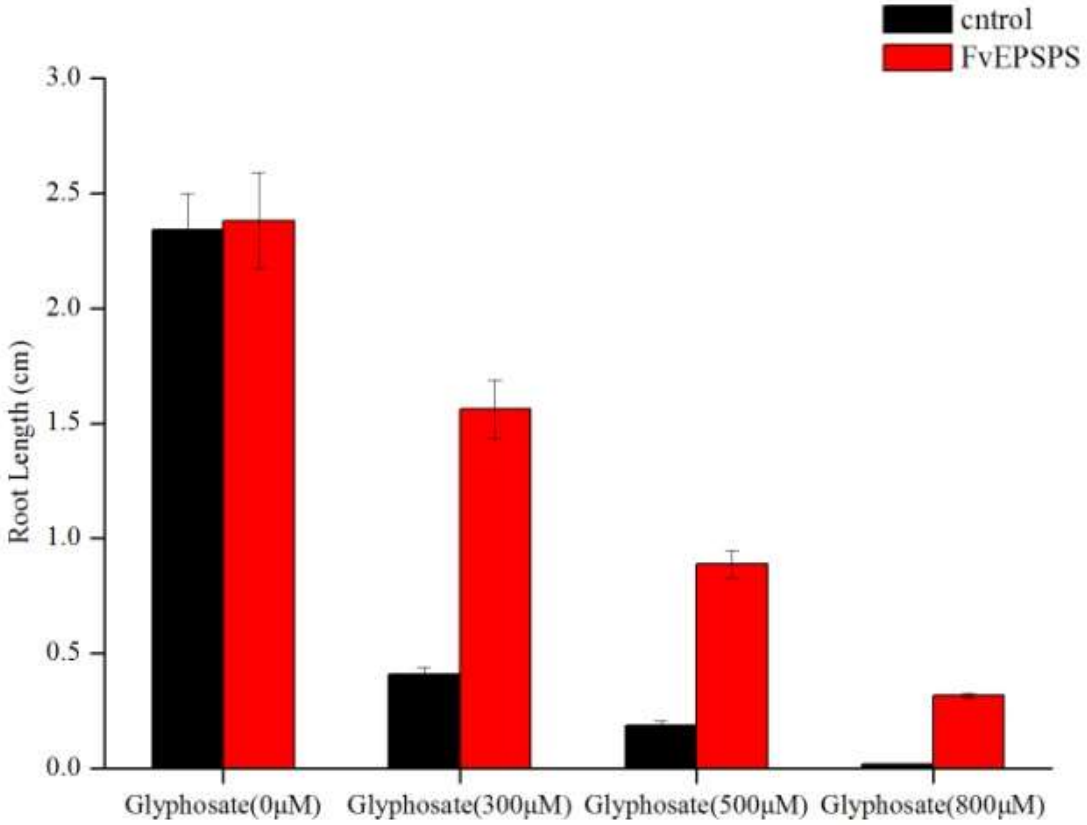

Figure 7. Root growth development of the transgenic plants under glyphosate stress.

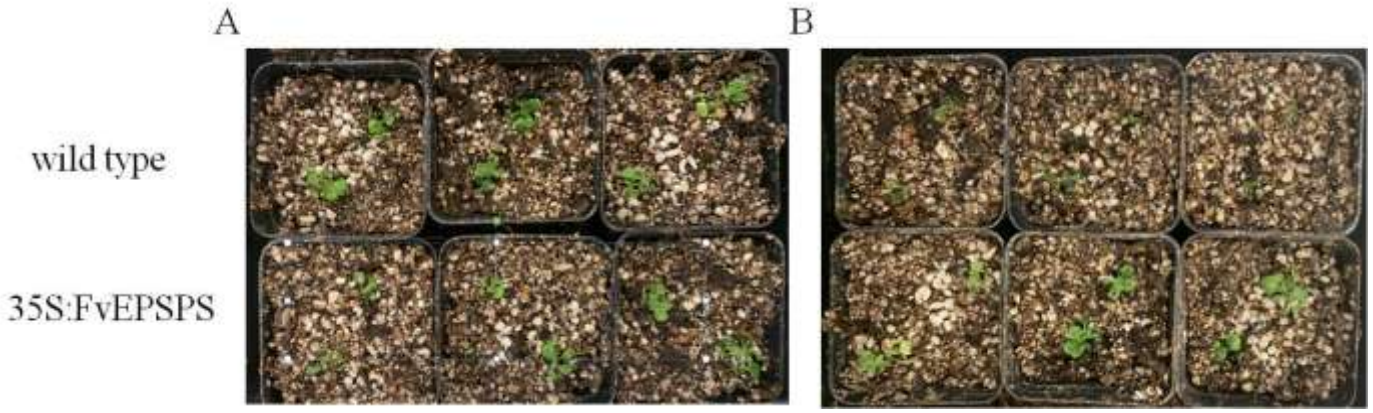

Figure 8. Photographs of *Arabidopsis thaliana* sprayed with 10 mM glyphosate. (A) Photograph taken before the spraying treatment. (B) Photograph taken 6 days after the spraying treatment.

DISCUSSION

Although *Fragaria vesca* is widespread in natural environment, there have been no previous reports of glyphosate resistance in *Fragaria vesca*. To our knowledge, this is the first report on the cloning and identification of EPSPS from *Fragaria vesca*.

Glyphosate efficiently restrains EPSPS by preventing the synthesis of aromatic amino acids, leading to plant death [14]. EPSPS gene has different transcription level in plant tissues [4]. The transcription level indicated that it is extensive for the expression of the FvEPSPS 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 *Camptotheca acuminata* [15,16]. Spraying 10 mM glyphosate after 36 h, transcription expression level of *FvEPSPS* is higher than the control group. The result is the same in field bindweed. These illustrated that the EPSPS played a key role in the reaction to glyphosate.

According to report, the amplification of EPSPS gene could enhanced plant resistance to the glyphosate . The petunia cells enhanced glyphosate to tolerance by overexpression wild-type EPSPS gene. In our study, the transgenic plants with *FvEPSPS* gene survive on 10 mM of glyphosate. These results indicated that *FvEPSPS* gene could improve the tolerance of transgenic plants to glyphosate. In the previous studies, the transgenic tobacco plants is 5 mM glyphosate to tolerance [17], while transgenic *FvEPSPS Arabidopsis thaliana*. is 10 mM glyphosate to tolerance. Therefore, this research offers a new glyphosate-resistant gene for development of transgenic crops.

Funding: This research was funded by foundation of Ningbo City College of Vocational Technology, grant number ZZX18123.

Conflicts of Interest: “The authors declare no conflict of interest.” “The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results”.

REFERENCES

1. Herrmann KM. The shikimate pathway: early steps in the biosynthesis of aromatic compounds. Plant Cell 1995 Jan:907-19.
2. Cao G, Liu Y, Zhang S, Yang X. A novel 5-enolpyruvylshikimate-3-phosphate synthase shows high glyphosate tolerance in *Escherichia coli* and tobacco plants. PLoS One**.** 2012 Mar;7:318-29.
3. Gao Y, Tao B, Qiu L.Role of physiological mechanisms and EPSPS gene expression in glyphosate resistance in wild soybeans (Glycine soja). Pestic Biochem Physiol.2014 Sep;109:6-11.
4. He M, Yang ZY, Nie YF. A new type of class I bacterial 5-en0opyruvylshikimate-3-phosphate synthase mutants with enhanced tolerance to glyphosate. Biochim Biophys Acta Gen Subj. 2001 Feb;68:1-6.
5. Liang A, Sha J, Lu W. A single residue mutation of 5-enoylpyruvylshikimate-3- phosphate synthase in *Pseudomonas stutzeri* enhances resistance to the herbicide glyphosate. Biotechnol Lett. 2008 Jan;30:1397-401.
6. Liu F, Cao YP. Cloning and characterization of 5-enopyruvylshikimate-3- phosphate synthase from *Pantoea* sp. GMR. 2015 Mar; 14:19233-41.
7. Yi SY, Cui Y, Zhao Y. A Novel Naturally Occurring Class I 5-Enolpyruvylshikimate-3-Phosphate Synthase from *Janibacter* sp. Confers High Glyphosate Tolerance to Rice. Sci Rep. 2016 Mar; 6:191-14.
8. Duke SO, Powles SB. Glyphosate: a once-in-a-century herbicide. Pest Manage Sci**.** 2008 Oct; 64:319-25.
9. Tian YS, Xu J, Han J. Complementary screening, identification and application of a novel class II 5-enopyruvylshikimate -3-phosphate synthase from *Bacillus cereus*. World J. Microbiol Biotechnol. 2013 Sep; 29:549-57.
10. Han J, Tian YS, Xu J, Wang LJ. Functional characterization of *aroA* from *Rhizobium leguminosarum* with significant glyphosate tolerance in transgenic *Arabidopsis*. J Microbiol Biotechnol.2014 Mar; 24:1162-9.
11. Baerson SR, Rodriguez DJ, Tran M, Feng Y. Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. Plant Physiol. 2002 Oct; 129:1265-75.
12. Huang ZF, Zhang CX, Huang HJ. Molecular cloning and characterization of 5-enolpyruvylshikimate -3-phosphate synthase gene from *Convolvulus arvensis* L. Mol Biol Rep. 2014 Sep;41:2077-84.
13. Liu F, Cao YP. Expression of a bacterial *aroA* gene confers tolerance to glyphosate in tobacco plants. Turk J Biol. 2018 Aug;42:187-94.
14. Liu F, Cao YP. Expression of the 5-enoylpyruvylshikimate-3-phosphate synthase domain from the *Acremonium sp.* aroM complex enhances resistance to glyphosate. Biotechnol Lett. 2018 Feb;40:855-64.
15. Binarová P, Cvikrová M, Havlický T. Changes of shikimate pathway in glyphosate tolerant alfalfa cell lines with reduced embryogenic ability. Biol. Plant. 1994 Mar;36:65-73.

16. Gong Y, Liao Z, Chen M. Characterization of 5-enolpyruvylshikimate-3-phosphate synthase gene from *Camptotheca acuminata*. Biol. Plant. 2014 Feb;50:542-50.
17. Peng RH, Tian YS, Xiong AS. A novel 5-enolpyruvylshikimate-3-phosphate synthase from *Rahnella aquatilis* with significantly reduced glyphosate sensitivity. PloS. One.2012 Oct;7(8):36-42.