Mol Breeding (2014) 33:197–208
DOI 10.1007/s11032-013-9944-7

Over-expression of the *Gr5$_{aroA}$* gene from glyphosate-contaminated soil confers high tolerance to glyphosate in tobacco

Jin Wang · Kaijing Zuo · Wei Lu · Yu Zhu · Chun Ye · Min Lin · Kexuan Tang

Received: 9 January 2013 / Accepted: 16 August 2013 / Published online: 6 October 2013

Abstract Herbicide resistance is the most widely used transgenic crop trait for broad-spectrum control of weeds. Here we report a novel 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene (*Gr5$_{aroA}$*) isolated from glyphosate-contaminated soil. The full *Gr5$_{aroA}$* gene was 1,819 bp and contained a 1,341-bp open reading frame encoding a 47-kDa protein. Phylogenetic analysis showed that Gr5$_{aroA}$ is a class I EPSPS even though most such enzymes are naturally sensitive to glyphosate. Interestingly, Gr5$_{aroA}$ protein contained highly conserved PEP and S3P binding residues (Glu-351) and several motifs insensitive to glyphosate. Transgenic *Gr5$_{aroA}$* plants (T_0) grew normally and produced seeds which we treated with a high-glyphosate solution (4× recommended spray). Analysis of the T_1 progenies showed that *Gr5$_{aroA}$* was inherited at a Mendelian 3:1 segregation ratios and that glyphosate tolerance in T_1 plants was unchanged. Our results show the *Gr5$_{aroA}$* gene to be a promising candidate for the development of commercial transgenic crops with high glyphosate tolerance.

Keywords Gr5$_{aroA}$ · Glyphosate tolerance · Transformation · Transgenic plant

Abbreviations

MS	Murashige and Skoog (1962) medium
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase

Introduction

Glyphosate (*N*-phosphonomethyl glycine) is one of the world's most extensively used broad-spectrum herbicides. It is effective against the majority of annual and perennial grasses and broad-leaved weeds. In plants, the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) enzyme normally catalyzes shikimate-3-phosphate (S3P) and phosphoenolpyruvate

Jin Wang, Kaijing Zuo, and Wei Lu have contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11032-013-9944-7) contains supplementary material, which is available to authorized users.

J. Wang · W. Lu · Y. Zhu · M. Lin (✉)
Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China
e-mail: linmin57@vip.163.com

J. Wang
e-mail: wjdsz@vip.sina.com

K. Zuo · K. Tang
Plant Biotechnology Research Center, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai 200030, China
e-mail: kxtang1@yahoo.com; kxtang1@sohu.com

C. Ye
School of Bioengineering, Southwest Jiaotong University, Chengdu 610064, China

(PEP) into EPSP and inorganic phosphate (Haslam 1993) [Electronic Supplementary Material (ESM) Fig. 1). Glyphosate cannot bind free EPSPS enzyme but instead forms a EPSPS–S3P–glyphosate complex, which ultimately stops aromatic amino acid biosynthesis by the plant via the shikimate pathway (Hollander-Czytko and Amrhein 1980; Herrmann 1995). Over-expression of the EPSPS gene could reduce this competitive binding affinity and allow the plant to synthesize aromatic amino acids and function normally in the presence of glyphosate (Padgette et al. 1995a, b; Priestman et al. 2005a, b).

Glyphosate resistance was first reported in transgenic tobacco expressing the $P^{101}S$ substitution mutant of *Salmonella typhimurium* EPSPS (Comai et al. 1985; Stalker et al. 1985). Many EPSPS genes, including the wild-type class II EPSPS and the mutant class I EPSPS gene (*aroA*), have been reported to improve glyphosate tolerance when engineered in transgenic plants (Barry et al. 1992; Chen et al. 1999; Ye et al. 2001; He et al. 2003; Wang et al. 2003). Class I EPSPS enzymes include those present in *Escherichia coli*, *Aeromonas salmonicida*, and *Arabidopsis thaliana*, all of which are naturally sensitive to glyphosate (Lewendon and Coggins 1983). Resistance to glyphosate can be achieved through mutation of the target enzyme, such as by mutating the residues around the glyphosate-binding site (Stalker et al. 1985; Padgette et al. 1991; Shuttleworth et al. 1999; Eschenburg et al. 2002; He et al. 2003; Priestman et al. 2005a, b; Haghani et al. 2008). Class II EPSPS enzymes are naturally tolerant to glyphosate, and some such enzymes have been identified in *Agrobacterium tumefaciens* CP4, *Bacillus subtilis*, and *Pseudomonas* sp. strain PG2982 (Fitzgibbon and Braymer 1990; Barry et al. 1992). Unfortunately, transgenic plants that over-express most of the EPSPS genes described above have failed to show sufficient glyphosate resistance for commercial utilization (Bradshaw et al. 1997). The transgene in most commercial glyphosate-resistant crops is the EPSPS gene from *Agrobacterium* spp. CP4 (Pilacinski 2002; Funke et al. 2006).

Structural studies have confirmed that PEP and glyphosate have the same binding site and that glyphosate inhibition is competitive with PEP (Boocock and Coggins 1983; McDowell et al. 1996; SchÖnbrunn et al. 2001; Funke et al. 2006, 2009; Pollegioni et al. 2011). Class I EPSPS enzyme mutants always exhibit decreased affinity for substrate PEP with an increased tolerance to glyphosate (Padgette et al. 1995a, b). Only those glyphosate-resistant EPSPS enzymes with a high PEP binding affinity are suitable for commercial plants (Funke et al. 2009). It has been reported that many organisms isolated from glyphosate-contaminated soil show the spontaneous occurrence of glyphosate resistance. For example, $AroA_{P.\ Putida}$ from glyphosate-contaminated soil was found to exhibit high tolerance to glyphosate without any decrease in its affinity for PEP (Sun et al. 2005). The T97I/P101S-mutated *E. coli* EPSP synthase, which was found to confer insensitivity to glyphosate, has been used to generate herbicide-resistant varieties of corn GA21 (Funke et al. 2009). In summary, novel EPSPS genes from glyphosate-contaminated soil and EPSPS genes mutated at multiple residues are potential candidates for use in the engineering of crops with acceptable levels of herbicide tolerance for commercialization (Sun et al. 2005; Yi et al. 2007; Li et al. 2009; Tian et al. 2011; Sun et al. 2012; Zhou et al. 2012).

In this study, we isolated a new EPSPS gene from glyphosate-contaminated soil. The full gene [$Gr5_{aroA}$; GenBank Acc. no. (Bankit tool) 1013095] was 1,819 bp and contained a 1,341-bp open reading frame (ORF) encoding a protein of 447 amino acids. Homology analysis and molecular modeling revealed that $Gr5_{aroA}$ has a low homology with other EPSPS genes, such as the *CP4 EPSPS* gene from *Agrobacterium* spp. and the *aroA* genes from *E. coli* and *Typhimurium.* We also identified the function of this EPSPS gene by generating transgenic $Gr5_{aroA}$ tobacco plants. The level of glyphosate tolerance and other agricultural characteristics of transgenic $Gr5_{aroA}$ tobacco plants were evaluated.

Materials and methods

Construction of the metagenomic library from soil heavily contaminated with glyphosate

Glyphosate-contaminated soil was collected from the ground near the glyphosate storage and transportation room of the Hebei Qixing Glyphosate Production Co. (Hebei Province, China). Metagenomic DNA was extracted from the soil according to the method described by Griffiths (Griffiths et al. 2000;

Kauffmann et al. 2004). Crude DNA was purified by agarose gel electrophoresis using a QIAEXII Gel Extraction kit (Qiagen, Venlo, The Netherlands). Purified DNA was then partially digested with *Sau3AI* (Promega, Madison, WI) and concentrated with electrophoresis on a 1 % agarose gel. DNA fragments of 3–5 kb were then ligated into *Sau3AI*-digested pACYC184 plasmid DNA using T4 DNA Ligase (Promega). The recombinant plasmids were electroporated into *E. coli* ER2799 (*aroA*-deleted mutant, New England Biolabs, Lpswich, MA) to generate a metagenomic library (Yanisch-Perron et al. 1985; Dower et al. 1988; Sambrook et al. 1989).

Cloning and sequence analysis of *aroA* gene

The DNA metagenomic library was screened on MOPS minimal medium supplemented with chloramphenicol, kanamycin, and 10 mM glyphosate. Positive clones were transferred onto MOPS minimal medium supplemented with chloramphenicol, kanamycin, and 150 mM glyphosate for the second screening. One colony harboring a plasmid with a 2-kb insert fragment was selected. The insertion fragment was then sequenced and named $Gr5_{aroA}$.

Structural analyses and phylogenic analysis of *aroA* genes

$Gr5_{aroA}$ protein was predicted based on sequence similarity by searching against the cDNA sequences in the GenBank database. For functional domain identification, we used Scan PROSITE on the ExPASy Web server for PROSITE motif database screening (http://ca.expasy.org/tools/scanprosite/) and NCBI RPS-BLAST for Pfam and SMART to screen conserved domain databases (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

EPSPS and AroA proteins from different organisms were used in our evolutionary analyses: *Escherichia coli* (P07638), *Aeromonas salmonicida* (Q03321), *Arabidopsis thaliana* (P05466), *Nicotiana tabacum* (P23981), *Petunia hybrida* (P11043), *Zea mays* (CAA44974), *Bordetella pertussis* (P12421), *Agrobacterium tumefaciens* CP4 (Q9R4E4), *Bacillus subtilis* (P20691), *Staphylococcus aureus* (Q05615), *Dichelobacter nodosus* (Q46550), *Streptococcus pneumoniae* (Q9S400), *Pseudomonas putida* (4G-1) (AJ812018), *Pseudomonas sp. strain* PG2982 (P56952), *P. fluorescens* Pf-5 (AAY93565), *P. entomophila* L48 (YP_607169), *P. aeruginosa* UCBPP-PA14 (ABJ12389), *P. mendocina ymp* (ZP_01526458), *P. syringae* pv. *phaseolicola* 1448A (YP_273200), and *P. putida* KT2440 (AAN67390). Deduced amino acid sequences of AroA were aligned by Clustal_X with manual adjustments (Jeanmougin et al. 1998). Phylogenetic trees were reconstructed using MEGA 4 (Kumar et al. 2004). These trees were based on the neighbor-joining method with the p-distance model. Gap sites in the alignment were not used in the phylogenetic reconstruction (the complete-deletion option). The reliability of the estimated trees was evaluated by the bootstrap method, with 1,000 pseudo-replications (Felsenstein 1985).

$Gr5_{aroA}$ protein expression

The DNA fragment containing the coding region of $Gr5_{aroA}$ was obtained by PCR using the pACYCG2 plasmid as the template with the following two primers: primer 1 (5′-CGGGA TCCAT GGCGT GTTTG CCTGA TGA-3′) and primer 2 (5′-CCAAG CTTTC AGGCA AACAC CTCGAG-3′). The fragment was digested with *Bam*HI and *Hin*dIII and then cloned into the corresponding restriction sites of the *pET-28a* vector (Novagen, Madison, WI). The expression vector *pET-28a* harboring $Gr5_{aroA}$ was confirmed by DNA sequencing. The plasmid was then transformed into *E. coli* BL21 (DE3) competent cells, and the positive *E. coli* BL21 clone was grown at 37 °C in 100 ml LB medium containing 50 μg/ml kanamycin. Cells were collected and diluted to a density 0.60 at OD600. Isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 mM for the induction of $Gr5_{aroA}$ protein expression. After 4 h, the cells were collected by centrifugation and re-suspended in 10 ml of 50 mM Tris buffer (pH 7.0) with 0.1 mM dithiothreitol. The cell suspension was frozen at −70 °C and then thawed to room temperature. Cells were lysed by sonication. The crude homogenate was clarified by centrifugation at 12,000 rpm for 30 min at 4 °C. The resulting supernatants were assayed for protein expression.

Enzyme activities assay of $Gr5_{aroA}$

$Gr5_{aroA}$ protein activity was measured by the production of inorganic phosphate using the malachite green dye assay method (Lanzetta et al. 1979). The standard reaction was carried out at 28 °C in a final reaction volume of 100 µl containing 50 mM HEPES, pH 7.0, 1 mM S3P, 1 mM PEP, 0.1 mM $(NH_4)_6Mo_7O_{24}{\cdot}2H_2O$, and purified enzyme. After incubation for 3 min, 1 ml of malachite green–ammonium molybdate colorimetric solution was added and the solution mixed thoroughly, following which 0.1 ml of 34 % sodium citrate solution was added. After a 30-min incubation at room temperature, samples were measured at 660 nm. The same reaction solution without S3P was used as the control.

Transformation and regeneration of transgenic $Gr5_{aroA}$ tobacco plants

The $Gr5_{aroA}$ coding sequence was inserted into the region between the CaMV35S promoter and the nopaline synthase terminator (Nos-ter) on *Agrobacterium* binary vector *pBI121* (Rogers et al. 1986). The resulting vector, *pBI-*$Gr5_{aroA}$, contained the selectable marker neomycin phosphotransferase gene (*npt*II) and the $Gr5_{aroA}$ gene driven by the CaMV35S promoter (ESM Fig. 2). The expression cassette was then introduced into *A. tumefaciens* strain EHA 105 by tri-parental mating using a previously described protocol (Hood et al. 1993; Horsch et al. 1988). and subsequently used to generate transgenic tobacco plants.

Tobacco (*Nicotiana tobacum*) cv. Petit Havana SR1 was used for transformation following the protocol described by Horsch et al. (1988) (see Murashige and Skoog 1962). The kanamycin-resistant plants were subjected to PCR, Southern blot, and quantitative real time (qRT)-PCR analyses to establish the presence and expression of the $Gr5_{aroA}$ gene.

Verification of transgenic plants

Genomic DNA was extracted from young kanamycin-resistant leaves according to the method described by Doyle and Doyle (1990), and then PCR analysis was carried out for the detection of the $Gr5_{aroA}$ gene in transgenic plants using the forward primer $Gr5_{aroA}$ F1 (5′-ATGGC GTGTT TGCCT GATGAT-3′) and the reverse primer $Gr5_{aroA}$ R1 (5′-GTGGA TTTGC TGACT GTGTGT-3′). The expected product size was 1.34 kb. The PCR reactions were carried out in a total volume of 25 µl containing 50 ng tobacco genomic DNA, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 µM dNTPs, 1.25 U of *Taq* DNA polymerase, and 25 pmol of each primer. For PCR amplification, DNA was denatured at 94 °C for 3 min followed by 30 amplification cycles of 94 °C for 50 s, 53 °C for 60 s, and 72 °C for 90 s, with a final extension at 72 °C for 10 min.

Southern blot analysis was further used to confirm the transgenic status of plants. Nine transgenic tobacco plants (No. 1, 3, 7, 12, 13, 15, 18, and 29) were analyzed by Southern blot hybridization for the presence of the $Gr5_{aroA}$ gene in the tobacco plant. Aliquots of genomic DNA (8 µg) were digested overnight at 37 °C with *Xba*I (which cuts only once in pBI $Gr5_{aroA}$), fractionated by 0.8 % agarose gel electrophoresis, and transferred to a Hybond-N^+ membrane (Amersham Bioscience, Amersham, UK). The $Gr5_{aroA}$ probe (ORF) was labeled with [^{32}P]dATP using a random-priming kit (Ready-to-Go; Pharmacia, Freiburg, Germany). Hybridization was carried out according to the method described by Thomas (Thomas 1980). Signals were visualized by exposure to Fuji X-ray film (Fuji, Tokyo, Japan) at −70 °C for 2 days.

The qRT-PCR analysis was used to investigate $Gr5_{aroA}$ gene expression according to the manual of SYBR premix Ex-Taq (Takara, Otsu, Japan). The specific primers (5′-CAC CACCT GGCGC GTCGC-3′, 5′-GGTCG GGATC GTATC CTG-3′) were used to amplify the $Gr5_{aroA}$ gene. The ubiquitin gene (Accession number: U66264.1) was used as the control. Each sample was tested three times. The results were analyzed by the comparative ΔCT method and Option 3 software.

Glyphosate tolerance assay of T_0 transgenic plants and T_1 transgenic lines

Nine independent transgenic tobacco T_0 lines (No. 1, 3, 7, 12, 13, 15, 18, and 29) were used in the glyphosate tolerance assay. Plants of the independent transgenic lines and wild-type (WT) (untransformed) plants were micro-propagated, and six plants of each were grown under a 14/10-h light/dark photoperiod with an irradiance of 50 µmol m^{-2} s^{-1} provided by white

fluorescent tubes. Both the transgenic and WT tobacco plants were then transferred into soil in the greenhouse to determine their glyphosate tolerance. In this assay, all plants were sprayed at the five- to six-leaf stage (about 2 weeks after transplanting) with a 41 % glyphosate salt solution (the active ingredient) to generate six levels of injury: 2.35 l/ha (1.0 kg a.i./ha; 0.5× recommended concentration), 4.7 l/ha (2.0 kg a.i./ha; 1×), 9.3 l/ha (3.9 kg a.i./ha; 2×), 14.0 l/ha (6.0 kg a.i./ha; 3×), 18.6 l/ha (7.8 kg a.i./ha; 4×), and 23.0 l/ha (9.8 kg a.i./ha; 5×). Vegetative injury was determined by visual observation 2 weeks after treatment. Agronomic characteristics were investigated at plant maturity.

T_1 seeds were harvested from different glyphosate-tolerant T_0 plants. Both WT and T_1 seeds were germinated in MS medium without kanamycin. Positive and negative plants were confirmed using PCR. At least ten plants from each independent transgenic line and WT and non-transgenic tobacco plants were transferred into soil in the greenhouse for a second glyphosate tolerance assay using the same procedure as in the assay of T_0 plants.

Analysis of herbicide resistance was performed according to the method described by Ye et al. (2001). Individual plants were scored for tolerance to glyphosate using a scale ranging from 0 (normal plant, fertile, no delay in maturity, seeds setting normally), through to 1 (small chlorosis on the leaves, fertile, no delay in maturity, seeds setting normally), 2 (less than one leaf wilted 2 weeks after treatment, fertile, no delay in maturity, seeds setting normally), 3 (two or more leaves wilted after treatment, stunt, delay in maturity, seeds setting un-normally), and 4 (wilt, dead plant). The injury data for all treated plants were averaged. Average values of <2.0 were considered to indicate tolerance relative to the treatment, while average values >2.0 were considered to indicate intolerance relative to the treatment.

Genetic analysis of segregation of the $Gr5_{aroA}$ gene in T_1 progenies

Seven independent T_0 transgenic plants (No. 1, 3, 7, 12, 13, 15, 18, and 29) were grown to maturity. T_1 seeds were harvested and sown in soil in the greenhouse. The germinated T_1 plants (2 weeks old) were analyzed for the presence of the $Gr5_{aroA}$ gene by the PCR method previously described for the segregation pattern analysis. Northern bolt analysis was performed to determine $Gr5_{aroA}$ gene expression in T_1 progenies of the same seven lines.

Results

Isolation of genes involved in glyphosate tolerance from the metagenomic library

In order to clone novel glyphosate tolerance genes, we constructed a soil metagenomic library with 5.06×10^5 clones. This library was then screened on glyphosate-resistant MOPS plates using mutant ER2799 as the recipient. After the first round of screening, 28 clones were found to tolerate 10 mM glyphosate. These positive clones were then transferred onto MOPS-minimal-medium plates containing chloramphenicol, kanamycin, and 150 mM glyphosate for the second screening. One colony harboring a plasmid with an insert of about 2 kb was selected based on the results of the second screening; the insert was named $Gr5_{aroA}$, and was subsequently sequenced and analyzed.

Bioinformatic analysis of $Gr5_{aroA}$ gene

Sequence analysis showed that the $Gr5_{aroA}$ gene was 1,819 bp and contained a 1,341-bp ORF encoding a protein of 447 amino acids (ESM Fig. 3). The deduced $Gr5_{aroA}$ aroA protein was found to have 30 % identity with *E. coli aroA* and 70 % identity with *Pseudomonas syringae* pv. B728a at the amino acid level.

To determine whether the $Gr5_{aroA}$ gene could be translated, we inserted the coding region of $Gr5_{aroA}$ into *pET-28a* (*E. coli* BL21 as the host cell) to detect protein expression. The soluble proteins in cell-free extracts were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the expression level of $Gr5_{aroA}$ protein. One major band appeared at around 45 kDa, and this band corresponded with the putative molecular weight of the protein detected in all induced samples (ESM Fig. 4).

Phylogenetic analysis revealed that the EPSPS and AroAs from different organisms could be grouped into two different classes. Class I AroA proteins consisted of typical AroA proteins from *E. coli*, *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Zea mays.* EPSP

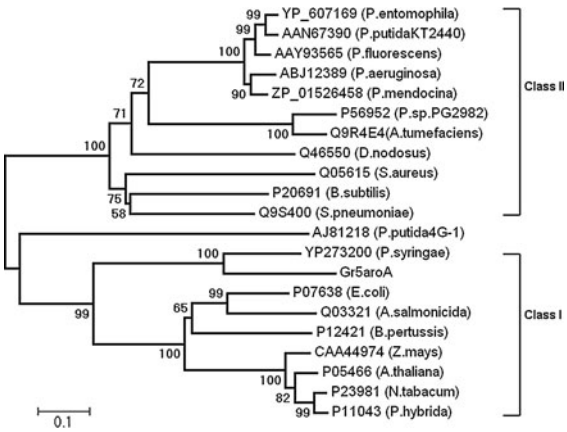

Fig. 1 A neighbor-joining (NJ) tree was constructed based on AroA amino acids sequences using the p-distance model. The complete deletion option was selected for the gaps or missing data. This tree is unrooted and bootstrapped 1,000 times

synthase proteins from *A. tumefaciens* CP4, *Bacillus subtilis*, and *Pseudomonas fluorescens* Pf-5 were grouped as class II AroA proteins. Based on the phylogenetic tree, we assigned $Gr5_{aroA}$ to class I (Fig. 1; ESM Fig. 5). $Gr5_{aroA}$ contains three conserved domains (^{49}GSKS^{52}, ^{196}SSQYV^{200}, and ^{319}SQMQ^{322}), which is characteristic of class I EPSPS enzymes.

In order to identify the residues implicated in the insensitivity of the $Gr5_{aroA}$ protein, we aligned Gr5aroA protein with other class I and II proteins from 14 different accessions. Among these reported residues (T42M, G96A, P101S, G101A/G137D, G101A/P158S, T97I/P101S) whose mutations could increase glyphosate tolerance, we identified the 101st amino acid of Gr5aroA protein to be phe (Padgette et al. 1991; Shuttleworth and Evans 1994; Eschenburg et al. 2002; He et al. 2003; Priestman et al. 2005a, b; Funke et al. 2009;). Gr5aroA protein was also found to have the residues Glu-351 and Arg-354, which are involved in PEP and S3P binding for CP4 EPSP synthase (SchÖnbrunn et al. 2001). $Gr5_{aroA}$ protein contains the strictly conserved EPSP synthase residues Glu-351 and Arg-354 in $Gr5_{aroA}$, which are involved in PEP glyphosate binding. Taken together, these results indicated that the $Gr5_{aroA}$ gene is an EPSPS gene and the result of extreme glyphosate selection.

Kinetic properties of $Gr5_{aroA}$ protein

Protein extracts prepared from *E. coli* BL21 cells containing the plasmids harboring $Gr5_{aroA}$ were used to analyze $Gr5_{aroA}$ enzyme activity. The *E. coli* EPSP synthase from the mutant AB2829 host cells was used as the control. The soluble proteins in cell-free extracts were separated by 10 % SDS-PAGE to determine the expression level of the $Gr5_{aroA}$ protein. One major band of protein was detected at about 45 kDa in all induced samples that corresponded with the molecular weight of EPSPS (ESM Fig. 4) (Lewendon and Coggins 1983). Kinetic analysis revealed the half maximal inhibitory concentration (IC_{50}) of $Gr5_{aroA}$ protein to be 24.06 mM, and its $Km_{(PEP)}$ and $Ki_{(glyphosate)}$ values were 220 and 217 μM, respectively. Relative to *E. coli* AroA, $Gr5_{aroA}$ protein showed a 360-fold higher $Ki_{(glyphosate)}$ value. Its catalytic efficiency relative to PEP utilization was increased by about threefold (Table 1). These results showed that $Gr5_{aroA}$ had a high tolerance to glyphosate and PEP catalytic efficiency.

Table 1 Kinetic constants of $Gr5_{aroA}$ and *Escherichia coli* EPSPS

Kinetic constants	G_{aroA}	*E. coli* aroA
IC_{50} (glyphosate) (mM)	24.061 ± 0.021	0.021 ± 0.005
K_m (PEP) (μM)	220 ± 13.01	86.2 ± 0.78
Sp act (nkat/mg)	22.83 ± 0.64	7.75 ± 0.61
K_i (glyphosate) (μM)	217 ± 2.00	0.6 ± 0.02

Generating transgenic $Gr5_{aroA}$ tobacco plants

To determine whether the $Gr5_{aroA}$ gene induced glyphosate tolerance in plants, we constructed an expressing vector, *pBI121-CaMV35S::*$Gr5_{aroA}$*::Nos*, and used *A. tumefaciens*-mediated transformation to generate transgenic $Gr5_{aroA}$ tobacco plants. Tobacco leaf discs were transformed with *A. tumefaciens* strain EHA 105 containing *pBI-*$Gr5_{aroA}$ (ESM Fig. 2) using the method described by Horsch et al. (1988). Following two rounds of kanamycin (50 mg/l) selection, 152 kanamycin-resistant plants were regenerated. Forty-eight plants showed amplification of the predicted fragment of $Gr5_{aroA}$, while no amplification was observed in the control plants (data not shown).

The results of Southern hybridization showed that each plant contained one or more $Gr5_{aroA}$-specific hybridizing bands, and the unique hybridization patterns observed indicated that each plant was derived from an independent transformation event

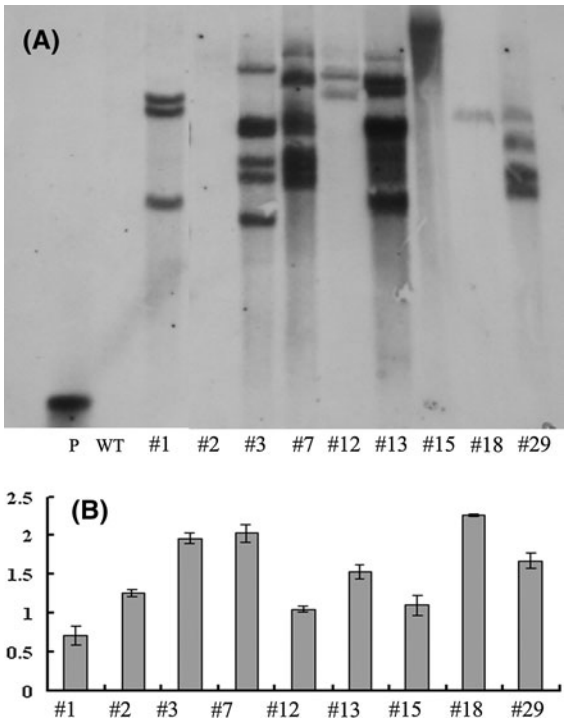

Fig. 2 Verification of transgenic $Gr5_{aroA}$ gene tobacco plants. a Representative Southern blot analysis for the presence of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene ($Gr5_{aroA}$) gene in transgenic tobacco plants. Plant genomic DNA (8 μg) and pBI $Gr5_{aroA}$ were digested with XbaI, separated by 1.0 % agarose gel electrophoresis, and hybridized to the ^{32}P-labeled $Gr5_{aroA}$ probe. *Lanes 1* $Gr5_{aroA}$ plasmid (positive control), 2 untransformed plant (negative control), *3–11* independent transgenic plants corresponding to lines No. 1, 2, 3, 7, 12, 13, 15, 18, and 29, respectively. b Representative real time-PCR analysis of the expression of $Gr5_{aroA}$ in transgenic tobacco plants

(representative sample shown in Fig. 2a). The copy number of $Gr5_{aroA}$ in the independent transgenic plants ranged from one to more than five.

The expression of $Gr5_{aroA}$ in independent transgenic lines was also detected by qRT-PCR analysis. The expression level varied across different lines, with the maximum expression level being about threefold higher than the lowest expression level (Fig. 2b).

Glyphosate tolerance in transgenic $Gr5_{aroA}$ plants

No significant differences were observed in the growth processes of WT and transgenic plants; therefore, the seedlings of both transgenic and WT plants were treated with six different concentrations of glyphosate salt solution. When treated with 0.5× the recommended dose of herbicide, WT plants maintained the same growth rate as transgenic plants even though their leaves showed some chlorosis. With treatment of 1× the recommended herbicide treatment, the leaves of the WT plants wilted after 7 days and no WT plant survived >14 days after treatment. In order to determine the highest concentration of glyphosate that transgenic plants could tolerate, we then sprayed 2×, 3×, 4×, and 5× the recommend dose of herbicide onto the plants. WT tobacco plants showed severe vegetative damage and died within 1 week. In contrast, transgenic tobacco plants showed no significant differences in newly emerged leaves, meristem tissues, or seed set characteristics following the 2× or 3× treatment, respectively. With the 4× the recommended dose of herbicide treatment, however, all transgenic plants showed a slight chlorosis on newly emerged leaves and their flowering times were delayed by about 2–3 days without stunting. Finally, when transgenic plants were sprayed with 5× the recommended dose of herbicide, growth was inhibited and severely stunted and no seeds were produced (Fig. 3; Table 2; ESM Table 1). In summary, the maximum glyphosate concentration endured by transgenic $Gr5_{aroA}$ plants was fourfold the recommended field dose. At this level, transgenic plants could still grow, flower, and produce seeds.

$Gr5_{aroA}$ glyphosate tolerance could be inherited in T_1 progenies

To prove that $Gr5_{aroA}$ glyphosate tolerance could be inherited in T_1 progeny, $Gr5_{aroA}$ gene inheritance was confirmed by PCR, gene expression, and glyphosate tolerance testing. The PCR analysis showed that the transgenes in five of the lines tested (No. 1, 12, 15, 18, and 29) were inherited at a segregation ratio of 3:1 (ESM Table 2). Inheritance of the transgenes was more complicated in the other two lines, indicating that the integration of the transgenes into tobacco genomes of the five lines was Mendelian.

Gene expression analysis was carried out to determine $Gr5_{aroA}$ expression in the seven transgenic T_1 progenies (No. 1, 3, 12, 13, 15, 18, and 29). The results showed that the transgenic T_1 lines containing $Gr5_{aroA}$ could express $Gr5_{aroA}$ at levels similar to their T_0 parental lines (data not shown).

Because T_1 seeds harvested from different glyphosate-tolerant T_0 plants were segregated, positive and

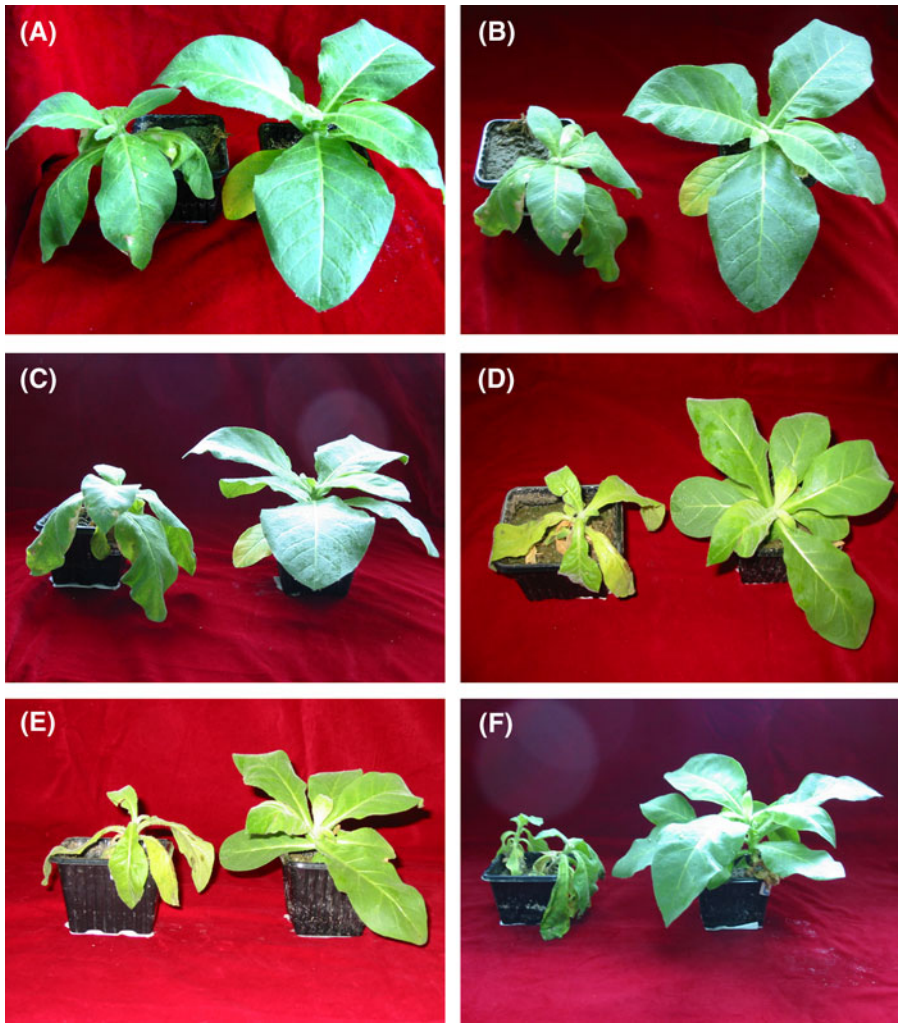

Fig. 3 Glyphosate tolerance assay of tobacco control and transgenic lines and their T_1 progenies. a Left wild-type (WT) control plant following treatment with 0.5× recommended herbicide dose, right transgenic plant No. 18 following treatment with 0.5× recommended herbicide dose. b Left WT control plant following treatment with 1× recommended herbicide dose, right representative transgenic T_1 plant (No. 18) following treatment with 1× recommended herbicide dose. c Left WT control plant following treatment with 2× recommended herbicide dose, right representative transgenic T_1 plant (No. 18) following treatment with 2× herbicide dose. d Left WT control plant following treatment with 3× recommended herbicide dose, right representative transgenic T_1 plant (No. 18) following treatment with 3× recommended herbicide dose. e Left WT control plant following treatment with 4× recommended herbicide dose, right representative transgenic T_1 plant (No. 18) following treatment with 4× recommended herbicide treatment. f Left Representative transgenic T_1 plant (No. 18) following treatment with 5× recommended herbicide treatment, right WT control plant following treatment with 5× recommended herbicide dose

negative seedlings were first confirmed by PCR amplification and then transferred into soil in the greenhouse for the glyphosate tolerance assay. The results of this assay showed that transgenic T_1 progenies and T_0 plants had the same level of glyphosate tolerance.

Under normal conditions (no glyphosate treatment), there was no significant difference in growth between WT and T_1 plants. Both WT and transgenic T_1 plants survived the application of 0.5× the recommended herbicide treatment. With the application of higher levels of herbicide (≥1×), the leaves of both WT and T_1 non-transgenic plants turned yellow and wilted quickly, and all plants in both of these groups died within 5–10 days. In contrast, T_1 transgenic plants grew normally after treatment with less than 4× the recommended herbicide

Table 2 Tolerance of transgenic $Gr5_{aroA}$ tobacco T_0 lines and wild-type line to different levels of glyphosate

Transgenic lines and WT	0.5×	1×	2×	3×	4×	5×
No. 1	0	0	0	1	2	4
No. 2	0	0	0	0	2	4
No. 3	0	0	0	0	1	4
No. 7	0	0	0	0	2	4
No. 12	0	0	0	0	1	4
No. 13	0	0	0	0	1	4
No. 15	0	0	0	1	2	4
No. 18	0	0	0	0	0	3
No. 29	0	0	0	0	2	4
WT.	1	4	4	4	4	4

Plants were treated with six different glyphosate solutions (0.5×, 1×, 2×, 3×, 4×, 5×, where 1× is the recommended glyphosate dose in the field)

The levels of resistance to the herbicide were analyzed according to the method described by to Ye et al. (2001). Individual plants were scored for tolerance to glyphosate using a scale ranging from 0 (normal plant, fertile, no delay in maturity, seeds setting normally) through to 1 (small chlorotic spots on leaves, fertile, no delay in maturity, seeds setting normally), 2 (less than one leaf wilted 2 weeks after treatment, fertile, no delay in maturity, seeds setting normally), 3 (two or more leaves wilted after treatment, stunted, delay in maturity, seeds setting abnormally), and 4 (wilted, dead plant). The injury data for all treated plants were averaged. Results with average values of <2.0 were considered to be tolerant to the treatment; those with average values of >2.0 were considered to be intolerant to the treatment

dose, and no significant differences in growth were observed. The agronomic characteristics of transgenic plants treated with 4× the recommended herbicide dose changed, with a delay in the flowering time and the development of a slight chlorosis of newly emerged leaves. No transgenic plant grew normally following treatment with 5× the recommended herbicide dose: The plants were severely stunted and no seeds were produced. These results supported the notion that $Gr5_{aroA}$ expression in tobacco plants enhances their glyphosate tolerance and that herbicide resistance is strictly conserved in their offspring.

Discussion

Expression of the EPSPS gene has been found to cause glyphosate tolerance in crop plants, enabling more effective weed control via the herbicide (Franz et al. 1997). Unfortunately, the transgene in most commercial glyphosate-resistant crops is the EPSPS gene derived from *Agrobacterium* spp. CP4 (Padgette et al. 1995a, b). This single source of the EPSPS gene is probably what has caused the decrease in herbicide tolerance that has become a concern in field management programs. Improving EPSPS gene diversity and generating multi-herbicide-resistant crops is an effective approach to this problem.

Recent studies have shown that continued glyphosate selection pressure on organisms will favor mutations that reduce glyphosate sensitivity while still maintaining catalytic efficiency in nature. In 2005, Sun et al. (2005) isolated an $Aroa_{p.putida}$ gene from *Pseudomonas putida 4G-1* in glyphosate-contaminated soil. The protein for which it coded showed a higher glyphosate tolerance and the same PEP binding activity as that of *E. coli*. Barry et al. (1997) isolated the widely used EPSPS gene from *Agrobacterium* sp. strain CP4 from a waste-fed column at a glyphosate production company. Taken together, these results show that there are EPSPS gene mutations which can occur spontaneously and that a large portion of EPSPS gene diversity exists in organisms exposed to highly glyphosate-contaminated environments.

Class I EPSPS mutant enzymes have been found to exhibit a decreased affinity for substrate PEP and an increased tolerance to glyphosate (Padgette et al. 1995a, b). Because glyphosate and PEP bind to the same site, the PEP binding ability of EPSPS enzyme is one of crucial factors that can determine its suitability for use in commercial glyphosate-resistant crops. $Gr5_{aroA}$ protein contains the strictly conserved EPSP synthase residue Glu-351 (corresponding to the *E. coli* Glu-341 residue), which is involved in stabilizing PEP binding (SchÖnbrunn et al. 2001). The $Gr5_{aroA}$ protein has also been shown to have a P101F mutation. Several studies have shown that P101 mutations can directly improve PEP binding ability and glyphosate tolerance (Comai et al. 1985; Stalker et al. 1985; Pilacinski 2002; Healy-Fried et al. 2007; Funke et al. 2009). Enzyme kinetic analysis has revealed that the glyphosate tolerance and PEP binding activity of $Gr5_{aroA}$ protein are about 360- and threefold higher than that of *E. coli* AroA. The glyphosate tolerance of $Gr5_{aroA}$ protein is fourfold higher than that of the AroA from *P. fluorescens* (Zhou et al. 2012). It is therefore reasonable to deduce that both of these two amino acid residues contribute to the glyphosate tolerance and PEP binding activity of the $Gr5_{aroA}$ protein.

Our analysis of transgenic tobacco plants showed that $Gr5_{aroA}$ protein can affect the plant genome. The results of our PCR and Southern blot analyses of transgenic $Gr5_{aroA}$ plants confirmed that the $Gr5_{aroA}$ gene was successfully inserted into the tobacco genome and highly expressed (Linn et al. 1990; Tang et al. 1999; Wang et al. 2004). Segregation analysis of T_1 progeny demonstrated that the $Gr5_{aroA}$ gene was inherited by Mendelian rules in most T_1 individuals. The expression level of $Gr5_{aroA}$ in the T_1 progeny was unchanged relative to the T_0 parental lines (Bano-Maqbool and Christou 1999; Tang et al. 1999; Wang et al. 2004).

$Gr5_{aroA}$ expression can significantly improve glyphosate tolerance in plants. In our study, following application of the recommended dose of herbicide, the leaves of both the WT plants and T_1 non-transgenic tobacco plants died within 5–10 days. In contrast, after being sprayed with the same dose, transgenic plants showed no significant effects of the herbicide. Transgenic plants can grow normally without agronomic characteristics under even higher concentrations of herbicide. In our study, the highest concentration that the transgenic plants could tolerate was 4× the recommended dose. The glyphosate treatment assay was repeated in T_1 plants with similar results. These results indicate that glyphosate tolerance in our transgenic $Gr5_{aroA}$ plants was much higher than the dose recommended (1×) for commercial crops. As such, they clearly demonstrate that there is a sufficient safety margin with regard to glyphosate spraying for $Gr5_{aroA}$ engineered crops to be used commercially in fields that rely on glyphosate as an herbicide.

In conclusion, we report the identification of the $Gr5_{aroA}$ gene from glyphosate-contaminated soil and confirm it as a promising candidate for the development of transgenic crops in the future.

Acknowledgments This work was supported by National Basic Research Program (973) of Chinaa 2013CB733903 and National High-Tech Program 2007AA021304), Research project 201103007, the National Natural Science Foundation of China (30470047 and 30200007) and the Sichuan Youth Science & Technology Foundation (No. 07ZQ026-002). We also thank the financial support from Sichuan Heben Bioengineering Co., Ltd.

References

Bano-Maqbool S, Christou P (1999) Multiple traits of agronomic importance in transgenic indica rice plants: analysis of transgene integration patterns, expression levels and stability. Mol Breed 5:471–480

Barry GF, Kishore GM, Padgette SR March (1992) DNA encoding class II 5-enolpyruvyl shikimate-3-phosphate synthase for producing plants and bacteria tolerant to glyphosate herbicides. World Patent WO 9204449

Barry GF, Kishore GM, Padgette SR, Stallings WC (1997) Glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthases. U.S. Patent 5633435

Boocock MR, Coggins JR (1983) Kinetics of 5-enolpyruvylshikimate-3-phosphate synthase inhibition by glyphosate. FEBS Lett 154:127–133

Bradshaw L, Padgette SR, Kimball SL, Wells BH (1997) Perspectives on glyphosate resistance. Weed Technol 11:189–198

Chen LH, Wang XW, Zhang WJ, Hu DF, Liu GT (1999) Transformation of common wheat (*Triticum aestivum* L.) with herbicide-resistant EPSPs gene. Acta Genet Sin 26:239–243 (in Chinese)

Comai L, Facciotti G, Hiatt WR, Thompson G, Rose RE, Stalker DM (1985) Expression in plant of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. Nature 317:741–744

Dower WJ, Miller JF, Ragsdale CW (1988) High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res 7:6127–6145

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Eschenburg S, Healy ML, Priestman MA, Lushington GH, SchÖnbrunn E (2002) How the mutation glycine 96 to alanine confers glyphosate insensitivity to 5-enolpyruvyl shikimate-3-phosphate synthase from *Escherichia coli*. Planta 216:129–135

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fitzgibbon JE, Braymer HD (1990) Cloning of a gene from *Pseudomonas* sp. strain PG2982 conferring increased glyphosate resistance. Appl Environ Microbiol 56:3382–3388

Franz JE, Mao MK, Sikorski JA (1997) Glyphosate's molecular mode of action. In: Franz JE (ed) Glyphosate: a unique global herbicide. American Chemical Society, Washington DC, pp 521–642

Funke T, Han H, Healy-Fried ML, Fischer M, Schönbrunn E (2006) Molecular basis for the herbicide resistance of Roundup Ready crops. Proc Natl Acad Sci USA 103:13010–13015

Funke T, Yang Y, Han HJ, Healy-Fried M, Olesen S, Becker A (2009) Structural basis of glyphosate resistance resulting from the double mutation $Thr^{97} \rightarrow Ile$ and $Pro^{101} \rightarrow Ser$ in 5-enolpyruvylshikimate-3-phosphate synthase from *Escherichia coli*. J Biol Chem 284:9854–9860

Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. Appl Environ Microbiol 66:5488–5491

Haghani K, Salmanian AH, Ranjbar B, Zakikhan-Alang K, Khajeh K (2008) Comparative studies of wild type *Escherichia coli* 5-enolpyruvylshikimate 3-phosphate synthase with three glyphosate-insensitive mutated forms: activity,

stability and structural characterization. Biochim Biophys Acta 1784:1167–1175

Haslam E (1993) Shimikic acid: metabolism and metabolites. Wiley, Chichester

He M, Nie YF, Xu PL (2003) A $T^{42}M$ substitution in bacterial 5-enolpyruvylskimate-3-phophsate synthase (EPSPs) generates enzymes with increased resistance to glyphosate. Biosci Biotechnol Biochem 67:1405–1409

Healy-Fried ML, Funke T, Priestman MA, Han H, Schonbrunn E (2007) Structural basis of glyphosate tolerance resulting from mutations of pro^{101} in *Escherichia coli* 5-enolpyruvylshikimate-3-phosphate synthase. J Biol Chem 282: 32949–32955

Herrmann KM (1995) The shikimate pathway: early steps in the biosynthesis of aromatic compounds. Plant Cell 7:907–919

Hollander-Czytko H, Amrhein N (1980) The site of the inhibition of the shikimate pathway by glyphosate. I. Inhibition by glyphosate of the phenylpropanoid synthesis in buckwheat (*Fagopyrum esculentum* Moench). Plant Physiol 66:823–829

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New Agrobacterium helper plasmids for gene transfer to plants. Transgenic Res 2:208–218

Horsch RB, Fry J, Hoffmann N, Neidermeyer J, Rogers SG, Fraley RT (1988) Leaf disc transformation. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual. Kluwer, Dordrecht, pp 1–9

Jeanmougin FJD, Thompson D, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. Trends Biochem Sci 23:403–405

Kauffmann IM, Schmitt J, Schmid RD (2004) DNA isolation from soil samples for cloning in different hosts. Appl Microbiol Biotechnol 64:665–670

Kumar SK, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA (1979) An improved assay for nanomole amounts of inorganic phosphate. Anal Biochem 100:95–97

Lewendon A, Coggins JR (1983) Purification of 5-enolpyruvylshikimate-3-phosphate synthase from *Escherichia coli*. Biochem J 213:187–191

Li L, Lu W, Han Y, Ping S, Zhang W, Chen M, Zhao Z, Yan Y, Jiang Y, Lin M (2009) A novel RPMXR motif among class II 5-enolpyruvylshikimate-3-phosphate synthases is required for enzymatic activity and glyphosate resistance. J Biotechnol 144:330–336

Linn F, Heidmann I, Saedler H, Meyer P (1990) Epigenetic changes in the expression of the maize *A1* gene in *Petunia hybrida*: role of numbers of integrated gene copies and state of methylation. Mol Gen Genet 222:329–336

McDowell LM, Schmidt A, Cohen ER, Studelska DR, Schaefer J (1996) Structural constraints on the ternary complex of 5-enolpyruvylshikimate 3-phosphate synthase from rotational-echo double resonance NMR. J Mol Biol 256:160–171

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Padgette SR, Re DB, Gasser CS, Eichholtz DA, Frazier RB, Hironaka CM, Levine EB, Shah DM, Fraley RT, Kishore GM (1991) Site-directed mutagenesis of a conserved region of the 5- enolpyruvylshikimate-3-phosphate synthase active site. J Biol Chem 266:22364–22369

Padgette SR, Kolacz KH, Delannay X, Re DB, LaVallee BJ, Tinius CN, Rhodes WK, Otero YI, Barry GF, Eichholtz DA, Peschke VM, Nida DL, Taylor NB, Kishore GM (1995a) Development, identification, and characterization of a glyphosate-tolerant soybean line. Crop Sci 35: 1451–1461

Padgette SR, Re DB, Barry GF, Eichholtz DE, Delannay X, Fuchs RL, Kishore GM, Fraley RT (1995b) In: Duke SO (ed) Herbicide-resistant crops: agricultural, environmental, economic, regulatory, and technical aspects. CRC Press, New York, pp 53–84

Pilacinski W (2002) Petition for determination of non-regulated status for Roundup Ready wheat event MON 71800. 02-WT-093F. Submitted to USDA December 18, 2002. Monsanto Company, St. Louis

Pollegioni L, Schonbrunn E, Siehl D (2011) Molecular basis of glyphosate resistance-different approaches through protein engineering. FEBS J 278:2753–2766

Priestman MA, Funke T, Singh IM, Crupper SS, SchÖnbrunn E (2005a) 5-Enolpyruvylshikimate-3-phosphate synthase from *Staphylococcus aureus* is insensitive to glyphosate. FEBS Lett 579:728–732

Priestman MA, Healy ML, Funke T, Becker A, Schönbrunn E (2005b) Molecular basis for the glyphosate-insensitivity of the reaction of 5-enolpyruvylshikimate 3-phosphate synthase with shikimate. FEBS Lett 25:5773–5780

Rogers SG, Horsch RB, Fraley RT (1986) Gene transfer in plants: production of transformed plants using Ti plasmid vectors. Methods Enzymol 118:627–640

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor

SchÖnbrunn E, Eschenburg S, Shuttleworth WA, Schloss JV, Amrheini N, Evans JNS, Kabsch W (2001) Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. Proc Natl Acad Sci USA 98:1376–1380

Shuttleworth WA, Evans JN (1994) Site-directed mutagenesis and NMR studies of histidine-385 mutants of 5-enolpyruvylshikimate-3-phosphate synthase. Biochemistry 33:7062–7068

Shuttleworth WA, Pohl ME, Helms GL, Jakeman DL, Evans JN (1999) Site-directed mutagenesis of putative active site residues of 5-enolpyruvylshikimate-3-phosphate synthase. Biochemistry 38:296–302

Stalker DM, Hiatt WR, Comai L (1985) A single amino acid substitution in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase confers resistance to the herbicide glyphosate. J Biol Chem 260:4724–4728

Sun YC, Chen YC, Tian ZX, Li FM, Wang XY, Zhang J, Xiao ZL, Lin M, Gilmartin N, Dowling DN, Wang YP (2005) Novel AroA with high tolerance to glyphosate encoded by a gene of *Pseudomonas putida* 4G-1 isolated from an extremely polluted environment in China. Appl Environ Microbiol 71:4771–4776

Sun H, Lang Z, Zhu L, Huang D (2012) Acquiring transgenic tobacco plants with insect resistance and glyphosate tolerance by fusion gene transformation. Plant Cell Rep 31:1877–1887

Tang KX, Tinjuangjun P, Xu Y, Sun XF, Gatehouse JA, Ronald PC, Qi H, Lu X, Christou P, Kohli A (1999) Particle bombardment-mediated co-transformation of elite Chinese rice cultivars with genes conferring resistance to bacterial blight and sap sucking insect pests. Planta 208:552–563

Thomas PS (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci USA 77:5201–5205

Tian YS, Xu J, Xiong AS, Zhao W, Fu XY, Peng RH, Yao QH (2011) Improvement of glyphosate resistance through concurrent mutations in three amino acids of the *Ochrobactrum* 5-enopyruvylshikimate-3-phosphate synthase. Appl Environ Microbiol 77:8409–8414

Wang HY, Li YF, Xie LX, Xu PL (2003) Expression of a bacterial aroA mutant, aroA-M1, encoding 5-enolpyruvylshikimate-3-phosphate synthase for the production of glyphosate-resistant tobacco plants. J Plant Res 116: 455–460

Wang J, Zuo KJ, Wu WS, Song J, Sun XF, Lin J, Li X, Tang KX (2004) Expression of a novel antiporter gene from *Brassica napus* resulted in enhanced salt tolerance in transgenic tobacco plants. Biol Plant 48:509–515

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119

Ye GN, Hajdukiewicz PT, Broyles D, Rodriguez D, Xu CW, Nehra NN, Staub JM (2001) Plastid-expressed 5-enolpyruvyl-shikimate-3-phosphate synthase genes provide high level glyphosate tolerance in tobacco. Plant J 25:261–270

Yi Y, Qiao D, Bai L, Xu H, Li Y, Wang X, Cao Y (2007) Cloning, expression, and functional characterization of the *Dunaliella salina* 5-enolpyruvylshikimate 3-phosphate synthase gene in *Escherichia coli*. J Microbiol 45:153–157

Zhou CY, Tian YS, Xu ZS, Zhao W, Chen C, Bao WH, Bian L, Cai R, Wu AZ (2012) Identification of a new gene encoding 5-enolpyruvylshikimate-3-phosphate synthase using genomic library construction strategy. Mol Biol Rep 39:10939–10947