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Physiological and molecular mechanisms of glyphosate tolerance in an *in vitro* selected cotton mutant

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ABSTRACT

We have selected an upland cotton (*Gossypium hirsutum* L.) cell line (R1098) that is highly tolerant to glyphosate. This cell line was developed by *in vitro* selection with gradually increasing glyphosate concentrations, and its mechanisms conferring glyphosate tolerance were studied. Based on a whole-plant dose–response bioassay, R1098 plants were tolerant to glyphosate at a concentration of 1500 g ae ha^{-1} glyphosate (1.5× the recommended field rate) whereas the control plants (Coker 312) were unable to survive at 150 g ae ha^{-1} glyphosate. Coker 312 accumulated 13.1 times more shikimate in leaves at 5 days after glyphosate treatment (1500 g ae ha^{-1}) than that of R1098. Two distinct cDNAs for 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), EPSPS-1 and EPSPS-2, were isolated from R1098. Both cDNAs were 97.7% identical within the common protein-coding region and the predicted sequences of the mature proteins were greater than 83% identical with EPSPS proteins from other known higher plants. In comparison to the glyphosate-susceptible cotton Coker 312, sequence analysis of the EPSPS-1 gene indicated that R1098 has an alanine insertion at nucleotide position 1216 resulting in frameshift. It leads to two copy functional EPSPS genes in R1098. There was no difference between R1098 and Coker 312 in EPSPS mRNA levels before glyphosate treatment. However, its treatment caused a 2–4 times increase in the basal EPSPS mRNA level in R1098.

1. Introduction

Glyphosate [*N*-(phosphonomethyl) glycine] is a nonselective foliar-applied herbicide that has been used for over several decades in the management of annual, perennial, and biennial herbaceous species of grasses, sedges, and broadleaf weeds, as well as woody brush and tree species [1,2]. It is well known that glyphosate exerts its effect through inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPS, EC 2.5.1.19) [3]. The enzyme catalyzes the formation of EPSP from the enolpyruvyl moiety of phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) [4]. This is a key step in the synthesis of aromatic acids phenylalanine, tryptophan and tyrosine as well as many important secondary compounds, such as indole acetic acid, lignin and phytoalexins.

Stepwise selection of a number of plant suspension cultures has been reported to obtain glyphosate-tolerant cell lines [5]. Increased EPSPS gene copy number, high mRNA levels and high specific activity have been conferred glyphosate tolerance. A carrot (*Daucus carota*) cell line was selected tolerant to 25 mM glyphosate and characterized as having a 12-fold higher activity of EPSPS and an 8- to 10-fold increase in gene copies [6,7]. A *Petunia hybrida* cell line was selected tolerant to glyphosate with 20-fold amplified EPSPS genes [8], Tobacco (*Nicotiana tabacum*) cell lines growing with 20 mM glyphosate had increased gene copy number, mRNA levels and EPSPS enzyme levels [9]. Tobacco, soybean (*Glycine max*) and alfalfa (*Medicago sativa*) cell lines also became glyphosate tolerant due to gene amplification [10]. However, in *Corydalis sempervirens*, suspension-cultured cells where EPSPS overexpression was due to a higher rate of gene transcription and stabilization of the EPSPS protein rather than gene amplification [11,12].

We obtained glyphosate-tolerant cotton mutant line (R1098) through tissue culture in 2002. R1098 is an excellent cotton germplasm with glyphosate tolerance to commercial levels (1500 g ae ha^{-1}) and it has been used to produce a commercial line of hybrid cotton [13,14]. The aims of the present studies were to study the physiological and morphological response of glyphosate-tolerant cotton (R1098) to various glyphosate treatments, as well to explore the molecular bases of the mechanisms being involved the tolerance against glyphosate.

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2. Materials and methods

2.1. Seed source and plant growth conditions

R1098 (*Gossypium hirsutum* L.) was a glyphosate-tolerant cotton mutant obtained via *in vitro* stepwise selection. Coker 312, the genetic background of R1098, is a glyphosate-susceptible cotton germplasm obtained from Cotton Germplasm Center in Cotton Research Institute of CAAS (Chinese Academy of Agricultural Science). Delinted seeds of R1098 and Coker 312 were washed thoroughly and were planted in 30 cm pots containing MetriMix 360 (Scotts-Sierra Horticultural Products Co., 14111 Scottslawn Rd., Marysville). Pots were thinned to two plants per pot at emergence and were grown in greenhouse maintained at 25 ± 2 °C/18 ± 2 °C day/night temperatures. The photoperiod was 16 h, and natural sunlight was supplemented by mercury halide lights yielding a photosynthetic photon flux density of 250 μmol m^{-2} s^{-1}.

2.2. Growth and morphological response of glyphosate tolerant and glyphosate susceptible seedlings to glyphosate

Seedlings of R1098 and Coker 312 were treated with glyphosate (Roundup, Monsanto) at rates of 0, 15, 150, 1500 and 15000 g acid equivalent per hectare (g ae ha^{-1}). The dose of 1000 g ae ha^{-1} approximately corresponded to the recommended field rate. Experimental design for dose–response tests was a randomized complete block with three replications of five plants for each glyphosate treatment. Symptom development was assessed each day for 15 days and above-ground fresh weight was recorded 15 days after treatment (DAT). Data for measuring the effect of glyphosate on above-ground fresh weight of seedlings was subjected to ANOVA (SAS Institute, Cary, NC) test and means were compared at $P = 0.05$ level using Tukey's multiple comparison procedure.

2.3. Analysis of shikimate accumulation

For the analysis of shikimate accumulation in the leaves, stems and roots of cotton seedlings, the extraction procedure similar to what has previously been reported was followed [15,16]. Frozen cotton tissue was finely ground in liquid nitrogen using a mortar and pestle. Then the grinding products were placed into centrifuge tubes, and 0.01 M H_2SO_4 was added at a ratio of 4 mL of H_2SO_4 solution per 1 g of tissue, each centrifuge tube contained about 0.2 g (fresh weight). Two hundred and fifty microliters of 0.4 M $NaHCO_3$ was added in each tube before centrifugation at 12,000*g* for 10 min at 4 °C. The supernatant was stored at −30 °C until used.

Samples were analyzed according to the methods of Singh and Shaner [16]. A volume of extract (20–50 μL) was reacted with 500 μL of 1% (w/v) periodic acid for 3 h. Samples were prepared for measurement by the addition of 500 μL of 1 M NaOH immediately followed by the addition of 300 μL of 0.1 M glycine to fix the color. Absorbance was read at 380 nm in a spectrophotometer. A standard curve was developed using commercial shikimate standards (Sigma–Aldrich, China) with known concentrations. The shikimate content in samples was quantified by comparison with the standard curves. Data were analyzed using SAS Proc GLM procedure (SAS Institute, Cary, NC) and the means were compared at $P = 0.05$ level using LSD.

2.4. Cloning and sequence analysis of full length EPSPS cDNA from G. hirsutum

Young leaves of R1098 and Coker 312 were ground into fine powder in liquid nitrogen and the total RNA was extracted with "RNAiso Reagent" (Catalog No. D312, TakaRa Bio Group, Japan) according to the manufacture's protocol. First-strand cDNA synthesis was performed using 0.5 μg of total RNA as the template, according to recommendations in the PrimeScript™ 1st Strand cDNA Synthesis Kit (Catalog No. D6110, TakaRa Bio Group, Japan). First-strand cDNA (2 μL) was used to generate EPSPS cDNA through PCR technique. According to *Arabidopsis thaliana* sequence (NCBI GenBank Accession No. AY040065), we obtained an assembled unique *Gossypium_hirsutum* transcript-fragment (Sequence ID: PUT-157a-Gossypium hirsutum 19465429) from Plant genome DataBase (www.plantgdb.org). The fragment was used to design the specific primer EPSPS-F (forward primer) and EPSPS-R (reverse primer) (Table 1). PCR amplifications were performed using the High Fidelity PCR System (Catalog No. DR010S, TaKaRa Bio Group, Japan). A thermal cycle program was performed in a thermal cycler as follows: 94 °C for 2 min, follow by 30 cycles at 94 °C for 30 s, 58 °C for 60 s, and 72 °C for, 40 s, and a final extension at 72 °C for 7 min. A 1.8 kb fragment was obtained and cloned into the pSIMPLE-18 EcoR V/BAP vector (Catalog No. D105A, TaKaRa Bio Group, Japan). The complete nucleotide sequence was determined using a terminator Cycle Sequencing Ready Reaction kit and an ABI 377 DNA Sequencer. DNA sequence comparison was carried out using the NCBI GenBank BLAST programs. RNA extraction and amplification of the EPSPS gene was performed from five plants of each cotton population. To exclude PCR errors, PCR products were pooled from a minimum of three independent reverse transcriptase-PCR reactions before ligation into vector, then five randomly selected isolates per construct were sequenced and aligned. The stereo view is drawn according to the crystal structure of *Escherichia coli* EPSPS enzyme [17–20].

2.5. Quantitative RT-PCR

2.5.1. RNA isolation and first strand cDNA synthesis

After the treatment with three glyphosate dose (0, 750, and 1500 g ae ha^{-1}), leaf samples were harvest from four plants of each replicate (three replicates). Total RNA was isolated from leaf samples as described above. First-strand cDNA was synthesized with a cDNA synthesis kit (Catalog No. DRR037S, Takara Bio Group, Japan). Total RNA (0.5–1.0 μg) was incubated with Random 9 primer and MMLV reverse transcriptase at 37 °C for 30 min according to the manufacturer's instructions.

2.5.2. Real-time quantitative PCR for EPSPS

Primers Actin-F and Actin-R (Table 1) specific for the constitutively expressed cotton actin gene were used in RT-PCR (Real Time-Polymerase Chain Reaction) to normalize the different samples for differences in the amounts of plant RNA. RT-PCR reactions were carried out in 0.2 mL tube (25 μL per tube) in a reaction buffer containing 1× SYBR Green I mix (Taq polymerase, dNTPs, SYBR Green dye), 300 nM primers qEPSPS-F and qEPSPS-R (Table 1), PCR conditions were 95 °C for 15 s and 60 °C for 1 min (annealing/extension) for 40 cycles on a BioRad sequence Detection System. PCR

Table 1
Primers for 5-enolpyruvylshikimate-3-phosphate synthase and actin gene.

Primer name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Products size (bp)
EPSPS	AGCCTTCAGATTTTGGTTGTTGGT	TTGATTGCCTTTCGTCCTATGATT	1829
QEPSPS	ACCTGGACCGAGAATAGCGTAA	ACCATCAGCGTAAAGAGCAACA	144
Actin	CCGATGCCTTGATGAAGATT	GCAGTCTCCAGTTCCTGCTC	133