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Role of physiological mechanisms and *EPSPS* gene expression in glyphosate resistance in wild soybeans (*Glycine soja*)

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ABSTRACT

The physiological mechanisms underlying glyphosate resistance in wild soybean germplasm and relevant *EPSPS* gene expression were evaluated. These germplasms were selected by gradually increasing glyphosate selection pressure started from 2010. As indicated by a whole-plant dose response bioassay, ZYD-254 plants were resistant to glyphosate at concentrations of 1230 g ae ha^{-1}, but the susceptible plants (ZYD-16) were unable to survive in the presence of 300 g ae ha^{-1} glyphosate. The ED_{50} values of resistant germplasm were approximately 8.8 times of the susceptible germplasm. Chlorophyll content was significantly decreased in ZYD-16 plants in comparison with ZYD-254 plants. ZYD-16 plants accumulated 10.1 times more shikimate in leaves at 5 days after glyphosate treatment at 1230 g ae ha^{-1} than ZYD-254 did. GST activity differed between ZYD-254 and ZYD-16 in three tissues. It was highest in leaves. There were no significant differences in *EPSPS1* or *EPSPS3* expression between two germplasms before exposure to glyphosate treatment. After glyphosate treatment, there was a 2- to 4-fold increase in *EPSPS1* mRNA levels in ZYD-254, but there was no change in *EPSPS3* mRNA levels in ZYD-254 or ZYD-16.

1. Introduction

Weeds can reduce farm income by up to 30% and the cost of developing new herbicides is 20–100 times higher than the cost of generating new varieties of herbicide-resistant crops. For this reason, the development of herbicide-resistant crops may be great significance to agriculture [1]. Glyphosate [N-(phosphonomethyl) glycine] is a wild-spectrum, non-selective post-emergence herbicide that has been used for over 30 years for the management of annual, perennial, and biennial herbaceous species of grasses, sedges, and broadleaf weeds, as well as woody brush and tree species [2,3].

The target enzyme of glyphosate is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) [4], which catalyzes the reaction of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to yield 5-enolpyruvilshikimate-3-phosphate [5]. Glyphosate treatments trigger the inhibition of this enzyme, the accumulation of shikimic acid and other precursors of the chorismate pathway and the depletion of aromatic amino acid pools [6]. How glyphosate-induced inhibition of the shikimate pathway actually kills plants is not entirely clear [7]. In 1986, scientists isolated the common soil bacteria, *Agrobacterium tumefaciens* strain *CP4* and inserted the *cp4 EPSPS* gene into the plant genome, producing glyphosate-resistant plants [8]. Two bean germplasms (*Phaselus vulgaris L.*) were screened for resistance to glyphosate in field experiments and subjected to chemical analysis [9]. A cotton mutant, R1098, which is resistant to glyphosate, was produced by somatic cell induction and continued directional selection [10]. Recently, stepwise selection of a number of whole-plant artificial selection has been reported to obtain glyphosate-resistant soybean lines [11]. The major resistance mechanisms that have been reported for GR weeds were restricted glyphosate translocation and target-site glyphosate resistance is due to an alanine substitution at Pro-106 [12,13]. Over-expression of the target site *EPSPS* has been reported in GR *Conyza Canadensis* [14]. However, mechanisms of resistance are still not completely understood although the efforts of several research groups [15].

The annual wild soybean (*Glycine soja*) is an original species of soybean. It has been suggested that the wild soybean may be a valuable genetic resource. It is distributed throughout East Asia, including Korea, Japan, eastern Russia, and central China [16]. We obtained GR wild soybean germplasm (ZYD-254) through

Abbreviations: EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GST, glutathione S-transferase; DAT, days after treatment; GR, glyphosate-resistant; GT, glyphosate treatment; R, resistant; S, susceptible.

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artificial glyphosate selection in 2010. The purpose of this work was to evaluate resistant and susceptible germplasm by determining the following: (a) the resistance index, (b) the amount of endogenous shikimic acid accumulated in R and S plants after glyphosate treatment, (c) chlorophyll content, (d) glutathione S-transferase (GST) activity, (e) the levels of *EPSPS* expression after glyphosate induction.

2. Materials and methods

2.1. Plant materials

The tested seeds were provided by the Institute of Crop Germplasm Utilization, Ministry of Agriculture of CAAS (Chinese Academy of Agricultural Sciences). The ZYD-254 GR strain was artificially obtained by glyphosate stepwise selection for 90 wild soybean germplasms in 2010. ZYD-16 is a glyphosate-susceptible wild soybean germplasm. Seeds of resistant and susceptible populations were germinated in 30 × 25 cm pots containing potting soil. Ten seedlings per pot were grown in a greenhouse at 20/15 °C day/night temperatures in natural sunlight.

2.2. Dose response to glyphosate and morphological determinations

The 41% isopropylamine salt of glyphosate (Roundup®, Monsanto) were sprayed at doses of 0, 300, 600, 900, and 1230 g acid equivalent per hectare (g ae ha^{-1}) at different growth stages. 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 each glyphosate dose. Plant symptoms were assessed at 14 days after treatment (DAT) and scored as dead or alive. Visible injuries and damage were evaluated on a scale of 0 to 5 (Table 1).

2.3. Analysis of degree of resistance to glyphosate-resistant germplasm in wild soybeans

To evaluate the relative number of seeds that germinated in the presence of different glyphosate concentrations, 10 seeds were used in a Petri dish containing a sheet of filter paper and 5 ml of the glyphosate solution (Roundup, Monsanto) at doses of 0, 10, 20, 40, and 80 mg ae L^{-1} [17]. There were five replicates per treatment. This experiment was carried out in a growth chamber with 100 μmol m^{-2} s^{-1} of photosynthetically active radiation with a 12/12 h day/night regime at temperatures of 25/15 °C. After 7 days, the lengths of the hypocotyls of plants exposed to different glyphosate concentrations were measured and ED_{50} values were calculated. For the plumule length test, the amount of herbicide required for 50% reduction in growth was referred to as ED_{50} (50% effective dose). To assess the accuracy of the model, an *F*-test was used for model significance, residual variance analysis, and coefficient of determination (R^2). In every case, the resistance index (RI) was calculated as the ratio of the ED_{50} of the *R* plants relative to those of the susceptible ones. For this data analysis, we used Data Processing System (DPS 7.05 version) statistical software.

2.4. Determination of chlorophyll content

Chlorophyll content was recorded with a portable chlorophyll meter (SPAD 502, Minolta®). This meter measures absorption at 650 and 940 nm wavelengths for estimation of chlorophyll levels [18]. The averages of three measurements of middle leaflet were taken as SPAD values. V_2 stage plants were sprayed with the Isopropylamine salt of glyphosate at doses of 0, 300, 600, 900, and 1230 g ae ha^{-1}. Ten replications were performed per treatment.

2.5. Shikimic acid accumulation studies

The resistant soybean germplasm ZYD-254 and the susceptible germplasm ZYD-16 were treated with glyphosate at rates of 0, 300, 600, 900, and 1230 g ae ha^{-1} at the V_2 leaf stage and shoot were sampled at 0, 1, 3, 7, and 10 d after treatment. Experimental design for dose-response tests was a randomized complete block with five replicates (1 plant for each replicate) for each glyphosate treatment. The samples were quickly dipped in liquid nitrogen and stored at −20 °C until use. The frozen tissue was finely ground in liquid nitrogen using a mortar and pestle. After grinding, the tissue was weighed into screw-cap tubes and 0.25 M HCl was added at a ratio of 5 ml HCl solution per 0.5 g (fresh weight) of tissue. Then 250 μl of 0.4 M $NaHCO_3$ was added to each tube. The tubes were centrifuged at 12,000 *g* for 15 min at 4 °C. The supernatant was then briefly re-centrifuged in a bench top centrifuge and the supernatant was used for the subsequent steps.

Samples were analyzed according to the methods described by Singh and Shaner [19]. Extract (20–30 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. Shikimic acid was quantified with a double-beam spectrophotometer at 382 nm. 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, U.S.) and the means were compared at $P = 0.05$ level using LSD.

2.6. Analysis of glutathione-S-transferase (GST) activities

The experiment was designed as a 2 × 5 × 5 factorial, replicated five times, with glyphosate dose 0, 300, 600, 900, and 1230 g ae ha^{-1}. GST activity was measured in different tissues such as leaves, stems, and roots of V_2 stage of each germplasm after 3 days of glyphosate treatment. The procedure for GSTs activities was determined as Hatton et al. [20]. In brief, frozen plant tissue (1 g) was ground to powder with a mortar and pestle using liquid nitrogen. The powder was then thawed in 0.1 M Tris HCl (pH = 7.5) containing 1 mM EDTA, 14 mM 2-mercaptoethanol, and 75 g kg^{-1} polyvinylpolypyrrolidone (PVPP). The supernatant

Table 1
Scoring criteria of glyphosate injury level.

HIS	Injury rate%	Symptoms of injury in glyphosate-treated plants
0	0–20	No symptoms
1	20.1–40	Slight inhibition of plant growth. The entire plant wilted, and all leaves drooped slightly, but no other symptoms were visible
2	40.1–60	The entire plant was wilted, including the young leaves. The leaf apexes curled and etiolated. Fewer than 40% of the leaves showed necrotic spots
3	60.1–80	Young leaves curled. The entire plant was etiolated, and 60–80% of the leaves were dead
4	80.1–100	Entire plant was completely wilted. The plant was either entirely dead or had more than 80% living leaves

HIS: glyphosate injury severity.