Proline-106 EPSPS Mutation Imparting Glyphosate Resistance in Goosegrass (*Eleusine indica*) Emerges in South America

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Abstract

Glyphosate-resistant (GR) goosegrass [*Eleusine indica* (L.) Gaertn.] was recently identified in Brazil, but its resistance mechanism was unknown. This study elucidated the resistance mechanism in this species and developed a molecular marker for rapid detection of this target-site resistance trait. The resistance factor for the resistant biotype was 4.4-fold compared with the glyphosate-susceptible (GS) in greenhouse dose–response experiments. This was accompanied by a similar (4-fold) difference in the levels of in vitro and *in planta* shikimate accumulation in these biotypes. However, there was no difference in uptake, translocation, or metabolism of glyphosate between the GS and GR biotypes. Moreover, both biotypes showed similar values for 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) copy number and transcription. Sequencing of a 330-bp fragment of the *EPSPS* gene identified a single-nucleotide polymorphism that led to a Pro-106-Ser amino acid substitution in the enzyme from the GR biotype. This mutation imparted a 3.8-fold increase in the amount of glyphosate required to inhibit 50% of EPSPS activity, confirming the role of this amino acid substitution in resistance to glyphosate. A quantitative PCR–based genotyping assay was developed for the rapid detection of resistant plants containing this Pro-106-Ser mutation.

Introduction

Brazil is one of the largest markets for transgenic glyphosate-resistant (GR) crops such as soybean [*Glycine max* (L.) Merr.] and corn (*Zea mays* L.), especially due to weather conditions that allow farmers to grow a second crop in the same year, which in most cases is GR (Roundup Ready® [RR]) corn (Lopez Ovejero et al. 2017). For the last 5 yr, more than 90% of soybean fields in South America have been planted with RR varieties (Peterson et al. 2018). The intense use of glyphosate with few herbicide-resistance mitigation practices has led to the selection of eight GR weed species over the past 12 yr in Brazil (Heap 2018). Among these species, goosegrass [*Eleusine indica* (L.) Gaertn.] has evolved multiple resistance to glyphosate and acetyl-CoA carboxylase (ACCase) inhibitors (Osuna et al. 2012; Takano et al. 2017).

While *E. indica* is native to Asia, this species has spread to most tropical and subtropical regions around the world (Mueller et al. 2011). Species of the genus *Eleusine* are diploid ($2n = 18$), self-pollinate, and produce large numbers of seeds (Ng et al. 2004a). *Eleusine indica* is a C_4 annual grass that can grow up to $0.8\,g\,d^{-1}$ under optimal temperatures (25 to 30 C) and soil moisture (Takano et al. 2016). When four or more *E. indica* plants per square meter infest annual crops [e.g., cotton (*Gossypium hirsutum* L.)], this species can cause more than 27% yield loss (Ma et al. 2015). Moreover, managing *E. indica* with herbicides is normally more effective on young plants, especially because its leaves accumulate a considerable wax layer as the plant matures, reducing herbicide absorption in older plants (Malpassi 2006).

The mechanism of glyphosate resistance in *E. indica* populations from other parts of the world (e.g., Asia and North America) has been associated with either a nonsynonymous single-nucleotide polymorphism (SNP) in *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) and/or *EPSPS* gene amplification (Baerson et al. 2002; Chen et al. 2015; Kaundun et al. 2008; Ng et al. 2004b). In such cases, a single mutation (Pro-106-Ser or Pro-106-Thr) and/or increased *EPSPS* copy number confer intermediate levels of resistance (resistance factor,

RF < 10) in this species. However, under very intense glyphosate selection pressure, *E. indica* can evolve a double mutation (Thr-102-Ile and Pro-106-Ser) (TIPS) in the *EPSPS* gene, conferring higher levels of resistance (RF > 10) (Yu et al. 2015). The occurrence of the TIPS mutation is very rare, because it carries significant fitness cost in resistant plants (Han et al. 2017).

Point mutations in the *EPSPS* gene have also been documented in other GR weeds such as Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam). Husnot], rigid ryegrass (*Lolium rigidum* Gaudin), waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer], junglerice [*Echinochloa colona* (L.) Link], sourgrass [*Digitaria insularis* (L.) Mez ex Ekman], and horseweed (*Erigeron canadensis* L.) (Gaines and Heap 2018). Considering that these mutations in the *EPSPS* gene are SNPs, their presence can be detected using quantitative PCR techniques (qPCR). Thus, a large number of samples can be screened for resistance at once with no need for full dose–response experiments, which take time and demand more resources. Genotyping assays can be employed for detection of resistance alleles, and they have been used to detect SNPs conferring resistance to acetolactate synthase (Warwick et al. 2008) and ACCase (Délye et al. 2010) inhibitors. qPCR assays usually use two probes, each with its own fluorochromes, to discriminate between two different alleles (susceptible and resistant in this case) (Délye et al. 2015).

The rapid detection of herbicide resistance is essential for development of weed management strategies to mitigate herbicide resistance. In these studies, we confirmed that the mechanism of glyphosate resistance in *E. indica* from Brazil is associated with a nonsynonymous SNP in the *EPSPS* gene resulting in a Pro-106-Ser amino acid substitution on the enzyme. Other target-site and non–target site resistance mechanisms were also evaluated, but no differences were found, suggesting that resistance is conferred solely by this *EPSPS* mutation. We also designed a qPCR-based genotyping assay for rapid detection of GR *E. indica* populations containing the resistant SNP in the *EPSPS* gene.

Material and Methods

Plant Material and Growth

Seeds of the GR *E. indica* biotype were collected in a soybean field with a 10-yr history of glyphosate application, located in Campo Mourão County, Paraná State, Brazil (24.15°S, 52.48°W). The GS seeds were collected in a neighboring field with no glyphosate spraying history (24.14 S, 52.40 W). The GR biotype was purified with glyphosate application (960 g ha^{-1}). We then performed a recurring selection of GR offspring for three self-pollinated generations to exclude potential contamination with susceptible seeds. Seeds were planted in pots (0.5-L volume) filled with potting soil. Three *E. indica* plants per pot were grown in the greenhouse under 25/20 C day/night with 12-h daylight until they reached the 3-leaf stage.

Glyphosate Dose Response

The experimental design was completely randomized in a 2 by 10 factorial. The first factor was *E. indica* biotypes (GR and GS), and the second factor was glyphosate doses (Roundup Transorb®, 480 g ae L^{-1}, Monsanto, São Paulo, Brazil): 0, 60, 120, 240, 480, 960, 1,920, 3,840, 7,680 and 15,360 g ha^{-1}. Four replications were used for each combination of biotype and dose. Application was with a pressurized backpack sprayer equipped with a 1.5-m-long bar fit with three AI 110-02 spray tips (0.5 m between tips). The sprayer was operated with 245 kPa pressure, providing a spray volume of 200 L ha^{-1}.

Eleusine indica survival was evaluated visually at 28 d after treatment using a scale of 0% to 100%, in which 100% represents no control, and 0% represents plant death. Data were subjected to ANOVA, and the nonlinear logistic regression model was fit using the DRC package in R (Ritz et al. 2015; Streibig 1988):

$$y = \frac{a}{\left[1 + \left(\frac{x}{b}\right)^c\right]} \qquad [1]$$

In this equation, y is survival, x is glyphosate dose (g ae ha^{-1}), a represents the maximum value, b is the dose providing 50% response (LD$_{50}$), and c is the curve slope around b. The RF was calculated as the ratio of LD$_{50}$ between the GR and GS biotypes (Burgos et al. 2013).

Absorption and Translocation

Glyphosate absorption and translocation experiments followed methods similar to those described in Figueiredo et al. (2017). *Eleusine indica* seeds were planted in potting soil and kept under controlled conditions in the greenhouse at 25 C with 80% relative humidity. One week after germination, seedlings were transferred to 15-ml Falcon tubes and filled with fine washed sand. Plants were then placed into a growth chamber under fluorescent and incandescent artificial light and the same temperature and humidity conditions as in the greenhouse. Plants were treated when they had four fully expanded leaves. The youngest expanded leaf was marked and covered with aluminum foil before the whole plant was sprayed with 960 g ha^{-1} glyphosate + 0.25% nonionic surfactant (NIS). The aluminum foil was then removed, and a solution containing [^{14}C]glyphosate and cold glyphosate (8 μg glyphosate cm^{-2}) was prepared and applied on this leaf as five 1-μl droplets. Total radioactivity applied per plant was 300,000 dpm (4.99 kBq).

Measurement time points were at 12, 24, 48, 96, and 192 h after treatment (HAT). For each time point, three replications (plants) were harvested simultaneously, and the experiment was repeated. Treated leaves were removed and washed with 5 ml of washing solution containing 10% methanol and 1% NIS. Scintillation cocktail (Ecoscint XR, National Diagnostics, Atlanta, GA, USA) was then added to the leaf rinse solution, and radioactivity was measured using liquid scintillation spectrometry (LSS) (Packard Tri-carb 2300TR, Packard Instrument, Meriden, CT, USA). Roots were washed with 10 ml of water to remove sand residues, and radioactivity was measured in a 2-ml aliquot with LSS.

Harvested plants were then oven-dried at 60 C for 96 h before exposure to Phosphor Screen film (GE Healthcare, Pittsburgh, PA, USA) for 4 d before being imaged with a Typhoon Trio Imager (GE Healthcare). Separate parts of the dried plant (treated leaf, other leaves, and roots) were oxidized in a biological oxidizer (OX500, RJ Harvey Instrument, Tappan, NY, USA); this was followed by radioactivity measurement with LSS. Absorption values were calculated as:

$$\% H_{abs} = [ot/(ot + wl)] \times 100 \qquad [2]$$

where $\% H_{abs}$ is the proportion of absorbed herbicide, ot is the amount of ^{14}C measured in oxidized tissue, and wl is the amount of ^{14}C detected in the treated leaf washing. For glyphosate

translocation, the following equation was used:

$$\% H_{tr} = 100 - [al/(tl + ol) \times 100] \qquad [3]$$

where $\%H_{tr}$ is the proportion of translocated herbicide, tl is the amount of ^{14}C measured in the treated leaf, and ol is the amount of ^{14}C detected in other leaves. Absorption and translocation over time were analyzed using R (Kniss et al. 2011).

Glyphosate Metabolism

Eleusine indica plants were grown in the greenhouse until the 4-leaf stage as described earlier and treated with $960\,g\,ha^{-1}$ glyphosate. Glyphosate and aminomethylphosphonic acid (AMPA) levels were quantified at 24, 48, 72, and 96 HAT. The extraction method started by grinding 2.5 g of leaf tissue with liquid nitrogen and placing ground tissue into 15-ml Falcon tubes. The tissue was then vortexed with 5 ml of water, shaken for 10 min, and centrifuged at $10,000 \times g$ for 5 min. A 1-ml aliquot was taken and used for derivatization with 0.5 ml of borate solution (5%) and 0.5 ml of fluorenylmethyloxycarbonyl chloride (FMOC-Cl) solution. The solution mixture was then vortexed and incubated at room temperature for 12 h. After this period, tubes were centrifuged at $10,000 \times g$ for 5 min and filtered through 0.2-μm nylon syringe filter into an ultra-high performance liquid chromatography vial. Blank samples were prepared by extracting and derivatizing untreated *E. indica* leaf tissue.

All samples were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS; Shimadzu Scientific Instruments, Columbia, MD, USA). The LC–MS/MS system consisted of a Nexera X2 UPLC with 2 LC-30 AD pumps, an SIL-30 AC MP autosampler, a DGU-20A5 Prominence degasser, a CTO-30A column oven, and an SPD-M30A diode array detector coupled to an 8040-quadrupole mass spectrometer. Glyphosate and AMPA were separated with a stationary-phase C18 column (100 by 2.1 mm, Alliance Atlantis T3, Phenomenex Co, 411 Madrid Avenue, Torrance, CA, USA) at a flow rate of $0.2\,ml\,min^{-1}$ using a linear gradient of acetonitrile (B) and 10 mM ammonium acetate (A): 2 min, 10% B; 10 min, 100% B; 10.1 min, 10% B; 17 min, 10% B. The multiple reaction monitorings (MRMs) were optimized to 390.13 > 168 and 331.9 > 110 for glyphosate-FMOC and AMPA-FMOC, respectively. Standard curves of serial dilutions of authentic standards were used for absolute quantification. Means were compared by *t*-test (P < 0.05) for each time point.

Shikimate Accumulation in vitro

Shikimate was quantified using leaf disks (5-mm diameter) from GR and GS plants in 96-well microtiter plates (Shaner et al. 2005). Six biological replications from each biotype were used. Each excised leaf disk was placed into an individual well containing 100 μl of 10 mM ammonium phosphate buffer (pH 4.4) plus 0.1% (v/v) Tween 80 surfactant. Each analytical-grade glyphosate at 10, 100, 250, and 500 μM was added to its corresponding well. Plates were closed with a lid, sealed with two Parafilm® layers, and incubated in a growth chamber at $150\,\mu M\,m^{-2}\,s^{-1}$ fluorescent light and 25 C for 16 h. Plates were then frozen at −80 C and thawed at 60 C for 30 min. After that, 25 μl of 1.25 N HCl was added to each well, and the plate was incubated at 60 C for 15 min. Aliquots of 15 μl were transferred into a new plate containing 100 μl of $2.5\,g\,L^{-1}$ periodic acid and $2.5\,g\,L^{-1}$ *m*-periodate. Following incubation at room temperature for 90 min, 100 μl of 0.6 N NaOH and 0.22 M Na_2SO_3 was added to each well. Absorbance was measured at 380 nm within 30 min, and

background values were subtracted from each of the treatments. Shikimate levels were quantified based on a standard curve with known shikimate concentrations.

Shikimate Accumulation in planta

Eleusine indica plants at the 3-leaf stage from GS and GR biotypes were treated with glyphosate ($960\,g\,ha^{-1}$) as described earlier. Shikimate levels were measured in four replications at 24, 48, 96, and 144 HAT. Shikimate extraction was based on the microwave-based method described by Matallo et al. (2009). Plants were dried at 65 C for 72 h and ground in a ball mill (MA250, Marconi, São Paulo, Brazil) for 40 s. Samples of 0.4 g of each plant were solubilized in 10 ml of phosphoric acid in water (pH 2), and the solution was homogenized for 30 min using a shaker (MaxQ 4000, Thermo Scientific, Waltham, Maryland, USA) at 32 rpm. Samples were then microwaved (NN-S62, Panasonic, São Paulo, Brazil) for 10 s and cooled at room temperature for 5 min. After extraction, samples were diluted in phosphoric acid (3.5 mM) at 1:50 and filtered through a 0.22-μm membrane (Millex-GV, Merck Millipore, Barueri, São Paulo, Brazil). Shikimic acid concentration was determined using high-performance liquid chromatography (HPLC; LC-20A Prominence, Shimadzu, Barueri, São Paulo, Brazil), with a total run time of 15 to 20 min and a shikimate retention time of 8.1 min. To determine the levels of shikimate accumulation, a calibration curve was generated using known concentrations of analytical shikimate standard. Means were compared by *t*-test (P < 0.05) for each time point.

EPSPS Relative Gene Copy Number and Transcription

Frozen leaf tissue (50 mg) from three plants of each biotype was ground using a Qiagen Tissuelyzer (Qiagen, Valencia, CA, USA). Total RNA was extracted using a Qiagen RNeasy Plant Mini Kit, treated with DNase I, quantified using a NanoDrop spectrophotometer (Thermo Scientific), and checked for quality and integrity by gel electrophoresis. *Eleusine indica* RNA (200 ng) was used for synthesizing cDNA using a Quanta qScript cDNA synthesis kit (Bio-Rad, 2000 Alfred Nobel Dr, Hercules, CA, USA) with oligo-dT and random primers. Total genomic DNA was also extracted with a DNeasy kit (Qiagen). DNA quality and quantification were measured using a NanoDrop spectrophotometer, and DNA integrity was evaluated by running samples through a 1% agarose gel. For both cDNA and genomic DNA, primers were designed for *EPSPS* and β-actin as internal standard, according to Chen et al. (2015): EPSPS_F (5′-CTGATGGCTGCTCCTTTAGCTC-3′) and EPSPS_R (5′-CCCAGCTATCAGAATGCTCTGC-3′), and β-actin_F (5′-AACAGGGAGAAGATGACCCAGA-3′) and β-actin_R (5′-GCCCACTAGCGTAAAGGGACAG-3′). Genomic DNA and cDNA (10 ng each) were amplified using quantitative real-time PCR with 25-μl total volume under the following conditions: 10 min at 95 C, 40 cycles of 95 C for 20 s, and 60 C for 1 min. After completing these cycles, the melting curve was obtained by increasing the temperature from 60 C to 95 C in increments of 0.5 C every 5 s. *EPSPS* quantification was calculated as $\Delta Ct = ($Ct for β-actin − Ct for *EPSPS*) (Gaines et al. 2010), and the relative increase in *EPSPS* copy number was expressed as $2^{\Delta Ct}$. Each sample was run in three replicates to calculate the mean and standard deviation. Results were represented as the fold increase in *EPSPS* copy number relative to β-actin. Means were compared

by *t*-test (P < 0.05) for both genomic copy number and transcription.

EPSPS Gene Sequencing

To investigate a possible mutation in the specific region of *E. indica EPSPS* where mutations are known to confer glyphosate resistance, genomic DNA was extracted from GR and GS biotypes. Approximately 0.2 g of leaf tissue was used for DNA extraction (Kaundun et al. 2008). Plant tissue was placed in 96 deep-well blocks and stored at −80 C. Tissue was then ground in a bead mill to a dry powder and centrifuged at 2,200 × *g* for 5 min. Genomic DNA was extracted using the Qiagen DNeasy plant DNA extraction kit as described earlier. A 300-bp *EPSPS* fragment was amplified using primers described by Kaundun et al. (2008): F-CTCTTCTTGGGGAATGCTGGA and R-TAACCTTGCCACCAGGTAGCCCTC. PCR was performed using a PCR kit (Multiplex, Qiagen) with 7.5 ml of Master Mix, 1.5 µl of Q-Solution, 1.5 µl of the primer mix (0.2 µM of final concentration of each primer, forward and reverse), 2.5 µl of ultrapure water, and 2.0 µl of DNA template (10 ng L^{-1}); this provided a final reaction volume of 15 µl. The PCR cycling settings were established in accordance with the specifications of the manufacturer: 1 cycle of 95 C for 15 min, 36 cycles of 94 C for 30 s, 60 C for 90 s, 72 C for 60 s, and a final extension at 72 C for 10 min. Before sequencing, PCR products were purified using a PCR Purification Kit (QIAquick, Qiagen). Once purified, the integrity of the PCR product was confirmed by capillary electrophoresis (QIAxel, Qiagen). The PCR products of three GS and three GR samples were sequenced using an ABI-Prism 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).
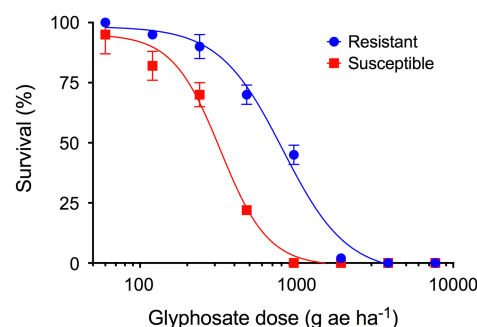
EPSPS Enzyme Activity

Protein extraction and EPSPS assay were conducted following the procedures developed by Monsanto and described in Dayan et al. (2015) and Salas et al. (2012). Initially, 20 g of young leaf tissue from GS and GR biotypes was ground in liquid nitrogen with a mortar and pestle. Ground tissue samples were homogenized for 5 min with constant stirring in cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mM KCl, and 0.5 mM benzamidine) with 1% polyvinylpolypyrrolidone (PVPP) and 10 µl of β-mercaptoethanol. Samples were centrifuged at 20,000 × *g* and 4 C for 40 min, and the supernatant was filtered through a cheesecloth into a cold beaker. Ammonium sulfate was slowly added to the solution to reach 450 g L^{-1} concentration, stirred for 30 min, and centrifuged at 30,000 × *g* and 4 C for 30 min. Ammonium sulfate was slowly added to the supernatant until 700 g L^{-1} concentration was reached, after which the

Figure 1. Survival (%) of glyphosate-resistant (blue) and glyphosate-susceptible (red) *Eleusine indica* biotypes at 28 d after treatment. Data were pooled from two experiments with three replications per treatment and biotype.

supernatant was centrifuged again at 30,000 × *g* and 4 C for 30 min. The pellet was dissolved in 2 ml of extraction buffer, but without EDTA and PVPP. Extracted samples were dialyzed overnight in dialysis buffer using Slide-A-Lyzer™ dialysis cassettes (Thermo Scientific) in 2 L of dialysis buffer (10 mM MOPS, 0.5 mM EDTA, 10% glycerol, and 5 mM β-mercaptoethanol) at 4 C on a stir plate.

EPSPS activity for GS and GR biotypes was measured in the presence of increasing concentrations of glyphosate (0, 0.1, 1, 10, 100, and 1000 µM). The assay for inorganic phosphate release was conducted using the EnzCheck phosphate assay kit (Invitrogen, Carlsbad, CA, USA) in three replications. The cuvette in which the reaction was conducted contained 500 µl of 2× assay buffer (100 mM of MOPS, 1 mM of MgCl$_2$, 10% glycerol, 2 mM of sodium molybdate, and 200 mM of NaF), 215 µl of HPLC-grade water, 200 µl of 1 mM 2-amino-6-mercapto7-methylpurine riboside, 10 µl 100 U ml^{-1} unit of purine-nucleoside phosphorylase, 25 µl of 50 mM phosphoenolpyruvate (PEP), 10 µl of glyphosate, and 10 µl of dialyzed EPSPS. After the solution was allowed to react for 5 min, 50 µl of 10 mM shikimate-3-phosphate was added. Inorganic phosphate release (µmol) per microgram of total soluble protein per minute was measured at 360 nm by calculating the slope of the phosphate release above background. Enzyme activity was expressed as µmol Pi µg^{-1} protein min^{-1} and values were regressed using the DRC package in R (Ritz et al. 2015).

SNP Genotyping for Pro-106-Ser on EPSPS Gene

The methodology developed in Warwick et al. (2008) was employed with some alterations for genotyping 240 GR plants and 60 GS plants totaling 300 individuals. To provide a quick technique for surveying GR *E. indica* populations, an SNP

Table 1. Primers and TaqMan probes used for single-nucleotide polymorphism genotyping assay in the *Eleusine indica* 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene.

Primer[a]	Sequence (5′ → 3′)	Region of homology
Forward	GGGAATGCTGGAACTGCA	1,051–1,069
Reverse	TTCCTCCAGCAGCAGTTACG	1,109–1,090
MGB probe for C allele	VIC-TGCTGTCAATGGTCGCAT	1,070–1,089
MGB probe for T allele	FAM-TGCTGTCAATGATCGCAT	1,070–1,089

[a]MGB, minor groove-binding.

Table 2. Lethal dose for 50% response (LD$_{50}$), estimated parameters of the model (Equation 1) and resistance factor (RF) for survival (%), in vitro shikimate assay (µg ml^{-1}), and EPSPS enzyme activity (µmol Pi µg^{-1} total soluble protein min^{-1}) of glyphosate susceptible (GS) and glyphosate resistant (GR) *Eleusine indica* biotypes.

Biotype	LD$_{50}$	*a*	*b*	*c*	RF
GR survival	741.9	102.1	762.4	− 2.1	4.2
GS survival	176.6	100.9	181.6	− 2.7	
GR in vitro shikimate	43.4	35.8	44.1	2.1	4.1
GS in vitro shikimate	10.5	86.4	10.9	2.6	
GR enzyme activity	23.4	0.2	23.6	− 2.1	3.7
GS enzyme activity	6.4	0.2	6.5	− 2.0	
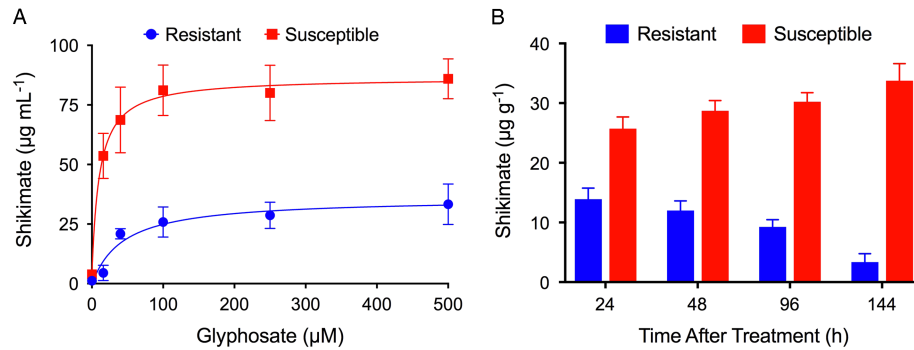
Figure 2. Shikimate accumulation in vitro (A) and *in planta* (B) for glyphosate-resistant and glyphosate-susceptible *Eleusine indica* biotypes. Samples for the *in planta* assay were extracted from plants treated with $960\,g\,ha^{-1}$ glyphosate and analyzed with high-performance liquid chromatography. The in vitro assay was conducted using *E. indica* leaf disks exposed to increasing glyphosate concentrations. Data were pooled from two experiments with three replications per treatment and biotype.

genotyping protocol was developed for the Pro-106-Ser target-site mutation in the *EPSPS* gene, considering that GS plants carry the codon CCA, whereas GR plants carry the codon TCA. Minor groove-binding group (MGB) probes and primers (Table 1) were custom designed and synthesized based on the *EPSPS* sequence provided (TaqMan, Applied Biosystems). Two primers were designed to amplify a 58-bp fragment containing the SNP, and the two MGB probes were designed to discriminate C or T alleles, that is, the Fluor VIC probe for the resistant T allele and the Fluor FAM probe for the susceptible C allele.

Each SNP genotyping PCR reaction (10 µl) contained 20 ng of genomic DNA, 0.5 µl of Custom TaqMan SNP Genotyping Assays containing primers and MGB probes, and 5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems). The product was amplified under the following conditions: 15 min at 95 C, followed by 36 cycles of 30 s at 94 C and 1 min at 72 C in a Viia 7 RT-PCR System (Thermo Scientific). Two technical replicates for

each of the 300 samples were performed to check technical consistency.

Results and Discussion

Both biotypes were completely controlled with glyphosate doses of $3,840\,g\,ha^{-1}$ or above (Figure 1). All plants of the GS biotype were killed by $960\,g\,ha^{-1}$ glyphosate, whereas 41% of the GR biotypes survived the same dose. The RF was 4.2, calculated from the relative LD_{50} values derived from the dose–response curves (Table 2). This RF is similar to those found in biotypes from other countries, including Malaysia (RF 8), the United States (RF 3 to 8), and China (RF 4 to 6) (Chen et al. 2015; Lee and Ngim 2000; Molin et al. 2013; Mueller et al. 2011). In such cases, resistance is conferred mainly by alterations in the target site that provide low to intermediate levels of glyphosate resistance. A similar range of RF values indicates that the resistance mechanism across these different locations could be the same.
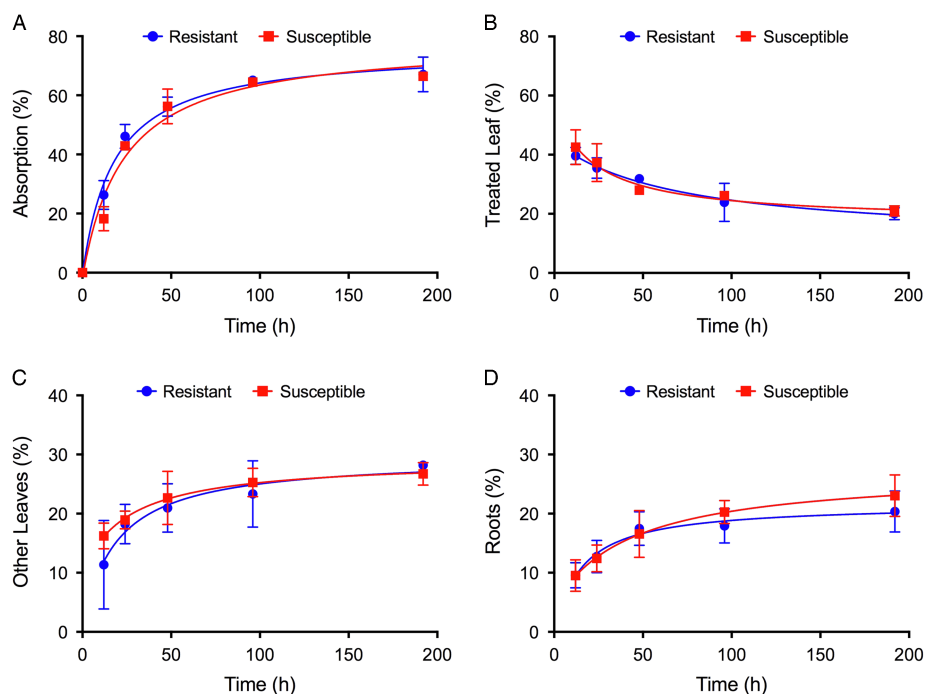
Figure 3. Absorption (A) and translocation of [^{14}C]glyphosate in the treated leaf (B), other leaves (C), and roots (D) of glyphosate-resistant and glyphosate-susceptible *Eleusine indica* biotypes from Brazil. Data were pooled from two experiments with three replications per treatment and biotype. Differences between biotypes were not significant ($P < 0.05$).
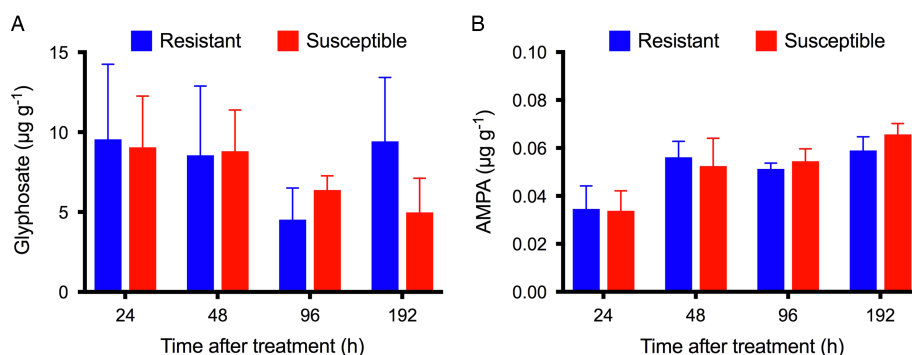
Figure 4. Levels of glyphosate (A) and aminomethylphosphonic acid (AMPA) (B) at 24, 48, 96, and 192 h after glyphosate treatment (960 g ha^{-1}) in glyphosate-resistant and glyphosate-susceptible *Eleusine indica*. Data were pooled from two experiments with three replications per treatment and biotype. Means did not differ by *t*-test (P < 0.05).

In vitro and *in planta* shikimate levels in the GS biotype were higher than those measured in the GR biotype, supporting the differences in glyphosate sensitivity observed with the whole-plant dose response (Figure 2). The average shikimate accumulation in GS divided by the average shikimate accumulation in GR provides an RF of 3.8-fold *in planta* and 4.1-fold based on LD$_{50}$ values in vitro (Table 2). These data are consistent with our whole-plant resistance to glyphosate (RF 4.2) and another GR *E. indica* biotype from Tennessee (Mueller et al. 2011). Glyphosate inhibits EPSPS, a key enzyme of the shikimate pathway, competing with PEP, one of the substrates for EPSPS. Inhibition of EPSPS leads to reduced feedback inhibition of the pathway, resulting in increased carbon flow to shikimate-3-phosphate, which is then converted into high levels of shikimate (Duke and Powles 2008). Because shikimate accumulation is normally proportional to EPSPS inhibition, greater accumulation of shikimate in GS than in GR indicates a stronger inhibition of EPSPS activity in GS than GR *E. indica*.

[^{14}C]glyphosate absorption increased similarly over time for both GS and GR biotypes (Figure 3). No significant difference was found for maximum absorption (A_{max}) between these two biotypes (GS: 66.9 ± 1.8; GR: 66.1 ± 1.7). The translocation of this herbicide throughout other parts of the plant was also very similar for GS and GR, suggesting that differential absorption and translocation do not contribute to glyphosate resistance in this *E. indica* biotype. This is supported by the times for 90% of total translocation (t_{90}) out of the treated leaf (GS: 32.5 ± 8.3; GR: 39.7 ± 11.6) and to roots (GS: 64.2 ± 12.9; GR: 55.9 ± 9.8), which were also similar between biotypes. These data corroborate previous studies that have not found differences in glyphosate

metabolism rate or absorption and translocation between GR and GS *E. indica* biotypes (Baerson et al. 2002; Kaundun et al. 2008; Ng et al. 2003, 2004a).

The levels of both glyphosate and AMPA were statistically similar for GS and GR biotypes, indicating that glyphosate metabolism is also not associated with the mechanism of resistance in this *E. indica* biotype (Figure 4). Metabolic degradation of glyphosate into less-toxic compounds does not occur very commonly in weeds, and the relationship between glyphosate metabolites and resistance levels is often unclear (reviewed in Sammons and Gaines 2014). However, glyphosate metabolism has been reported in other species such as *D. insularis* (Carvalho et al. 2012). Some recent research using RNA sequencing to investigate the mechanism of GR in a *E. indica* population from China found overexpression of a glutathione transferase gene family in the resistant biotype compared with the susceptible biotype (Chen et al. 2017a). However, the hypothesis of whether or not there is a connection between this gene family and glyphosate resistance remains to be proven.

The first case of *EPSPS* gene amplification conferring glyphosate resistance in a weedy species was reported in Palmer amaranth (*Amaranthus palmeri* S. Watson) (Gaines et al. 2010). Since then, this mechanism has been documented in other GR species such as *A. tuberculatus* (Lorentz et al. 2014), spiny amaranth (*Amaranthus spinosus* L.) (Nandula et al. 2014), kochia [*Bassia scoparia* (L.) A. J. Scott] (Jugulam et al. 2014; Wiersma et al. 2015), *L. multiflorum* (Salas et al. 2012), ripgut brome (*Bromus diandrus* Roth) (Malone et al. 2016), and windmillgrass (*Chloris truncata* R. Br.) (Ngo et al. 2018). While relative *EPSPS* genomic copy number was 16.8-fold higher in a GR compared with a GS *E. indica* biotype from China (Chen et al. 2017b), both the biotypes from Brazil had only one copy of *EPSPS* and similar levels of the *EPSPS* transcript (Figure 5). Consequently, *EPSPS* gene amplification is not associated with the mechanism of glyphosate resistance in *E. indica* from Brazil.

A nonsynonymous mutation in a codon encoding for Pro-106 (CCA) to a serine (TCA) (Pro-106-Ser) was observed in a 330-bp *EPSPS* fragment from the GR biotype (Figure 6). This mutation is prevalent in other GR *E. indica* populations around the world, as well as in other GR species (Baerson et al. 2002; Chen et al. 2015; Kaundun et al. 2008; Molin et al. 2013; Ng et al. 2004b). This amino acid substitution confers low to intermediate levels of glyphosate resistance, supporting dose–response and shikimate data reported in this research. Other GR species such as *E. colona*, *L. perenne* ssp. *multiflorum*, and *L. rigidum* that harbor the same *EPSPS* mutation (Pro-106-Ser) also have similar levels of resistance to glyphosate (Sammons and Gaines 2014).
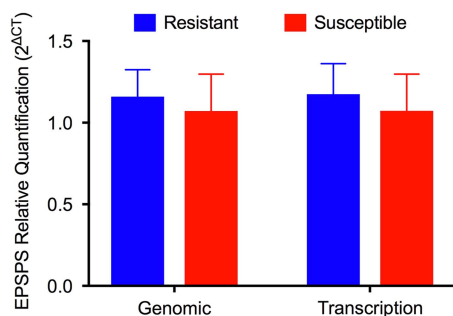
Figure 5. Relative quantification of *EPSPS* genomic copy number and *EPSPS* transcription in glyphosate-resistant and glyphosate-susceptible *Eleusine indica* from Brazil, using β-actin as the reference gene. Data were pooled from two experiments with three replications per treatment and biotype. Means did not differ by *t*-test (P <0.05).

```
              096   098   100   102   104   106   108   110   112   114   116
A. thaliana   CTCTTCTTGGGGAATGCTGGAACTGCAATGCGACCATTGACAGCAGCCGTAACTGCTGCTGGAGGAA

AY157642  MS  CTCTTCTTGGGGAATGCTGGAACTGCAATGCGACCATTGACAGCAGCCGTAACTGCTGCTGGAGGAA

AY157643  MR  CTCTTCTTGGGGAATGCTGGAACTGCAATGCGATCATTGACAGCAGCCGTAACTGCTGCTGGAGGAA

E. indica BS  CTCTTCTTGGGGAATGCTGGAACTGCAATGCGACCATTGACAGCAGCCGTAACTGCTGCTGGAGGAA

E. indica BR  CTCTTCTTGGGGAATGCTGGAACTGCAATGCGATCATTGACAGCAGCCGTAACTGCTGCTGGAGGAA

               097   099   101   103   105   107   109   111   113   115
```

Figure 6. *EPSPS* gene fragment comparison of *Arabidopsis thaliana EPSPS* sequence [GenBank: CAA29828.1]; susceptible (S) and resistant (R) biotypes of *Eleusine indica* from Malaysia (AY157642 MS and AY157643 MR) and from Brazil (BS and BR). The colored bases show a proline (red) to a serine (blue) codon substitution at position 106 in the GR biotype.

As expected, EPSPS activity from the GR biotype was less inhibited than in GS plants (Figure 7). The LD_{50} values for GS and GR were 6.4 and 23.4, respectively, providing an RF of 3.8, which is consistent with shikimate accumulation (RF = 3.4 to 3.8) and whole-plant dose response (RF = 4.2) (Table 2). The single point mutation Pro-106-Ser decreases the affinity of glyphosate (K_i) to its binding site in *E. indica* EPSPS (Baerson et al. 2002; Kaundun et al. 2008). Because this target-site mutation confers intermediate levels of resistance, it may not be enough to provide full dose resistance in polyploid species (e.g., *E. colona*) due to a dilution effect (Alarcón-Reverte et al. 2015). Therefore, polyploid plants containing Pro-106-Ser in only one of their genomes can become susceptible when glyphosate is applied under mild temperatures (Han et al. 2016).

A qPCR-based SNP genotyping assay was developed to discriminate individuals from GS (homozygous CCA) and GR (homozygous TCA) nucleotides for the Pro-106 codon (Figure 8). Plants of the GS biotype carried the homozygous susceptible allele (C), whereas those of the GR biotype contained the homozygous resistance allele (T). Therefore, this assay can be useful for the detection of the Pro-106-Ser mutation and, consequently, glyphosate resistance in *E. indica*. Rapid SNP genotyping assays, like the one described here, should be adopted more widely in other species and with other herbicides for which the mechanism of resistance involves point mutations [e.g., ALS-resistant *B. scoparia* (Warwick et al. 2008) and ACCase-resistant blackgrass (*Alopecurus myosuroides* Huds.) (Délye et al. 2010)].

The development of a simple, rapid, and accurate diagnostic test is needed to detect resistant biotypes before herbicide application. Genotyping assays are inexpensive and suitable for the screening of large numbers of individuals to detect even low-frequency resistant alleles in individuals within a population. Such assays can inform herbicide applicators to make better decisions when choosing herbicides. Hopefully, these decisions would minimize the spread of the resistant biotypes by encouraging farmers to adopt more proactive approaches (Corbett and Tardif 2006). SNP genotyping assays, like the TaqMan assay used herein, are high-throughput techniques, and they have already been widely applied in human genetics and molecular plant breeding (Giancola et al. 2006; Ranade et al. 2001). This technology has been adopted in a number of areas in weed science, especially for diagnosing herbicide resistance and identifying genetically related species (Küpper et al. 2017; Patterson et al. 2017).

The GR *E. indica* biotype from Brazil had a resistance factor of 4.2 in greenhouse dose–response studies. There were no differences between susceptible and resistant biotypes for glyphosate metabolism, absorption, or translocation. The two biotypes also had a similar *EPSPS* gene copy number and *EPSPS* transcription. However, the resistant biotype has an SNP resulting in a Pro-106-Ser amino acid substitution in the EPSPS, conferring similar resistance levels for enzyme activity and shikimate accumulation. The EPSPS of susceptible plants was 3.8-fold more sensitive to glyphosate inhibition than the enzyme from resistant plants, while shikimate accumulation was 3.8- to 4.1-fold greater in the susceptible than in the resistant biotype. Consistent with biotypes from other regions of the globe, the Pro-106-Ser is associated with the mechanism of glyphosate resistance in *E. indica* from Brazil, characterizing the first report of this target-site mutation for this species in South America. Therefore, a qPCR-based SNP genotyping assay was developed based on this amino acid substitution for the rapid and precise identification of glyphosate-resistant *Eleusine indica* populations.
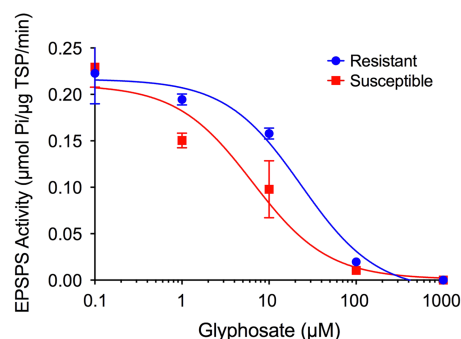
Figure 7. EPSPS enzyme activity of glyphosate-resistant and glyphosate-susceptible *Eleusine indica* biotypes from Brazil. Enzyme activity is represented in amount of inorganic phosphate (Pi) released (µmol) per µg of total soluble protein (TSP) per minute. Means are of three biological replications per glyphosate dose and biotype.
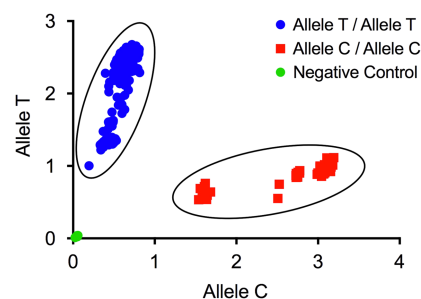
Figure 8. Single-nucleotide polymorphism genotyping assay developed in this study using quantitative PCR for the C-to-T substitution resulting in a Pro-106-Ser mutation in *Eleusine indica*. The *y*-axis corresponds to fluorescence measures for the Fluor VIC probe for the T allele, and the *x*-axis corresponds to the Fluor FAM probe for the C allele. The three clusters correspond to genotypes: TT-homozygote (GR), CC-homozygote (GS), and the negative control.

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