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Gene Amplification of *EPSPS* with a Mutation in Conserved Region: The Evolved Glyphosate Resistance Mechanism in *Eleusine indica*

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Abstract: Goosegrass (*Eleusine indica* (L.) Gaertn.) is one of the most vicious weeds worldwide, competing with crops and greatly reducing their yields. Glyphosate, a non-selective, broad spectrum, post-emergence herbicide has inevitably induced severe resistance in many weeds owing to its intensive use. Additionally, control strategies rely on the clarity of resistance mechanisms. In this study, we aimed to investigate the resistance levels and potential resistance mechanisms of two goosegrass populations collected from orchards. Results showed that the resistance indexes of LL and SS populations were 3.8 and 1.9, respectively. A single nucleotide change led to a Pro106Leu (P106L) mutation in the LL population and the SS population had a Pro106Ser (P106S) amino acid substitution. The *EPSPS* expression in both populations was 2.6 times that of the wild-type population. However, the relative copy number and EPSPS protein content in the LL population were higher than those of the SS population, as indicated by immunoblot analysis and enzyme-linked immunosorbent assays. Overall, we confirmed *EPSPS* amplification with a P106L mutation, resulting in overproduction of this mutated EPSPS protein, which conferred moderate glyphosate resistance. This study details a case of simultaneous evolution of mutation and amplification in *EPSPS* of glyphosate resistance weeds.

Keywords: *EPSPS*; goosegrass; glyphosate; mutation; amplification

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1. Introduction

Glyphosate (N-phosphonomethyl-glycine) is a systemic and broad spectrum herbicide that has been used to effectively control weeds in tea gardens, orchards, and non-crop areas [1]. Glyphosate inhibits the target enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; E.C. 2.5.1.19), complexly affecting the shikimate pathway and disturbing the biosynthesis of phenylalanine, tyrosine, tryptophan, and other secondary aromatic products [2]. Since its commercial introduction in the early 1970s and the promotion of glyphosate-resistant (GR) crops, reliance on glyphosate has become increasingly critical, and 56 weed species, including goosegrass, have evolved resistance [3]. Target-site (TS) and non-target-site (NTS) resistance are reported as the two main mechanisms of glyphosate resistance in weeds [4–6]. Single, double, and triple amino acid substitutions in the conserved region of EPSPS (Pro 106, Thr 102, and Gly 101) have been reported to be one of the target site mechanisms [7–9]. In addition to *EPSPS* gene mutations, amplification of this gene is another target site alteration mechanism, which subsequently leads to overproduction of the EPSPS enzyme and varying resistance levels [6]. Since the discovery of *EPSPS* gene amplification in Palmer amaranth (*Amaranthus palmeri* S. Watson) [10], several other evolved GR weed species have been reported to possess this resistance mechanism [4,11–13]. Some GR weeds with moderate *EPSPS* expression levels, but not corresponding copies, sometimes show insufficient resistance levels, probably caused by differences in transcript regulation to control gene expression [14]. Non-target site resistance mechanisms are caused by the reduction in glyphosate translocation or absorption, enhanced herbicide metabolism, and

the sequestration of herbicides, indicating greater complexity in determining the comprehensive mechanism [15–17]. NTSR mechanisms to glyphosate have typically been reported in several different glyphosate-resistance biotypes of *Aster squamatus*, *Conyza bonariensis*, and *Bidens pilosa* L. [18–20].

Goosegrass (*Eleusine indica* (L.) Gaertn.), one of the most problematic weeds worldwide, is mainly distributed in temperate and tropical regions, including Brazil, the United States, Europe, Australia, and China [21,22]. Since the first identification of a resistance-endowing EPSPS point mutation, P106S, several other resistance-endowing single amino acid substitutions at P106 (P106T, P106A, P106S, and P106L) have been reported in GR goosegrass [7,23,24]. In addition, a double mutation identified in EPSPS (Thr102Ile + Pro106Ser) was first found in a goosegrass population in Malaysia [25]. A mechanism of *EPSPS* amplification was also found in some goosegrass populations [26,27].

In China, goosegrass populations distributed across many provinces have evolved resistance to glyphosate; however, few studies have been conducted to investigate the resistance mechanisms [26–29]. In this study, two suspected GR goosegrass populations were collected from banana plantations in Guangdong Province which were not well controlled under the recommended dose. The aim of the present study was to clear the resistance level of these two populations, and investigate the potential resistance mechanisms to glyphosate. A new target site resistance mechanism was found, wherein amplification and *EPSPS* mutation (P106L) coexisted in the same individuals and conferred moderate glyphosate resistance.

2. Materials and Methods

2.1. Plant Materials and Dose–Response Test

Seeds (at least 100 individuals) of two suspected GR goosegrass populations, LL (113°52′23.7″ E, 23°11′15.6″ N) and SS (113°47′50.5″ E, 23°9′31.4″ N), were collected from banana plantations in Guangzhou, Guangdong Province. Additionally, seeds of a glyphosate-susceptible (GS) population WT (113°48′14.3″ E, 23°9′25.8″ N) were also collected in a field without herbicide application history near the GR populations. Seeds of the three populations were soaked in gibberellin (1%) for 24 h to break dormancy, then cultured in plastic pots (9 × 9 cm). All the pots were kept in a greenhouse, watered as needed, and the temperature was controlled at 30 ± 2 °C in the day with a 14 h photoperiod, and at 27 ± 2 °C overnight [30]. Different doses (0, 112.5, 225, 450, 900, and 1800 g ae ha^{-1} for the GS population; 0, 225, 450, 900, 1800, and 3600 g ae ha^{-1} for the suspected GR populations) of glyphosate with a recommended rate of 900 g ae ha^{-1} were designed and applied to individuals with 5- to 7-leaf stage (four replicates for each treatment). Glyphosate (Roundup, 300 g ae L^{-1} glyphosate, 410 g ae L^{-1} isopropylamine salt of glyphosate, Bayer Crop Science, 800 North Lindbergh Blvd, Saint Louis, MO 63167, USA) was applied using a moving cabinet sprayer (TeeJet® XR8002) and a compressed air cabinet sprayer (3WPSH-500D) [31]. The fresh weights of the shoots in each pot (8 plants) were measured at 14 days after treatment (DAT). The experiment of whole plant assay was repeated twice in a completely randomized design.

2.2. Shikimate Accumulation Experiment

Plants were cultivated to the 4- to 6-leaf stage (8 plants per pot) and glyphosate was applied in a dose of 1800 g ae ha^{-1}. Treated leaf tissues were collected at 2, 4, 6, 8, 10, and 12 DAT and stored at −80 °C until used for shikimate detection. Shikimate accumulation was determined according to the method previously described [29]. In brief, leaf samples (0.5 g) were finely ground in liquid nitrogen and added to 1.0 mL of 0.25 mol L^{-1} HCl and centrifuged at 12,000 rpm for 30 min. The supernatant (0.2 mL) was mixed with 2 mL periodic acid (1%). After 3 h, the mixtures were supplemented with 2 mL of 1 mol L^{-1} NaOH and 1.2 mL of 0.1 mol L^{-1} glycine. Subsequently, 0.2 mL of each mixture were added to 96-well plate to analyze the shikimate accumulation. Sample absorbance was measured

at 380 nm using a Tecan Infinite 200 Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland). This experiment was repeated twice.

2.3. EPSPS Sequencing

The leaf tissues of twenty plants without glyphosate treatment from each population were sampled and stored at −80 °C when the plants reached the 5- to 7-leaf stage. Genomic DNA was extracted using a plant DNA extraction kit (Tiangen Biotech Beijing Co., Ltd., Beijing, China). Primers were used to amplify a fragment of 310 bp in length which included the conserved region of *EPSPS* (Table S1). PCR was performed as described previously, with the annealing temperature set at 60 °C [27]. The PCR product was then loaded onto a 1% agarose gel and sequenced using Sanger sequencing by Sangon Biotech (Sangon Biotech Shanghai Co. Ltd., Beijing, China). The *EPSPS* sequences of all populations were aligned using DNAMAN software (Lynnon BioSoft, San Ramon, CA, USA) to detect mutations in the target gene.

2.4. EPSPS Gene Expression and Copy Number Detection

The leaf tissues of twenty individuals were randomly collected from each population without glyphosate treatment, and RNA was extracted using a plant RNA extraction kit (Tiangen Biotech Beijing Co., Ltd., Beijing, China). First-strand complementary DNA (cDNA) synthesis was performed using the EasyScript All-in-One First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) with 1000 ng RNA in a total volume of 20 µL, according to the manufacturer's instructions. Primer pairs described in a previous study were used to detect the relative expression and copy number of the *EPSPS* gene and acetolactate synthase (*ALS*) was used as the reference gene which has already been screened in our previous study (Table S1) [30]. The relative expression of *EPSPS* was analyzed using the $2^{-\Delta\Delta Ct}$ method. The relative copy number was calculated using the $2^{\Delta Ct}$ method [10,32,33]. This experiment was repeated twice.

2.5. Protein Extraction and Immunoblot Analysis

Protein extraction and immunoblotting analysis were performed as described previously [13,28,34]. In brief, leaf tissues (0.2 g) were collected for each population and were cultured under the conditions described above. Protein extraction for the leaf tissue was performed using the MinuteTM Total Protein Extraction Kit for Plant Tissues (Invent Biotechnologies, Inc., Eden Prairie, MN, USA) following the manufacturer's protocol. The protein concentration was determined, followed by separation using 10% SDS-PAGE and then transferred to nitrocellulose membranes (0.45 µm). Additionally, the proteins were probed with the primary anti-EPSPS antibody (Shanghai Youlong Biotech Co., Ltd., Shanghai, China) at a dilution of 1:5000 overnight at 4 °C. The membranes were then incubated with the rabbit secondary antibodies diluted 1:5000 at room temperature for 1 h (ABclonal Biotech Co., Ltd., Cambridge, MA, USA). Finally, immunoreactive bands were visualized using ECL (enhanced chemiluminescence).

2.6. Detection of EPSPS Protein Content Using an Enzyme-Linked Immunosorbent Assay

The leaf tissues were collected for each population and thoroughly ground in liquid nitrogen. Ground leaves (0.1 g) were added to 1.0 mL sample extract and centrifuged at 12,000 rpm for 10 min. EPSPS content was detected using an enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Youlong Biotech Co., Ltd., China) according to the manufacturer's protocol. Briefly, 0.1 mL supernatants were protected from light for 45 min in an antibody-coated plate. After washing the plate, 0.1 mL monoclonal and multi-antibodies as reagents were added to the ELISA plate to react for 30 min under strict exclusion of light. Washing the plate again, 0.1 mL chromogenic agent was added to the ELISA plate to react for 15 min under strict exclusion of light. Sample absorbance was measured using a Tecan Infinite 200 Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland) at 450 nm after adding the stop solution. A standard curve was constructed based on the OD values of the

EPSPS standards. The EPSPS concentration was calculated by extrapolating the OD value of the test sample to the standard curve. This experiment was repeated twice.

2.7. Statistical Analysis

Dose–response data were subjected to nonlinear regression analysis using a log-logistic equation: $Y = y_0 + a/[1 + (X/X_0)^b]$. In this model, y is the inhibition rate, X is the glyphosate dose (g ae ha^{-1}), b is the curve slope around X_0, y_0 is the lower limit, a is the difference between the upper and lower limits, and X_0 is the herbicide dose required for 50% plant growth reduction (GR_{50}) [35]. The GR_{50} was used to calculate the resistance index (RI) as the ratio of the GR_{50} of the resistant population to that of the WT population.

EPSPS expression, shikimate accumulation, relative copy numbers, and EPSPS protein content data were subjected to ANOVA and analyzed using a non-parametric Kruskal–Wallis H test with SPSS (version 13.0; SPSS, Chicago, IL, USA).

3. Results

3.1. Dose–Response Assays

The biomass of the three goosegrass populations decreased with increasing glyphosate doses (Figure 1). At 14 DAT, all plants from the WT population were in a state of death with a dose of 900 g ae ha^{-1}, whereas all plants of the GR populations (LL and SS) were killed by 3600 g ae ha^{-1} glyphosate (Figure 1). The GR_{50} values, indicating the herbicide doses required for 50% growth reduction in the LL and SS populations, were 878.3 and 442.6 g ae ha^{-1}, respectively (Table 1). Compared with the WT population (GR_{50} = 233.1 g ae ha^{-1}), the RIs of the LL and SS populations were 3.8 and 1.9, suggesting a moderate and low level of resistance, respectively.

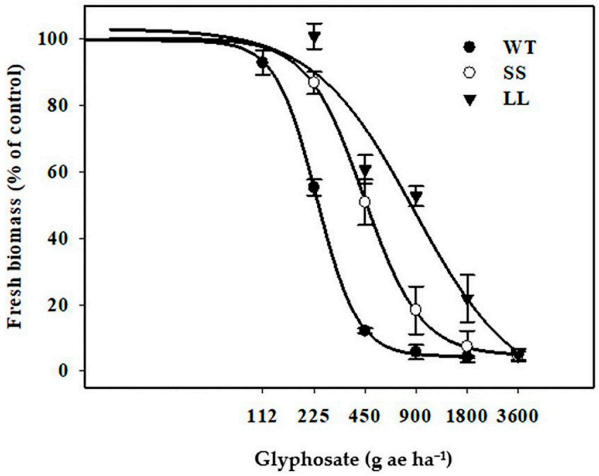


Figure 1. Response of three populations to different doses of glyphosate at 14 days after treatment (DAT). The dose–response curve of these three populations calculated by nonlinear regression analysis, vertical bars represent the standard errors of the means (SEM).

Table 1. Parameter estimates (SE) by nonlinear regression for the dose–response assay using fresh shoot weights, and four samples were designed and selected for each treatment.

Population	*b*	y_0	*a*	x_0	R^2	*p*	*RI* [1]
LL	1.5 ± 0.3	−6.7 ± 11.6	109.9 ± 13.4	878.3 ± 173.6	0.95	<0.0001	3.8
SS	2.6 ± 0.3	4.7 ± 1.9	95.6 ± 3.2	442.6 ± 19.4	0.98	<0.0001	1.9
WT	3.6 ± 0.3	4.4 ± 1.1	95.3 ± 2.0	233.1 ± 5.0	0.99	<0.0001	-

[1] GR_{50} of the resistant population divided by GR_{50} of the susceptible population. WT is the susceptible population. Parameter y is the inhibition rate, X is the glyphosate dose (g ae ha^{-1}), b is the curve slope around X_0, y_0 is the lower limit, a is the difference between the upper and lower limits, and X_0 is the herbicide dose required for 50% plant growth reduction (GR_{50}).

3.2. Shikimate Accumulation

After spraying the herbicide at 1800 g ae ha^{-1}, slight shikimate accumulation was observed in the LL and SS populations, whereas the WT population showed significant shikimate accumulation, reaching 3381.0 μg g^{-1} at 12 DAT, which was 39.3 times higher than that at the first detection (2 DAT) with fatal damage to the plants (Table 2). The LL and SS populations showed similar changes in shikimate accumulation, with moderate increases after glyphosate treatment. However, the final shikimate accumulation remained at approximately 200–300 μg g^{-1} in the LL and SS populations. These results suggested that shikimate accumulation provided evidence of evolved resistance in both of the two populations.

Table 2. Shikimate accumulation in the leaf tissues of three populations at different days after glyphosate treatment under a dose of 1800 g ae ha^{-1}. Letters indicates differences among values according to Student's *t*-test at $p < 0.05$. Data are means ± standard error of four replicates samples.

	Shikimate Accumulation (μg g^{-1})					
Populations	**2 DAT**	**4 DAT**	**6 DAT**	**8 DAT**	**10 DAT**	**12 DAT**
SS	47.5 ± 6.6 b	72.4 ±7.0 c	72.6 ± 5.4 c	195.1 ± 10.9 b	130.1 ± 8.0 b	288.1 ± 12.2 b
LL	50.3 ± 4.8 b	100.7 ± 7.0 b	141.9 ± 5.7 b	134.9 ± 8.8 b	103.5 ± 11.1 b	200.1 ± 13.5 b
WT	85.9 ± 12.6 a	133.3 ± 6.5 a	477.1 ± 46.8 a	2635.5 ± 55.5 a	2962.2 ± 48.7 a	3381.0 ± 65.9 a

3.3. EPSPS Gene Sequence Analysis

To determine the existence of a target site resistance mechanism in plants, we examined a conserved region of the *EPSPS* gene. Designed primer pairs were used to obtain an *EPSPS* DNA fragment of approximately 310 bp covering the Gly101, Thr102, and Pro106 positions (Figure 2). The PCR products of 20 individuals were sequenced for each population. After sequence alignment, all LL and SS populations exhibited a codon change at position 106, resulting in an amino acid change. In the LL population, a single nucleotide polymorphism (CCA to CTA) resulted in a proline to leucine substitution (P106L), and another nucleotide substitution from CCA to TCA at codon 106 in all SS individuals resulted in an amino acid substitution from proline to serine (P106S).

3.4. EPSPS Expression and Relative Copy Number

EPSPS gene expression and copy number were evaluated in 20 random individuals without glyphosate treatment from each population after nucleic acid extraction (Figure 3). The reference gene, *ALS*, was used to normalize and guarantee accuracy. *EPSPS* expression in the LL and SS populations was 2.6 times higher than that in the WT population. The average copy number of *EPSPS* was 3.0 and 1.3 in the LL and WT individuals, respectively, according to the relative expression of *EPSPS*. Although the expression of *EPSPS* in the SS population was the same as that in the LL population, the average copy number of *EPSPS* in this population was only 1.6, showing no significant difference to the WT population.

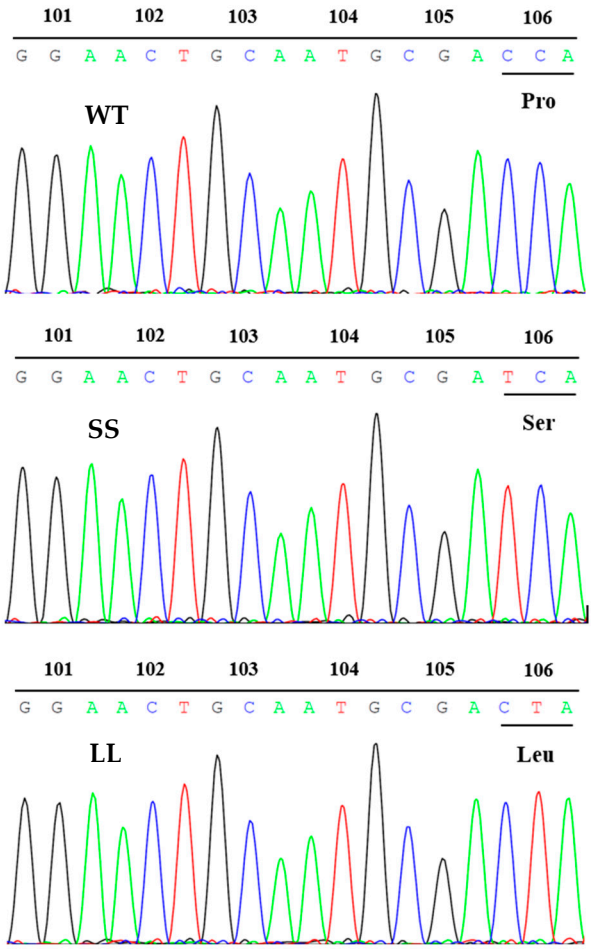


Figure 2. Partial nucleotide sequence results of *EPSPS* gene for the WT, SS, and LL populations, respectively. A homozygous mutation of Pro106Ser was detected in the SS population and a homozygous mutation of Pro106Leu was identified in the LL population by sequencing chromatograms.

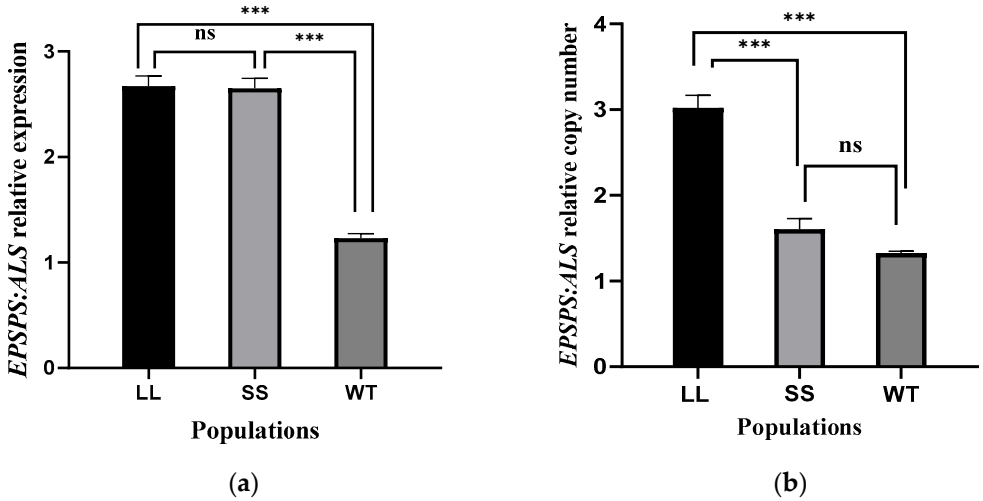


Figure 3. *EPSPS* gene expression (**a**) and copy number (**b**) relative to acetolactate synthase (*ALS*) in the leaf tissue of three goosegrass populations (LL, SS, WT) without glyphosate treatment. Twenty individuals were selected from each population and vertical bars show the SEM for the replicates. The non-parametric Kruskal-Wallis H test ($p < 0.05$) was selected to analyze the data. Asterisks indicate significant differences based on data of expression or copy number among goosegrass populations, and ns indicates no significant differences.

3.5. EPSPS Protein Abundance by Immunoblot Analysis and Enzyme-Linked Immunosorbent Assay

Anti-EPSPS immunoblotting was used to detect EPSPS protein content in the three goosegrass populations. EPSPS protein can be probed with signals from specific binding with antibodies. The results showed that signals from the LL population with a higher copy number and expression were stronger than those from the SS population with a lower copy number, and the signals from the sensitive population were weaker than those from the GR populations (Figure S1). In addition, an enzyme-linked immunosorbent assay was used for the quantitative detection of EPSPS protein. Unlike anti-EPSPS immunoblotting, this method allows quantitative analysis of EPSPS proteins by measuring the $OD_{450\ nm}$ of the EPSPS protein standard to obtain a regression equation: $y = 10.441x - 2.1484$, $R^2 = 0.9913 > 0.99$, where x is the $OD_{450\ nm}$ value and y is the EPSPS protein content. Accordingly, the average EPSPS protein content in LL and WT populations was 1.7 and 0.6 μg g^{-1}, respectively (Figure 4). The EPSPS protein content in the SS population was only 0.4 μg g^{-1}, which was significantly lower than that in the LL population and inconsistent with the gene expression results. Compared to the glyphosate sensitive population (WT), the protein content in the SS population had no obvious difference. These results indicate that other biochemical reactions, such as translation, might affect EPSPS protein content.

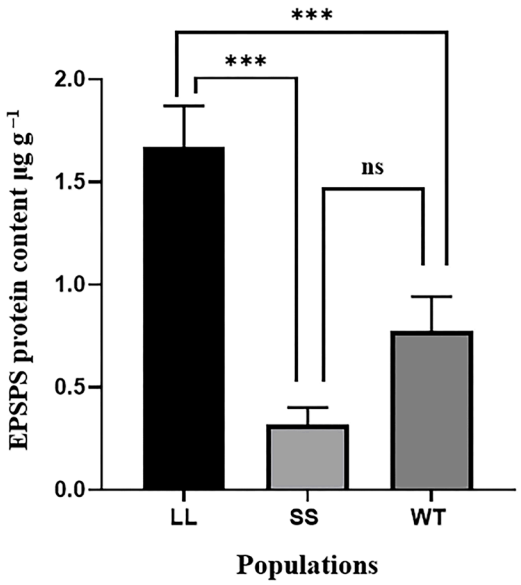


Figure 4. EPSPS protein content in leaf tissues of three goosegrass populations using enzyme-linked immunosorbent assay. Ten individuals were selected from each population. Vertical bars show the standard error of the mean for the replicates. Asterisks indicate significant differences in protein content when analyzed using the non-parametric Kruskal–Wallis H test ($p < 0.05$) and ns indicates no significant differences.

4. Discussion

Glyphosate inhibits EPSPS activity in the shikimate pathway, an important biological metabolic pathway that links carbohydrate metabolism and aromatic compound biosynthesis [36]. This leads to reduced feedback inhibition in this pathway, resulting in high levels of shikimate accumulation in tissues [2]. In this study, shikimate accumulation in the GS population was more than 11.3 times those of the LL and SS populations, which had RI values of 3.8 and 1.9, respectively, indicating the evolution of resistance. Generally, the GR populations accumulated lower levels of shikimate compared to the GS populations, which changed with time under the effect of glyphosate [13,37,38].

As often observed in studies on GR goosegrass, glyphosate resistance in this study was attributed to target site resistance [25,27,39,40]. *EPSPS* gene sequencing demonstrated that the LL and SS populations possessed a mutation at the Pro106 codon, resulting in amino

acid substitutions at this genomic EPSPS position. Mutations at the Pro106 position have also been found in other weeds, such as *Lolium rigidum*, *Lolium perenne* ssp. *multiflorum*, and *Amaranthus tuberculatus* [41–44]. Substitutions of proline at position 106 cause a slight structural change in the EPSPS active site, which has different effects on catalytic activity and glyphosate resistance [45,46]. Many studies have demonstrated that a single mutation at position 106 results in decreased glyphosate resistance levels (approximately 2-fold) than those obtained with double (more than 180-fold) or triple mutations (84-fold) because of changes in the EPSPS enzyme [25,39,40,47]. In our previous study, individuals with the P106L mutation showed 2.1–3.0-fold resistance relative to that of the sensitive biotype, and showed GR_{50} values of approximately 700 g ae ha^{-1} [23].

Gene amplifications or duplications of the *EPSPS* gene have been associated with the glyphosate resistance mechanism since the first report on *Amaranthus palmeri* in 2010 [10]. Others studies have reported the increase in *EPSPS* gene expression, and overproduction of the EPSPS protein might correspond positively to the level of glyphosate resistance in plants of other weed species (*Lolium perenne* ssp. *multiflorum*, *Amaranthus palmeri*, and *Kochia scoparia*), with *EPSPS* gene amplification ranging from 2- to 100-fold [48–51]. Gene amplification can produce extra EPSPS enzyme products that are inhibited by glyphosate, allowing increased synthesis of amino acid. In this study, the average relative expression of EPSPS in individuals was similar between the LL and SS populations, which were collected from the same area. However, the copy numbers and EPSPS protein content in the LL population were both higher than those in the SS population, and the GR_{50} of LL was 2.0 times than that of the SS biotype. The slight increase observed in *EPSPS* gene expression in the SS population did not affect its resistance level. This phenomenon also occurred in other GR goosegrass populations; the expression and copy numbers of *EPSPS* were reported to be 5.7 and 15.4 times higher, respectively, than those in the susceptible population; however, this resulted in a relatively low resistance level, with a resistance index of 3.4 [26]. In some species of *Lolium perenne* ssp. *multiflorum* and *Amaranthus palmeri*, > 10 and 30–50 *EPSPS* copies enabled the conferral of different levels of glyphosate resistance [10,45]. Furthermore, the number of *EPSPS* copies is not always positively correlated with expression and transcript abundance [37,52]. *EPSPS* overexpression is influenced by gene duplication as well as by efficient transcriptional regulation of the promoter or increased transcription factor activity [53]. Zhang et al. reported differences in EPSPS promoter activity among sensitive and resistant goosegrass, attributed to transcriptional regulation [54].

In recent years, some studies have revealed that resistance in GR weeds is conferred by more than one resistance mechanism. In this study, we found that EPSPS P106L mutation and overexpression coexisted within the same individual. Reduced glyphosate binding but enhanced PEP binding depends on the three-dimensional conformation of the enzyme, as well as the availability of excess EPSPS protein to combine with glyphosate to induce higher resistance levels. Target site mechanisms involving P106S mutation and overexpression were first reported in goosegrass [55]. Subsequently, we have found that the *EPSPS* amplification mechanism also existed in mutated (Pro106Ala) GR goosegrass [28]. A deeper understanding of resistance alleles and amplification mechanisms within these plant populations is thus critical for predicting weed occurrence and combating weeds efficiently.

5. Conclusions

In conclusion, two goosegrass populations (LL and SS) from banana plantations in Guangdong Province were confirmed to have evolved resistance to glyphosate in our study. A single nucleotide change at position 302 (C to T) and 301 (C to T) led to a P106L and P106S amino acid substitution in LL and SS populations, respectively. In addition, we found that *EPSPS* amplification and mutation (P106L) coexisted in the same individuals, resulting in overproduction of the mutated EPSPS protein, which conferred moderate glyphosate resistance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030699/s1, Figure S1: Immunoblot analysis of EPSPS protein in leaf tissue of plants from the three goosegrass populations. The primary anti-EPSPS antibody at a dilution of 1:5000. The molecular weight of EPSPS protein in goosegrass was 50 kD.; Table S1: The designed primers for *EPSPS* gene clone, qPCR, and the reference genes for *E. indica*.

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Conflicts of Interest: The authors declare no conflict of interest.

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