Evolution of multiple target-site resistance mechanisms in individual plants of glyphosate-resistant *Eleusine indica* from China

Runing title: Multiple TSR mechanisms in glyphosate-resistant goosegrass

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Author contribution statement

C. Z., Q. Y. and X. T. conceived, designed the experiments and wrote the paper. C. Z. and C. Y. performed the experiments and analyzed the data. W. G. and T. Z. contributed materials. The manuscript has been read, edited and approved by all authors.

Abstract

BACKGROUND: Glyphosate has been used for weed control in South China in various situations for four decades, and most *Eleusine indica* populations are suspected to have evolved resistance to glyphosate. This research investigated underling target-site glyphosate resistance mechanisms in six field-collected, putative glyphosate-resistant (R) *E. indica* populations.

RESULTS: The six R *E. indica* populations were confirmed to be low (1.8 to 2.6-fold) to moderately (5.6- to 8.4-fold) resistant to glyphosate relative to the susceptible (S) population. Sixty-seven glyphosate-surviving plants from the six R populations were used to examine target-site resistance mechanisms. Target-site 5-enolpyruvylshikimate3-phosphate synthase (EPSPS) overexpression (OE) (plus further induction by glyphosate treatment) and gene copy number variation (CNV) occurred in 94% R plants, and among them, 16% had the P106A mutation and 49% had the heterozygous double TIPS (T102I+P106S) mutation (plus P381L). In addition, a low number of R plants (6%) only had the homologous TIPS (plus P381L) mutation. The $(CT)_6$ insertion mutation in the *EPSPS* 5'-UTR always associates with *EPSPS* OE and CNV. Progeny plants possessing *EPSPS* OE/CNV (and P106A) displayed low level (up to 4.5-fold) glyphosate resistance. In contrast, plants homozygous for the TIPS mutation displayed higher (25-fold) resistance to glyphosate and followed by plants heterozygous for this mutation plus *EPSPS* OE/CNV (12-fold).

CONCLUSIONS: Target-site glyphosate resistance in *E. indica* populations from South China is common with prevalence of *EPSPS* OE/induction/CNV conferring low level resistance. Individual plants acquiring both the TIPS mutation and *EPSPS* OE/CNV are favored due to evolutionary advantages. The role of $(CT)_6$ insertion mutation in *EPSPS* CNV is worth further investigation.

Keywords: *Eleusine indica*; glyphosate resistance; *EPSPS* gene overexpression; copy number variation (CNV); *EPSPS* gene mutation; $(CT)_n$ insertion

1 INTRODUCTION

Eleusine indica is a highly invasive and adaptive global agriculture weed[1] with evolution of resistance to multiple herbicides. [2-5] *E. indica* is widely distributed in southern China especially in Guangdong Province, where it can flourish for up to 2-3 generations a year and is the worst weed in both farm and non-farm situations. Glyphosate is one of the main herbicides for *E. indica* control. However, since the first case of glyphosate resistance,[6] populations of this species from other provinces have been also reported to be resistant to glyphosate.[7]

Glyphosate is the most widely used herbicide in global agriculture.[8-9] Glyphosate inhibits 5-enolpyruvylshikimate3-phosphate synthase (EPSPS; EC 2.5.1.19), a key enzyme in the shikimate pathway. Like resistance to other major herbicides, field-evolved glyphosate resistance weed species are conferred by target or non-target-site mechanisms.[10-11] Target-site resistance include *EPSPS* mutations, overexpression (OE) and (or due to) copy number variation (CNV).[12-13] In *E. indica*, Baerson *et al*. [14] first identified and characterized

the single resistance mutation P106S, and demonstrated that the P381L mutation alone does not confer glyphosate resistance. Since then, other single *EPSPS* resistance mutations (P106L, P106T, P106S, P106A, T102I) were also reported in *E. indica.*[7, 15-18] In particular, the double mutation T102I + P106S (TIPS) (also containing the P381L mutation) conferring higher level glyphosate resistance than 106 single mutations was first identified and characterized also in *E. indica.*[19] Interestingly high resistance cost for the homozygous TIPS mutation was observed at the *EPSPS* level as reduced catalytic efficiency [19] and this is translated to severe fitness cost at the whole plant level.[20] Therefore, the authors predicted a good recipe for glyphosate target-site resistance would be evolution of both *EPSPS* OE/CNV and the TIPS mutation to mitigate fitness cost. *EPSPS* CNV alone or coexisting with P106A has been reported in glyphosate-resistant *E. indica* populations.[7, 16, 21] *EPSPS* OE in combination with P106S has also been described conferring glyphosate resistance in *E. indica*.[17] In addition, *EPSPS* OE caused by the insertion of a $(CT)_6$ tandem repeat in the 5'-untranslated region (UTR) of the *EPSPS* was identified in glyphosate-resistant *E. indica*.[22]

In South China, especially in Guangdong province, glyphosate has been used frequently on small-scale farmland by unprofessional application to control *E. indica*, and glyphosate control failure is observed in more and more *E. indica* populations. We hypothesise that the increase in glyphosate resistance in this species is related to evolution of multiple target-site mechanisms likely involving *EPSPS* OE/CNV and *EPSPS* single and double mutations. Therefore, this study aims to survey several *E. indica* populations suspect of glyphosate resistance, confirm resistance status, quantify resistance levels and investigate target-site resistance mechanisms, and also with a goal to identify populations for further study of target-site resistance genomics taking advantage of our inhouse *E. indica* genome data information.

2 MATERIALS AND METHODS

2.1 Plant material and glyphosate dose-response assay

Six putative glyphosate-resistant (R) *E. indica* populations were collected from different crop fields in Southern China (Table 1). A glyphosate-susceptible (S) population characterized in our previous study [6] was used as control. Seeds were sown on wet filter paper in petri dishes in a climate chamber at 28-30°C, with 12/12h light/dark period and 70% relative humidity. Seedlings at the two-leaf stage were transplanted into 28 x 54 cm trays (50 plants per tray) filled with potting soil and grown outdoors during the normal growing season. At the 5-6 leaf stage, seedlings were treated with commercial glyphosate (41% glyphosate isopropylamine salt; Roundup, Shanghai, China) at rate of 0, 270, 540, 1080, 2160, 4320, and 8640 g ha^{-1} for R populations; and 0, 135, 270, 540, 1080, 2160, and 4320 g ha^{-1} for the S population using an in-house sprayer (ASS-4 automatic spray tower bioassay, Beijing Research Center for Information Technology in Agriculture. Beijing, China). The fresh weight of above-ground material was measured 21 days after treatment. The herbicide rate causing 50% plant growth reduction (GR_{50}) was estimated using a four-parameter logistic curve model (Sigma Plot 12.0), and resistance index (RI) calculated based on the R/S GR_{50} ratio. Each treatment had three replicate trays and the GR_{50} values were calculated using pooled data from two independent experiments.

Sixty-seven plants that survived the 540-8640 g ha^{-1} glyphosate treatment from the six

populations, together with five S plants, were grown in greenhouses until the tillering stage. Three tillers from each plant were isolated and transplanted to 12-cm- diameter pots (one tiller per pot). Two tillers of each plant were used for molecular mechanism analysis, and the third one was grown individually for seeds to produce mechanism-based resistance groups. Four lines of each resistance mechanism group were randomly selected for glyphosate dose response experiments (using the same rates as above) to determine resistance levels. Each treatment had three replicate pots (10 seedlings per pot) and the GR_{50} values of each resistance mechanism group were averaged from the four examined lines. The resistance index of each glyphosate resistance mechanism group in *E. indica* was obtained based on the averaged data of four progeny lines.

2.2 Analysis of *EPSPS* gene overexpression (OE)

For analysis of *EPSPS* OE, one tiller of each S and R *E. indica* plant was treated with the recommended field rate of 1000 g ha^{-1} glyphosate, and another tiller without glyphosate treatment was used as control. Because *EPSPS* expression was significantly higher in leaf sheath than in leaves, [21] the leaf sheath material from each individual R tiller, and from bulked material of five S tillers, were used for RNA extraction according to manufacturer's instructions (TaKaRa, Co. 9769, Dalian, China). Reverse transcription of each RNA sample was performed using the Reverse Transcriptase M-MLV kit (TaKaRa, Co. 639522, Dalian, China). The primer pairs used for *EPSPS* amplification were: 5'- AAGGAGACCGAGAGGATGG-3' (forward) and 5'-CGGCAGGAGAGCAAAAGAG-3' (reverse). The primers for the reference gene *EF1α*[21] were: 5'-TGGTGGTTTTGAGGCTGGTA-3' (forward) and 5'-TCATCTGCTTCACTCCAAGAG-3 (reverse). *EPSPS* expression was determined by RT-qPCR using SYBR Master Mix (TaKaRa, Co. 639676, Dalian, China). PCR conditions were: 95°C for 10 min, followed by 40 cycles of 95°C for 5 s, 58°C for 30 s, and 72°C for 30 s for each cycle, according to manufacturer's instruction for SYBR Green kit (TaKaRa, RR820L, Dalian, China) using the Bio-Rad fluorescence quantitative PCR system (CFX96 Touch, Bio-Rad, Guangzhou, China). The *EPSPS* expression levels were expressed as $2^{-\Delta Ct}$ where ΔCt is the difference between the *EPSPS* and *EF1α* Ct values.

2.3 Analysis of *EPSPS* gene copy number variation (CNV)

Genomic DNA was extracted using the Plant Genomic DNA kit (Trans Gen Biotech Beijing Co., LTD), and qPCR preformed to measure the *EPSPS* CNV relative to the acetolactate synthase (*ALS*) gene that is confirmed to be a single copy from our unpublished inhouse *E. indica* genome information. The primer pairs were 5´-AAGGAGACCGAGAGGATGG-3´ (forward) and 5´-CGGCAGGAGAGCAAAAGAG-3´ (reverse). For each sample, a 100-ng genomic DNA sample was used as template and the PCR reaction conditions were as described above for *EPSPS* gene expression. The specificity of each PCR reaction was examined by the melt curve analysis, which showed only one PCR product for each of the *EPSPS* and *ALS* primer pairs. Relative *EPSPS* gene copy number was calculated based on the $2^{-\Delta Ct}$ where ΔCt is the difference between the *EPSPS* and *ALS* Ct values.

2.4 *EPSPS* gene sequencing

To identify the diversity of target-site glyphosate resistance mechanisms among the R *E.*

indica plants, full *EPSPS* coding sequence (1176 bp) were amplified and sequenced. The cDNA templates used were the same as for the *EPSPS* gene expression. The primer pairs used were: 5'-TTAGTTCTTGACGAAAGTGCTGA-3' (forward) and 5'-GTGACGTGAACGAACTGCAAC-3' (reverse). PCR was performed in a 25 µL reaction mixture according to the manufacturer's instructions (Tiangen Biotech Beijing CO., Ltd., Beijing, China). The amplified fragment length was confirmed by agarose gel (1%) electrophoresis and sequenced by Sanger sequencing technology (Sangon Biotech Shanghai Co., Ltd, China). Sequence analysis was performed using the DNAStar software (DNAStar, Inc., Madison, WI).

2.5 Genotype analysis of the *EPSPS* 5'-UTR $(CT)_6$ insertion mutation

A CT tandem repeat sequence (12bp) insertion in the *EPSPS* 5'-UTR of R *E. indica* plants possessing *EPSPS* OE has been identified in our previous study.[22] Genomic DNA PCR was carried out to identify the presence and absence of this insertion in glyphosate R plants of populations examined. The templates were the same as used for the CNV assay. The primer pairs used to amplify the *EPSPS* gene fragment (120 bp) containing the $(CT)_6$ insertions were: CT-F: 5'-GCGGCGCACGCCTCAGCTCA-3' (forward) and CT-R: 5'- GTCGAGGTTGGTTTGGCTGC-3' (reverse). The PCR conditions were: 95°C for 3 min, 32 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 60 s, and a final extension at 72°C for 10 min. The presence of a 119 bp band in polyacrylamide gel (0.8%) electrophoresis (by silver staining) indicates existence of the $(CT)_6$ insertion.

3 RESULTS

3.1 Glyphosate dose response of putative *E. indica* populations

The S population was controlled by glyphosate with a low GR_{50} value of 281 g ha^{-1}. The R populations exhibited GR_{50} values ranging from 507 to 2364 g glyphosate ha^{-1}, resulting in resistance index (RI) of 1.8 to 8.4 (Table 1). Results confirmed that all the field-collected populations tested have developed resistance to glyphosate. Then, 67 plants that well survived 540-8640 g ha^{-1} glyphosate treatment were selected from the six R populations to identify target-site resistance mechanisms.

3.2 *EPSPS* expression and copy number variation (CNV)

Expression of *EPSPS* was determined in 67 individual R samples and one bulked S sample. Most of the R plants (63 out of 67 plants, except plants 26, 41, 49 and 52) exhibited higher *EPSPS* gene expression (3.2 to 34 times) than the S (Fig. 1A). Expression of *EPSPS* in these 63 R plants was further up-regulated (8- to 127-fold) 24 h after glyphosate treatment. It seemed that the higher the basal level of *EPSPS* gene, the greater the induction level detected (Fig. 1B). Similarly, relative *EPSPS* gene copy numbers were higher (6 to 42 copies) in the 63 R plants than in the S and the four R plants (26, 41, 49 and 52) (Fig. 1C). Population R2 had the highest, and R4 the lowest, expression and CNV of the *EPSPS* gene (Fig. 1, 2). Generally, higher *EPSPS* gene expression levels were correlated with higher levels of CNV (R^2=0.94, P=0.001) (Table 2, Fig. 2), although population R5 showed variations in *EPSPS* expression, induction and CNV due to limited numbers of plants analyzed (Table 2, Fig 2). This result indicates that *EPSPS* overexpression (OE, under no glyphosate treatment) was likely the consequence of *EPSPS* CNV. However, the magnitude of increase in *EPSPS* OE was

lower than that in *EPSPS* CNV (Table 2).

3.3 Genotype analysis of *EPSPS* 5'-UTR $(CT)_6$ insertion mutation

The $(CT)_6$ insertion mutation occurred in the 63 R plants with *EPSPS* OE and CNV, but not in the four R plants (26, 41, 49 and 52) (Fig. 3) and in the S plant. Interestingly, all the 63 R plants were heterozygous (+/-) for this insertion mutation. This result is in line with our previous study showing the $(CT)_6$ insertion is largely associated with *EPSPS* OE.[22]

3.4 *EPSPS* gene sequencing

Sixteen percent of R plants had the homologous P106A mutation and 46% had the heterozygous TIPS double mutation (Table 3). It was noted that an additional mutation P381L was linked to the TIPS mutation (Table 3), and this was also reported in previously studies [14, 18-19], although it has been demonstrated that the P381L alone does not confer glyphosate resistance [14]. Only the four R plants (26, 41, 49 and 52) that showed no *EPSPS* OE and CNV had the homologous TIPS mutation, accounting for 6% of the total. Based on the above data, glyphosate R *E. indica* populations from South China have evolved at least four target-site resistance mechanisms: Type I) *EPSPS* OE/CNV; Type II) *EPSPS* OE/CNV plus P106A; Type III) *EPSPS* OE/CNV plus TIPS (heterozygote) and Type IV) TIPS (homozygote) (Fig. 4).

3.5 Glyphosate resistance levels conferred by different resistance mechanisms

Glyphosate resistance levels were determined using four progeny lines of each resistance mechanism group. Based on R/S GR_{50} values, type IV R lines (TIPS homozygote) had a higher level of resistance followed by type III (OE/CNV plus TIPS heterozygote), and type I (OE/CNV) and II lines (OE/CNV plus P106A) displayed a lower level of resistance, relative to the S line. The P106A mutation in type II lines did not provide additional resistance to type I, likely due to a lower level of *EPSPS* OE/CNV of involving plants (No. 1 to 10) (Table 4, Fig. 1). Segregation of the S individuals in the progeny due to heterozygosity of the TIPS mutation is likely responsible for a lower level resistance (12-fold) in type III than type IV (25-fold).

The field-collected populations R1 and R2, which were dominated by type I and II mechanisms, had relatively lower glyphosate resistance indices (1.8 and 2.6, respectively) (Table 1). However, populations R3, R4, R5 and R6, which were dominated by type III and type IV mechanisms (Table 3), exhibited a relatively higher level of resistance to glyphosate (Table 1). In these glyphosate R *E. indica* populations although the frequency of *EPSPS* OE/CNV was high, it was only associated with low-level glyphosate resistance, and higher-level resistance was observed with plants homologous for the TIPS mutation alone or plants heterozygous for TIPS plus *EPSPS* OE/CNV.

4 DISCUSSION

EPSPS OE/induction/CNV is a basal target-site glyphosate resistance mechanism in E. indica

Gene CNV can be a primary step in resistance evolution in bacteria and then followed by point mutations in the amplified gene.[23] This may hold true for plants. For example, a recently study showed that *EPSPS* CNV increased only after one generation of glyphosate selection. [24] Indeed, convergent evolution of *EPSPS* CNV in response to glyphosate selection has been reported in populations of at least eight glyphosate R weed species. [13, 25-26]

Likewise, *EPSPS* OE (plus further up-regulation by glyphosate treatment) and/or due to CNV was also found to be common in glyphosate R *E. indica* individuals/populations (Fig. 1, 2, Table 2). Generally, the resistance level conferred by *EPSPS* CNV (ranging from 2 to more than 100 relative copies) is low to moderate. Similarly, a 4-fold resistance was observed in one R *E. Indica* population involving only *EPSPS* OE/CNV (Table 4). Usually, *EPSPS* OE and CNV is positively correlated. [27-28] However, in some studies, either the correlation was not examined, or no correlation was found [26] or it was negative.[29] This might be due to (1) regulation of duplicated gene copies, and (2) challenges in CNV quantification especially in polyploid species due to the lack of single copy gene as a reference. In this study, fold-change in *EPSPS* OE was found to be generally lower than CNV (Table 2). Genome re-sequencing versus RNA-sequencing can be used to confirm qPCR results for weed species with available genome information. Currently, genetic and genomic mechanisms of *EPSPS* CNV have only been studied in two weed species: tandem duplication in *K. scopara* and dispersed duplication via a large extrachromosomal circular DNA (eccDNA) in *Amaranthus palmeri*.[30] Genomic mechanisms enabling *EPSPS* CNV in *E. indica* remain to be revealed.

The $(CT)_6$ insertion mutation associated with *EPSPS* OE/induction is consistent with our previous [22] and current studies (Fig. 1B, 4). The 5'-UTR Py-rich stretch element is a typical transcriptional regulatory element, which contains GA/CT repeat sequences. [31] Promoters with the $(CT)_n$ showed higher transcriptional efficiency than those without the repeats, [32] and the length of $(CT)_n$ was positively correlated with gene expression. [33] The $(CT)_6$ insertion in the *EPSPS* 5'-UTR resulting in significant enhancement of promoter activity was also demonstrated in our previous study. [22] Therefore, the $(CT)_6$ insertion was likely involved in *EPSPS* gene expression. In the current study, the $(CT)_6$ insertion was also found to be associated with *EPSPS* OE/induction/CNV (Fig. 1, 3). At present it is unclear if the $(CT)_6$ insertion occurred in all or part of the duplicated and expressed *EPSPS* copies, although genotype analysis showed 63 plants were heterozygous for this insertion (Fig. 3). Genome resequencing and RNA-Seq may help determine whether the $(CT)_6$ insertion is involved in regulating expression of duplicated *EPSPS* copies.

Evolution of EPSPS OE/induction/CNV plus EPSPS mutations in individual E. indica plants

One of the consequences of gene CNV is to increase the likelihood of mutation in the amplified copies. In plants, co-existence of *EPSPS* OE/CNV and *EPSPS* single 106 mutations has been reported in *E. indica* [16, 17] and *Poa* annua.[34] In addition, both *EPSPS* CNV and the triple mutation (TAP-IVS) were found in *A. hybridus.*[35-36] In this study, co-existence of *EPSPS* OE/induction/CNV and the P106A mutation in individual *E. indica* plants was identified (Fig. 4, Table 3). Most importantly, individual plants possessing both the *EPSPS* OE/CNV and the heterozygous double TIPS mutation were detected. Indeed, plants heterologous for the TIPS mutation have been found to have minimum fitness cost and higher fitness benefit (growth and fecundity) respectively in the absence and presence of glyphosate selection,[20,37] compared to plants homozygous for the same mutation. Likewise, in the current study, plants heterozygous for the TIPS mutation plus *EPSPS* OE/CNV (type III) showed sufficiently high level of resistance to glyphosate (Table 4) and occurred at a higher frequency (49%) while displaying better growth than plants homozygous for the TIPS mutation (type IV) (Fig. 5). Together with the lack of consistent evidence for universal high fitness cost for *EPSPS* CNV,

[25, 38] type III resistance plants will be likely dominant both in the presence and absence of glyphosate selection. Fitness cost of *EPSPS* OE/CNV (Type I) in *E. indica* is yet to be determined, although no effect on plant growth was observed (Fig. 5). In addition, antibiotic resistance studies reveal that bacterial gene CNV is usually unstable and can be rapidly lost if selection pressure is not maintained. [23] It is worth to examine if this is true in plants by monitoring *EPSPS* CNV frequency over several generations in the absence of glyphosate selection, and to relate results with genomic mechanisms of *EPSPS* CNV (e.g., tandem vs dispersed). Nevertheless, in the presence of glyphosate selection, *EPSPS* CNV is increased.[24, 39]

In this study, population R1 and R2 were collected from taro and banana cropping fields, respectively. Because banana and taro are susceptible to glyphosate, glyphosate is usually used at the low end of the recommended rate range and no more than two applications once a year pre crop planting. Consequently, type I and II resistance mechanisms involving *EPSPS* OE/CNV and P106A and conferring lower-level resistance were favored. However, populations R4, R5 and R6 were collected from maize fields and orchards, where glyphosate was applied frequently (7-8 times a year) and often at high doses. As a result, glyphosate resistance mechanisms involving *EPSPS* OE/CNV and the TIPS mutation and endowing higher level resistance were selected. In particular, plants homozygous for the TIPS mutation were identified at a low frequency (6%) and exhibited relatively high-level glyphosate resistance (Table 4) but had the poorest growth in the absence of glyphosate (Fig. 5). This is in line with previous studies.[19, 20] As *E. indica* is a self-pollinated species, accumulation of multiple resistance mechanisms can be due to sequential evolution of each mechanism or can be enabled by a low rate of crossing. Non-target-site resistance mechanisms involving ABC transporter [40] and AKR [41] was not examined in this study, and hence the possibility of their contribution to glyphosate resistance cannot be excluded in the studied populations.

In summary, this study showed the adaptability of *E. indica*, a C_4 species, to abiotic stress (herbicide) by evolving multiple target-site resistance mechanisms, especially the predicted *EPSPS* OE/CNV plus the TIPS mutation. Apart from glyphosate resistance, resistance to paraquat, [42] glufosinate and *ALS*- and *ACCase*-inhibiting herbicides is also evolving in China. Therefore, non-chemical weed control tactics need to be incorporated to diversify control methods and to delay or mitigate resistance evolution. Currently, mechanical, hot flame weeding and weed control film are in trials and their weed control efficacies are being assessed and tailored to suit small scale farmland and diverse plantations in South China.

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CONFLICT OF INTEREST DECLARATION

The authors declare that they have no conflict of interest.

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Table 1. Sampling information and glyphosate resistance levels of field-collected, glyphosate-resistant (R) versus the susceptible (S) *Eleusine indica* populations

Population	Location	GR_{50} (g ha^{-1})	Resistance index
S	Fallow field, Panyu, Guangzhou (22°8'N, 113°44' E)	281±6	1.0
R1	Taro field, Dongyuan, Heyuan (24°15'N, 114°4' E)	507±8	1.8
R2	Banana orchard, Wanqingsha, Guangzhou (23°48'N, 114°46' E)	732±24	2.6
R3	Carambola orchard, Yangcun, Huizhou (23°1'N, 114°91' E)	1576±22	5.6
R4	Litchi orchard, Boluo, Huizhou (23°38'N, 114°5' E)	1688±13	6.0
R5	Corn field, Baiyun, Guangzhou (23°39'N, 113°42' E)	2364±22	8.4
R6	Guava orchard, Yangcun, Huizhou (22°95'N, 114°65' E)	2307±22	8.2

Table 2. Relative *EPSPS* gene expression, induction and copy number variation (CNV) in glyphosate-resistant (R) *E. indica* populations

Population	*EPSPS* expression	*EPSPS* induction	*EPSPS* CNV
S	1	1	1
R4	3.2 $\pm$ 0.6	8 $\pm$ 1.9	6 $\pm$ 1
R6	3.5 $\pm$ 0.7	6.8 $\pm$ 1.2	9 $\pm$ 1.7
R1	4.9 $\pm$ 1	17 $\pm$ 3.9	8 $\pm$ 1.6
R3	12 $\pm$ 1.8	34 $\pm$ 7.8	12 $\pm$ 1.4
R5	14 $\pm$ 5	51 $\pm$ 22.8	24 $\pm$ 8.9
R2	34 $\pm$ 2.1	127 $\pm$ 7.9	42 $\pm$ 3.3

Data are means ± SE of all plant samples that showed *EPSPS* over expression (OE)/CNV/induction in each population. *EPSPS* gene expression, induction and CNV values were set to 1 for the glyphosate-susceptible (S) plant.

Table 3. Analysis of resistance mutations at 102, 106 and 381 sites of the *EPSPS* gene for glyphosate-resistant (R) versus susceptible (S) *E. indica* populations

Population	Plants involved	Amino acid		
		102	106	381
S	1	Thr	Pro	Pro
R1	1-10	Thr	Pro/Ala	Pro
R2	11-25	Thr	Pro	Pro
R3	27,29,32,35	Thr	Pro	Pro
	28, 30-31,33-34,36-37	Thr/Ile	Pro/Ser	Pro/Leu
	26	Ile	Ser	Leu
R4	42	Thr	Pro	Pro
	38-40,43-47	Thr/Ile	Pro/Ser	Pro/Leu
	41	Ile	Ser	Leu
R5	48,51	Thr	Pro	Pro
	50	Thr/Ile	Pro/Ser	Pro/Leu
	49, 52	Ile	Ser	Leu
R6	53-67	Thr/Ile	Pro/Ser	Pro/Leu

Table 4. Glyphosate resistance levels of *E. indica* plants possessing different target-site-resistance (TSR) mechanisms, relative to the susceptible (S) population

Type of TSR	Resistance mechanism	Plants involved	GR_{50} (g ha^{-1})	Resistance index
S	/	/	119 ± 18	1
Ⅰ	*EPSPS* OE/CNV	11-25,27,29,32,35,42, 48,51	484 ± 71	4
Ⅱ	*EPSPS* OE/CNV plus P106A	1-10	535 ± 19	4.5
Ⅲ	*EPSPS* OE/CNV plus TIPS (heterozygotes)	28,30-31,33-34,36-37, 38-40,43-47,50,53-67	1464 ± 398	12
Ⅳ	TIPS (homozygotes)	26, 41, 49, 52	2921 ± 665	25

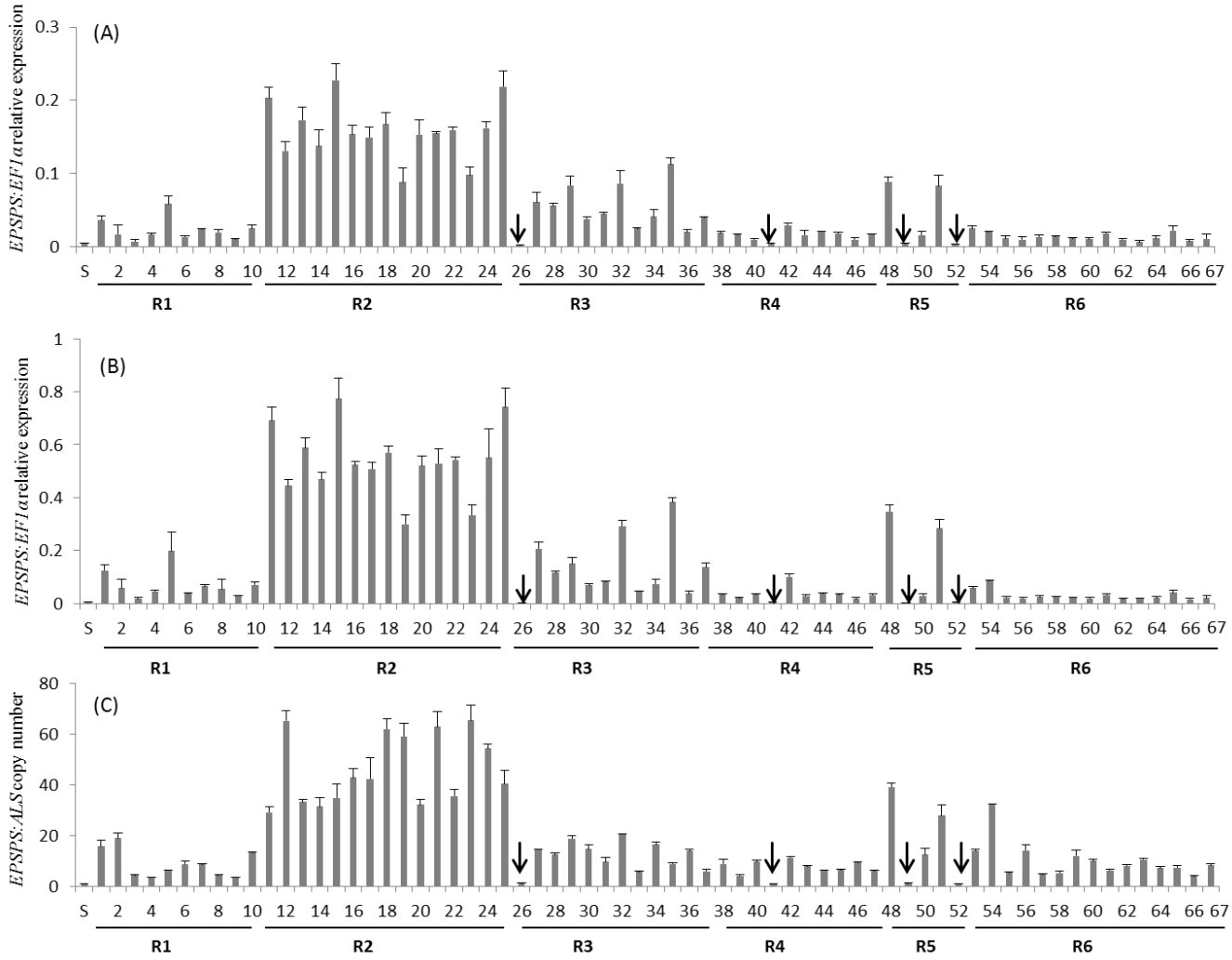

Figure 1. Analysis of *EPSPS* expression, induction and copy number variations (CNV) in bulked glyphosate susceptible (S) and 67 resistant (R) *E. indica* individuals from six field-collected populations (R1 to R6) by RT-qPCR. *EPSPS* expression (A) and induction 24 h after glyphosate treatment (B) was normalized with the internal control *EF1a*, and *EPSPS* CNV (C) was determined relative to *ALS*. Arrows indicate plants without *EPSPS* overexpression/CNV/induction. Data are means ± SE of three technical replicates for each sample.

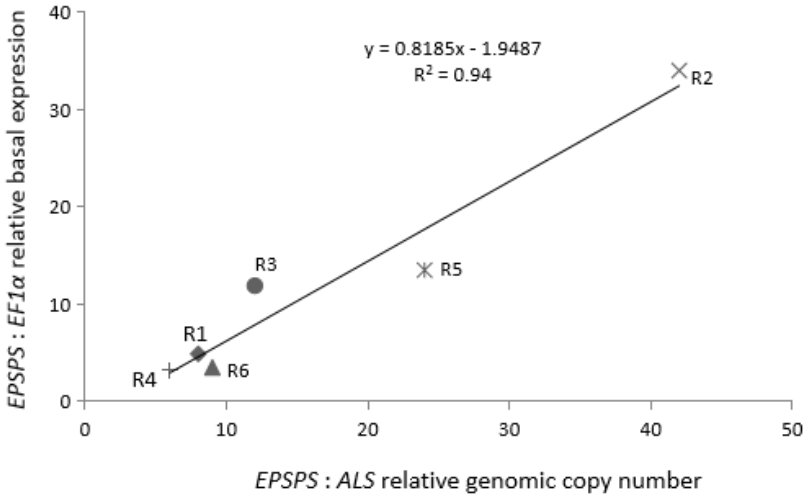

Figure 2. Correlation of fold-change in relative gene expression (R^2=0.94, *p*=0.001) and copy number variation (CNV) of *EPSPS* in the six glyphosate-resistant (R) populations (based on Table 2).

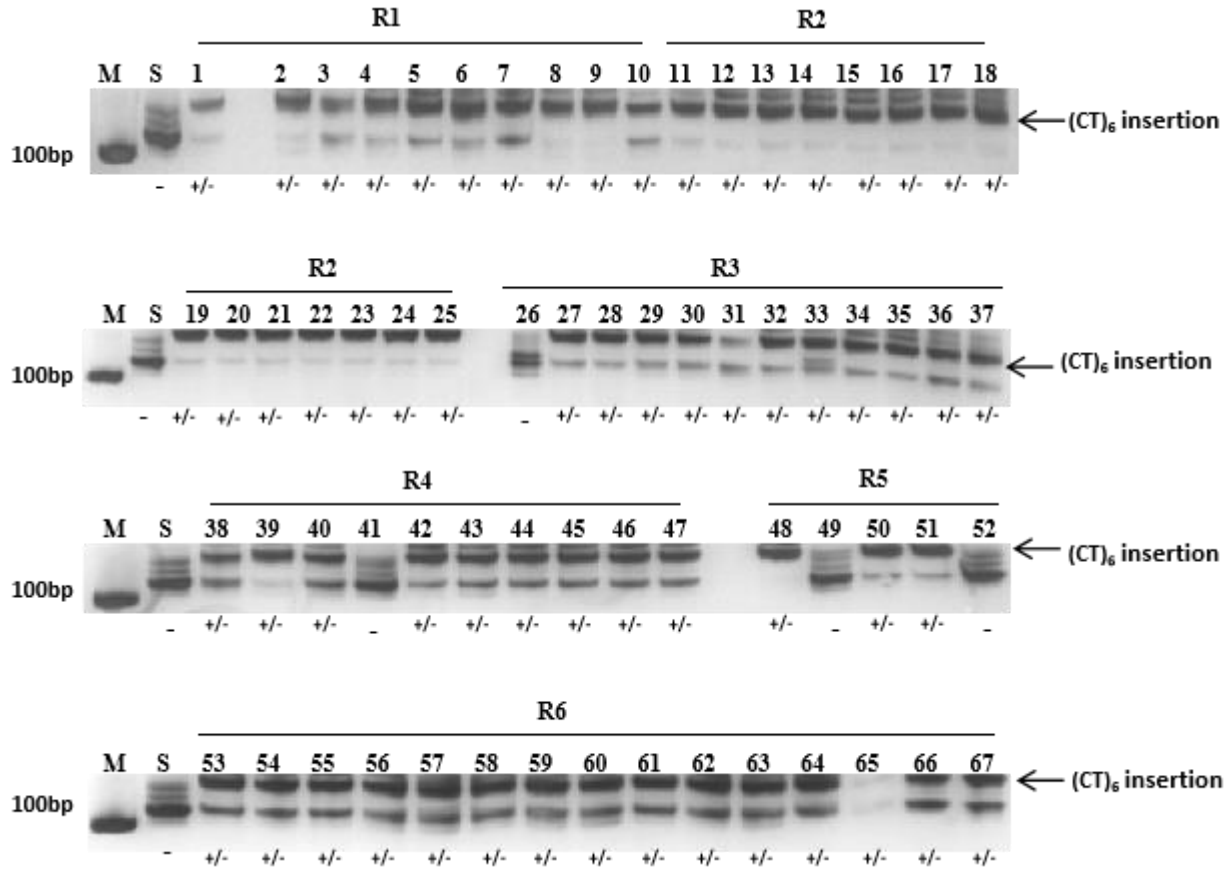

Figure 3. Analysis of the 5' untranslated region (UTR) Py-rich stretch in the *EPSPS* gene in bulked susceptible (S) and individual resistance (R) *E. indica* plants by polyacrylamide gel electrophoresis and silver staining using DNA samples. The presence (+) and absence (-) of the $(CT)_6$ insertion mutation (as arrowed) in the 5' UTR element was indicated. M: molecular size marker; S: the susceptible plant.

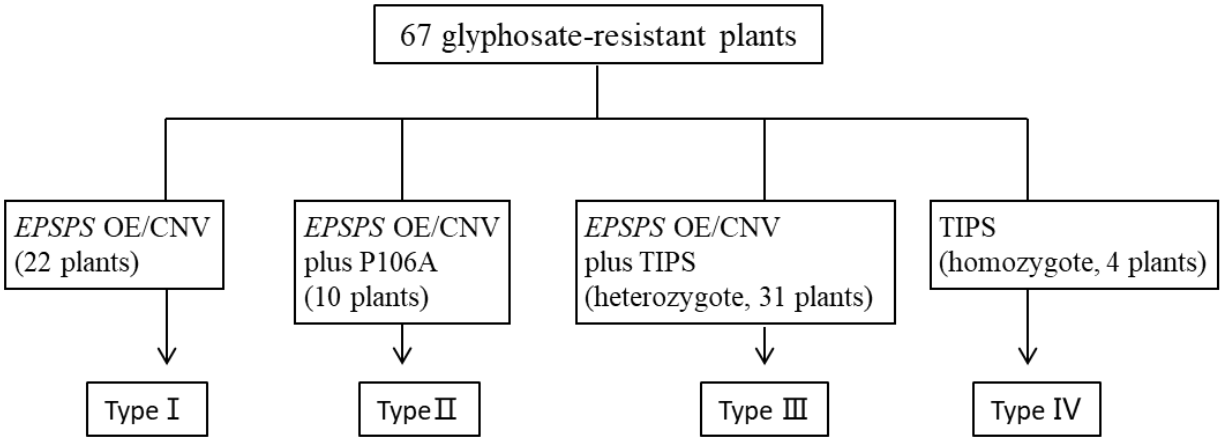

Figure 4. Four types of target-site resistance mechanisms in the 67 glyphosate-resistant *E. indica* plants. OE: *EPSPS* gene overexpression; CNV: copy number variation; TIPS: EPSPS T102I+P106S double mutation.

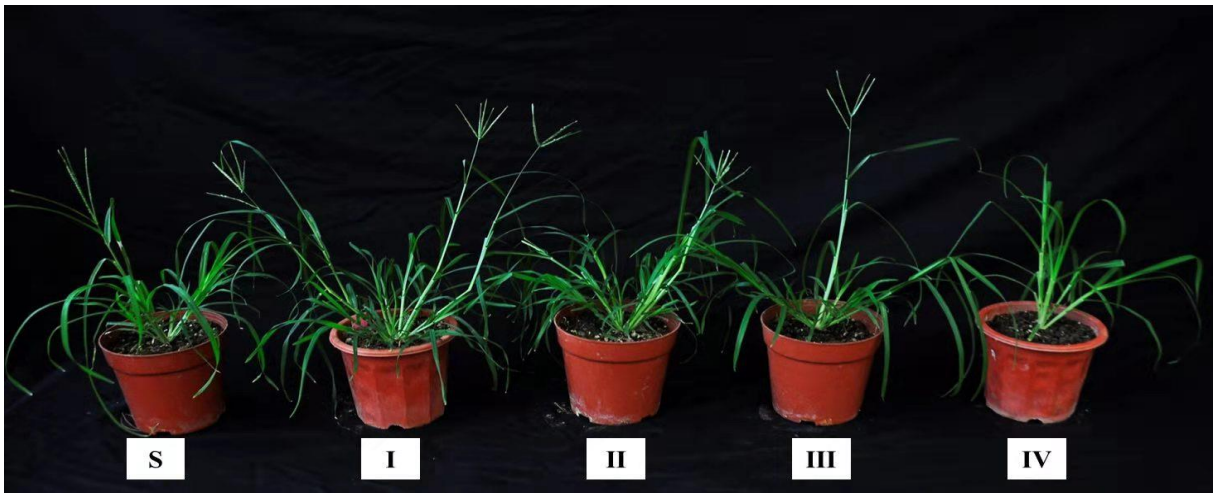

Figure 5. Growth of the four types of (I-IV) glyphosate-resistant (R) versus the susceptible (S) *E. indica* plants. Photo of representative plants was taken two months after transplanting.