Research Article

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A novel triple amino acid substitution in the EPSPS found in a high-level glyphosate-resistant *Amaranthus hybridus* population from Argentina

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Abstract

BACKGROUND: The evolution of herbicide-resistant weeds is one of the most important concerns of global agriculture. *Amaranthus hybridus* L. is a competitive weed for summer crops in South America. In this article, we intend to unravel the molecular mechanisms by which an *A. hybridus* population from Argentina has become resistant to extraordinarily high levels of glyphosate.

RESULTS: The glyphosate-resistant population (A) exhibited particularly high parameters of resistance (GR_{50} = 20 900 g ai ha^{-1}, Rf = 314), with all plants completing a normal life cycle even after 32X dose application. No shikimic acid accumulation was detected in the resistant plants at any of the glyphosate concentrations tested. Molecular and genetic analyses revealed a novel triple substitution (TAP-IVS: T102I, A103V, and P106S) in the 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) enzyme of population A and an incipient increase on the *epsps* relative copy number but without effects on the *epsps* transcription levels. The novel mechanism was prevalent, with 48% and 52% of the individuals being homozygous and heterozygous for the triple substitution, respectively. *In silico* conformational studies revealed that TAP-IVS triple substitution would generate an EPSPS with a functional active site but with an increased restriction to glyphosate binding.

CONCLUSION: The prevalence of the TAP-IVS triple substitution as the sole mechanism detected in the highly glyphosate resistant population suggests the evolution of a new glyphosate resistance mechanism arising in *A. hybridus*. This is the first report of a naturally occurring EPSPS triple substitution and the first glyphosate target-site resistance mechanism described in *A. hybridus*.

Supporting information may be found in the online version of this article.

Keywords: *Amaranthus quitensis*; glyphosate-resistance; EPSPS triple substitution; TIPS mutation, high resolution melting analysis; single-seed DNA extraction

1 INTRODUCTION

The high selection pressure exerted by the dominance of chemical control strategies during recent decades has led to a severe problem regarding the evolution of herbicide-resistant weeds in crop fields. Currently, 255 different species have evolved resistance to at least one herbicide across the world, evidencing a growing evolution rate in recent years.[1] In particular, the glyphosate resistance (GR) evolution is one of the most worrying challenges for modern agriculture. As many as 42 species were reported to exhibit resistance to this single herbicide in the last two decades.[1] Consequently, the study of the mechanisms endowing GR has grown significantly, as described in review articles.[2,3]

Briefly, a target-site resistance (TSR) mechanism associated with a mutation causing a P106S substitution in the target enzyme of glyphosate (5-enol-pyruvylshikimate-3-phosphate synthase,

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EPSPS) was first elucidated in *Eleusine indica*[4] and then reported in many other species, including new substitutions (P106T, P106L or P106A).[5–10] Moreover, a new EPSPS T102S substitution involved in GR was recently discovered.[11] These single *epsps* gene mutations generally confer low levels of resistance while preserving EPSPS catalytic efficiency. Curiously, the artificial double amino acid substitution in the EPSPS (called TIPS), which is known for conferring high GR levels in commercial transgenic maize lines,[12] has been found in two weed species (*Eleusine indica* and *Bidens pilosa*),[13,14] evidencing the power of natural evolution on a persistent selection pressure.

Another TSR mechanism, which involves the overexpression of *epsps* produced by gene amplification, was discovered in *Amaranthus palmeri*.[15] This mechanism endows high levels of GR and seems to be the predominant TSR evolved in several resistant weeds.[16–20]

The growing interest in the molecular characterization of non-target-site resistance (NTSR) allowed the identification of some mechanisms associated with active sequestration in the vacuole and limited translocation of glyphosate.[21–23] Moreover, a novel NSTR mechanism related to a rapid necrosis of the glyphosate-exposed tissue, known as the pheonix phenomenon, has recently been described.[24,25]

Finally, the accumulation of multiple mechanisms, mainly in cross-pollinated species, produces higher resistance levels and is a dangerous consequence of continuous glyphosate selection pressure.[9,26]

Amaranthus hybridus L. is a broadleaf weed native to South America and is currently most problematic in Argentina.[27] The first worldwide herbicide resistance case in *A. hybridus* was reported in 1972 for the herbicide atrazine.[1] Currently, this species has become resistant to herbicides with six different sites of action (SOAs), and numerous populations presenting multiple resistance have been characterized.[1] Although the mechanisms of GR have been described in some species of the gender, up to now the mechanism by which *A. hybridus* has evolved GR is still unknown. In this work, we intend to unravel the molecular mechanisms by which an *A. hybridus* population from Argentina has become resistant to extraordinarily high levels of glyphosate.

2 MATERIALS AND METHODS

2.1 Plant material and growth conditions

A. hybridus seeds were collected in 2015, sampling at least 100 plants from two neared farms (Córdoba province): A (33° 33′ S and 62° 53′ W) and B (33° 39′ S and 63°38′ W). These samples were considered resistant and susceptible, respectively, since plants from farm A had survived the application of the recommended dose (1X rate) of glyphosate (540 g ai ha^{-1}, Roundup Full II®) during several consecutive field seasons (at least 10 years), while the population from farm B was susceptible to this herbicide. Progeny from each population (seeds from the field population sampling) were grown in a growing chamber at 25 °C and 8/16 photoperiod and GR was confirmed by 1X rate applications and survival assessment 21 days after treatment. Eight of these surviving plants (selected by their vigorousness after glyphosate application) were grown up to the reproductive phase. All the assays (except from dose–response and genotypic frequency screening) were carried out using the eight selected plants, which were called GR_1 plants, and those from the original sampling of B as a control, which were referred as GS plants.

2.2 *In vivo* dose–response assays

Seeds from the A and B population samplings were grown and treated as described in Supporting information Appendix S1. When the plants reached 20 cm in height, ten doses (0.0625X, 0.125X, 0.250X, 0.5X, 1X, 2X, 4X, 8X, 16X and 32X, with X = 540 g ai ha^{-1}) of glyphosate were differentially sprayed to obtain a good curve fit for each population. Dose–response curves were analyzed according to Larran *et al.*[28]

2.3 *In vivo* shikimate accumulation assay

Ten leaf discs of 4 mm in diameter were collected separately from each plant (eight GR_1 and eight GS) for each test condition and were placed in 20-mL vials containing 1 mL of the assay solution, according to the protocol described by Shaner *et al.*[29] Control vials contained 10 mM ammonium phosphate (pH 4.4) plus 0.1% (v/v) Tween 80. Glyphosate treatment vials contained 10 mM ammonium phosphate (pH 4.4), 0.1% (v/v) Tween 80, plus glyphosate at 200, 400 or 800 μM. Vials were capped, transferred to a growth chamber and incubated under light (150 mM m^{-2} s^{-1}) for 16 h. After incubation, 250 μL of 1.25 N HCl were added into each vial and they were placed at −20 °C until assayed for shikimic acid. Shikimate levels were measured according to Cromartie and Polge.[30] Absorbance was read at 380 nm in a spectrophotometer (PerkinElmer Lambda Bio+, Waltham, Massachusetts, U.S.) and values were converted to nanograms of shikimic acid per gram of fresh weight by a shikimate standard (Sigma-Aldrich, St. Louis, MO, USA) curve. Each treatment was replicated at least three times.

2.4 *epsps* partial sequencing

Genomic DNA was obtained from fresh leaf tissue of plants (eight GR_1 and eight GS) using a Wizard Genomic DNA Purification Kit® (Promega, Madison, Wisconsin, USA). Egf (5′-ATGTTGGACGCTCTCAGAACTCTTGGT-3′) and Egr (5′-TGAATTTCCTCCAGCAACGGCAA-3′) primers were used to amplify 195 bp, according to the experimental design performed on *Amaranthus palmeri* by Gaines *et al.*[15] PCRs were performed with Q5® High-Fidelity DNA polymerase (New England Biolabs, Inc., Ipswich, MA, USA). Reactions were prepared as follows: ∼100 ng DNA, 200 µM dNTPs, 1.5 mM $MgCl_2$, 500 nM primers, one polymerase unit, 1X buffer, and distilled H_2O to 50 μL. The amplification program was 95 °C for 1 min, 30 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, 72 °C for 7 min, and a 12 °C hold. Amplification products were sequenced through the Macrogen service (Marcogen Inc., Seoul, South Korea). Sequencing reads were aligned with a reference *epsps* sequence from *A. palmeri* (FJ861243.1). The alignment was performed in Unipro Ugene v1.11.2 software using the MUSCLE algorithm. Nucleotide sequences were translated and subsequently aligned to search for amino acid substitutions.

2.5 dCAPS for detecting mutations at P106

Based on the *epsps* sequence data obtained from GR_1 and GS plants of *A. hybridus*, we developed a derived cleaved amplified polymorphic sequence (dCAPS) test to detect mutations at P106. DNA from GR_1 plants was used to determine the zygosis of the P106 mutation in each single *A. hybridus* plant. PCR and digestion were performed as described previously,[31] with modifications in the reverse primer in order to make it specific for local *Amaranthus* spp. populations (EPSdcapsR1: 5′-TCCAGCAACGGCAACCGCAGCTGTCCATG-3′). The restriction enzyme used was NcoI (Promega), which specifically recognizes

and cuts the sequence C^CATGG, which is only present in the wild-type (wt) sequence. DNA samples were subjected to an electrophoresis on 2% agarose gel stained with SYBR Safe® (Invitrogen, Carlsbad, CA, USA). *A. palmeri* control DNAs were included to validate the fidelity of the test. The wt sequence produces a single 156-bp digested band, whereas a mutation causes the loss of the restriction site and the visualization of a single 181-bp undigested band. Therefore, plants presenting only the undigested 181-pb band or both bands can be classified as homozygous or heterozygous for the Pro106 mutation, respectively.

2.6 RNA extraction and cDNA synthesis

Young leaves of GR_1 and GS plants were used to carry out the RNA extraction using the Spectrum Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer's instructions. An additional step consisting of a 20-min DNase treatment (1 unit/reaction) was performed at 25 °C. The resulting RNA was dissolved in sterile HPLC-grade water, quantified by spectrophotometry, and assessed for quality and integrity through gel electrophoresis. cDNA was synthesized by incubating 25-µl reaction mixtures at 42 °C for 1 h. Reactions contained 200 pmol of oligo(dT) primer, 0.25 µg of mRNA, and 200 U of M-MLV reverse transcriptase (Promega).

2.7 Quantitative PCR

Quantitative real-time PCR (qPCR) was used to estimate the *epsps* genomic relative copy number and the *epsps* relative cDNA expression. In both cases, the *als* (acetolactate synthase) gene was used as reference, prior to validation of its fidelity using the actin gene as a second reference. The primer sets Egf × Egr mentioned in 2.4, giving a 195-bp product, and Af (5′-GCTGCTGAAGGCTACGCT-3′) × Ar (5′- GCGGGACTGAGTCAAGAAGTG-3′), giving a 118-bp product, were used for qPCR on both genomic DNA and cDNA.

qPCR reactions were prepared as follows: 200 nM of gene-specific primers, 1X Realmix qPCR (Biodynamics, Buenos Aires, Argentina), 50 ng of genomic DNA or 0.5 µL of cDNA, distilled H_2O to 13 µL. Eight samples from GR_1 and GS plants were processed in triplicate. Amplifications were carried out in a Rotor-Gene Q thermocycler (Qiagen) as follows: 2 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C. Melting curves (86 cycles of 10 s from 72 to 95 °C, with a 0.5 °C temperature increase after cycle 2) were performed at the end of the cycling to control primer amplification specificity. Amplification efficiency was independently calculated in each reaction and was considered in data processing. Comparative Ct quantifications were made with REST software (REST V 2.0.7 for Rotor Gene, Corbett Life Sciences)[32] using the *als* gene as reference, given its low-copy number and monogenic inheritance in other *Amaranthus* species.[33] Results were expressed as fold increase in *epsps* copy number in GR_1 plants relative to the *epsps* copy number in GS plants. A similar relative quantification was made for the fold increase in *epsps* expression.

2.8 Single-seed DNA extraction

DNA from single seeds of *A. hybridus* population A was extracted as described in Supporting information Appendix S1.

2.9 High-resolution melting analysis

Molecular screening of TAP-IVS triple substitution (T102I + A103V + P106S) in population A was carried out using a high-resolution melting analysis (HRMA) approach.[34] Primers and the qPCR program were identical to those used in the *epsps* copy number study, using 1 µL of single-seed DNA as template. Since TAP-IVS multiple substitution derives from three C→T transitions, it causes a ~1.2 °C decrease in the melting temperature of the *epsps* amplified fragment with respect to the wt amplified sequence. This shift of the unique peak in the melting curve from ~84 to ~82.8 °C was observed during the qPCR experiments using GS and GR_1 plants, respectively, and confirmed through sequencing. To check the ability of the HRMA assay to detect heterozygous variants, artificial heterozygous samples were created by mixing genomic DNA from GS and GR_1 samples (mixing ratio 1:1) (Fig. 5(A)).

Melting curves were generated for 130 seeds from the original sampling of population A. The different patterns in the melting curves (I, II and III in Fig. 5(A)) were correlated to the absence/presence of the triple EPSPS substitution in homozygosis or heterozygosis. A subset of representative samples (including a few ambiguous results that were slightly different from the profiles represented in I, II, and III) were validated through dCAPS (wt allele presence/absence) and sequencing.

2.10 Structural TAP-IVS mutant analysis

Structural molecular modelling was performed with a SWISS-PROT server to model the wt, double mutant (TIPS), and triple mutant (TAP-IVS) EPSPS from *A. hybridus*. EPSPS *E. coli* structure (1G6S PDB) was used as template for wt sequence modelling, and EPSPS TIPS *E. coli* structure (3FK1 PDB) was used as template for the double and triple mutants. For all models a global RMSD lower than 0.2 and global secondary structure conformations similar to their templates were guaranteed. To analyze local differences in the glyphosate binding site, the distance between this ligand and a previously reported key residue (G96 in *E. coli*, equivalent to G101 in plants)[35] was determined with the Pymol Molecular Graphics System. Finally, residues comprising the binding site were predicted using the LIDIA application of COOT Program.[36]

2.11 Statistical analysis

Data from all the experiments were tested through one-way analysis of variance (ANOVA). The Holm–Sidak test ($\alpha = 0.05$) was used to calculate minimum significant differences using the Sigma Stat Package.

3 RESULTS AND DISCUSSION

3.1 Population A presents an unusually high GR

This experiment confirmed the resistance of population A to glyphosate. Population B displayed a large biomass reduction at low glyphosate doses in comparison to the glyphosate-resistant population (Fig. 1; Supporting information Figure S1). The GR_{50} value for the susceptible population was 66.6 g ai ha^{-1}, whereas the resistant population exhibited GR_{50} = 20 900 g ai ha^{-1}, with an Rf value of 314 (Table 1). Population A showed a GR_{50} value remarkably higher (19.6-fold) than the glyphosate field rate recommended (540 g ia ha^{-1}). Furthermore, the highest dose tested (32X) was not effective in controlling this population, since 100% of the plants survived and continued growing to complete the life cycle.

3.2 Intact EPSPS activity in GR_1 plants in the presence of glyphosate

As a result of EPSPS inhibition, shikimate-3-phosphate (one of its substrates) accumulates and is converted to shikimate faster than

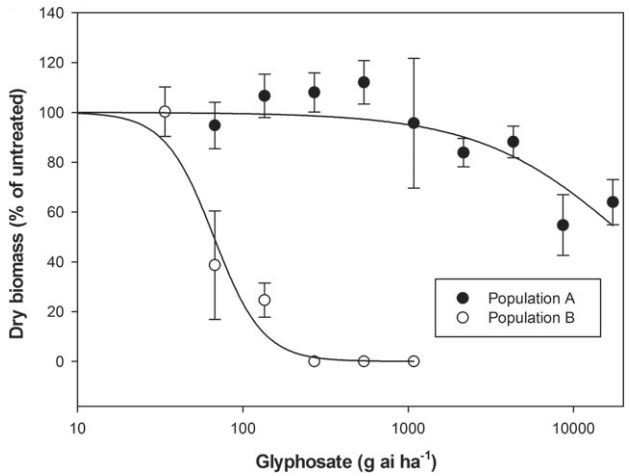


Figure 1. *In vivo* dose–response assay. Plants from populations A and B were subjected to the application of different doses of glyphosate (see Materials and Methods). Results are expressed as percentage of remaining biomass relative to untreated controls from each population.

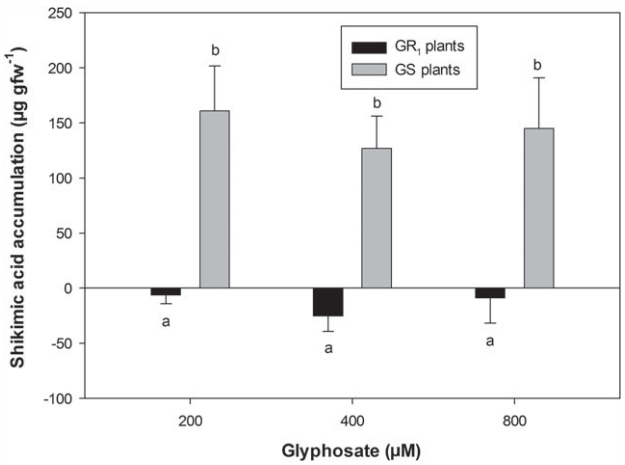


Figure 2. Shikimic acid accumulation in GS versus GR_1 plants. Each determination was performed after leaf disc incubation at three different glyphosate concentrations (200, 400, and 800 μM) in triplicate. Vertical bars represent the standard error of the mean. Different letters indicate statistically significant differences.

Table 1. *In vivo* glyphosate resistance parameters for *A. hybridus* populations

Population	GR_{50}			R^2
B	66.6[a]	(7.4)	Rf	0.9639
A	20900[b]	(8600)	314	0.7526

Standard errors are between parentheses. [a] and [b] indicate statistically significant differences. Rf is the ratio between GR_{50} values from the resistant (A) and the susceptible (B) populations.

it can be consumed by alternative metabolic pathways.[37] The leaf disc bioassay showed that GS plants accumulated shikimate after glyphosate treatment (ranging between 125 and 160 μg shikimic acid per gram of fresh weight), whereas GR_1 plants did not accumulate at any of the different glyphosate concentrations tested (200, 400 and 800 μM) (Fig. 2). These results are consistent with an intact EPSPS activity in GR_1 plants in the presence of glyphosate, even at a high herbicide concentration.

3.3 A novel triple EPSPS substitution (TAP-IVS) is present in all GR_1 plants in homozygosis

To determine whether or not target-site mutations were present in glyphosate-resistant *A. hybridus*, we decided to amplify a highly conserved *epsps* region in which several point mutations conferring GR in both plants and bacteria had been found.[2,35] Sequence analysis revealed a triple substitution of amino acids in the EPSPS fragments from all GR_1 plants (Fig. 3(A)). The four nucleotide replacements produced the following codon changes: ACA for ATA (T102I), GCG for GTC (A103V), and CCA for TCA (P106S). This triggers a triple substitution that hereinafter is referred to as TAP-IVS mutation. Substitutions occurring simultaneously at positions 102 and 106 are known as TIPS, and their contribution to GR was well characterized and even commercially used.[12,38,39] Up to now, the TIPS natural evolution has only been documented in two weedy species,[13,14] the *A. hybridus* population being the third worldwide case in which these substitutions are reported. However, the third substitution found here is produced by two consecutive mutations (see Fig. 3(A)) and, to our knowledge, has never been described before. The partial *epsps* sequences obtained here were annotated in the Gen Bank database as MH482843 (from GR_1 plants) and MH482844 (from GS plants).

Although the consequences of the novel substitution at position 103 on the EPSPS kinetic and regulatory properties have not yet been elucidated, its localization within the conserved region suggests that it could be contributing to the extremely high levels of GR observed in *A. hybridus*, as discussed in Sections 3.5 and 3.6.

Additionally, dCAPS assays were performed on the eight GR_1 plants carrying the TAP-IVS mutation in order to distinguish homozygous from heterozygous samples. All the GR_1 plants tested were homozygous for the triple mutation, while all the GS samples showed the same digested pattern observed for S1 and S2 (Fig. 3(B)). Given that *A. hybridus* is a self-pollinated species, homozygosis can be increased in a small number of generations. Therefore, this result would be expected, considering that TAP-IVS mutation is probably not a recent event in GR evolution (see Section 3.5).

3.4 Amplification in the *epsps* gene is not correlated with *epsps* cDNA expression levels in GR_1 plants

Gene amplification of *epsps* was estimated through qPCR. Plants from population B exhibited an average of 1.4 copies of *epsps* relative to the *als* control gene, while GR_1 plants displayed a slightly higher number, ranging from 1.8 to 3.7 (Fig. 4(A)). However, *epsps* mRNA was not found to be significantly increased in these GR_1 plants, which presented very similar expression levels to those from susceptible individuals (Fig. 4(B)). Similarly, no direct correlation between *epsps* expression levels and copy number was observed in a previous report of GR in *Bromus diandrus*, which was attributed to not all *epsps* copies being transcriptionally active.[20]

It is important to highlight that both glyphosate TSR mechanisms acting together in the same plant were only found recently in *Eleusine indica*.[26] Therefore, this is the second report of target-site mutations and *epsps* amplification evolving together against this herbicide. We hypothesize that the *epsps* amplification could be an emergent mechanism in this population (given the low number of *epsps* copies) and thus its effect over the expression levels may not be detected yet.

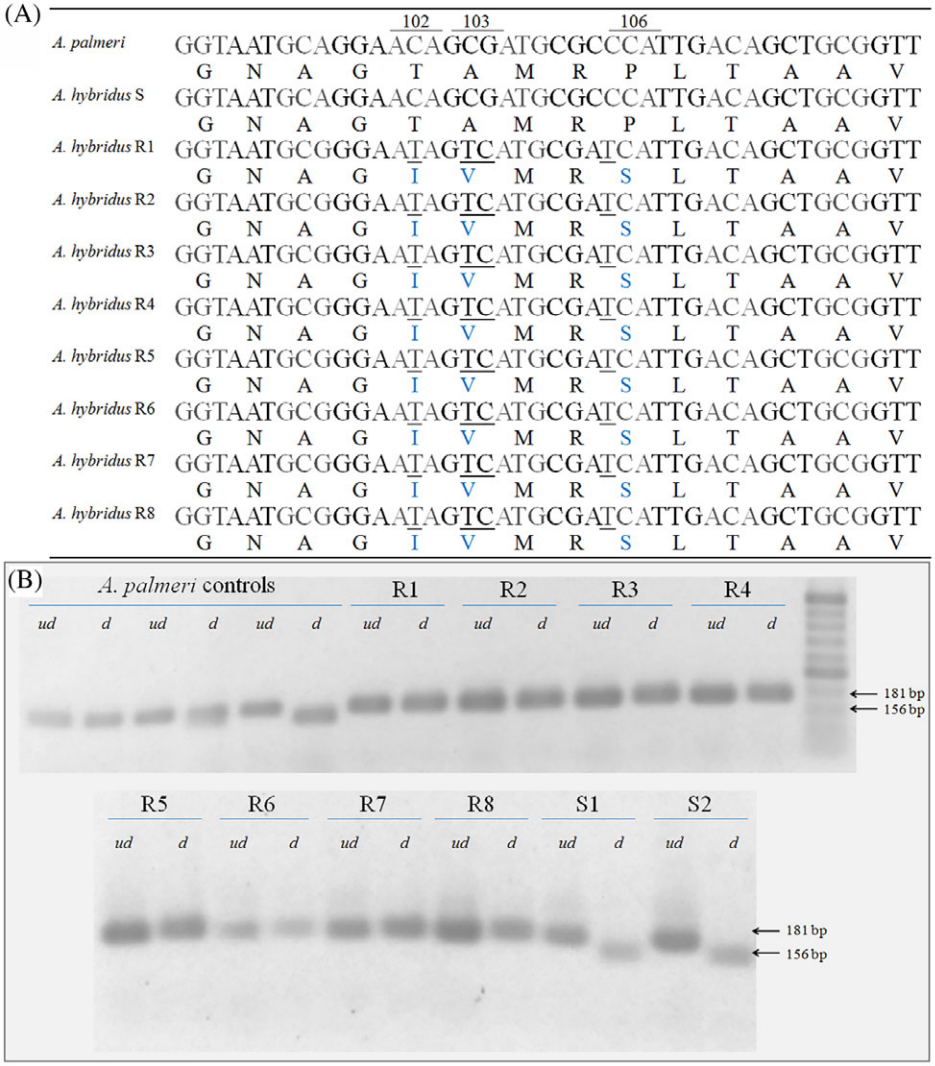


Figure 3. Partial *epsps* gene sequencing and genotyping by dCAPS. (A) Sequence analysis of the *epsps* fragment derived from GR_1 plants. The four mutations found are underlined. Amino acids are numbered according to *Z. mays epsps* sequence. (B) Genotyping by dCAPS markers developed for P106 EPSPS mutations in *A. hybridus*. The restriction enzyme NcoI was used. Control DNAs (*A. palmeri* sequenced samples) were included to validate the fidelity of the test. The wt sequence produces a single digested 156-bp band (S1 and S2 samples), whereas the mutant sequence at 106 produces an undigested 181-bp band (R1-8 samples). *d*, digested; *ud*, undigested.

3.5 TAP-IVS mutation is prevalent in population A, supporting its role in GR

Based on the sequence information obtained, we designed an experiment to determine the frequency of the mutant TAP-IVS allele (R) in population A. HRMA, dCAPS, and/or sequencing (see Materials and Methods) were employed to classify the genotype of 130 seeds from the original sampling of the resistant population A. Profiles obtained from HRMA are illustrated in Fig. 5(C). Partial chromatograms derived from sequencing are shown in Fig. 6.

This assay revealed that 48% of the samples were homozygous for R (referred to as the RR genotype) and the remaining 52% were classified as heterozygous for this allele (referred to as the RS genotype) (Fig. 6). Considering that the *epsps* gene could be duplicated (see section 3.4), it cannot be discarded *a priori* the presence of the IAS, TVS, and TAS alleles in RS genotypes, but they should occur in such a combination that chromatograms from sequencing are indistinguishable from heterozygous TAP/IVS (Fig. 6, bottom chromatogram). However, no homozygous single or double mutants were found in all the samples analyzed. Therefore, only two alleles (R and S) were considered. Since it was not possible to find any individual homozygous for the wt EPSPS (SS genotype), gene duplication most probably occurred after the mutation. Therefore, plants classified as RS could also include RS/RR or SS/RR possible genotypes, bearing in mind the presence of at least one extra *epsps* copy.

The high percentage of resistant individuals homozygous for the TAP-IVS EPSPS (RR) also suggests that the additional A103V mutation is not a recent event. The absence of homozygous individuals for P106S mutation can be attributed to the low levels of GR tolerated by the single mutant. Additionally, the non-detection of homozygous TIPS mutants could be rationalized as the consequence of a notable resistance cost associated with the presence of this double substitution.[35,40] In any case, there should be an adaptive advantage in TAP-IVS mutants over TIPS and P106S mutants that may explain the prevalence of the first allele in population A. This advantage could be that (i) TAP-IVS mutants may undergo less or little fitness cost and thus proliferate within the population, even in absence of herbicide, and/or (ii)

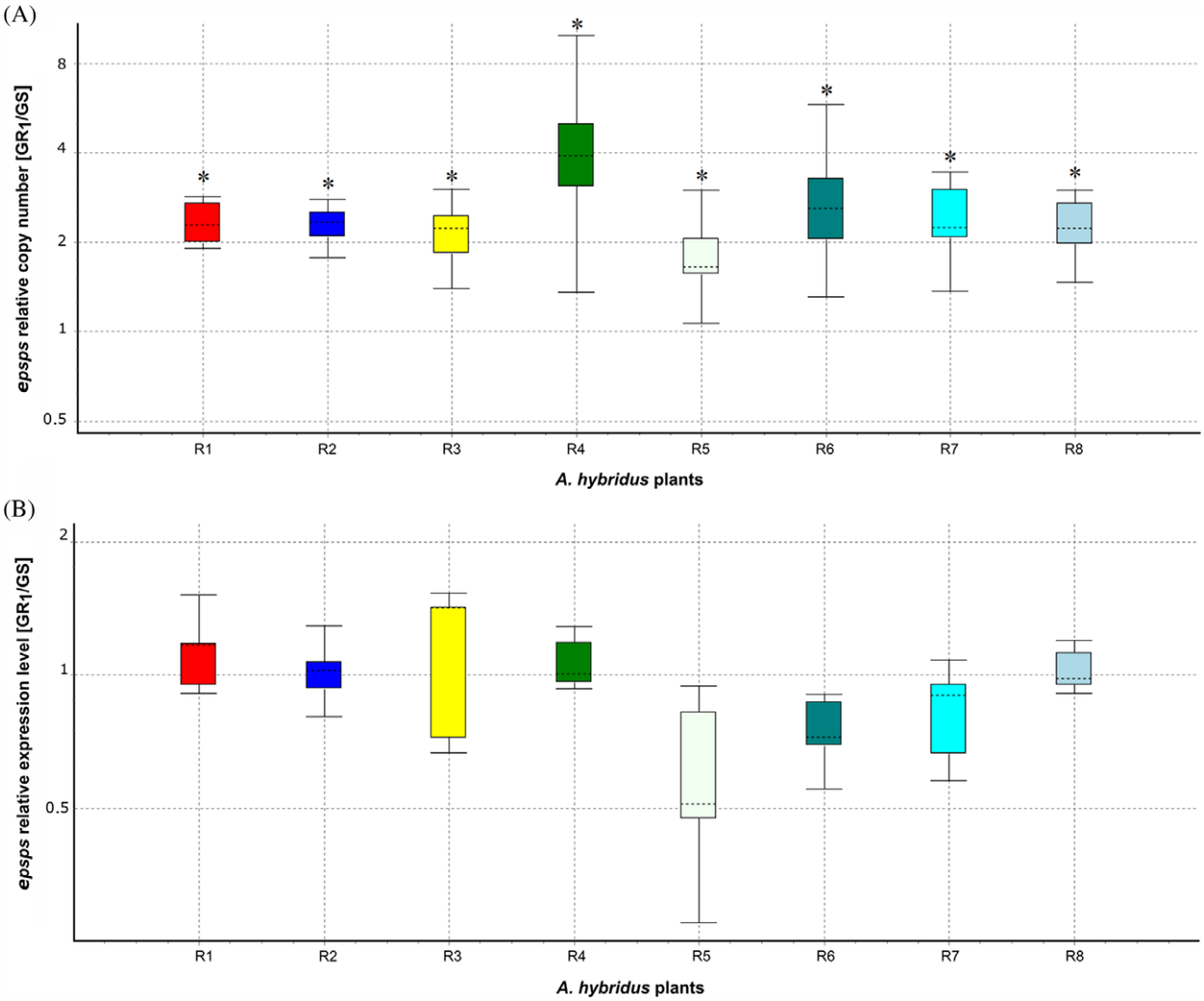


Figure 4. EPSPS gene expression and copy number in GR_1 plants. Genomic copy number (A) and expression level (B) of *epsps* relative to *als* control gene were determined by qPCR. The results were normalized using the GS sample determinations as reference. Vertical bars represent the standard error of the mean. * indicates statistically significant differences.

TAP-IVS mutants could tolerate higher glyphosate doses and more frequent herbicide applications. Considering the history of farm A (see Materials and Methods) and the GR_{50} determined for the population, the second scenario (ii) seems to be the most feasible. However, a lower fitness cost associated with TAP-IVS mutation cannot be discarded, as discussed in the following paragraphs.

As a final point, we decided to evaluate if the GR_1 plants (characterized as RR in this work) could tolerate the highest dose of glyphosate used in the *in vivo* dose–response assay for the original population A. The behavior observed was actually concluding: all GR_1 plants survived the application of a 32X dose, producing viable progeny. Thus, a higher resistance of RR plants versus RS plants could explain why all GR_1 plants, originally selected by their best recovering after glyphosate application (see Materials and Methods), resulted be homozygous RR plants, when each genotypic frequency in the population was around 50%. This observation together with the normal development of all GR_1 plants in the absence of glyphosate (data not shown) would support the non-existence of a strong fitness cost associated with this genotype. However, future studies are necessary to accurately evaluate this presumption since many different considerations must be taken into account to determine fitness cost, as was carefully detailed by Vila-Aiub *et al.*[41]

Interestingly, when our dose–response results are compared with those from other molecular-characterized glyphosate-resistant populations from different species around the world (see Supporting information Table S1), the GR_{50} from population A is by far the highest, being an *E. indica* population the unique report with at least a similarly high LD_{50}.[13] This population contained the TIPS mutation as the unique mechanism of GR. Although *E. indica* and *A. hybridus* are different species, the only difference between the GR mechanisms found in these populations is the A103V mutation, strengthening the hypothesis of triple substitution contributing to the extremely high GR observed. Empirical studies are necessary to evaluate this hypothesis, as discussed next.

3.6 TAP-IVS EPSPS structure could explain the high GR

In a previous work, the TIPS version of *E. coli* EPSPS was crystallized and its effect on the functionality and glyphosate-susceptibility of the enzyme was studied.[35] This deep molecular insight showed

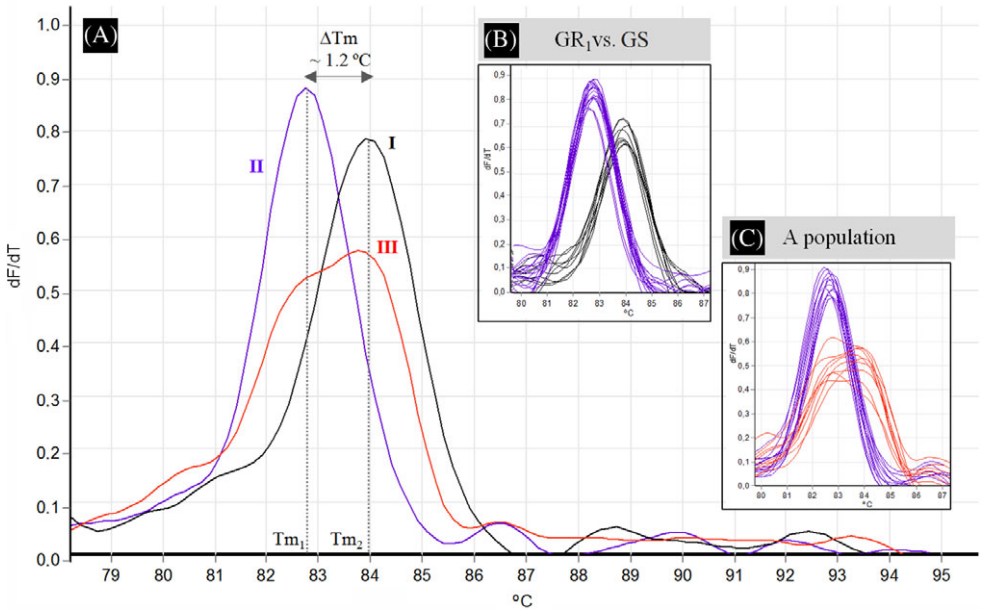


Figure 5. Detection of the triple TAP-IVS EPSPS substitution through HRMA. (A) Representative profiles of the melting curves for TAP homozygous samples (I, black line), IVS homozygous samples (II, violet line), and artificial heterozygous samples (III, red line). The triple substitution causes a ~1.2 °C shift in the melting temperature of the *epsps* amplified fragment. (B) Melting curves for GR_1 plants (violet line) and GS plants (black line) showing all IVS homozygous and all TAP homozygous genotypes, respectively. (C) Melting curves for A population samples, all of them found to be either IVS homozygous (violet line) or heterozygous (red line).

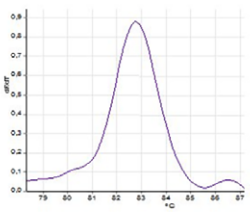
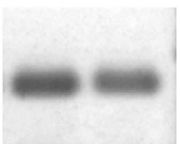
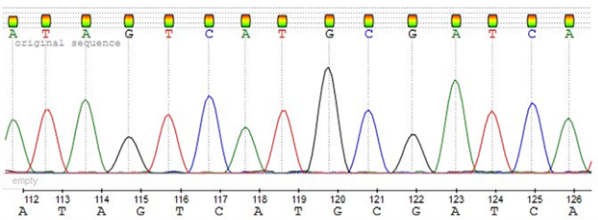
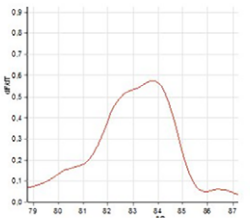
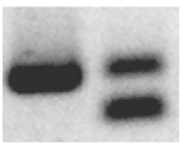
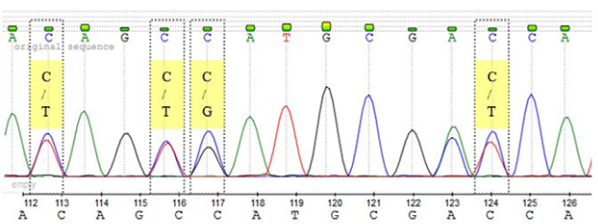
Genotype	HRMA	dCAPS	Sequence	%
RR				47.6
RS†				52.4

Figure 6. Steps for the genotypic classification of samples from population A. R accounts for the IVS mutant allele and S accounts for the wt allele. A single peak (HRMA), no digestion (dCAPS), and/or unambiguous sequence assignments in the region of the triple substitution are observed for IVS homozygous samples (RR line). Double peaks (HRMA), partial digestion (dCAPS), and/or ambiguous sequence assignments in the four nucleotides causing the triple substitution (dotted line boxes) are observed in IVS heterozygous samples (RS line). †The diploid approach addressed here may be an oversimplification of the real genotypes, as gene amplification may be present. The presence of IAS, TAS, and TVS alleles was dismissed based on the absence of homozygous samples for this genotype.

that the substitution T97I (T102 in plants) in the presence of S101 (S106 in plants) produces a shift of the G96 toward the glyphosate binding site, causing a reduction in the affinity for this herbicide at the time that the affinity for phosphoenolpyruvate (PEP) is also reduced. As a consequence, higher levels of GR are produced at the expense of a lower catalytic efficiency of the enzyme, which can be finally translated into a plant fitness cost, as was described for an *E. indica* population where TIPS mutation has evolved.[40]

As no *A. hybridus* EPSPS structure has yet been solved, we performed structure-homology modelling to model the wt, TIPS, and TAP-IVS EPSPS variants using *E. coli* EPSPS templates. The templates were selected because they have around 54% identity with the *A. hybridus* EPSPS and the possibility to have several crystal structures variants in the presence of both glyphosate and shikimate-3-phosphate (Fig. 7(A)).

In order to analyse the structural modification of the glyphosate binding site produced by TAP-IVS mutation, we compared the

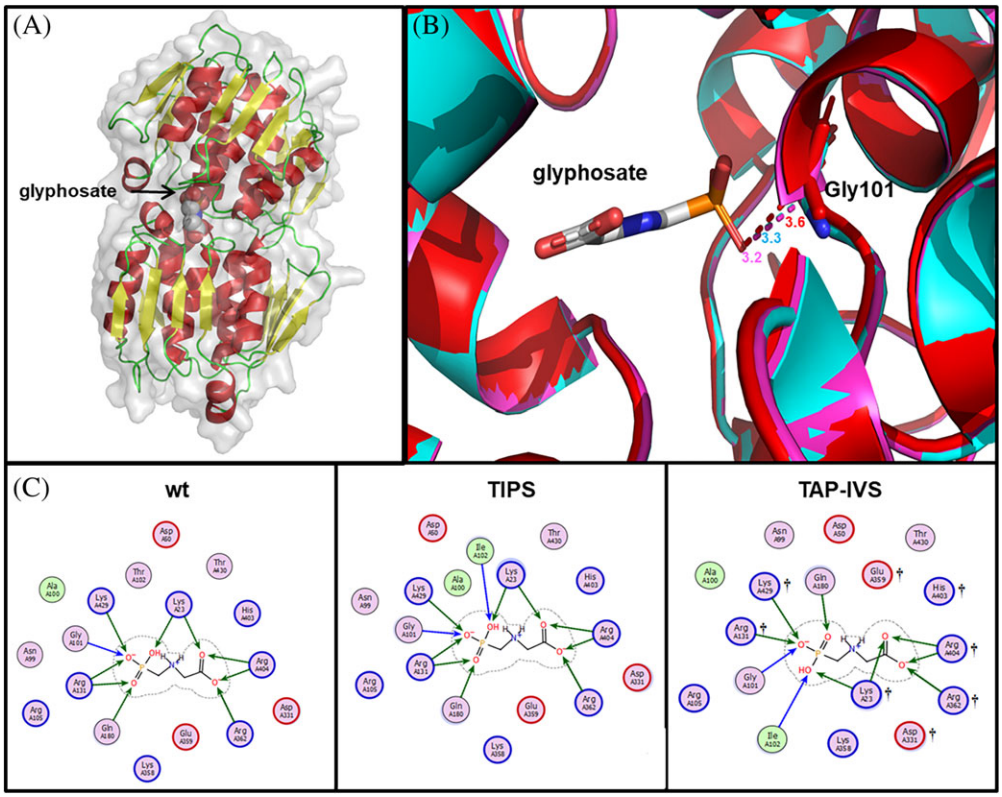


Figure 7. Structural homology modelling of wt, TIPS, and TAP-IVS EPSPS variants. (A) Structural homology model of wt using EPSPS *E. coli* (1G6S pdb code) as template in the Swiss-Prot server. (B) Interaction distance between glyphosate and G101 residue in the wt (red), TIPS (cyan), and TAP-IVS (pink) EPSPS variants. (C) Residues involved in the glyphosate/PEP binding site in wt, TIPS, and TAP-IVS versions. Basic residues are shown in blue, acid in red, apolar residues in green, and polar in pink. Blue and green arrows indicate sidechain and backbone donors, respectively. † In TAP-IVS scheme indicates the eight essential amino acids for the binding of PEP.

distance between glyphosate and the G101 residue (equivalent to G96 from *E. coli*), as was previously described in TIPS mutant.[35] The results show that the additional substitution at position 103 causes a slight enclosure of the PEP and glyphosate binding site, even with respect to the TIPS structure, which is evidenced by the shortening of the distance between glyphosate and the key residue G101 (Fig. 7(B)). As can be seen, the distance from glyphosate to G101 is reduced from 3.6 (red dashed line) to 3.3 (cyan dashed line), and 3.2 (pink dashed line) in the wt, TIPS, and TAP-IVS EPSPS models, respectively. In relation to this, we performed a second analysis using LIDIA application of the COOT program[36] to predict the residues involved in the structural conformation of the glyphosate binding site in the three variants. The comparative analysis of the glyphosate binding site shows that there is a rearrangement of the amino acids that participate in the primary and secondary coordination sphere with glyphosate (Fig. 7(C)), but the eight essential amino acids for the binding of PEP[42,43] (marked with dagger) are still present in all versions. In particular, when the glyphosate interactions predicted for TIPS mutant are compared with those predicted for TAP-IVS mutant, the arginine at position 131 (equivalent to R124 in *E. coli*) would only keep one of the two possible interactions with glyphosate in the triple mutant.

Our hypothesis is that this new conformational structure has a smaller active site, causing a higher exclusion of glyphosate. This steric impediment could probably be eluded by PEP due to its lower molecular size. The normal plant development observed in population A would support this presumption. However, a precise kinetic and structural characterization of the recombinant TAP-IVS mutant in comparison to the TIPS mutant is essential for confirming the role of the third substitution discovered here.

4 CONCLUSIONS

The prevalence of the TAP-IVS triple substitution as the sole mechanism detected in the highly glyphosate-resistant population suggests the evolution of a new GR mechanism arising in *A. hybridus*. This work is the first report of a naturally occurring EPSPS triple substitution and the first glyphosate TSR mechanism described in *A. hybridus*. New chemical tactics together with non-chemical practices must necessarily be integrated into weed management programs in order to slow down the evolution of herbicide resistance and prevent the emergence of new or stacked mechanisms.

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CONFLICT OF INTEREST

None.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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