

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

Novel triple EPSPS substitution in *A. hybridus*

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Abstract

BACKGROUND:

The evolution of herbicide-resistant weeds is one of the most important concerns of the 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 ($GR_{50} = 20900 \text{ g ai ha}^{-1}$, $R_f = 314$), 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 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 the glyphosate binding.

CONCLUSION:

The prevalence of the TAP-IVS triple substitution as the solely mechanism detected in the highly glyphosate resistant population suggest the evolution of a new GR mechanism arising in *A. hybridus*. This is the first report of a naturally-occurring EPSPS triple substitution and the first glyphosate TSR mechanism described in *A. hybridus*.

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 the last decades has led to a severe problem regarding the evolution of herbicide resistant weeds in crop fields. Nowadays, 255 different species have evolved resistance to at least one herbicide across the world, evidencing a growing evolution rate in the last years.¹ Particularly, the glyphosate resistance (GR) evolution is one of the most worrying challenges for the modern agriculture. As much 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 it is properly 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, EPSPS) was early 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- known for conferring high GR levels in commercial transgenic maize lines,²³ has been found in two weed species (*Eleusine indica* and *Bidens pilosa*),^{24,25} evidencing the power of the natural evolution upon a persistent selection pressure.

Other TSR mechanism, which involves the overexpression of *epsps* produced by gene amplification, was discovered in *Amaranthus palmeri*.¹² This mechanism endows high levels of GR and seems to be the predominant TSR evolved in several resistant weeds.¹³⁻¹⁷

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.^{18–20} Moreover, a novel NSTR mechanism related to a rapid necrosis of the glyphosate-exposed tissue, known as "pheonix phenomenon", has been recently described.^{21,22}

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

Amaranthus hybridus L. is a broadleaf weed native to South America and is currently the 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 6 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 a.i. 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) was grown on growing chamber at 25 °C and 8/16 photoperiod and glyphosate resistance 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 those 8 selected plants -which were called GR₁ 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” populations sampling were grown and treated as described in Supplemental Material-Protocols. Basically, when 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, in order 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 diameter were collected separately from each plant (8 GR₁ and 8 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 ($150 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$) for 16 h. After incubation, 250 μl of 1.25 N HCl were added into each vial and they were placed at $-20 \text{ } ^\circ\text{C}$ 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+) and values were converted to ng of shikimic acid per g of fresh weight by a shikimate standard (Sigma-Aldrich) curve. Each treatment was replicated at least three times.

2.4 *epsps* partial sequencing

Genomic DNA was obtained from fresh leaf tissue of plants (8 GR₁ and 8 GS) using Wizard Genomic DNA Purification Kit[®] (Promega). Egf (5'-ATGTTGGACGCTCTCAGAACTCTTGGT-3') and Egr (5'-TGAATTCCTCCAGCAACGGCAA-3') primers were used to amplify 195 bp, according to the experimental design performed on *Amaranthus palmeri* by Gaines *et al.*¹² PCRs were performed with Q5[®] High-Fidelity DNA polymerase (New England Biolabs, inc.). Reactions were prepared as follows: ~100 ng DNA, 200 μM dNTPs, 1.5 mM MgCl_2 , 500 nM primers, 1 polymerase unit, 1X buffer, and distilled H_2O to 50 μL . Amplification program was: 95 $^\circ\text{C}$ for 1 min, 30 cycles of 95 $^\circ\text{C}$ for 30 s, 58 $^\circ\text{C}$ for 30 s, and 72 $^\circ\text{C}$ for 1 min, 72 $^\circ\text{C}$ for 7 min, and a 12 $^\circ\text{C}$ hold. Amplification products were sequenced through Macrogen service (Macrogen Inc., Seoul, 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, in order to search for amino acid substitutions.

2.5 dCAPS for detecting mutations at P106

Based on the *epsps* sequence data obtained from GR₁ and GS plants of *A. hybridus*, we developed a derived Cleaved Amplified Polymorphic Sequence (dCAPS) test to detect mutations at P106. DNA from GR₁ plants was used to determine the zygosity of the P106 mutation in each single *A. hybridus* plant. PCR and digestion were performed as described previously³¹, 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 only present in the wild-type sequence. DNA samples were subjected to an electrophoresis on 2% agarose gel stained with SYBR Safe[®] (Invitrogen). *A. palmeri* control DNAs were included to validate the fidelity of the test. The wild-type 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-bp 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 GR₁ 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. RNA resulting was dissolved in sterile HPLC-grade water, quantified by spectrophotometry, and assessed for quality and integrity through gel electrophoresis. cDNA was synthesized incubating 25- μ l 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 *epsps* genomic relative copy number and *epsps* relative cDNA expression. In both cases, the *als* (acetolactate synthase) gene was used as reference, prior validation of its fidelity using the actin gene as a second reference. The primer sets Egf x Egr mentioned in 2.4, giving a 195-bp product, and Af (5'-GCTGCTGAAGGCTACGCT-3') x 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 H₂O to 13 µL. Eight samples from GR₁ 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 increasing 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 GR₁ plants relative to *epsps* copy number in GS plants. A similar relative quantification was made for 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 Supplemental Material-Protocols.

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 qPCR program were identical to the one set for *epsps* copy number study, using 1 μ L of single-seed DNA as template. Since TAP-IVS multiple substitution derives from three C \rightarrow T transitions, it causes a \sim 1.2 $^{\circ}$ C decrease in the melting temperature of the *epsps* amplified fragment respect to the wild-type amplified sequence. This shift of the unique peak in the melting curve from \sim 84 to \sim 82.8 $^{\circ}$ C was appreciated during the qPCR experiments using GS and GR₁ plants, respectively, and confirmed through sequencing. To check the ability of the HRMA assay to detect heterozygous variants, artificial heterozygous were created by mixing genomic DNA from GS and GR1 samples (mixing ratio 1:1) (Figure 5A).

Melting curves were generated for 130 seeds from the original sampling of the population “A”. The different patterns in the melting curves (I, II and III in Figure 5A) 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 profiles represented in I, II, and III) were validated through dCAPS (wild-type allele presence/absence) and sequencing.

2.10 Structural TAP-IVS mutant analysis

Structural molecular modelling was performed with SWISS-PROT server to model the wild-type (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. It was guaranteed -for all models- a global RMSD lower than 0.2 and global secondary structure conformations similar to their templates. 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 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 (Figure 1; Supplemental Material-Figure). The GR₅₀ value for the susceptible population was 66.6 g ai ha⁻¹, whereas the resistant population exhibited a GR₅₀ = 20900 g ai ha⁻¹, with a Rf value of 314 (Table 1). Population “A” showed a GR₅₀

value remarkably higher (19.6-fold) than the glyphosate field rate recommended (540 g ia ha⁻¹). Even more, the highest dose tested (32X) was not effective in controlling this population, since 100 % of the plants survived and continued growing up to complete the life cycle.

3.2 Intact EPSPS activity in GR₁ 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 it can be consumed by alternative metabolic pathways.³⁷ The leaf disc bioassay showed that GS plants accumulated shikimate after glyphosate treatment (ranged between 125-160 µg shikimic acid g⁻¹ of fresh weight), whereas GR₁ plants did not accumulate at any of the different glyphosate concentrations tested (200, 400 and 800 µM) (Figure 2). These results are consistent with an intact EPSPS activity in GR₁ 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 GR₁ plants in homozygosis

To define whether 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 both in plants and bacteria had been found.^{2,35} Sequence analysis revealed a triple substitution of amino acids in the EPSPS fragments from all GR₁ plants (Figure 3A). 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

glyphosate resistance was well characterized and even commercially used.^{23,38,39} Up to now, the TIPS natural evolution has only been documented in two weedy species,^{24,25} being this *A. hybridus* population the third worldwide case in which these substitutions are reported. However, the third substitution found here is produced by two consecutive mutations (see Figure 3A) and, to our knowledge, has never been described before. The partial *epsps* sequences obtained here were annotated in Gen Bank database as MH482843 (from GR₁ plants) and MH482844 (from GS plants).

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

Additionally, dCAPS assays were performed on the eight GR₁ plants carrying the TAP-IVS mutation in order to distinguish homozygous from heterozygous samples. All the GR₁ plants tested were homozygous for the triple mutation, while all the GS samples showed the same digested pattern observed for S1 and S2. (Figure 3B). Given that *A. hybridus* is a self-pollinated species, homozygosis can be increased in a small number of generations. Therefore, this result would be expectable, considering that TAP-IVS mutation is not probably a recent event in the glyphosate resistance evolution (see section 3.5).

3.4 Amplification in *epsps* gene is not correlated with *epsps* cDNA expression levels in GR₁ 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 GR₁ plants displayed a slightly higher number, ranging from 1.8 to 3.7 (Figure 4A). However,

epsps mRNA was not found to be significantly increased in these GR₁ plants, which presented very similar expression levels to those from susceptible individuals (Figure 4B). 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.¹⁷

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.

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 the 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 Figure 5C. Partial chromatograms derived from sequencing are shown in Figure 6.

This assay revealed that 48% of the samples were homozygous for R (referred to as the RR genotype); and the 52% remained were classified as heterozygous for this allele (referred to as the RS genotype) (Figure 6). Considering that the *epsps* gene could be duplicated (see preceding section), 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 undistinguishable from heterozygous TAP/IVS (Figure 6, bottom chromatogram). However, none homozygous single or double mutants were found from the total of the samples analyzed. Therefore, only two alleles (R and S) were considered. Since it was not possible to find any individual homozygous for the wild type 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 *epsps* extra 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 the 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) TAP-IVS mutants could tolerate higher glyphosate doses and more frequent herbicide applications. Considering the history of the farm “A” (see Materials and Methods) and the GR₅₀ 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 as follows.

As a final point, we decided to evaluate if the GR₁ 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 GR₁ 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 GR₁ 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 a normal development of all GR₁ plants in the absence of glyphosate (data not shown) would support the nonexistence of a strong fitness cost associated to 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 it 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 Supplemental Material-Table), the GR₅₀ from population “A” is by far the highest, being an *E. indica* population the unique report with at least a similarly high LD₅₀.²⁴ 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 the 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 has been crystallized and its effect on functionality and glyphosate-susceptibility of the enzyme has been studied.³⁵ This deep molecular insight showed 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 PEP, is also reduced. As a consequence, higher levels of GR are produced at the expense of a lower catalytic efficiency of the enzyme, what can be finally translated into a plant fitness cost, as it was described for an *E. indica* population where TIPS mutation has evolved.⁴⁰

As there is not any *A. hybridus* EPSPS structure solved yet, we performed a structure-homology modelling to model the wild-type (wt), TIPS and TAP-IVS EPSPS variants using *E. coli* EPSPS templates. The templates were selected considering that they have around 54% identity with the *A. hybridus* EPSPS, and the possibility to have several crystal structures variants in presence of both glyphosate and shikimate-3-P (Figure 7A).

In order to analyse the structural modification of the glyphosate binding site produced by TAP-IVS mutation, we compared the distance between glyphosate and the G101 residue (equivalent to G96 from *E. coli*), as it was previously described in TIPS mutant.³⁵ 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 (Figure 7B).

As it 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 COOT program³⁶ to predict the residues involved in the structural conformation of the glyphosate binding site in the three variants. The comparative analysis of glyphosate binding site shows that there is a re-arrangement of the amino acids that participate in the primary and secondary coordination sphere with glyphosate (Figure 7C), but the eight essential amino acids for the binding of PEP^{60,61} (marked with an asterisk) are still present in all versions. Particularly, when glyphosate-interactions predicted for TIPS

mutant are compared with those predicted for TAP-IVS mutant, the arginine at the 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 appreciation of a normal plant development in the 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 here discovered.

4 CONCLUSIONS

The prevalence of the TAP-IVS triple substitution as the solely mechanism detected in the highly glyphosate resistant population suggest 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 emerging of new or stacked mechanisms.

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SUPPORTING INFORMATION

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

Supplemental Material-Protocols

Supplemental Material-Figure

Supplemental Material-Table

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

Population	GR₅₀	Rf	R²
“B”	66.6 ^a (7.4)		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 GR₅₀ values from the resistant (A) and the susceptible (B) populations.

Figure legends**Figure 1.** *In vivo* dose-response assay.

Plants from population “A” and “B” were subjected to the application of different doses of glyphosate (see Materials and Methods). Results are expressed as percent of remaining biomass relative to untreated controls from each population.

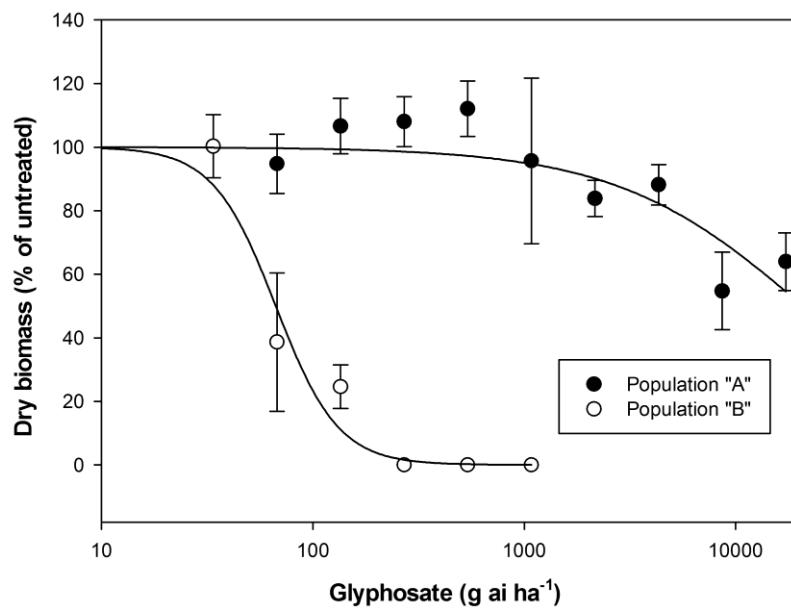


Figure 2. Shikimic acid accumulation in GS versus GR₁ plants.

Each determination was performed after leaf discs incubation at three different glyphosate concentrations (200, 400 and 800 μM) by triplicate. Vertical bars represent the standard error of the mean. Different letters indicate statistically significant differences.

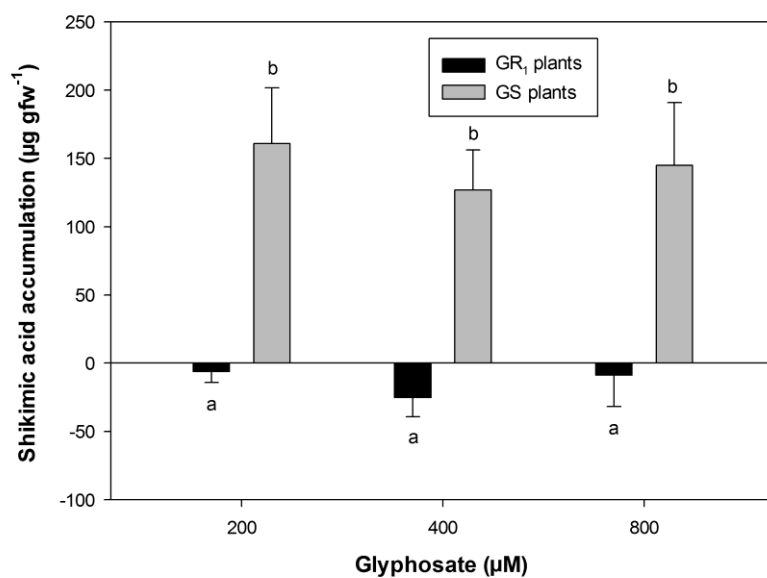


Figure 3. Partial *epsps* gene sequencing and genotyping by dCAPS.

A) Sequence analysis of the *epsps* fragment derived from GR₁ 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 wild-type 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.

Figure 4. EPSPS gene expression and copy number in GR₁ 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.

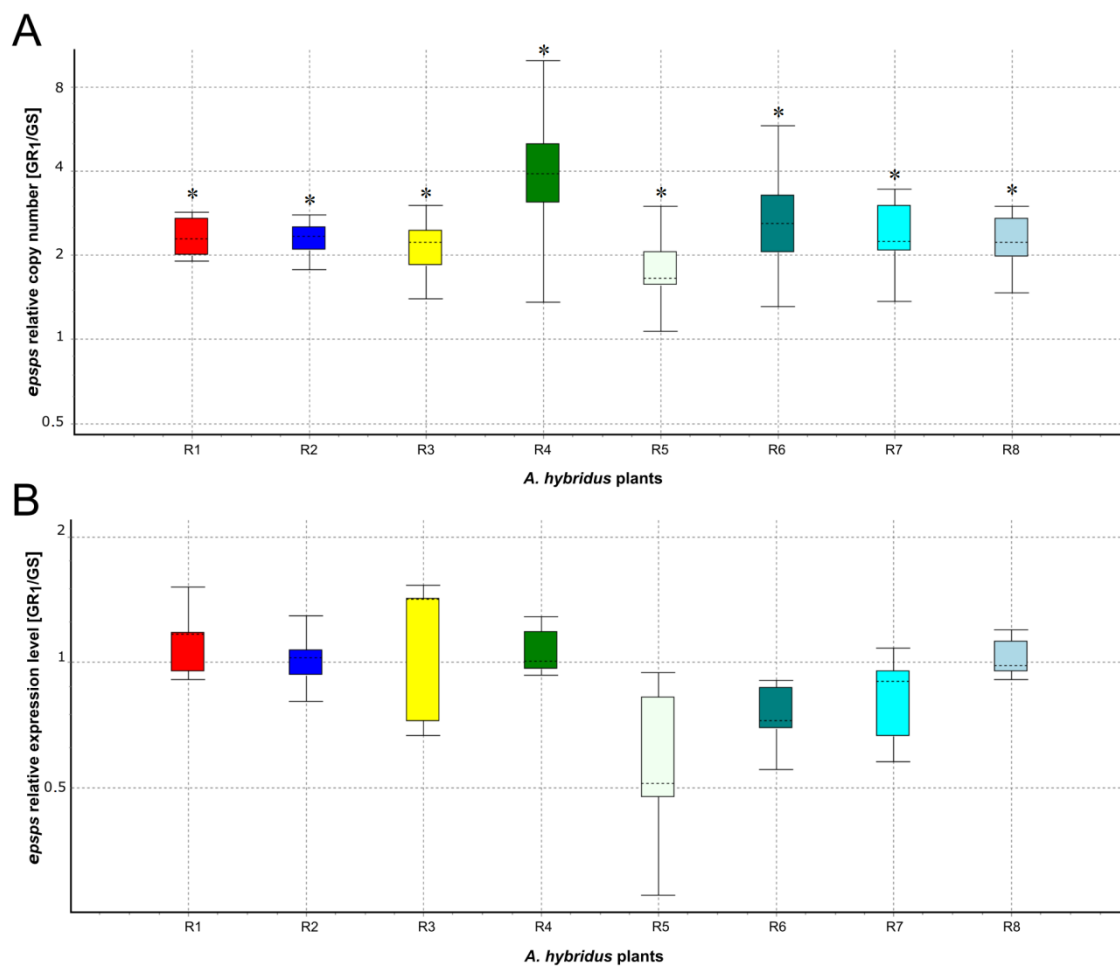


Figure 5. Detection of the triple TAP-IVS EPSPS substitution through High Resolution Melting Analysis (HRMA). A) Representative profiles of the melting curves for TAP homozygous samples (I, black line), IVS homozygous samples (II, violet line), and artificial heterozygous samples (III, red line). The triple substitution causes a ~ 1.2 °C shift in the melting temperature of the *epsps* amplified fragment. B) Melting curves for GR₁ 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).

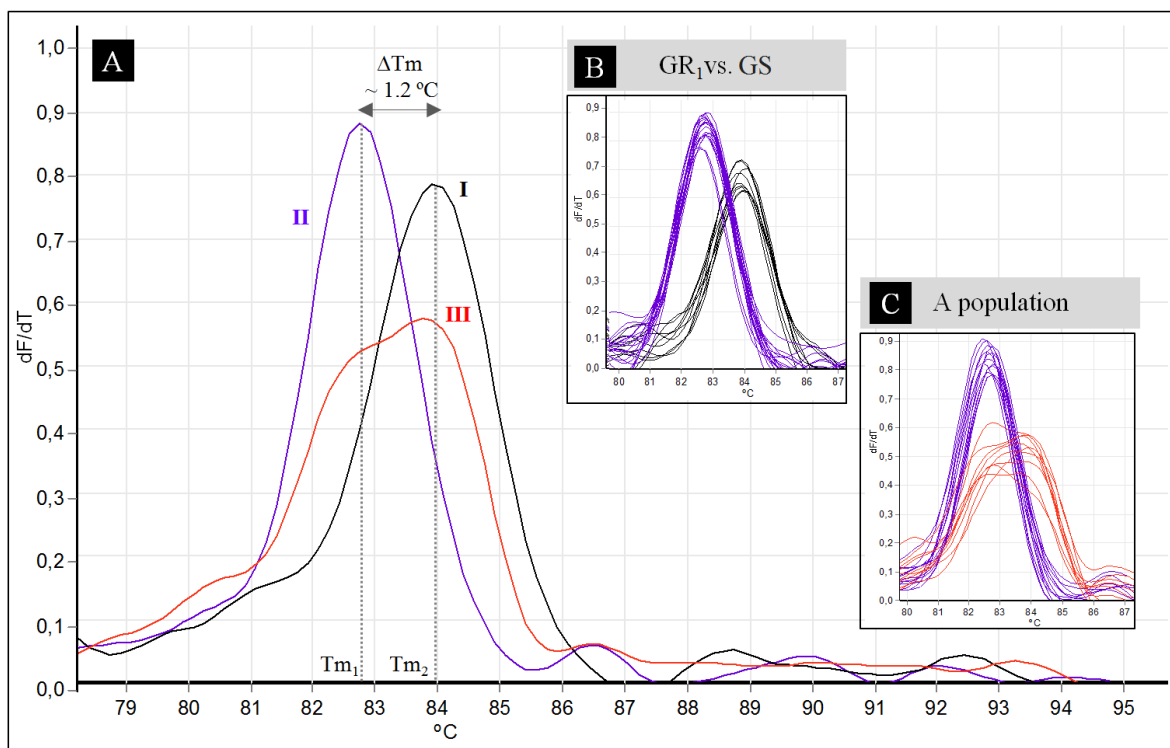


Figure 6. Steps for the genotypic classification of samples from population “A”.

"R" accounts for the IVS mutant allele and "S" accounts for the wild-type 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 genotypes.

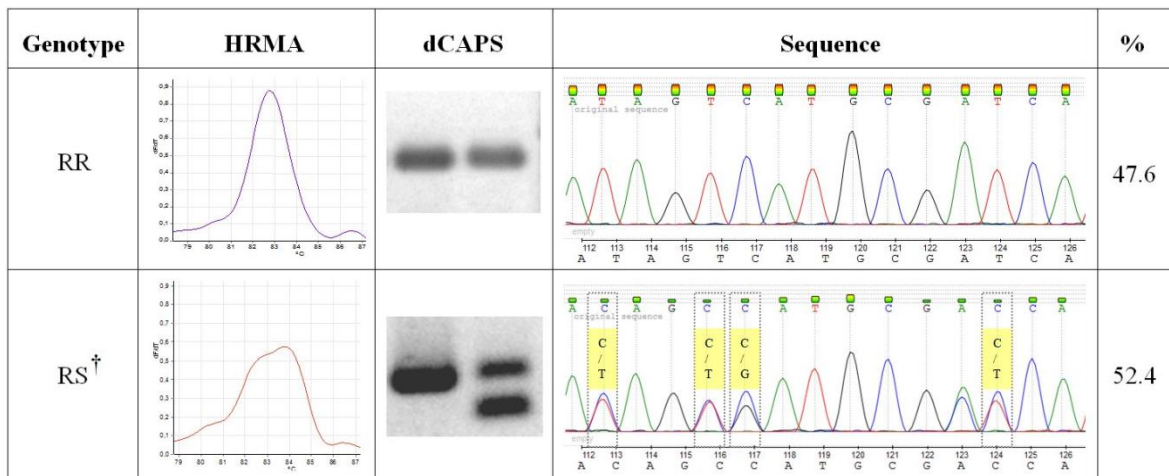
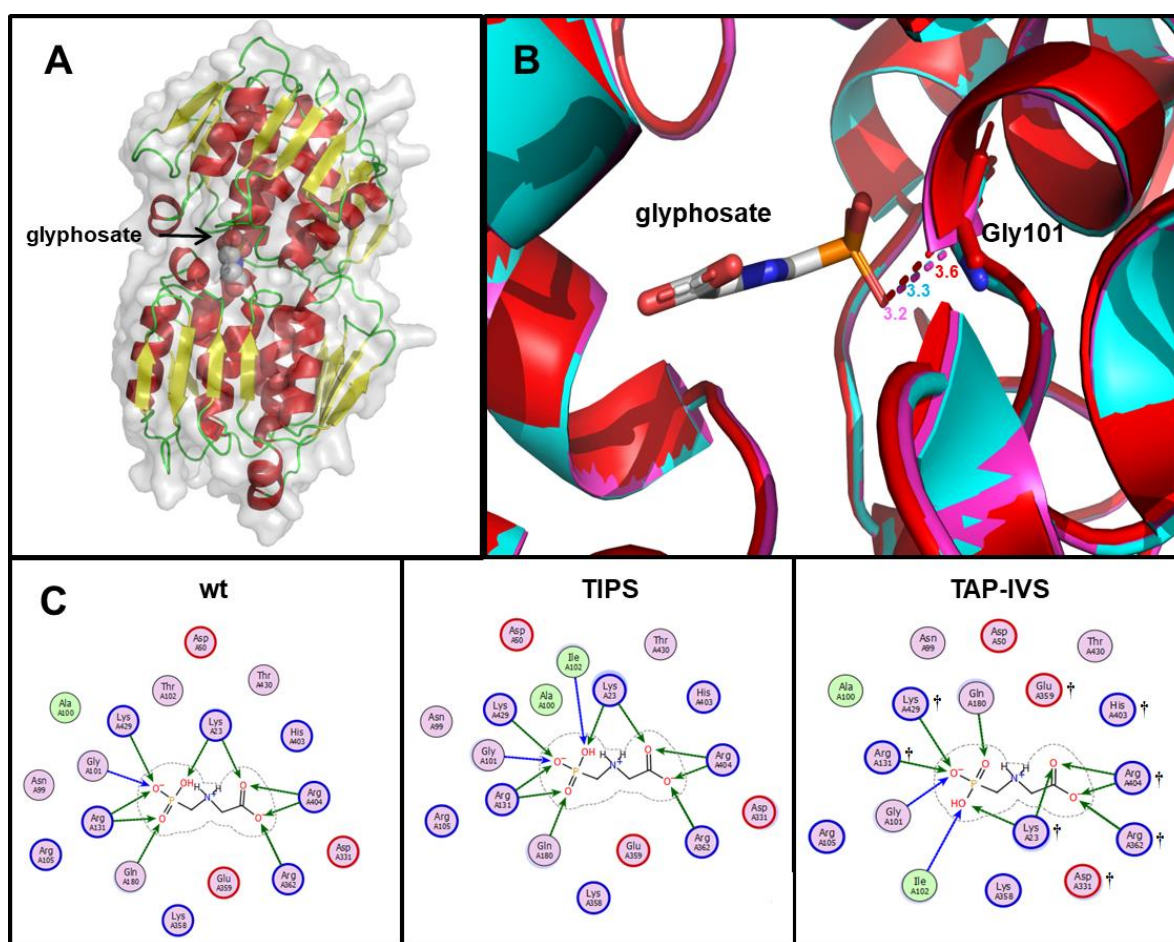


Figure 7. Structural homology modelling of wild type (wt), TIPS and TAP-IVS EPSPS variants. A) Structural homology model of wt using EPSPS *E. coli* (1G6S pdb code) as template in Swiss-Prot Server. B) Interaction distance between glyphosate and G101 residue in the wt (red dashed line), TIPS (cyan dashed line) and TAP-IVS (pink dashed line) EPSPS variants. C) Residues involved in the glyphosate/PEP binding site in wt, TIPS and TAP-IVS versions. Basic residues are coloured 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.



Supplementary Figure 1. Glyphosate dose-response of susceptible (S) and resistant (R) plants from the *A. hybridus* populations “B” and “A”, respectively.