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# A double EPSPS gene mutation endowing glyphosate resistance shows a remarkably high resistance cost

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## Abstract

A novel glyphosate resistance double point mutation (T102I/P106S, TIPS) in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene has been recently identified for the first time only in the weed species *Eleusine indica*. Quantification of plant resistance cost associated with the TIPS and the often reported glyphosate resistance single P106S mutation was performed. A significant resistance cost (50% in seed number currency) associated with the homozygous TIPS but not the homozygous P106S EPSPS variant was identified in *E. indica* plants. The resistance cost associated with the TIPS mutation escalated to 85% in plants under resource competition with rice crops. The resistance cost was not detected in nonhomozygous TIPS plants denoting the recessive nature of the cost associated with the TIPS allele. An excess of 11-fold more shikimate and sixfold more quinate in the shikimate pathway was detected in TIPS plants in the absence of glyphosate treatment compared to wild type, whereas no changes in these compounds were observed in P106S plants when compared to wild type. TIPS plants show altered metabolite levels in several other metabolic pathways that may account for the expression of the observed resistance cost.

**KEYWORDS**

aromatic amino acids, glyphosate resistance cost, Pro-106-Ser, TIPS

## 1 | INTRODUCTION

Agro-ecosystems for crop growth are fertile productive environments and this is why infestation by weedy plant species is a constant problem. In the modern era, crop-infesting weeds are mostly battled with herbicides. However, herbicides impose strong evolutionary selection pressure, and consequently, herbicide resistant weed populations have evolved worldwide.

The evolutionary trajectory of herbicide resistant weed populations is very dependent on herbicide selection regimes, characteristics of particular herbicide resistance alleles as well as being influenced by several molecular, genetic, physiological, and agro-ecological factors (see reviews by Beckie & Tardif, 2012, Jasieniuk, Brûlé-Babel, & Morrison, 1996, Maxwell & Mortimer, 1994, Powles & Yu, 2010, Vila-Aiub, Neve, & Powles, 2009b, Yu & Powles, 2014). Herbicide resistance-endowing gene traits obviously confer a benefit in the presence of the herbicide (resistance benefit) but can, in some cases, confer a cost (resistance cost) in the absence of herbicide. Resistance benefit and resistance cost strongly influence the evolutionary trajectory of resistance alleles (Maxwell & Mortimer, 1994; Simms & Rausher, 1987).

Herbicide resistance-endowing mutations/alleles may be associated with a resistance cost (also termed reduced fitness) when compared to herbicide susceptible wild-type alleles in the absence of herbicide selection (see reviews by Bergelson & Purrington, 1996, Herms & Mattson, 1992, Vila-Aiub et al., 2009b). Costs have been shown to arise depending on the particular resistance gene and mutation, number of mutant alleles, genetic background, and abiotic/biotic environmental conditions (Beckie & Tardif, 2012; Bergelson & Purrington, 1996; Bostamam, Malone, Dolman, Boutsalis, & Preston, 2012; Christopher, Powles, Liljegren, & Holtum, 1991; Han et al., 2012; Preston & Powles, 1998; Purba, Preston, & Powles, 1995; Roux, Gasquez, & Reboud, 2004; Roux, Matéjicek, Gasquez, & Reboud, 2005; Tardif & Powles, 1994; Vila-Aiub et al., 2009b; Vila-Aiub, Gundel, Yu, & Powles, 2013; Vila-Aiub, Neve, & Roux, 2011; Vila-Aiub, Neve, Steadman, & Powles, 2005; Yu et al., 2012; Yu, Han, Vila-Aiub, & Powles, 2010).

  

Rapid herbicide resistance evolution is expected if the resistance mutation endows clear resistance benefit under herbicide selection but shows no or negligible resistance cost in herbicide free environments (e.g., many of the acetolactate synthase [ALS] gene mutations endowing resistance to ALS-inhibiting herbicides). However, if the resistance-endowing mutation incurs a significant resistance cost, then resistance evolution will be slower.

Glyphosate (N-[phosphonomethyl]-glycine) is a broad spectrum, globally dominant herbicide (Duke & Powles, 2008) that inhibits the chloroplastic enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme involved in the synthesis of aromatic amino acids (Herrmann & Weaver, 1999; Steinrücken & Amrhein, 1980). As a result of intense glyphosate use, glyphosate resistance has evolved in many weed species (Heap, 2014; Powles, 2008; Sammons & Gaines, 2014). Glyphosate resistance can be endowed by one or more of several different resistance mechanisms, including target site resistance due to specific EPSPS gene mutations. Target-site mutations at EPSPS Pro-106 (changing Pro106 to any of Ala/Leu/Ser/Thr) are known to endow moderate glyphosate resistance in a number of field evolved resistant weed species (Baerson et al., 2002; Sammons & Gaines, 2014). Given the competitive nature between glyphosate and the EPSPS substrate phosphoenolpyruvate for binding at the EPSPS catalytic site, the above mentioned amino acid substitutions at the EPSPS Pro-106 only confer moderate level resistance benefit (typically two- to fourfold relative to the susceptible counterpart). At normal field glyphosate doses, population survival rates of plants possessing Pro-106 resistance-endowing mutations vary dependent on the weed species, specific EPSPS 106 mutation, heterozygous or homozygous status of the mutation and environmental condition (Kaundun et al., 2008; Kaundun et al., 2011; Wakelin & Preston, 2006). Estimation of the resistance cost endowed by these specific target site EPSPS gene mutations evolved in arable weeds has not been reported.

Early site-directed mutagenesis work revealed a double EPSPS gene mutation (Thr-102-Ile + Pro-106-Ser, hereinafter termed TIPS) endowing high level glyphosate resistance in a commercial corn line (GA21). Now, this double EPSPS TIPS resistance mutation has evolved in a field *Eleusine indica* population under intense glyphosate selection (Yu et al., 2015). Here, we assess the resistance cost associated with both the naturally evolved double TIPS and single Pro-106-Ser (P106S) EPSPS glyphosate resistance-endowing gene mutations. The results of this study enable an understanding of the physiological and ecological bases of resistance cost associated with EPSPS target site resistance alleles endowing glyphosate resistance in a global agricultural weed species.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material: Purification and selection of glyphosate resistance EPSPS variants

All work reported here was conducted with a field evolved glyphosate resistant *E. indica* population (Jalaludin, Ngim, Bakar, & Alias, 2010; Jalaludin, Yu, & Powles, 2014; Yu et al., 2015). Plants were grown outdoors simulating field conditions at the University of Western Australia (31°58′52″S, 115°49′11″E).

Individuals within the *E. indica* population with the normal glyphosate susceptible wild type (Thr-102/Pro-106 [WT]) or either of the two resistant mutant EPSPS alleles 102-Ile/106-Ser (termed *R* because of strong resistance) or Thr-102/106-Ser (termed *r* because of weak resistance) were identified by DNA sequencing and dCAPS markers (Yu et al., 2015). About 200 plants were individually genotyped, and four genotypes were identified and purified (Table 1): plants possessing susceptible Pro-106 WT (16%), homozygous resistant 106-Ser (rr, 33%) or homozygous resistant TIPS (RR, 2%) alleles. A fourth compound heterozygous genotype (Rr) segregating only at Thr-102 was identified in 49% of the plants within the field collected glyphosate resistant *E. indica* population (Table 1; Yu et al., 2015). The RR TIPS mutation has been shown to endow a higher glyphosate resistance level ($LD_{50}$-based resistance factor > 182) than the single P106S mutation ($LD_{50}$ = 5.6) in *E. indica* (Yu et al., 2015). The compound heterozygous Rr TIPS plants exhibit similar survival (100%) to plants with the homozygous RR TIPS mutation when exposed to glyphosate recommended field dose (1,080 g/ha) to control *E. indica* (data not shown).

The genotyped homozygous plants were bulked in isolation in glasshouse conditions to produce seeds that resulted in three purified subpopulations containing plants with homozygous genotypes of WT, 106-Ser (rr) or TIPS (RR; Table 1). Progeny plants ($n$ = 12) from each of the purified genotypic populations were randomly marker-analysed to confirm their genotype and homozygosity prior to use for subsequent studies. The Rr TIPS individuals were identified (Yu et al., 2015) in seedlings derived from a bulked progeny of Rr x Rr crossing and immediately used for subsequent experiments.

### 2.2 | Estimation of resistance cost

To evaluate the expression of a glyphosate resistance cost associated with the three glyphosate resistant EPSPS genotypes (RR, Rr TIPS, and P106S) various experiments were conducted to assess the

**TABLE 1** EPSPS genotypes, alleles, and mutations found in glyphosate resistant *Eleusine indica* used in this study after genotyping 200 plants

| Mutation | Allele | Genotype* (%) | Zygosity | Subpopulation |
|---|---|---|---|---|
| | Thr-102/Pro-106 (WT) | WT (16) | Homozygous | WT |
| Pro-106-Ser | Thr-102/106-Ser (r) | rr (33) | Homozygous | P106S (rr) |
| Thr-102-Ile/Pro-106-Ser | 102-Ile/106-Ser (R) | RR (2) | Homozygous | TIPS (RR) |
| Thr-102-Ile/Pro-106-Ser Pro-106-Ser | 102-Ile/106-Ser (R) Thr-102/106-Ser (r) | Rr (49) | Compound heterozygous | TIPS (Rr) |

*Note*: WT = wild type; TIPS = Thr-102-Ile + Pro-106-Ser.

*Numbers in brackets denote the genotypic frequency found in the genotyped natural field collected *E. indica* population.

vegetative and reproductive growth and compared to the WT genotype in the absence of glyphosate selection. The magnitude (%) of a resistance cost was estimated as (Vila-Aiub, Gundel, & Preston, 2015)

$$RC = 1 - \frac{W_R}{W_S} x\ 100 \quad (1)$$

where $W$ denotes the quantitative estimation of a fitness trait of resistant ($W_R$) rr, RR, or Rr TIPS and susceptible ($W_S$) WT EPSPS gene variants.

## 2.3 | Physiological resistance cost

Direct or physiological resistance costs originate directly from pleiotropic effects of the resistance trait itself on resource allocation patterns that compromise growth and reproduction (Strauss, Rudgers, Lau, & Irwin, 2002). Plants were grown from seedling to maturity in the absence of competitive interactions, and resource allocation to vegetative and reproductive biomass was estimated. One-way analysis of variance (ANOVA) was performed to determine main genotype effects on vegetative and reproductive growth traits (α = 0.05).

## 2.4 | Early seedling growth

Seeds of similar size from the purified WT, RR TIPS, and P106S were germinated on 0.6% (w/v) agar and incubated at fluctuating temperature regime of 30/20 °C with 12 hr photoperiod at photosynthetically active radiation (PAR) of 530 μmol·m$^{-2}$·s$^{-1}$. At the two-leaf stage, seedlings from each genotype were transferred to petri dishes (120 mm × 120 mm) containing 0.8% (w/v) agar and half strength nutrient solution containing 200 μM $K_2SO_4$, 100 μM $MgCl_2$, 100 μM $NH_4NO_3$, 700 μM $Ca(NO_3)_2$, 20 μM $KH_2PO_4$, 500 μM MES, 20 μM FeNaEDTA, 2 μM $H_3BO_3$, 0.25 μM $MnSO_4$, 0.3 μM $ZnSO_4$, 0.1 μM $CuSO_4$, 0.03 μM $Na_2MoO_4$, 0.2 μM $CoSO_4$, and 0.1 μM $NiSO_4$ (pH to 5.5–6.0). Seedlings (seven seedlings per petri dish) were incubated at the same environmental conditions as described above. Seedling fresh weight was evaluated 11 and 15 days after germination. Five to eight replicates per treatment were used.

## 2.5 | Dynamics of vegetative plant growth

Seeds of WT, P106S, and RR TIPS plants were germinated as described above. Two-leaf stage seedlings were transplanted into pots containing a substrate of composted fine pine bark (50%), coco-peat (30%), and washed river sand (20%) and grown in glasshouse conditions during the summer growing season (fluctuating temperature of 30/24 °C). A total of 25 individual plants per genotype were grown in pots (Ø = 20 cm, height = 20 cm) and relative growth rate (RGR) of above-ground biomass (leaf and stem) during the vegetative stage was weekly estimated during a 34-day growing period after seedling transplanting. The unbiased formula proposed by Hoffmann and Poorter (2002) was used to determine the RGR for each genotype. The variance ($V$) associated with RGR was estimated according to Causton and Venus (1981).

Resource allocation to vegetative biomass was estimated by weighing harvested plants that had been oven-dried at 60 °C for 3 days. Plants were daily watered and fertilized and frequently rearranged to randomize environmental differences in the glasshouse. Final resource allocation to vegetative tissues was estimated at the end of the growth cycle in single plants ($n$ = 25) of each genotype growing in pots (Ø = 30 cm, height = 30 cm) containing the above mentioned substrate.

## 2.6 | Plant growth at maturity

Repeated experiments under glasshouse ($n$ = 25; WT vs. P106S vs. RR TIPS) and outdoors summer ($n$ = 10; WT vs. RR TIPS vs. Rr TIPS) conditions were conducted to assess the reproductive growth of individual plants growing in the above mentioned substrate.

Inflorescences produced by individuals from each genotype were sequentially collected from first maturity. Inflorescences from each individual plant were threshed to separate the seed from chaff and rachis material, and total seed mass was quantified. The number of seeds ($S_n$) produced per *E. indica* plant was estimated as

$$S_n = \frac{TS_w\ 100}{S_w} \quad (2)$$

where $TS_w$ denotes the total seed weight produced per plant, and $S_w$ represents the weight of 100 seeds per plant ($n$ = 3).

The relative proportion of resources allocated to seeds (i.e., reproductive effort) in individual plants of each genotype was estimated according to Reekie and Bazzaz (2005):

$$RE\ (\%) = \frac{Sb}{Tb} 100 \quad (3)$$

where $Sb$ denotes the biomass contained in all seeds produced, and $Tb$ is the total (stems, leaves, inflorescences, and seeds) biomass produced per plant.

The experimental design enabled the quantification of the dominance ($h$) of the resistance cost (Menchari, Chauvel, Darmency, & Delye, 2008; Roux et al., 2004) associated with the EPSPS resistance allele 102-Ile/106-Ser (R). By convention, the resistance TIPS allele (R) is dominant, semidominant, or recessive when $h$ approaches to 1, 0.5, or 0, respectively. The dominance ($h$) is calculated as

$$h = \frac{W_{WT} - W_{RrTIPS}}{W_{WT} - W_{RR\ TIPS}} \quad (4)$$

where $W_{WT}$, $W_{RrTIPS}$, and $W_{RRTIPS}$ is the mean fitness trait of the WT, Rr, and RR TIPS genotypes, respectively.

## 2.7 | Ecological resistance cost

Ecological resistance costs arise when ecological interactions such as competition or herbivory trigger or amplify the expression of resistance costs (Strauss et al., 2002). Plants were grown from seedlings to maturity under competition from a rice crop, and estimation of resource allocation to vegetative and reproductive biomass was conducted. One-way ANOVA was performed to determine main genotype effects on vegetative and reproductive growth traits (α = 0.05).

A target-neighbourhood design was conducted to evaluate the competitive responses of P106S and RR TIPS plants under crop competition in an experimental garden under the normal summer growing

conditions for this species. This experimental design evaluates the vegetative and/or reproductive growth of a target plant under increasing density of neighbour crop plants (Goldberg & Werner, 1983; Weiner, 1982). In the absence of neighbour plants, the response of the target plant is expected to be maximum, and the continuous increase of neighbour density/biomass is likely to reduce the target plant response. The experimental design allows the quantification of ecological resistance costs associated with the particular *E. indica* EPSPS gene variants (Vila-Aiub, Neve, & Powles, 2009a). To achieve this goal, vegetative and reproductive growth of individuals from the EPSPS variants (RR TIPS, P106S) and WT under size symmetric competition with a rice (*Oriza sativa*) crop (Yunlu 29 cv.) was assessed. Experiments were conducted outdoors during the normal summer growing season.

Rice seeds were soaked in tap water overnight and germinated on 0.6% (w/v) agar for 6 days at fluctuating temperature 30/20 °C with 12 hr photoperiod and PAR of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Two days later, seeds of WT, P106S, and RR TIPS plants were also germinated in the same environmental conditions. Two rice densities (0, 5 in each experimental unit), which equal to 0, 71 rice plants $m^{-2}$, were spatially arranged (Supporting Information). Two days later, after rice transplanting, individual two-leaf stage *E. indica* seedlings were transplanted into single plastic pots (Ø = 30 cm, height = 30 cm) containing the above mentioned organic substrate. Plants were regularly watered and fertilized.

Above-ground vegetative biomass of target plants (WT, P106S, and RR TIPS genotypes) competing with neighbouring rice plants was evaluated at maturity. Biomass of target plants was oven-dried as previously described. Treatments (3 genotypes × 2 rice densities) were replicated nine times comprising 54 experimental units. Reproductive biomass produced by individuals from each genotype (WT, P106S, and RR TIPS) was sequentially collected from first maturity, up to 99 days after seed germination. Seed heads produced from *E. indica* genotypes were collected and reproductive traits estimated as described above.

## 2.8 | GC-MS analysis of polar metabolites, in particular phenylalanine, tyrosine, tryptophan, shikimic acid, and quinic acid

Plants of the three genotypes (WT, P106S, and RR TIPS) were grown in a phytotron room under 30/25 °C day/night temperature and light intensity of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The six to seven leaf stage plants were harvested from above-ground and snap-frozen in liquid $N_2$. There are four biological replicates each with eight to 10 plants. Plant material (2 g) from each genotype was ground in liquid $N_2$ with a mortar and pestle into fine powder. About 50 mg was subsampled into 2 ml Eppendorf extraction tubes and kept in liquid nitrogen until extraction. Chloroform (100 µl) was added to the tubes and vortexed for 30 sec in a MultiTube-Vortexer and followed by addition of 300 µl of 90% methanol containing three internal standards (100 µM $^{13}C_5$, $^{15}N$-valine, 10 µM $^{13}C_6$-sorbitol and 10 µM ribitol). The samples were vortexed for 1 min and incubated with shaking for 15 min at 500 rpm. Then 200 µl of water was added and vortexed for 1 min and centrifuged for 3 min at 20,800 g. The aqueous supernatant (100 µl) was transferred into a 300 µl glass insert in a 2 ml GC vial and evaporated to dryness for 3 hr in a vacuum evaporator. The vials were capped with magnetic crimp tops.

## 2.9 | TMS derivatization for the analysis of polar metabolites, in particular shikimic acid and quinic acid

A calibration curve of pure standards was prepared for the organic shikimic and quinic acids with a concentration range of 0, 0.2, 1, 5, 20, and 50 nmol/mg fresh weight and extracted as described above. Samples were derivatized using a CTC autosampler, and 20 µl of methoxylamine hydrochloride (20 mg/ml) in pyridine was added. The samples were incubated at 37 °C for 2 hr with agitation at 750 rpm. Then 20 µl N-methyl-N-(trimethylsilyl) trifluoroacetamide was added and incubated at 37 °C for 30 min with agitation. After 1 hr, incubation at RT 1 µl sample extract was injected onto the GC/MS in the splitless mode.

The samples were analysed on a 7890A GC coupled to a 5975C MSD (Agilent). The column used was a VF 5 ms 30 m × 250 µm × 0.25 µm with a 10 m guard column (Agilent J&W). The carrier gas was helium, and the column flow was 1 ml/min. The temperature gradient of the oven was 70 °C for 1 min, then 7 °C per minute to 325°C. The inlet, thermal auxiliary, MS source, and MS quadrupole temperatures were set to 250 °C, 280 °C, 230 °C, and 150 °C, respectively. The scan range was m/z 50–600. One-way ANOVA was performed to determine differences in shikimic and quinic acid concentration among WT, P106S, and TIPS genotypes ($\alpha = 0.05$).

## 2.10 | TBS derivatization for the analysis of amino acids, in particular phenylalanine, tyrosine, and tryptophane

A calibration curve of pure standards was prepared for the amino acids phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) with a concentration range of 0, 1, 2, 5, 10, 15, and 20 nmol/mg fresh weight and extracted as described above. The samples were derivatized using a CTC autosampler, and 20 µ of methoxylamine hydrochloride (20 mg/ml) in pyridine was added. The samples were incubated at 75 °C for 20 min with agitation at 750 rpm. Then 40 µl N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane was added and incubated at 75 °C for 20 min with agitation. After 1 hr, incubation at RT 1 µl sample extract was injected onto the GC/MS in the splitless mode. The temperature gradient of the oven was 70 °C for 1 min, then 1 °C per minute to 75 °C and finally 6 °C per minute to 325 °C for 5 min. The inlet, thermal auxiliary, MS source, and MS quadrupole temperatures were set to 300 °C, 250 °C, 230 °C, and 150 °C, respectively. The scan range was m/z 70–650. One-way ANOVA was performed to determine differences in Phe, Tyr, and Trp pools among WT versus P106S and TIPS genotypes ($\alpha = 0.05$).

Polar metabolites were identified in ADMIS Version 2.72 (NIST) using the Metabolomics Australia in-house library and NIST 14 library Version 2.2 for mass spectral comparison, and quantified in MassHunter Workstation Software Quantitative Analysis Version B.06.00 for GCMS (Agilent). Eighty-two compounds were identified or putatively identified in AMDIS and used for statistical analysis.

Statistical and metabolic pathway analysis was performed using the online software MetaboAnalyst 3.0 (Xia Lab/McGill University). All data were normalized to the three internal standards and the fresh weight, log transformed and auto-scaled (mean-centred and divided by the standard deviation of each variable). Compounds with fold change ≥2 and $p \leq .05$ in the *t* test performed for P106S versus WT, and RR TIPS versus WT data set were visualized via Volcano Plot analysis.

## 3 | RESULTS

In a series of experiments in both glasshouse and field conditions, the vegetative and reproductive growth of *E. indica* plants was quantified without any glyphosate treatment. This enabled assessment of the expression of ecophysiological resistance costs associated with the homozygous RR and compound heterozygous Rr TIPS and P106S glyphosate resistance genotypes.

### 3.1 | Physiological resistance cost

#### 3.1.1 | Plant growth at early seedling stage

Despite optimal growing conditions (nutrient, temperature and light, no competition), the RR TIPS seedlings produced much less biomass than the WT or the P106S resistant plants during early vegetative growth (Table 2). Just 11 days after seed germination, RR TIPS seedlings exhibited 30% less total biomass than WT and P106S seedlings (Table 2). Over the first 35 days of growth (glasshouse, summer growing season, 30/24 °C), the RR TIPS plants displayed a significantly ($p < .001$) lower RGR (0.25/day) than WT (0.31/day) or P106S (0.32/day) plants (Figure 1). As a result, RR TIPS plants showed a reduction in the amount of resources allocated to above-ground vegetative biomass compared to both WT and P106S plants that showed similar resource acquisition (Figure 1). After 35 growing days, the RR TIPS plants produced 1 g of above-ground biomass whereas both the WT and P106S plants exhibited 4 g biomass (Figures 1 and 2).

**TABLE 2** Estimated growth of *Eleusine indica* seedlings in absence of glyphosate selection carrying the wild type, P106S, and RR TIPS EPSPS gene variants

| | Total biomass (mg/plant) | |
|---|---|---|
| | Days since germination | |
| Genotype | 11 | 15 |
| WT | 108[a] (10) | 209 [a] (9) |
| P106S | 104[a] (12) | 275[a] (35) |
| RR TIPS | 70[b] (4) | 151[b] (12) |
| *p* | *.02* | *.002* |

*Note*. Growth was assessed as individual total fresh weight after 2 weeks since germination at fluctuating 30/20 °C (12 h photoperiod) temperature. Values are mean fresh weight ($n$ = 5–8) ± *SE* in brackets; *p* values after ANOVA. Different letters indicate significant differences between genotypes within each growth time, according to Tukey's honest significant difference (HSD) test (α = 5%). WT = wild type; TIPS = Thr-102-Ile + Pro-106-Ser; R = 102-Ile/106-Ser; r = Thr-102/106-Ser.

Different letters indicate significant differences between genotypes within each growth time, according to Tukey's honest significant difference test (α = 5%)

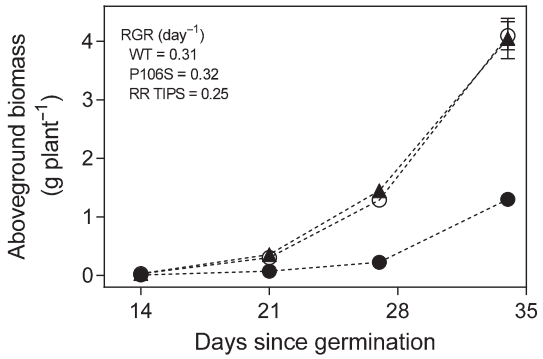


**FIGURE 1** Estimated vegetative biomass and relative growth rate (RGR) of *Eleusine indica* plants under no glyphosate treatment carrying the wild type (WT [○]), P106S (▲), and homozygous RR TIPS (●) EPSPS gene mutations over 4 weeks since seed germination under glasshouse conditions (January–February 2014). Data are mean ($n$ = 19–25) with standard errors as vertical bars

#### 3.1.2 | Plant growth at maturity

As experiments under glasshouse and field conditions yielded similar results, only glasshouse results are shown. At plant maturity (105 growing days), remarkable differences in the vegetative and reproductive growth were evident among the EPSPS gene variants (Figure 3). The RR TIPS plants produced only half of the aboveground biomass and only a third of the number of seeds compared to the WT or the P106S plants (Figure 3). Considering the number of produced seeds, a glyphosate resistance cost of 69% was associated with the RR TIPS plants. It is noteworthy that RR TIPS plants also showed a change in the reproductive effort denoted by 5% less resources allocated to reproductive organs compared to P106S plants that exhibited a similar RE to WT plants (Figure 3). Resource allocation patterns to vegetative and reproductive organs of the WT and P106S plants were similar (Figure 3).

Remarkably, especially given the strong resistance cost of the RR homozygous TIPS double mutation, the compound heterozygous Rr TIPS plants exhibited similar vegetative and reproductive growth estimates to the WT denoting the absence of any glyphosate resistance cost (Figure 4). The Rr TIPS variant showed no decrease in the size of plants, number of inflorescences and seeds, and reproductive effort when compared to the WT variant (Figure 4). The dominance index of the resistance cost (*h*) associated with the 102-Ile/106-Ser TIPS alleles approached zero for all estimated growth traits, highlighting that the resistance cost associated with TIPS EPSPS gene mutation is expressed when only in the homozygous state.

#### 3.1.3 | Ecological resistance cost

The effect of resource competition from a competing rice crop on the vegetative and reproductive growth of WT, RR TIPS, and P106S plants grown under field conditions was evaluated in a glyphosate free environment. During the course of the experiment and as a measure of the intensity of the plant competition for light, PAR reaching the *E. indica* EPSPS variants was quantified. Only 80% and 5% of the PAR observed in competition free plants (1,650 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was available for

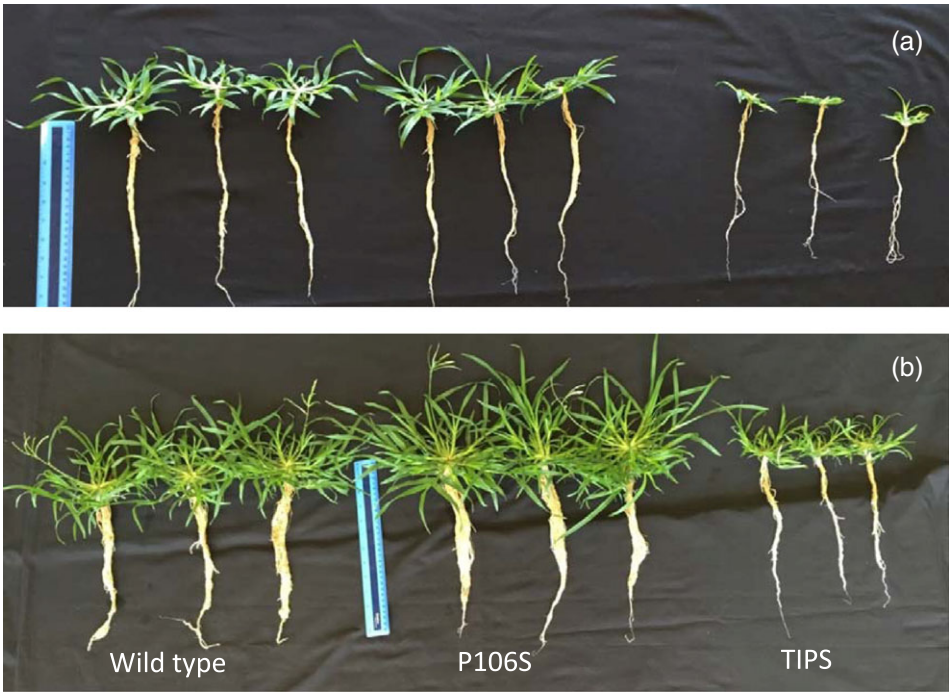


**FIGURE 2** (a) Vegetative growth of *Eleusine indica* wild type, P106S, and homozygous RR TIPS plants, 24 days and (b) 34 days after germination growing outdoor under summer conditions in the absence of glyphosate treatment [Colour figure can be viewed at wileyonlinelibrary.com]

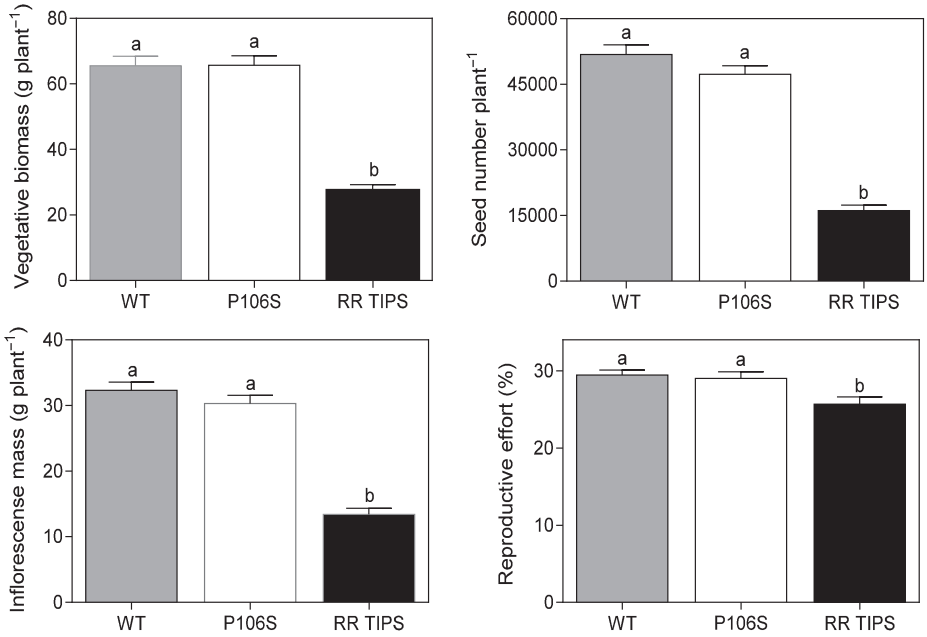


**FIGURE 3** Fitness traits of *Eleusine indica* wild type (WT), P106S, and RR TIPS in a glyphosate free environment. Traits were estimated at plant maturity (105 days of growth since germination) under glasshouse conditions. Vertical bars denote standard errors of the mean ($n$ = 25–26). Different letters indicate significant differences according to Tukey's multiple comparison test (α = 0.01, except for the reproductive effort trait, α = 0.05)

*E. indica* plants competing under the rice canopy during the vegetative and reproductive stage, respectively (Supporting Information).

Understandably, regardless of genotype, rice crop competition was a major force ($p$ < .001) in determining overall *E. indica* vegetative and reproductive growth (Figure 5). After nearly 100 days of growth in competition from a rice crop, total above-ground biomass, inflorescence biomass, and seed number were reduced in all the three *E. indica* genotypes. Rice competition reduced the relative proportion of resources allocated to *E. indica* reproductive tissues (i.e., reproductive effort) in RR TIPS but not in P106S and WT plants (Figure 5). Despite the large rice competition effect, *E. indica* WT, P106S, and RR genotypes showed significant differences in their vegetative and

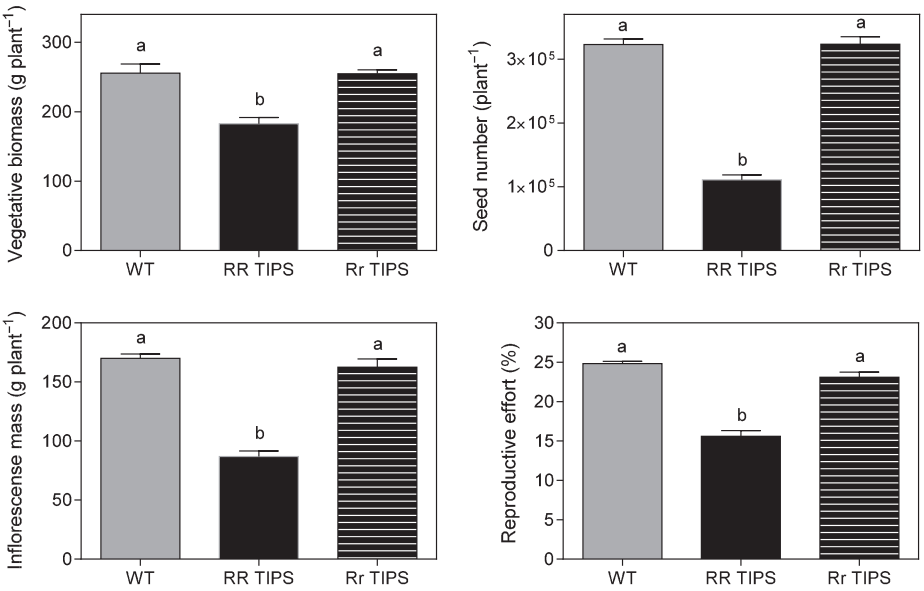


**FIGURE 4** Fitness traits of *Eleusine indica* plants with the RR TIPS or Rr TIPS EPSPS gene mutations compared to wild type (WT) under no glyphosate treatment. Fitness traits were estimated at plant maturity (103 days of growth since germination) under natural conditions. Vertical bars denote standard errors of the mean ($n$ = 10). Different letters indicate significant differences among genotypes according to Tukey's honest significant difference test (α = 5%). TIPS = Thr-102-Ile + Pro-106-Ser; R = 102-Ile/106-Ser; r = Thr-102/106-Ser

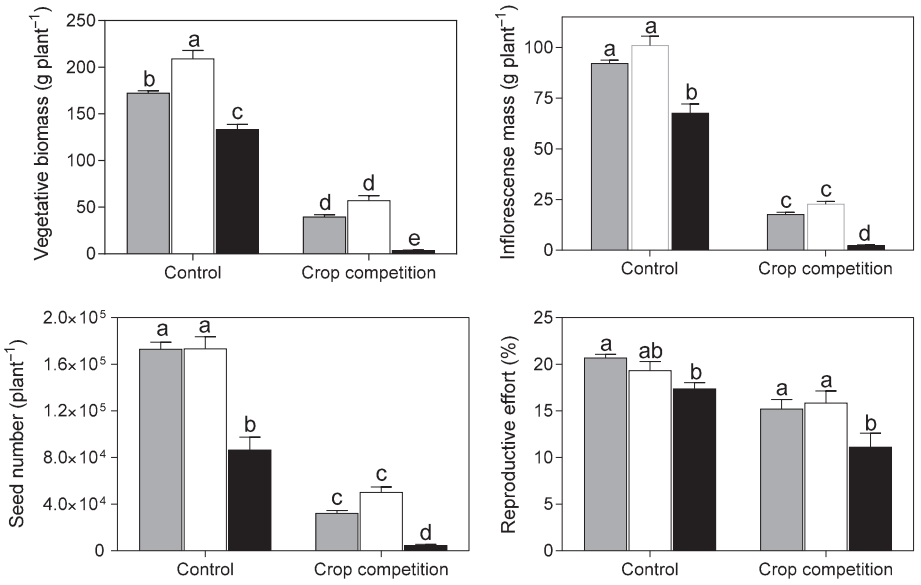


**FIGURE 5** Fitness traits of *Eleusine indica* wild type (WT [■]), P106S (□), and homozygous RR TIPS (■) plants under resource competition from rice in a glyphosate free environment. Traits were estimated at plant maturity after 98 days of growth since germination under field conditions (November 2014–February 2015). Vertical bars denote standard errors of the mean ($n$ = 10, except for reproductive effort $n$ = 5). Different letters indicate significant differences according to Tukey's multiple comparison test (α = 0.05)

reproductive growth (Figure 5). As observed in previous experiments under controlled (Table 2), glasshouse (Figures 1--3) and field (Figure 4) conditions, the total vegetative above-ground biomass, inflorescence biomass, number of seeds produced, and overall amount of resources allocated to reproduction were significantly impaired in RR TIPS plants compared to WT and P106S plants growing without rice competition (Figure 5).

The impaired growth in RR TIPS plants constituted a significant resistance fitness cost that was magnified in the presence of a competitive rice crop (Table 3). When considering the biomass of inflorescences and number of seeds produced under no competition, a resistance cost of approximately 30% and 50% in RR TIPS plants was, respectively, identified (Table 3). However, this cost increased significantly under rice competition, accounting for approximately 90% and 85% in both reproductive traits, respectively (Table 3). Similarly, the glyphosate resistance cost increased up to 90% and 40% from 23% and 16% for vegetative growth and reproductive effort, respectively (Table 3). On the contrary, the P106S plants had no resistance cost evident either with or without rice competition (Table 3).

**TABLE 3** Estimated ecological resistance cost associated with the *Eleusine indica* P106S and RR TIPS EPSPS gene variants

| | Control | Crop competition |
|---|---|---|
| Genotype | Vegetative biomass | |
| P106S | 0% | 0% |
| RR TIPS | 23% | 90% |
| | Inflorescence biomass | |
| P106S | 0% | 0% |
| RR TIPS | 27% | 87% |
| | Seed number | |
| P106S | 0% | 0% |
| RR TIPS | 50% | 85% |
| | Reproductive effort | |
| P106S | 7% | 0% |
| RR TIPS | 16% | 40% |

*Note*. Resistance cost was evaluated in terms of final above-ground vegetative and inflorescence biomass, seed number, and reproductive effort under rice competitive and uncompetitive conditions. Resistance cost ranges from 99% (significantly high cost) to 0% (no cost). TIPS = Thr-102-Ile + Pro-106-Ser; R = 102-Ile/106-Ser.

### 3.1.4 | GC-MS analysis of polar metabolites, in particular phenylalanine, tyrosine, tryptophan, shikimic, and quinic acid

EPSPS is a key enzyme in the shikimate pathway and target of glyphosate herbicide (Herrmann & Weaver, 1999). As the TIPS mutation has been shown to significantly reduce EPSPS Vmax (Yu et al., 2015), potential changes in metabolite levels in the shikimate pathway likely have a causal effect on the resistance cost observed in RR TIPS plants. Eighty-two polar metabolites were analysed in RR TIPS, rrP106S, and WT, with particular attention to those involved in the shikimate pathway.

In the absence of glyphosate treatment, a major difference was found in the concentration of shikimic and quinic acids of RR TIPS plants compared to WT and P106S plants (Figure 6). The TIPS plants exhibited 11- and sixfold, respectively, more shikimic and quinic acids than WT, whereas no significant difference in the concentration of these EPSPS substrate precursors was observed between P106S and WT plants (Figure 6). The downstream biosynthesis of the three aromatic amino acids was little affected (Figure 6). Whereas a marginal increase of Phe was observed in TIPS compared to WT, no change in Tyr and Trp content was found in either the TIPS or P106S compared to WT (Figure 6).

In addition to the significant changes in the background concentration of shikimic and quinic acids in the TIPS plants, significant ($p < .05$) ≥2 fold-changes in the concentration of 25 polar metabolites in TIPS, and only two metabolites in P106S were identified as shown in the Volcano Plot analysis when compared to WT (Figure 7, Tables S1 and S2). These include a higher level of eight metabolites (shikimic acid, L-homoserine, quinic acid, L-asparagine, L-glutamine, L-alanine, glycine, and L-serine) and a lower level of 17 metabolites (fructose-6-phosphate, threonic acid, ascorbic acid, glucose-6-Phosphate, mannose-6-Phosphate, sinapic acid, gentiobiose, galactose, cis-aconitic acid, myo-Inositol, maltose, malonic acid, 3-phosphoglyceric acid, trehalose, mannitol, arbutin, and galactinol) were detected in TIPS compared to WT.

Metabolic pathway analysis was performed in a pairwise manner comparing TIPS versus WT and P106S versus WT genotypes. To account for a significant pathway change among the EPSPS gene variants all factors (Hits, Raw $p$ value related to $-\log_{10}(p)$, Holm adjusted $p$, false discovery rate and impact) were considered using the MetaboAnalyst software. Whereas P106S versus WT analysis resulted in no significant changes in metabolic pathways (Table S3), significant disturbance in at least 10 metabolic pathways was found in TIPS versus WT (Table S4). TIPS plants showed changes not only in the shikimate pathway but also in the metabolism of galactose, methane, pyruvate, glyoxylate, dicarboxylate, inositol phosphate, starch, sucrose, ascorbate and aldarate, citrate cycle, and pantothenate and CoA biosynthesis (Table S4).

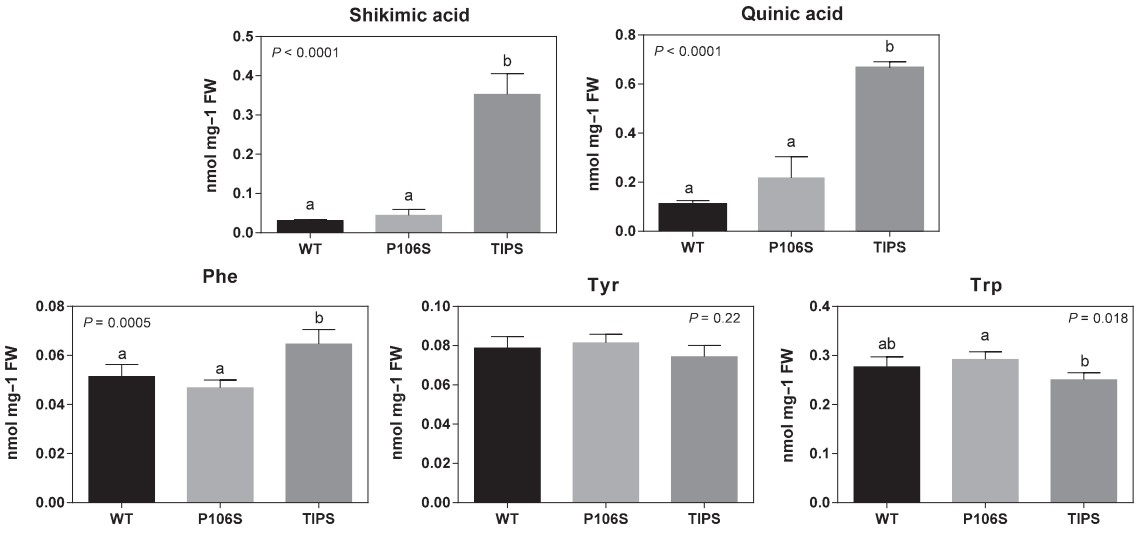


**FIGURE 6** Tissue contents of shikimic and quinic acids, and aromatic amino acids Phe, Tyr, and Trp in *Eleusine indica* wild type (WT), homozygous P106S, and TIPS plants in the absence of glyphosate treatment by GC-MS analysis. Vertical bars denote standard errors of the mean ($n = 4$); $p$ values after analysis of variance, and different letters indicate significant differences among genotypes according to Tukey's honest significant difference test (α = 5%). Phe = phenylalanine; Tyr = tyrosine; Trp = tryptophan; TIPS = Thr-102-Ile + Pro-106-Ser

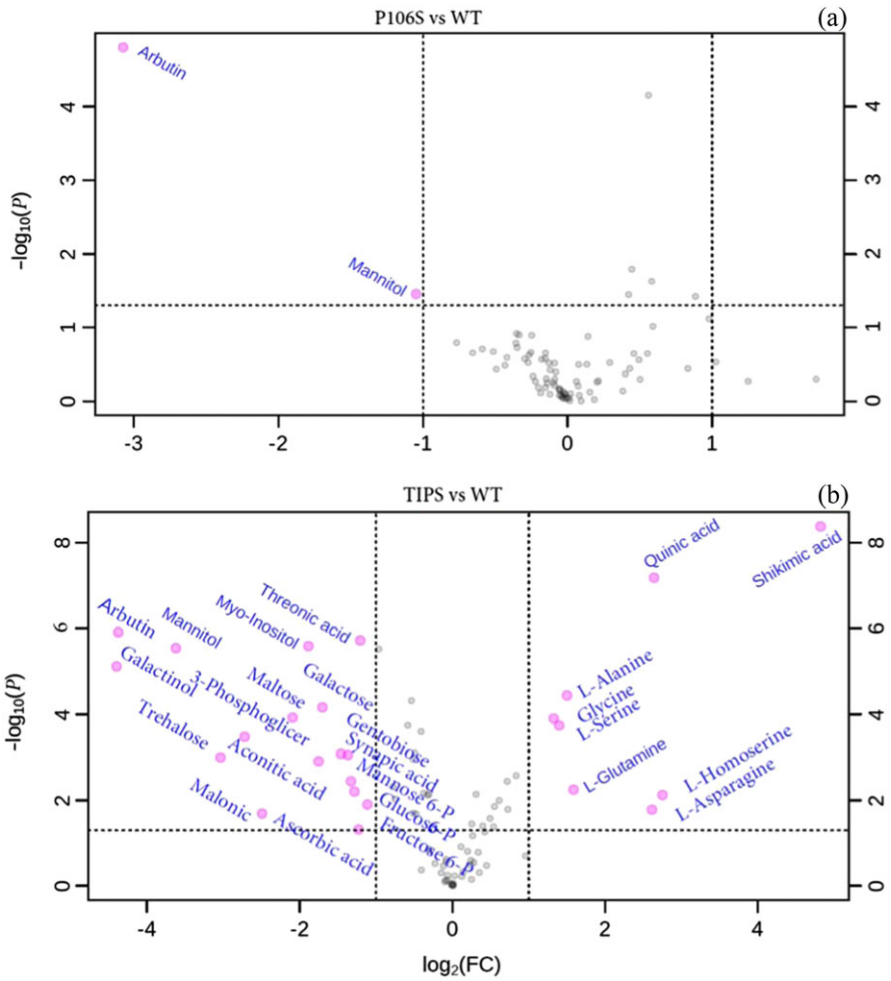


**FIGURE 7** Identified polar metabolites with significant quantitative changes in *Eleusine indica* (a) homozygous P106S versus WT and (b) homozygous TIPS versus WT. Changes were detected by volcano plot analysis that combines both significant fold changes (FC ≥2) in metabolites and *t* test ($p \leq .05$). TIPS = Thr-102-Ile + Pro-106-Ser; WT = wild type [Colour figure can be viewed at wileyonlinelibrary.com]

## 4 | DISCUSSION

The present study shows that there is a clear expression of a significant resistance fitness cost associated with the homozygous RR TIPS glyphosate resistance-endowing double mutation that is not evident in the homozygous P106S nor the compound heterozygous Rr TIPS EPSPS variants in *E. indica*. Only 11 days after seed germination, the homozygous TIPS seedlings expressed a reduced RGR that resulted in mature plants of limited size yielding 30% less inflorescences and 60% less seeds compared to the WT or to the homozygous P106S mature plants (Figure 3, 4). Resource allocation theory predicts that resistance fitness costs will be magnified under stressful conditions (Bergelson & Purrington, 1996; Marak, Biere, & Van Damme, 2003; Van Dam & Baldwin, 2001). Accordingly, under competition from a vigorously growing rice crop (severely limiting light penetration), the glyphosate resistance cost associated with the homozygous RR TIPS variant increased to 85%, 90%, and 40% in relation to seed number, inflorescence biomass, and reproductive effort traits, respectively (Table 3). The very high resistance fitness cost of the homozygous RR TIPS mutation endowing glyphosate resistance has only been matched by the 95% cost associated with the Trp-574-Leu ALS gene mutation in *Amaranthus powellii* (Tardif, Rajcan, & Costea, 2006). Thus, the TIPS double mutation is the most severe resistance cost reported in naturally evolved herbicide resistant weed species in agriculture.

Notably, the high resistance cost associated with the homozygous RR TIPS mutation is not expressed in plants with the Rr TIPS mutation, which show equal fitness to WT (Figure 4). This result denotes the recessive nature of the resistance cost associated with the TIPS allele (102-Ile/106-Ser). This is likely due to the two different EPSPS alleles in compound heterozygous Rr TIPS plants, which encode two different EPSPS enzymes with equal amount: TIPS enzyme conferring high level glyphosate resistance compensated by P106S enzyme functioning normally with no resistance cost.

Along with the results reported here, Li et al. (2016) have recently shown that a CRISP/Cas9 edited homozygous TIPS mutation in rice is very likely to be lethal as they did not find homozygous TIPS mutants in 93 T2 plants analysed. This is, in essence, in line with our observation that in 193 *E. indica* plants analysed only 1.6% were homozygous RR TIPS mutants (Yu et al., 2015). The Li et al. (2016) study also reports that the heterozygous TIPS mutation, unlike the results presented here, also attracts a resistance cost in the EPSPS edited rice plants. However, it must be noted that results are not comparable as our so called heterozygote TIPS plants (i.e., containing a TIPS allele and a P106S allele) is different from the true heterozygote TIPS in Li et al. (2016) paper (i.e., containing a TIPS allele and a WT allele).

### 4.1 | Physiological basis of fitness cost associated with RR TIPS

Adaptive resistance mutations in bacteria (Cohan, King, & Zawadzki, 1994; Melnyk, Wong, & Kassen, 2015), insects (Chevillon, Pasteur, Marquine, Heyse, & Raymond, 1995; Groeters, Tabashnik, Finson, & Johnson, 1994), and plants (Darmency, 2013; Vila-Aiub et al., 2009b) have been shown to incur resistance costs when they compromise normal function and metabolism (Uyenoyama, 1986). In particular, herbicide resistance point mutations may potentially trigger a number of biochemical changes at the herbicide target enzyme impairing enzyme catalytic capacity, reducing substrate affinity, and/or altering feedback inhibition, resulting in insufficient or excessive product biosynthesis and thus accounting for a resistance cost (Purrington & Bergelson, 1999; Vila-Aiub et al., 2009b; Vila-Aiub, Yu, Han, & Powles, 2015; Yu et al., 2010). We have recently investigated the impact of the homozygous TIPS and P106S EPSPS glyphosate resistance-endowing gene mutations on EPSPS catalytic properties. When expressed in *Escherichia coli*, the TIPS mutation resulted in 15.5-fold lower EPSPS activity ($V_{max}$), whereas no impact on EPSPS activity was observed for the P106S mutation, relative to the WT (Yu et al., 2015).

Results reported here show that the reduced EPSPS catalytic efficiency of the TIPS mutation (Yu et al., 2015) causes increased concentration of shikimate (11-fold) and quinate (sixfold; Figure 6, 7) in the shikimate pathway. The higher levels of these two carbon compounds may arise as a consequence of both the reduced EPSPS $V_{max}$ and increase of carbon flux from photosynthetically fixed carbon (Herrmann & Weaver, 1999; Maeda & Dudareva, 2012; Steinrücken & Amrhein, 1980). Despite the higher background concentration of these metabolites in TIPS plants, the downstream biosynthesis of the aromatic amino acids Phe, Tyr, and Trp was little affected (Figure 6) suggesting that TIPS plants are somehow compensating the reduced EPSPS efficiency by recycling aromatic amino acids from destroyed proteins (Teixeira et al., 2017). This process would maintain the production of these essential aromatic amino acids for the biosynthesis of downstream secondary compounds such as lignin, alkaloids, and flavonoids (Maeda & Dudareva, 2012).

Thus, the high glyphosate resistance cost evident as reduced growth and reproduction in TIPS plants is not directly due to limited aromatic amino acid biosynthesis, especially the biosynthesis of the IAA precursor tryptophan (Figure 6) but likely due to altered concentration of carbon metabolites caused primarily by disturbance of shikimate and then other related metabolic pathways such as glycolysis and starch and sucrose metabolism among others (Figure 7, Table S3). A more thorough metabolomics approach is needed for better understanding of the resistance cost associated with the TIPS mutation.

### 4.2 | The evolutionary significance of results

Increase and fixation of novel herbicide resistance mutations in agro-ecosystems depends on their impact on plant survival and fecundity in both the presence (resistance benefit) and absence (resistance cost) of herbicide selection (see reviews by Bergelson & Purrington, 1996, Jasieniuk et al., 1996, Vila-Aiub et al., 2015a, Vila-Aiub et al., 2009b, Vila-Aiub et al., 2011). It is clear that herbicide resistance fitness benefit and cost play opposite evolutionary roles in favouring the genetic fixation or loss of novel resistance alleles. Whereas differences in resistance benefit among resistance traits define the rate of resistance allele enrichment in areas under herbicide selection, the expression of resistance costs define their equilibrium frequency in herbicide free areas.

Both at the enzyme and plant level, the RR TIPS mutation has been shown to endow a higher glyphosate resistance benefit than the P106S gene mutation in *E. indica* (Yu et al., 2015). Thus, in persistently glyphosate treated fields the TIPS (R) allele will exhibit a higher rate of enrichment than the P106S (r) alleles although both will increase their frequency at the expense of the WT (S) allele. In an agro-ecological scenario defined by the early evolutionary appearance of the novel *E. indica* EPSPS gene variants, it is possible to predict that the specific allele (r) encoding for the P106S variant will enrich and coexist with WT (S) alleles in glyphosate untreated areas whereas the same environment will pose severe limitations for the evolution of the TIPS allele (R).

Assuming a different agro-ecological scenario in which the R (TIPS) and r (P106S) glyphosate resistance EPSPS alleles have already enriched in an *E. indica* population and the glyphosate selection intensity is discontinued, the RR TIPS plants are likely to go extinct due to the observed major resistance fitness cost whereas the P106S and compound heterozygous Rr TIPS plants will likely continue to persist over time. Although an empirical validation for the above predictions is required, interestingly, in the field-collected glyphosate resistant *E indica* population, the majority (49%) of the resistant individuals found were compound heterozygous Rr with only minority of homozygous RR TIPS plants (<2%; Yu et al., 2015).

It is interesting that it does not appear to be a fitness cost of the TIPS mutation in genetically modified glyphosate resistant GA21 corn, which may be due to the integration (by biolistic transformation) at a single site of three TIPS EPSPS gene copies (Spencer, Mumm, & Gwyn, 2000) in addition to the endogenous WT EPSPS. Compensatory evolution of the herbicide resistance cost associated with the RR TIPS variant is possible in nature (Bergelson & Purrington, 1996; Darmency, Menchari, Le Corre, & Délye, 2015; Menchari et al., 2008; Paris, Roux, Berard, & Reboud, 2008; Vila-Aiub et al., 2009b; Yu et al., 2010). Natural selection of modifiers at other genetic loci leading to such as EPSPS amplification or over-expression may compensate and/or overcome the associated reduced EPSPS activity found in RR TIPS plants. This is possible as the TIPS mutation and increased EPSPS gene expression/amplification has been reported respectively in different glyphosate-resistant *E. indica* populations from China (Chen et al., 2015). If these traits are coexpressed in plants, then resistance cost of the TIPS mutation can be mitigated, assuming EPSPS gene amplification/overexpression does not incur significant resistance cost as demonstrated in *A. palmeri* (Vila-Aiub et al., 2014).

## 5 | CONCLUSION

The naturally evolved glyphosate resistance-endowing single (P106S) and double (TIPS) EPSPS gene mutations endow dramatically contrasting glyphosate resistance costs in the globally important *E. indica*.

Plants with the homozygous RR TIPS genotype incur a significantly high glyphosate resistance fitness cost as denoted by a 20% RGR reduction in isolated plants and 85% seed reduction when competing with a rice crop. However, this resistance cost associated with the homozygous TIPS mutation is not expressed in plants with the homozygous rr P106S genotype nor with the compound heterozygous (Rr TIPS mutation). This shows the recessive nature of the cost associated with the TIPS allele, which is triggered by the Thr-102-Ile mutation. Polar metabolite profiling reveals extensive perturbations of many metabolic pathways in the TIPS plants, which might be primarily caused by the metabolic changes in the shikimate pathway, and all these contribute to the observed glyphosate resistance-fitness trade off associated with the homozygous EPSPS TIPS mutation in *E. indica*.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Table S1. Identified polar metabolites with significant ≥2 fold changes in *Eleusine indica* P106S vs WT. Changes were detected after Volcano Plot analysis which combines both significant fold changes (FC ≥2) in metabolites and t-test ($P \leq 0.05$).

Table S2. Identified polar metabolites with significant ≥2 fold changes in *Eleusine indica* RR TIPS vs WT. Changes were detected after Volcano Plot analysis which combines both significant fold changes (FC) in metabolites (≥50% threshold value) and t-test ($P \leq 0.05$).

Table S3. Metabolic pathway analysis in P106S vs WT *EPSPS* gene variants. Changes in metabolic pathways are denoted when significant changes are met in all analysis criteria (Total compounds, Hits, Raw *P* value related to $-\log_{10}(P)$, Holm adjusted *P*, False Discovery Rate (FDR) and Impact) after analysis by MetaboAnalyst 3.0 software.

Table S4. Metabolic pathway analysis in RR TIPS vs WT *EPSPS* gene variants. Highlighted metabolic pathways correspond to significant changes in all analysis criteria (Total compounds, Hits, Raw *P* value related to $-\log_{10}(P)$, Holm adjusted *P*, False Discovery Rate (FDR) and Impact) reported by MetaboAnalyst 3.0 software.

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