RUNNING TITLE: Evolution of a highly glyphosate-resistant EPSPS in *Eleusine indica*

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Evolution of a Double Amino Acid Substitution in the EPSP Synthase in *Eleusine indica* Conferring High Level Glyphosate Resistance

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**ONE-SENTENCE SUMMARY:** This paper identifies and characterises crop field-evolved EPSPS double mutants (TIPS) that mimic engineered glyphosate-tolerant EPSPS in crops and confers high level glyphosate resistance.
Foot notes:

Our research is supported by the Grains Research and Development Corporation of Australia (GRDC) and the Australian Research Council (ARC).
Glyphosate is the most important and widely used herbicide in world agriculture. Intensive glyphosate selection has resulted in the widespread evolution of glyphosate resistant weed populations, threatening the sustainability of this valuable once-in-a-century agrochemical. Field-evolved glyphosate resistance due to known resistance mechanisms is generally low to modest. Here, working with a highly glyphosate-resistant Eleusine indica population, we identified a double amino acid substitution (T102I+P106S)(TIPS) in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene in glyphosate resistant individuals. This TIPS mutation recreates the biotechnology-engineered commercial first generation glyphosate-tolerant EPSPS in corn and now other crops. In E. indica, the naturally evolved TIPS mutants are highly (>180-fold) resistant to glyphosate compared to the wild type (WT), and more resistant (>32-fold) than the previously known P106S mutants. The E. indica TIPS EPSPS showed very high level (2647-fold) in vitro resistance to glyphosate relative to the WT, and is (600-fold) more resistant than the P106S variant. The evolution of the TIPS mutation in crop fields under glyphosate selection is likely a sequential event with the P106S mutation being selected first and fixed, and then followed by the T102I mutation to create the highly resistant TIPS EPSPS. The sequential evolution of the TIPS mutation endowing high level glyphosate resistance is an important mechanism by which plants adapt to intense herbicide selection and a dramatic example of evolution in action.
INTRODUCTION

Modern herbicides make major contributions to global food production by easily removing weeds whilst maintaining sustainable soil conservation practices. However, persistent herbicide selection of huge weed numbers across vast areas has resulted in the widespread evolution of herbicide resistant weed populations. Worldwide, there are currently more than 433 unique cases of herbicide resistance, with on average about 11 new cases reported annually (Heap, 2014 online). Target-site resistance due to target gene mutation is one of the major mechanisms enabling resistance evolution (Gressel, 2002; Powles and Yu, 2010).

The most important and globally used herbicide in crop fields is glyphosate (Duke and Powles, 2008). Glyphosate disrupts the shikimate pathway by specifically inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Steinrucken and Amrhein, 1980). Glyphosate resistance was initially considered to be unlikely to evolve in nature based on the facts that intentional selection for glyphosate tolerance using whole plants and cell/tissue culture was unsuccessful and laboratory-generated highly resistant EPSPS mutants displayed undesirable enzyme kinetics (Bradshaw et al., 1997; reviewed by Pline-Srnic, 2006). This seemed to be true as resistance was not found during the first 15 years of glyphosate use, primarily as a non-selective herbicide. However, unprecedented intensive glyphosate use for controlling large numbers of weeds over massive areas, especially after the introduction of glyphosate-resistant transgenic crops imposed high selection pressure on weeds, resulting in the evolution of glyphosate resistance in populations of 31 weed species (Heap, 2015 online). Since the first identification of a resistance-endowing EPSPS point mutation, P106S in a glyphosate-resistant Eleusine indica population (Baerson et al., 2002) several other resistance-endowing single amino acid substitutions at P106 (P106T, P106A, P106L) have been reported in glyphosate-resistant weeds (e.g. Ng et al., 2004; Yu et al., 2007; Kaundun et al., 2011; reviewed by Sammons and Gaines, 2014). These single-codon EPSPS resistance mutations only endow low level glyphosate resistance (2-3 fold the recommended rates). This is not surprising, because glyphosate is a competitive inhibitor of the second substrate, phosphoenolpyruvate (PEP) (Boocock and Coggins, 1983) and is
considered a transition state mimic of the catalysed reaction course (Schönbrunn et al., 2001). Indeed, highly glyphosate-resistant EPSPS variants (e.g. mutants at G101 or T102) have greatly increased Km’s (decreased affinity) for PEP when expressed in *E. coli* (Eschenburg et al., 2002; Funke et al., 2009; reviewed by Sammons and Gaines et al., 2014).

In contrast, P106 substitutions confer weak glyphosate resistance but preserve adequate EPSPS functionality (Healy-Fried et al., 2007; reviewed by Sammons and Gaines, 2014). Aside from P106 EPSPS gene mutations there are other glyphosate resistance mechanisms, including EPSPS gene amplification, and non-target reduced glyphosate translocation/increased vacuole sequestration (Lorraine-Colwill et al., 2003; Gaines et al., 2010; Ge et al., 2010; reviewed by Powles and Preston 2006; Shaner, 2009; Powles and Yu 2010; Sammons and Gaines, 2014). Generally, each of these mechanisms endows moderate level (4-8 fold the recommended rates) glyphosate resistance.

Low level glyphosate resistance due to the EPSPS P106 mutations was reported in Malaysian *E. indica* (Baerson et al., 2002; Ng et al., 2004). Recently we reported a highly (>10 fold the recommended rates) glyphosate-resistant Malaysian *E. indica* population (Jalaludin et al., 2014). This paper investigates the high level glyphosate resistance in this population and reveals for the first time the sequential evolution of a double amino acid substitution in EPSPS.

**RESULTS**

**EPSPS Gene Sequencing Revealed a Double Amino Acid Substitution in EPSPS**

To identify the basis of very high level glyphosate resistance, a 301 bp EPSPS DNA fragment covering the highly conserved region (95LFLGNAGTAMRPL107) of the EPSPS gene was analysed from 43 resistant plants. These resistant individuals were found to have the known weak resistance mutation at codon 106 (CCA to TCA), but importantly, also display a very rare mutation at codon 102 (ACT to ATT). Therefore, in this naturally evolved, highly glyphosate-resistant *E. indica* population there are two resistance-endowing EPSPS amino acid substitutions, T102I and P106S. Cloning of the EPSPS cDNA fragment covering the 102 and 106 codons from resistant individuals revealed the two mutations were always present.
in the same EPSPS gene fragment, confirming the double amino acid substitution in a single
EPSPS allele. This double amino acid substitution, T102I and P106S is hereinafter referred to
as the TIPS mutation. Based on the sequence information obtained, dCAPS markers for the
T102I and P106S mutations were developed (Figure S1). Analysis (by the dCAPS markers and
sequencing) of 193 individuals (untreated) in the resistant population (Table 1) revealed that
84% of the individuals are resistant mutants and 16% are WT. Only a very small percentage
(1.6%) of the plants analyzed were homozygous mutants for the TIPS mutation (referred to
as RR genotype), about 30% were homozygous solely for the P106S mutation (rr), and nearly
half were the resistant mutants of Rr. Importantly, neither the single T102I mutants, or
heterozygous P106S single mutants (r/WT), or heterozygous TIPS mutants (R/WT) were
found from the samples analyzed. Therefore, only three alleles (R, r and WT) were found in
the samples examined and the frequency of mutant TIPS allele (R) is only half of the P106S
allele (r). To better understand the resistance allele frequencies a more detailed analysis of
the field population together with herbicide histories is needed. The full EPSPS cDNA
sequences (1338 bp) were compared among individuals of WT, P106S and the TIPS mutant
(GenBank accession number KM078728). Except for the SNPs at the 102 and 106 codons,
there was only one SNP that resulted in an amino acid change, a P381 in WT individuals, but
a L381 in mutant individuals. However, blast results showed that this amino acid residue is
not conserved in plant EPSPS, and the P381L mutation has been previously proven to be
irrelevant to glyphosate resistance in E. indica (Baerson et al., 2002)

Plants Homozygous for the TIPS Mutation Displayed a High Level Glyphosate Resistance

To characterise the glyphosate resistant genotypes we produced from within the
resistant population three purified sub-populations with individuals homozygous for WT,
P106S and TIPS EPSPS, respectively and conducted detailed glyphosate dose response
studies. To examine the possible involvement of any other glyphosate resistance
mechanisms in these purified sub-populations, a herbicide susceptible (S) E. indica
population was also included as a further reference. The S and WT populations were found
to be identically fully susceptible to glyphosate (Table 2, Fig. 1A, 1B, Fig.2), indicating no
major additional glyphosate resistance mechanisms present in the purified sub-populations.
As expected, the P106S population is only moderately resistant to glyphosate, with 30%
survival at the recommended field rate. Conversely, homozygous TIPS mutant plants were

found to be highly glyphosate-resistant such that a LD$_{50}$ could not be determined and therefore must be greater than the highest glyphosate rate used (25,900 g ha$^{-1}$) (Fig. 1A, Fig S2). Based on the glyphosate LD$_{50}$ ratios (Table 2), homozygous TIPS mutants are highly (more than 180-fold) resistant, whereas, as expected, homozygous P106S mutant are only moderately (5.6-fold) resistant. The homozygous TIPS plants, therefore, can tolerate more than 20-fold the recommended glyphosate rate of 1080 g ha$^{-1}$. While the TIPS mutants survived high glyphosate doses, their growth was affected (Fig. 1B), resulting in a lower glyphosate GR$_{50}$ ratio than the LD$_{50}$ ratio (Table 2).

**TIPS Encodes a Highly Glyphosate-resistant EPSPS**

To further characterise the TIPS mutation at the EPSPS level, the WT, P106S and TIPS *E. indica* EPSPS (EiEPSPS) were expressed in *E. coli*, and the activity and IC$_{50}$ (herbicide dose causing 50% *in vitro* inhibition) of the His-tagged recombinant EiEPSPS variants determined (Table 3). As expected, based on glyphosate IC$_{50}$ ratios, the *E. coli* expressed P106S variant is moderately (4.3-fold) resistant to glyphosate, while the TIPS variant is highly (2647-fold) resistant (Table 3, Fig. 3). These results confirm that TIPS EiEPSPS is essentially insensitive to glyphosate, with an IC$_{50}$ value of 54 mM, and therefore, responsible for very high level glyphosate resistance as observed at the whole plant level (Fig.1). Notably, while incurring no significant changes in the Km (PEP), the *E. coli* expressed TIPS variant displayed an EPSPS specific activity greatly (16-fold) lower than the WT (Table 3), indicating a resistance cost at the enzyme level due to reduced catalytic efficiency.

**DISCUSSION**

In the biotechnology search for glyphosate tolerant crops, various EPSPS double mutations have been generated using site directed mutagenesis and expressed in *E. coli* and plants (e. g. Spencer et al., 2000; Howe et al., 2002; Lebrun et al., 2003; Kahrizi et al., 2007; Alibhai et al., 2010). The P106 and then later the TIPS mutation were first found empirically in a mutational screen in *Salmonella sp.* (Comai et al. 1983; Stalker et al., 1985). The TIPS mutation was engineered into tobacco (Arnaud et al., 1998) and field tested for glyphosate tolerance (Lebrun et al., 2003). The TIPS EPSPS was then used to produce the first generation commercially successful glyphosate-tolerant transgenic corn (GA21) (Spencer et al., 2000). Here, we demonstrate that this TIPS mutation has now evolved in nature.
In target-site resistance evolution for acetolactate synthase (ALS)- and acetyl coenzyme A carboxylase (ACCase)-inhibiting herbicides, highly resistant yet fit individuals with single target-site mutations are common (Vila-Aiub et al., 2009), as these herbicides have large binding sites in and adjacent to the enzyme catalytic site resulting in contacts with amino acids that are non-essential for structure or function (Sammons et al., 2007; Powles and Yu, 2010). The most dramatic example is the multiple (more than 10) different amino acid substitutions at P197 in ALS (Tranel and Wright, 2002; Tranel et al. 2015 online). The transition state inhibitor designation for glyphosate comes from the observation that PEP contacts 17 amino acids responsible for catalysis which necessarily prevents any substitutions of these essential amino acids (Schönbrunn et al., 2001). The P106S EPSPS provides relatively low glyphosate resistance (Arnaud et al., 1998), whereas the T102I EPSPS alone endows high level resistance, but with drastically decreased affinity for the second substrate PEP (Kishore et al., 1992; Funke et al., 2009). The concomitant mutations at the 106 and 102 codons are merely adjacent to the active site and together make very small fractional Angstrom modifications structurally to the EPSPS active site, therefore selectively impacting glyphosate binding more than PEP (Funke et al., 2009). Therefore, the TIPS mutation endows high level glyphosate resistance with acceptable affinity for PEP.

Multiple mutations of a single pesticide target site gene are known in adaptive evolution of fungicide or insecticide resistance (Brunner et al., 2008; Karasov et al., 2010). Accumulation of multiple mutations in a single allele in insects and fungi can be achieved via intragenic recombination between pre-existing resistant alleles in natural populations, in response to increased selective pressure (Mutero et al., 1994; Brunner et al., 2008). However, this is unlikely to occur in *E. indica*, as the single-codon mutation T102I was not detected in the resistant population, therefore, unlikely pre-existing in the population. Indeed the very poor fitness of the kinetics of the T102I mutant enzyme (Alibhai et al., 2010) suggests this mutation would be unfit and even lethal when obtained alone. This lack of fitness of the T102I mutation explains why this single mutation has not been observed in nature.
The notion that compensatory mutations may require a particular evolution trajectory to prevent lethal mutants is discussed by Weinreich et al. (2006) where a series of 5 amino acid point mutations providing 100,000-fold resistance (compared to susceptible counterparts) to beta-lactamase in a matrix of combinations were studied to reveal a defined successful evolutionary pathway. Here, for the glyphosate-resistant *E. indica*, our data suggests that the TIPS evolved sequentially under intense glyphosate selection. First, the weak P106S mutation was selected, enriched and reached homozygosity and then was followed by the T102I mutation, to create the highly resistant TIPS EPSPS. This TIPS EPSPS enables plants to survive high glyphosate rates. Indeed, many glyphosate resistant *E. indica* populations in Malaysia and other countries have been found to possess mutations at P106 (Baerson et al., 2002, Ng et al., 2003, 2004; Kaundun et al., 2008) and in Malaysia, glyphosate was used frequently (every month) and continually (5-10 years) at increased glyphosate rates (0.72-1.92 k/ha) to control *E. indica* (Ng et al., 2004). Therefore, evolution of at least the TIPS mutation can be expected from other glyphosate resistant *E. indica* populations in Malaysia and other countries.

Will there be other EPSPS double mutations in nature? In addition to the TIPS mutant, various EPSPS double mutants at 102 and 106 were intentionally produced and the kinetics of *E. coli*-expressed EPSPS variants studied. Compared to the WT and T102I mutant alone, double mutants such as TIPA (T102I + P106A), TIPT (T102I +P106T) or TLPA (T102L + P106A) also show favourable kinetics comparable to or even better than TIPS (Alibhai et al., 2010; reviewed by Sammons and Gaines, 2014). As various amino acid substitutions at P106 have been identified (e.g. P106A, P106S, P106T or P106L) in glyphosate-resistant weed species (reviewed by Sammons and Gaines, 2014), the evolution and selection for other EPSPS double variants is also possible where glyphosate selection is intense.

Does the decreased catalytic efficiency of TIPS EPSPS have a whole plant resistance cost? The very low percentage (1.6%) of resistant individuals homozygous for the TIPS EPSPS (RR) as compared to the higher percentage (49%) of resistant individuals of Rr genotype (Table 1) may suggest (1) the additional T102I mutation is a recent event, and given that *E. indica* is a self-pollinated species, homozogosity at 102 can be increased in a few generations, and/or (2) a significant resistance cost is associated with homozygous TIPS.
mutants when glyphosate selection is relaxed. This latter correlates with the measured low catalytic turnover of the \textit{E. coli} expressed TIPS EiEPSPS (Vmax in Table 3). This decreased catalytic efficiency then translates to the significantly reduced plant growth (Figure 4, above ground dry weight per plant of WT (4.14 g ± 0.24), P106S (4.06 g ± 0.34) and TIPS (1.29 g ± 0.05) (n=20-25)) and fecundity we have observed for the homozygous TIPS plants (data not shown). Consequently, RR TIPS mutants are outperformed over time by Rr TIPS mutants which may suffer less or little fitness cost and therefore proliferate in the population. Due to predominate self-pollination in \textit{E. indica}, a very low level of out crossing (if any) between WT and homozygous P106S (rr) mutants may produce a small number of heterozygous P106S (r/wt) mutants. However, as expected, these individuals are unable to survive the field or higher glyphosate rates and hence are selected against. This would explain why the r/wt mutants were not detected in the resistant population. We have underway fitness studies with the WT, P106S (rr) and TIPS (RR, Rr) EPSPS mutants. However, if this fitness cost from decreased catalytic efficiency is offset, for instance, by gene duplication of the TIPS gene as required for commercial crops (CaJacob et al., 2004) then evolution of “Roundup Ready Like” \\textit{E. indica} may be expected in nature. Especially in species exhibiting EPSPS gene amplification (reviewed by Sammons and Gaines, 2014) where the tandem repeat nature of the duplication (Jugulam et al., 2014) may facilitate incorporation of the necessary point mutations, and gene duplication is free of fitness cost (Vila-Aiub et al., 2014).

Therefore, the evolutionary recipe to high level glyphosate resistance in weedy plant species under glyphosate selection may have these primary components: (1) overexpression of EPSPS, as already reported in 4 weed species with gene duplication; (2) P106S (or T/A)-EPSPS, as documented in 6 weed species (reviewed by Sammons and Gaines, 2014); (3) acquiring the second EPSPS T102I mutation, as described here for the first time, and (4) combining with other glyphosate resistance-endowing mechanisms that would have additive impact on the resistance magnitude, as demonstrated in glyphosate resistant \textit{L. rigidum} (Yu et al., 2007; Ge et al., 2012; Nandula et al. 2013).

In summary, this research reports for the first time the evolution of an EPSPS double mutation (TIPS) conferring very high level glyphosate resistance in crop fields. The TIPS mutation mimics the biotechnology-derived glyphosate tolerant EPSPS, demonstrating that laboratory and field selection methods are linked. This is a dramatic manifestation of the
power of evolution in action and how nature responds and adapts to manipulated
environment stresses. This is also a very clear example that herbicide sustainability demands
much greater diversity in weed control tactics than reliance on a single herbicide.
MATERIALS AND METHODS

Plant Material

The glyphosate-resistant *E. indica* population was originally collected from several patches within a palm oil nursery in Jerantut, Malaysia (Jalaludin et al. 2010), and the resistant and susceptible populations used in the current study were further characterised by Jalaludin et al. (2014). Seeds were germinated on 0.6% agar for 4-7 days and germinating seedlings transplanted into plastic pots (12-20 per pot) filled with potting mix (50% moss peat and 50% river sand) and grown in a glasshouse during the summer growing season (Dec to March) with average day/night temperature of 30/24°C and 13-h photoperiod under natural sunlight. When seedlings reach the 3-4 leaf stage they were treated with various rates of commercial glyphosate using a cabinet sprayer with a spray volume of 112 L ha\(^{-1}\) at a pressure of 200 kPa and a speed of 1 m s\(^{-1}\). Each treatment contained at least 3 to 4 replicate pots. Visual assessment for mortality was made 3-4 weeks after treatment. Plants were recorded as alive if they were actively growing and tillering after herbicide treatment and as dead if there was little new growth and no new tiller formation.

EPSPS Sequencing and cDNA Cloning

Genomic DNA was extracted from the leaf tissue of resistant and susceptible plants and total RNA isolated using the Plant RNeasy Mini Kit (Qiagen). Genomic DNA contamination was removed using the TURBO-DNA free kit (Ambion). For EPSPS DNA partial sequencing a pair of published primers (Ng et al., 2003) was used to amplify a highly conserved region (\(^{95}\)LFLGNAGTAMRPL\(^{107}\), refer to plant EPSPS numbering system) in which point mutations conferring glyphosate resistance in plants and bacteria have been found (Sammons and Gaines, 2014). The forward primer EleuEPSPS-F (5’-GCGGTAGTTGTGGCTGTGGTGG-3’) and the reverse primer EleuEPSPS-R (5’-TCAATCCGACAACCAAGTGC-3’) (Ng et al., 2003) amplify a 301 bp DNA (includes 99 bp intron) fragment covering the potential mutation sites. Using the same primer pairs a 202 bp (without intron) cDNA fragment was amplified from pre-genotyped plants and cloned into the pGEM-T vector (Promega) and transformed into *E. coli*. White colonies with putative inserts were used as templates for PCR re-amplification and sequencing of the 202
bp fragment. The PCR was conducted in a 25 µl volume that consisted of 1-2 µl of genomic DNA or cDNA, 0.5 µM of each primer, and 12.5 µl of 2× GoTaq Green Master Mix® (Promega). The PCR was run with the following profile: 94°C for 4 min; 40 cycles of 94°C for 30s, 57°C (annealing temperature) for 30s, and 72°C for 30-50s; followed by a final extension step of 7 min at 72°C. For EPSPS full cDNA sequencing, a 1338 bp cDNA was amplified with Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Inc.) using the primer pair (EiRT1 and EiLFT1, Baerson et al., 2002).

The PCR was conducted in a 20 µl volume that consisted of 1µl (80 ng) of cDNA, 0.5 µM of each primer, and 4 µl of 5× Phusion HF buffer, 200 µM dNTPs and 0.2 µl Phusion DNA Polymerase. The PCR was run with the following profile: 98°C for 30 sec; 40 cycles of 98°C for 10 sec, 72°C for 50 sec; followed by a final extension step of 10 min at 72°C. The PCR product was purified from agarose gel as above and sequenced with the primers (EiRT1 and EiLFT1) and a internal forward primer (5’-CTCTTCTTGGGAATGCTGGA-3’, Kaundun et al., 2008).

The PCR product was purified from agarose gel with Wizard® SV Gel and PCR Clean-up System (Promega Co., Madison, WI USA) and sequenced by commercial services (LotteryWest, State Biomedical Facility Genomics, Western Australia). All sequence chromatograms were visually checked for quality and consistency before sequences were assembled and aligned.

Derived Cleaved Amplified Polymorphic Sequences (dCAPS) Marker Development and Genotyping

Based on the EPSPS sequence information obtained from the susceptible (20 plants) and resistant samples (at least 80), we developed dCAPS markers for detecting mutation(s) at 102 and 106 using the web-based dCAPS finder 2.0 software. The A to T mismatch was introduced in the forward primer RsaIF (5’-TGCAGCTCTTGGGAATGCTGGA-3’) two nucleotide upstream of the 102 codon (i.e. N+2 position) to create a restriction site for RsaI (GT AC) in the WT sequence. Any nucleotide mutations resulting in substitution of the T102 would abolish the restriction site. Therefore, the primer pair RsaIF and EleuEPSPS-R was used to amplify a 234 bp fragment which was then digested with RsaI. The WT sequence would generate a single digested 208 bp band, while the mutant sequence at 102 would generate an undigested 234 bp band (Fig S1 A). Heterozygous sequence at 102 would
produce both the 208 and 234 bp bands (Fig S1 B). Similarly, a G to C mismatch was
introduced in the forward primer Sau96IF (5’-CTCTTCTTGGGGAATGCTGGAACTGCAATGGAGA-
3’) at N+3 position of the 106 codon to create a restriction site for Sau96I (G GNCC) in the
WT sequence. Any mutations resulting in substitution of the P106 would abolish the
restriction site. Therefore, the primer pair Sau96IF and EleuEPSPS-R was used to amplify a
233 bp fragment which was then digested with Sau96I. The WT sequence would produce a
single digested 202 bp band, while the mutant sequence at 106 would produce an
undigested 233 bp band (Fig S1 A). Heterozygous sequence at 106 would produce both the
202 and 233 bp bands (Fig S1 B) (no heterozygous sequences at 106 were detected). PCR
conditions were similar to above except that the annealing temperature was 62°C.
Restriction digestions were carried out according to the manufacturer’s recommendations
(New England BioLabs) and digestion patterns were viewed on 2% agarose gels
(electrophoresis at 90-100 V for 50-80 min) stained with ethidium bromide. The accuracy of
the two markers was confirmed by comparing sequencing and marker analysis results of
over 40 samples.

It is noticed that the two dCAPS markers were designed to only detect mutant and
WT sequences at the 102 and 106 codons without knowing the nature of the specific
mutation. As we confirmed that the resistant population only possessed the T102I and
P106S mutations, so the two dCAPS markers can be used for genotyping in the population. If
used in other uncharacterized E. indica populations, the specific mutations have to be
determined by sequencing.

**Generation of Purified Sub-populations**

Plants (7-12) that were confirmed by sequencing and marker analysis to be
homozygous for the WT, P106S, or TIPS mutation were bulk selfed in isolation to produce
seeds to enable respective sub-populations. Progeny plants (10-12) from each of these
purified sub-populations were randomly marker-analysed to confirm their genotype and
homozygosity prior to use for subsequent glyphosate dose response.

**E. coli Transformation**
Total RNA was isolated from *E. indica* (abbreviated as Ei) P106S EPSPS mutant. cDNA was synthesized and mature P106S EiEPSPS cassette was PCR amplified using the primers pair 1 and 2 (Table S1, Baerson et al., 2002). The PCR product was inserted into the pCR Blunt II TOPO vector and verified by sequencing. The P106S EiEPSPS was converted to WT (primer pair 3 and 4, Table S1) and the double mutant TIPS EiEPSPS (primer pair 5 and 6, Table S1) using the Phusion site-directed mutagenesis kit (Thermo Scientific). After sequencing verification, these 3 genes were PCR amplified using the primer pair 7 and 8 and the PCR products are digested by Ndel and then inserted into the Ndel site of pET19-b vector to form an N-terminal His-Tag fusion to facilitate the downstream purification of the enzymes.

**EPSPS Purification and Activity Assay**

The BL21 (DE3) cells (Invitrogen) harboring the EiEPSPS constructs in pET-19b vector were cultured in the MagicMedia *E. coli* Expression Medium (Invitrogen) according to the dual temperature protocol provided by the media supplier. Soluble proteins were extracted from frozen cells using the B-per bacterium extraction reagent supplied with DNase I, lysozyme and Halt Protease inhibitor cocktail (Thermo Scientific). After centrifugation at 21,000 g for 5 min the supernatant fraction was used to purify His-Tagged EPSPS with HisPur Ni-NTA resin (Thermo Scientific). The binding buffer was made of 20 mM Tris (pH 8.0), 500 mM NaCl, 10 mM imidazole and 0.03% Triton X-100; washing buffer contained 20 mM Tris (pH 8.0), 500 mM NaCl, 50 mM imidazole and 0.03% Triton X-100; and elution buffer contained 20 mM Tris (pH 8.0), 500 mM NaCl, 500 mM imidazole. The eluted enzyme was kept in storage buffer (10 mM MOPS, 10% Glycerin, 0.5mM EDTA, 2.5 mM beta-mercaptoethanol) after buffer exchange using Amicon Ultra 0.5 mL centrifugal filters (MWCO 30 kDa) 3 times 5 min at 14,000g. The protein content was quantified using the Pierce 660 nm Protein Assay kit (Thermo Scientific) and purification results analyzed by SDS-PAGE electrophoresis.

Activity of purified EPSPS variants was measured by the coupled assay that measures continuous release of inorganic phosphate using the EnzChek Phosphate Assay Kit (E-6646, Invitrogen) (Gaines et al., 2010). For IC₅₀ measurement of EiEPSPS variants various concentrations of glyphosate were used (WT: 0, 0.1, 5, 10, 20, 50, 150, 500 μM; P106S: 0, 1, 10, 25, 50, 80, 150, 500, 1250 μM; TIPS: 0, 12.5, 1250, 15000, 25000, 35000, 50000, 70000, 23, 442x449).
10000 \mu M) holding shikimate 3-phosphate (S3P) constant at 0.1 mM. To measure the $K_m$ (PEP) of the EiEPSPS variants, S3P concentration was fixed at 0.1 mM and various amounts of PEP (2.5, 5, 10, 15, 20, 25, 40, 60, 80 \mu M) were used.

**Statistics**

The LD$_{50}$, GR$_{50}$ or IC$_{50}$ was estimated by non-linear regression using the 3 parameter logistic curve model $y = a/(1+(X/X_0)^b)$, where $a$ is the maximum plant response close to untreated controls, $X_0$ is the dose giving 50% response and $b$ is the slope around $X_0$. The estimates were obtained using the Sigmaplot software (version 12.3, Systat Software, Inc) and the test ($\alpha=0.05$) was used to test significance of the regression parameters. The $K_m$ (PEP) and $V_{max}$ were calculated by non-linear fit of the data to the Michaelis-Menten equation $v = VS/(Km + S)$, where $S$ is the concentration of the substrate pyruvate, $v$ is the reaction velocity at any PEP concentration, and $V$ is the maximal reaction velocity. The kinetic values were obtained using GraFit (version 7.0.3, Erithacus Software Ltd) and the Chi-square ($\chi^2$) ($\alpha=0.05$) was used to test goodness of fit. Glyphosate dose response experiments were repeated at least twice with similar results so all data were pooled and evaluated for a composite line fit. Each EPSPS kinetic assay contained three technical replicates and three independent enzyme extracts were used for each assay set.

The *E. indica* TIPS cDNA sequence information can be found in GenBank with an accession number KM078728.

**LITERATURE CITED**


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Table 1. Genotype and allele frequencies determined for 193 *Eleusine indica* individuals by the dCAPS method developed for the T102I and P106S mutations (see Fig S1).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>No of individuals</th>
<th>Genotype frequency</th>
<th>Alleles</th>
<th>Allele frequency</th>
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<tr>
<td>RR</td>
<td>3</td>
<td>1.6%</td>
<td>102I-106S (R)</td>
<td>26%</td>
</tr>
<tr>
<td>rr</td>
<td>65</td>
<td>34%</td>
<td>T102-106S(r)</td>
<td>58%</td>
</tr>
<tr>
<td>WT</td>
<td>31</td>
<td>16%</td>
<td>T102-P106</td>
<td>16%</td>
</tr>
<tr>
<td>Rr</td>
<td>94</td>
<td>49%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R/WT</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r/Wt</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Parameter estimates of the non-linear regression analysis (the logistic 3 parameter model) of herbicide rates causing 50% plant mortality (LD\textsubscript{50}) or growth reduction (GR\textsubscript{50}) for glyphosate susceptible, EPSPS WT, and homozygous P106S and TIPS EPSPS \textit{Eleusine indica} mutants. SE is in parentheses.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>a</th>
<th>b</th>
<th>(X_0) (g ha(^{-1}))</th>
<th>P value for (X_0)</th>
<th>Ratio to S of (X_0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible (S)</td>
<td>99.7(0.22)</td>
<td>8.71 (1.61)</td>
<td>142 (1.3)</td>
<td>&lt;0.0001</td>
<td>1</td>
</tr>
<tr>
<td>WT</td>
<td>99.7(0.22)</td>
<td>8.71 (1.61)</td>
<td>142 (1.3)</td>
<td>&lt;0.0001</td>
<td>1</td>
</tr>
<tr>
<td>P106S</td>
<td>101(1.78)</td>
<td>3.14 (0.78)</td>
<td>798 (29)</td>
<td>&lt;0.0001</td>
<td>5.6</td>
</tr>
<tr>
<td>TIPS</td>
<td>&gt;25900</td>
<td>&gt;</td>
<td></td>
<td></td>
<td>&gt;182</td>
</tr>
<tr>
<td>Susceptible (S)</td>
<td>98.3 (8.42)</td>
<td>1.65(0.43)</td>
<td>65 (8.0)</td>
<td>0.0013</td>
<td>1</td>
</tr>
<tr>
<td>WT</td>
<td>97.8 (6.12)</td>
<td>1.98 (0.37)</td>
<td>57 (10.8)</td>
<td>0.0063</td>
<td>0.88</td>
</tr>
<tr>
<td>P106S</td>
<td>100 (1.96)</td>
<td>1.76 (0.12)</td>
<td>173 (7.3)</td>
<td>&lt;0.0001</td>
<td>2.67</td>
</tr>
<tr>
<td>TIPS</td>
<td>99.8 (3.87)</td>
<td>0.85 (0.08)</td>
<td>2023 (299)</td>
<td>0.0005</td>
<td>31.1</td>
</tr>
</tbody>
</table>
Table 3. Glyphosate IC50 (herbicide dose causing 50% inhibition of in vitro enzyme activity) and EPSPS activity, Km (PEP) of *E. coli* expressed EiEPSPS variants. SE from the non-linear regression analysis is in parentheses. The parameter estimates for IC50 can be found in Table S2. The Chi-square for goodness of fit of the kinetic data (Vmax and Km) is 0.8 (α=0.05).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>IC50 (µM)</th>
<th>Ratio to WT</th>
<th>EPSPS Vmax (nmol Pi µg⁻¹ min⁻¹)</th>
<th>Ratio to WT</th>
<th>Km(PEP) (µM)</th>
<th>Ratio to WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible (S)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>20 (0.84)</td>
<td>1</td>
<td>28.0 (0.84)</td>
<td>1</td>
<td>11.6 (1.1)</td>
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</tr>
<tr>
<td>P106S</td>
<td>87 (2.15)</td>
<td>4.3</td>
<td>27.5 (0.55)</td>
<td>1</td>
<td>10.0 (0.68)</td>
<td>0.9</td>
</tr>
<tr>
<td>TIPS</td>
<td>52938 (1206)</td>
<td>2647</td>
<td>1.8 (0.04)</td>
<td>0.06</td>
<td>9.8 (0.72)</td>
<td>0.8</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Glyphosate dose response (A: mortality, B: dry mass) of susceptible (S), EPSPS WT, and homozygous P106S and TIPS mutant *Eleusine indica*.

Figure 2. Glyphosate dose response of susceptible (S), EPSPS WT, homozygous P106S and TIPS mutant *Eleusine indica*.

Figure 3. Glyphosate dose response of *E. coli* expressed *Eleusine indica* EPSPS variants.

Figure 4: Reduced growth of the homozygous EPSPS TIPS mutants, as compared to the WT and homozygous P106S mutants. *Eleusine indica* plants are five weeks after transplanting.
Figure 1. Glyphosate dose response (A: mortality, B: dry mass) of susceptible (S), EPSPS WT, and homozygous P106S and TIPS mutant *Eleusine indica*.
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Karasov T, Messer PW, Petrov DA (2010) Evidence that adaptation in Drosophila is not limited by mutation at single sites. PLoS Genetics 6: e1000924


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