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# A Novel P106L Mutation in EPSPS and an Unknown Mechanism(s) Act Additively To Confer Resistance to Glyphosate in a South African *Lolium rigidum* Population

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**ABSTRACT:** Glyphosate resistance evolution in weeds is a growing problem in world agriculture. Here, we have investigated the mechanism(s) of glyphosate resistance in a *Lolium rigidum* population (DAG1) from South Africa. Nucleotide sequencing revealed the existence of at least three *EPSPS* homologues in the *L. rigidum* genome and identified a novel proline 106 to leucine substitution (P106L) in 52% DAG1 individuals. This mutation conferred a 1.7-fold resistance increase to glyphosate at the whole plant level. Additionally, a 3.1-fold resistance increase, not linked to metabolism or translocation, was estimated between wild-type P106-DAG1 and P106-STDS sensitive plants. Point accepted mutation analysis suggested that other amino acid substitutions at EPSPS position 106 are likely to be found in nature besides the P106/S/A/T/L point mutations reported to date. This study highlights the importance of minor mechanisms acting additively to confer significant levels of resistance to commercial field rates of glyphosate in weed populations subjected to high selection pressure.

**KEYWORDS:** *Lolium rigidum* (rigid ryegrass), glyphosate resistance, 3-phosphoshikimate 1-carboxyvinyltransferase (EC 2.5.1.19), target site mutation, P106L, PASA method

## INTRODUCTION

Glyphosate is by far the most important nonselective, systemic herbicide for postemergence control of a wide range of grass and broadleaved weeds.[1] It exerts its herbicidal activity by inhibiting 3-phosphoshikimate 1-carboxyvinyltransferase (EPSPS; EC 2.5.1.19), an important enzyme in the synthesis of essential aromatic amino acids.[2] Inhibition of EPSPS results in the depletion of L-phenylalanine, L-tyrosine, and L-tryptophan and accumulation of shikimic acids, leading to plant death. In addition to being very versatile, glyphosate is characterized by a very favorable environmental profile and low mammalian toxicity.[3] When introduced to markets in 1974, glyphosate was mainly employed in noncrop systems and as a preplant burn down herbicide. In crops, glyphosate usage was limited to directed or postharvest applications. Glyphosate use has increased dramatically in the past decade following the development of glyphosate tolerant crops. This has allowed for the selective in-crop application of glyphosate for managing a plethora of weeds. Provided with a very simplified and cost-effective solution, farmers in North and South America have rapidly and overwhelmingly adopted glyphosate tolerant technology in over 80% soybean, cotton and corn acreages.[4,5]

Compared to other single site herbicide modes of action such as acetolactate synthase, acetyl-CoA carboxylase and photosystem II inhibitors, glyphosate is considered low risk for resistance evolution in weeds.[6] This is because glyphosate is not significantly metabolized in plants. Also target site resistance was deemed very unlikely given that glyphosate binds to a few and highly conserved amino acids in EPSPS. In addition, it closely mimics the EPSPS substrate phospho-*enol*pyruvate in such a way that it has not been possible to alter any of the critical amino acids for glyphosate binding without incurring a significant fitness cost to EPSPS.[6,7] For over 20 years of use, glyphosate resistance was not documented in weeds. However, subjected to high selection pressure, a first case of glyphosate resistance was reported in a *Lolium rigidum* population exposed to two to three glyphosate applications per year for 15 years.[8,9] To date, glyphosate resistance has evolved in seven grass and 11 broadleaved weeds across the world, and among these, 11 occur in glyphosate tolerant cropping systems.[10]

The first elucidated glyphosate resistance mechanism consisted of reduced herbicide translocation to meristematic tissues and increased acropetal movement to the leaf tips in a *Lolium rigidum* population.[11] This impaired translocation mechanism has thus far been described in several *Lolium*[12−14] and *Conyza*[15,16] populations. In both species inheritance studies have demonstrated that a major partially dominant nuclear allele is involved in conferring around 8−12-fold resistance to glyphosate.[17−19]

Target site mutations near the EPSPS active site have also been linked to glyphosate resistance and involved a proline to serine, alanine or threonine change at position 106 of the EPSPS in *Eleusine indica*[20−23] and *Lolium* species.[24−26] The level of resistance is relatively low, in the order of 2−4-fold, and its

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relevance to overall field performance has been questioned.[6] More recently, a third resistance mechanism has been reported in *Amaranthus palmeri* consisting of gene amplification on multiple chromosomes and concomitant overexpression of EPSPS target.[27]

With increasing glyphosate selection pressure, multiple resistance mechanisms acting additively have also been reported in a *Lolium* population endowed by a mutated target site and impaired glyphosate translocation.[14] Similarly resistance in a Chilean *Lolium multiflorum* population was attributed mainly to impaired glyphosate translocation but also to a lower spray retention and foliar uptake.[28] Polygenic resistance to glyphosate has also been found in *Amaranthus tuberculatus* for which the precise mechanisms are yet to be elucidated.[29,30]

After 17 years of use as primary method for grass weed management, moderate levels of glyphosate resistance were reported in a *Lolium rigidum* population (DAG1) from a South African vineyard. Today glyphosate remains an important component for weed control in the vineyard in question but has to be complemented with ACCase inhibiting herbicides for controlling evolved glyphosate resistance in DAG1. The objectives of this study were to confirm glyphosate resistance in this population and investigate the mechanism(s) involved.

## MATERIALS AND METHODS

**Plant Materials.** The suspected resistant *L. rigidum* population (DAG1) originated from a vineyard in the Riebeeck Kasteel District in the Western Cape, South Africa. A second Western Cape *L. rigidum* population (AFRL2), from the Tulbagh Valley, was also included as control in uptake, metabolism and translocation studies. This AFRL2 population was previously confirmed resistant due to impaired glyphosate translocation to meristematic tissues.[14] A standard sensitive *L. rigidum* population (STDS) was acquired from a local distributor (Herbiseed, Twyford, U.K.) and was used for comparison in all studies.

**Initial Glyphosate Resistance Confirmation Test.** Seeds from STDS and DAG1 were sown separately in a soil medium (John Innes, North Yorkshire, U.K.) containing a 1:1 ratio of compost and peat and were irrigated as required. The emerged plants were maintained in controlled greenhouse conditions set to 24 °C/16 h day, 18 °C/8 h night, 65% relative humidity, and a photon flux density of approximately 250 $\mu$mol quanta $m^{-2}$ $s^{-1}$. Ten days after sowing, seedlings were transplanted into individual pots (75 mm diameter) with the aforementioned soil medium; pots were irrigated and plants fertilized as necessary. At the two leaf stage, fifty plants each from STDS and DAG1 were treated with the recommended field rate of 860 g acid equivalents (ae) glyphosate $ha^{-1}$ (Touchdown Total, Syngenta, NC, USA) using a precision $CO_2$-powered laboratory sprayer (Thurnall Inc., Manchester, U.K.) equipped with a flat fan spray nozzle and delivering a spray volume of 200 L $ha^{-1}$. Fifty additional plants from STDS and DAG1 were unsprayed and used as control. Plant mortality was recorded 21 days after glyphosate treatment (21 DAT).

***EPSPS* Sequencing.** *DNA Extraction.* Sixteen untreated plants from the DAG1 population were analyzed individually. Approximately 0.25 g of plant tissue was excised per plant, placed in a single well in 96-deep-well blocks and stored at −80 °C. The tissue was then ground in a bead mill to a dry powder and centrifuged at 2200*g* for 5 min. Finally, Magnesil Plant DNA Extraction kit (Promega, Madison, WI, USA) was used to extract the genomic DNA using a Biomek FX automation workstation (Beckman Coulter Inc., CA, USA).

*PCR Amplification and EPSPS Sequencing.* PCR reactions were performed with Ready-To-Go Taq Beads (Amersham Biosciences, NJ, USA) in a volume of 25 $\mu$L, consisting of a sample of genomic DNA (10−50 ng) and a primer concentration of 20 pmol $\mu L^{-1}$. The Mastercycle Gradient Thermocycler model 96 machine (Eppendorf AG, Hamburg, Germany) was used, and PCR was conducted on genomic DNA with *Lolium* EPSPS F (TCTTCTTGGGGAACGCTGGA) and *Lolium* EPSPS R (TAACCTTGCCACCAGGTAGCCCTC) primers to amplify a fragment covering the *EPSPS* region containing the critical 106 amino acid position. PCR conditions included 1 cycle of 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min and a final extension cycle of 72 °C for 10 min. The PCR fragments were cloned into the TOPO 2.1 TA vector (Invitrogen, CA, USA) and sequenced using M13F and M13R primers. To minimize the risk of identifying a sequencing artifact as a mutation, a nucleotide change was recorded only when it was present on more than one clone per individual plant sequenced.

*EPSPS P106L Expression.* To confirm that the P106L allele was actually expressed, RNA was extracted from liquid $N_2$ homogenized *Lolium* leaf tissue using the TRIzol (Invitrogen, CA, USA) reagent. The RNA was precipitated with isopropanol and washed with 80% ethanol. cDNA was made using the Superscript III kit (Invitrogen, CA, USA), and PCR, cloning and sequencing were carried out as described above.

**Development of P106L PASA Method.** Four primers were designed for PCR amplification of specific alleles (PASA) analysis. These comprised two external nonallele specific primers, LOL-EPSPS F (ATAAGGTTGCAAAAAGAGCTGTAG) and LOL-EPSPS R (TAACCTTGCCACCAGGTAGCCCTC), and two allele specific primers, LOL- EPSPS P (GAACGCTGGAACTGCGATGCGGTC) and LOL-EPSPS L (CAGCTACTACAGCAGCCGTCAAGA), to positively identify the wild-type $prolyl^{106}$ (P106) and mutant $leucyl^{106}$ (L106) alleles. PCR was conducted with Ready-To-Go Taq Beads in a volume of 25 $\mu$L; 10−50 ng of genomic DNA was used in each reaction with a primer concentration of 20 pmol $\mu L^{-1}$. The PASA analysis was conducted on a Tgradient PCR machine (Biometra, Gottingen, Germany) with the following conditions: 1 cycle of 95 °C for 5 min, followed by 20 cycles of 95 °C for 30 s, 61.5 °C for 30 s (−0.5 °C per cycle) and 72 °C for 60 s then 15 cycles of 95 °C for 30 s, 51.5 °C for 30 s and 72 °C for 60 s and a final extension cycle of 72 °C for 5 min. The PASA products were then resolved in 2% agarose gels in a 1 × TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA; pH 8.0) running buffer.

**Evaluation of Glyphosate Resistance on Predetermined P106 and L106 Genotypes.** A glyphosate dose response test was carried out on previously characterized P106 and L106 genotypes from DAG1 and P106 individuals from STDS. The plants were then treated at the two leaf stage with glyphosate at 0 (untreated control), 150, 210, 300, 430, 610, 860, 1230, and 1750 g ae glyphosate $ha^{-1}$ under the aforementioned conditions. Fifty-six individual replicate plants per genotype were sprayed per herbicide rate. Following glyphosate treatment, plants were arranged in a randomized complete block (RCB) design and placed in the aforementioned greenhouse conditions. Percent mortality was recorded 21 DAT.

The relationship between percent survival ($P$) and glyphosate rate was modeled by a regression analysis appropriate to quantal response data[31] and in which identical slopes were fitted to each of the three genotypes. This was found to fit the data adequately and allowed a straightforward interpretation of the differences between genotypes in terms of their resistance factors. The resistance factor between two genotypes was therefore estimated as the ratio of their respective $LD_{50}$ values. Since the fitted regression lines are parallel, the estimated resistance factors are independent of the response level. The model is described by the equation

$$P = \frac{100}{1 + e^{-\beta(x - \mu_i)}}$$

where $x$ denotes $\log_{10}$(rate); $\mu_i$ denotes the log $LD_{50}$ for genotype $i$; and $\beta$ denotes the common slope fitted to all three genotypes.

**Metabolism, Uptake and Translocation Studies.** Uptake, metabolism and translocation studies were conducted on three plant genotypes including wild-type P106 plants from DAG1, STDS (standard sensitive) and AFLR2, the latter used as a positive control for impaired glyphosate translocation. Throughout the experiments, plants were grown hydroponically in sterilized sand, fertilized and irrigated as necessary in a growth chamber with alternative 20 °C/12 h light and 15 °C/12 h dark conditions. At two−three leaf stage, 15 plants per population were treated with a 2 $\mu$L herbicide droplet at the adaxial surface of the youngest leaf with a microsyringe. Each 2 $\mu$L droplet contained 1.62 kBq of [$^{14}$C]-glyphosate for a total of 9.2 $\mu$g ae (8.84 $\mu$g of nonradioactive plus 0.36 $\mu$g of [$^{14}$C]-glyphosate). The experimental design was a randomized complete block (RCB) with 3 populations and three replicates of 5 plants ($n$ = 15). The plants were harvested at 3 and 7 DAT, leaf surfaces were washed with methanol−water (1:9, V/V) and unabsorbed radioactivity was subsequently quantified by liquid scintillation spectroscopy (LSS) (1409 Liquid Scintillation Analyzer; Wallac). In view of investigating the relative upward and downward glyphosate movement, plants were then dissected and analyzed into two sections: leaves and culm + roots. The different plant sections were weighed, frozen in liquid nitrogen, ground with a pestle and mortar and extracted with ultrapure water (1:4 g fresh weight mL$^{-1}$). After centrifugation (15000$g$, 10 min), the supernatant was assayed for radioactivity by LSS. Plant debris contained in the centrifugation pellet was dried and combusted in a Packard 387 oxidizer (Packard Instrument Co., Downers Grove, IL). The nonextracted radioactivity was then quantified by LSS.

For metabolism studies, the major metabolite aminomethylphosphonic acid (AMPA) was separated from the parental glyphosate molecule by thin layer chromatography (TLC; SG60 with fluorescent marker; Merck). TLC analysis was carried out by combining plant extracts within each block (5 plants). Electronic autoradiography and image analysis of TLC plates were then performed using a Molecular Imager (Bio-Rad, Hercules, CA). Glyphosate and AMPA were identified by comparing their relative retention factor ($R_f$) values in reference to their commercial standards.

Data were analyzed by analysis of variance appropriate to a randomized complete block design. Where there was evidence of an overall effect of genotype (as provided by the $F$-test for the genotype effect), individual genotype comparisons were carried out using $t$ tests.

All statistical tests were carried out with SAS software (SAS Institute, Cary, NC).

## RESULTS

**Confirmation of Glyphosate Resistance.** The standard sensitive population STDS was effectively controlled at the single glyphosate rate of 860 g ae ha$^{-1}$. On the other hand, 10 out of 50 suspected resistant DAG1 plants survived the latter glyphosate treatment. The survivors were generally stunted and possessed less than 50% biomass relative to untreated controls. Based on our knowledge of glyphosate resistance mechanisms, a target site mutation and/or other minor resistance mechanisms were suspected in DAG1 given the low levels of observed glyphosate resistance. Higher resistance levels were reported for impaired glyphosate translocation[13] or EPSPS overexpression.[27]

**Investigation of the Resistance Mechanism(s) in DAG1.** *Partial Sequencing of the EPSPS Gene.* A highly conserved *EPSPS* region previously found to contain mutations linked to glyphosate resistance was sequenced.[12,14,24−26] Using genomic DNA from 16 untreated DAG1 plants as template, PCR amplified a DNA fragment of around 331 bp encompassing glycyl$^{101}$ to glycyl$^{162}$ in the mature EPSPS. This fragment also contained the variable *EPSPS* intron #2 of around 98 bp. Comparison of the 238 bp coding sequences from 16 DAG1 plants to a previously reported sensitive *EPSPS* nucleotide sequence from *Lolium multiflorum* (GenBank accession DQ153168) showed over 95% homology, thus confirming the identity of EPSPS gene amplified. The genomic sequence comparison of intron #2 revealed the presence of up to five different *EPSPS* alleles per *Lolium* plant, suggesting the presence of at least three *EPSPS* copies in the *Lolium* genome. Eight nucleotide differences were observed in the 238 EPSPS coding region between the 16 DAG1 plants and a wild-type sensitive *Lolium multiflorum* (GenBank accession: DQ153168). Of these eight changes, seven were identified among the 16 DAG1 plants and only one mutation was between the DAG1 and the published EPSPS sequence. Six nucleotide changes were synonymous and involved third bases of codon triplets at positions A109, A118, A145, L151, P156 and N161. The remaining two nucleotide changes were in the second and third bases of codon 106 and consisted of CCA to CTG transversions. These two changes resulted in a novel proline to leucine mutation at EPSPS position 106 (P106L). Partial EPSPS gene amplification and sequencing via RT-PCR confirmed the expression of the novel P106L allele in DAG1. The wild-type and mutant EPSPS nucleotide sequences from DAG1 were deposited in GenBank under the accessions GU594896 and GU594897, respectively.

*Development of PASA for P106L EPSPS Genotyping.* Since several hundred plants were required to confidently assess the impact of the P106L mutation on glyphosate efficacy, a simple, expeditious and cost-effective PASA[32] was developed to genotype DAG1 individuals at EPSPS position 106. The PASA primers were purposely destabilized at the nucleotide minus one position (N−1) from the 3′ end to increase the specificity of the assay (Liu et al., 1997).[33] By nature of the PASA assay all plant samples had a nonspecific 410 PCR fragment. Wild-type plants contained an additional 320 bp fragment while mutant plants had the 320 bp fragment and a third 138 bp mutant band. The EPSPS genotypes identified by PASA analysis were totally correlated with nucleotide sequencing results. Large scale genotyping of over 1000 DAG1 plants revealed that 51.7% individuals contained at least one mutant L106 allele. It is noteworthy that the PASA method could not differentiate between homozygous and heterozygous mutant plants due to the presence of multiple EPSPS copies in *Lolium*. It can nevertheless be inferred that the majority of mutant L106 EPSPS plants were at the heterozygous state for the single variable 106 EPSPS locus given that 48% of individuals from this mixed resistant population were homozygous wild-type PP106. In a panmictic situation, where crossing between complete outbreeding *Lolium* plants occurs freely in the field, the genotypic frequencies are given by the Hardy−Weinberg equation: $(p + q)^2 = 1$ where $p$ and $q$ are the allelic frequencies for the wild-type prolyl$^{106}$ and mutant leucyl$^{106}$ EPSPS alleles respectively. The value $p$, deduced from the frequency of homozygous wild-type plants in DAG1, was calculated as 0.69 (square root of 0.48), and consequently the frequency of the mutant L106 allele was equal to 0.31. The genotypic frequency ($q^2$) of homozygous mutant plants (LL106) is thus estimated at 0.09, which represents less than 10% of plants in DAG1.

**Glyphosate Efficacy on Predetermined P106 and L106 Genotypes.** A glyphosate dose response test was conducted on previously characterized P106 and L106 DAG1 plants (Figure 1). Comparison of plants within DAG1 permitted for a

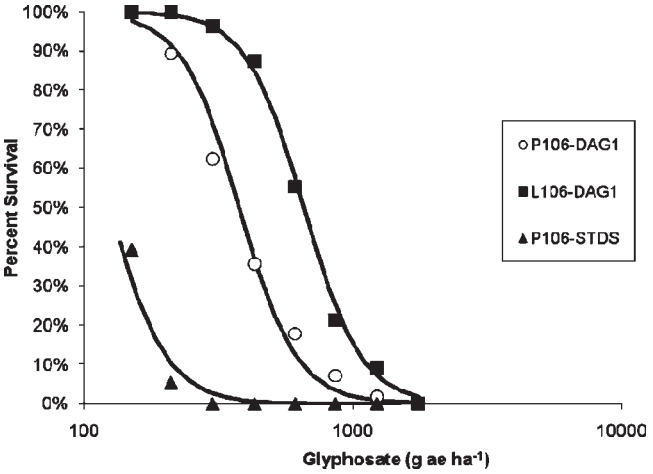


**Figure 1.** Glyphosate rate response on three previously characterized *Lolium rigidum* genotypes at EPSPS position 106. P106-STDS = homozygous wild-type P106 plants from the standard sensitive population STDS. P106-DAG1 = homozygous wild-type P106 plants from the Riebeeck Kasteel population. L106-DAG1 = Riebeeck Kasteel plants containing at least one mutant L106 EPSPS allele.

**Table 1. Estimated $LD_{50}$s for the Three Genotypes and Corresponding 95% Confidence Intervals (CI)**

| genotype | estimated $LD_{50s}$ (95% confidence intervals) |
|---|---|
| P106 (STDS)[a] | 123.6 (109.1−138.5) |
| P106 (DAG1)[b] | 376.6 (348.3−407.0) |
| L106 (DAG1)[c] | 653.0 (604.5−705.7) |

[a] Homozygous wild-type P106 EPSPS plants from the standard sensitive population (STDS). [b] Homozygous wild-type P106 EPSPS plants from the Riebeeck Kasteel population. [c] Mutant plants from the Riebeeck Kasteel population with at least one L106 EPSPS allele at position 106 of EPSPS.

more accurate assessment of the impact of the P106L mutation on glyphosate efficacy since the individuals had similar genetic backgrounds. P106 plants from STDS were also included to investigate whether other underlying glyphosate resistance mechanisms exist in DAG1. P106-STDS plants were effectively controlled at 300 g ae glyphosate $ha^{-1}$and above. At this same rate 63% and 96% of P106-DAG1 and L106-DAG1 plants survived respectively. Significant differences ($p < 0.001$) between STDS plants and P106-DAG1 or L106-DAG1 individuals were also found at 430 g ae glyphosate $ha^{-1}$. At the recommended field rate of 860 g ae $ha^{-1}$, a considerable reduction in plant biomass was observed and survival decreased to 21% in L106-DAG1 and 7% in P106-DAG1 genotypes. Overall, the levels of resistance were relatively low with only one and eight P106-DAG1 and L106-DAG1 plants respectively surviving 1230 g ae glyphosate $ha^{-1}$; no single plant survived 1750 g ae glyphosate $ha^{-1}$. $LD_{50}$ values estimated from the logistic model were 653, 377, and 124 g ae glyphosate $ha^{-1}$ for L106-DAG1, P106-DAG1 and P106-STDS plants respectively (Table 1).

Pairwise comparison of $LD_{50}$ values between P106-DAG1 and L106-DAG1 plants estimated a resistance factor of 1.7 (1.55−1.94) fold due to P106L mutation. Interestingly a 3.1 (2.66−3.53) fold increase in the level of glyphosate resistance was observed between wild-type P106 plants from DAG1 and STDS, indicating that other non target site based resistance mechanism(s) exist in DAG1. The divergence in response between L106-DAG1 and P106-STDS plants was even more evident (5.3 (4.61−6.13) fold increase) suggesting that the effects of multiple glyphosate resistance mechanisms were additive in DAG1.

**Uptake, Metabolism and Translocation.** Considering the target site and other resistance mechanism(s) uncovered by the whole plant dose response test, potential differences in uptake, metabolism and translocation of glyphosate were further explored between P106-DAG1 and P106-STDS plants. A second glyphosate resistant South African population (AFRL2) was used as positive control for impaired glyphosate translocation. On average 76% and 85% of the applied glyphosate was absorbed by the plants at three and seven DAT, and this response was not significantly different between populations ($P > 0.5$) (Table 2). Similarly no significant difference in glyphosate metabolism was observed between populations with more than 92% of total radioactivity detected as unmodified parental molecule. Glyphosate movement in P106-DAG1 and P106-STDS plants was similar at the two time points, suggesting that impaired translocation is not associated with the glyphosate resistant phenotype in DAG1. As expected, three DAT, significant differences were observed between P106-STDS or P106-DAG1 plants and the standard resistant population AFRL2. The magnitude of the difference was greater seven DAT with 39% and 45% downward translocation toward meristematic tissues for P106-STDS and P106-DAG1 and only 14% for AFLR2 plants.

## DISCUSSION

Despite the global importance of glyphosate and increasing number of weed species and populations evolving resistance, the underlying mechanisms are yet to be elucidated in most cases.[10] Confirmation of glyphosate resistance itself can sometimes be difficult given the relatively low levels of resistance involved. This is particularly the case with EPSPS target site modifications which endow a 2−4-fold resistance increase. In contrast, target site resistance to ACCase, ALS and PSII inhibiting herbicides can result in 20−100-fold resistance increase[34] and therefore resistance confirmation in these cases is relatively straightforward.

To date the naturally evolving amino acid substitutions associated with glyphosate resistance consist of prolyl[106] substitution into seryl, alanyl or threonyl in *E. indica* and *Lolium* spp. The importance of these mutations on glyphosate efficacy is well established for the P106S mutation in *E. indica* only.[35,36] Correlating EPSPS genotypes and glyphosate phenotypes at the whole plant level was facilitated by the fact that EPSPS exist as a single copy gene in *E. indica*.[20]

In *Lolium* species EPSPS exists as a small gene family,[37] and no direct correlation was made between mutated EPSPS genotypes and resistant glyphosate phenotypes in any of the populations studied.[12,14,24,26] The studies concluded that the reported prolyl[106] mutations were responsible for the resistant phenotype citing the findings in *Escherichia coli*[20] and *E. indica*.[36] Such statements may be ambiguous since the effects of point mutations on herbicide efficacies can be specific and dependent on the herbicide tested, the number of mutant alleles and weed species involved.[38−40]

In this study we detected a cysteine to thymine transversion at the second nucleotide base of the EPSPS 106 codon triplet resulting in a novel proline to leucine mutation in EPSPS enzyme in around half of the DAG1 plants. In contrast to the P106A/S/T

**Table 2. Estimate of Glyphosate Uptake, Upward Translocation (Acropetal) and Downward Translocation (Basipetal) in Three Plant Groups and Two Sample Timings**

| | 3 days after treatment | | | 7 days after treatment | | |
|---|---|---|---|---|---|---|
| population | uptake % of applied dose | % upward translocation | % downward translocation | uptake % of applied dose | % upward translocation | % downward translocation |
| P106-STDS[a] | 76.4 | 70.1 | 29.9 | 85.9 | 61 | 39 |
| P106-DAG1[b] | 76.3 | 74.9 | 25.1 | 85.6 | 55 | 45 |
| AFRL2[c] | 77.7 | 81.6 | 18.4 | 85 | 85.6 | 14.4 |
| *F*-test probability | 0.864 | <0.001 | <0.001 | 0.741 | <0.001 | <0.001 |
| 5% LSD | | 5.7 | 5.7 | | 6.4 | 6.4 |

[a] Homozygous wild-type P106 plants from the standard sensitive population. [b] Homozygous wild-type P106 plants from the Riebeeck Kasteel population. [c] Standard resistant plants from the Tulbagh valley population characterized by impaired glyphosate translocation.

reported to date, the P106L mutation consists of a nonconservative change of a proline to the hydrophobic leucine residue. We clearly established the importance of this mutation by comparing wild and mutant plants from the similar DAG1 genetic background and found that the P106L mutation conferred 1.7-fold resistance to glyphosate.

An analogous proline to leucine mutation at EPSPS position 106 was created via site directed mutageneis in rice cell lines.[41] Kinetic studies demonstrated that the mutant P106L EPSPS had a Michaelis−Menten constant ($K_m$) of 88.3 $\mu$M for PEP, a 4.4 increase compared to the wild-type EPSPS, and a 70-fold increase in dissociation constant $K_i$ for glyphosate. The mutant P106L EPSPS was therefore capable of endowing high glyphosate resistance while retaining appreciable catalytic activity. Similarly *E. coli* expressing the P106L and grown in the presence of glyphosate showed a 3-fold increase in glyphosate resistance as compared to the wild-type strain. Further evidence of the importance of the P106L mutation on glyphosate efficacy was provided at the whole plant level with transgenic tobacco lines expressing the mutated P106L enzyme displaying significant levels of resistance to glyphosate in comparison with the non-transformed wild-type plants.

Topological analysis of a recently elucidated EPSPS crystal structure revealed that prolyl[106] is not involved in catalysis or glyphosate binding.[42] Therefore mutations at this position very likely alter glyphosate binding by modifying the steric forces of vicinal residues. Prolyl[106] resides in the amino terminus of an α-helix and adjacent to the highly conserved and structural arginyl[105]. Prolyl residues are typically found in loops guiding the architecture of $\beta$-turn motifs and in helix caps or helix terminator residues. Proline is unique since the five-membered cyclic nature of the residue precludes torsion around the Φ peptide bond (Cα−N) and significantly reducing the flexibility of polypeptide chains. Mutations in prolyl residues often add flexibility to these secondary structures, in particular α-helixes.[43] We therefore hypothesize that the P106L mutation alters the spatial orientation of the guanidinium arginyl[105] group required for the structural integrity of EPSPS. The mutation could also alter the orientation the vicinal asparaginyl[99] and glycyl[101] residues which are essential for hydrogen bonding with the glyphosate phosphonate group.

Researchers often question whether resistance mutations different from prolyl[106] in EPSPS exist in nature.[44] Such occurrences are very unlikely for amino acids that are critical for glyphosate binding as these are also essential for the catalytic activity and integrity of EPSPS.[6] However, at EPSPS position 106 other mutations could be expected following our findings of a naturally occurring and nonconservative P106L mutation in DAG1. This is predicted by point accepted mutation (PAM) analysis which consists of a set of matrices to score sequence alignments.[45] Each matrix is twenty-by-twenty and represents the probability of a substitution of one amino acid for another. This matrix indicates that a proline to alanine change is most often encountered in nature followed very closely by serine and threonine. It is noteworthy that these same three mutations have been detected at EPSPS prolyl[106] in several *Lolium* and *Eleusine* populations. Conversely, a proline to leucine mutation is estimated as being 54 times less likely to occur than a proline to serine substitution. Between the two extremes represented by P106S and P106L mutations, PAM analysis predicts the occurrence of other amino acid substitutions including proline to arginine, asparagine, aspartate, glutamic acid, glutamine, glycine, lysine and valine. Of these, arginine and glutamine changes are more likely to be encountered as it would require a single base change at the second position of the codon triplet, namely cysteine to guanine and adenine respectively.

While being predicted by PAM analysis, to date, however, the P106 mutations have been detected in only three out of 19 grass and broadleaved species that have developed glyphosate resistance worldwide. The lack of reports on P106-EPSPS variations is surprising given the relatively conserved nature of the EPSPS gene across grass and broadleaved weed species. This could be due to P106 mutations being masked when direct sequencing methods (or insufficient number of clones) are employed for *EPSPS* sequencing in suspected resistant species. Primarily though, it could be because of the low level of resistance conferred by this resistance mutation in species characterized by multiple EPSPS. Indeed the three species in which the P106 mutations have been detected to date are characterized by a single *EPSPS* (*E. indica*), thus resulting in more dramatic effect on glyphosate efficacy upon Prolyl[106] mutations and a complete outbreeder (*Lolium* spp.) that can accumulate minor resistance genes, when subjected to high glyphosate selection pressure. Correspondingly a relatively small number of plants from a sensitive *Lolium rigidum* population has been shown to evolve significant levels of resistance in a few generations when sprayed at sublethal doses of glyphosate.[46] This implies that sensitive *Lolium* plants naturally possess genes that can confer low levels of glyphosate resistance, when acting additively can cause a significant decrease in glyphosate efficacy.

Comparison of wild-type plants from STDS and DAG1 populations in a whole plant dose response assay indicated other mechanism(s) conferring higher levels (3.1-fold) of resistance to glyphosate than the P106L target site mutation. Detailed studies showed that resistance was not due to differences in uptake and translocation mechanisms detected previously in *Lolium* populations. This could constitute a single new glyphosate resistance mechanism in *Lolium* that remains to be investigated. The unknown resistance mechanism(s) could also be a result of a combination of minor glyphosate resistant genes that exist in this species prone to evolving resistance to many different herbicide modes of action.[47,48] Multiple minor resistance mechanisms were very probably present in another *Lolium multiflorum* population in which a P106S mutation was identified and characterized by 5-fold level of resistance to glyphosate.[12] Other contributing mechanisms were undetected as the authors did not compare wild-type EPSPS plants from the mixed resistant population with a standard sensitive population.

In conclusion, we have detected a novel EPSPS mutation in *L. rigidum* and clearly quantified its importance in conferring resistance to glyphosate. In addition the population contains other non target site mechanism(s) that are yet to be determined. Based on our results, as glyphosate selection pressure intensifies, so does the potential for multiple resistance mechanisms to act additively, particularly in species with diverse genetic background, prolific and allogamous as *Lolium* spp. It also highlights the importance of diversity in weed control methods for preserving glyphosate for present and future generations.

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