



Mechanism of Resistance to Glyphosate in *Lolium perenne* from Argentina

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In Argentina, glyphosate resistance was reported in a *Lolium perenne* population after 12 years of successful herbicide use. The aim of the current paper was to put in evidence for the mechanism of glyphosate resistance of this weed. Susceptible leaves treated with different doses of glyphosate and incubated *in vitro* showed an accumulation of shikimic acid of around three to five times the basal level, while no changes were detected in leaves of glyphosate-resistant plants. The resistance mechanism prevents shikimate accumulation in leaves, even under such tissue-isolation conditions. The activity of the glyphosate target enzyme (EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase) was quantified at different herbicide concentrations. EPSPS from resistant plants showed no difference in glyphosate-sensitivity compared to EPSPS from susceptible plants, and, accordingly, no amino acid substitution causing mutations associated with resistance were found. While the glyphosate target enzymes were equally sensitive, the basal EPSPS activity in glyphosate resistant plants was approximately 3-fold higher than the EPSPS activity in susceptible plants. This increased EPSPS activity in glyphosate resistant plants was associated with a 15-fold higher expression of EPSPS compared with susceptible plants. Therefore, the over-expression of EPSPS appears to be the main mechanism responsible for resistance to glyphosate. This mechanism has a constitutive character and has important effects on plant fitness, as recently reported.

Keywords: glyphosate resistance, shikimate assay, EPSPS activity, EPSPS overexpression, perennial ryegrass

INTRODUCTION

Glyphosate-resistant weeds put at risk the efficacy of glyphosate, an important herbicide used worldwide in current cropping systems. Since the first report of glyphosate resistance in 1998, the evolution of glyphosate resistant weed populations has rapidly escalated (Powles et al., 1998; Duke and Powles, 2008). At present, glyphosate resistance has been documented in 36 species (Heap, 2017). Herbicide-resistant weeds have several negative effects on farms and surrounding

regions. They would increase the costs and difficulties for weed control (Norsworthy et al., 2012). Accordingly, the evolution of glyphosate-resistant weeds threatens the long-term efficacy of the world's most important herbicide resource (Duke and Powles, 2008).

The target enzyme of glyphosate is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC. 2.5.1.19) that catalyses the reaction of shikimate-3-phosphate and phosphoenolpyruvate to yield 5-enolpyruvylshikimate-3-phosphate (Franz et al., 1997). This is the sixth enzyme on the shikimate pathway, which is essential for the synthesis of aromatic amino acids (Schönbrunn et al., 2001). The inhibition of EPSPS triggers the accumulation of shikimate, a substrate of the enzyme. Therefore, the effect of glyphosate on plants can be easily measured by monitoring the accumulation of this substrate of EPSPS (Dayan et al., 2015).

After EPSPS inhibition, several physiological processes are affected by glyphosate. The effect of the herbicide is not restricted to the inhibition of aromatic amino acid biosynthesis alone. It also disrupts the *de novo* biosynthesis of most non-aromatic amino acids, coupled with decreased protein synthesis (Maroli et al., 2016). In consequence, growth is inhibited (Pline et al., 2002; Orcaray et al., 2012), the transport of assimilates is reduced (Yanniccari et al., 2012a) and photosynthetic CO₂ assimilation decreases significantly (Olesen and Cedergreen, 2010). Several of these effects are limited or absent in glyphosate-resistant weed plants treated with the herbicide (Yanniccari et al., 2012a,b,c; Maroli et al., 2016).

Although more than 200 weed populations have shown glyphosate resistance in 26 countries (Heap, 2017), mechanism of plant survival was determined in a few cases. These mechanisms can be grouped into target-site and non-target-site based mechanisms (Powles and Yu, 2010). In the first category, the resistance can occur due to a gene mutation conferring an amino acid change that prevents herbicide binding. EPSPS target site mutations have been reported in six species, most frequently in the genus *Lolium* (Sammons and Gaines, 2014). Amino acid substitutions at Pro-106 have been the most common target site mutations in glyphosate-resistant *Lolium* spp. (Wakelin and Preston, 2006; Perez-Jones et al., 2007; Jasieniuk et al., 2008; Simarmata and Penner, 2008; Kaundun et al., 2011; Collavo and Sattin, 2012; Gonzalez-Torralva et al., 2012). However, Pro-106 substitutions confer only a modest degree of glyphosate resistance (Powles and Yu, 2010). Mutations in the EPSPS gene at Gly-101, Thr-102, Gly-144, and Ala-192 have been detected as sources of glyphosate resistance when expressed in *Escherichia coli*, but have not yet been reported in glyphosate-resistant weeds (Sammons and Gaines, 2014).

In crop fields under glyphosate selection, the evolution of a double amino acid substitution at Thr-102 and Pro-106 in the EPSPS in *Eleusine indica* conferring a high level of glyphosate resistance was recently communicated (Yu et al., 2015). In this species, other mechanisms of glyphosate resistance have also been found, associated with the over-expression of EPSPS (Chen et al., 2015). Target-gene duplication was previously reported in *Amaranthus palmeri* and *Lolium perenne* ssp. *multiflorum* (Gaines et al., 2010; Salas et al., 2012) and today it is a common and robust glyphosate resistance mechanism

(Sammons and Gaines, 2014). On the other hand, non-target-site mechanisms of glyphosate resistance based on exclusion systems such as decreased herbicide retention and foliar uptake (Michitte et al., 2007), or reduced glyphosate translocation and vacuolar sequestration (Lorraine-Colwill et al., 2003; Perez-Jones et al., 2007; Ge et al., 2010; Vila-Aiub et al., 2012; Ghanizadeh et al., 2016) are well documented.

In the genus *Lolium*, allogamous species have demonstrated an unprecedented capacity to evolve resistance to multiple herbicides on five continents (Heap, 2017). In Argentina, glyphosate resistance was reported in a *L. perenne* population after 12 years of successful use (Yanniccari et al., 2012d). In this population, a 10.8-fold greater dose of glyphosate was necessary to match the control efficiency of a susceptible population (Yanniccari et al., 2012d). Nonetheless, the underlying mechanism of herbicide resistance has not been studied. The aim of the current paper was to determine the mechanism of glyphosate resistance in *L. perenne* from Argentina.

MATERIALS AND METHODS

Plant Material

Perennial ryegrass seeds from a glyphosate-resistant population were collected in a field in the south of Buenos Aires province (37°S, 62°W) in 2009. The fields had a history of 12 years of no-tillage agriculture, with weed control based on three applications of glyphosate per year, at doses ranging from 360 to 720 g ae ha⁻¹ (Yanniccari et al., 2012d). One hundred field-collected seeds from 50 randomly chosen plants were germinated in Petri dishes containing filter paper with distilled water. The germination occurred in a growth chamber with 75 μmol m⁻² s⁻¹ of photosynthetically active radiation, in a regime of 12/12 h of light/darkness and temperatures of 25/15°C day/night. After 7 d, 25 seedlings taken at random were transferred to 250 cm³ pots (contained in a plastic tray) filled with soil. The plants were grown in a greenhouse and the pots were randomized and subirrigated daily maintaining a water level of about 10 mm in the irrigation tray throughout the assay. Fertilizer (12:10:20, Nitrofoska, Compo Argentina) (2 g L⁻¹) was added every 15 d. Plants were grown for 8 weeks and vegetative clones of individual plants were propagated by tiller partition in order to obtain four ramets per plant. When individual ramets reached the BBCH 23–24 scale, each one was treated with glyphosate (isopropylamine salt of glyphosate, Roundup, 360 g L⁻¹, Monsanto Argentina) at 0, 500, or 2,000 g acid equivalent (ae) ha⁻¹. To this end, a backpack sprayer equipped with flat-fan nozzles (Teejet 11002) was used, delivering 150 L ha⁻¹. At 21 d post-application, plants were characterized as glyphosate susceptible (with no survivors at 500 g ae ha⁻¹ and higher doses) or glyphosate resistant (surviving plants at 2,000 g ha⁻¹ and lower doses).

Shikimate Accumulation Test

Six plants of each susceptible and glyphosate-resistant phenotype were employed to evaluate the shikimate accumulation in glyphosate-treated leaves incubated under *in vitro* conditions. Four last full-expanded leaves were cut at 0.5 cm above the ligule from tillers of each plant. Subsequently, the leaves of each

plant were randomly placed vertically on metal lab racks and treated with different doses of glyphosate (0, 500, 1,500 and 3,000 g ae ha⁻¹) using a manual sprayer calibrated to deliver 200 L ha⁻¹. After 10 min of application, each leaf was placed in a 20 cm³ glass test tube, where the base of the leaf was immersed in with 1 mL of Hoagland nutrient solution diluted 1:3 with distilled water. The tubes were closed with flexible thermoplastic (Parafilm®) in which a 1 mm-diameter hole was made to promote gas exchange between the confined atmosphere and the exterior. Immediately, the tubes were incubated for 72 h under continuous light (300 μmol m⁻² s⁻¹ of photosynthetically active radiation) at 20°C (tubes were randomly placed every 24 h). After that, 0.05 g of fresh weight from the middle third of the each leaf was used for shikimic acid extraction, following the methodology of Singh and Shaner (1998) with the modifications of Perez-Jones et al. (2007). Shikimic acid was quantified with a double beam spectrophotometer (Shimadzu UV-160A, Shimadzu Corporation) at 382 nm. The determination of the concentration of shikimic acid was based on a shikimate (3α,4α,5β-Trihydroxy-1-cyclohexene-1-carboxylic acid, 99%, Sigma Aldrich, Inc.) standard curve. The experiment was conducted as a completely randomized design and was repeated twice.

ANOVA was performed to evaluate the differences between experiment replicates, phenotypes, herbicide treatments, and interactions. Residual plots indicated that the variances were normally distributed and homogeneous. Means were compared using Fisher's LSD test ($p < 0.05$) when there were significant differences.

Enzyme Assay

The activity of EPSPS from glyphosate-susceptible and -resistant plants was evaluated under increasing glyphosate concentrations. The EPSPS extraction and assay protocols were modified from procedures described by Sammons et al. (2007). Forty grams of fresh unexpanded leaves were harvested from non-glyphosate treated clones of both phenotypes. Using liquid nitrogen, the samples were ground with 8 g of polyvinyl pyrrolidone. Subsequently, 200 mL of extraction buffer (100 mM Tris-HCl, 5 mM EDTA, 10% (v/v) glycerol, 50 mM KCl, 10 mM 2-mercaptoethanol, 2 mM benzamide, 0.5 mM phenylmethylsulfonyl fluoride and 10 μM leupeptine, pH 7.0) was added. Then, the crude extract was homogenized at 1,000 rpm for 5 min using a vertical axis micromotor with steel blades. After this, the extract was centrifuged at 800 × g for 45 min at 4°C and was subsequently filtered using a muslin cloth. Finally, the supernatant was fractionated with ammonium sulfate, and the 45–70% fraction was collected by centrifugation at 20,000 × g for 45 min. The pellet obtained was resuspended in a minimal volume of extraction buffer (2–3 mL) and desalted on a Sephadex G-25 column (PD-10, GE®) pre-equilibrated with extraction buffer according to the manufacturer's instructions. The eluate obtained was stored at –20°C until use in EPSPS activity determinations. For every fraction obtained, protein quantifications were carried out using the method of Bradford (1976) with bovine serum albumin as a reference standard.

EPSPS activity was assayed in 100 μL of 100 mM HEPES 100 mM, pH 7.0; 2 mM (NH₄)₆Mo₇O₂₄, 10 mM KF, 1 mM MgCl₂, 10% v/v glycerol, 0.5 mM shikimate-3-phosphate, 1.25 mM phosphoenolpyruvate (using ultrapure water (Milli-Q®) at 25°C by determining the amount of inorganic phosphate produced in the reaction following Eschenburg et al. (2002). EPSPS activity was expressed in enzyme unit (U) per milligram of protein (1 U stands for 1 nmol of phosphate produced per minute in the assayed reaction). The activities of glyphosate-susceptible and -resistant plants were determined at 0, 1, 5, 25, 50, 500, 1,000, 10,000 μg L⁻¹ glyphosate [96% N-(phosphomethyl) glycine, Sigma Aldrich] concentrations.

EPSPS activity data was used to build dose response curves with a non-linear log-logistic regression model of standard slope:

$$y = C + \frac{D - C}{1 + \frac{x}{I50}}$$

where y represents the EPSPS activity at the glyphosate concentration x ; C is the lower asymptote, D is the upper asymptote and $I50$ is the herbicide concentration required to achieve 50% of the maximum response. To assess the accuracy of the model, an F -test for model significance, residual variance analysis and coefficient of determination (R^2) were calculated. Finally, the parameters from glyphosate-susceptible and -resistant models were contrasted using an F -test ($P < 0.05$).

EPSPS Partial Sequencing

Total RNA was extracted from leaf tissue of both glyphosate-susceptible and -resistant plants (S1, S2, S3 and R1, R2, R3; respectively) using the Trizol® reagent (Invitrogen) method according to the manufacturer's protocol. DNA in the RNA samples was degraded using DNase (RQ1 RNase-Free DNase kit, Promega) for 30 min at 37°C. Subsequently, the RNA was quantified spectrophotometrically at 260 nm.

First strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA. Reverse transcription was carried out in a 25 μL reaction mixture containing 1 μg oligo d(T)₁₅ primer, M-MLV reverse transcriptase (200 U), RNasin (25 U), 8 mM dNTPs and M-MLV reverse transcriptase reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂ and 50 mM DTT) at 42°C for 1 h.

The resulting cDNA was used as a template for amplification of the EPSPS sequence. Forward primer (5'-AAAGGATGCCAAGGAGGAAGTAA-3') and reverse primer (5'-GTACTTCTGTCCTCCTTTAATG-3') were employed to amplify a highly conserved region in which point mutations conferring glyphosate resistance have been reported (Sammons and Gaines, 2014). A 466-bp fragment was obtained in PCR reactions (initial denaturation at 94°C for 2 min and 30 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min) containing: 250 ng cDNA template, 0.4 μM of each primer, 0.2 mM dNTPs, 0.5 mM MgCl₂, 1X reaction buffer (Inbio Highway), and 0.5 U Taq polymerase (Inbio Highway) in a 25 μL reaction mix. PCR products were purified by precipitation at 20°C with three parts of absolute ethanol and 0.1 parts of sodium acetate (3 M) for each PCR

product. The samples were centrifuged at 32,000 g for 20 min at 4°C and then washed three times with 70% ethanol. Each DNA pellet was diluted in ultrapure water and sequenced from both ends. DNA sequences obtained were cleaned, aligned, translated and compared at the 101, 102, 106, 144, and 192 codons (numbers based on the plant EPSPS numbering system used by Padgett et al., 1996).

EPSPS Expression

The cDNAs obtained from three plants of both phenotypes were employed as a template for two step qPCR reactions using an StepOnePlus™ Real-Time PCR System (Life Technologies) and FastStart Universal SYBR Green Master (Roche). *Cinnamoyl-CoA reductase* (*CCR*, AY061888.1) was used for normalization (forward primer: 5'-AGCAGCCATACAAGATGT-3' and reverse primer: 5'-AGCTAGGGTTTCCTTGTC-3'). Primers specific to *EPSPS* were designed (forward: 5'-CTTGAGTTCCTTGCTGATG-3' and reverse: 5'-GTA CTCTGTCCTCCTTAATG-3') and the following program was used: one cycle at 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Real time fluorescence data were captured at the end of each cycle of 15 s. Melt-curve analysis was conducted by holding the samples at 95°C for 15 s, then reducing the temperature to 60°C for 1 min, and then increasing the temperature to 95°C for 15 s. During this change of temperature fluorescence was measured continuously. Negative controls consisting of primers with no template were included. Each measurement was performed in triplicate. Results were expressed as fold increases in *EPSPS* expression relative to *CCR* and data were analyzed using ANOVA. Residual plots corroborated that the variances were normally distributed and homogeneous. The means were compared using the LSD test ($P < 0.05$).

RESULTS

Shikimate Accumulation Test

Shikimate concentration in leaves was strongly affected by the herbicide treatment depending on the phenotype studied. The interaction between the phenotype and the treatment showed highly significant differences (Table 1). Both experiments carried out had similar results ($P > 0.05$, Table 1) and data from them were pooled.

Shikimic acid levels quantified in glyphosate treated leaves were significantly different between samples from glyphosate-susceptible and -resistant plants (Figure 1). Leaves from susceptible plants increased their shikimate concentration by around 3.5-fold in response to glyphosate application at 500 or 1,500 g ae ha⁻¹ (Figure 1). When 3,000 g ae ha⁻¹ was sprayed the susceptible leaves increased their shikimate levels 5-fold compared with controls without glyphosate (Figure 1). Neither of the glyphosate doses applied significantly affected the shikimic acid concentration of leaves from glyphosate-resistant plants (Figure 1).

TABLE 1 | Summary of ANOVA: mean square (MS), degrees of freedom (df), and probability values (P) for the effect of replication of the experiment comparing the effect of *Lolium perenne* phenotype (glyphosate-susceptible and -resistant phenotypes), treatment (dose of glyphosate: 0, 500, 1,500, and 3,000 g ae ha⁻¹) and their interaction on shikimate concentration.

Source of variation	df	MS	P
Experiment	1	194,002	0.7213
Phenotype	1	114,065,468	<0.0001
Treatment	3	25,795,044	<0.0001
Experiment*Phenotype	1	4,220	0.9580
Experiment*Treatment	3	495,236	0.8065
Phenotype*Treatment	3	15,145,488	<0.0001
Experiment*Phenotype*Treatment	3	235,714	0.9262
Error	144	1,518,769	
Total	159		

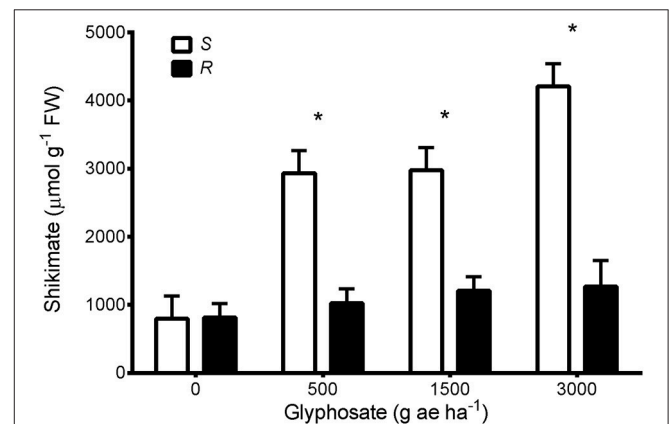


FIGURE 1 | Shikimate accumulation in foliar tissues of glyphosate-susceptible (S) and -resistant phenotypes (R) after glyphosate treatments. Vertical bars represent ± 1 standard error of the mean. Asterisks indicate significant differences between phenotypes within each dose.

EPSPS Activity

Based on the EPSPS activity data, a model was fitted for each biotype ($P < 0.001$ in both cases) and the parameters of glyphosate-susceptible and -resistant models were contrasted (Table 2, Figure 2). Comparison of the lower asymptotes (C), i.e., the level of EPSPS activity at infinitely high concentrations of the inhibitor, showed no significant differences between phenotypes. The upper asymptote (D), which is associated with EPSPS activity without glyphosate in the reaction medium, was significantly different between phenotypes ($P = 0.0018$). In this sense, basal EPSPS activity from glyphosate-resistant plants was approximately 3-fold higher than the EPSPS activity from susceptible plants. The half-maximal inhibitory concentration (I50) was not significantly different between the glyphosate-susceptible and -resistant models ($P = 0.93$) (Table 2, Figure 2).

EPSPS Partial Sequencing

A cDNA fragment of the *EPSPS* gene was sequenced and no nucleotide differences were identified between the sequences

TABLE 2 | Non-linear regression parameters for the activity of EPSPS from glyphosate-susceptible (S) and -resistant (R) phenotypes and *P*-values for model accuracy.

Phenotype	D (U mg ⁻¹ protein)	C (U mg ⁻¹ protein)	I50 (μg L ⁻¹ glyphosate)	<i>P</i>
S	3.2	-0.1	430.0	<0.001
R	8.7*	-0.1	462.8	<0.001

Asterisks indicate significant differences between phenotypes.

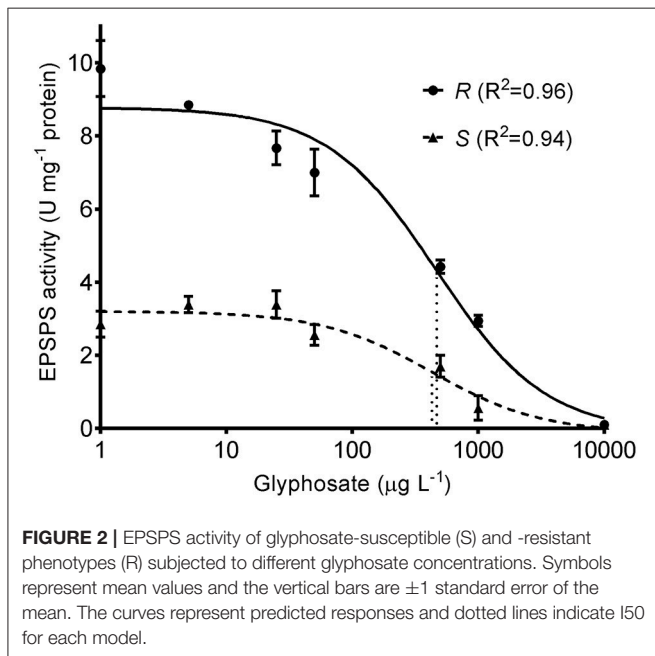


FIGURE 2 | EPSPS activity of glyphosate-susceptible (S) and -resistant phenotypes (R) subjected to different glyphosate concentrations. Symbols represent mean values and the vertical bars are ± 1 standard error of the mean. The curves represent predicted responses and dotted lines indicate I50 for each model.

from glyphosate-resistant and -susceptible individuals. Therefore, no substitutions were recorded at Gly-101, Thr-102, Pro-106, Gly-144, or Ala-192 residues, which have been associated with a mechanism of resistance to glyphosate (Sammons and Gaines, 2014; **Figure 3**).

EPSPS Expression

EPSPS transcript abundance was measured relative to *cinnamoyl-CoA reductase*. The relative expression levels of EPSPS were significantly different between phenotypes ($P < 0.01$) wherein glyphosate-resistant plants showed around 15-fold higher expression than susceptible plants (**Figure 4**).

DISCUSSION

The quantification of shikimic acid in glyphosate-treated plants has been used as metabolic marker of glyphosate sensitivity (Dayan et al., 2015). The shikimate pathway consumes $\geq 30\%$ of carbon fixed through the Calvin cycle (Maeda and Dudareva, 2012) and the inhibition of EPSPS causes a non-regulated carbon flux into the shikimate pathway

through induction of the expression of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, the first enzyme of this metabolic pathway (Zabalza et al., 2017). These alterations are reflected in a massive carbon flow to shikimate-3-phosphate, which is converted into high levels of shikimate (Duke and Powles, 2008).

The shikimic acid concentration measured in susceptible *L. perenne* plants sprayed with glyphosate was approximately three to five times the basal level while no changes were detected in glyphosate-resistant counterparts (Yanniccari et al., 2012d). In the current results, susceptible leaves treated with different doses of glyphosate and incubated *in vitro* showed shikimic acid accumulation, but leaves from glyphosate-resistant plants showed no change in shikimate concentration. This could be considered as a semi-destructive method in order to evaluate the glyphosate sensitivity of plant materials, where susceptible genotypes could be tested without killing them.

While *in vitro* methods based on shikimate quantification have been proposed for identifying glyphosate-resistant plants, in most methods leaf fragments are immersed in a medium with glyphosate and thus incubated (Shaner et al., 2005). This methodology has been effective to quickly detect differences between glyphosate-susceptible and -resistant plants (Perez-Jones et al., 2005). However, the doses used in these methodologies cannot be compared to the doses sprayed under field conditions. Moreover, the process of herbicide absorption in a system where the leaves are immersed could be very different to the foliar uptake that occurs in treated plants under field conditions. In this regard, the shikimate accumulation test in the current work successfully addressed these limitations.

The glyphosate-resistant and -susceptible plants showed differential glyphosate sensitivity when the whole plant was sprayed with the herbicide (Yanniccari et al., 2012d), as when a sample of leaf was treated and shikimate concentration was recorded. In both cases, the mechanism of resistance put into play would prevent shikimate accumulation in glyphosate-resistant plants.

The activity of herbicide target enzymes at different herbicide concentrations has been used to determine if a target site mechanism provokes low sensitivity in resistant plants (Baerson et al., 2002; Burke et al., 2006; Yu et al., 2010; Cruz-Hipolito et al., 2011). Following this approach, glyphosate-susceptible and -resistant models of EPSPS activity showed no significant differences in half maximal inhibitory concentration (I50) (**Table 2**). As a consequence, EPSPS from glyphosate-resistant plants was not different in glyphosate sensitivity compared to EPSPS from susceptible plants. Accordingly, no changes in the nucleotide sequences associated with Gly-101, Thr-102, Pro-106, Gly-144, and Ala-192 were found in either case. The affinity between the glyphosate molecule and EPSPS from resistant plants was not affected, unlike in other cases of glyphosate resistance (Ng et al., 2004; Yu et al., 2007; Simarmata and Penner, 2008; Kaundun et al., 2011).

While the glyphosate target-site was equally sensitive, differential levels of EPSPS activity was detected when comparing the plant materials. At a glyphosate concentration of 1,000 $\mu\text{g L}^{-1}$, when the EPSPS activity of susceptible plants was inhibited

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aaggatgccaaggaggaagtaaagctcttcttggggaacgctggaactgcgatgcgccattgacggctgctgtagta
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·M·E·R·F·G·V·T·A·E·H·S·D·S·W·D·R·F·Y·I·K·G·G·Q·K·Y·

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FIGURE 3 | Sequence of *EPSPS* cDNAs isolated from the glyphosate-resistant phenotype and conceptual translation of the amino acid sequence. Resistance conferring codons are shown in boxes (numbers refer to amino acid positions in the *EPSPS* plant sequence according to Padgett et al. (1996)). No mutations were detected in positions reported as sources of glyphosate resistance.

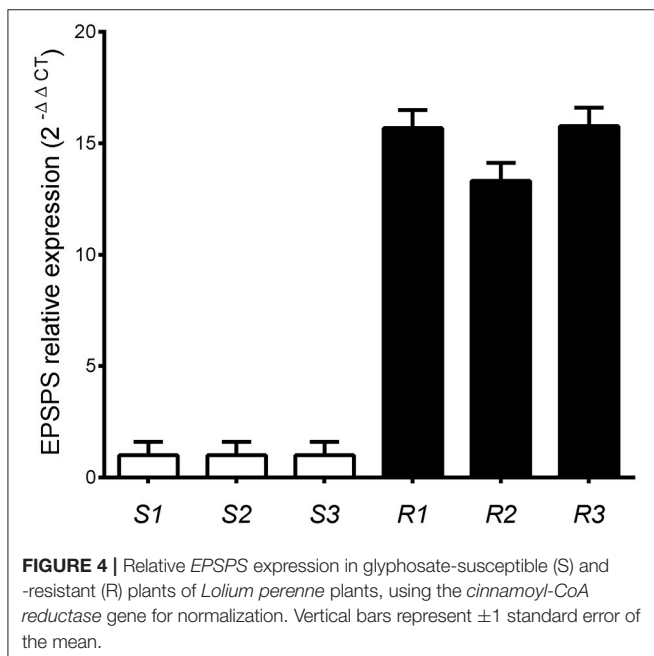


FIGURE 4 | Relative *EPSPS* expression in glyphosate-susceptible (S) and -resistant (R) plants of *Lolium perenne* plants, using the *cinnamoyl-CoA reductase* gene for normalization. Vertical bars represent ± 1 standard error of the mean.

by ~90%, the level of *EPSPS* activity measured in the crude extract from glyphosate-resistant plants was not different to the *EPSPS* activity of the susceptible material without glyphosate (Figure 2). This higher *EPSPS* activity likely ensures maintenance of a functional shikimate pathway after glyphosate treatment in the glyphosate-resistant plants.

The increased *EPSPS* activity may be due to increased transcription of the *EPSPS* gene or greater stability of the *EPSPS* enzyme at a similar transcription rate compared with susceptible plants. In the current work, the glyphosate-resistant

plants showed 15-fold higher expression of *EPSPS* than the susceptible plants and this explains the differential glyphosate sensitivity between individuals. In this sense, the glyphosate effect on net carbon assimilation, chlorophyll fluorescence, and production and transportation of assimilated carbon would be prevented by the high activity of the target enzyme (Yannicari et al., 2012a,b).

This mechanism of resistance is constitutive in character, i.e., it does not depend of a glyphosate treatment to be induced. In consequence, the high level of expression of *EPSPS* likely has important effects on plant fitness, as have recently been detected on the inhibition of growth and seed production (Yannicari et al., 2016). Considering that molecules from the shikimate pathway can account for up to 60% of the total plant dry weight (Haslam, 1993), the over-expression of *EPSPS* likely affects the regulation of the shikimate pathway and would therefore provoke pleiotropic effects on several physiological processes associated with growth and seed production.

Although is not possible to rule out that other resistance mechanisms also operate, the over-expression of *EPSPS* appears to be the main mechanism responsible for resistance to glyphosate. This mechanism would be controlled by a single locus with incomplete dominance (Yannicari et al., 2015) and would provoke low glyphosate sensitivity in plants treated in the field as observed for foliar tissues sprayed with glyphosate and incubated *in vitro*.

AUTHOR CONTRIBUTIONS

MY, CI, CN, DG, and AC conceived and designed the experiments; MY and MG performed the experiments; MY, DG, and AC analyzed the data and MY wrote the paper.

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