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Glyphosate Resistance in *Tridax procumbens* via a Novel EPSPS Thr-102-Ser Substitution

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ABSTRACT: This study confirmed the first case of glyphosate resistance in *Tridax procumbens* and investigated the glyphosate-resistance mechanisms. Sequencing and cloning of the full 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) coding sequences revealed a point mutation (ACC to TCC) at amino acid position 102, resulting in a novel Thr-102-Ser substitution. Other possible resistance mechanisms (i.e., target-site EPSPS-gene overexpression, nontarget-site differential glyphosate uptake and translocation) were also examined and were unlikely to be involved in resistance in this population. Structural modeling of the wild-type and mutant EPSPS in complex with glyphosate and phosphoenolpyruvate (PEP) revealed that the Thr-102-Ser substitution weakly decreased EPSPS affinity to glyphosate, but sharply increased EPSPS affinity to the natural substrate, PEP. Therefore, this novel mutation is very likely responsible for the observed glyphosate resistance in this tetraploid weed species via dual mechanisms of reducing glyphosate binding and favoring PEP binding to EPSPS.

KEYWORDS: *Tridax procumbens, glyphosate resistance, EPSPS mutation, polyploidy*

INTRODUCTION

Glyphosate is globally the most important and widely used herbicide, controlling broad-spectrum weeds with minimum human and environmental toxicity.[1] Glyphosate is a potent inhibitor of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which catalyzes the reaction converting shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to 5-enolpyruvylshikimate-3-phosphate (EPSP) in the plant shikimate pathway. Glyphosate competitively inhibits the substrate PEP binding in EPSPS, reducing EPSP production and leading to plant death.[2] Glyphosate use has increased greatly in the last few decades, particularly where there is the adoption of glyphosate-resistant crops. Overreliance on glyphosate has resulted in the evolution of glyphosate resistance in at least 41 weed species[3] and the selection of many glyphosate-resistance mechanisms.[4,5]

Target-site EPSPS-gene mutations are known to endow glyphosate resistance. Since the first resistance-conferring Pro-106-Ser substitution was reported in glyphosate-resistant *Eleusine indica*,[6] several other single resistance mutations at the Pro-106 site (e.g., Thr-, Leu-, and Ala-106) have been identified in glyphosate-resistant weed species.[5,7−10] These single EPSPS-gene mutations generally endow low-level glyphosate resistance while preserving EPSPS catalytic efficiency. Recently, a double EPSPS mutation of Thr-102-Ile and Pro-106-Ser (TIPS) was identified in *E. indica*, endowing high-level glyphosate resistance.[11] Apart from EPSPS point mutations, other major glyphosate-resistance mechanisms have also been identified,[4,5] typically target-site EPSPS-gene amplification,[12−14] nontarget-site vacuolar sequestration,[15] and reduced glyphosate translocation.[16−18] More glyphosate-resistance mechanisms and genes are expected to be identified in the future due to the increasing selective pressure from continued glyphosate use.

Tridax procumbens is native to the tropical Americas and now a global weed of at least 31 crops in 60 countries. It is a major weed, infesting vegetables, pastures, and irrigated and rainfed crops in northern Australia, and it is often controlled with glyphosate. Recently, we suspected that a *T. procumbens* population from Kununurra, in Western Australia had evolved glyphosate resistance. This population had been managed with repeated glyphosate treatments over several years, with poor control only evident recently. In order to better understand and manage glyphosate resistance in *T. procumbens*, this study aimed to confirm glyphosate resistance by determining the glyphosate rate causing 50% plant mortality and growth reduction in the resistant versus susceptible populations and to reveal the biochemical and molecular basis of glyphosate resistance by identifying target-site EPSPS-gene mutations or overexpression and analyzing nontarget-site glyphosate uptake and translocation. In addition, the ploidy of the Australian *T. procumbens* was also determined by chromosome-number

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counting to gain insight on glyphosate resistance in a tetraploid ($2n = 4x = 36$) background.

MATERIALS AND METHODS

Plant Material. The putative glyphosate-resistant (R) *Tridax procumbens* population was collected from tropical Kununurra (15.78 °S, 128.74 °E), Western Australia, in 2014, and the susceptible (S) population was collected from Queensland in 2016 from an area with no previous exposure to glyphosate.

Herbicide Treatment. Seeds of the R and S populations were germinated on wet filter paper at 30 °C for 5 days, and germinating seedlings were transplanted to plastic pots (12 seedlings per pot for the glyphosate-dose−response experiment) or to plastic trays (25−30 seedlings per tray for a single-rate herbicide test). Both the pots and trays contained a potting mix of 50% composted fine pine bark, 30% coco peat, and 20% river sand. The pots and trays were placed in a heated glasshouse with average day and night temperatures of 33 and 25 °C, respectively. When seedlings reached the two- to four-leaf stage, the pots were treated with varying rates of glyphosate (Roundup Powermax, 540 g a.e. L^{-1}, Monsanto), and the trays were treated with the recommended field rates of seven other herbicides with different modes of action (Table 1). Each treatment contained three replicate pots for the glyphosate-dose−response experiment. The herbicides were applied using a cabinet sprayer with a spray volume of 118 L ha^{-1} at a pressure of 200 kPa and a speed of 1 m s^{-1}. Plant mortality was determined 3 weeks after treatment, and aboveground material was harvested and oven-dried (65 °C for 2 days) for dry-weight measurements. Plants that produced no new growth after treatment were recorded as dead.

Table 1. Susceptibility (S) of the Glyphosate-Resistant *T. procumbens* Population to Herbicides with Different Modes of Action

herbicide chemical class	active ingredient	herbicide mode of action	rate (g ha^{-1})	resistance status
trizinones	metribuzin	PS II	200	S
triazines	atrazine	PS II	1800	S
ureas	diuron	PS II	1800	S
bypyridyliums	paraquat	PS I	250	S
nitriles and other	bromoxynil and pyrasulfotole	PS II and HPPD	158 and 28	S
phenoxy-carboxylic acids	2,4-D	synthetic auxins	500	S

Chromosome Counting of *T. procumbens*. Somatic chromosomes of *T. procumbens* were counted in the root-tip meristems when the roots were 0.3 cm in length. Root tips were pretreated in a saturated 1,4-dichlorobenzene solution at 18 °C in darkness for 3 h, fixed in methanol/acetic acid (3:1) at 4 °C for 30 min, and then washed in distilled water for 30 min. After enzymolysis in a solution of 2.5% cellulose and 2.5% pectinase for 45 min, the root tips were washed in distilled water for 30 min and then refixed in methanol/acetic acid (3:1) at 4 °C for 30 min. Finally, the root tips were placed on a slide and macerated in a drop of methanol/acetic acid (3:1) before being squashed, dried, and subsequently stained with Giemsa for 10 min. At least 10 metaphase cells were observed under a Nikon 80i microscope. Well-dispersed somatic chromosomes were photographed and counted using a SPOT RT KE CCD camera (Diagnostic Instruments Inc.).

Partial EPSPS-cDNA and -DNA Sequencing. For EPSPS-cDNA partial sequencing, total RNA was isolated from leaf tissue of individual R and bulked S plants using the Isolate II RNA plant Kit (Bioline). Genomic-DNA contamination was removed using the TURBO DNA-free kit (Ambion). A pair of designed primers was used to amplify a highly conserved region (^{95}LELGNAGTAMRPL^{107}) of the EPSPS gene. The forward primer TrEPSPSF1 (5′-AAGTCTTTGTCTAATCGGAT-3′) and the reverse primer TrEPSPSR1 (5′-CAGCTAGCCACGTCTCTAATG-3′) amplify a 998 bp fragment covering the known mutation sites. The amplified cDNA fragment was cloned into the pGEM-T vector (Promega) and transformed into competent *Escherichia coli* cells (strain JM109). White colonies with putative inserts were used as templates for PCR reamplification. The PCR was conducted in a 25 μL volume including 1−2 μL of cDNA, 1 μL 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 30 s, 54 °C for 30 s, and 72 °C for 1.5 min; and a final extension step of 72 °C for 7 min. Plasmids from white colonies containing the right insert were extracted with a Wizard plus SV Minipreps DNA Purification System (Promega) and sequenced.

For EPSPS-DNA partial sequencing, total DNA was extracted from the leaf tissue of single R plants using the CTAB method. On the basis of the two different EPSPS transcripts identified from our EPSPS-cDNA sequencing, two primer pairs were designed, which included two forward primers (TrEPSPSF2, 5′-CAGTTGTGGA-AGGTTGCG-3′, and TrEPSPSF3, 5′-CGGGCAGTTG-TGGAAGGTTGTA-3′) and a shared reverse primer (TrEPSPSR2, 5′-CCACCTCCAACTACACGCACG-3′). These primers were used to amplify the DNA fragments flanking the potential mutation site. The PCR system was run with the following profile: 94 °C for 4 min; 40 cycles of 94 °C for 30 s, 62 and 65 °C for primers TrEPSPSF2/R2 and TrEPSPSF3/R2 for 30 s, and 72 °C for 1 min; and a final extension step of 72 °C for 7 min. The PCR product was purified from agarose gel with Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced by a commercial service. Sequence chromatograms were visually checked for quality and clarity prior to alignment and comparison.

Full EPSPS-cDNA Sequencing. Total RNA was isolated from leaf tissue of individual R and S plants, using the NucleoZOL reagent (Macherey-Nagel). Genomic-DNA contamination was removed using the TURBO DNA-free kit (Ambion). The SMARTer RACE 5′/3′ Kit (Takara) was used for cloning the EPSPS 5′- and 3′-cDNA-flanking sequences according to the manufacturer's instructions. The gene-specific reverse primer TrEPSPSRACER1 (5′-GATTACGCCA-AGCTTGTAGCTTGAGTTGCCACCAGCAGCAG-3′) and the forward primer TrEPSPSRACEF1 (5′-GATTACGCCA-AGCTTGGTGGCACCATCACTGTTGAAGGTTGT-3′) were designed to generate 5′-RACE and 3′-RACE cDNA fragments, respectively. The PCR products were cloned into the linearized pRACE vector and transformed into Stellar competent cells. Putative inserts within colonies were reamplified using the universal M13 primers. The PCR products were sequenced by a commercial service, and sequence chromatograms were visually checked for quality and clarity.

On the basis of assembled full EPSPS-cDNA sequences, the forward primer (TrEPSPSF4, 5′-ATGGCAGCAATCACCAAC-3′) and the reverse primer (TrEPSPSR3, 5′-TAAACACATC-AATGCTTGGTG-3′) were designed to amplify the full cDNA sequences. The PCR products were cloned into the PMD18-T vector and transformed into DH5a competent cells. Putative inserts were reamplified using the M13 universal primers, and sequences obtained were compared to the assembled full EPSPS-cDNA sequence.

EPSPS Expression. Total RNA isolation from R and S plants was conducted using the same method as described above for full EPSPS-cDNA sequencing and quantified using a Qubit 3.0 Fluorometer prior to reverse transcription. For measuring EPSPS-cDNA expression, the forward (5′-GCCCTTGGAGATGTAGAGATTG-3′) and reverse (5′-TGACTTGTACTTTTGACCGCC-3′) primers were designed on the basis of the sequenced *T. procumbens* EPSPS gene in this study, and a 156 bp cDNA fragment was amplified. The quantitative-PCR system containing 50 ng of cDNA, each primer at 0.5 μM concentrations, and 10 μL of SYBR Green in a total volume of 20 μL was run using the following standard procedure from 7500 Software (Life Technologies): 20 s at 50 °C, 10 min at 95 °C, 40 cycles of 95 °C for 15 s and 60 °C for 1 min; then, the temperature

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

was gradually increased (by 0.5 °C every 5 s) to 95 °C for the generation of melting curves. Melting-curve analysis of quantitative-PCR products confirmed the specificity of the EPSPS PCR product. The primer efficiency and slope were determined to be 87% and −3.63 for the EPSPS gene from the R samples. Each assay included three biological replicates and two technical replicates. The cDNA-expression level was expressed as mean C_T ± standard error.

Glyphosate Uptake and Translocation. Germinated seedlings of the R and S populations were transplanted into plastic cups (60 × 60 × 100 mm, one seedling per cup) filled with potting mixture and grown in a controlled-environment room with 35 and 25 °C day and night temperatures, a 12−12 h day−night photoperiod, a 650 μmol m^{-2} s^{-1} light intensity, and 75% relative humidity.

The application of the ^{14}C-glyphosate treatment solution, sample harvest and combustion, and the visualization and quantification of ^{14}C-glyphosate uptake and translocation are essentially the same as the published method in ref 19, except that the four-leaf-stage seedlings were treated at the midpoint of one fully expanded leaf (the first pair of opposite leaves), and the ^{14}C-glyphosate treatment solution prepared by dilution of ^{14}C-glyphosate in glyphosate formulation (Roundup Powermax) yielded a final glyphosate concentration of 22 mM (equivalent to the field rate of 540 g a.e. ha^{-1}).

Five treated plants from each R and S population were harvested 24, 48, and 72 h after treatment. The average recovery of applied ^{14}C-glyphosate (from leaf and root wash and combustion) across the three time points in the R and S samples was 82 ± 1.2%. Glyphosate leaf uptake was expressed as a percentage of the total recovered, and translocation was expressed as a percentage of the total absorbed.

Structural Modeling of EPSPS Variants. The spatial structure of the glyphosate-susceptible wild-type (WT) isoform of *T. procumbens* EPSPS was reconstructed on the basis of *TrEPSPS1* by a homology-modeling approach[20] using the MODELER software.[21] The 1.02 Å resolution crystal structure of EPSPS from *Vibrio cholerae* in complex with S3P and glyphosate (PDB[22,23] ID: 3NVS) was used as template for *T. procumbens* reconstruction according to the highest score among all possible structural templates. To generate the promodels of mutant *T. procumbens* EPSPS isoforms, the appropriate amino acids at position 102 (Thr to Ser, Thr to Ile) were changed using the Discovery Studio Visualizer software, version 4.5.2. (www.accelrys.com). The spatial geometry of obtained promodels of the WT and mutant EPSPS isoforms was optimized via energy minimization using the L-BFGS algorithm[24] and OPLS-AA force field[25] with the gmx mdrun module of the GROMACS software.[26]

The spatial localization of the glyphosate molecule in the *T. procumbens* EPSPS binding site was defined from its position in the above-mentioned template complex, and the appropriate localization of the natural EPSPS substrate, PEP, was defined from its arrangement in complex with EPSPS from *Mycobacterium tuberculosis* (PDB ID: 2O0E). The topologies of both glyphosate and PEP for application in molecular-dynamics (MD) simulations were performed via the web-based tool LibParGen.[27]

The position-restrained MD within a 100 ps time interval (to achieve the equilibrium state) and the unrestrained (productive) MD within a 100 ns time interval at 300 K were calculated in realistic intracellular conditions for all studied complexes. Computational details correspond to a procedure described in our previous work.[28]

Free interaction energy was estimated using a custom-made tool, gr3 (T.K. and A.N., unpublished data). Shifts of equilibrium constants in solution were calculated from the equation $\ln \frac{K1}{K2} = \frac{G1 - G2}{RT}$, where $K1$ and $K2$ are equilibrium constants, $G1$ and $G2$ are the appropriate free energies, R is the universal gas constant, and T is the absolute temperature.

Statistics. The herbicide rate causing 50% plant mortality (LD_{50}) or 50% growth reduction (GR_{50}) was estimated by nonlinear regression using the four-parameter logistic model $y = C + (D - C)/[1 + (X/I_{50})^b]$, where D is the upper limit, close to the values of the untreated controls; C is the lower limit, close to the values from infinitely large herbicide rates; and b is the slope of the best-fitting curve through LD_{50} or GR_{50}. The estimates were obtained using the Sigma plot software (version 12.3, Systat Software, Inc.). Significant differences in the estimated LD_{50} and GR_{50} values and other data (i.e., glyphosate uptake and translocation and gene expression) between the R and S populations was determined by the t test ($a = 0.05$) using the software Prism (version 5.0, GraphPad Software, Inc.). Glyphosate-dose−response experiments were repeated at slightly different herbicide doses with similar results (in terms of LD_{50} and GR_{50} ratios), and therefore, only results from a single experiment were presented.

RESULTS

Glyphosate-Resistance Confirmation. The susceptible *T. procumbens* population was well controlled with no survivors at the rate of 450 g ha^{-1} glyphosate (Figure 1), with a LD_{50} value of 283 g ha^{-1}. However, 95% of the plants from the R population survived this glyphosate rate and were controlled at ≥1620 g ha^{-1}, with a LD_{50} value of 916 g ha^{-1}. On the basis of the glyphosate LD_{50} ratios in the R versus S populations, the resistance level is estimated to be a moderate 3.2-fold. Glyphosate-dose−response analysis based on plant dry mass (GR_{50}) displayed a similar level (3.9-fold) of resistance (Table 2). Therefore, glyphosate resistance is evident ($p < 0.001$) in this *T. procumbens* population from Western Australia, albeit at a relatively low level. Other commonly used herbicides with different modes of action were tested for *T. procumbens* control

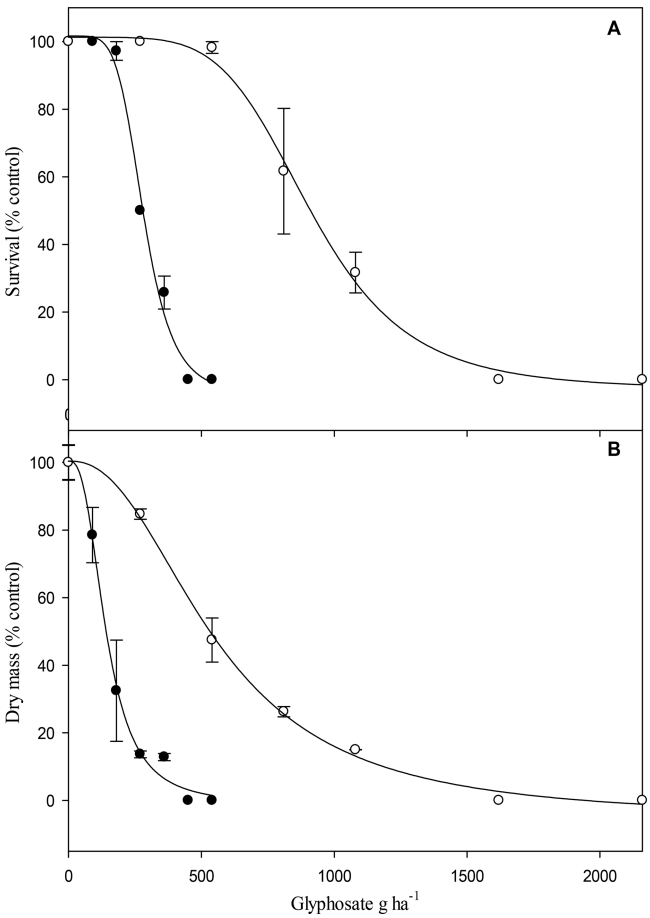

Figure 1. Plant-mortality (A) and dry-mass (B) responses to glyphosate in resistant (○) and susceptible (●) *T. procumbens* populations 3 weeks after treatment.

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 2. Parameter Estimates of Nonlinear Logistic Analysis of Dose Responses to Glyphosate for the Resistant (R) and Susceptible (S) *T. procumbens* Populations[a]

population	*C*	*D*	*b*	R^2 (coefficient)	LD_{50} or GR_{50} (g ha^{-1})	R/S ratio of LD_{50} or GR_{50}
Plant-Survival Dose−Response Curves						
S	−4.61 (4.35)	101.64 (2.27)	5.12 (0.74)	0.99	283.05 (8.31) a	
R	−2.90 (6.86)	101.26 (4.69)	5.02 (1.44)	0.94	916.27 (52.49) b	3.2
Plant-Dry-Mass Dose−Response Curves						
S	−1.19 (6.54)	100.39 (6.35)	2.70 (0.69)	0.93	142.47 (16.34) a	
R	−5.41 (4.03)	100.42 (3.06)	2.32 (0.29)	0.98	554.31 (32.66) b	3.9

[a]Standard errors are in parentheses. LD_{50} or GR_{50} values labeled with different letters for the S and R populations indicate significant difference (*t* test, $p < 0.001$).

at the recommended field rates, and the glyphosate-resistant population remained susceptible to all of them (Table 1).

Tetraploidy of *T. procumbens*. To determine ploidy, nearly 100 root-tip samples of resistant and susceptible *T. procumbens* were analyzed, and 10 metaphase cells with well-dispersed chromosomes were used for chromosome counting. Both resistant and susceptible plants were found to have 36 chromosomes (Figure 2). An earlier study determined that the

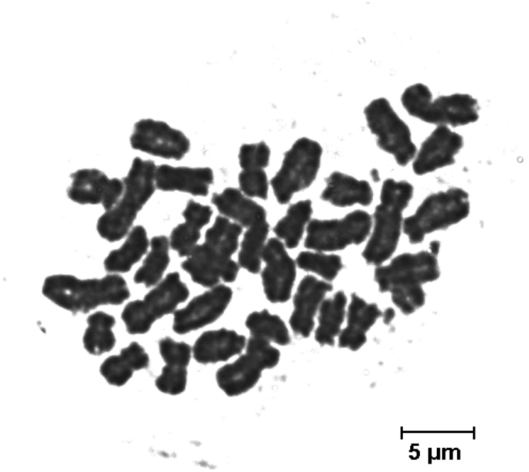

Figure 2. Somatic chromosome counting of *T. procumbens* ($4n = 36$) using the root-tip samples.

T. procumbens basic chromosome number (x) is 9;[29] therefore, the R and S plants are very likely tetraploid with chromosome numbers of $2n = 4x = 36$.

Gene Cloning and Sequencing and Analysis of EPSPS. A 998 bp EPSPS-cDNA fragment was amplified from the R and S samples using the primer pair flanking the most conserved EPSPS region. Direct sequencing revealed an A to T nucleotide mutation resulting in a Thr-102-Ser substitution in EPSPS in all five analyzed R plants as compared with the sequence of the bulked S plants. However, at this 102 codon, both mutant and wildtype nucleotides coexisted in all sequences from R individuals, indicating multiple copies of EPSPS transcripts in this tetraploid *T. procumbens*. This 998 bp fragment was then cloned from both the R and S plants and sequenced. At least two EPSPS transcripts were revealed with 35 SNPs resulting in 5 amino acid changes. However, no amino acid differences were found in sequences of the R and S plants except at Thr-102.

The 1533 bp long full coding sequences of the two EPSPS transcripts (*TrEPSPS1* and *TrEPSPS2*) were obtained from the R and S plants, coding for 510 amino acids including the transit peptide. The two translated *T. procumbens* EPSPS protein sequences share 96% identity with only 11 amino acid differences. Three amino acid changes are in the transit peptide region, six are in unconserved positions, and two are in relatively conserved positions with no reports of resistance mutations. These two homologous *T. procumbens* EPSPS protein sequences share >88% homology to *Conyza canadensis* EPSPS and 93% homology to *Helianthus annuus* EPSPS. Alignment of the *TrEPSPS1* and *TrEPSPS2* transcripts of 20 R and 20 S samples revealed no other nucleotide changes, except at the 102 codon, in either of the two transcripts. These results further confirmed that the deduced Thr-102-Ser substitution is the only amino acid change in the R EPSPS transcripts.

Furthermore, transcript-specific primers were designed to amplify EPSPS-DNA fragments flanking the 102 site. The primer pair TrEPSPSF2 and TrEPSPSR2 amplified a partial EPSPS fragment of 703 bp, and the primer pair TrEPSPSF3 and TrEPSPSR2 amplified a fragment of 350 bp (Figure 3),

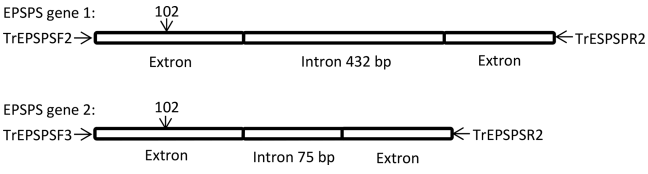

Figure 3. Two identified partial EPSPS-DNA sequences containing the Thr-102-Ser substitution but differing in intron length in *T. procumbens* plants.

and these two partial fragments differed remarkably by a length of 357 nucleotides in the intron sequences, indicating that these two transcripts are two distinct EPSPS genes. Transcript *TrEPSPS1* corresponds to the EPSPS gene with a longer intron (432 bp), whereas transcript *TrEPSPS2* matches the EPSPS gene with a shorter intron (75 bp). By using these transcript-specific primer pairs, no nucleotide heterozygosity was observed in the amplified-DNA-sequence chromatographs at the 102 site, and all the 20 R plants analyzed were found to be homozygous for the Thr-102-ser substitution on either one of these two EPSPS transcripts.

Identification of Other Possible Mechanisms. To determine if other possible mechanisms are involved in glyphosate resistance in this *T. procumbens* population, EPSPS-gene expression and glyphosate uptake and translocation were investigated. The EPSPS-gene-expression analysis revealed no significant differences in the R and S plants (Table 3). In addition, no significant difference was found in the amount of ^{14}C-glyphosate foliar uptake between the R and S plants, with 30−46% of the applied ^{14}C-glyphosate absorbed into the leaf tissue 24 to 72 h after treatment (Table

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 3. EPSPS-cDNA Expression Levels in Glyphosate-Resistant (R) and -Susceptible (S) *T. procumbens* Plants[a]

population	relative EPSPS-gene-expression level
S	19.2 (0.35) a
R	18.3 (0.05) a

[a]Standard errors are in parentheses. Means with the same letters are not significantly different (t test, $p > 0.05$).

4). Glyphosate translocation from treated leaves to untreated leaves, stems, and roots was statistically similar (Figure 4 and Table 4) in R and S plants, except at 48 h after treatment, when less ^{14}C-glyphosate was translocated away from the treated leaves and into the roots in the S plants than in the R plants. This is likely due to some self-limitation in glyphosate translocation within the S plants. Therefore, glyphosate resistance in this *T. procumbens* population is unlikely to involve changes in EPSPS-gene expression or glyphosate uptake or translocation.

EPSPS Structural Modeling. As expected, the glyphosate molecule shares the binding site with one of the EPSPS natural substrates, PEP (Figures 5 and 6), and thus is a competitive inhibitor of EPSPS activity. Glyphosate closely contacts several amino acid residues (i.e., Lys-23, Asp-50, Gly-101, Thr-102, Arg-105, Arg-131, Asp-331, Glu-359, Arg-362, Arg-404, and Lys-429; Figure 5). Glyphosate forms 13 regular hydrogen bonds with the surrounding residues (5 of them are salt bridges) and 5 carbon−hydrogen bonds; glyphosate is also involved in forming 7 attractive electrostatic interactions (Figure 7). Over the studied MD interval, the glyphosate molecule also interacts with residues Asn-99, Ala-100, Val-357, Lys-358, Asp-402, and His-403, and all of them make a significant contribution to the free interaction energy between the EPSPS protein and glyphosate (Table 5). It seems to be very intriguing that the three contact residues Thr-102, Arg-362, and Lys-429 do not make essential energy contributions to the EPSPS−glyphosate interaction in WT EPSPS, as their free energies of ligand interaction are −0.67, 0.48, and 2.78 kJ mol^{-1}, respectively. In contrast, the three amino acids Pro-132, Asp-252, and Asp-283, which are not in direct contact with glyphosate and interact only with some residues from the above contact list, do make essential contributions to the free energy of the EPSPS−glyphosate interaction.

The substitution of Thr-102-Ser has several important consequences. Despite the similarity in the chemical properties of the threonine and serine residues, this substitution results in the essential redistribution of free interaction energies in component amino acids in the binding site (Table 5). The most significant changes in the interaction energies occur with

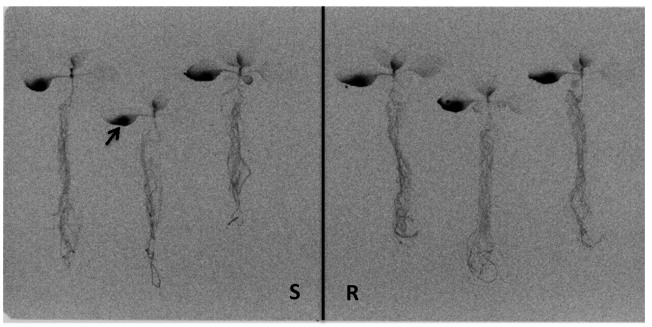

Figure 4. Translocation of ^{14}C-glyphosate from the application leaf (arrowed) to untreated parts of glyphosate-resistant (R) and -susceptible (S) *T. procumbens* plants 24 h after treatment.

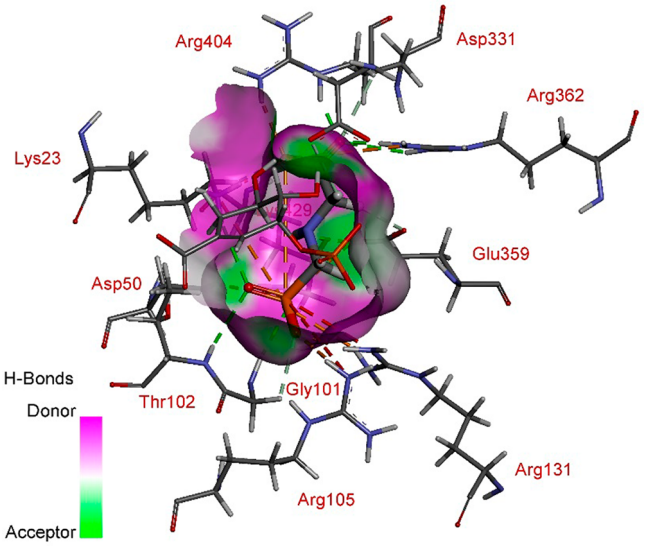

Figure 5. Spatial structure of contact interface between glyphosate and wild-type (WT) *T. procumbens* EPSPS. The protein-contact surface is colored by the H-bond-donor and -acceptor distribution, binding-site amino acids are represented by sticks, and intermolecular contacts are indicated by dotted lines.

contact residues Ala-100, Gly-101, Arg-131, Gln-180, Asp-331, Asp-402, and the "remote residue" Asp-252. The contribution of the binding-site residue Arg-362 becomes essential, as its free interaction energy reduces from 0.48 to −34.31 kJ mol^{-1}, as a result of the Thr-102-Ser replacement. The general pattern of individual energy changes (Table 5) is complex. However, analysis of the total free interaction energy of EPSPS−glyphosate (Table 6) testifies a rise to −215.8 kJ mol^{-1} in the

Table 4. Absorption and Translocation of ^{14}C-Glyphosate in Glyphosate-Resistant (R) and -Susceptible (S) *T. procumbens* Plants 24, 48, and 72 h after Treatment[a]

			translocation (% absorbed)		
treatment	treatment duration (h)	absorption (% applied)	root	untreated leaf and stem	treated leaf
R	24	29.2 (2.6) a	27.9 (2.3) a	13.6 (1.8) a	58.5 (3.6) a
S		36.8 (3.4) a	22.9 (1.5) a	10.6 (1.3) a	66.5 (2.5) a
R	48	37.0 (3.0) a	32.3 (3.6) a	16.5 (2.0) a	51.2 (4.9) a
S		46.1 (4.7) a	19.9 (1.2) b	13.5 (2.7) a	66.6 (2.5) b
R	72	43.1 (1.7) a	26.4 (3.1) a	21.4 (2.2) a	52.2 (2.7) a
S		42.4 (4.4) a	24.9 (2.2) a	15.5 (2.5) a	59.7 (4.6) a

[a]Standard errors are in parentheses. Means with the same letters in a column for each time point are not significantly different (t test, $p > 0.05$).

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

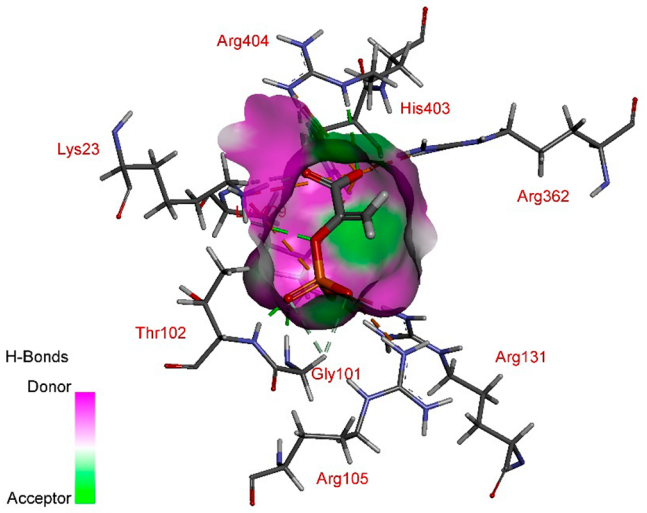

Figure 6. Spatial structure of contact interface between phosphoenolpyruvate (PEP) and wild-type (WT) *T. procumbens* EPSPS. The protein-contact surface is colored by the H-bond-donor and -acceptor distribution, binding-site amino acids are represented by sticks, and intermolecular contacts are indicated by dotted lines.

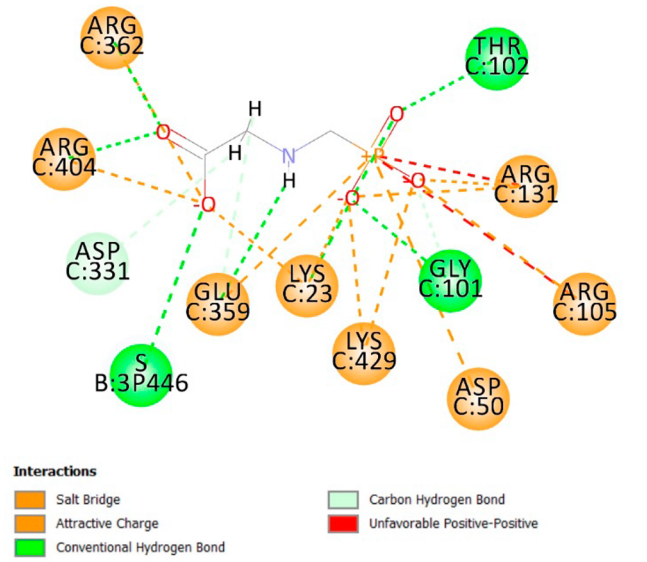

Figure 7. Diagram of intermolecular interactions (shown by dotted lines) between glyphosate and WT *T. procumbens* EPSPS. (Note that not all the interactions are visible in this figure as some are masked by the others.)

Thr-102-Ser mutant compared with −219.3 kJ mol^{-1} in the WT and the shift of the equilibrium constant to the unbound form of glyphosate. More importantly, the Thr-102-Ser substitution appears to be effective in enhancing the binding of the natural EPSPS substrate, PEP, as the free interaction energy of EPSPS−PEP sharply decreases to −403.7 from −358.3 kJ mol^{-1} in the WT, resulting in a shift of the equilibrium constant (in solution) to the bound form of PEP (Table 6). Thus, glyphosate resistance endowed by the Thr-102-Ser replacement in *T. procumbens* is likely due to at least slightly reduced glyphosate binding and sharply increased PEP binding to EPSPS.

In addition to the Thr-102-Ser substitution, we also modeled the Thr-102-Ile substitution from our previously discovered double EPSPS mutant, TIPS (Thr-102-Ile, Pro-106-Ser), in *E. indica*.[11] The Thr-102-Ile substitution in *T. procumbens* causes essential rearrangement of the free-energy contributions of individual amino acid residues (Table 5). The most significant energy changes occur in residues Lys-23, Ala-100, Gly-101, Arg-131, Gln-180, Asp-331, Arg-362, and Asp-402. In clear contrast to the Thr-102-Ser substitution, the Thr-102-Ile substitution results in greatly reduced EPSPS affinity both to glyphosate and to PEP, with significant free interaction energy increases of 29.3 and 110.3 kJ mol^{-1}, respectively. Thus, it is expected that Thr-102-Ile substitution, although endowing glyphosate resistance, will incur a plant-fitness cost because of its impact on the binding of the substrate PEP.

DISCUSSION

This research established low level (3- to 4-fold) glyphosate resistance in a *T. procumbens* population from Western Australia. The resistance is very likely to be endowed by the novel EPSPS Thr-102-Ser substitution. Target-site EPSPS mutations that confer low to moderate levels of resistance to glyphosate have been identified only at the Pro-106 position (e.g., Pro-106-Ala, -Leu, -Ser, or -Thr) in other glyphosate-resistant weed species.[5]

To our knowledge, single mutations in EPSPS that confer resistance, other than those at Pro-106, have not been reported in naturally glyphosate-resistant plants. Early EPSPS mutagenesis studies showed that the single Thr-102-Ile substitution endows a high level of glyphosate resistance but with greatly reduced affinity for the substrate PEP.[5,30] Therefore, the single Thr-102-Ile substitution is unlikely to evolve in the wild, likely because of an associated high resistance cost.[5,11] Indeed, the Thr-102-Ile substitution alone has not been reported in naturally occurring glyphosate-resistant plants. However, the Thr-102-Ile substitution has been identified as a double mutation of Thr-102-Ile and Pro-106-Ser with reduced EPSPS catalytic capacity[11] and plant fitness.[31] Instead, a different mutation (Thr-102-Ser) was identified in this study at this same site. There are several possible reasons why this novel 102 mutation may confer glyphosate resistance: (1) No other amino acid differences were found within the EPSPS full coding sequences of the R and S plants. (2) The 102 site is within the highly conserved and catalytically important EPSPS region, and the Thr-102 residue is absolutely conserved in all forms of EPSPS. (3) No other resistance mechanisms were evident in the R plants. (4) In the Thr-102-Ser substitution, the mutant amino acid Ser is very similar to the wild-type Thr in several aspects: the polarity (both are polar), the number of potential side-chain H-bonds (three), the isoelectric point (5.6−5.7), the hydrophobicity (both are hydrophilic, 0.60−0.63), and the interaction modes (both interact via H-bonds and van der Waals). One difference is that the mutant Ser residue is smaller than the wild-type Thr residue (molecular weight of 87 vs 101). These mild changes in the properties of the mutant amino acid 102-Ser may be sufficient to disturb glyphosate binding and endow glyphosate resistance, while having a minimal impact on EPSPS functionality. Hence, the single Thr-102-Ser substitution is likely to be a weak mutation, similar to the various Pro-106 mutations, which confer a low level of resistance with low or no fitness cost.

Next we took a structural-modeling approach to understand how this Thr-102-Ser substitution impacts glyphosate and PEP binding in *T. procumbens* EPSPS. EPSPS from *Vibrio cholerae* in complex with S3P and glyphosate was used as a template.

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 5. Amino Acid Residues with the Most Significant Free-Energy Contributions to the EPSPS−Glyphosate Interaction and Their Free-Energy Changes (kJ mol^{-1}) As Influenced by the Thr-102-Ser and Thr-102-Ile Substitutions

	glyphosate			phosphoenolpyruvate (PEP)		
residue	Thr-102 (WT)	102-Ser	102-Ile	Thr-102 (WT)	102-Ser	102-Ile
Lys-23	−47.18	−46.91	−94.82	−152.71	−5.28	−158.92
Asp-50	121.7	116.7	110.28	153.57	132.25	171.08
Asn-99	20.26	30.4	14.4	38.37	37.43	24.05
Ala-100	−27.52	−2.39	−1.00	−21.07	2.71	−2.78
Gly-101	−34.93	−4.85	−3.73	−40.78	−6.40	−25.23
Thr-102	−0.67	−5.85	−10.54	−11.4	−6.58	−2.72
Arg-105	−41.73	−33.33	−18.38	−37.5	−83.65	−34.18
Arg-131	−105.12	−0.82	−6.92	−178.82	−46.02	−134.64
Pro-132	12.36	7.21	4.72	6.48	8.33	5.06
Gln-180	−31.24	4.57	12.18	4.56	7.88	4.08
Asp-252	62.86	112.11	66.2	29.78	14.87	19.67
Asp-283	51.13	30.31	30.52	1.49	6.82	3.52
Asp-331	42.04	73.43	143.07	10.14	165.78	73.31
Val-357	17.48	14.77	8.82	6.02	21.13	7.72
Lys-358	−15.5	−30.44	−4.38	−3.54	−26.44	−32.14
Glu-359	141.93	144.35	153.61	82.81	70.4	138.21
Arg-362	0.48	−34.31	3.74	−2.63	−10.92	−5.85
Asp-402	28.66	88.11	125.86	9.96	67.02	108.25
His-403	−11.56	−8.16	−22.24	−6.24	−33.84	−47.8
Arg-404	−54.04	−51.1	−67.57	−3.65	−38	−23.18
Lys-429	2.78	0.43	−3.95	−99.47	12.89	−14.07

Table 6. Estimation of Total Free Interaction Energy for the Wild Type (WT) and Mutant (Thr-102-Ser) EPSPS Variants in *T. procumbens* in Comparison with Those of the Thr-102-Ile EPSPS Variant

	free interaction energy (kJ mol^{-1})		equilibrium-constant shift	
EPSPS variants	glyphosate−EPSPS	PEP−EPSPS	bound/unbound glyphosate	bound/unbound PEP
Thr-102 (WT)	−219.3	−358.3		
Thr-102-Ser	−215.8	−403.7	3.9×10^{1}	1.1×10^{-8}
Thr-102-Ile	−190	−248	1.35×10^{5}	2.2×10^{19}

Unexpectedly the Thr-102-Ser substitution only slightly reduced EPSPS affinity to glyphosate but sharply increased EPSPS affinity to the natural substrate, PEP (Table 6), rendering glyphosate less competitive than PEP. Subject to further confirmation by in vitro EPSPS kinetic studies, such a structural interaction endowing target-site herbicide resistance represents a novel mechanism, and it should be sufficient in endowing glyphosate resistance when glyphosate concentration in chloroplasts is not very high. This structural-modeling result is in line with our experimental observation that plants homozygous for the Thr-102-Ser substitution in either gene copies only displayed low-level (3- to 4-fold) resistance to glyphosate (Table 2 and Figure 1). In addition, the EPSPS structural modeling also reveals that the Thr-102-Ile substitution has a negative impact both on glyphosate binding and especially on PEP binding (Table 6), thus likely incurring a fitness disadvantage to plants. This may explain why the selection of Thr-102-Ser was favored by glyphosate in *T. procumbens*.

In addition, it is noticed that the amino acid residue in position 102 immediately contacts the phosphate group shared by both glyphosate and PEP. From this point of view, resistance mutations at Thr-102 cannot be highly effective a priori due to fitness costs. Instead, it is possible that mutations of amino acid residues contacting moieties that differ in the glyphosate and PEP structures (e.g., Asp-331 and Glu-359, Figures 5 and 6) will be more effective and selective and have less of an impact on plant fitness.

T. procumbens can be diploid or tetraploid, with a haploid chromosome number of $n = 9$ or 18.[29] In this study, we determined the chromosome number in somatic cells of the R and S plants to be $2n = 36$, and hence, it is likely that *T. procumbens* from Australia is a tetraploid ($2n = 4x = 36$). Correspondingly, at least two expressed EPSPS transcripts were cloned in *T. procumbens* in this study, corresponding to two EPSPS genes with different intron lengths. The EPSPS Thr-102-Ser substitution was found on either of the two EPSPS genes. As demonstrated in our earlier studies for herbicide target-site resistance in the polyploid weed species *Avena fatua* and *Echinoclona colona*, plant ploidy can have an impact on the level of resistance endowed by target-site resistance mutations, largely as a result of the dilution of mutant alleles by multiple wild-type alleles.[32,19] For example, even two expressed EPSPS mutations (e.g., Pro-106-Thr and Pro-106-Leu) only confer 2-fold resistance to glyphosate in the hexaploid *Echinochloa colona*.[19] Depending on the dominant nature of the resistance mutation (allele), the impact of the dilution effect on resistance level can be different in tetraploids and hexaploids. Therefore, lower-level glyphosate resistance will be expected from the EPSPS-gene mutation in the tetraploid *T. procumbens*, unless other resistance mechanisms coevolve in individuals or populations. As *T. procumbens* can be both self- or cross-pollinated,[33] evolution and accumulation of multiple glyphosate-resistance mechanisms, as evidenced in other weed species,[5,8,34,35] are possible with continued glyphosate use.

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

In summary, the first case of glyphosate resistance in tetraploid *T. procumbens* was confirmed, and the resistance established as due to a novel single EPSPS Thr-102-Ser substitution in either of the two EPSPS transcripts (*TrEPSPS1* and *TrEPSPS2*). The 3D modeling of the *T. procumbens* EPSPS variants reveals novel structural interactions of reduced glyphosate binding together with enhanced PEP binding by the Thr-102-Ser substitution. Compared with more frequently occurring single resistance mutations at the EPSPS Pro-106 site, single resistance mutations at the Thr-102 site are rare. However, sole and continued reliance on glyphosate without diversity in weed-control tactics will facilitate rare-gene selection, as has been demonstrated in this study and in *E. indica* with the in-field selection of a rare TIPS double mutation.[11]

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Notes

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