Gene amplification confers glyphosate resistance in *Amaranthus palmeri*


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The herbicide glyphosate became widely used in the United States and other parts of the world after the commercialization of glyphosate-resistant crops. These crops have constitutive overexpression of a glyphosate-insensitive form of the herbicide target site gene, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Increased use of glyphosate over multiple years imposes selective genetic pressure on weed populations. We investigated recently discovered glyphosate-resistant *Amaranthus palmeri* populations from Georgia, in comparison with normally sensitive populations. EPSPS enzyme activity from resistant and susceptible plants was equally inhibited by glyphosate, which led us to use quantitative PCR to measure relative copy numbers of the EPSPS gene. Genomes of resistant plants contained from 5-fold to more than 160-fold more copies of the EPSPS gene than did genomes of susceptible plants. Quantitative RT-PCR on cDNA revealed that EPSPS expression was positively correlated with genomic EPSPS relative copy number. Immunoblot analyses showed that increased EPSPS protein level also correlated with EPSPS genomic copy number. EPSPS gene amplification was heritable, correlated with resistance in pseudo-F2 populations, and is proposed to be the molecular basis of glyphosate resistance. FISH revealed that EPSPS genes were present on every chromosome and, therefore, gene amplification was likely not caused by unequal chromosome crossing over. This occurrence of gene amplification as an herbicide resistance mechanism in a naturally occurring weed population is particularly significant because it could threaten the sustainable use of glyphosate-resistant crop technology.

The molecular target of glyphosate (10) is the chloroplastic enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19), a component of the shikimate pathway (11). In crop species, resistance to glyphosate has been conferred by expression of bacterial genes that metabolize glyphosate (12), overexpression of sensitive EPSPS, expression of glyphosate-resistant EPSPS from bacteria, and expression of glyphosate-resistant plant EPSPS containing one or more target-site mutations (13). After step-wise glyphosate selection, EPSPS gene amplification has occurred in plant cell lines, resulting in glyphosate resistance in cell culture (12).

Glyphosate resistance has been confirmed in 16 weed species as of 2009 (14). In weed species that have evolved glyphosate resistance, the resistance mechanisms thus far elucidated are reduced glyphosate translocation and/or target-site mutations in the EPSPS gene (15). Reduced glyphosate translocation is a common resistance mechanism in *Conyza canadensis* and *Lolium rigidum* and this mechanism provides a higher level of resistance (7- to 11-fold) than do known EPSPS mutations in weedy species (16). EPSPS mutations at Pro106 (using the maize mature EPSPS numbering system) confer glyphosate resistance in several glyphosate-resistant weed species, including *Eclisus indicus* (17), *L. rigidum* (18), and *L. multiflorum* (19). The lower levels of resistance (2- to 3-fold) provided by the Pro106 mutations are sufficient for weeds to survive typical glyphosate application rates (18). To date, increased EPSPS expression has not been identified as a resistance mechanism in glyphosate-resistant weeds.

CROP yield loss due to *A. palmeri* is particularly problematic (20), in part because *A. palmeri* populations previously evolved herbicide resistance to photosystem II inhibitors, acetolactate synthase (ALS) inhibitors, and dinitroanilines (21). The first reported glyphosate-resistant *A. palmeri* population was 6- to 8-fold more resistant than a susceptible population (3), and the glyphosate resistance mechanism in this population was previously unknown but is not due to differences in absorption or translocation of glyphosate (3). The


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mechanism is also not due to a ploidy change (3), because glyphosate-resistant individuals had the reported A. palmeri genome size (22). Here, we use genetic and molecular analyses of EPSPS genes and proteins from glyphosate-resistant and -susceptible A. palmeri populations and demonstrate that amplification of the EPSPS gene is the glyphosate resistance mechanism.

Results

EPSPS cDNA Sequencing. Target site mutations in the EPSPS gene confer 2- to 3-fold glyphosate resistance in several other weedy species (15). To determine whether a target site mutation was present in glyphosate-resistant A. palmeri, full-length cDNA of EPSPS was obtained by PCR from seven glyphosate-resistant (R) and two glyphosate-susceptible (S) A. palmeri plants collected from Georgia (United States). Sequence analysis did not reveal mutation in the R cDNA at the Pro106 residue known to confer glyphosate resistance in other weed species (Fig. S1). An SNP occurred in position 316 of all EPSPS fragments from R individuals (Fig. S1), resulting in a substitution of a lysine for arginine. Some plant species susceptible to glyphosate contain a lysine at this position, suggesting that this polymorphism is not conferring glyphosate resistance.

Effect of Glyphosate on EPSPS cDNA and Shikimate Levels. Shikimate accumulates in plants when EPSPS is inhibited by glyphosate because shikimate-3-phosphate, a substrate in the reaction catalyzed by EPSPS, converts to shikimate and accumulates faster than it can be consumed in other metabolic pathways (11). Glyphosate R and S plants originating from Georgia populations were sampled for shikimate accumulation and RNA before and 8 h after treatment (HAT) with water or 0.4 kg ha−1 glyphosate. The S plants accumulated shikimate after glyphosate treatment, whereas R plants did not (Table 1). Using quantitative RT-PCR, EPSPS transcript abundance was measured relative to ALS (EC 4.1.3.18), a low-copy gene with known monogenic inheritance in Amaranthus species (23). Compared with S plants, R plants had, on average, 35-fold higher EPSPS expression relative to ALS (Table 1), and expression was unaffected by glyphosate treatment.

EPSPS Gene Copy Number Correlates with Glyphosate Resistance. DNA blot hybridizations indicated an increase in EPSPS copy number in R relative to S plants (Fig. S2). We used quantitative PCR to more accurately measure relative genomic copy numbers of the EPSPS gene relative to ALS in R and S individuals. Genomic EPSPS copy numbers relative to ALS ranged from 1.0 to 1.3 (n = 12) for S plants, whereas relative copy numbers for R plants were much higher, varying from 5 to more than 160 (n = 12) (Fig. 1).

In a leaf disk assay using 250 μM glyphosate, all 12 S plants accumulated shikimate, an indication that EPSPS was inhibited, whereas 10 of 12 R plants did not accumulate shikimate, indicating that EPSPS was still functioning (Fig. 1). The R plant with the lowest relative EPSPS copy number accumulated a modest amount of shikimate, the R plant with a relative EPSPS copy number of 65 accumulated shikimate to levels only slightly above background, and both accumulated much less shikimate than the S plants (Fig. 1).

To determine whether the association between glyphosate resistance and increased EPSPS copy number was heritable, two pseudo-F2 populations were generated, one by hand-pollinating F1 plants that were verified resistant by treatment with 0.4 kg ha−1 glyphosate. The F1 plants had a glyphosate R male parent and an S female parent. EPSPS relative copy number was determined for the parents of the hand-pollinated pseudo-F2 population, in which the F1 male parent had 18 relative EPSPS copies and the F1 female parent had 39 relative EPSPS copies. The pseudo-F2 populations segregated for both relative EPSPS copy number and glyphosate resistance, and these two traits were strongly associated (Fig. 2 A and B). Relative EPSPS copy number ranged from one to greater than the sum of copy numbers from both parents (Fig. S4). Generally, pseudo-F2 individuals with increased copy number did not accumulate shikimate at 250 μM glyphosate, indicating that they were resistant to that glyphosate dose, although a few individuals with >20 relative copies accumulated shikimate at levels slightly higher than background after treatment with 250 μM glyphosate. All pseudo-F2 individuals with 1 relative EPSPS copy were distinguishable by high shikimate accumulation, indicating that they were susceptible to glyphosate and that the population was segregating for glyphosate resistance (Fig. 2 A and B).

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Glyphosate</th>
<th>Shikimate 8 HAT (Δ ng shikimate μL−1)</th>
<th>EPSPS expression relative to ALS 8 HAT [2ΔΔCt]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>–</td>
<td>0.5 (0.3)</td>
<td>0.8 (0.1)</td>
</tr>
<tr>
<td>Susceptible</td>
<td>+</td>
<td>15.0 (1.8)</td>
<td>0.8 (0.1)</td>
</tr>
<tr>
<td>Resistant</td>
<td>–</td>
<td>–0.9 (0.6)</td>
<td>35.1 (4.7)</td>
</tr>
<tr>
<td>Resistant</td>
<td>+</td>
<td>–0.5 (0.3)</td>
<td>35.0 (5.7)</td>
</tr>
</tbody>
</table>

EPSPS cDNA was measured relative to ALS using quantitative PCR and expressed as 2ΔΔCt (threshold cycle), where ΔΔCt = (Ct, ALS − Ct, EPSPS). The + glyphosate data were obtained 8 HAT with 0.4 kg ha−1 glyphosate, and the – glyphosate data were obtained 8 HAT with water. Means and standard errors (in parentheses) are from two experimental runs with four biologic replicates each.
copy number of 1:1 had a relative EPSPS:ALS transcript abundance of $\approx 1:1$ (Fig. 3), whereas plants with increased relative EPSPS genomic copy number had increased EPSPS relative transcript abundance (Fig. 3). There was a strong correlation ($r = 0.76$, $P < 0.0001$) between relative EPSPS genomic copy number and transcript abundance (Fig. 3).

EPSPS Quantity and Activity Correlate with EPSPS Genomic Copy Number. EPSPS protein quantity was measured with immunoblotting. The EPSPS signal in plants with increased EPSPS relative copy number rapidly saturated, preventing quantification relative to plants with lower copy number. Thus, we loaded half as much total soluble protein (TSP) for plants with $> 20$ relative EPSPS copies as for plants with $< 20$ relative copies. EPSPS signal intensity had a significant positive relationship ($R^2 = 0.85$, $P < 0.0001$) with EPSPS genomic copy number in S, R, and pseudo-F$_2$ plants (Fig. 4).

We conducted an EPSPS activity assay to compare the EPSPS activity in resistant pseudo-F$_2$ plants with increased EPSPS genomic copy number relative to a susceptible pseudo-F$_2$ plant with no increase in relative copy number. EPSPS activity was measured using phosphate released by EPSPS and was much lower in the susceptible pseudo-F$_2$ plant than in resistant pseudo-F$_2$ plants (Fig. 5), because the pseudo-F$_2$ plant with 54 relative copies exhibited $\approx 20$ times more EPSPS activity than the plant with 1 relative copy. The IC$_{50}$ values (glyphosate dose that inhibited 50% of EPSPS activity) for three samples with greater than 1 relative EPSPS copy were lower but not statistically different ($\alpha = 0.05$) from the IC$_{50}$ for the sample with 1 relative copy (Fig. 5), indicating that EPSPS from plants with increased EPSPS copy number is as sensitive to glyphosate inhibition as EPSPS from plants lacking increased EPSPS copies.

Distribution of the Amplified EPSPS Gene in the A. palmeri Genome. We used FISH to determine the chromosomal locations and distributions of the amplified EPSPS genes. A 1-kb fragment from the EPSPS gene was used as the FISH probe. The reported chromosome number for A. palmeri is 2n = 34 (24), and we observed FISH signals dispersed throughout the genome in an R individual (Fig. 6). A uniform FISH signal pattern was also observed in most interphase nuclei (Fig. 6). These results suggest that the amplified EPSPS genes were randomly inserted into the A. palmeri genome. In contrast, we did not observe unambiguous FISH signals in the majority of metaphase or interphase cells prepared from an S individual. One or few putative FISH signal spots were observed in some interphase nuclei from the S individual. However, it was technically difficult to distinguish such putative FISH spots from background signals. The EPSPS copy number was not characterized in the R individual plant, but the average copy number in R plants ($n = 12$) was 77 ($\pm 14$) (Fig. 1).

Discussion

We demonstrate that the recent evolution of glyphosate resistance in a weed population is due to EPSPS gene amplification and increased EPSPS expression. Increased expression of EPSPS as a molecular glyphosate resistance mechanism has been reported to endow relatively low-level glyphosate resistance in laboratory studies (25–27), but this report concerns a field weed population. The data reported here indicate that an EPSPS gene amplification in glyphosate-resistant A. palmeri from Georgia results in high levels of EPSPS expression and that this mechanism imparts high-level glyphosate resistance. The gene amplification is not due to genome duplication (i.e., ploidy change) (3). EPSPS is normally a low-copy gene in plants: rice has one EPSPS locus (28) and Arabidopsis has two loci (29).

It is unknown whether the EPSPS gene amplification existed within the A. palmeri population in Georgia before glyphosate selection pressure or whether EPSPS gene amplification occurred during a period of $< 7$ years over which glyphosate was
repeatedly applied. Interesting future questions include whether other loci are duplicated and whether this *A. palmeri* biotype carries a genetic trait that endows high levels of gene amplification without an increase in chromosome number. FISH analysis revealed that the amplified *EPSPS* genes were dispersed throughout the genome. Lack of large tandem arrays of the *EPSPS* gene suggests that the amplification is not due to unequal crossing-over or rolling circle replication-based mechanisms. The high number of copies and their location throughout the genome suggest that the amplification could have originated via a transposon- or RNA-mediated mechanism, followed by selection of a highly amplified individual from the population. Most transposons in plant genomes are inactive but may be activated by various conditions, including abiotic stress (30). Therefore, a testable hypothesis is that the original *EPSPS* locus was associated with a mobile genetic element that activated and amplified the *EPSPS* gene.

The most common glyphosate resistance mechanism selected in plant cell culture is increased *EPSPS* activity, typically due to gene amplification (12). There is evidence for enhanced *EPSPS* expression in glyphosate-resistant weeds, but no previous evidence for *EPSPS* gene amplification. Two- to threefold elevated *EPSPS* expression and enzyme activity were found in glyphosate-resistant *L. rigidum*, and *EPSPS* from glyphosate-resistant and -susceptible plants were equally sensitive to glyphosate (31). However, the elevated expression was not due to gene amplification because *EPSPS* gene copy number in *L. rigidum* was examined using DNA blot hybridizations and glyphosate-resistant lines did not have increased *EPSPS* gene copy number in comparison with glyphosate-susceptible lines. In glyphosate-resistant biotypes of *C. canadensis* and *C. bonariensis*, basal *EPSPS* mRNA levels were double the levels in susceptible biotypes, but the resistant biotypes also had reduced glyphosate translocation (32, 33).

In our studies of a segregating *A. palmeri* pseudo-F₂ population, increasing *EPSPS* gene copy number correlated with increased *EPSPS* mRNA, increased *EPSPS* protein activity, and glyphosate resistance. The higher quantity of *EPSPS* in glyphosate-resistant pseudo-F₂ plants was equally sensitive to glyphosate inhibition as *EPSPS* from glyphosate-susceptible pseudo-F₂ plants, in contrast with *E. indica*, in which the IC₅₀ for glyphosate-resistant lines with a Pro106 mutation was 5-fold higher than in S lines (17).

*EPSPS* protein levels and activity both increased as the number of *EPSPS* genomic copies increased. Therefore, the effect of *EPSPS* copies is additive, and additional copies confer higher levels of resistance. We measured the resistance phenotype with 250 μM glyphosate in an in vivo leaf disk assay, and this dose did not induce shikimate accumulation in most individuals with *EPSPS* gene amplification. This result should not be interpreted to indicate that plants with a 20-fold increase in copy number are as resistant as plants with a 60- or 100-fold increase in copy number. *EPSPS* activity can be reduced to nearly zero in plants with increased copy number, but the dose required to eliminate *EPSPS* activity increases with increasing copy number, indicating that additional *EPSPS* gene copies have an additive effect in conferring resistance.

The stability of *EPSPS* gene amplification in *A. palmeri* is unknown, because the extent of *EPSPS* gene amplification varied greatly in plants from the R field population. Additionally, one individual in an *A. palmeri* pseudo-F₂ had a higher relative *EPSPS* copy number than the sum of the relative copy number from both parents, indicating that additional copies may be gained during recombination. Even if the *EPSPS* gene amplification is unstable during sexual recombination, apomixis may occur in *A. palmeri* (34), which could function to maintain the large amplification in the population. Further contributing to the dynamics of *EPSPS* copy number, its amplification and increased expression could have a fitness penalty in the resistant biotype in the absence of glyphosate selection (35).

Although not previously reported in naturally occurring plant populations, large gene amplifications that confer resistance to xenobiotic compounds have occurred in other organisms. Large tandem gene amplifications of metabolic genes confer insecticide resistance in *Culex* mosquitoes and *Myzus* aphids (36, 37). Organophosphate-resistant mosquitoes had ≥80-fold more copies of esterase genes than susceptible mosquitoes (37). Resistance to methotrexate in mammalian cancer cells is due to overproduction of the target enzyme, dihydrofolate reductase, from gene amplification (38). This adaptation occurred during step-wise selection with increasing methotrexate doses and resulted in gene amplification and overproduction of normal dihydrofolate reductase.
Our data demonstrate that glyphosate resistance in a Georgia *A. palmeri* population is due to many-fold amplification of the EPSPS gene on multiple chromosomes. This occurrence of gene amplification as an herbicide resistance mechanism was observed in a naturally occurring weed population. It remains to be seen whether the same mechanism exists in other glyphosate-resistant *A. palmeri* populations or in other glyphosate-resistant species. The occurrence of the EPSPS gene amplification in *A. palmeri* raises many questions about how the amplification occurred initially and has been subsequently maintained, including the frequency of other gene amplifications across the genome and the role of this process in the evolution of *A. palmeri* as an economically damaging weed with a history of multiple herbicide resistance traits.

**Materials and Methods**

**Plant Material and Genetic Populations.** Seeds of *R. A. palmeri* were collected from a field site in Macon County, Georgia (3), whereas seeds of a known S *A. palmeri* population were collected from the University of Georgia Ponder Farm Research Station. Seeds of R and S were germinated and transplanted into large pots for growth in a greenhouse. The resistance phenotype of each plant was confirmed using an in vivo leaf disk assay (39). Each plant was covered with polination bags before flowering. R males were placed next to 5 females to create an F1 generation (S/R). Plants were shaken daily to ensure adequate pollination.

Seeds from the S female plants were stored at 4°C for 2 months, then germinated and grown to the four-leaf stage. These S/R F1 plants were sprayed with a low rate (0.4 kg ae ha⁻¹) of formulated glyphosate (potassium salt, Roundup Weather Max, Monsanto) to select for heterozygous resistant progeny, because apomixis may occur in *A. palmeri* (34). One R F1 male was selected for hand crossing to one R F1 female to generate a hand-pollinated pseudo-F2 through half-sibling mating. Both parents of the hand-pollinated pseudo-F2 were sampled for DNA extraction (see below). Polination bags were placed over female inflorescences before emergence, and pollen from the resistant male was applied by hand daily for 2 weeks. An open-pollinated pseudo-F2 population was generated by placing different R female and male half-siblings from the S/R F1 next to each other in the greenhouse. Seeds from female plants were stored at 4°C for 2 months.

**EPSPS cDNA Sequencing.** The EPSPS sequence from *A. tuberculatus* (FJ859880) was obtained by 5′ and 3′ RACE (40) and used to design primers for *A. palmeri* EPSPS. The following primer sets were used to amplify overlapping fragments of the central, 5′, and 3′ regions, respectively, of the EPSPS gene from resistant and susceptible cDNA: EPSF1 (5′-ATGTGGAGCGTCTCAGAATCTGGT-3′) and EPSR1 (5′-GTCAAGTTCCAATGGCGGTGG-3′); EPSF5 (5′-GCCAGAAACA- CAAAGGGAAAA-ATCTCAGAG-3′) and EPSR5 (5′-TCTTACCCACAGGGAAAACA- GACACCAC-3′) (41); and EPSF6 (5′-GGAGGAATCTCGAGGGAAAAATTTG-3′) and EPSR6 (5′-CTTATAGCTGAAATCAAAACCCCTGGGGG-3′). PCRs contained 1 μL cDNA; 400 nM each of forward and reverse primers; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 1.5 mM MgCl₂; and 1 U of high-fidelity Taq polymerase (Invitrogen) with a 1 x concentration of supplied buffer in a final volume of 25 μL. The thermaprofile included 5 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, with a final extension of 10 min at 72°C. The EPSF1 × EPSR1 PCR product contained the Pro106 codon. Seven R individuals and two S individuals were used. The EPSF1 × EPSR1 PCR product was ligated into pGEM-T Easy plasmids (Promega). Plasmids were transferred into *Escherichia coli* cells, and transformed cells were cultured overnight in liquid LB media. Plasmids from six clones of each individual were isolated for Sanger sequencing using the M13F and M13R primers. EPSPS × EPSR5 and EPSP6 × EPSR6 PCR products were isolated by gel electrophoresis for direct sequencing. Consensus sequences for each biotype were assembled using Lasergene v. 7.0 SeqMan (DNASTAR). Multiple sequence alignments of plant EPSPS, including selected accessions from GenBank, *A. tuberculatus*, and both *A. palmeri* biotypes were constructed using ClustalW2 (European Bioinformatics Institute).

**Effect of Glyphosate on EPSPS cDNA and Shikimate Levels.** Seeds from the R and S populations were germinated in small pots and grown to the four-stage leaf. Five plants each of R and S were sampled for one 4-mm leaf disk for in vivo measurement of background absorbance in a leaf disk shikimate assay (39) and one leaf disk for RNA extraction (see below). Four plants each of R and S were then treated with 0.4 kg ae ha⁻¹ glyphosate, and one plant of each was treated with water. At 8 HAT, all plants were again sampled for one leaf disk for shikimate measurement and one leaf disk for RNA extraction, to measure EPSPS cDNA expression level (see below). The 8 HAT leaf disk samples were taken from both sides of the midvein at the base of the leaf, and the 0 HAT leaf disk samples were taken distal to the 8 HAT location. The experiment was conducted twice.

**EPSPS Gene Copy Number.** Seeds from the hand-pollinated and open-pollinated pseudo-F2 populations, along with R and S seeds, were germinated and grown in small pots. Fifty-four plants of each pseudo-F2 and 12 plants of each R and S were grown to the four-stage leaf stage. One leaf of each plant was used for in vivo leaf disk shikimate accumulation assay (39) with glyphosate concentrations of 0 and 250 μM in 10 mM ammonium phosphate buffer. A shikimate standard curve was used to calculate the ng shikimate μL⁻¹ accumulation above the background level. Each plant was assayed in duplicate. One leaf from each plant was sampled for genomic DNA extraction and one leaf for RNA extraction for subsequent measurement of genomic EPSPS copy number and EPSPS cDNA expression level (see below).

**DNA and RNA Extraction and cDNA Synthesis.** Tissue samples were immediately frozen in liquid nitrogen, ground in a 1.5-mL microcentrifuge tube, and stored at −80°C. Genomic DNA was extracted using the Qiagen DNEasy Plant Mini Kit (Qiagen), quantified using a NanoDrop spectrophotometer (Thermo Scientific), and checked for quality by gel electrophoresis. DNA concentrations were adjusted to 1 ng μL⁻¹ in sterile HPLC grade water. RNA was extracted using TRIzol reagent (Invitrogen), dissolved in sterile HPLC water, quantified using a NanoDrop spectrophotometer, and checked for quality and integrity by gel electrophoresis.

* A. palmeri RNA (200 ng for time course treatments and 700 ng from each of 20 pseudo-F2 individuals) was used for cDNA synthesis with oligo-DT primers and the Verso cDNA kit (Thermo Scientific). This kit includes a DNase treatment. Final cDNA volume was 20 μL.

**Quantitative PCR.** Quantitative real-time PCR was used to measure EPSPS genomic copy number relative to ALS and cDNA expression level of EPSPS.
relative to ALS. Primer efficiency curves were conducted for each primer set using a 1x, 1.5x, 2x, and 1.25x dilution series of resistant genomic DNA. The efficiency of the EPSPS × EPSPR (S. TGAATTTTCTTCCAGCAAAGCCGAA-3′) (195-bp product) and ALSF2 (S′-GCTGTCGAAGCTGACTC-3′) × ALSR2 (S′-GCCGGAACCTGAAGCAATGCT-3′) (118-bp product) were used for quantitative PCR on genomic DNA and cDNA. ALS primers were designed on the basis of conserved regions of published plant ALS gene sequence (41).

Triplicate genomic DNA templates (10 ng) or triplicate cDNA templates (1 µL) were amplified in a 25-µL reaction volume using Syber-Green master mix (Bio-Rad Laboratories) by the following thermoprofile on a MyQ real-time PCR detection system (Bio-Rad): 95°C for 15 min, then 30 cycles of 95°C for 30 s and 60°C for 1 min. Real-time fluorescence data were captured during the amplification cycles. Melt-curve analysis was conducted by holding the samples at 95°C for 5 min, then reducing the temperature to 55°C for 5 min, followed by increasing the temperature by 0.5°C every 10 sec to 95°C. The same condition of template with no primers or template with no template were included. Threshold cycles (Ct) were calculated using Cycler iQ v. 3.1 (Bio-Rad). Melt-curve analysis of quantitative PCR products showed that no primer-dimers formed with either primer set.

Relative quantification using a modification of the 2-ΔΔCt method (42) was used to analyze data from the quantitative PCR experiments. The ALS gene was used as a low-copy control gene with known monogenic inheritance in other Amaranthus species (23). Relative quantification of EPSPS was calculated as ΔCt = (Ct, ALS – Ct, EPSPS). Increase in EPSPS copy number was expressed as 2ΔΔCt. Each individual sample was run in triplicate, and the average increase in EPSPS copy number and standard deviation were calculated for each sample. Results were expressed as fold increase in EPSPS copy number relative to ALS. The same relative quantification calculation was used for fold increase in EPSPS expression.

**EPSPS Quantification and Activity Assay.** Young expanding leaf tissue was sampled from selected R, S, and pseudo-F2 plants for protein extraction and EPSPS quantification (SI Materials and Methods). A continuous assay for inorganic phosphate release (43) was conducted with a phosphate detection kit (Molecular Probes) to assay for EPSPS activity (SI Materials and Methods). Phosphate release above background level was measured for 10 min, and a slope was calculated to determine micromoles of phosphate released per reaction. The response analysis in R was used to compare the IC50, the glyphosate concentration that inhibited EPSPS activity by 50%, and to statistically compare IC50 values (44).

**FISH Mapping of the EPSPS Gene.** FISH was conducted according to published protocols (45). The probe (1,044 bp) was synthesized using EPSPF1 × EPSPR primers from an R plant cDNA, cloned, sequenced, and then PCR amplified from the plasmid.

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