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-F_2 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.

5-enolpyruvylshikimate-3-phosphate synthase | herbicide resistance | mobile genetic element | evolution | Palmer amaranth

Global adoption of transgenic crops has been rapid, reaching 120 million ha in 2008. Approximately 85% of this area has been planted with herbicide-resistant crops, nearly all of which are glyphosate-resistant (1). Evolution of resistance to the widely used, nonselective herbicide glyphosate (N-[phosphonomethyl] glycine) in weedy species endangers the continued success of transgenic glyphosate-resistant crops and the sustainability of glyphosate as the world's most important herbicide (2). Since commercialization of glyphosate-resistant cotton in the U.S. in 1997, some growers have relied exclusively on multiple glyphosate applications each season in a monoculture system to manage weeds including *Amaranthus palmeri* (Palmer amaranth) (3). *A. palmeri* is dioecious (4) and is an economically troublesome weed threatening the sustainability of cotton production in the southeastern United States (5), where glyphosate has been the principal tool for *A. palmeri* control since 1997. Unfortunately, glyphosate resistance has now evolved in *A. palmeri* populations within glyphosate-resistant cotton fields reported in Georgia (3), Tennessee (6), North Carolina (7), South Carolina (8), and Arkansas (9). In 2009, glyphosate-resistant *A. palmeri* was projected to occur on at least 250,000 ha of crop land (8).

The molecular target of glyphosate (10) is the chloroplast-targeted 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 *Eleusine indica* (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).

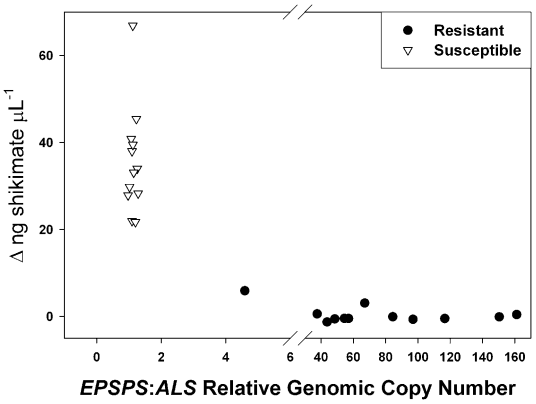

Fig. 1. Increase in genomic copy number of *EPSPS* correlates with reduced shikimate accumulation in 12 individuals each of glyphosate-resistant (filled circles) and -susceptible (open triangles) *A. palmeri* plants. Increase in genomic copy number of *EPSPS* is relative to *ALS* as measured using quantitative PCR on genomic DNA. Shikimate accumulation was measured after incubation in 250 μM glyphosate in an in vivo leaf disk assay.

To determine whether the association between glyphosate resistance and increased *EPSPS* copy number was heritable, two pseudo-F_2 populations were generated, one by hand-pollinating and one by open-pollinating F_1 plants that were verified resistant by treatment with 0.4 kg ha^{-1} glyphosate. The F_1 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-F_2 population, in which the F_1 male parent had 18 relative *EPSPS* copies and the F_1 female parent had 39 relative *EPSPS* copies. The pseudo-F_2 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. 2*A*). Generally, pseudo-F_2 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-F_2 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*).

***EPSPS* Transcript Abundance Correlates with *EPSPS* Genomic Copy Number.** Selected individuals from pseudo-F_2 and parental populations were measured for *EPSPS* transcript accumulation using quantitative RT-PCR. Plants with a relative *EPSPS*:*ALS* genomic

Table 1. Expression of *EPSPS* cDNA in glyphosate-resistant and -susceptible *A. palmeri* is not affected by glyphosate treatment

Biotype	Glyphosate	Shikimate 8 HAT (Δ ng shikimate μL^{-1})	EPSPS expression relative to *ALS* 8 HAT [$2^{(\Delta Ct)}$]
Susceptible	–	0.5 (0.3)	0.8 (0.1)
Susceptible	+	15.0 (1.8)	0.8 (0.1)
Resistant	–	−0.9 (0.6)	35.1 (4.7)
Resistant	+	−0.5 (0.3)	35.0 (5.7)

EPSPS cDNA was measured relative to *ALS* using quantitative PCR and expressed as 2^ΔCt (threshold cycle), where ΔCt = (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.

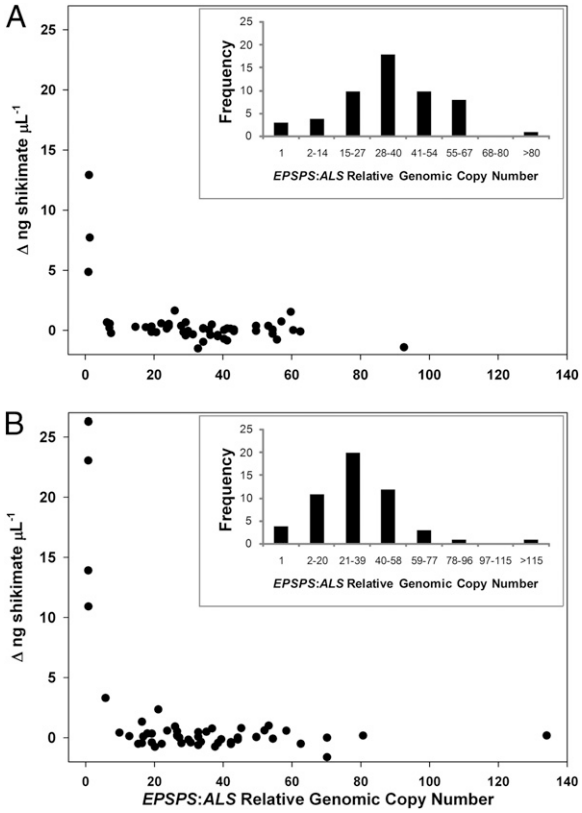

Fig. 2. *EPSPS* genomic copy number and glyphosate resistance cosegregate in pseudo-F_2 *A. palmeri* populations. *EPSPS* copy number relative to *ALS* and accumulation of shikimate were determined as described in *Materials and Methods*. *Insets:* Relative copy number histograms in pseudo-F_2 populations generated using (*A*) hand pollination (F_1 male parent 18 relative *EPSPS* copies and F_1 female parent 39 relative *EPSPS* copies) and (*B*) open pollination (parental relative copy number not measured).

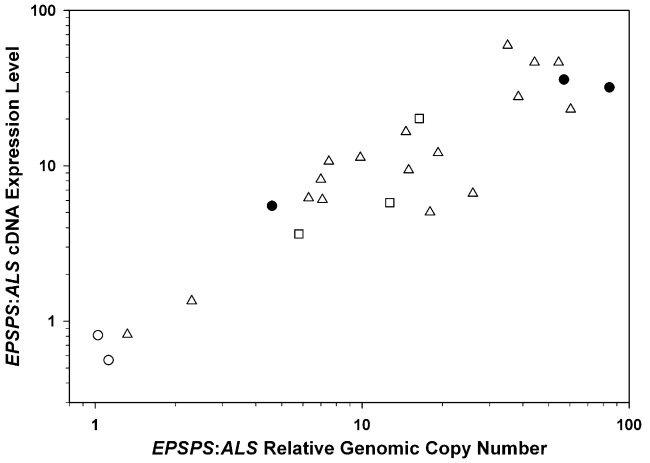

Fig. 3. Increase in *EPSPS* genomic relative copy number is positively correlated with increase in *EPSPS* cDNA expression levels in selected *A. palmeri* glyphosate-resistant (filled circles), -susceptible (open circles), and pseudo-F_2 individuals [pseudo-F_2 hand pollinated (open triangles), open pollinated (open squares)]. Genomic copy numbers and expression levels are relative to *ALS* and were determined by quantitative PCR as described in *Materials and Methods*.

copy number of 1:1 had a relative *EPSPS*:*ALS* transcript abundance of ≈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 ≈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 (±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

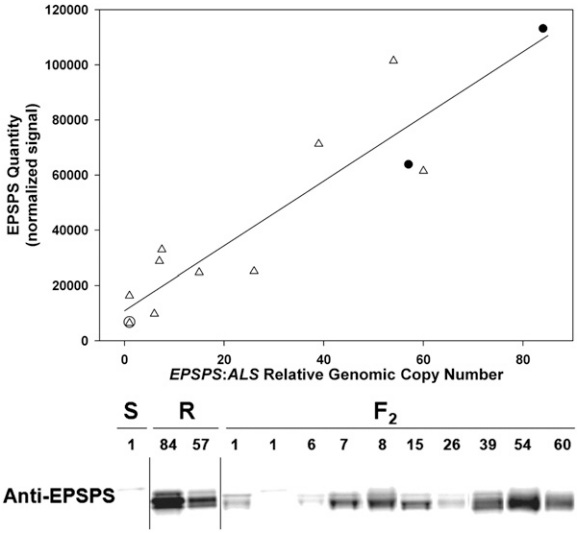

Fig. 4. EPSPS protein levels in glyphosate-susceptible (S), glyphosate-resistant (R), and pseudo-F_2 *A. palmeri* plants are correlated with relative *EPSPS* genomic copy number. *Top:* Regression of normalized EPSPS quantity on increase in relative *EPSPS* genomic copy number; open circles: S; filled circles: R; open triangles: F_2. *Bottom:* Samples with <20 relative *EPSPS* copies had 30 μg TSP loaded per lane, and samples with >20 relative *EPSPS* copies had 15 μg TSP loaded per lane. Increase in relative *EPSPS* genomic copy number is indicated above each sample lane.

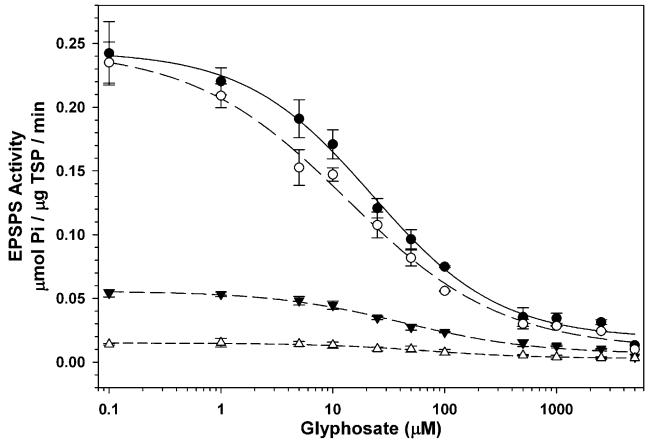

Fig. 5. Increased EPSPS enzyme activity is positively correlated with *EPSPS* relative genomic copy number in four pseudo-F_2 *A. palmeri* plants. Glyphosate inhibition assays were normalized for TSP quantity. Data points are means and standard errors of three replications. Filled circles: 54 relative *EPSPS* copies, IC_{50} (glyphosate concentration that reduced enzyme activity by 50%) = 22 μM; open circles: 39 relative copies, IC_{50} = 15 μM; filled triangles: 8 relative copies, IC_{50} = 36 μM; open triangles: 1 relative copy, IC_{50} = 66 μM.

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-F_2 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-F_2 plants was equally sensitive to glyphosate inhibition as EPSPS from glyphosate-susceptible pseudo-F_2 plants, in contrast with *E. indica*, in which the IC_{50} 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 additional *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-F_2 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.

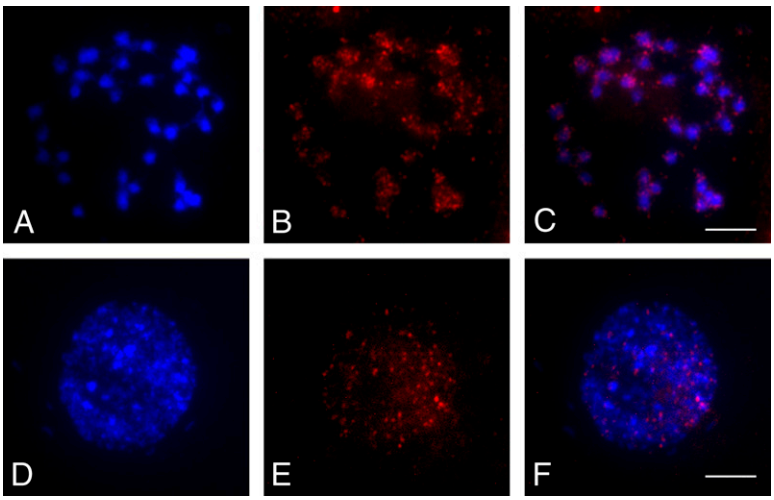

Fig. 6. FISH mapping in an *A. palmeri* glyphosate-resistant individual. (*A*) Somatic metaphase chromosomes of glyphosate-resistant *A. palmeri*. (*B*) FISH signals from the *EPSPS* gene probe. (*C*) Merged image from *A* and *B*. Dispersed signals can be observed on every chromosome. (*D*) An interphase nucleus of glyphosate-resistant *A. palmeri*. (*E*) FISH signals from the *EPSPS* gene probe. (*F*) Merged image from *D* and *E*. (Scale bars, 5 μm.)

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 pollination bags before flowering. R males were placed next to S females to create an F_1 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 F_1 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 F_1 male was selected for hand crossing to one R F_1 female to generate a hand-pollinated pseudo-F_2 through half-sibling mating. Both parents of the hand-pollinated pseudo-F_2 were sampled for DNA extraction (see below). Pollination 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-F_2 population was generated by placing different R female and male half-siblings from the S/R F_1 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* (FJ869880) 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′-ATGTTGGACGCTCTCAGAACTCTTGGT-3′) EPSR1 (5′-GTCATAAGTTTCAATGGCGGTGG-3′); EPSF5 (5′-GCCAAGAACACAAAGCGAAATTCAGAG-3′) × EPSR5 (5′-TCTTTACCAACAGGAAACAGACCACCAC-3′); and EPSF6 (5′-CAGGGAATCATCTGGAAGGAAACATTTG-3′) × EPSR6 (5′- CTATTAGTCTCAAATCAAAACCTTCGGCG-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 $MgCl_2$; and 1 U of high-fidelity Taq polymerase (Invitrogen) with a 1× concentration of supplied buffer in a final volume of 25 μL. The thermoprofile 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 transformed 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. EPSF5 × EPSR5 and EPSF6 × 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-leaf stage. 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-F_2 populations, along with R and S seeds, were germinated and grown in small pots. Fifty-four plants of each pseudo-F_2 and 12 plants each of R and S were grown to the four-leaf stage. One leaf of each plant was used for an 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-F_2 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 1×, 1/5×, 1/25×, and 1/125× dilution series of resistant genomic DNA. The primer sets EPSF1 × EPSR8 (5′- TGAATTTCCTCCAGCAACGGCAA-3′) (195-bp product) and ALSF2 (5′-GCTGCTGAAGGCTACGCT-3′) × ALSR2 (5′- GCG GGACTGAGTCAAGAAGTG-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 MyiQ 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 s to 95°C. Negative controls consisting of template with no primers and primers with no template were included. Threshold cycles (Ct) were calculated using iCycler iQ v. 3.1 (Bio-Rad). Melt-curve analysis of quantitative PCR products showed that no primer-dimers formed with either primer set. The melting peak for products of both primer sets was 86.0°C. Primer efficiency and slope were 100.2% and −3.318 for *EPSPS* and were 103.8% and −3.235 for *ALS*. No amplification products were observed in any controls lacking template.

Relative quantification using a modification of the $2^{-\Delta\Delta Ct}$ 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 $2^{\Delta Ct}$. 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-F_2 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 microgram TSP per minute. Dose–response analysis in R was used to calculate the IC_{50}, the glyphosate concentration that inhibited EPSPS activity by 50%, and to statistically compare IC_{50} values (44).

FISH Mapping of the *EPSPS* Gene. FISH was conducted according to published protocols (45). The probe (1,044 bp) was synthesized using EPSF1 × EPSR1 primers from an R plant cDNA, cloned, sequenced, and then PCR amplified from the plasmid.

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