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EPSPS Gene Amplification Primarily Confers Glyphosate Resistance among Arkansas Palmer amaranth (*Amaranthus palmeri*) Populations

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Abstract

Research was conducted to determine whether resistance to glyphosate among Palmer amaranth (*Amaranthus palmeri* S. Watson) populations within the U.S. state of Arkansas was due solely to increased *EPSPS* gene copy number and whether gene copy number is correlated with resistance level to glyphosate. One hundred and fifteen *A. palmeri* accessions were treated with 840 g ae ha⁻¹ glyphosate. Twenty of these accessions, selected to represent a broad range of responses to glyphosate, underwent further testing. Seven of the accessions were controlled with this dose; the rest were resistant. The effective dose to cause 50% injury (ED₅₀) for susceptible accessions ranged from 28 to 207 g ha⁻¹. The glyphosate-resistant (GR) accessions had ED₅₀ values ranging from 494 to 1,355 g ha⁻¹, a 3- to 48-fold resistance level compared with the susceptible standard (SS). The 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene relative copy number was determined for 20 accessions, 4 plants accession⁻¹. Resistant plants from five GR accessions (38% of resistant plants tested) did not have increased *EPSPS* gene copies. Resistant plants from the remaining eight GR accessions (62% of resistant plants tested) had 19 to 224 more *EPSPS* gene copies than the SS. Among the accessions tested, injury declined 4% with every additional *EPSPS* copy. ED₅₀ values were directly correlated with *EPSPS* copy number. The highly resistant accession MIS11-B had an ED₅₀ of 1,355 g ha⁻¹ and 150 gene copies. Partial sequences of *EPSPS* from GR accessions without *EPSPS* amplification did not contain any of the known resistance-conferring mutations. Nearly 40% of GR accessions putatively harbor non-target site resistance mechanisms. Therefore, elevated *EPSPS* gene copy number is associated with glyphosate resistance among *A. palmeri* from Arkansas.

Introduction

Glyphosate, a nonselective herbicide, is the most widely used herbicide worldwide, due to its use in glyphosate-resistant (GR) crops (Duke and Powles 2008). Resistance to glyphosate was thought unlikely to develop because of the critical role of the shikimate pathway in plant function, the high conservation of the *EPSPS* binding domain, and inability of plants to detoxify glyphosate (Bradshaw et al. 1997). However, with persistent broadscale use, resistance to glyphosate has evolved in 36 weed species, including common ragweed (*Ambrosia artemisiifolia* L.), hairy fleabane (*Erigeron bonariensis* L.), horseweed (*Erigeron canadensis* L.), goosegrass [*Eleusine indica* (L.) Gaertn.], Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot], rigid ryegrass (*Lolium rigidum* Gaudin), kochia [*Bassia scoparia* (L.) A. J. Scott], tall waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer], and Palmer amaranth (*Amaranthus palmeri* S. Watson) in North America (Heap 2017).

To date, resistance to glyphosate in weeds has been reported to be primarily conferred either by target-site (TSR) or non-target site (NTSR) mechanisms in the *EPSPS* gene (Fernández-Moreno et al. 2016; Shaner et al. 2012). NTSR mechanisms with respect to glyphosate include reduced absorption and translocation (Fernández-Moreno et al. 2017; Vila-Aiub et al. 2012), increased vacuolar sequestration (Ge et al. 2012), degradation to nontoxic compounds (Rojano-Delgado et al. 2012), and rapid tissue necrosis followed by regrowth (Moretti et al. 2017; Van Horn et al. 2017). Reduced glyphosate translocation is the most commonly reported NTSR mechanism (Dinelli et al. 2008; Feng et al. 2004; Perez et al. 2004; Wakelin et al. 2004). TSR mechanisms confer resistance to glyphosate as a result of

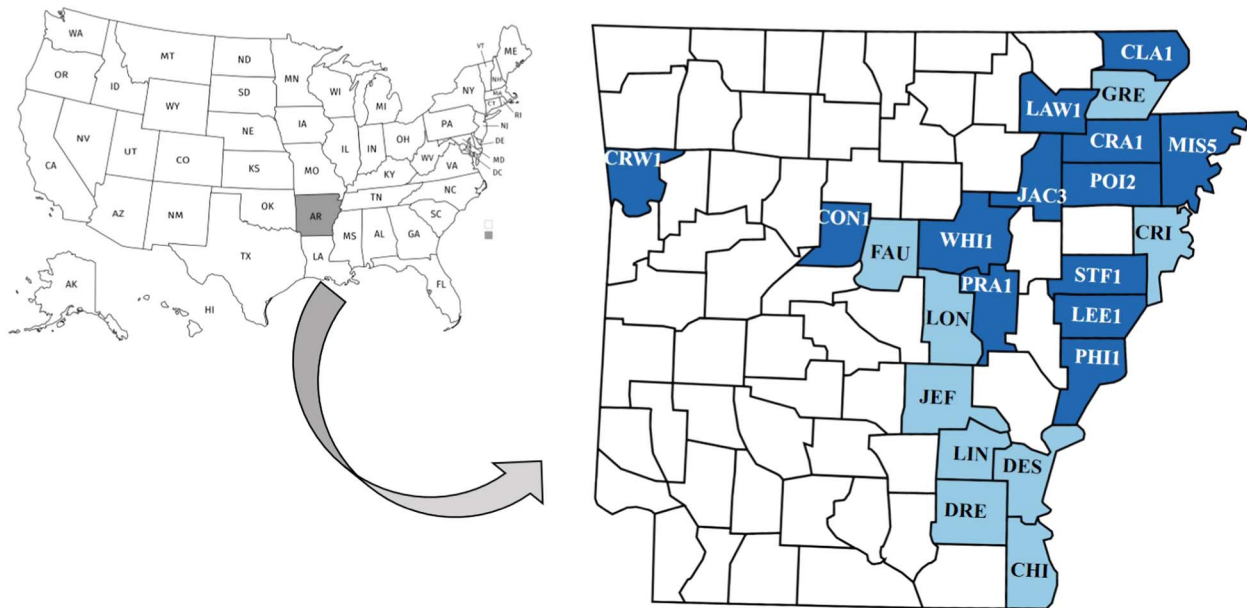


Figure 1. Map of the U.S. state of Arkansas showing counties where *Amaranthus palmeri* accessions were collected between 2008 and 2014. The 20 selected accessions used in this research were from CLA (Clay), CON (Conway), CRA (Craighead), CRW (Crawford), JAC (Jackson), LAW (Lawrence), LEE (Lee), MIS (Mississippi), PHI (Phillips), POI (Poinsett), PRA (Prairie), STF (St Francis), and WHI (White) counties. The number of accessions selected per county is also indicated.

either changes in the herbicide-binding site in the EPSPS (Fernández-Moreno et al. 2016; Yu et al. 2015) or overexpression of the EPSPS protein by gene amplification (Gaines et al. 2010; Ribeiro et al. 2014; Salas et al. 2012). Mutations at Pro-106 and Thr-102 of EPSPS endow low-level resistance to glyphosate and used to be the most commonly reported TSR mechanisms in GR weeds (Baerson et al. 2002; Kaundun et al. 2011; Ng et al. 2004; Yu et al. 2007). Reduced translocation results in a higher level of resistance (7- to 11-fold) than the level of resistance (2- to 3-fold) afforded by the EPSPS mutations mentioned (Preston and Wakelin 2008). Lately, the amplification of EPSPS has been reported as a TSR mechanism conferring moderate to high level of resistance in three *Amaranthus* species (Chandi et al. 2012; Chatham et al. 2015; Gaines et al. 2010; Nandula et al. 2014), *L. perenne* (Salas et al. 2012), *B. scoparia* (Wiersma et al. 2015), and riggut brome (*Bromus diandrus* Roth) (Malone et al. 2016).

Amaranthus palmeri is highly competitive, has extremely rapid growth, high fecundity, and extended emergence behavior, which allows it to escape weed management tactics (Monks and Oliver 1988; Ward et al. 2013). It is the number one most troublesome and difficult-to-control weed in the United States (WSSA 2016). *Amaranthus palmeri* is among the most herbicide resistance-prone dicots, with resistance to six herbicide sites of action in the United States (Heap 2017). Intensive use of glyphosate has resulted in the evolution of GR *A. palmeri* in 30 states, including Arkansas. Multiple resistance to glyphosate and acetolactate synthase (ALS) and protoporphyrinogen oxidase (PPO) inhibitors is increasing (Salas-Perez et al. 2017), making weed management difficult. Although GR *A. palmeri* is widespread in Arkansas, the mechanisms involved are not yet known. This study was conducted to (1) survey the occurrence of EPSPS gene amplification and target-site mutation(s) among GR *A. palmeri* accessions from Arkansas and (2) determine the correlation of EPSPS copy number with resistance level to glyphosate.

Materials and Methods

Plant Material

Amaranthus palmeri plants remaining in the fields at the end of the growing season were sampled in late summer between 2008 and 2014 from crop production fields in Arkansas, mostly cotton (*Gossypium hirsutum* L.) and soybean [*Glycine max* (L.) Merr.]. Inflorescences of at least 10 plants field⁻¹ were collected (Burgos et al. 2013), air-dried, and manually threshed. A composite seed sample from each field (hereafter referred to as an “accession”) was prepared by mixing 500 mg of seed plant⁻¹. A total of 115 accessions were tested with glyphosate (unpublished data). Out of 115 accessions, 20 were selected from 13 counties (Figure 1) to represent a broad range of responses to glyphosate. These accessions included the susceptible standard (SS), which was collected from a field historically planted mostly with watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] and vegetables.

General Testing for Resistance to Glyphosate

The composite seed samples of 20 accessions were planted in 50-cell trays (Redway Feed Garden and Pet Supply, Redway, CA) filled with potting medium (Sunshine® Premix #1, Sun Gro Horticulture, Bellevue, WA). Plants were grown in a greenhouse with temperature maintained at 32/25 ± 5 C day/night and a 16-h photoperiod. The experiment was set up as randomized complete block design with two replications (50 plants replication⁻¹), with each tray being a replication with 1 seedling cell⁻¹. The bioassay was conducted twice. Thus, 200 plants representing a field were treated with the recommended dose of glyphosate at 840 g ae ha⁻¹ (Roundup PowerMax®, Monsanto, St Louis, MO). Seedlings, 7- to 10-cm tall, were treated in a spray chamber using a boom fitted with two 800067 flat-fan nozzles (TeeJet® spray nozzles, Spraying Systems, Wheaton, IL) delivering 187 L ha⁻¹ at 269 kPa. Plants were labeled and leaf tissues were collected before herbicide treatment for use in succeeding experiments. At 21 d after

treatment (DAT), each plant was evaluated visually for injury relative to the nontreated control. Injury was rated on a scale of 0% to 100%, where 0% and 100% represented no injury and complete death, respectively. Data were analyzed using ANOVA in JMP Pro v. 12 (SAS Institute, Cary, NC). Hierarchical clustering of accessions was done using injury and mortality data (S Singh, NR Burgos, V Singh, EAL Alcober, R Salas-Perez, V Shivrain, unpublished data).

Evaluation of Level of Resistance to Glyphosate

Twenty *A. palmeri* accessions were used in this study. Seeds of each accession were planted in 11 by 11 cm square pots filled with Sunshine® Mix LC1 potting soil (Sun Gro Horticulture Canada, Vancouver, BC, Canada). Seedlings (5 pot⁻¹, 7- to 10-cm tall) of resistant accessions were treated with eight doses of glyphosate: 0, 110, 220, 420, 840, 1,680, 3,360, and 6,720 g ha⁻¹; and the susceptible accessions were treated with 0, 27.5, 55, 110, 220, 420, 840, and 1,680 g ha⁻¹. Glyphosate was applied following the procedure outlined in the previous section. The experiment was conducted in a randomized complete block design with six replications. The number of survivors and injury were evaluated at 21 DAT using the same scale described previously. Data were analyzed using SigmaPlot v. 13 (Systat Software, San Jose, CA). Dose-response data were subjected to nonlinear regression analysis and fit with a three-parameter log-logistic (Equation 1) to determine the glyphosate dose that would cause 50% and 90% control as follows:

$$Y = c / \left[1 + e^{-a(x-b)} \right] \quad [1]$$

where Y is the % injury; a is the asymptote; b is the slope of the line; c is the inflection point; and x is the glyphosate dose.

Determination of Relative EPSPS Gene Copy Number

After glyphosate application (840 g ha⁻¹), tissues from surviving plants with injuries ranging from 10% to 70%, were collected at 21 DAT to determine the relative *EPSPS* gene copy number. For susceptible accessions, leaf tissues from nontreated plants were used. Leaf tissues were frozen immediately in liquid nitrogen and stored at -80 C until processed. Genomic DNA was extracted from approximately 100 mg of leaf tissue using a modified CTAB protocol (Doyle and Doyle 1990), quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and checked for quality by gel electrophoresis.

The *EPSPS* gene copy number was determined by quantitative real-time polymerase chain reaction (qPCR) relative to the *A36* gene, a RNA dead-box helicase from woolflower (*Celosia trigina* L.) (PI649298). This reference gene was used because the *A. palmeri* accessions were also resistant to ALS-inhibiting herbicides; should resistance involve any *ALS* amplification, it could result in underestimation of *EPSPS* gene copy. The *ALS* gene has been used by Gaines et al. (2010) and others as a reference to analyze the *EPSPS* gene copy number. The *A36* primers were designed using Biolign and Primer 3 software from sequences of the *Amaranthus* genus: *A36_F244* (5'TTGGAACCTGTCAGAGCAACC3') and *A36_R363* (5'GAACCCACTT CCA CCAAAAAC3'). To amplify the *EPSPS* gene, the primer sets *EPSPF1* (5'ATGTTGGACGCT CTCAGAACTCTTGGT3') and *EPSPR8* (5'TGAATTTCTCCAGCAACGGCAA3') designed by Gaines et al. (2010) were used. For the qPCR, 25- μ l reactions were prepared using 12.5 μ l of Bio-Rad iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 1 μ l of the forward and reverse primers (10 μ M), and

10 ng gDNA. The thermoprofile consisted of 15 min denaturation at 95 C, 40 cycles of 95 C for 30 s, and a single cycle of 60 C for 60 s. This program was followed by a melt-curve analysis of 81 cycles at 55 C for 30 s (Chandi et al. 2012). A negative control consisting of primers with no template DNA was included. No amplification products were observed in negative control reactions. Data were analyzed using a modified $2^{-\Delta\Delta C_t}$ method to express genomic copy number of *EPSPS* relative to *A36*, as $\Delta C_t = (C_t A36 - C_t EPSPS)$. The relative increase in genomic *EPSPS* copy number was expressed as $2^{\Delta C_t}$ (Gaines et al. 2011). Each population had four biological replicates, and each sample was run in triplicate (for each primer pair).

Partial Sequencing of EPSPS

A 150-bp fragment of the *EPSPS* gene was sequenced from five GR *A. palmeri* accessions (4 plants accession⁻¹) that did not have *EPSPS* amplification to determine whether any of the known resistance-conferring amino acid substitutions at Thr-102 or Pro-106 were present. The *EPSPS* gene of SS was also sequenced. Forward and reverse primers (*EPSPSF*: 5'CCAAAAGGCGAGTCGTAGAG3'; *EPSPSR*: 5'ACCTTGAATTTCTCCAGCA3') designed by Varanasi et al. (2015) were used to amplify a short fragment of *EPSPS* from genomic DNA. The 25- μ l PCR reaction consisted of 12.5 μ l 2X PCR master mix (Takara Bio USA, Mountain View, CA), 2.5 μ l of both the forward and reverse primers (5 μ M), 4 μ l gDNA (50 ng μ l⁻¹), and 3.5 μ l of water. The PCR was performed with the following conditions: initial denaturation at 95 C for 3 min, followed by 40 cycles of denaturation at 95 C for 30 s, annealing at 53.5 C for 45 s, final extension at 72 C for 7 min, and infinite hold at 4 C. The PCR product was run on a 1% agarose gel to verify the fragment size and purified using a NucleoSpin® Gel and PCR Clean-up kit (Takara Bio USA). The purified DNA was sequenced with the same primers used for PCR and were prepared with ABI BigDye® Terminator v. 3.1 (Thermo Fisher Scientific, Waltham, MA) for sequencing at Institute for Plant Genomics and Biotechnology, Texas A&M University, College Station, TX. The nucleotide sequences were aligned using BioEdit software (Hall 1999). Sequences from SS and GR plants were aligned to the *EPSPS* sequences of *A. palmeri* (FJ861242.1 and FJ861243.1) and *A. tuberculatus* (FJ869881.1) from GeneBank.

Results and Discussion

Response of *Amaranthus palmeri* to Glyphosate

The *A. palmeri* accessions used in this study differentiated into three clusters based on frequency of survivors and levels of injury from treatment with 840 g ha⁻¹ glyphosate (Table 1). The first cluster consisted of seven susceptible accessions, including accession SS, with mortality and injury averaging 98% and 99%, respectively, on the remaining plants at 3 wk after treatment. All individuals of the SS accession died at this dose. The second cluster consisted of six slightly resistant accessions with 63% mortality and an average injury of 80%. This cluster was distinct from the susceptible cluster, exhibiting an early phase of selection for glyphosate resistance. The third cluster was composed of seven highly resistant accessions with an average mortality of 32% and 50% injury. The majority of survivors in this cluster incurred 0% to 10% injury. Of the 200 plants accession⁻¹ treated with the 1X rate of glyphosate, the most resistant survivors (0% to 10% injury) were from Conway (112 plants), Mississippi (91), Lee (87), Poinsett (75), and St Francis (70) counties (Table 2). One

Table 1. Response of selected *Amaranthus palmeri* accessions to glyphosate (840 g ae ha⁻¹), Arkansas, USA.

Cluster	Accession ^a	Injury (%) ^b			Mean frequency of survivors ^c					Mortality	Survivor
		Mean	Min	Max	HR	R	MR	SR	S		
										-----%	
1	CRA08-A	98	0	100	0	0	0	8	192	96	4
1	JAC08-B	96	30	100	0	0	0	10	190	95	5
1	JAC11-A	100	100	100	0	0	0	0	200	100	0
1	LAW11-A	100	100	100	0	0	0	0	200	100	0
1	MIS11-D	100	100	100	0	0	0	0	200	100	0
1	PRA11-B	97	70	100	0	0	0	10	190	95	5
1	SS ^d	100	100	100	0	0	0	0	200	100	0
	Cluster average	99	71	100	0	0	0	4	196	98	2
2	PHI08-A	81	0	100	15	8	14	30	133	67	33
2	POI08-A	83	0	100	10	1	4	29	156	78	22
2	CLA11-A	85	5	100	12	4	8	20	156	78	22
2	JAC11-B	76	15	100	17	12	30	41	100	50	50
2	MIS11-C	74	5	100	24	10	24	38	104	52	48
2	WHI11-A	80	10	100	10	0	19	64	107	54	46
	Cluster average	80	6	100	15	6	17	37	126	63	37
3	LEE08-A	52	0	100	87	3	2	19	89	45	55
3	MIS08-B	65	0	100	59	3	5	18	115	58	42
3	STF08-A	46	0	100	70	22	20	44	44	16	84
3	CON09-A	36	0	100	112	7	12	14	55	28	72
3	MIS11-A	52	10	100	66	24	19	48	43	22	78
3	MIS11-B	60	5	100	91	30	15	10	54	27	73
3	POI11-B	42	5	100	75	10	24	31	60	30	70
	Cluster average	50	3	100	80	14	14	26	66	32	68

^aAccessions were selected to represent different levels of resistance. Abbreviations: CL, Clay; CON, Conway; CRA, Craighead; JAC, Jackson; LAW, Lawrence; LEE, Lee; MIS, Mississippi; PHI, Phillips; POI, Poinsett; PRA, Prairie; STF, St. Francis; WHI, White.

^bPlants were sprayed when 7- to 10-cm tall and evaluated 21 d after herbicide application.

^cSurvivors were categorized based on visible injury: HR, highly resistant (0–10% injury); MR, moderately resistant (31–60% injury); R, resistant (11–30% injury); S, sensitive (90–100% injury); and SR, slightly resistant (61–89% injury).

^dSusceptible standard accession.

accession from Mississippi County had the most number of survivors and the lowest injury (5% to 10%). These fields had been planted with GR crops for at least a decade. Field population-level resistance is expected to increase with time of exposure to glyphosate. Once a resistant individual is selected, and the individual is fit enough to compete and survive, each selection cycle is expected to increase the frequency of resistant alleles in the population, leading to the evolution of a highly resistant population.

Resistance Level to Glyphosate

The glyphosate dose that killed 90% (ED₉₀) of SS was 193 (±16) g ha⁻¹, about one-fourth of the field use rate (Table 2).

Other accessions in the susceptible cluster also had low ED₉₀ values. The ED₉₀ values for accessions in the slightly resistant group (Cluster 2) approached the 1X rate of glyphosate; such field populations may have already harbored a few resistant individuals that could tolerate the field use rate. Accessions represented by Cluster 2 do not pose an immediate economic problem, but without mitigation, will adapt to become a problematic weed population perhaps in three or four more cycles of selection. This was demonstrated by the progression of resistance to PPO-inhibitor herbicides after first detection (Salas et al. 2016). Thus, glyphosate is still effective on some populations but should be monitored closely. Some resistant accessions (CLA11-A; CON09-A; MIS11-A,-B,-C; MIS08-B; PHI08-A; and STF08-A) could not be controlled 90% by the highest dose tested; therefore,

Table 2. ED₅₀ and ED₉₀ values for selected glyphosate-resistant *Amaranthus palmeri* accessions (7- to 10-cm tall) from Arkansas, USA.

Accession	ED ₅₀ (g ae ha ⁻¹) ^a	R/S ^b	ED ₉₀ (g ae ha ⁻¹) ^a	R/S ^b
CRA08-A	177 (±14)	6	797 (±74)	4
JAC08-B	207 (±15)	7	757 (±61)	4
LEE08-A	522 (±23)	19	1,403 (±152)	7
MIS08-B	1,153 (±72)	41	ND	ND
PHI08-A	494 (±18)	18	ND	ND
POI08-A	525 (±20)	19	1,459 (±101)	8
STF08-A	834 (±57)	30	ND	ND
CON09-A	1,219 (±60)	44	ND	ND
CLA11-A	761 (±34)	27	ND	ND
JAC11-A	93 (±6)	3	211 (±17)	1
JAC11-B	322 (±16)	12	1,063 (±155)	5
LAW11-A	100 (±5)	4	225 (±20)	1
MIS11-A	1,305 (±83)	47	ND	ND
MIS11-B	1,355 (±78)	48	ND	ND
MIS11-C	1,204 (±74)	43	ND	ND
MIS11-D	84 (±18)	3	432 (±39)	2
POI11-B	355 (±18)	13	1,264 (±134)	7
PRA11-B	147 (±20)	5	795 (±76)	4
WHI11-A	392 (±15)	14	1,118 (±545)	6
SS ^c	28 (±6)	—	193 (±16)	—

^aED₅₀ (effective dose to cause 50% injury) and ED₉₀ (effective dose to cause 90% injury) values. Values in parentheses represent SEs. ND (not determined): the highest rate applied resulted in less than 90% control of the accession.

^bResistance levels (R/S) were calculated using the estimated doses relative to the susceptible standard.

^cSusceptible standard accession.

the ED₉₀ value was not determined. The resistance levels varied widely among GR accessions (i.e., effective dose to cause 50% injury [ED₅₀] values from 969 g ha⁻¹ to 1,459 g ha⁻¹). Large variation in resistance levels to glyphosate among field populations of *A. palmeri* indicate the occurrence of different resistance mechanisms or different phases of resistance evolution. Given the same resistance mechanism, populations approaching homozygosity in resistance allele(s) would have higher resistance levels than those with low frequency of resistance allele(s). The ED₅₀ values for GR *A. palmeri* in Arkansas were similar to those reported previously for other GR *A. palmeri* (Gaines et al. 2010; Ribeiro et al. 2014) and *A. tuberculatus* (Nandula et al. 2013).

EPSPS Gene Copy Number Relative to A36

The susceptible *A. palmeri* accessions from Arkansas had a single copy of *EPSPS* (Figure 2). Survivor plants from eight GR accessions CLA11-A, CON09-A, LEE08-A, MIS08-B, MIS11-A, MIS11-B, MIS11-C, and POI11-B (Clusters 2 and 3) had 19 to 224 maximum

copies of *EPSPS*, with population means of 6 to 149 copies. Thus, *EPSPS* copies also differed within resistant populations. For instance, the relative copy number in MIS11-A ranged from 78 to 109; in MIS11-B, 61 to 224; and in MIS11-C from a single copy to 182 copies with SDs of 15, 75, and 84, respectively (Figure 3). In a resistant plant, gene amplification results in abundant *EPSPS* enzymes, which compensates for the units inactivated by glyphosate. Therefore, resistant plants are still able to produce aromatic amino acids and survive glyphosate application. Resistant plants with a single *EPSPS* copy most likely harbor a different resistance mechanism. *EPSPS* gene amplification is a common resistance mechanism among GR *Amaranthus* spp. In the first case of *EPSPS* amplification observed in *A. palmeri* from Georgia, GR plants had up to 160 *EPSPS* copies that resulted in 40-fold overexpression of the gene (Gaines et al. 2010). Glyphosate-resistant *A. palmeri* from North Carolina had 22 to 63 *EPSPS* copies (Chandi et al. 2012); in Kansas, 50 to 140 copies (Varanasi et al. 2015); in Mississippi, 33 to 59 average copies (Ribeiro et al. 2014); in New Mexico, up to 8 copies (Mohseni-Moghadam et al. 2013); and recently, in Nebraska, 32 to 105 copies (Chahal et al. 2017). It has been reported that fewer than 30 *EPSPS* copies result in resistance to field-recommended rates of glyphosate (Gaines et al. 2011), but the minimum number of copies needed to confer resistance to glyphosate is not known. In current research, the level of injury from glyphosate application was negatively strongly correlated ($r = -0.78$) with genomic *EPSPS* copy number (Figure 3). Resistance to glyphosate increased with *EPSPS* gene copy number. Seven susceptible accessions (including SS) that had >60% injury had only one copy, whereas those with <60% injury (resistant) had higher *EPSPS* gene copy numbers. The highly resistant accessions MIS11-A, MIS11-B, and MIS11-C, with average 30%, 31%, and 26% injury at the 1X rate had 87, 149, and 118 *EPSPS* copies, respectively. Injury declined by 4% with each additional copy of *EPSPS*. The *EPSPS* copy number was also strongly correlated with the ED₅₀ values of the GR accessions ($r = 0.72$). Sixty-two percent of the GR accessions had 10 or more *EPSPS* gene copies and survived the field rate of glyphosate (840 g ha⁻¹). The *EPSPS* gene duplication mechanism has been studied extensively. Gaines and colleagues (2010) reported that 30 to 50 *EPSPS* copies enabled plants to survive treatment with 0.5 to 1 kg ha⁻¹ glyphosate. Glyphosate-resistant spiny amaranth (*Amaranthus spinosus* L.) had up to 37 more *EPSPS* copies, resulting in a 5-fold resistance to glyphosate (Nandula et al. 2014), and *A. tuberculatus* had up to eight extra copies of *EPSPS* compared with the SS and 5- to 7-fold glyphosate resistance (Lorentz et al. 2014). *Bromus diandrus* from Australia had 5-fold resistance with 10 to 30 *EPSPS* copies (Malone et al. 2016). In *L. perenne*, up to 30 *EPSPS* copies conferred 13-fold resistance to glyphosate (Salas et al. 2012). Thus, *EPSPS* gene amplification is becoming the predominant resistance mechanism among more recently evolved GR weed populations.

Taken together, studies indicate that weedy species have evolved various mechanisms to survive glyphosate application. It appears that GR plants (across species) harboring the *EPSPS* gene amplification mechanism have at least eight *EPSPS* copies, resulting in a wide range of resistance levels. In *A. palmeri* and *L. perenne*, at least 10 relative copies of the *EPSPS* gene confer resistance to glyphosate. The correlation between *EPSPS* gene copy number and resistance level had not been assessed in GR species except for *L. perenne* and *A. palmeri*; hence, a broad conclusion is not supported. At least two factors can modify the resulting whole-plant resistance level from increased *EPSPS* gene copies: (1) the realized level of protein expression and (2) the presence of other resistance mechanisms in the same plant. Different TSR

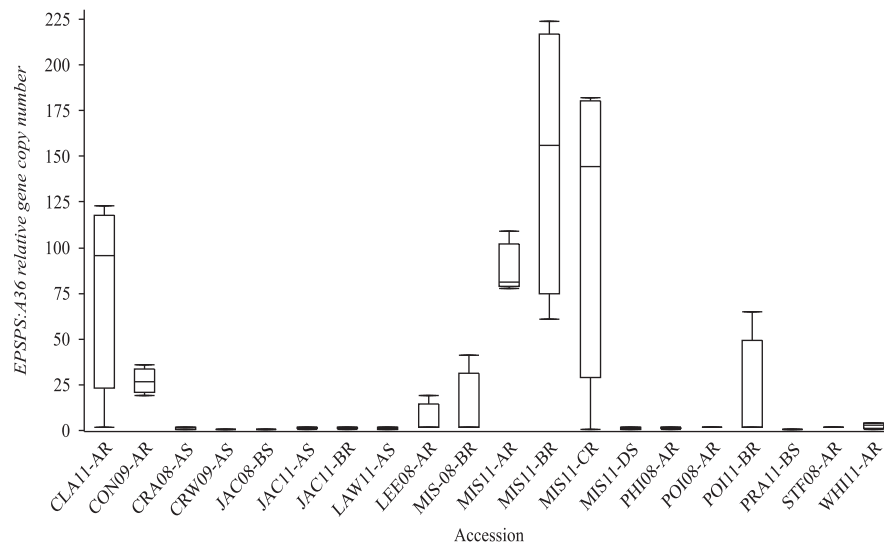


Figure 2. Variability in relative *EPSPS:A36* gene copy number among susceptible (S) and resistant (R) *Amaranthus palmeri* accessions. Box plot shows median values (horizontal line inside the box), first and third quartile values (box outlines), and minimum and maximum values (whiskers).

mechanisms can occur in one species, as observed in *E. indica*. One GR *E. indica* accession had 28 more *EPSPS* gene copies than the SS, while other GR accessions had mutations at the target site (Chen et al. 2015). If plants with different resistance mechanisms are selected and survive to reproduce in one population, then multiple, separate mechanisms could accumulate in a plant via gene flow.

Partial EPSPS Gene Sequencing

Resistance-conferring point mutations at Pro-106 and Thr-102 have been documented in GR weed species. Substitutions of Pro-106-Ala in *L. rigidum* in Australia (Yu et al. 2007), Pro-106-Leu in *L. rigidum* from South Africa (Kaundun et al. 2011), and Pro-106-Ser (Baerson et al. 2002) or Pro-106-Thr (Ng et al. 2004) in *E. indica* from Malaysia all resulted in low-level resistance to glyphosate. High resistance to glyphosate has been observed in the presence of a double mutation Thr-102-Ile and Pro-106-Ser (TIPS) in the corn (*Zea mays* L.) *EPSPS* gene (Eichholtz et al. 2001).

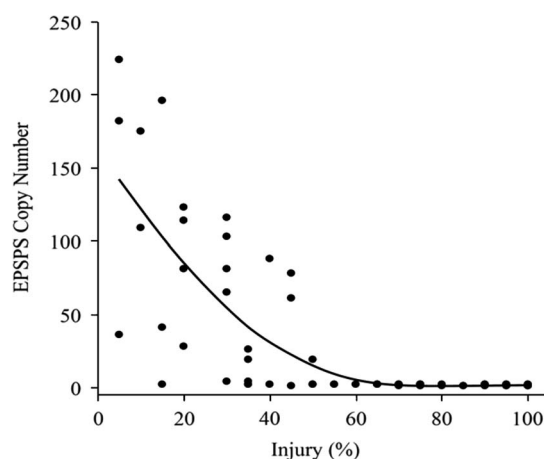


Figure 3. *EPSPS* gene copy number versus level of injury in glyphosate-resistant and glyphosate-susceptible *Amaranthus palmeri*. Data were subjected to the quadratic regression equation $a + b \cdot \text{injury} + c \cdot \text{injury}^2$, where a = intercept, b = slope of the line, c = quadratic. The correlation between percent injury (x -axis) and *EPSPS:A36* relative gene copy number (y -axis) was ($r = -0.78$).

The same TIPS double mutation was discovered in a rare population of *E. indica*, resulting in high-level resistance to glyphosate (Yu et al. 2015). In our study, 38% of GR accessions did not have gene amplification (Figure 3). The GR plants did not contain any changes in amino acid sequence in the conserved region of *EPSPS* (unpublished data). Some silent mutations were observed in plants POI08-A1, STF08-A1, and STF08-A4 at Pro-106 (CCA to CCG) and in five plants from PHI08-A1, PHI08-A3, STF08-A1, STF08-A3, and WHI11-A1 at Leucine-107 (TTG to TTA). Therefore, none of the known target-site mutations were involved in resistance to glyphosate in these accessions.

The analysis of multiple accessions from different counties of Arkansas indicated variability within and among field populations for resistance level and resistance mechanisms. Additionally, there is a correlation between *EPSPS* gene copy number and resistance level to glyphosate. Resistance to glyphosate in *A. palmeri* is rampant in Arkansas. Nonetheless, 30% of the populations can still be controlled with glyphosate and should be managed proactively to delay and isolate resistance evolution. Large-scale surveys are informative in making appropriate management decisions. In conclusion, resistance to glyphosate among *A. palmeri* populations was conferred by amplification of the *EPSPS* gene among 62% of the GR populations studied. The GR plants showing neither *EPSPS* gene amplification nor resistance-conferring mutations indicate the putative involvement of NTSR mechanisms, which requires further investigation.

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