Research Article

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EPSPS amplification in glyphosate-resistant spiny amaranth (*Amaranthus spinosus*): a case of gene transfer via interspecific hybridization from glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*)

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Abstract

BACKGROUND: *Amaranthus spinosus*, a common weed of pastures, is a close relative of *Amaranthus palmeri*, a problematic agricultural weed with widespread glyphosate resistance. These two species have been known to hybridize, allowing for transfer of glyphosate resistance. Glyphosate-resistant *A. spinosus* was recently suspected in a cotton field in Mississippi.

RESULTS: Glyphosate-resistant *A. spinosus* biotypes exhibited a fivefold increase in resistance compared with a glyphosate-susceptible biotype. *EPSPS* was amplified 33–37 times and expressed 37 times more in glyphosate-resistant *A. spinosus* biotypes than in a susceptible biotype. The *EPSPS* sequence in resistant *A. spinosus* plants was identical to the *EPSPS* in glyphosate-resistant *A. palmeri*, but differed at 29 nucleotides from the *EPSPS* in susceptible *A. spinosus* plants. PCR analysis revealed similarities between the glyphosate-resistant *A. palmeri* amplicon and glyphosate-resistant *A. spinosus*.

CONCLUSIONS: Glyphosate resistance in *A. spinosus* is caused by amplification of the *EPSPS* gene. Evidence suggests that part of the *EPSPS* amplicon from resistant *A. palmeri* is present in glyphosate-resistant *A. spinosus*. This is likely due to a hybridization event between *A. spinosus* and glyphosate-resistant *A. palmeri* somewhere in the lineage of the glyphosate-resistant *A. spinosus* plants.

Keywords: *Amaranthus spinosus*; *EPSPS*; gene amplification; gene transfer; glyphosate; herbicide resistance; interspecific hybridization; introgression

1 INTRODUCTION

Glyphosate, a non-selective, broad-spectrum, systemic, post-emergence herbicide, has been used extensively throughout the world in both crop and non-crop lands since its commercialization in 1974. With the introduction of glyphosate-resistant (GR) crops in the mid-1990s, glyphosate has been used selectively and predominantly for weed control in GR crops. The widespread adoption of GR crops around the world, with the associated use of recurring glyphosate applications, has resulted in the evolution of several GR weed biotypes, with 25 weed species documented worldwide.[1]

Spiny amaranth (*Amaranthus spinosus* L.), variously referred to as hogweed, needle burr, spiny pigweed or stickerweed[2] (Dodd J, Randall RP and Lloyd SG: http://www.agric.wa.gov.au/PC_93102.html) is usually problematic in pastures.[3] It is an annual and can grow up to 1.5 m tall.[3] Mature plants have two sharp spines at most nodes. Plants are monoecious in nature (male and female inflorescences separated, but on the same plant), with inflorescence spikes numerous, 5–15 cm, 6–10 mm thick, the terminal often wholly or chiefly staminate and the basal part and axillary clusters mostly pistillate.[4] *A. spinosus* seeds germinate over a wide range of temperatures.[3] Uninhibited *A. spinosus* plants are known to accumulate 0.55 kg dry weight of plant material in a 14 week period.[3] This rate of growth is comparable with that of waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer.] and smooth pigweed (*Amaranthus hybridus* L.), but is less than the rate of growth of Palmer amaranth (*Amaranthus palmeri* S. Wats.) and redroot pigweed (*Amaranthus retroflexus* L.).[5] Further, *A. spinosus* can produce over 100 000 seeds per plant.[5,6]

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Interspecific hybridization between weed species belonging to the *Amaranthus* genus has been well documented. For example, hybridization of *A. palmeri* has been reported with *A. hybridus*,[7] *A. spinosus*[7] and *A. tuberculatus*.[8,9] Also, *A. tuberculatus* has been shown to cross with *A. hybridus*[10–12] in addition to *A. palmeri*. Populations of GR *A. palmeri* were first documented in Georgia.[13] These and other GR *A. palmeri* populations around the United States have gained notoriety owing to their aggressive growth habit and fecundity, their economic impact on row-crop production systems of the southeastern United States and their propensity to develop multiple herbicide resistance. In addition, they interbreed with other *Amaranthus* spp. A recently discovered glyphosate resistance mechanism, amplification of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene,[14] in GR *A. palmeri* has been shown to introgress into *A. spinosus*.[7]

A. spinosus populations have anecdotally been reported to exhibit reduced sensitivity to glyphosate since the mid-2000s (Robinson E: http://deltafarmpress.com/bruce-bond-high-cotton-winner). In 2011, a grower from Lafayette County in northeastern Mississippi reported lack of control of *A. spinosus* from glyphosate applications in GR cotton (*Gossypium hirsutum* L.). The present article reports on research conducted to confirm and quantify the magnitude of glyphosate resistance in *A. spinosus* from northeastern Mississippi, to characterize the molecular mechanism of glyphosate resistance and to investigate the possible role of field-level interspecific hybridization between this *A. spinosus* and natural infestations of GR *A. palmeri*.

2 EXPERIMENTAL METHODS

2.1 Seed collection, storage, germination, planting, growth and herbicide treatment

In the summer of 2011, mature *A. spinosus* plants suspected to be resistant to glyphosate were identified in a field in Lafayette County, Mississippi (34.23450 N, 89.63433 W), that had been continuously planted to GR cotton in 2011 and preceding years. *A. spinosus* plants were collected along with roots, transferred to 10 L pots containing field soil and allowed to grow outdoors until they reached reproductive maturity. *A. spinosus* inflorescence spikes containing seeds were collected, kept separate for each individual plant (not bulked) and air dried in a greenhouse (25/20 °C day/night, 12 h photoperiod under natural sunlight conditions) for 7 days, cleaned and stored at 2–8 °C until further use.

Seeds were planted at 0.25–0.5 cm depth in 50 × 20 × 6 cm plastic trays with holes that contained a commercial potting mix (Metro-Mix 360; Sun Gro Horticulture, Bellevue, WA). Two weeks after emergence, *A. spinosus* seedlings were transplanted into 6 × 6 × 6 cm pots containing the potting mix. Trays and pots were maintained in a greenhouse set to a temperature of 25/20 °C (day/night) and a 12 h photoperiod under natural sunlight conditions supplemented with high-pressure sodium lights providing 400 mmol m^{-2} s^{-1}. One week after transplanting, plants were fertilized once with a nutrient solution (Miracle-Gro; The Scotts Company LLC, Marysville, OH) containing 200 mg L^{-1} each of N (both ammonia and urea forms), P_2O_5 and K_2O, and were subirrigated as needed thereafter. All herbicide treatments were applied with a moving nozzle sprayer equipped with 8002E nozzles (Spraying Systems Co., Wheaton, IL) delivering 140 L ha^{-1} at 280 kPa to *A. spinosus* plants that were 10 cm tall and at the 4–6-leaf stage. Percentage control [visible estimate of injury on a scale of 0 (no injury) to 100 (plant death)] was recorded 3 weeks after treatment (WAT). A glyphosate-susceptible (GS) *A. spinosus* biotype, GS1, was collected from Yazoo County, Mississippi (32.37137 N, 90.922066 W), and grown, processed and screened as with the suspected GR plants. A second glyphosate-susceptible biotype, GS2 (also from Yazoo County), was included in the molecular studies. A third glyphosate-susceptible biotype, GS3, was selected from seed collections originating from North Carolina and obtained from the National Plant Germplasm System (North Central Regional Plant Introduction Station, Iowa State University, Ames, IA); this biotype represents *A. spinosus* from years prior to the commercialization of GR crops (pre-1996). All studies were conducted from 2011 to 2013 at the Jamie Whitten Delta States Research Center of USDA-ARS in Stoneville, Mississippi.

2.2 Screening with a discriminating glyphosate dose

In preliminary resistance screening studies, several *A. spinosus* plants from suspected GR and GS groups were treated with a 0.84 kg AE ha^{-1} rate of glyphosate (potassium salt, Roundup WeatherMAX®; Monsanto Company, St Louis, MO) (data not shown). Plants that survived 3 WAT were allowed to grow and mature to produce the second-generation seed. Two GR biotypes, GR1 and GR2, were thus developed. Additional screening experiments indicated that all the second-generation plants survived a glyphosate treatment of 0.84 kg ha^{-1} (data not shown). This second-generation seed was used in all subsequent studies.

2.3 Glyphosate dose response

GR1 and GR2 *A. spinosus* plants were treated with glyphosate at 0, 0.21, 0.42, 0.84, 1.68 and 3.36 kg ha^{-1}. GS1 plants were treated with glyphosate at 0, 0.03, 0.05, 0.11, 0.21, 0.42, 0.84 and 1.68 kg ha^{-1}. Percentage control ratings (0 = no effect on growth, 100 = complete kill) were recorded 3 WAT. The 0.84 kg ha^{-1} rate represents a 1× field application rate. There were three replications per treatment, and the experiment was conducted 2 times.

2.4 Shikimate assay with leaf discs

A shikimate assay of *A. spinosus* biotypes was conducted using a leaf-disc assay following previously reported protocols.[15,16]

2.5 *EPSPS* sequence analysis

RNA was extracted from five accessions of spiny amaranth, GR1, GR2, GS1, GS2 and GS3, by incubating leaf tissue in RNA Later (Life Technologies, Grand Island, NY) at 4 °C for 4 h and then extracting RNA with RNeasy Plant Mini kit (Qiagen, Valencia, CA). One-step RT-PCR kit (Thermo Scientific Verso RT-PCR system; Thermo Fisher Scientific Inc., Pittsburgh, PA) with *EPSPS* primers AW263 and AW266 (see Table 1 for sequences) was used to amplify most of the *EPSPS* cDNA. Each reaction contained ~50 ng of RNA, 5 μM of primer, 1× master mix, 0.5 μL of RT enhancer, 0.4 μL of enzyme mix and water to 10 μL. Cycle conditions were as follows: 50 °C for 10 min, 95 °C for 2 min, 35 cycles of 95 °C for 20 s, 55 °C for 20 s and 72 °C for 2 min, and 72 °C for 5 min. A second round of amplification was performed with Takara LA PCR kit v.2.1 (Thermo Fisher Scientific Inc., Pittsburgh, PA) to generate a PCR product with overhanging A residues for TOPO TA cloning. Reactions were prepared as follows: 0.5 μL of the initial PCR reaction, 4 μmol of AW263 and AW266, 2.5 mM of Mg^{2+}, 1× buffer, 400 μM of dNTPs, 2.5 U of polymerase and water to 25 μL. Cycle conditions were as follows: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, and 72 °C for 5 min. After PCR, the *EPSPS* cDNA was gel purified (GenElute Gel Extraction kit; Sigma Aldrich, St Louis,

Table 1. Names and sequences of primers used in PCR

Name	Sequence
ALSF2	GCT GCT GAA GGC TAC GCT[14]
ASLR2	GCG GGA CTG AGT CAA GAA GTG[14]
AW22	GTG ACA GTC CCA CCA GTG
AW66	GTA TGG ACA CCA TTG TTG ATG
AW67	CGT CAC TAT CCT TGA TCC G
AW90	GTT GTG AGT TCG ATA CAC TGC
AW100	GAG TTC TGA GAG CGT CCA
AW130	CTC TCT GGA TCG GTT AGT AGC
AW140	GTA CAG CCA AAA GGG CAG
AW145	CTC GTT TCC GTT GCA TG
AW146	CAA CAG TTG AGG AAG GAT CTG
AW147	CAG CAA GAG AGA ATG CCA T
AW155	CAG TAG GTA AAC CGT GTT G
AW157	GAT GTA CAA GTT CAG ACT AGG TTG
AW173	GTC GGG TCA TTT TCG GA
AW203	CAA AAG GAC TGG GGT AGG GT
AW215	GAC CTT CAG CTC TAT AGT CAG
AW263	GCT CAA GCT ACT ACC ATC AAC AAT
AW266	CGG ATC AAG GAT AGT GAC
AW313	GTT GGT GGG CAA TCA TCA AT
AW366	GTA GCG TTC GTG ATT CTG
AW367	CTC TTA AGC GGC TAT AAA TGA CT

MO). The fragment was ligated into the pCR™2.1 vector using TA Cloning® kit (Life Technologies, Grand Island, NY). Reactions were prepared as follows: 3:1 ratio of insert to vector (25 ng), 1× buffer, 5 U of ligase and water to 10 μL. Reactions were incubated at room temperature for 1 h. Chemically competent TOP10 cells were prepared and transformed as described previously.[17] The transformants were screened by PCR amplification of the insert with primers AW263 and AW266. Glycerol stocks of positive transformants were prepared by adding 800 μL of an overnight culture to 200 μL of 80% glycerol and were stored at −80 °C. Three positive clones per *A. spinosus* accession were selected for sequencing. Cultures were prepared by inoculating 500 μL of LB media (1% w/v tryptone, 0.5% w/v yeast extract and 0.5% w/v NaCl) with 50 μg mL^{-1} of ampicillin with 10 μL of an overnight culture. The cultures were submitted to the Genomics and Bioinformatics Research Unit, USDA-ARS, Stoneville, Mississippi, for plasmid isolation and sequencing of the insert. Sequences were analyzed in Geneious (v.5.6.5; Auckland, New Zealand).[18]

2.6 *EPSPS* copy number and expression

DNA was extracted using DNeasy Plant Mini kit (Qiagen, Valencia, CA). RNA was extracted as described above, and DNAse was treated using the RNase free DNase set (Qiagen, Valencia, CA). RNA was incubated with the DNAse for 1 h and then repurified on a column according to kit instructions. cDNA was generated using High Capacity cDNA Reverse Transcription kit (Life Technologies, Grand Island, NY). Reactions contained 400 ng of RNA, 4 mM of dNTPs, 1× buffer, 1× random primers, 2.5 U μL^{-1} of reverse transcriptase and water to 20 μL. 'No RT' controls, in which the enzyme was excluded from the reaction, were included to confirm removal of DNA. Cycle conditions were as follows: 25 °C for 10 min, 37 °C for 2 h and 85 °C for 5 s. Copy number and gene expression assays were performed using *EPSPS* primer pairs AW146 and AW147 and ALS primer pairs developed previously.[14] Reactions contained 50 ng of cDNA, 200 μM of primers, 1× *Power* SYBR® Green PCR Master mix (Life Technologies, Grand Island, NY) and water to 50 μL. Cycle conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and 95 °C for 15 s. Reactions were performed in triplicate on the ABI 7500 Real Time PCR system (Life Technologies, Grand Island, NY). Data were analyzed using the relative standard curve method, with ALS serving as a reference, and standard deviations were calculated as per ABI recommendations (Applied Biosystems User Bulletin No. 2: http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf).

2.7 Screening for the *EPSPS* amplicon

Primers designed to amplify regions of the *EPSPS* amplicon in *A. palmeri*[19] were used to detect the presence of the *EPSPS* amplicon in resistant and sensitive *A. spinosus* accessions. Reactions were performed as described above with the Takara LA PCR kit v.2.1 (Thermo Fisher Scientific Inc., Pittsburgh, PA) using primer pairs AW130 and AW22, AW203 and AW66, AW90 and AW155, AW140 and AW157, AW67 and AW145 and AW173 and AW215 (Table 1). PCR products were analyzed on 1% agarose gels. For GR1, fragments amplified with primer pairs AW203 and AW66 and AW67 and AW145 were cloned and sequenced as described above. Additionally, intron 1 was amplified, cloned and sequenced for GR1, GS1 and GS3 as described above with primers AW100, AW313, AW366 and AW367.

2.8 Statistical analysis

The glyphosate dose response and shikimate experiments were conducted using a completely randomized design. Data from these experiments were analyzed by ANOVA via the PROC GLM statement using SAS software (v.9.2; SAS Institute, Inc., Cary, NC), and treatment means were separated using Fisher's Protected LSD test at $P = 0.05$. No significant experimental effect was observed in repeated experiments; therefore, data from experiments were pooled. Non-linear regression analysis was applied to fit a sigmoidal log-logistic curve of the form

$$y = \frac{a}{1 + \exp\left[-\left(x - x_0\right)/b\right]} \quad (1)$$

where a is an asymptote, x and x_0 are the upper and lower response limits, with the latter approaching 0, and b is the slope of the curve around x_0, to relate the effect of glyphosate dose on *A. spinosus* control, and glyphosate concentration on shikimate accumulation. Equation parameters were computed using SigmaPlot (v.11.0; Systat Software, Inc., San Jose, CA).

3 RESULTS AND DISCUSSION

3.1 Glyphosate dose response

The response of *A. spinosus* biotypes to glyphosate dose is presented in Fig. 1. GR_{50} (dose required to reduce plant growth by 50%) values for the GR1, GR2 and GS1 *A. spinosus* biotypes, based on percentage control, were 0.66, 0.70 and 0.14 kg ha^{-1} glyphosate respectively. This indicates that the GR1 and GR2 biotypes were both fivefold (4.7–5.0) less sensitive to glyphosate compared with the GS1 biotype. In comparison with other GR *Amaranthus* spp., *A. spinosus* resistance levels reported here are similar to those of *A. tuberculatus* from Mississippi (fivefold)[20] and *A. palmeri* from Georgia (6.2-fold),[13] but less than those of *A. tuberculatus* from

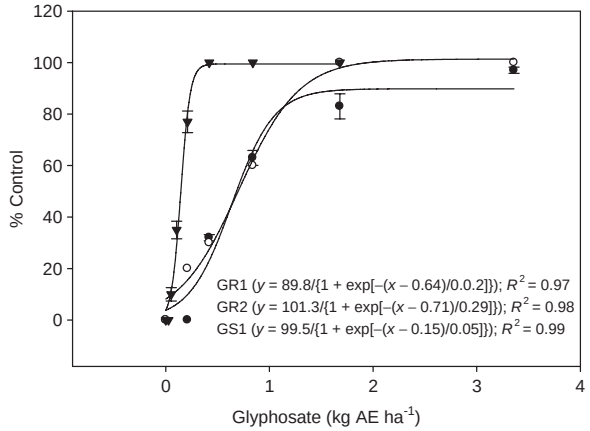

Figure 1. Glyphosate dose response on control (0 = no effect on growth, 100 = complete kill) of glyphosate-resistant (GR1, closed circles; GR2, open circles) and glyphosate-susceptible (GS1, closed triangles) *A. spinosus* biotypes 3 weeks after treatment. Vertical bars represent the standard error of the mean ($n = 6$).

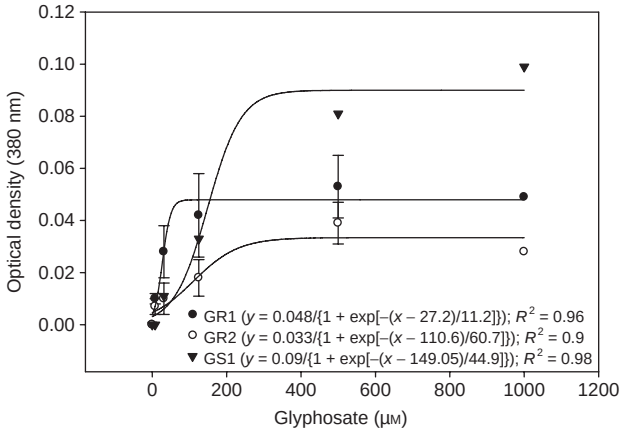

Figure 2. Effect of glyphosate concentration on shikimate levels in excised leaf discs of glyphosate-resistant (GR1, closed circles; GR2, open circles) and glyphosate-susceptible (GS1, closed triangles) *A. spinosus* biotypes. Vertical bars represent the standard error of the mean ($n = 6$).

Missouri (9.2–19.2)[21] and *A. palmeri* from Mississippi (14–17-fold),[22] and greater than the resistance level of *A. tuberculatus* from Texas (2.5-fold).[23] The GR plants survived glyphosate treatment and continued growth to the reproductive phase at all rates of glyphosate, except for GR2 which was completely controlled at the 3.36 kg ha^{-1} rate. The GS biotype did not survive glyphosate at 0.21 kg ha^{-1} or higher rates.

3.2 Shikimate assay with leaf discs

The shikimate (shikimic acid) accumulation patterns in the GR and GS1 *A. spinosus* biotypes are shown in Fig. 2. The level of shikimate accumulation in the GR1 biotype tended to be higher than in the GS1 biotype when the glyphosate concentration was 31.25–125 μM, but the GS1 biotype accumulated more shikimate than either of the GR biotypes when glyphosate was applied at 500–1000 μM. Variable shikimate accumulation patterns have been observed in other *Amaranthus* spp. in response to glyphosate treatment. A GR *A. palmeri* biotype accumulated low levels of shikimate, whereas a second GR biotype had a similar pattern to a GS biotype.[22] In another report on *A. palmeri*, shikimate was detected in a GS biotype after glyphosate treatment, but none in a resistant biotype.[13] Also, shikimate accumulation was apparent in both GR and GS *A. palmeri* biotypes from Tennessee.[24] In *A. tuberculatus*, shikimate buildup was higher in a GS population compared to a GR population.[20]

Inhibition of EPSPS in susceptible plants by glyphosate results in the accumulation of shikimate-3-phosphate and shikimate.[25] In general, GR plants of a weed or crop species would be expected to accumulate negligible or less shikimate compared with their GS counterparts. According to Shaner,[25] if shikimate accumulation is lower in GR plants compared with their GS equivalents at both low and high levels of glyphosate, this may indicate that the mechanism of resistance in GR plants is target-site based (mutation, gene amplification or other unknown processes), reflecting a functional EPSPS enzyme. Conversely, if shikimate accumulation is much lower in GR plants compared with GS plants at low glyphosate levels but similar at high glyphosate levels, the glyphosate resistance mechanism could be assumed to be reduced translocation. It was reasoned that, at low external glyphosate concentrations, the herbicide absorption is blocked, whereas at higher concentrations of glyphosate the herbicide can enter the cells through passive diffusion and inhibit EPSPS.[26] Shikimate assays provide some insights into the mechanism of glyphosate resistance, but are not definitive.[25] Nevertheless, based on the pattern of accumulation of shikimate in the GR and GS *A. spinosus* biotypes, especially at the higher (500–1000 μM) levels of glyphosate, it is hypothesized that the resistance mechanism could be target-site based.

3.3 *EPSPS* copy number and expression

Copy number and gene expression assays were performed in triplicate for three plants in each biotype, and those data were averaged together for each biotype. Copy number analysis showed that the GR1 biotype had 33 copies of *EPSPS*, and biotype GR2 had 37 copies (Fig. 3). Expression assays also showed that the resistant biotypes exhibited increased *EPSPS* expression, with both the GR1 and GR2 biotypes having 37-fold increase (Fig. 3). *EPSPS* gene amplification in other GR weed species has been documented previously. For example, *EPSPS* was amplified by 5–160[14] and by 32–59[27] copies in *A. palmeri*[14,27] and *A. tuberculatus*,[28] by 25 copies in Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot][29] and by three copies in kochia [*Kochia scoparia* (L.) Schrad.].[30] Gene amplification was not detected in a GR *A. tuberculatus* population from Mississippi.[20]

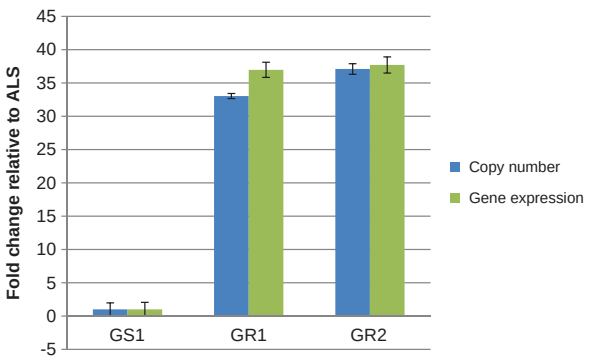

Figure 3. Copy number and expression assays on glyphosate-resistant (GR1 and GR2) and glyphosate-susceptible (GS1) *A. spinosus* biotypes. Values represent an average of three plants per biotype, with reactions performed in triplicate. Vertical bars represent the standard deviation of the mean ($n = 9$).

It is possible that either or both of the GR *A. spinosus* biotypes reported here could have more than one glyphosate resistance mechanism. Involvement of at least two different resistance mechanisms in GR *Amaranthus* spp. has previously been demonstrated.[20] An *A. palmeri* biotype from Mississippi absorbed and translocated less glyphosate than a GS biotype[22] and exhibited EPSPS gene amplification.[27] A GR *A. tuberculatus* population from Mississippi had a proline-to-serine substitution at position 106 of its *EPSPS*, relative to *Arabidopsis thaliana*, as well as reduced absorption and translocation of glyphosate compared with a GS population.[20] Additionally, a GR *A. tuberculatus* from Illinois was speculated to have another resistance mechanism in addition to gene amplification.[28]

3.4 *EPSPS* sequence analysis

The *EPSPS* sequences for GR1, GR2, GS1, GS2 and GS3 were analyzed for potential point mutations that may contribute to resistance. Sequences for GR1 (KF569211), GS1 (KF569212) and GS3 (KF569213) have been submitted to GenBank. There was no mutation at the Pro_{106} site of the *EPSPS* in the GR1 and GR2 biotypes. The only report of an altered *EPSPS* with a mutation of any sort at the Pro_{106} site of *EPSPS* (position 180 in the *A. palmeri* sequence) of GR *Amaranthus* spp. was a proline-to-serine substitution in *A. tuberculatus*.[20] The GR1 and GR2 sequences were identical to each other, and the GS1 and GS2 sequences were identical to each other, but different from the GR biotypes. Interestingly, alignment of the GR and GS *A. spinosus* biotypes with GR *A. palmeri EPSPS* (accession number FJ861243) revealed that the *EPSPS* of the resistant *A. spinosus* biotype is identical to that of GR *A. palmeri* (Fig. 4). There are 29 nucleotide differences between the *EPSPS* sequences of the GS and the GR *A. spinosus* sequences. One of these differences is missing in GS3, and there is one additional nucleotide change found only in GS3. Between GS and GR biotypes there are five polymorphisms resulting in differences in the amino acid sequence: a His to Gln and a Glu to His in the transit peptide and a Val75Ile, a Lys215Arg and a Thr395Ser in the mature protein.

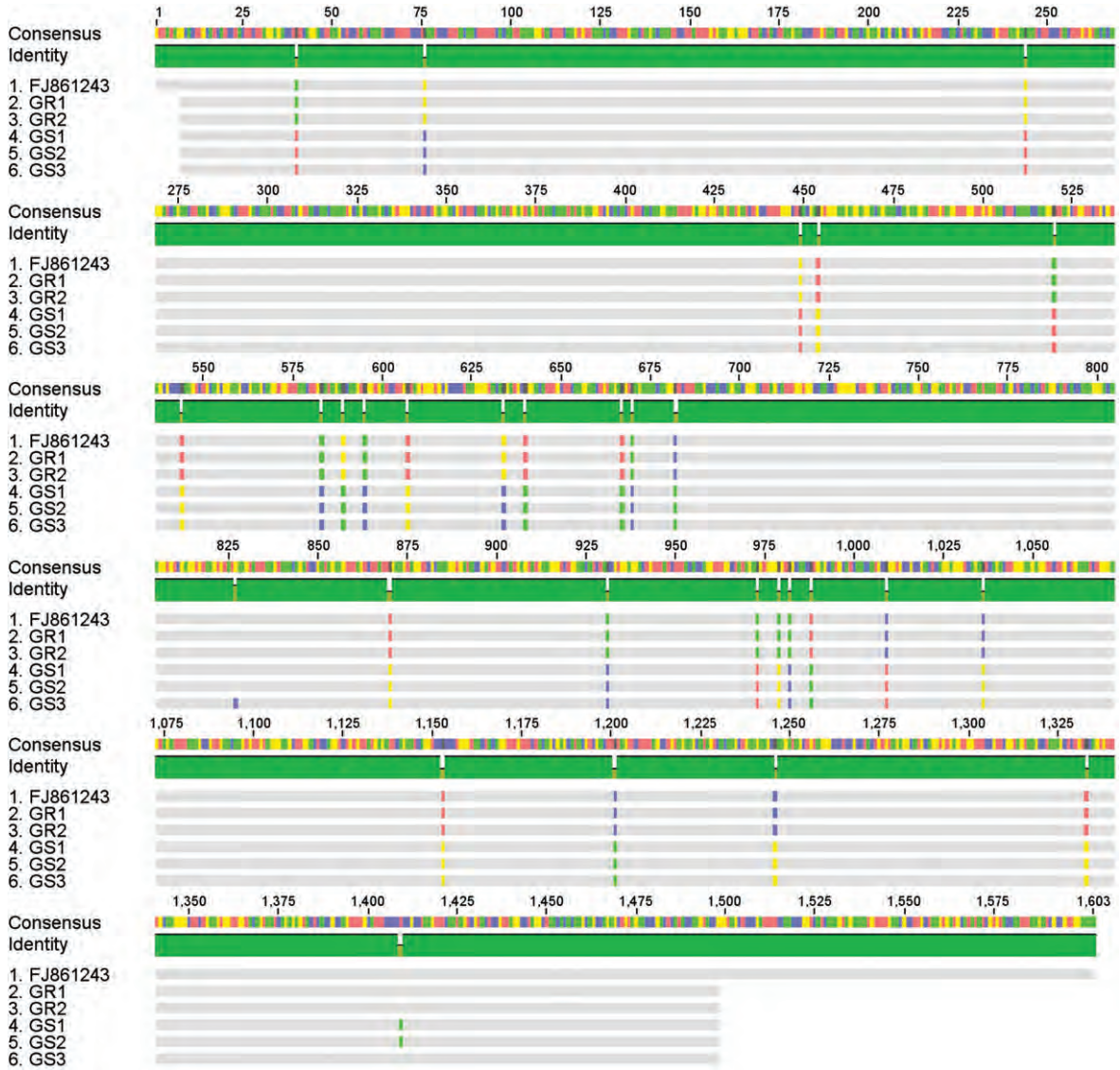

Figure 4. Alignment of glyphosate-resistant (GR1 and GR2) and glyphosate-susceptible (GS1, GS2 and GS3) *A. spinosus* biotypes and *A. palmeri* EPSPS cDNA. The green bar indicates consensus, whereas brown gaps indicate differences. The gray lines are the sequences. The red (A), yellow (G), blue (C) and green (T) marks on the gray line are color-coded markers for nucleotide differences.

The relationship of these amino acid sequence polymorphisms to glyphosate resistance was not investigated. It is known that *A. palmeri* and *A. spinosus* can hybridize, and this can be a means of transferring glyphosate resistance to *A. spinosus*.[7] Given that the *EPSPS* sequence of the GR *A. spinosus* biotypes is identical to the *A. palmeri* sequence (Fig. 4), it is possible that the observed similarity arose out of a hybridization event in the lineage of the resistant *A. spinosus* plants.

3.5 Examination of the *EPSPS* amplicon

As the mechanism of resistance in *A. spinosus* is due to amplification of the *EPSPS*, and the *EPSPS* sequence in the GR accessions is identical to *A. palmeri*, PCR was used to investigate the possibility of transfer of the *EPSPS* amplicon from *A. palmeri* to one of the GR *A. spinosus* biotypes. A large section of the amplicon is known,[19] and primers for regions of the amplicon are available. Primers were used to amplify specific regions of *EPSPS* and the flanking sequence to determine whether the same sequence might be present in the GR but absent in the GS biotypes. A map of the regions is provided in Fig. 5A. Each primer set was tested on MS-R (GR *A. palmeri* exhibiting *EPSPS* amplification),[19] GR1, GS1 and GS3. MS-R and GR1 produced similar bands, except in Fig. 5B, where GR1 produced a band of greater intensity, and in Fig. 5F, where GR1 had an extra band. GS1 and GS3 failed to produce bands with primer pairs AW203 and AW66, AW90 and AW155 and AW173 and AW215 (Figs 5B, C and G). With primer pair AW67 and AW145, a barely discernible band was produced in GS1, but was absent in GS3 (Fig. 5F). Both primers from the AW90 and AW155 primer pair annealed to a sequence in intron 1. Amplification in the resistant biotypes but failure to amplify in the susceptible biotypes indicates that there are differences in intron 1 between resistant and susceptible biotypes. Primers AW203 and AW145 annealed to regions upstream and downstream of the *EPSPS*. The reactions containing these primers (Figs 5B and F) failed in the susceptible biotypes, thereby suggesting that the upstream and downstream regions differ between the resistant and susceptible biotypes. Primer pair AW140 and AW157 produced a band in both resistant and susceptible biotypes (Fig. 5D), even though AW157 anneals within intron 2, suggesting that at least some of the intron sequence is similar between biotypes. Primer pair AW175 and AW215 failed to produce a band in the susceptible (Fig. 5G) biotypes, demonstrating that there are differences between the resistant and susceptible biotypes in the region downstream of *EPSPS*. These data show a striking similarity between GR1 and

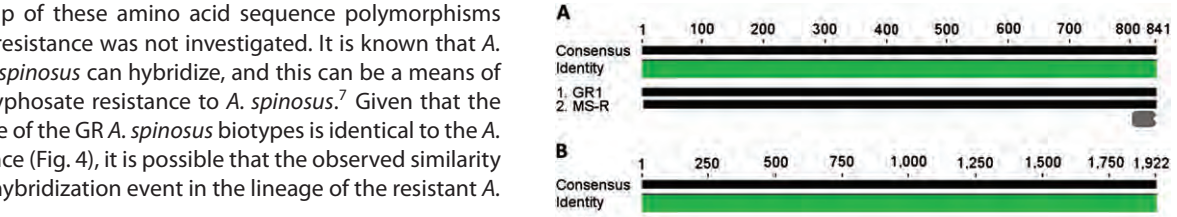

Figure 6. Alignment of (a) upstream and (b) downstream regions of *EPSPS* in glyphosate-resistant (GR) *A. palmeri* and GR *A. spinosus*. The gray bar and arrow represent (a) the beginning of exon 1 and (b) the end of exon 8. The green shading indicates homology. The black bars represent the nucleotide sequence.

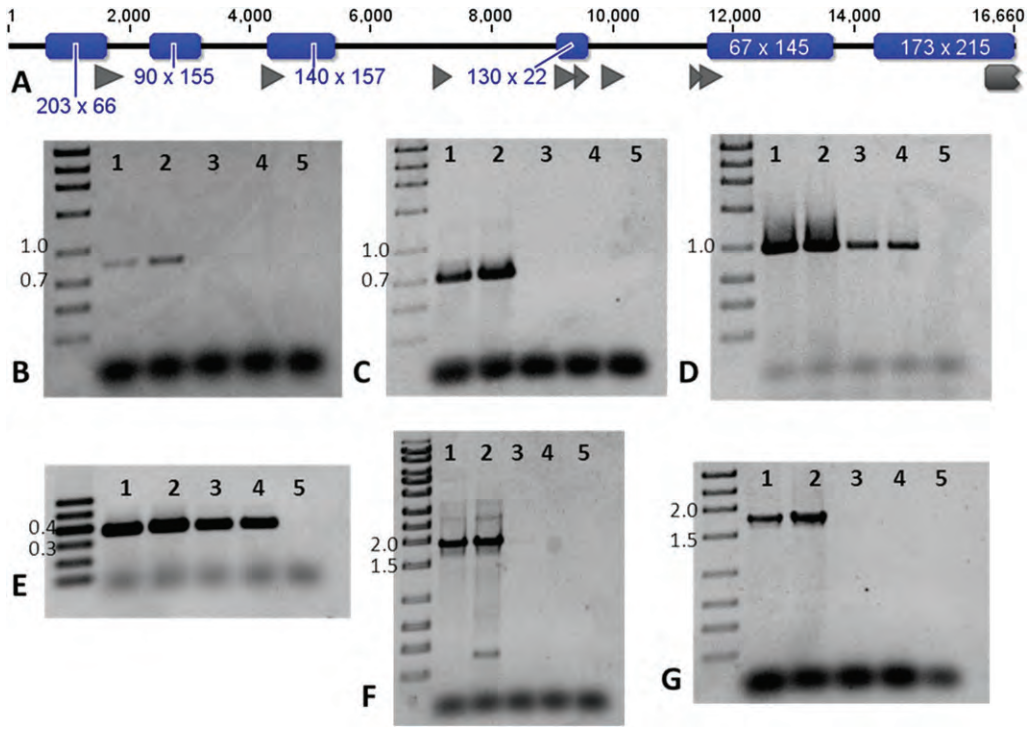

Figure 5. (A) Map of part of the EPSPS amplicon in *A. palmeri*. The gray arrows are the EPSPS exons, and the gray box at the right end is part of the first exon of a putative transposase. In blue are the regions amplified by PCR, labeled with the primer names. (B) to (G) Agarose gels of PCR products for each primer pair. The template used for the reaction in lane 1 is glyphosate-resistant *A. palmeri* MS-R, lane 2 is glyphosate-resistant *A. spinosus* GR1, lane 3 is glyphosate-susceptible *A. spinosus* GS1, lane 4 is glyphosate-susceptible *A. spinosus* GS3 and lane 5 is a negative control. Sizes on relevant bands on the ladder are to the right of the ladder and are given in kb. (B) Primer pair AW203 and AW66. (C) Primer pair AW90 and AW155. (D) Primer pair AW140 and AW157. (E) Primer pair AW130 and AW22. (F) Primer pair AW67 and AW145. (G) Primer pair AW173 and AW215.

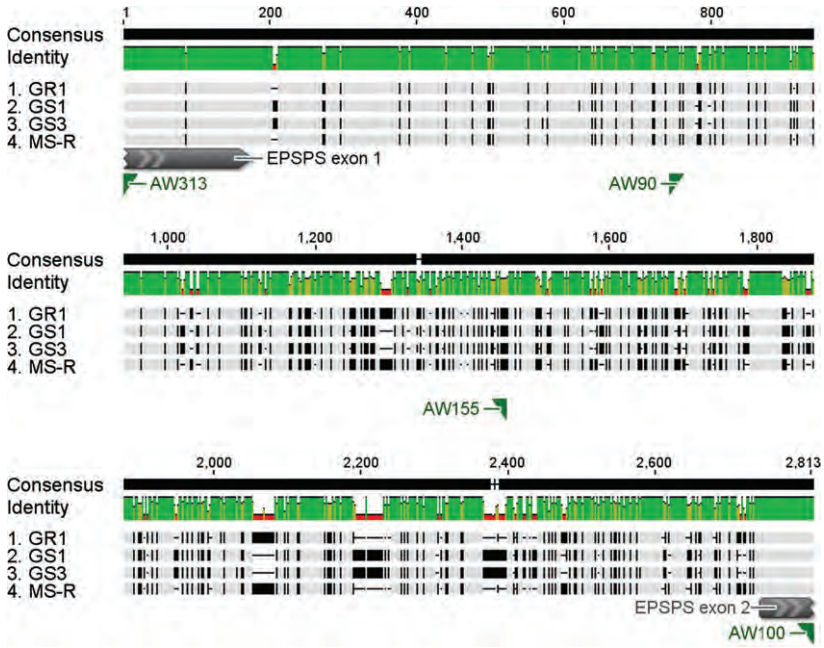

Figure 7. Alignment of intron 1 for glyphosate-resistant *A. spinosus* GR1 biotype, glyphosate-susceptible *A. spinosus* biotypes GS1 and GS3 and glyphosate-resistant *A. palmeri* biotype MS-R. Green indicates consensus, brown indicates differences between sequences and red indicates gaps in the consensus. The light-gray lines are the sequences, with black bars indicating differences and black lines indicating the corresponding sequence for the alignment that is absent. The dark-gray arrows indicate exon 1 and exon 2. The green arrows are for primers AW100 and AW313 used to amplify the intron and for primers AW155 and AW90 internal to intron 1. The sequences for MS-R and GR1 are nearly identical. GS1 and GS3 are nearly identical to each other and differ greatly from the intron 1 sequence for the glyphosate-resistant plants. The differences in sequence at AW155 and to a lesser extent at AW90 make it highly unlikely that PCR including these two primers would have ever worked in glyphosate-sensitive *A. spinosus*.

MS-R, thereby suggesting that the *EPSPS* amplicon from *A. palmeri* may be present in GR1.

The downstream and upstream regions amplified by primer pairs AW203/AW66 and AW67/AW145 (Figs 5B and F) were sequenced to investigate further the similarity between *A. spinosus* GR1 and *A. palmeri* MS-R. Alignment of the sequences shows that the upstream and downstream regions of GR1 are identical to MS-R (Fig. 6). Additionally, owing to the absence of a band for the GS biotypes when using primers AW90 and AW155, intron 1 was sequenced for GR1, GS1 and GS2 (Fig. 7). GR1 was nearly identical to intron 1 from *A. palmeri*, and the susceptible *A. spinosus* biotypes were nearly identical to each other. However, the consensus sequences for the glyphosate-resistant and glyphosate-susceptible biotypes were very different. The binding sites for primers AW90 and AW155 were compared between susceptible and resistant biotypes. There were two nucleotide differences at the AW90 binding site, and seven at the AW150 binding site. The sequence differences between susceptible and resistant biotypes at these primer binding sites make it highly unlikely that the PCR would have ever generated a product in the susceptible biotypes. These findings are in line with the PCR data (Fig. 5) and support the hypothesis that the *EPSPS* amplicon from *A. palmeri* is present in GR1. The *EPSPS* amplicon in *A. palmeri* has only been partially defined;[19] however, as new findings about the amplicon in *A. palmeri* arise, they will be used in future studies to determine whether the full amplicon is present in GR1 and whether the entire mechanism, not just the amplicon, is present.

In summary, a fivefold glyphosate resistance in *A. spinosus* from Mississippi has been confirmed, and the mechanism of resistance has been shown to be gene amplification of *EPSPS*. The regulation of this mechanism is unknown. However, some progress has been made on this front. Sequences with homology to miniature inverted-repeat transposable elements (MITEs) were identified next to *EPSPS* gene copies in GR *A. palmeri* plants, and not in GS *A. palmeri* individuals.[19] In addition, a putative activator (Ac) transposase and a repetitive sequence region were associated with amplified *EPSPS* genes.[19] The presence of putative MITEs and transposase may indicate a role in the regulation of *EPSPS* amplification. The sequence homology of *EPSPS* between glyphosate-resistant *A. spinosus* and glyphosate-resistant *A. palmeri* and the present demonstration by PCR that the GR *A. spinosus* biotypes contain a sequence specific to the *A. palmeri* amplicon supports the hypothesis that the *EPSPS* amplicon in *A. spinosus* originated from *A. palmeri*. Introgressive hybridization has been shown to occur between *Amaranthus* spp. (see Section 1), including *A. spinosus* and *A. palmeri*. The authors have initiated research on several fronts to investigate further whether the *EPSPS* gene amplification trait has been transferred via hybridization, as some of the data presented in this report indicate.

The authors are currently examining, using morphological and molecular analytical methods, hybrids generated in the greenhouse by crossing glyphosate-resistant male *A. palmeri* plants with glyphosate-susceptible *A. spinosus* plants that have had male flowers removed. Also, we have collected plants morphologically 'resembling' *A spinosus* in the fall of 2013 from the original field where initial reports of glyphosate-resistant *A. spinosus* arose. Several important questions remain to be answered regarding the phylogenetic relationship between *A. palmeri* and *A. spinosus*, which is not limited to *EPSPS* amplification and glyphosate resistance. For example, how similar are the whole genomes of *A. palmeri* and *A. spinosus*? How much genetic exchange is occurring between these two species with each event of hybridization? What other genes, in addition to the *EPSPS* amplicon (much of which is still not understood), are introgressing into *A. spinosus*

from glyphosate-resistant *A. palmeri*? Can and do hybrids of *A. palmeri* and *A. spinosus* backcross with either parents? How many generations of hybridization does it take to acquire resistance to glyphosate at field labeled rates? The evidence of putative introgression in the field that is reported in this research should alert private and public land managers to possible gene transfer between species of other weed genera, with profound implications for biodiversity and weed management and other unknown consequences.

4 CONCLUSIONS

A. spinosus that is resistant to glyphosate will remain a weed of minor importance compared with GR *A. palmeri* or *A. tuberculatus*. However, given the ability to germinate over a broad range of temperatures, prolific seed production and the ability to hybridize with *A. palmeri*, *A. spinosus* populations resistant to glyphosate and/or other herbicides may pose management problems in the future. Entomologists have been observed harvesting staminate inflorescences of *A. palmeri* plants for use as a food source for rearing selected insects (personal observation). Thus, it is very likely that *A. spinosus* plants could be fertilized by pollen transferred by insect vectors migrating from *A. palmeri* plants. An additional consideration is that herbicide-resistant *A. spinosus* plants could serve as an alternative host for certain insect pests and diseases.

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