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ORIGINAL ARTICLE

Involvement of facultative apomixis in inheritance of *EPSPS* gene amplification in glyphosate-resistant *Amaranthus palmeri*

Daniela N. Ribeiro · Zhiqiang Pan · Stephen O. Duke · Vijay K. Nandula · Brian S. Baldwin · David R. Shaw · Franck E. Dayan

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Abstract The inheritance of glyphosate resistance in two *Amaranthus palmeri* populations (R1 and R2) was examined in reciprocal crosses (RC) and second reciprocal crosses (2RC) between glyphosate-resistant (R) and -susceptible (S) parents of this dioecious species. R populations and Female-R × Male-S crosses contain higher 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene copy numbers than the S population. *EPSPS* expression, EPSPS enzyme activity, EPSPS protein quantity, and level of resistance to glyphosate correlated positively with genomic *EPSPS* relative copy number. Transfer of resistance was more influenced by the female than the male parent in spite of the fact that the multiple copies of *EPSPS* are amplified in the nuclear genome. This led us to hypothesize that this perplexing pattern of inheritance may result from apomictic seed production in *A. palmeri*. We confirmed that reproductively isolated R and S female plants produced seeds, indicating that *A. palmeri* can produce seeds both sexually and apomictically (facultative apomixis). This apomictic trait accounts for the low copy number inheritance in the Female-S × Male-R offsprings. Apomixis may also enhance the stability of the glyphosate resistance trait in the R populations in the absence of reproductive partners.

Keywords *Amaranthus palmeri* · Apomixis · Asexual reproduction · 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) · Facultative apomixis · Glyphosate · Herbicide resistance · Palmer amaranth

Abbreviations

ALS	Acetolactate synthase
CNT	Counts per mm^2
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
GR	Glyphosate-resistant
GS	Glyphosate-susceptible

Introduction

The non-selective herbicide glyphosate (*N*-(phosphonomethyl) glycine) has become the leading herbicide worldwide, largely because of its use with transgenic, glyphosate-resistant (GR) crops (Duke and Powles 2008). Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway, thereby, preventing the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan (Siehl et al. 1997; Steinrücken and Amrhein 1980). GR crop technology was utilized on ca. 128 million ha in 2012 worldwide (James 2012), boosting farm income globally by $32.2 billion (Brookes and Barfoot 2013) and significantly reducing environmental impact of weed management (Bonny 2011; Gardner and Nelson 2008; Kleter and Kuiper 2003).

Although strong arguments were made against the likelihood of weeds evolving resistance to glyphosate (Bradshaw

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D. N. Ribeiro · B. S. Baldwin · D. R. Shaw
Department of Plant and Soil Sciences, Mississippi State University, Starkville, MS 39762, USA

Z. Pan · S. O. Duke · F. E. Dayan (✉)
Natural Products Utilization Research Unit, United States Department of Agriculture, Oxford, MS 38677, USA
e-mail: fdayan@olemiss.edu; franck.dayan@ars.usda.gov

V. K. Nandula
Crop Production Systems Research Unit, United States Department of Agriculture, Stoneville, MS 38776, USA

et al. 1997), the first case of a GR weed was reported in *Lolium rigidum* (Gaud) about 20 years following the introduction of the herbicide glyphosate (Heap 2013; Powles et al. 1998; Pratley et al. 1999). Resistance to glyphosate has now been confirmed in 23 other weed species (Heap 2013). Evolved glyphosate resistance has recently emerged in *Amaranthus palmeri* (S. Wats.) populations (Culpepper et al. 2006).

Amaranthus palmeri is a tall, upright, and dioecious summer annual species that is native to the Mexican states of Sonora and Baja California, as well as parts of southern Arizona and California, USA (Ehleringer 1983). *A. palmeri* was first described from specimens collected from San Diego County, CA, and along the banks of the Rio Grande River (Watson 1877). The species has since spread into the southeastern United States, where it has become a common and competitive weed in row crop production. Interference from *A. palmeri* affects the growth and yield of several crops that are now mostly GR crops in the USA: maize (*Zea mays* L.) (Massinga et al. 2001), soybean [*Glycine max* (L.) Merr.] (Bensch et al. 2003), and cotton (*Gossypium hirsutum* L.) (Morgan et al. 2001). Currently, GR *A. palmeri* infests more than two million ha in 17 US states (Heap 2013). GR *A. palmeri* has become the single greatest threat to the economic sustainability of cotton production in infested fields, largely due to the lack of adequate control provided by available post emergence herbicides (Mayo et al. 1995).

The steady increase in the number of weed species with evolved resistance to glyphosate has been exacerbated with the introduction and widespread adoption of GR crops (Duke and Powles 2009; James 2012). In evolved resistance, single base pair mutations in *EPSPS* have not provided a high level of resistance (Baerson et al. 2002; Ng et al. 2003; Perez-Jones and Mallory-Smith 2010). Alteration of more than one codon, as with site-directed mutagenesis to produce the GA21 version of maize *EPSPS*, has resulted in commercial GR maize with a much higher level of resistance (Green 2009). GA21-type mutations resulting from the double Thr to Ile101 and Pro to Ser 106 (TIPS) mutation was recently reported in a Malaysian goosegrass biotype (Jalaludin et al. 2013). The most recently evolved GR weed populations have other mechanisms of resistance based on amplification of the *EPSPS* gene(s) (Gaines et al. 2010) or sequestration of glyphosate in the vacuole (Ge et al. 2010, 2011, 2012).

Gene amplification of target site genes is a known resistance mechanism for insecticides (Bass and Field 2011) and fungicides (Selmecki et al. 2008). In the laboratory, step-wise increases in glyphosate concentrations of plant cell cultures has been selected for gene amplification of *EPSPS* (Pline-Srnic 2006). Amplification of the *EPSPS* gene is a mechanism for field-evolved glyphosate resistance in *A. palmeri* (Gaines et al. 2010), *Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot (Salas et al. 2012) and *Kochia scoparia* (L.) Schrad. (Wiersma 2012). Glyphosate is the only herbicide for which there is field-evolved amplification of a target gene known to confer resistance.

In the case of *A. palmeri*, glyphosate resistance correlates with increased copy number of *EPSPS* genes, transcripts, protein content, and enzymatic activity (Gaines et al. 2011). These correlations indicate that between 30 and 50 *EPSPS* copies endow complete resistance to the normal field rate of glyphosate (0.5–1 kg ha^{-1}). These extra copies are found throughout the genome, on every chromosome (Gaines et al. 2010). The mechanism leading to the multiplication of gene copies has recently been linked to sequences with homology to miniature inverted-repeat transposable elements (MITEs) flanking the *EPSPS* gene copies in R individuals (Gaines et al. 2013). Other genetic elements such as a putative activator (Ac) transposase and a repetitive sequence region were also reported, though the precise mechanism of gene amplification is yet to be fully determined. Studies by Gaines and co-workers were all conducted on an *A. palmeri* population that evolved in the southeastern coastal plain of the USA. GR populations of this species were later found in 17 other US states extending throughout the south and as far west as California and as far north as Michigan (Heap 2013). This GR trait can be transferred via pollen to at least three other *Amaranthus* species (Gaines et al. 2012).

Very little is understood about the inheritance of this novel mode of resistance to glyphosate. The objectives of this research were to investigate the molecular mechanism of resistance to glyphosate in *A. palmeri* populations from Mississippi and investigate the mode of inheritance of this novel mechanism of resistance in this GR weed.

Materials and methods

Plant material and general experimental conditions

Seeds of suspected GR (R) populations of *A. palmeri* were collected from 35 GPS site coordinates across the northwestern region of Mississippi (MS), USA, in 2007 (Nandula et al. 2012). Each location corresponded to a population, with each population defined as a group of seed collected from 10 to 20 randomly selected female plants. A preliminary screening with 0.84-kg glyphosate ha^{-1} was conducted on these 35 populations. The C1B1 and T4B1 accessions were selected for subsequent research because they had the greatest number of surviving individuals. A known susceptible (S) population, collected in Washington County, MS, was included

for comparison in all experiments. Seeds were stored at 10 °C until further use.

Experiments were carried out as described by Nandula et al. (2012). Seeds were planted at 1-cm depth in 50-cm by 20-cm by 6-cm plastic trays with holes containing a commercial potting mix (Metro-Mix 360®, Sun Gro Horticulture, Bellevue, WA, USA). Two weeks after emergence, *A. palmeri* plants were transplanted into 6-cm by 6-cm by 6-cm pots containing potting mix. Plants were greenhouse grown under 30/20 °C day/night temperature with a 14-h photoperiod, sub-irrigated as needed and fertilized with a nutrient solution (All Purpose Miracle-Gro®, The Scotts Company, Marysville, OH, USA) containing 200 mg L^{-1} each of N, P_2O_5, and K_2O 1 week after transplanting and then once per month. Plants were sprayed at the four- to six-leaf stage with glyphosate (Roundup WeatherMAX®, Monsanto Company, St. Louis, MO, USA) using an 8002E nozzle and overhead compressed-air sprayer calibrated to deliver 140 L ha^{-1} at 280 kPa.

Production of genetic populations

The first controlled crosses (Female-R × Male-R, R/R) were produced by first treating the C1B1 or T4B1 accessions (Nandula et al. 2012) with 0.84-kg glyphosate ha^{-1}, as previously described. At least 100 surviving individuals of each gender were selected and grown together in isolation from other populations in different greenhouses to ensure genetic purity of each controlled cross. Pollen from the male plants was spread on the female plants every morning over a period of 2 months. *A. palmeri* inflorescence spikes were hand-harvested where the majority of the seed-possessed coats were black in color (seed maturity). Seeds were air-dried in a greenhouse (25/20 °C day/night, 12-h photoperiod under natural sunlight conditions) for 7 days, cleaned, and stored at 10 °C until further use. Resistance was confirmed by treating a subset of whole plant first R/R controlled cross progeny with 0.84-kg glyphosate ha^{-1} before proceeding with the second controlled crosses (data not shown). Second controlled crosses (R/R) were developed using at least 100 first R/R controlled crosses surviving individuals that were treated with 0.84-kg glyphosate ha^{-1}, as described for the development of the first R/R controlled crosses. A subset of second R/R controlled cross plants was grown and their resistance to glyphosate was confirmed following the previously described procedure (data not shown). Seeds from the C1B1 or T4B1 accessions having been through the first and second controlled crosses were, respectively, defined as resistant parents R1 and R2 accessions to generate subsequent genetic populations.

Reciprocal crosses (Female-S × Male-R, S/R, and Female-R × Male-S, R/S) were made by growing S females in isolation with each R male (R1 and R2), and vice versa in separated greenhouses with the aim of generating four reciprocal crossed (RC) populations, hereafter referred to as S/R1, S/R2, R1/S, and R2/S. Plants were pollinated as previously described and resistance confirmed by the application of glyphosate at 0.42 kg ae ha^{-1} to a subset of the RC generations. The majority (≥85 %) of S/R1 and S/R2 cross plants treated with glyphosate at the described rate were susceptible (data not shown) indicating homozygous susceptible individuals.

Second reciprocal crosses (Female-S/R × Male-S/R, S/R//S/R, and Female-R/S × Male-R/S, R/S//R/S) were made by growing each RC female in isolation with each RC male in separated greenhouses with the aim of creating four second reciprocal crossed (2RC) populations, hereafter referred to as S/R1//S/R1, S/R2//S/R2, R1/S//R1/S, and R2/S//R2/S. Plants were pollinated as previously described and resistance confirmed by the application of glyphosate at the low rate of 0.42 kg ae ha^{-1} to a subset of the 2RC generations. The majority (≥70 %) of plants of S/R1//S/R1 and S/R2//S/R2 populations were susceptible, some plants of all four 2RC populations had an intermediate level of injury, and the majority (≥80 %) of plants of R1/S//R1/S and R2/S//R2/S were resistant (data not shown).

These experiments were designed to determine the inheritance of glyphosate resistance at a population level, rather than at the level of individuals. Therefore, crosses were performed with groups of plants with predetermined sensitivity or resistance to glyphosate. For these experiments, we assumed that little or no reproductive fitness penalty resulted in generating these crosses.

DNA, RNA isolation and cDNA synthesis

Total DNA and RNA were extracted from frozen 2- to 4-leaf stage tissue of S, R1, R2, RC, and 2RC plants. Genomic DNA (gDNA) was extracted using DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions and treated with the RNase-Free DNase (Qiagen). The quality and quantity of prepared genomic DNA and total RNA were assessed according to the MIQE Guidelines (Bustin et al. 2009). The DNA and RNA were quantified using a NanoDrop (ND-1000) spectrophotometer (Thermo Scientific, Wilmington, DE 19810) and checked for quality and integrity by TAE agarose gel electrophoresis. For quantitative real-time PCR (qPCR), cDNA was synthesized from 2 μg of total RNA in a 20 μL reaction volume using a proprietary blend of oligo(dT) and random hexamer primers according to the manufacturer's instructions (iScript cDNA Synthesis Kit, Bio-Rad Laboratories, Hercules, CA, USA).

EPSPS cDNA sequencing

Total RNAs for cDNA cloning were isolated from S, R1 and R2 populations as described above. First strand cDNA synthesis was then performed using 1 μg total RNAs and M-MuLV reverse transcriptase in a final volume of 20 μL using oligo$(dT)_{23}$VN according to the manufacturer's instructions (New England BioLabs, Ipswich, MA, USA). A pair of primers (sense: 5′-TGGCTCAAGCTACTACCATC AAC-3′; antisense: 5′-ATATAGCTACTCAATGCTTGGCG AAC-3′) was designed based on the *EPSPS* coding sequence from *A. palmeri* (GenBank accession number FJ861242) (Gaines et al. 2010). PCR reaction contained 1 μL cDNA; 0.1 mM each of forward and reverse primers; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 2 mM $MgCl_2$; and 1 U of proof reading PfuUltra high-fidelity DNA polymerase (Stratagene, La Jolla, CA, USA) with a 1× concentration of supplied buffer in a final volume of 50 μL. The cycling conditions were 2 min at 95 °C followed by 30 cycles of 20 s at 95 °C, 20 s at 55 °C, and 1 min at 72 °C, with a final extension of 3 min at 72 °C. PCR products were ligated into pCR Blunt TOPO vector using Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA), transformed into *Escherichia coli* TOP10 cells and grown on selection media. Single colonies of six transformants of R1 and R2, and 11 transformants from S were cultured overnight in liquid LB media for plasmid extraction. Plasmid DNAs were sequenced and assembled using Lasergene version 10.0 SeqMan (DNASTAR, Madison, WI, USA). Multiple DNA sequence alignments of *EPSPS*, including sequences from both R and S *A. palmeri* populations (Gaines et al. 2010), GenBank accession numbers FJ861242 and FJ861243, were constructed using ClustalW version 10.0 (DNASTAR). RNA extraction and amplification of the *EPSPS* gene was performed on pooled leaf samples from S, R1 and R2 populations.

Quantitative PCR

Quantitative real-time PCR (qPCR) was used to measure *EPSPS* genomic copy number relative to the gene for acetolactate synthase (*ALS*) and cDNA expression level of *EPSPS* relative to *ALS* in S, R1, R2, RC, and 2RC *A. palmeri* plants according to previously described procedures (Gaines et al. 2010). The *ALS* gene was used as a low-copy control gene with known monogenic inheritance in other *Amaranthus* species (Trucco et al. 2005). The first experiment measured *EPSPS* genomic copy number and expression level in the population (i.e., combined leaf material from at least ten individuals per population studied). The second experiment measured *EPSPS* genomic copy number by individuals of each population (i.e., 30 individuals per population were analyzed separately).

Protein extraction and EPSPS enzyme activity assay

Ten grams of fresh leaf tissue was frozen in a −80 °C refrigerator prior to being ground to fine powder in a chilled mortar. Total soluble protein (TSP) was extracted from 2- to 4-leaf stage tissue of S, R1, R2, RC, and 2RC plants. Protein extraction and EPSPS assay were conducted following published protocols (Sammons et al. 2007; Webb 1992) and described in detail in Dayan et al. (2013).

The specific activity of EPSPS from *A. palmeri* plants was determined using EnzCheck phosphate assay kit (Invitrogen) with a UV-3101 spectrophotometer (Shimadzu North America, Columbia, MD, USA) as described in detail elsewhere (Dayan et al. 2013). The effect of glyphosate was tested in triplicates at concentrations ranging from 0.3 to 1,000 μM in half-log intervals. Two experiments were conducted with three replications per population. The collected samples consisted of combined leaf material from at least ten individuals per population studied.

Western blot analysis for the detection of EPSPS protein

Total cellular protein was isolated from 2- to 4-leaf stage tissue of S, R1, R2, RC, and 2RC plants. A quantity of 0.25 g of fresh leaf tissue was ground to fine powder in a chilled mortar as described above. Powdered tissues were homogenized in 500 μL cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10 % glycerin, and 50 mM KCl, pH 7.0) with freshly added protease inhibitor (1 tablet per 10 mL of buffer) (Roche Applied Science, Indianapolis, IN, USA). The samples were thawed at room temperature and vortexed for 10 s. The mixtures were kept cold and placed on a Geno/Grinder mechanical shaker (SPEX SamplePrep, Metuchen, NJ, USA) for 1 min at 1,750 strokes per min. This step was repeated three times. Subsequently, the extract was centrifuged for 5 min at 18,000*g*. The supernatant was transferred to a new vial and it was centrifuged for 5 min at 18,400*g*. This step was repeated one more time and extract was stored at −80 °C. Protein concentrations were determined using a Bradford assay (Bradford 1976).

Western blot analysis for the detection of EPSPS protein was performed diluting soluble protein in 950-μL Laemmli premixed protein sample buffer (Bio-Rad Laboratories) and 0.71 M β-mercaptoethanol to reach a final concentration of 15 μg μL^{-1}. The sample was transferred to a heating block at 90 °C for 4 min and allowed to cool to room temperature. The soluble protein was separated on a 12 % Tris–HCl precast polyacrylamide gel (Bio-Rad Laboratories). After separation, the proteins were blotted onto 0.45 μM Immobilon-FL PVDF membranes (EMD Chemicals, San Diego, CA, USA) and the membrane was equilibrated with transfer buffer [0.2 % methanol and Tris/glycine buffer (2.5 mM Tris, 19.2 mM glycine, pH 8.3)]. The membrane

was washed with Tris-buffered saline (TBS) (20 mM Tris, pH 7.5 and 500 mM NaCl) and incubated overnight at 4 °C with 3 % gelatin from cold water fish skin. Western blot was probed with an EPSPS specific antibody (2 mg mL^{-1}) developed against recombinant maize EPSPS (Monsanto Company) at dilution 1:2,000 and re-probed against Alexa Fluor 635 goat anti-rabbit (Invitrogen) at dilution 1:2,000. Within 20 min of the final wash with TBS buffer and Tween-20, the array was scanned and analyzed on a Pharos FX Plus Molecular Imager system equipped with an external 635-nm laser and quantified by densitometric analysis using Quantity One analysis software (Bio-Rad Laboratories) and represented as CNT (counts mm^{-2}). The collected samples consisted of combined leaf material from at least ten individuals per population studied with three replications per population.

Glyphosate dose–response bioassay

Plants from parental lines (R1 and R2) and from the first and second reciprocal crosses were submitted to a dose–response experiment by applying glyphosate at 0, 0.052, 0.105, 0.21, 0.42, 0.84, 1.68, 3.36, and 6.72 kg ae ha^{-1} at the 3- to 4-leaf growth stage. Two weeks after treatment (WAT) shoots were clipped at soil level and fresh weight was determined. Biomass data are reported as percentage of the non-treated control. The experiment was conducted two times and arranged in a completely randomized design with triplicates.

Shikimate bioassay

Shikimate content of tissues of *A. palmeri* populations was determined spectrophotometrically in microtiter plates according to Shaner et al. (2005). Glyphosate concentrations ranged from 10 to 333 μM. The absorbance at 380 nm was measured within 30 min in a PowerWave XS microplate reader (Biotek, Winooski, VT, USA). Shikimate in μg mL^{-1} was determined based on a standard curve. Two experiments were conducted with four replications per population.

Testing for facultative apomixis

To test for apomixis in *A. palmeri* parent (S, R1 and R2) populations, pollination bags were placed over main inflorescences prior to emergence and were examined daily to determine their sex. The male plants were discarded and the female plants were isolated in different geographically located greenhouses to ensure repeatability of the apomixis test and exclude external sources of pollen contamination. The inflorescences were enclosed in pollination bags for the whole duration of the experiments. A total of 44 S individuals were grown, Spring/Summer, 2011, in Oxford (MS), 36 R1 individuals in Starkville (MS), and 38 R2 individuals in Abbeville (MS). *A. palmeri* inflorescence spikes were hand-harvested where the majority of the seed-possessed coats were black in color (seed maturity). *A. palmeri* seeds were air-dried, cleaned, and stored at 10 °C as previously described. The sex segregation ratio of the offsprings was determined by germinating the apomictically produced seeds and growing the plants until their sex could be determined by observing their flowers. A similar experiment was carried out during the winter, when no *A. palmeri* plants remained alive outside the greenhouse, to insure that no pollen could accidentally fertilize our reproductively isolated female plants. The inflorescence were also enclosed in pollination bags to further prevent pollination.

Intraspecific genetic diversity and relationships

Genetic markers that might confirm apomixis in *A. palmeri* were selected based on information from available literature (Chan and Sun 1997; Chandi et al. 2013; Lanoue et al. 1996; Lee et al. 2009; Ma et al. 2008; Popa et al. 2010; Wassom and Tranel 2005; Wetzel et al. 1999; Xu et al. 2011). RAPD (random-amplified polymorphic DNA) and ISSR (inter-simple sequence repeat) methods were used because they can provide up to a population level of identification of intraspecific variation.

DNA extraction was carried out as previously described from three different plants of the R1 population ($n = 3$) and diluted to 2 ng μL^{-1}. RAPD analyses were performed using 12 decamer primers (Online Resource 1) randomly selected from a list of 100 primers (NAPS Unit, University of British Columbia, Biotechnology Laboratory, Vancouver, BC, Canada). ISSR analyses were performed using 15 primers (Online Resource 1) randomly selected from a list of 100 primers (NAPS Unit, University of British Columbia) and five provided by Natascha Techen (National Center for Natural Products, University of Mississippi, Oxford, USA). PCR reaction contained 10 ng DNA; 0.1 μM each of forward and reverse primers; 200 μM each of dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI, USA); 1.5 mM $MgCl_2$; and 1 unit of Platinum Taq DNA polymerase (Invitrogen) with a 1× concentration of supplied buffer in a final volume of 13.6 μL. The cycling conditions included 3 min at 94 °C followed by 45 cycles of 30 s at 94 °C, 30 s at 50 °C, and 3 min at 72 °C, with a final extension of 7 min at 72 °C in a thermal cycler (PTC-225, MJ Research, Waterton, MA, USA). PCR products were analyzed by electrophoresis on a 2 % TAE agarose gel stained with ethidium bromide and visualized under UV light using Quantity One (Bio-Rad, version 4.3.0). The sizes of the PCR products were compared to the molecular size standard 1 kb plus DNA ladder (Invitrogen).

Statistical analysis

Data from *EPSPS* genomic copy number and gene expression, EPSPS protein quantity and shikimate levels were analyzed by ANOVA via the PROC GLM statement using SAS software (version 9.3, SAS Institute, Cary, NC, USA) to determine the main effects and interactions of the factors at $P < 0.05$. No significant experiment effect was observed in repeated experiments; therefore, data from those experiments were pooled.

Where ANOVA indicated significant differences between treatments, means separation were performed using Fisher's protected least significant difference (LSD) test at $P = 0.05$ using SAS software. The data from *EPSPS* relative copy number were regressed against *EPSPS* cDNA relative expression level and against EPSPS enzyme activity.

The whole plant glyphosate dose–responses were analyzed using a four parameters log-logistic model (Seefeldt et al. 1995), whereas the in vitro dose–responses were analyzed with a three parameters log-logistic model using R software (version 2.15.2, R Foundation for Statistical Computing, Vienna, Austria) using package drc, drm and modelFit function. The graphs were generated with Sigma Plot (version 11, Systat Software, San Jose, CA, USA). Subsequently, the Student *t* test at $P < 0.05$ was used to determine any differences in potency between populations and the S population at the respective GR_{50} or IC_{50} effect level. The null hypothesis, H_0: GR_{50} or IC_{50} populations/GR_{50} or IC_{50} S population = 1, was tested against the alternative hypothesis, H_a: GR_{50} or IC_{50} populations/GR_{50} or IC_{50} S population ≠ 1. This test was performed using the open-source R software using package drc, drm function, and the comparisons were given by means of the selectivity index (SI) function.

Results

EPSPS cDNA sequencing

There was little difference among *EPSPS* sequences from the R1 (six clones) and R2 (six clones) populations. Consequently, the consensus sequence represents residues common to all GR sequences (R_consensus) (Online Resource 2). Alignment of consensus sequences from R and S individuals had several polymorphisms in all S sequences in the alignment contig when compared with the reference S sequence (FJ861242, Gaines et al. 2010). This may be the result of combining seeds from several plants at the time of initial collection of this population.

The cDNA sequence analysis of the *EPSPS* gene in both GR (R1 and R2) populations revealed several nucleotide substitutions resulting in silent mutations when compared with the S population sequence. The S and R sequences had 99 and 100 % homology to the *A. palmeri* reference sequence FJ861242 (S) of Gaines et al. (2010), respectively (online resource 2). Only one of the three single nucleotide polymorphisms (SNP) between S and R clones (contig position 866) resulted in a non-synonymous mutation (arginine to lysine amino acid substitution) at position 215, based on the maize mature EPSPS numbering system (Fig. 1), in the GS *A. palmeri* (Lebrun et al. 1997).

EPSPS gene copy number correlates with *EPSPS* gene expression

Analysis of the genomic DNA extracted from pooled samples of several individuals for each population showed that on average, R1 and R2 populations contained 59 and 33 copies of the *EPSPS* gene, whereas the S population contained a single copy (Fig. 2b). Quantitative RT-PCR on cDNA revealed that the single copy of *EPSPS* gene was proportionally

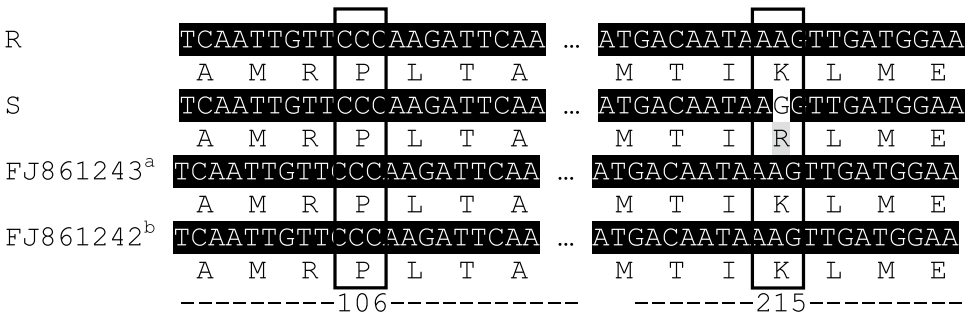

Fig. 1 Partial and deduced amino acid sequence alignment of the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene of glyphosate-susceptible (*S*) and glyphosate-resistant (*R*) *A. palmeri* populations. The *boxed codon* shows the substitutions of arginine (*R*) to lysine (*K*) at amino acid 215 and no substitution at proline (*P*) 106, when using the maize mature EPSPS numbering system. [a]FJ861243, glyphosate-resistant *A. palmeri* from Georgia (Gaines et al. 2010) sequence reference, sequence information can be found at http://www.ncbi.nlm.nih.gov/nuccore/ [b]FJ861242, glyphosate-susceptible *A. palmeri* from Georgia (Gaines et al. 2010) sequence reference, sequence information can be found at http://www.ncbi.nlm.nih.gov/nuccore/

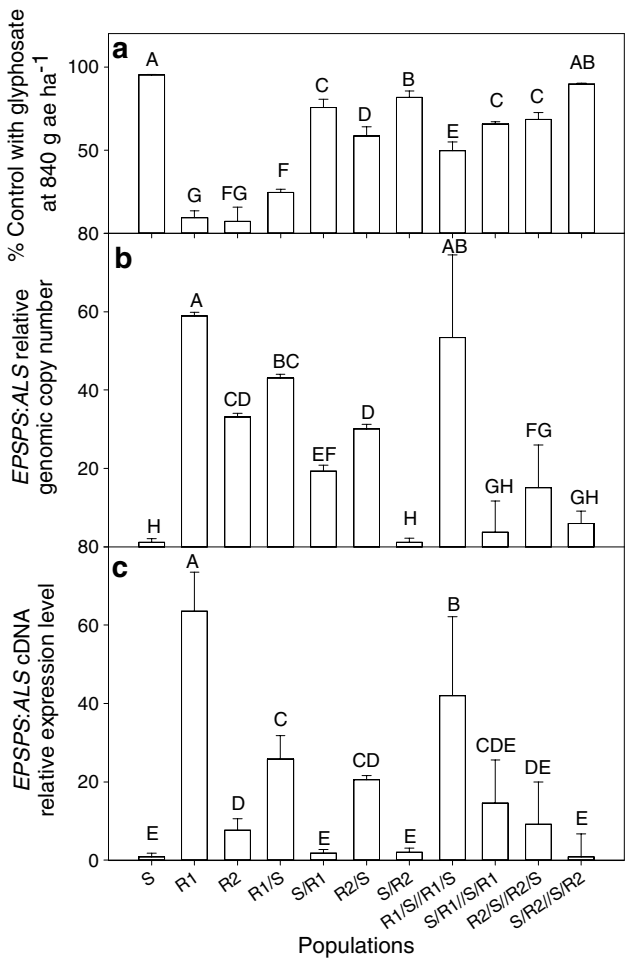

Fig. 2 Glyphosate effect (**a**), genomic copy number of *EPSPS* (**b**), and relative transcription level of *EPSPS* (**c**) in glyphosate-susceptible (*S*), -resistant (*R*1 and *R*2), and first (*R*/*S* and *S*/*R*) and second (*R*/*S*//*R*/*S* and *S*/*R*//*S*/*R*) reciprocal crosses of *A. palmeri* populations. *Vertical bars* represent ± standard error of the mean ($n = 20$). *Letters* represent statistical difference based on Fisher's LSD at $P = 0.05$

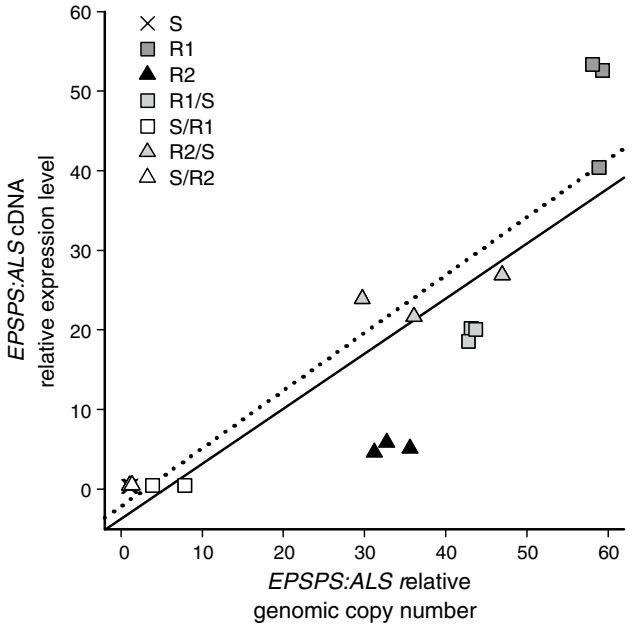

Fig. 3 Positive correlation between increase in 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) relative to acetolactate synthase (*ALS*) genomic copy number with increase in *EPSPS: ALS* cDNA expression levels in glyphosate-susceptible (*S*), -resistant (*R*1 and *R*2), and first reciprocal crosses (*R*/*S* and *S*/*R*) of *A. palmeri* populations. Regression of the entire dataset indicated a good relationship between mRNA levels and copy numbers (*solid line*, $r = 0.87$, $P < 0.0001$). The *R*2 population did not fit the model as well as the other populations. Excluding this population from the dataset improved the strength of that relationship (*dotted line*, $r = 0.94$, $P < 0.0001$)

expressed, with a 1:1 correlation to *EPSPS* gene copy:*EPSPS* transcript richness (Figs. 2, 3). The *EPSPS* copy number was positively correlated with the gene expression level ($r = 0.94$, $P < 0.0001$, excluding R2 data, Fig. 3), and a pattern was observed where the reciprocal crosses generated by Female-S × Male-R (S/R) and by Female-S/R × Male-S/R (S/R//S/R) had lower *EPSPS* expression level than the reciprocal crosses generated by Female-R × Male-S (R/S) and by Female-R/S × Male-R/S (R/S//R/S). While the R2 population is shown in the graph (Fig. 3), it was excluded from the data used for the regression because it did not have a strong correlation between its relatively high number of gene copies and unexpectedly low number of mRNA transcript.

When individuals from each population were analyzed separately, there was a strong ($P < 0.0001$) relationship between *EPSPS* copy number and the populations (Fig. 4), with S, S/R1 and S/R2 with the fewest copy numbers. In fact, all of the S and 73 % of S/R1 and 70 % of S/R2 individuals analyzed had a single copy of the *EPSPS* gene. R1 and R2 populations had the highest copy numbers, and R1/S and R2/S had intermediate numbers (Fig. 4).

EPSPS gene copy number correlates with EPSPS enzyme activity and protein quantity

EPSPS specific activity in each *A. palmeri* population was inhibited by glyphosate concentrations (Online Resource 3, Fig. 5). The amounts of glyphosate needed to reduce the EPSPS activity by 50 % (IC_{50}) ranged from 5.5 to 55 μM glyphosate (Online Resource 3), but there was no clear relationship between the IC_{50} values at the enzymatic level and in vivo sensitivity to glyphosate (i.e., the sensitive biotype had the highest IC_{50} value). Consequently, the *R*/*S* ratios for all populations were smaller than 1, suggesting that EPSPS from the GR plants was more sensitive to glyphosate than in the susceptible population.

The specific activity of EPSPS varied greatly between populations in the absence of glyphosate. The S biotype had

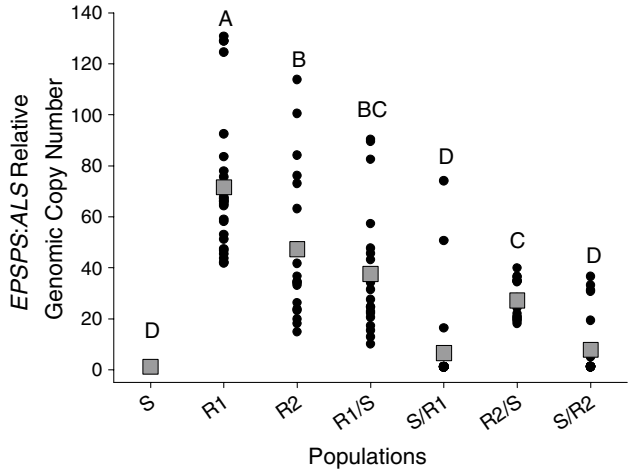

Fig. 4 Genomic copy number of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) relative to acetolactate synthase (*ALS*) genomic copy of 30 ($n = 30$) sampled individuals per *A. palmeri* population (*dark circle*), glyphosate-susceptible (*S*), -resistant (*R*1 and *R*2), and first (*R*/*S* and *S*/*R*) reciprocal crosses. Means of *EPSPS* relative gene copy number followed by the *same letter* are not significantly different by LSD test at 0.05. The *EPSPS* copy number segregation pattern observed in Fig. 2b, when combined sample population, is observed when averaging sampled individuals (*dark gray square*)

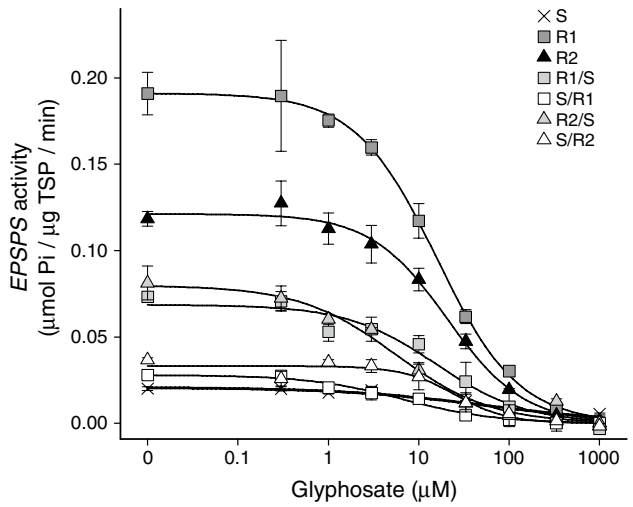

Fig. 5 Dose–response of glyphosate against EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) enzyme activity of glyphosate-susceptible (*S*), -resistant (*R*1 and *R*2), and first reciprocal cross (*R*/*S* and *S*/*R*) of *A. palmeri* populations. Glyphosate inhibition assays were normalized for total soluble protein (TSP) quantity. S *black cross*, 1 relative *EPSPS* copy, IC_{50} (glyphosate concentration that reduced enzyme activity by 50 %) = 55 µM; R1 *dark gray square*, 59 relative copies, IC_{50} = 16 µM; R2 *black triangle*, 33 relative copies, IC_{50} = 21 µM; R1/S *light gray square*, 43 relative copies, IC_{50} = 15 µM; S/R1 *open square*, 19 relative copies, IC_{50} = 7 µM; R2/S *light gray triangle*, 30 relative copies, IC_{50} = 6 µM; S/R2 *open triangle*, 1 relative copy, IC_{50} = 26 µM. *Pi* inorganic phosphate. *Vertical bars* represent ± standard error of the mean ($n = 6$)

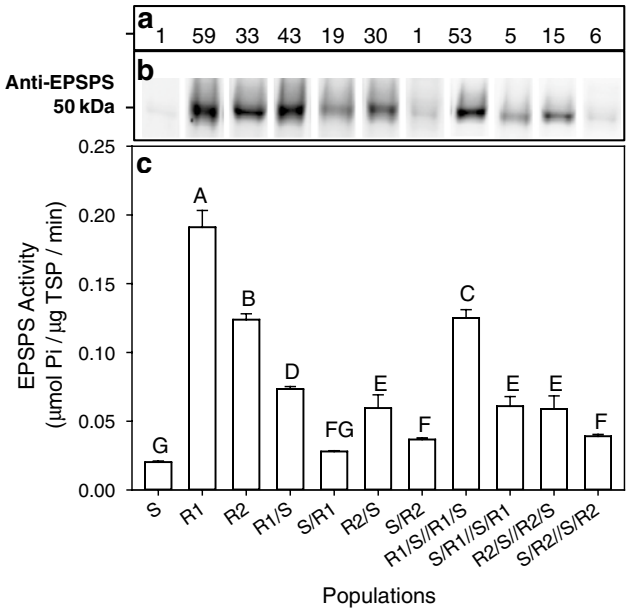

Fig. 6 Positive correlation among *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) genomic copy number (**a**), EPSPS quantity (**b**), and specific activity of EPSPS enzyme (absence of inhibitor glyphosate) in glyphosate-susceptible (*S*), -resistant (*R*1 and *R*2), and first (*R*/*S* and *S*/*R*) and second (*R*/*S*//*R*/*S* and *S*/*R*//*S*/*R*) reciprocal crosses of *A. palmeri* populations. *Pi* inorganic phosphate; *TSP* total soluble protein. *Vertical bars* represent ± standard error of the mean ($n = 6$). Means of EPSPS activity with the *same letter* are not significantly different by Fisher's LSD test at 0.05

the lowest activity (0.02 µmol μg^{-1} protein min^{-1}), whereas both R1 and R2 parent populations had high activity (0.19 to 0.12 µmol μg^{-1} protein min^{-1}, respectively) (Fig. 6c). In the RC populations, the R1/S and R2/S had intermediate EPSPS activity and had consistently higher activity than the S/R1 and S/R2 crosses ($r = 0.87$, $P < 0.0001$, including and excluding R2 data) and *EPSPS* expression level relative to *ALS* ($r = 0.97$, $P < 0.0001$, excluding R2 data; $r = 0.84$, $P < 0.0001$, including R2 data) (Fig. 7). In the second reciprocal crosses (2RC), R1/S//R1/S had a level of EPSPS activity similar to R1. The other 2RC populations had intermediate levels of EPSPS specific activity, which was positively correlated with *EPSPS* relative copy number. Consistent with these observations, EPSPS protein level, as measured with immunoblotting, correlated strongly with other EPSPS-related parameters such as *EPSPS* gene copy number and enzymatic activity (Fig. 6).

EPSPS gene copy number correlates with level of glyphosate resistance

The shoot biomass of all *A. palmeri* population decreased in a dose-dependent manner with increasing glyphosate dose (Online Resource 4). Consistent with our preliminary data, the S population was the most sensitive to glyphosate,

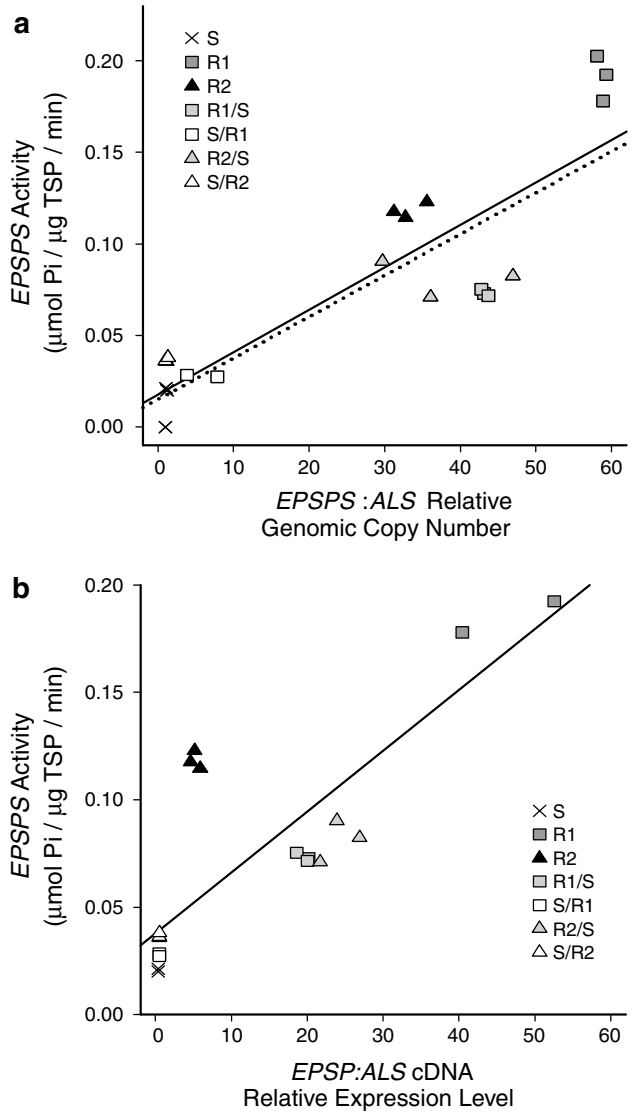

Fig. 7 Positive correlation between specific activity of EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) enzyme and *EPSPS* relative to acetolactate synthase (*ALS*) genomic copy number (**a**) and *EPSPS*: *ALS* cDNA expression levels (**b**) in glyphosate-susceptible (*S*), -resistant (*R*1 and *R*2), and first reciprocal cross (*R*/*S* and *S*/*R*) of *A. palmeri* populations. **a** regression of the entire dataset indicated a strong relationship between enzyme activity and copy number (*solid and dotted line*, $r = 0.87$, $P < 0.0001$). **b** Regression of the entire dataset indicated a good relationship between mRNA levels and enzyme activity (line not shown $r = 0.84$, $P < 0.0001$). The R2 population did not fit the model as well as the other populations. Excluding this population from the dataset improved the strength of that relationship (*solid line*, $r = 0.97$, $P < 0.0001$)

with a GR_{50} of 94.4 g ae ha^{-1} (Table 1). The R1 and R2 populations were 17 and 14.5 times more resistant, respectively, to glyphosate than S.

The S biotype had the highest shikimate accumulation at all glyphosate treatment concentrations, differing from the grouped R1 and R2 populations, which had no accumulation except for the R2 at 333-µM glyphosate (Fig. 8). Shikimate accumulation was highly dependent on glyphosate concentration and between populations ($P < 0.005$ for both).

In all cases, the progeny of the RC and 2RC crosses had less resistance to glyphosate (lower GR_{50}) than the resistant parent (Table 1). Interestingly, the GR_{50} for the RC were closer to their respective maternal parent than the midpoint (Table 1). The 2RC dose–response was intermediate between those of the R and S populations, containing both highly susceptible and highly resistant individuals and a range of intermediate phenotypes (Table 1). Consistent with the pattern observed with the number of gene copy numbers, mRNA levels and protein expression, the greenhouse data suggest that the mode of inheritance of GR trait was maternally affected.

Facultative apomixis and intraspecific genetic diversity

Involvement of apomixis in glyphosate resistance inheritance was tested by measuring seed production in reproductively isolated female plants. In that experiment, reproductively isolated female individuals (44 S, 36 R1 and 38 R2) were studied. In all cases, seeds were produced with the exception of one R1 plant. From 60 to 100 % (depending on the population) of individuals studied produced 1–1,000 seeds, and some S individuals produced as many as 6,000 seeds (Table 2). The female:male ratio of the apomictically produced offspring was close to 1:1.

Attempts to monitor apomixis using RAPD and ISSR genetic markers were unsuccessful (Online Resources 5 and 6). No significant RAPD and ISSR polymorphism was observed in a preliminary test experiment on a subset of three R1 individuals. Therefore, none of these markers would have been useful in differentiating apomictically produced seeds from seeds resulting from a fertilized egg through sexual reproduction (Chandrika et al. 2010; Lata et al. 2010, 2011).

Discussion

Analysis of the *EPSPS* sequences from the S and R populations of *A. palmeri* from Mississippi revealed several polymorphisms. Only one of these SNPs resulted in a non-synonymous mutation (arginine to lysine amino acid substitution) at position 215 (Fig. 1). However, this mutation was in the *EPSPS* sequence from the glyphosate sensitive wild-type and is therefore not linked to resistance. Importantly, all the sequences possessed the normal proline 106 residue.

These *A. palmeri* populations from Mississippi evolved resistance to glyphosate by gene amplification of *EPSPS* as

Table 1 Glyphosate dose–response parameters and variables in the log-logistic model estimates for parents, reciprocal crosses and second reciprocal crosses of *A. palmeri* at 14 days after treatment

Population code[a, **]	R^2	% Fresh weight reduction (SE)[d]			GR_{50}^{c}	GR_{50} ratio[c]
		L^c	U^c	s^c	g ae ha^{-1} (SE)[d]	
R1	0.99	16.56* (2.59)	100.24* (1.18)	3.42* (0.51)	1,623.3* (68.5)	17.2
R1/S	0.99	8.05* (2.07)	97.92* (1.21)	3.70* (0.41)	1,138.1* (44.7)	12.1
S/R1	0.99	3.69 (2.29)	100.75* (1.90)	1.69* (0.16)	464.2* (29.1)	4.9
R1/S//R1/S	0.98	6.63* (2.74)	97.98* (1.41)	2.20* (0.24)	976.4* (56.7)	10.4
S/R1//S/R1	0.99	7.08* (2.01)	98.02* (1.48)	2.65* (0.32)	663.6* (29.9)	7.0
R2	0.98	16.85* (2.72)	100.67* (1.23)	2.85* (0.37)	1,368.8* (71.3)	14.5
R2/S	0.99	3.54 (2.12)	97.85* (1.48)	2.35* (0.23)	758.9* (36.5)	8.0
S/R2	0.99	1.78 (1.79)	99.89* (1.88)	2.02* (0.18)	363.0* (18.7)	3.9
R2/S//R2/S	0.99	4.81* (1.91)	98.65* (1.51)	2.49* (0.25)	569.7* (26.5)	6.0
S/R2//S/R2	0.99	2.08 (1.55)	97.65* (1.53)	3.35* (0.41)	433.1* (14.8)	4.6
S	0.99	1.07 (1.37)	98.86* (2.58)	1.75* (0.16)	94.36* (5.6)	–

Model proposed by Seefeldt et al. (1995): y [fresh weight (% of untreated control)] $= L + \{(U - L)/[1 + (D/GR_{50})^s]\}$

[a] Resistant parents (R1 and R2), susceptible parent (S), reciprocal crosses (Female-S × Male-R, S/R, and Female-R × Male-S, R/S), second reciprocal crosses (Female-S/R × Male-S/R, S/R//S/R, and Female-R/S × Male-R/S, R/S//R/S)

[c] The parameter estimates are L, lower limit of response; U, upper limit of response; s, slope of the curve around the point of inflexion (GR_{50}); GR_{50}, glyphosate dose required to cause a 50 % reduction in plant growth and GR_{50} dose was estimated using responses to nine glyphosate doses (D) (0, 52, 105, 210, 420, 840, 1,680, 3,360, and 6,720 g ha^{-1}); and GR_{50} ratio, GR_{50} populations/GR_{50} susceptible population

[d] SE represents the standard error of the mean where $n = 6$ (polled data from two experiments)

* Estimated parameters of the log-logistic model are different according to Student t test at $P < 0.05$; accept alternative hypothesis, H_a: parameter $\neq 0$

** Relative potencies between populations and susceptible population at GR_{50} response level are different according to Student t test at $P < 0.05$; accept alternative hypothesis, H_a: relative potency $\neq 1$. The two exceptions, where the relative potencies of S/R1 vs. S/R2//S/R2 and R2/S vs. S/R1//S/R1 at GR_{50} response level are not different according to Student t test at $P < 0.05$; accept null hypothesis, H_0: relative potency $= 1$

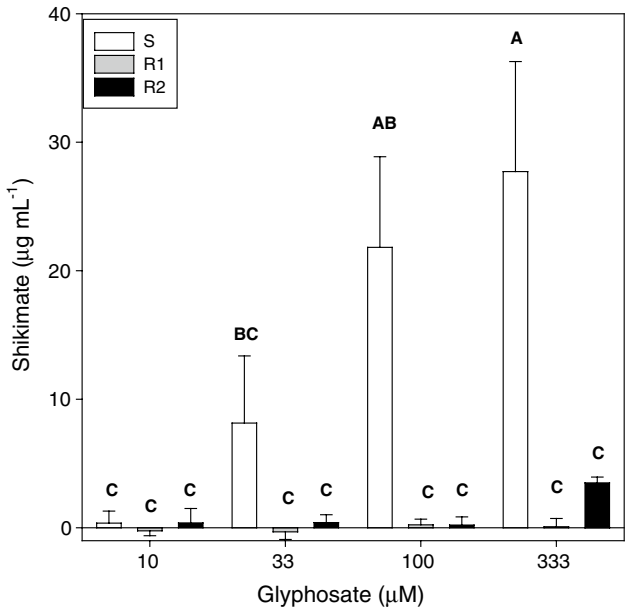

Fig. 8 Effect of glyphosate concentration on shikimate levels in excised leaf discs of glyphosate-resistant (*R1* and *R2*) and -susceptible (*S*) *A. palmeri* populations. Means of shikimate level with the *same letter* are not significantly different by Fisher's LSD test at 0.05. *Vertical bars* represent ± standard error of the mean ($n = 8$)

Table 2 Seed production of reproductively isolated female plants due to the effect of agamospermy/apomixis of glyphosate-susceptible (S) and -resistant (R1 and R2) *A. palmeri* plants used as parents to generate first reciprocal crosses (R/S and S/R)

Number of seeds	Biotypes		
	Number of individuals		
	S	R1	R2
0	0	0	0
1–999	29	25	38
1,000–1,999	8	5	0
2,000–2,999	3	4	0
>3,000	4	2	0

first reported by Gaines et al. (2010) in Georgia and in other species by others (Salas et al. 2012; Wiersma 2012). The populations have as many as 59 copies of *EPSPS* (Fig. 2b), which is similar to that reported in other GR biotypes of *A. palmeri*. The biotypes from Georgia had individual plants with as many as 160 copies of the *EPSPS* gene (Gaines et al. 2010), and six individuals from the S × R cross had 1–39 copies (Gaines et al. 2011). A GR population from North Carolina possessed 22–63 copies of *EPSPS* gene (Chandi et al. 2012).

As with other cases where resistance is associated in amplification of multiple *EPSPS* gene copy numbers, the specific activity of EPSPS in our R1 and R2 populations was six- to tenfold higher than in S plants (Fig. 6c and Online Resource 3). Similar differences in specific enzyme activity were observed in GR and GS *A. palmeri* from Georgia (Gaines et al. 2010), *L. perenne* L. ssp. *multiflorum* from Arkansas (Salas et al. 2012) and *K. scoparia* from the US central Great Plains (Wiersma 2012). R1 and R2 plants had a nine- to sixfold increase, respectively, in EPSPS enzyme activity relative to that of the S plants. Other *A. palmeri* populations had a 16-fold increase in specific activity between GR and GS (Gaines et al. 2010) and there was an average sixfold higher basal enzyme activity in GR *L. perenne* compared to the GS biotype (Salas et al. 2012).

In our study, the 2RC genomic copy number varied greatly and was an intermediate value between those of the R and S populations (R1/S//R1/S: 53, S/R1//S/R1: 5, R2/S//R2/S: 15, and S/R2//S/R2: 6), containing both highly susceptible and highly resistant individuals and a range of intermediate genotypes (Fig. 2b). The molecular mechanism leading to the insertion of multiple *EPSPS* copies throughout the genome of *A. palmeri* (Gaines et al. 2010) is not fully understood, but it is linked to the presence of MITEs flanking the *EPSPS* gene copies in R individuals (Gaines et al. 2013). Furthermore, this process is very dynamic, where one individual *A. palmeri* (S/R × S/R) had a higher relative copy number than the sum of their relative copy number from both parents (Gaines et al. 2010).

Reciprocal crosses generated by Female-R × Male-S (R/S) and by Female-R/S × Male-R/S (R/S//RS) of the populations tended to have higher *EPSPS* copy number than the other crosses (Fig. 2b). This pattern of inheritance where the direction of the cross affected the level of resistance suggests a maternal influence.

While the transfer of glyphosate resistance via pollen movement is evident in *A. palmeri*, the rate of transfer is inversely proportional to the distance from a R male parent (Sosnoskie et al. 2012). In fact, that study in which only GS female plants surrounded a central group of GR male plants, no resistance was transferred to the seeds of female plants located 600 m or more from the resistant male pollen donor. Nevertheless, GS seeds were produced by plants at all distances from the GR male plants (40 % at 5 m to 80 % at 250 m), and there was little difference in the number of seeds produced at distances of 50 m and farther from the pollen source. The authors proposed that these findings may be due to fertilization by autopollination or from pollen providers outside the boundary of the experiment. These authors discounted agamospermy (apomixis) because the proportion of male to female offsprings approximated 1:1. However, all but one of the reproductively isolated *A. palmeri* plants in our study underwent apomixis and produced seeds (Table 2) that yielded a similar ratio of male to female offspring.

Little is known about the inheritance of this incompletely dominant, nuclear encoded GR trait, but it might follow a polygenic additive pattern in populations of *A. palmeri* from Georgia and North Carolina (Chandi et al. 2012; Gaines 2009). However, both of these studies observed an unpredictable behavior in the inheritance of glyphosate resistance in some populations, which may be the result of facultative apomixis, even though the overall contribution of this process may be small at the population level under most conditions. Indeed, a relatively small number of seeds are produced by apomixis (Table 2) when compared with 'normal' seed production per female plant in the field reaching 200,000–600,000 seeds (Keeley et al. 1987). Nonetheless, production of apomictic seeds would be sufficient to maintain the resistant trait in a population.

Our observations that *A. palmeri* can produce seeds both sexually and apomictically (facultative apomixis) are consistent with some of the data by other groups (Chandi et al. 2012; Gaines 2009; Gaines et al. 2010, 2011; Sosnoskie et al. 2012). The occurrence of agamospermy in *A. palmeri* is also evident in several studies demonstrating that crosses between *A. palmeri* and *A. rudis* can yield non-hybrid progeny (Franssen et al. 2001; Steinau et al. 2003; Trucco et al. 2007; Wetzel et al. 1999). The ability of plants to reproduce apomictically is particularly advantageous because it "guarantees a protracted existence to the individual genotypes, over long periods and over wide areas" (Gustafsson 1947).

The current understanding of sex determination in dioecious species suggests that the change from unisexuality to bisexuality has been a short step in evolution (Chattopadhyay and Sharma 1991). Apomictic seeds derived from unreduced cells (apomeiosis) should be 100 % female. However, reproductively isolated *A. palmeri* individuals produced 1:1 female:male ratio of offspring apomictically. The production of both male and female apomictic offspring would require meiotic events and subsequent fusion (to restore the somatic number). Such events are known among apomictic species as synergid or antipodal apogamy. If the Y male sex determinant in *A. palmeri* were a non-heteromorphic chromosome, then it would segregate and assort during meiosis I with the other chromosomes of the genome. If gender of this dioecious species were determined by homozygous presence or absence of genes, then synergids or antipodals with W (conferring femaleness) or Y (conferring maleness) would produce genotypes; WW (a female) or YY (a male) in a 1:1 phenotypic ratio. While Westergaard (1958) reports the normal (sexual) male of *Acnida* spp. (an amaranth) to be heterozygous (XY); he also reports 8 of 18 plant families investigated showing possibility of the homozygous genotype (however, viability

is unknown for this amaranth species). It is possible that the offsprings we are observing in this 1:1 ratio are homozygous (normal) females and homozygous males.

Alternatively, if gender were determined by an X:autosome balance as found in *Rumex* and *Humulus* (Parker and Clark 1991; Jacobsen 1957, respectively), a 1:1 ratio of male to female would be possible in apomictic offspring. In this system, the X:autosome ratio determines the sexual fate of floral primordial, and thus gender of the plant, rather than the presence or absence of the Y chromosome. In diploid *Rumex*, XX are female and XYY are male. In polyploidy species of the genus *Rumex*, an X:autosome ratio of 1.0 or higher is female; a ratio of 0.5 or lower is male (Parker and Clark 1991). In domestic hops (*Humulus* spp.) a XY system is known. XX is female while XY is male. Polyploid variants (multiple X) of hops show XXXX to be female and XXYY to be male. In the above synergid/antipodal apogamy system proposed, meiosis I could change the ratio by eliminating the X chromosome from (half) the megaspore mother cells being generated, thereby generating males (00:autosomes) and females (XX:autosomes) in a 1:1 ratio.

A third possibility is that the amplification of *EPSPS* copy number is altering gene sequences that determine gender. Since *A. palmeri* does not have a heteromorphic sex chromosome (Grant 1959a; Westergaard 1958), it is possible that glyphosate-induced *EPSPS* gene copy number amplification occurs concurrent with or adjacent to segments of the genome that cause female/maleness. Selection for high EPSPS copy number would alter the ratio of sex factor:autosome resulting in the production of male and female apomictic offspring, however, a fixed ratio of 1:1 is unlikely.

However, *A. palmeri/A. rudis* non-hybrid apomictic offsprings were all females (Trucco et al. 2007). *A. palmeri* does not have a heteromorphic sex chromosome (Grant 1959a; Westergaard 1958), and the factors that determine sex are unknown. The dioecious condition may have evolved in Amaranthus after the split of 32/34 chromosomes, and sex expression is likely under the control of one or more genes (Grant 1959b). It is possible that the sex determination factors are present in both female and male dioecious plants. Glyphosate could affect sex expression by effects on sex-modifying hormones. Environmental stress on female *A. palmeri* is known to modulate hormonal balance (abscisic acid levels) in seeds (Jha et al. 2010). Therefore, the strong selection pressure imposed by glyphosate combined with its ability to reduce of indole-3-acetic acid (IAA) levels (e.g., Lee 1984) may contribute to this phenomenon.

The level of R alleles in a population under the selection pressure of glyphosate is dependent on the mode of inheritance of the resistance trait. Facultative apomixis would maintain the presence of individuals with high levels of *EPSPS* gene copy number in *A. palmeri* populations with GR individuals, even in the presence of overwhelming amounts of GS pollen or absence of pollen donors. A deeper understanding of the mechanism of gene amplification (Gaines et al. 2013) and the regulation of apomixis and sex determination factors in *A. palmeri* populations are essential for understanding the stability of multiple *EPSPS* gene copies in populations.

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