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ORIGINAL ARTICLE

Interspecific hybridization transfers a previously unknown glyphosate resistance mechanism in *Amaranthus* species

Todd A. Gaines,[1] Sarah M. Ward,[2] Bekir Bukun,[1] Christopher Preston,[3] Jan E. Leach[1] and Philip Westra[1]

1 Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO, USA
2 Department of Soil and Crop Sciences, Colorado State University, Fort Collins, CO, USA
3 School of Agriculture, Food, and Wine, University of Adelaide, Glen Osmond, SA, Australia

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Correspondence
Todd A. Gaines, Australian Herbicide Resistance Initiative, School of Plant Biology, M086, University of Western Australia, Perth, WA 6009, Australia.
Tel.: +61 8 6488 7872;
fax: +61 8 6488 7834;
e-mail: todd.gaines@uwa.edu.au

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Abstract

A previously unknown glyphosate resistance mechanism, amplification of the 5-enolpyruvyl shikimate-3-phosphate synthase gene, was recently reported in *Amaranthus palmeri*. This evolved mechanism could introgress to other weedy *Amaranthus* species through interspecific hybridization, representing an avenue for acquisition of a novel adaptive trait. The objective of this study was to evaluate the potential for this glyphosate resistance trait to transfer via pollen from *A. palmeri* to five other weedy *Amaranthus* species (*Amaranthus hybridus*, *Amaranthus powellii*, *Amaranthus retroflexus*, *Amaranthus spinosus*, and *Amaranthus tuberculatus*). Field and greenhouse crosses were conducted using glyphosate-resistant male *A. palmeri* as pollen donors and the other *Amaranthus* species as pollen recipients. Hybridization between *A. palmeri* and *A. spinosus* occurred with frequencies in the field studies ranging from <0.01% to 0.4%, and 1.4% in greenhouse crosses. A majority of the *A. spinosus* × *A. palmeri* hybrids grown to flowering were monoecious and produced viable seed. Hybridization occurred in the field study between *A. palmeri* and *A. tuberculatus* (<0.2%), and between *A. palmeri* and *A. hybridus* (<0.01%). This is the first documentation of hybridization between *A. palmeri* and both *A. spinosus* and *A. hybridus*.

Introduction

Herbicide resistance in weeds is an excellent model system for studying applied evolutionary concepts including rapid adaptive change. Evolution of resistance to glyphosate, the world's most widely used herbicide, is a significant problem facing world agriculture (Powles 2008). A recently reported novel mechanism of glyphosate resistance involves amplification of the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) gene (Gaines et al. 2010), with resistant individuals possessing from 40-fold to more than 100-fold more copies of the EPSPS gene than susceptible individuals. If this type of rapid gene amplification is especially rare or unique to a particular species, then interspecific hybridization, a process of evolutionary significance, provides the potential for the novel trait to transfer to related species exposed to similar selection pressure (Trucco et al. 2009).

Glyphosate-resistant (GR) crops have been rapidly adopted since their introduction in 1996 and provide many economic and agronomic benefits (Dill et al. 2008). In these crops, glyphosate has essentially replaced most other weed control tactics. This extensive reliance on a single weed control tactic has resulted in intense selection pressure for any traits enabling survival. Shifts in weed species community composition have occurred (Westra et al. 2008), and glyphosate resistance in weeds is evolving rapidly (Powles and Yu 2010). Glyphosate resistance now has been reported in 21 species globally (Heap 2011).

One weed species that had been well controlled with glyphosate in GR cotton and soybean systems is *Amaranthus palmeri* S. Wats., a dioecious plant that has spread from its origin in the southwestern United States and Mexico to become a major weed pest throughout the southeastern United States (Steckel 2007). Glyphosate resistance in *A. palmeri* first occurred in GR cotton

dominated systems (Culpepper et al. 2006) where the very high level of glyphosate resistance was found to be conferred by EPSPS gene amplification, a herbicide resistance mechanism unknown prior to its discovery in *A. palmeri* (Gaines et al. 2010). Glyphosate resistance in *A. palmeri* was subsequently reported in numerous additional US GR cotton and soybean regions including Tennessee (Steckel et al. 2008), Arkansas (Norsworthy et al. 2008), North Carolina, New Mexico, Alabama, Mississippi, Missouri, and South Carolina (Heap 2011). This species previously evolved resistance to several different herbicides, including triazine, acetolactate synthase (ALS) inhibitor, and dinitroaniline herbicides (Gossett et al. 1992; Horak and Peterson 1995; Sprague et al. 1997; Vencill et al. 2008; Wise et al. 2009; Heap 2011).

A key evolutionary concept is the relative importance of acquiring a novel adaptive trait via interspecific gene transfer versus evolution of the same adaptive trait within a species. Interspecific hybridization can influence evolution if hybrid genotypes are more fit than one or both parents (Abbott 1992; Barton 2001). In the case of herbicide resistance genes, the hybrid would clearly have higher fitness when exposed to herbicide selection than the susceptible parental species. Much attention has been given to the transfer of herbicide resistance genes from crops to related weed species (e.g., Ellstrand et al. 1999; Legere 2005; Gaines et al. 2008), but far less information is available on interspecific hybridization between weedy species. Examples of interspecific hybridization in weedy species include the genera *Linaria* (Ward et al. 2009), *Avena* (Cavan et al. 1998), *Ambrosia* (Vincent and Cappadocia 1987), *Helianthus* (Natali et al. 1998), and *Conyza* (Zelaya et al. 2007). Intraspecific hybridization has been shown to transfer herbicide resistance at the commercial field scale in *Lolium* (Busi et al. 2008) and *Amaranthus* (Sosnoskie et al. 2009).

Interspecific hybridization has been investigated among most of the *Amaranthus* species (Sauer 1950), and some have even referred to the genus as 'promiscuous' (Trucco et al. 2005b). Hybridization occurs between *Amaranthus tuberculatus* and *A. hybridus*, although genetic introgression between these species occurs only in one direction, from *A. hybridus* to *A. tuberculatus* (Trucco et al. 2009). Interspecific hybridization was experimentally documented under field conditions for these two species (Trucco et al. 2005a,b). Chromosome number varies among weedy *Amaranthus* species (Table 1). Based on reported interspecific hybridizations in *Amaranthus*, equal chromosome number is not a prerequisite for hybridization. However, hybrid progeny appears to be more viable and fertile when their parental species have the same chromosome number, as in the case of hybridization between *A. hybridus* and *A. tuberculatus* (Trucco et al. 2009). Floral structure may also influence hybridization, as pollen from other species would face greater pollen competition in self-pollinating species than in dioecious species.

There is concern that glyphosate resistance owing to EPSPS gene amplification could be introgressed from GR *A. palmeri* to other weedy *Amaranthus* species (Culpepper et al. 2006). However, *A. palmeri* has not been studied for interspecific compatibility with other important weedy

Table 1. Chromosome numbers, flowering type (D, dioecious; M, monoecious), fertilization type (O, outcrossing; S, primarily selfing), and previous reports of interspecific hybridization among selected *Amaranthus* species. F_1 hybrids known to occur (Y) or unknown whether hybrids occur (U).

	Flowering type and fertilization	Chromosome number (2*n*)*	*Amaranthus palmeri*	*Amaranthus hybridus*	*Amaranthus powellii*	*Amaranthus retroflexus*	*Amaranthus spinosus*	*Amaranthus tuberculatus*
A. palmeri	D, O	34, 32†	–	U	U	U	U	Y‡,§,¶,**
A. hybridus	M, S	32		–	Y††	Y††	Y††	Y††,‡‡,§§,¶¶
A. powellii	M, S	34			–	Y††	U	Y††
A. retroflexus	M, S	34				–	Y††	Y††
A. spinosus	M, O	34					–	Y††
*A. tuberculatus****	D, O	32						–

*Reported in Grant (1959c).
†Reported in Rayburn et al. (2005).
‡Wetzel et al. (1999b).
§Trucco et al. (2007).
¶Steinau et al. (2003).
**Franssen et al. (2001b).
††Murray (1940).
‡‡Trucco et al. (2005b).
§§Tranel et al. (2002).
¶¶Trucco et al. (2005a).
****A. tuberculatus* includes former designations *Acnida tamariscina*, *Acnida tuberculata*, and *Amaranthus rudis*.

Amaranthus species, with the exception of *A. tuberculatus* (Table 1). Hybridization between *A. palmeri* and *A. tuberculatus* following controlled pollinations has been previously reported (Wetzel et al. 1999b; Franssen et al. 2001b; Steinau et al. 2003; Trucco et al. 2007).

The novel glyphosate resistance trait from *A. palmeri*, amplification of the EPSPS gene, would have adaptive potential in related weedy *Amaranthus* species under glyphosate selection pressure. The potential interspecific transfer of EPSPS gene amplification and glyphosate resistance within the genus *Amaranthus* therefore has considerable evolutionary and agronomic significance. Greater knowledge about evolutionary processes such as interspecific hybridization and the potential effects on evolution of weedy traits and herbicide resistance is essential for the future of weed management (Neve et al. 2009). Given the extensive reliance on glyphosate for control of economically damaging weedy *Amaranthus* species and the unusual nature of glyphosate resistance owing to EPSPS gene amplification in *A. palmeri*, the objective of this research was to determine whether EPSPS gene amplification and glyphosate resistance could transfer under pollen-competition conditions from *A. palmeri* to other weedy *Amaranthus* species.

Materials and methods

Field pollen-mediated gene transfer study

The globally distributed genus *Amaranthus* contains several economically important weed species (Sauer 1950). The agronomically troublesome *Amaranthus* species sympatric with *A. palmeri* in North America include *A. tuberculatus* (Moq.) Sauer [including the former designation *Amaranthus rudis* (Pratt and Clark 2001)], *A. hybridus* L., *Amaranthus retroflexus* L., *Amaranthus powellii* S. Wats., and *Amaranthus spinosus* L. (Mosyakin and Robertson 2003). Three of these species (*A. retroflexus*, *A. powellii*, and *A. hybridus*) are monoecious and are largely self-pollinated, while *A. spinosus* is monoecious and has higher levels of cross-pollination (Grant 1959b). Both *A. tuberculatus* and *A. palmeri* are dioecious (Grant 1959a). Seeds of *A. hybridus*, *A. palmeri*, *A. powellii* S. Wats. ssp. *powellii*, *A. powellii* S. Wats. ssp. *bouchonii* (Thell.) Costea & Carretero, *A. retroflexus*, *A. tuberculatus*, and *A. spinosus* were obtained from the United States Department of Agriculture – Agriculture Research Service National Plant Germplasm System North Central Regional Plant Introduction Station in Ames, IA (Table S1 for accession details). Additional accessions of *A. hybridus* and *A. tuberculatus* were obtained from Kansas State University. Seeds of glyphosate-susceptible and GR *A. palmeri* were obtained from the Ponder and Macon populations, respectively, reported in Culpepper et al. (2006).

For all accessions, 40 seeds were germinated on moistened blotter paper at 20°C in the dark for 7 day, transplanted to small pots, and then repotted after 3 weeks in 4 L pots with commercial potting soil and slow-release granular fertilizer (17-11-10). Plants were grown in a greenhouse under natural light conditions with supplemental lighting to provide a 14-h day length. Day mean temperatures were approximately 24°C, and night mean temperatures were approximately 18°C. All accessions were screened for glyphosate susceptibility by treating seedlings at the two true leaf stages with 0.4 kg a.e. ha^{-1} commercially formulated glyphosate in a pressurized spray chamber calibrated to deliver 187 L ha^{-1} at 206 kPa, on a minimum of 15 seedlings per accession. All accessions (except GR *A. palmeri*) were equally sensitive to glyphosate, with 100% mortality at 0.4 kg a.e. ha^{-1}, one-half the commercially recommended glyphosate rate for *Amaranthus* species.

In early summer (June) 2006 and 2007, potted plants of each species were moved to a field site in Colorado spatially isolated by 15 km from agronomic crop production fields containing *Amaranthus* weed species. The plot was established prior to the plants initiating flowering. A central pollen-donor row was established in a north–south orientation consisting of GR *A. palmeri* with unknown EPSPS copy number, but which had been classified as highly GR based on the leaf disk shikimate accumulation assay described by Shaner et al. (2005). Upon flowering initiation, female *A. palmeri* were removed from the central row to leave 20 male GR *A. palmeri* plants as pollen donors. Parallel rows of potted plants were placed 1 and 3 m on both sides from the pollen-donor row, with each row containing two glyphosate-susceptible plants of each species for eight plants of each species total. The continuous flowering period of the male *A. palmeri* plants permitted overlap with anthesis of all the recipient plants. All the related species in the study are capable of self-pollination, with the exception of *A. tuberculatus*. Any male *A. tuberculatus* were removed upon the identification of floral structure, leaving two female *A. tuberculatus* in each pollen-recipient row. Two glyphosate-susceptible female *A. palmeri* were included in each parallel recipient row to measure intraspecific pollination. Plants within each 10-m row were spaced 0.5 m apart and maintained by watering daily. The plot area was maintained by mowing and hand weeding to eliminate weed competition.

Plants were harvested in the autumn after seed maturation, and seeds were manually threshed from the plants. Seed biomass was recorded for each plant, and seeds were stored in sealed plastic bags at 4°C until use. Seeds were counted from a 0.1-g sample from each plant, and the number of seeds per plant was estimated from total seed weight. The 2006 field experiment was repeated in 2007 at the same location.

Screening for hybrids from field study

The glyphosate resistance trait from *A. palmeri* was used as a phenotypic marker to screen progeny from the glyphosate-susceptible *Amaranthus* species for GR hybrids. Each seed sample was screened for GR hybrids in the greenhouse during the winter following each summer field season. Five replications, each consisting of 1 g of seed (about 1000 seeds), were used for each sample, and the experiment was repeated. Seeds were spread on moistened potting soil in plastic germination boxes and covered with a thin layer of potting soil. Sealed boxes were placed in a 4°C cold room for 7 day. The boxes were then transferred to germination chambers for two cycles of the following temperate regime: 18°C for 6 h, 30°C for 6 h, 42°C for 6 h, and 30°C for 6 h, along with 18-h light. Germinated seedlings were spread onto moistened potting soil in 25 by 50 cm flats, covered with an additional 0.5 cm of soil, placed in a greenhouse, and watered daily. Liquid fertilizer (24-8-16) was applied once after emergence.

Owing to the large number (typically more than 500) of emerged plants in each flat, the total number of emerged plants was estimated by counting plants in two 1.5-cm-wide strips along the length of each flat. Percent emergence was calculated by dividing the total number of emerged plants by the estimated seed number planted. Seedlings at two true leaves were treated with 0.4 kg a.e. ha^{-1} glyphosate in a pressurized spray chamber calibrated to deliver 187 L ha^{-1} at 206 kPa, a dose previously determined to be lethal to 100% of susceptible but 0% of resistant plants. Plants were evaluated for survival 14 day after treatment, and surviving plants were considered putative hybrids.

Putative hybrids were transplanted into individual pots and grown to maturity for scoring of floral phenotype. Leaf tissue from these plants was sampled for DNA extraction as described below. All monoecious putative hybrids were covered with pollinating bags to exclude foreign pollen and determine whether seed would be produced from self-pollination. Progeny produced from self-pollinated monoecious hybrids were subsequently germinated and treated as previously described with 0.4 kg a.e. ha^{-1} glyphosate, to test whether glyphosate resistance was inherited in the self-pollinated progeny.

Greenhouse crosses

Greenhouse crosses were made between GR male *A. palmeri* and the five *Amaranthus* species used in the field pollination study. All *A. palmeri* plants used as pollen donors were confirmed as GR using an *in vivo* leaf disk method (Shaner et al. 2005). The small, closely grouped flowers of the monoecious *Amaranthus* species make emasculation extremely difficult (Murray 1940). Because pistillate flowers in monoecious *Amaranthus* species emerge and are receptive to pollen several days prior to the opening of staminate flowers (Murray 1940), crosses were attempted by applying GR *A. palmeri* pollen to four susceptible recipient plants of each of the five other *Amaranthus* species from the first pistillate flower emergence and daily thereafter for 2 weeks. Pollen was applied by shaking intact *A. palmeri* plants actively shedding pollen directly on the inflorescences of the other species, and no other method was used to reduce self-pollination in the monoecious species. Only female plants were used for the dioecious species, *A. tuberculatus*.

Screening for hybrid progeny from greenhouse crosses

Seeds (5 g per plant) from the five species pollinated by male *A. palmeri* were germinated and screened for glyphosate resistance as previously described for seeds from the field study. Leaf tissue was sampled from each surviving putative hybrid plant for DNA extraction as described below. Flowering phenotype was scored, and any monoecious hybrids were covered with pollination bags upon flowering initiation to ensure self-pollination. Dioecious hybrids were not able to be self-pollinated. Seed produced from putative hybrid plants was germinated and treated with 0.4 kg a.e. ha^{-1} glyphosate as a progeny test for whether glyphosate resistance was inherited in the progeny of self-pollinated hybrid plants.

Verification of putative hybrids from field study and greenhouse crosses

All putative hybrid plants that survived glyphosate application were further screened using species-diagnostic molecular markers, with the exception of *A. spinosus* hybrids. Owing to the large number of putative *A. spinosus* hybrids obtained, three putative *A. spinosus* × *A. palmeri* hybrids from the 2007 field study and four putative hybrids from greenhouse crosses were selected for marker-based screening. Genomic DNA of putative hybrids was extracted from leaf tissue using a modified CTAB (Hexadecyl trimethylammonium bromide) method (Murray and Thompson 1980). The internal transcribed spacer (ITS) region was amplified and digested using the PCR–RFLP method of Wetzel et al. (1999a) to generate distinguishing ncDNA polymorphisms between *A. palmeri* and *A. hybridus*, *A. powellii*, *A. retroflexus*, and *A. spinosus*; aliquots of the amplified ITS PCR product were digested with *Bsa*AI, *Hae*II, and/or *Xho*I, depending on the species. The presence of ITS restriction polymorphisms from both parental species was evaluated in putative hybrids between *A. palmeri* and those four species. A different marker system able to clearly distinguish between the ALS

gene from *A. palmeri* and *A. tuberculatus* was used to verify putative hybrids between these two species using the method of Tranel et al. (2002). A partial ALS gene fragment was amplified with forward primer 5′-GCTGCTGAAGGCTACGCT-3′ and reverse primer 5′-CACTTCTTGACTCAGTCCCGC-3′ and digested with *Eco*RV to confirm the presence of ALS alleles from *A. tuberculatus* (contains an *Eco*RV restriction site) and *A. palmeri* (lacks restriction site) (Tranel et al. 2002). Digested and undigested ITS and ALS amplicons were analyzed on ethidium bromide-stained gels.

Determination of glyphosate resistance transfer

Amplification of the EPSPS gene was used to verify that putative hybrids had inherited the glyphosate resistance trait. Quantitative PCR was used to measure genomic EPSPS copy, as described by Gaines et al. (2010); in this study, glyphosate-susceptible *A. palmeri* had one EPSPS copy relative to ALS, and GR *A. palmeri* had from 40- to more than 100-fold more EPSPS copies relative to ALS. We hypothesized that GR hybrids between GR *A. palmeri* and other *Amaranthus* species would inherit additional copies of the EPSPS gene. Additionally, putative hybrids were further tested for glyphosate resistance using an *in vivo* leaf disk shikimate assay (Shaner et al. 2005).

Data analysis

Seed production data were analyzed using ANOVA and means compared using an adjustment for multiple comparisons (SAS 2004). Means and standard errors were calculated for percent emergence, percent confirmed hybrids, and percent survival of hybrid F_2 progeny. Emergence data were analyzed using chi-square tests, and 95% confidence intervals were calculated for percent confirmed hybrid data. Seedling emergence rates of 20% (field) or 1% (greenhouse crosses) were tested, and these rates were chosen based on model sensitivity analysis and literature reviewed by Neve et al. (2011). Percent survival of hybrid F_2 progeny was analyzed using chi-square tests for 75% survival, based on previously observed 75% survival at 400 g a.e. ha^{-1} glyphosate in F_2 *A. palmeri* from a resistant by susceptible cross (Gaines et al. 2011).

Chromosome counts

Chromosome counts of $2n = 34$ (Grant 1959c) and $2n = 32$ (Rayburn et al. 2005) have been reported for *A. palmeri*. To ascertain the chromosome number for the *A. palmeri* plants used in this study, actively growing root tips from greenhouse-grown plants were collected and pretreated in 0.05% aqueous colchicine for 4 h at room temperature. The root tips were then fixed for 24 h in a 3:1 mixture of absolute ethanol and glacial acetic acid, hydrolyzed for 6 min in 1 N hydrochloric acid at 60°C, and stained in Feulgen's solution (Jones 1947) for 45 min in the dark. The stained portion of the root tip was excised in 45% acetic acid and squashed on a glass slide in a drop of acetocarmine solution. Chromosome counts were recorded for a minimum of two mitotic spreads from each of five plants from the *A. palmeri* population identified as GR by Culpepper et al. (2006).

Results

Field pollen-mediated gene transfer study

Male *A. palmeri* plants shed pollen during the entire experimental period (approximately 12 weeks) in both years, enabling temporal overlap in pollination with all other species. The accessions grown in 2006 and 2007 varied in seed production (Table S1), although there was no particular trend across accessions for any one species to produce the most seed.

More than 400 000 progeny in total were screened for glyphosate resistance from all *Amaranthus* species in both years of the field study. GR plants were found in progeny of susceptible female *A. palmeri* (Table 2), indicating that pollination was sufficient for intraspecific gene transfer up to 3-m distance. We did not expect 100% resistance in the progeny of susceptible *A. palmeri* in this study because inheritance of the gene amplification does not follow a predictable Mendelian pattern: F_1 progeny of resistant by susceptible *A. palmeri* crosses inherit a range of EPSPS copy numbers (Gaines et al. 2011). Therefore, the detection of glyphosate resistance transferred from *A. palmeri* to progeny of other *Amaranthus* species reported here does not indicate absolute hybridization rates, but pollen-mediated transfer of a sufficient number of EPSPS gene copies to confer glyphosate resistance. Seedling emergence rates ranged from 20% to 96%, and all species exceeded the expected 20% emergence rate with the exception of *A. tuberculatus*, which had emergence much lower than expected (Table 2). High seed production was observed on *A. tuberculatus* (Table S1), but these seeds were not measured for viability, so it is possible that nonviable or aborted seeds were counted as part of the total seed production. In contrast with the other species that had self-pollination available, no *A. tuberculatus* pollen was present at the study site and this may explain the very low seedling emergence for this species.

Amaranthus spinosus had the highest hybridization rates of the species tested, averaging <0.01% (accession 8 in 2006) and 0.4% (accession 9 in 2007) (Table 2). Seven of eight *A. spinosus* plants in 2007 and two of eight in 2006 produced resistant hybrid offspring. Hybridization

Table 2. Hybridization in field (F) and greenhouse (GH) studies between a glyphosate-resistant *Amaranthus palmeri* pollen source and related *Amaranthus* species. Glyphosate treatment was used to identify hybrid plants expressing glyphosate resistance inherited from *A. palmeri*. Emerged plants were screened with glyphosate, and molecular markers were used to confirm hybrid plants.

Maternal parent	Study	Total emerged progeny screened	Mean % emergence ± SE	χ^2 test for expected emergence (20% F, 1% GH)	Mean % hybrids ± SE	Total hybrids	95% confidence interval (% hybrids)
1-*A. palmeri*	F	21 800	36 ± 6	3488; $P < 0.0001$	27.90 ± 2.80	–	(22.4, 33.4)
2-*A. palmeri*	F	3700	32 ± 6	333; $P < 0.0001$	21.10 ± 2.46	–	(16.3, 25.9)
3-*Amaranthus hybridus*	F	91 450	40 ± 3	22862; $P < 0.0001$	0.008 ± 0.006	2	(−0.004, 0.02)
	GH	462	5.2 ± 2	1561; $P < 0.0001$	0.0	0	
4-*A. hybridus*	F	52 900	41 ± 4	14580; $P < 0.0001$	0.0	0	
	GH	12	0.1 ± 0.2	72; $P < 0.0001$	0.0	0	
5-*Amaranthus powellii* ssp. *bouchonii*	F	8700	20 ± 2	0; n.s.	0.0	0	
6-*A. powellii* ssp. *powellii*	F	44 150	24 ± 2	441; $P < 0.0001$	0.0	0	
	GH	33	0.4 ± 0.2	39; $P < 0.0001$	0.0	0	
7-*Amaranthus retroflexus*	F	65 200	39 ± 4	14710, $P < 0.0001$	0.0	0	
8-*Amaranthus spinosus*	F	60 400	96 ± 9	218 044; $P < 0.0001$	0.005 ± 0.004	2	(−0.003, 0.01)
9-*A. spinosus*	F	42 350	27 ± 2	1296; $P < 0.0001$	0.43 ± 0.07	211*	(0.29, 0.57)
	GH	533	7.9 ± 4	3266; $P < 0.0001$	1.44 ± 0.86	21*	(−0.25, 3.13)
10-*Amaranthus tuberculatus*	F	3350	3 ± 1	604; $P < 0.0001$	0.079 ± 0.059	2	(−0.04, 0.19)
	GH	1	0.02 ± 0.05	43; $P < 0.0001$	0.0	0	
11-*A. tuberculatus*	F	3400	8 ± 2	306; $P < 0.0001$	0.19 ± 0.13	4	(−0.06, 0.44)
	GH	1	0.01 ± 0.03	69; $P < 0.0001$	0.0	0	
12-*A. tuberculatus*	F	5550	17 ± 4	31; $P < 0.0001$	0.0	0	

*Subset of surviving plants tested to confirm hybrid identity.

occurred at 1- and 3-m distance from the pollen source (Table 3). All *A. spinosus* × *A. palmeri* hybrids examined were confirmed as having increased EPSPS gene copy number (Table 3), ITS alleles from both parents, and were classified GR using the *in vivo* leaf disk shikimate assay. The EPSPS gene copy number in these hybrids from the field study ranged from 19 to 64 (Table 3), consistent with the number of EPSPS gene copies required to confer resistance in *A. palmeri* F_1 progeny (Gaines et al. 2011). These hybrids were either monoecious or androecious (Table 3), and their morphological phenotype was intermediate to the two parents. Most had spines at the base of leaf petioles and an intermediate leaf shape; some had long petioles similar to *A. palmeri* and others had short leaf petioles similar to *A. spinosus*. Floral structure arrangement in the monoecious hybrids was similar to *A. spinosus* as staminate flowers were distal to pistillate flowers on an inflorescence. Monoecious *A. spinosus* × *A. palmeri* hybrids were self-fertile. Progeny produced from seven of these monoecious hybrid plants had survival rates following glyphosate treatment ranging from 48% to 90% (Table 3), generally consistent with the expected survival rate of 75%, indicating that the EPSPS gene amplification glyphosate resistance mechanism functions in an *A. spinosus* genetic background.

Glyphosate-resistant hybrids were also confirmed with *A. hybridus* at a rate of <0.01% and in two *A. tuberculatus* accessions at 0.08% and 0.19% (Table 2). All of these hybrids occurred in 2007, with four of 16 *A. tuberculatus* female plants and two of eight *A. hybridus* plants having resistant hybrid offspring, and hybridization occurring at 1- and 3-m distance from the pollen source (Table 3). Hybrids with *A. hybridus* were confirmed using EPSPS copy number and ITS alleles, and these hybrids had a similar morphological appearance to *A. palmeri*. Hybrids with *A. tuberculatus* were confirmed using EPSPS copy number and ALS alleles (Table 3), and these hybrids were morphologically intermediate between *A. palmeri* and *A. tuberculatus*. All hybrids were classified GR using the *in vivo* leaf disk shikimate assay. GR hybrids with *A. hybridus* and *A. tuberculatus* had a range of EPSPS copy numbers from 22 to 122 (Table 3). Hybrids of *A. palmeri* with *A. tuberculatus* and *A. hybridus* were dioecious and were not backcrossed to the susceptible recurrent parent, so their fertility levels are unknown. However, reduced viability and fertility of *A. tuberculatus* × *A. palmeri* hybrids has been reported previously (Wetzel et al. 1999b; Franssen et al. 2001b; Steinau et al. 2003; Trucco et al. 2007).

Greenhouse crosses

Hybridization was detected in a greenhouse cross between maternal *A. spinosus* and paternal *A. palmeri* (Table 2) at a rate of 1.4%. This rate was higher than the field study, possibly due in part to the ability to directly pollinate pistillate

Table 3. Confirmation of hybrids between *Amaranthus palmeri* and related *Amaranthus* species from field and greenhouse studies using 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) relative copy number, flowering phenotype, and glyphosate resistance in progeny of self-pollinated hybrids.

Maternal parent	Year*	Distance from pollen source (m)	EPSPS copy no.†	Hybrid plant flowering phenotype	No. progeny tested	Mean % resistance in progeny ± SE‡	χ^2 for 75% survival
3-*Amaranthus hybridus*	2007	1	104	A		n/a	
	2007	3	67	G		No seed	
8-*Amaranthus spinosus*	2006	1	64	M		Not tested	
	2006	1	22	A		n/a	
9-*A. spinosus*	2007	3	40	M	49	70 ± 16	0.17; n.s.
	2007	3	49	M		Not tested	
	2007	3	19	A		n/a	
	2007	3		M	24	61 ± 15	0.16; n.s.
	2007	1		M	50	94 ± 4	7.7; $P = 0.006$
	2007	1		M	8	50 ± 0	2.7; n.s.
	2007	1		M	49	95 ± 3	11; $P = 0.0007$
	2007	1		M	14	92 ± 8	0.9; n.s.
	2007	3		M	20	48 ± 16	17; $P < 0.0001$
	GH		29	M	14	78 ± 22	0.86; n.s.
	GH		34	A		n/a	
	GH		77	A		n/a	
	GH		67	M	10	83 ± 17	1.2; n.s.
10-*Amaranthus tuberculatus*	2007	1	99	A		n/a	
	2007	1	58	A		n/a	
11-*A. tuberculatus*	2007	1	79	A		n/a	
	2007	1	58	G		No seed	
	2007	3	122	A		n/a	
	2007	3	70	A		n/a	

G, gynoecious; A, androecious; M, monoecious.

*Year of field study or GH, greenhouse study; underlined within a species indicates hybrids originated from the same maternal plant.

†EPSPS copy number measured relative to acetolactate synthase copy number using quantitative PCR on genomic DNA.

‡If confirmed hybrid produced seed through self-pollination, mean of three replications following 0.4 kg a.e. ha^{-1} glyphosate application

A. spinosus flowers with *A. palmeri* pollen. Hybrids from the cross with *A. spinosus* were confirmed with EPSPS copy number (Table 3) and ITS alleles, and the hybrids showed the same variation for flowering phenotype observed in hybrids from the field trial, with both androecious and monoecious individuals (Table 3). No confirmed hybrids were found in greenhouse crosses with the other *Amaranthus* species tested. This difference in hybridization rates between the field and greenhouse environments may be due to the smaller number of individuals included in the greenhouse study, lower seed production on plants in the greenhouse, and the relatively lower probability of obtaining hybrids for the other species compared to the *A. spinosus* × *A. palmeri* cross. Other environmental differences between the two studies, including wind, humidity, and insect activity, all may have contributed to higher observed hybridization rates in the field study.

Seedling emergence from seed produced in greenhouse crosses between *A. palmeri* and other *Amaranthus* species was less than the expected 1% (Table 2), possibly owing to different dormancy conditions generated in the greenhouse growing environment. Emergence of *A. tuberculatus* seed was especially low, similar to observations from the field study (Table 2), indicating that most of the *A. tuberculatus* seed produced was likely not viable.

Chromosome counts of *Amaranthus palmeri*

At least two spreads in each of five plants from the GR population contained 34 chromosomes, indicating that the *A. palmeri* plants used in this study were $2n = 34$, as reported by Grant (1959c). As Rayburn et al. (2005) reported *A. palmeri* counts of $2n = 32$ and Pal et al. (1982) reported that, within the *Amaranthus* genus, both $n = 16$ and $n = 17$ occasionally occur in the same species, it is possible that more than one cytotype exists within *A. palmeri*, but our GR population appears to have $2n = 34$.

Discussion

Species in the *Amaranthus* genus are consistently listed among the top 10 most troublesome weeds in soybeans

and cotton in the southeast United States (Webster 2009). Of particular concern for glyphosate resistance gene transfer is *A. spinosus*, a troublesome species in Asian rice production (Chauhan and Johnson 2009) and in South America (de Carvalho and Christoffoleti 2008), a region where GR soybeans are extensively cultivated and where the transfer of glyphosate resistance to *A. spinosus* would cause extensive economic harm. We have demonstrated interspecific hybridization between *A. spinosus* and *A. palmeri*, a previously unknown hybrid combination of considerable evolutionary and agronomic significance. Given that *A. palmeri* shares geographic habitat range in the United States with *A. spinosus* and hybridization between *A. palmeri* and *A. spinosus* appears to have limited pre- or postzygotic barriers (given the self-fertility of the monoecious *A. spinosus* × *A. palmeri* F_1 hybrids), the novel adaptive trait of EPSPS gene amplification may transfer into a species in which this trait may not otherwise evolve. Further work is needed to examine the dynamics of EPSPS gene amplification introgression into the *A. spinosus* genome via repeated backcrossing. Persistence of the *A. spinosus* × *A. palmeri* hybrids will also depend on their fecundity and other fitness components, and further work is needed to characterize the seed production on GR *A. spinosus* × *A. palmeri* hybrids.

Amaranthus palmeri and *A. spinosus* may share a more recent common ancestor than the other *Amaranthus* species we tested, as they have the same chromosome number ($2n = 34$), pollen morphological similarities (Franssen et al. 2001a), and similar genome sizes (Rayburn et al. 2005). Additionally, *A. palmeri* and *A. spinosus* have been assigned as sister taxa in phylogenetic analyses in which all other taxa from our study were included, indicating the other species are more distantly related to both *A. palmeri* and *A. spinosus* (Wassom and Tranel 2005; Riggins et al. 2010). Greater genetic introgression of adaptive traits from *A. palmeri* to *A. spinosus* may also occur because *A. spinosus* is cross-pollinated and subsequent gene flow between populations may occur more rapidly than for the primarily self-pollinated *Amaranthus* species.

We have also documented transfer of the EPSPS gene amplification and glyphosate resistance from *A. palmeri* to both *A. hybridus* and *A. tuberculatus*. The *A. hybridus* × *A. palmeri* event has not been previously reported. Evolved glyphosate resistance has been reported in some populations of *A. tuberculatus* (Legleiter and Bradley 2008), but the molecular basis of this resistance is not known. Resistance to ALS-inhibiting herbicides was previously shown to transfer from *A. palmeri* to *A. tuberculatus* via interspecific hybridization (Franssen et al. 2001b). Subsequent genetic introgression from *A. palmeri* to *A. tuberculatus* may be limited, as Trucco et al. (2007) established that interspecific hybrids had developmental problems and died before flowering or did not produce flowers. The potential for genetic introgression from *A. palmeri* to *A. hybridus* is unknown and warrants further investigation. Interspecific hybridization with *A. retroflexus* and *A. powellii* was not demonstrated experimentally in our study.

5-enolpyruvyl shikimate-3-phosphate synthase gene amplification in *A. palmeri* may be associated with a mobile genetic element (Gaines et al. 2010); such transposons are known to proliferate following interspecific hybridization (Kawakami et al. 2010). At present, we do not know whether pollination of *A. spinosus*, *A. hybridus*, and *A. tuberculatus* by *A. palmeri* simply transfers additional EPSPS gene copies of paternal origin to the hybrid offspring. An alternative mechanism could be that interspecific pollination by *A. palmeri* stimulates EPSPS gene amplification in hybrid progeny, possibly by transposon transfer or activation. Some of the observed hybrids had EPSPS gene copy numbers higher than 100 (Table 3), suggesting that further gene amplification may have occurred following hybridization. Further investigation is needed to clarify whether mobile genetic elements are involved in EPSPS gene amplification in *A. palmeri*, and to determine the evolutionary consequences of transferring a highly active mobile genetic element from *A. palmeri* into related *Amaranthus* species.

The novel form of glyphosate resistance owing to EPSPS gene amplification that has evolved in *A. palmeri* is transmissible via pollen-mediated gene flow to other weedy *Amaranthus* species, particularly *A. spinosus*. The observed hybridization rates were higher in the field study, where the environmental conditions more accurately reflect conditions that would occur in field sites where *A. palmeri* is present with other *Amaranthus* species. Thus, interspecific hybridization may be expected to occur in field conditions and represents a potential avenue of evolutionary adaptive change for related species currently lacking this novel resistance mechanism, with considerable agronomic relevance for managing *Amaranthus* weeds. Evolved glyphosate resistance in *A. palmeri* is widespread, and the potential exists for the rapid selection and spread of glyphosate resistance in other troublesome *Amaranthus* species via interspecific hybridization with *A. palmeri*. In the case of hybrids with *A. spinosus*, GR hybrid progeny are self-fertile and their progeny are viable and GR. Both *A. tuberculatus* and *A. hybridus* provide an evolutionary linkage for the *A. hybridus*/*A. powellii*/*A. retroflexus* complex (Franssen et al. 2001b; Trucco et al. 2007) to acquire glyphosate resistance via interspecific hybridization from *A. palmeri* prior to glyphosate resistance evolution within those species. Despite intensive glyphosate use on *Amaranthus* species, only *A. tuberculatus*

and *A. palmeri* have evolved glyphosate resistance (Culpepper et al. 2006; Legleiter and Bradley 2008; Norsworthy et al. 2008; Steckel et al. 2008), so interspecific hybridization is a potential evolutionary route for glyphosate resistance to occur in related *Amaranthus* species more rapidly than by independent evolution within each species. The agronomic implications of this are that the major troublesome *Amaranthus* weed species would not be manageable with glyphosate, and the evolutionary implications are the potential transfer of adaptive traits among related *Amaranthus* species (Trucco et al. 2009). Additional work is needed to analyze the fitness components and persistence of the GR hybrid progeny reported in this study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article

Table S1. Origin and United States Department of Agriculture – Agriculture Research Service National Plant Germplasm System plant introduction (USDA-ARS NPGS PI) numbers of accessions used in hybridization studies.

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