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Transfer and Expression of ALS Inhibitor Resistance from Palmer Amaranth (*Amaranthus palmeri*) to an *A. spinosus* × *A. palmeri* Hybrid

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Transfer of herbicide resistance among closely related weed species is a topic of growing concern. A spiny amaranth × Palmer amaranth hybrid was confirmed resistant to several acetolactate synthase (ALS) inhibitors including imazethapyr, nicosulfuron, pyrithiobac, and trifloxysulfuron. Enzyme assays indicated that the ALS enzyme was insensitive to pyrithiobac and sequencing revealed the presence of a known resistance conferring point mutation, Trp574Leu. Alignment of the ALS gene for Palmer amaranth, spiny amaranth, and putative hybrids revealed the presence of Palmer amaranth ALS sequence in the hybrids rather than spiny amaranth ALS sequences. In addition, sequence upstream of the ALS in the hybrids matched Palmer amaranth and not spiny amaranth. The potential for transfer of ALS inhibitor resistance by hybridization has been demonstrated in the greenhouse and in field experiments. This is the first report of gene transfer for ALS inhibitor resistance documented to occur in the field without artificial/human intervention. These results highlight the need to control related species in both field and surrounding noncrop areas to avoid interspecific transfer of resistance genes.

Nomenclature: Imazethapyr; nicosulfuron; pyrithiobac; trifloxysulfuron; Palmer amaranth *Amaranthus palmeri* S. Wats.; spiny amaranth (*Amaranthus spinosus* L.).

Key words: Acetolactate synthase, ALS enzyme, cross resistance, hybrid, mutation.

Glyphosate, a nonselective, broad spectrum, systemic, POST herbicide has been used extensively throughout the world in both crop and noncrop lands since its commercialization in 1974. With the introduction of glyphosate-resistant (GR) crops in the mid 1990s, glyphosate was used selectively and predominantly for weed control in GR crops without concern for crop injury. The widespread adoption of GR crops around the world has led to overuse of the herbicide and reduced crop rotation, which resulted in the evolution of several GR weed biotypes. As of August 2015, GR populations have been reported for 32 weed species worldwide (Heap 2015), including an *Amaranthus spinosus* × *A. palmeri* hybrid (Nandula et al. 2014).

Interspecific hybridization between weed species belonging to the *Amaranthus* genus with transfer of herbicide resistance has been well documented. For example, hybridization of Palmer amaranth has been reported with smooth pigweed (*Amaranthus hybridus* L.) and spiny amaranth (Gaines et al. 2011), and tall waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] (Franssen et al. 2001). Also, tall waterhemp has been shown to cross with smooth pigweed (Trucco et al. 2005a,b, 2009) in addition to Palmer amaranth. Except in the work by Gaines et al. (2011), who used glyphosate resistance, ALS inhibitor resistance from one of the parent species was used as a marker to track hybridization.

Use of ALS inhibitors to control weeds is widespread. Examples of ALS inhibitors include pyrithiobac, imazaquin, and trifloxysulfuron, and nicosulfuron, which belong to pyrimidinylthiobenzoate, imidazolinone, and sulfonylurea herbicide families, respectively (Shaner 2014) and represent traditional POST weed control options in the major crops of the Midsouth, corn (nicosulfuron), cotton (pyrithiobac and trifloxysulfuron), and soybean (imazaquin) (Mississippi State University 2015). These herbicides, according to labels, control both Palmer amaranth and spiny amaranth. Their loss, in combination with the increase in glyphosate resistance, would seriously hamper control efforts.

A recently discovered glyphosate resistance mechanism, amplification of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene (Gaines et al. 2010), in GR Palmer amaranth has been shown to introgress into spiny amaranth (Gaines et al. 2011) under controlled greenhouse and field experimental conditions. A naturally occurring GR spiny amaranth population in Mississippi was shown to have an increased copy number of EPSPS (Nandula

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et al. 2014), with evidence pointing towards a likely interspecific hybridization event between spiny amaranth and GR Palmer amaranth. In addition to resistance to glyphosate, one of the Mississippi GR spiny amaranth accessions displayed resistance to ALS-inhibiting herbicides, based on preliminary herbicide treatments (data not shown). Resistance to multiple herbicides, such as glyphosate and ALS inhibitors, has been documented in Palmer amaranth (Nandula et al. 2012; Sosnoskie et al. 2011).

The objective of this research was to confirm and characterize the nature and origin of resistance to ALS inhibitors in a field population of spiny amaranth (a putative hybrid between spiny amaranth and Palmer amaranth) collected in 2013, nearly 2 yr after the original plant/seed of GR spiny amaranth (Nandula et al. 2014) was collected. Whole plant dose-response experiments with multiple ALS inhibitors, ALS enzyme assays with pyrithiobac, and molecular sequence analysis of ALS gene constructs were conducted to address resistance in these hybrids and Palmer amaranth.

Materials and Methods

Seed Collection, Storage, Germination, Planting, Growth, and Herbicide Treatment Conditions. In the summer of 2013, spiny amaranth plants, hereafter referred to as SA-R, were collected from the same field near Water Valley, MS, where the original GR spiny amaranth plants were documented (Nandula et al. 2014). Spiny amaranth plants were collected along with roots and transferred to 10-L pots containing field soil and allowed to grow indefinitely (*Amaranthus* species such as Palmer amaranth, spiny amaranth, etc. can be regenerated by cloning; see below) in a greenhouse set to 25/20 C day/night, 12-h photoperiod under natural sunlight conditions supplemented with high pressure sodium lights providing 400 mmol m^{-2} s^{-1} of light intensity. All greenhouse studies were conducted under these growing conditions.

Clones of spiny amaranth plants were generated following a modified cloning procedure by Teaster and Hoagland (2014). Briefly, 2.5- to 5-cm long lateral/axillary stem segments were excised from an actively growing field-collected plant and immediately placed in a paper towel wetted with distilled water. The cut end of each segment was dipped in a thin layer of rooting hormone powder (1.6%; Hormex; Brooker Chemicals, Hollywood, CA, USA) and planted in 6-cm by 6-cm by 6-cm pots containing a thoroughly wetted commercial potting mix (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA, USA). Root initiation occurred within 1 wk. The segments were allowed to grow at least 10 cm before transplanting to a larger 4-L pot containing the above-mentioned potting mix. This procedure provided a supply of cloned plants for various research studies described herein.

Seeds of wild type/susceptible spiny amaranth (SA-S) and wild type/susceptible Palmer amaranth (PA-S), and ALS inhibitor-resistant Palmer amaranth (PA-R) biotypes 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). Two weeks after emergence, SA-S, PA-S, and PA-R seedlings were transplanted into 6-cm by 6-cm by 6-cm pots containing the potting mix. Trays and pots were maintained in a greenhouse set to conditions described previously. Plants were fertilized once with a nutrient solution (Miracle-Gro, The Scotts Company LLC, Marysville, OH, USA) containing 200 mg L^{-1} each of N, P_2O_5, and K_2O 1 wk after transplanting and subirrigated as needed, thereafter. All herbicide treatments were applied with a moving nozzle sprayer equipped with 8002E nozzles (Spraying Systems Co., Wheaton, IL, USA) delivering 140 L ha^{-1} at 280 kPa to SA-R, SA-S, PA-S, and PA-R plants that were 10-cm tall and at the four- to six-leaf stage. Above ground shoot tissue was collected 3 wk after treatment, dried in an oven at 60 C for 72 to 96 h, and weighed. Dry shoot weights are expressed in terms of percent of nontreated control (no herbicide check). All studies were conducted from 2014 to 2015 at the Jamie Whitten Delta States Research Center of US Department of Agriculture–Agricultural Research Service in Stoneville, MS.

SA-S was selected from seed collections originating from North Carolina and obtained from the National Plant Germplasm System (PI 632248, North Central Regional Plant Introduction Station, Iowa State University, Ames, IA, USA). This biotype represents spiny amaranth from years prior to the commercialization of GR crops (pre-1996). PA-S was a previously characterized (susceptible to all commercial herbicide chemistries labeled for POST Palmer amaranth control; Charles Bryson, personal communication) biotype accession from Washington County, MS. PA-R was from seed collected from a single resistant Palmer amaranth plant from Washington County, MS.

Herbicide Dose Response. Plants of SA-R and PA-R were treated with pyrithiobac (0, 0.11, 0.21, 0.43,

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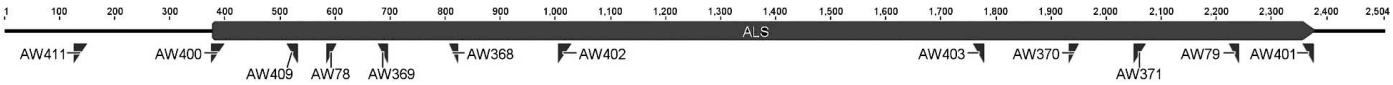

Figure 1. Map of the primer binding sites used for PCR relative to ALS gene sequence.

0.85, and 1.7 kg ai ha^{-1}), imazaquin (0, 0.14, 0.28, 0.56, 1.12, and 2.24 kg ai ha^{-1}), trifloxysulfuron (0, 0.008, 0.015, 0.031, 0.062, and 0.12 kg ai ha^{-1}), and nicosulfuron (0, 0.034, 0.07, 0.13, 0.27, and 0.54 kg ai ha^{-1}). SA-S and PA-S plants were also treated with the same herbicides, but at the following rates: pyrithiobac (0, 0.002, 0.007, 0.03, 0.11, and 0.43 kg ha^{-1}), imazaquin (0, 0.002, 0.009, 0.04, 0.14, and 0.56 kg ha^{-1}), trifloxysulfuron (0, 0.0001, 0.0005, 0.002, 0.008, and 0.031 kg ha^{-1}), and nicosulfuron (0, 0.001, 0.002, 0.008, 0.034, 0.13 kg ha^{-1}). The respective rates of the various herbicides used represent 0, 1X, 2X, 4X, 8X, and 16X for SA-R and PA-R and 0, 1/64 X, 1/16 X, 1/4 X, 1X, and 4X for SA-S and PA-S biotypes. A nonionic surfactant (Induce, Helena Chemical Co., Collierville, TN, USA) was included with all herbicide treatments at 1% v/v. There were three replications per treatment and all experiments were repeated once.

ALS Assay. SA-R, SA-S, PA-R, and PA-S plants were grown as described before. ALS enzyme activity from four- to six-leaf plants was assayed in vitro using procedures similar to previous descriptions (Nandula and Messersmith 2000; Ray 1984). Pyrithiobac (Dupont Crop Protection, Newark, DE, USA) concentrations used were 0, 0.1, 1, 10, 100, and 1,000 μM. This assay measured acetoin concentration that was formed from acid decarboxylation of acetolactate. Background acetoin sources were included as controls. There were three replications per treatment per biotype and the experiments were repeated once.

***ALS* Sequence Analysis.** DNA was extracted from leaf tissue by homogenization in a mortar and pestle with an extraction buffer as described by Paterson et al. (1993). Following homogenization, the lysate was transferred to a 1.5-mL microcentrifuge tube and incubated at 65 C for 1 h. After incubation, the cellular debris was collected by centrifugation at 13,000 rpm for 4 min and 266 μL of the supernatant was transferred to a 1.5-mL microcentrifuge tube. From this point, the DNEasy plant mini kit (Qiagen, Valencia, CA, USA) was used to finish the DNA extraction. Quantity of DNA was assessed by an A_{260} reading, and the quality was determined by an A_{260}/A_{280} ratio and by agarose gel electrophoresis. A portion of the ALS gene was amplified with primers AW78 and AW79 (designed based on the tall waterhemp ALS sequence, accession number EF157821; Table 1; Figure 1) using the Takara LA PCR kit V2.1 (Clontech, Mountain View, CA, USA). Reactions consisted of ~50 ng DNA, 200 nM primers, 2.5 mM $MgCl_2$, 400 uM dNTPS, 1 × buffer, 1.25 units polymerase, and H_2O to 25 μL. Cycle conditions were as follows: 94 C for 1 min, 30 cycles of 94 C for 30 s, 55 C for 30 s, and 72 C for 3 min, 72 C for 5 min, and a 4 C hold. Generation of the expected polymerase chain reaction (PCR) product was confirmed by agarose gel electrophoresis. PCR products were purified by gel extraction using the GenElute™ gel extraction kit (Sigma Aldrich, St. Louis, MO, USA). Purified PCR products were ligated into the pCR®2.1 vector using the TA cloning kit (Life Technologies, Grand Island, NY, USA). Ligation reactions contained an insert to vector ratio of 2 : 1 with 25 ng vector, 1 × ligase buffer, 4 units of ligase, and H_2O to 10 μL. Reactions were incubated overnight at 14 C. Chemically competent TOP10 cells were prepared according to Sambrook et al. (1989). Chemically competent cells were incubated on ice with 2 μL of ligation reaction for 30 min and heat shocked at 42 C for 90 s. Cells were immediately transferred to ice and recovered in 250 μL super optimal broth with catabolite repression (SOC) (2% w/v tryptone, 0.5% w/v yeast extract, 0.05% w/v NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose). Cells were incubated at 37 C and 250 rpm for 1 h and then spread onto Luria broth (LB) 50 μg/mL

Table 1. Primers used in polymerase chain reactions.

Name	Sequence
AW78	CACCTTCGTCTCTCACCG
AW79	CAGCCCTTAAATCGCTCAC
AW368	GTAGCCTTCAGCAGCGA
AW369	CACCTTCACGTTCAAGAG
AW370	GCTATTGGAGCTGCTGTTG
AW371	CATGCTCTTGAACAATCAACA
AW400	CAATGGCGTCCACTTCAACAAAC
AW401	GCCCTTCTTCCATCACCCTC
AW402	CTCGATCCATTACTAAGCATAAT
AW403	GAATGGCGTATTGCGGAGGA
AW409	GATGAGATTTGAAGAGGGCG
AW411	GCAATGAGATGAATCAAACAAGAT

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carbenicillin plates (1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, and 1.5% w/v agar). Plates were incubated at 37 C overnight and the next day colonies were selected for isolation on LB 50 μg mL^{-1} carbenicillin plates. Colonies were screened by PCR for the insert as described above. For colonies with the insert, overnight cultures of LB 50 μg mL^{-1} carbenicillin were inoculated and incubated at 37 C and 250 rpm. Glycerol stocks were prepared by adding 800 μL of overnight culture to 200 μL of 80% glycerol and storing at −80 C. Clones were turned over to the Bioinformatics and Genomics Research Unit in Stoneville, MS, for plasmid isolation and sequencing with M13 primers. Sequencing data were analyzed in Geneious version 7.1.4 (Kearse et al. 2012).

The full ALS sequence and flanking sequence was obtained through the Genome Walker kit (Clontech) by using the partial ALS sequence to design primers directed towards the 3′ and 5′ ends. Briefly, genomic DNA was digested in separate reactions by DraI, EcoRV, PvuII, and StuI. Reactions consisted of 2.5 μg genomic DNA, 1× buffer, 10 units of enzyme, and H_2O to 100 μL and were incubated at 37 C overnight. Following confirmation of digestion by gel electrophoresis, adaptors were ligated to the restriction fragments. Ligation reactions consisted of 4 μL of the digest, 25 μM adaptor, 2× ligation buffer, and 3 units of T4 DNA ligase. After an overnight incubation at 16 C, the reaction was stopped by incubating at 70 C for 5 min and then diluted by addition of 72 μL Tris EDTA (10 mM Tris-Cl pH 7.5, 1 mM EDTA pH 8.0). A primary PCR reaction was performed to amplify the flanking sequence using the Advantage® 2 PCR kit (Clontech). Reactions consisted of 1 μL of the ligation reaction, 1× PCR buffer, 10 mM dNTPs, 10 μM primer AP1, 200 nM AW368, or AW370 (Table 1; Figure 1; upstream and downstream, respectively), 1× polymerase mix, and H_20 to 50 μL. Cycle conditions were as follows: seven cycles of 94 C for 25 s and 72 C for 3 min, 32 cycles of 94 C for 25 s and 67 C for 3 min, 67 C for 7 min, and 4 C hold. A secondary PCR was performed as described above with 1 μL of the primary reaction, 10 mM AP2, and 200 nM AW369 or AW371 (Table 1; Figure 1; upstream and downstream, respectively). Cycle conditions were as follows: five cycles of 94 C for 25 s and 72 C for 3 min, 20 cycles of 94 C for 25 s and 67 C for 3 min, 67 C for 7 min, and a 4 C hold. PCR products were gel purified and cloned as described above.

To sequence the ALS for parental species and hybrids, the ALS was amplified with the Takara LA PCR kit V2.1 (Clontech) as described above using primers AW400 and AW401. These primers were designed from the full ALS sequence that was completed with the Genome Walker kit. Cycle conditions were as follows: 94 C for 3 min; 30 cycles of 94 C for 30 s, 55 C for 30 s, and 72 C for 2 min; and 72 C for 5 min. Cloning and sequencing was performed as described above, except that primers AW402 and AW403 (Table 1; Figure 1) were used in addition to M13 primers. To clone the upstream flanking sequence, primers AW409 and AW401 (Table 1; Figure 1) were used and cloning and sequencing were performed as described above. All sequences were analyzed and aligned in Geneious version 7.1.4.

Statistical Analysis. All experiments were conducted using a completely randomized design. Data were analyzed by ANOVA via the PROC GLM statement using SAS software (version 9.2; SAS Institute, Inc., Cary, NC 27513). No significant experiment effect at a P value of 0.05 was observed in repeated experiments; therefore, data from experiments were pooled. Nonlinear regression analysis was applied to fit a sigmoidal 3 parametric logistic curve of the form:

$$y = a/\left(1+(x/x_0)^b\right) \quad [1]$$

where a is the upper response limit, x_0 is the GR_{50} (herbicide dose required to cause a 50% reduction in shoot dry weight of test plants) or I_{50} (herbicide concentration required to cause a 50% reduction in ALS enzyme activity), and b is the slope of the curve to relate effect of herbicide dose and concentration, x, on growth of *Amaranthus* plants and ALS activity, y, respectively. Equation parameters were computed using SigmaPlot (version 11.0, Systat Software, Inc., San Jose, CA, USA).

Results and Discussion

Herbicide Dose Response. Herbicide GR_{50} values for all biotypes are presented in Table 2. GR_{50} values, where the estimates generated from the nonlinear regression model are outside the range of tested doses, the GR_{50} value is reported as < lowest tested dose. In cases where the GR_{50} values for SA-S and PA-S are reported as < lowest tested dose, R/S ratio is reported as greater than the GR_{50} value of respective resistant counterpart divided by the lowest tested dose. GR_{50} values of SA-R and SA-S biotypes for pyrithiobac, imazaquin, trifloxysulfuron, and

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Table 2. Nonlinear regression equation parameters and herbicide dose required for 50% inhibition of shoot dry weight of ALS-inhibiting herbicide-resistant and –susceptible *Amaranthus* biotypes.[a]

Herbicide	Biotype	Regression parameters[b]			(R/S)[c]
		x_0 (GR_{50})	a	b	
Pyrithiobac	SA-R	0.65 ± 0.44	100	0.17	> 325
	SA-S	< 0.002	100	0.52	
	PA-R	1.12 ± 0.0.46	101	0.63	112
	PA-S	0.01 ± 0.005	105	0.90	
Imazaquin	SA-R	8.56 ± 2.52	100	0.23	856
	SA-S	0.01 ± 0.001	100	0.56	
	PA-R	—	—	—	—
	PA-S	0.02 ± 0.01	99	0.53	
Trifloxysulfuron	SA-R	0.16 ± 0.04	100	0.23	> 1,600
	SA-S	< 0.0001	100	0.47	
	PA-R	0.07 ± 0.02	100	0.19	> 700
	PA-S	<0.0001	100	0.35	
Nicosulfuron	SA-R	0.03 ± 0.01	100	0.34	30
	SA-S	0.001 ± 0.0006	100	0.51	
	PA-R	0.15 ± 0.05	101	0.75	> 150
	PA-S	<0.001	100	0.15	

[a] Abbreviations: ALS, acetolactate synthase; GR_{50}, herbicide dose required to cause a 50% inhibition of shoot dry weight of test plants; PA-R, resistant Palmer amaranth; PA-S, susceptible Palmer amaranth; R/S, resistant GR_{50} divided by susceptible GR_{50}; SA-R, resistant spiny amaranth; SA-S, susceptible spiny amaranth.

[b] Regression equation parameters were generated by fitting a nonlinear 3 parametric logistic regression equation of the form $y = a/(1 + (x/x_0)^b)$ to response of *Amaranthus* biotypes to herbicide dose. Values after ± indicate standard error. In cases where the x_0 estimates are outside the range of tested doses, the GR_{50} value is reported as < lowest tested dose.

[c] R/S value was calculated by dividing the GR_{50} values of the SA-R and PA-R biotypes by those of SA-S and PA-S biotypes, respectively. In cases where the GR_{50} values for SA-S and PA-S are reported as < lowest tested dose, R/S ratio is reported as greater than the GR_{50} value of respective resistant counterpart divided by the lowest tested dose.

Table 3. Nonlinear regression equation parameters and pyrithiobac concentration required for 50% inhibition of ALS enzyme activity of ALS-inhibiting herbicide-resistant and –susceptible *Amaranthus* biotypes.[a]

Biotype	Regression parameters[b]			(R/S)[c]
	x_0 (I_{50})	a	b	
SA-R	35.53 ± 2.42	101	1.01	> 355
SA-S	< 0.1	100	0.03	
PA-R	65.62 ± 7.82	100	0.86	> 656
PA-S	< 0.1	100	0.07	

[a] Abbreviations: ALS, acetolactate synthase; I_{50}, herbicide concentration required to cause a 50% inhibition of ALS enzyme activity; PA-R, resistant Palmer amaranth; PA-S, susceptible Palmer amaranth; R/S, resistant I_{50} divided by sensitive I_{50}; SA-R, resistant spiny amaranth; SA-S, susceptible spiny amaranth.

[b] Regression equation parameters were generated by fitting a nonlinear 3 parametric logistic regression equation of the form $y = a/(1 + (x/x_0)^b)$ to response of ALS activity of *Amaranthus* biotypes to pyrithiobac concentration. Values after ± indicate standard error. In cases where the x_0 estimates are outside the range of tested doses, the I_{50} value is reported as < lowest tested concentration.

[c] R/S was calculated by dividing the I_{50} values of the SA-R and PA-R biotypes by those of SA-S and PA-S biotypes, respectively. Since the I_{50} values for SA-S and PA-S are reported as < lowest tested concentration, R/S ratio is reported as greater than the I_{50} value of respective resistant counterpart divided by the lowest tested concentration.

nicosulfuron were 0.65, 8.56, 0.16, and 0.03 kg ha^{-1} and < 0.002, 0.01, < 0.0001, and < 0.001 kg ha^{-1}, respectively. These results indicate that the SA-R biotype is > 325-fold, 856-fold, > 1,600-fold, and 30-fold more resistant (or less sensitive) to pyrithiobac, imazaquin, trifloxysulfuron, and nicosulfuron, respectively, than the SA-S biotype.

GR_{50} values of PA-R and PA-S biotypes for pyrithiobac, trifloxysulfuron, and nicosulfuron were 1.12, 0.07, and 0.15 kg ha^{-1} and 0.01, < 0.0001, and < 0.001 kg ha^{-1}, respectively. These results indicate that the PA-R biotype is 112-fold, > 700-fold, and > 150-fold more resistant (or less sensitive) to pyrithiobac, trifloxysulfuron, and nicosulfuron, respectively, than the PA-S biotype. GR_{50} value of PA-S for imazaquin was 0.02 kg ha^{-1}. The GR_{50} value of the PA-R biotype for imazaquin could not be computed because the chosen nonlinear regression model did not fit the dose response, most likely, due to the reduction in shoot dry weight being only 20% of nontreated control, even at the highest rate of 2.24 kg ha^{-1} (16 ×) (data not shown).

These results are similar to other *Amaranthus* species where high levels of ALS inhibitor resistance have been observed. A prostrate pigweed (*Amaranth blitoides* S. Wats.) biotype in Israel had 790-fold

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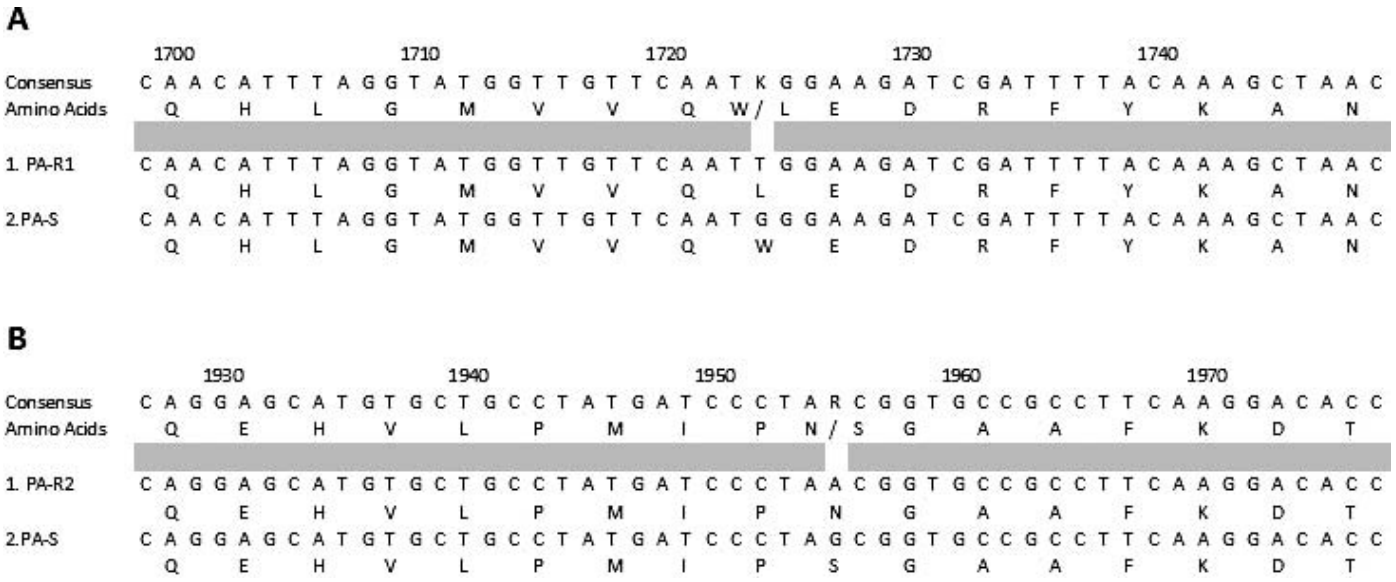

Figure 2. Sequencing of multiple Palmer amaranth plants with ALS inhibitor resistance revealed the presence of two resistant alleles (PA-R1 and PAR-2) in the population. (A) A guanine to thymine substitution results in a tryptophan to leucine substitution in the amino acid sequence at positon 574 (W574L). (B) A guanine to adenine substitution results in a serine to asparagine substitution at position 653 (S653N).

resistance to ALS inhibitors (Sibony and Rubin 2003). Resistance to imazethapyr of 2,800-fold has been reported in Palmer amaranth (Sprague et al. 1997). Some smooth pigweed populations exhibited up to 537-fold resistance to imazethapyr (Whaley et al. 2006).

ALS Assay. I_{50} values and related regression parameters are presented in Table 3. As with GR_{50} values, where the estimates generated from the nonlinear regression model are outside the range of tested doses, the I_{50} value is reported as $<$ lowest tested concentration. In cases where the GR_{50} values for SA-S and PA-S are reported as $<$ lowest tested concentration, R/S ratio is reported as greater than the I_{50} value of respective resistant counterpart divided by the lowest tested concentration. I_{50} values of SA-R and SA-S biotypes for pyrithiobac were 35.53 and < 0.1 μM, indicating that the ALS enzyme of the SA-R biotype is > 355 more resistant to pyrithiobac than the SA-S biotype under our experimental conditions. Similarly, the PA-R biotype was > 656-fold resistant to pyrithiobac compared to the PA-S biotype. I_{50} values for pyrithiobac of PA-R and PA-S biotypes were 65.62 and < 0.1 μM. The difference in fold resistance between the resistant and sensitive plants derived via the enzyme assay indicates that it is the enzyme that is insensitive to the herbicide. This implies a target site based mechanism of resistance.

Table 4. GenBank Accession numbers.

Sequence	Accession number
Palmer amaranth sensitive allele	KT833339
Palmer amaranth resistant allele (W574L)	KT833337
Palmer amaranth resistant allele (S653N)	KT833336
Spiny amaranth sensitive allele	KT833338
Spiny amaranth × Palmer amaranth resistant allele	KT833335
Palmer amaranth ALS[a] upstream region	KT833347
Spiny amaranth × Palmer amaranth ALS upstream region	KT833349
Spiny amaranth ALS upstream region	KT833350

[a] Abbreviation: ALS, acetolactate synthase.

***ALS* Sequence Analysis.** Sequencing of ALS in ALS inhibitor resistant Palmer amaranth revealed the presence of a tryptophan to leucine substitution at position 574 (Figure 2A), a point mutation well known to confer herbicide resistance in many species (see Table 4 for all GenBank accession numbers). A second point mutation was identified in a different plant, consisting of a guanine to adenine substitution and resulted in a serine to asparagine substitution at position 653 (S653N; Figure 2B). In sequencing of numerous *als* alleles from individual plants, this second point mutation was found less frequently than the W574L. Only the first point mutation was found in the hybrids. Since glyphosate resistance in these hybrids came from Palmer amaranth, this new data posed the question: did ALS inhibitor resistance also originate in Palmer amaranth and was it

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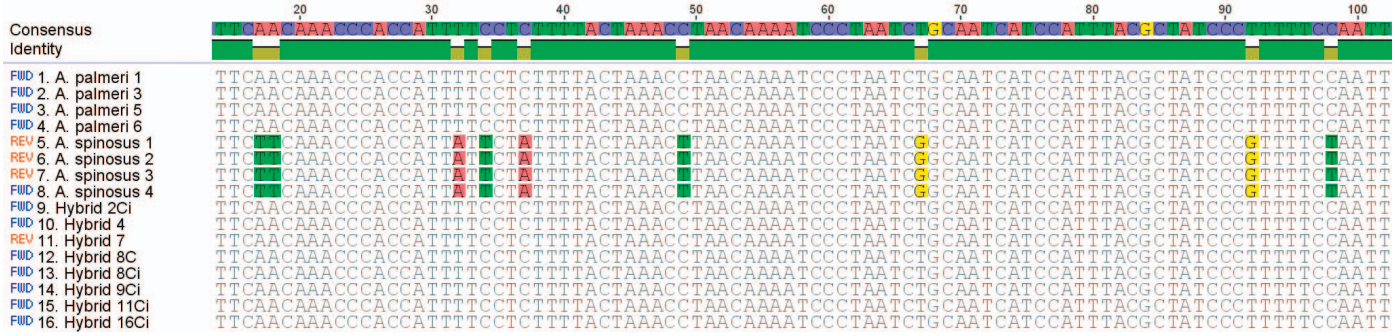

Figure 3. Alignment of part of *als* for Palmer amaranth, spiny amaranth, and hybrids. Sequences are identified according to species on the left and quality of the alignment is represented by the green bar at the top (brown indicates a difference). Sequence differences are highlighted according to nucleotide difference. A total of 15 nucleotide positions were consistently different between Palmer amaranth and spiny amaranth; only nine are shown here. (Color for this figure is available in the online version of this article.)

transferred to spiny amaranth by hybridization? To answer this question, several ALS sequences from Palmer amaranth, spiny amaranth, and the hybrids were aligned (Figure 3). Fifteen nucleotide differences were consistent in spiny amaranth and were absent from the hybrids when compared to Palmer amaranth. In other words, at those locations the hybrids were indistinguishable from Palmer amaranth. The sequence immediately upstream and downstream of ALS was sequenced. The downstream sequence was similar between Palmer amaranth and spiny amaranth. However, in the upstream region, there were several differences between Palmer amaranth and spiny amaranth, with the hybrids indistinguishable from Palmer amaranth (Figure 4). These data indicate that the ALS in the hybrids originated in Palmer amaranth and that parent was the source of ALS inhibitor resistance.

It is not surprising that the Trp574Leu point mutation was present. This point mutation is known to confer resistance to multiple classes of ALS inhibitors (Heap 2015) and that was the phenotype observed in these plants. This mutation has been found in other weedy *Amaranthus* species, including prostrate pigweed (Sibony and Rubin 2003), tall waterhemp, redroot pigweed (*Amaranthus retroflexus* L.), Powell amaranth (*Amaranthus powellii* L.), and smooth pigweed (Heap 2015). The presence of this point mutation in so many *Amaranthus* species limits control options for growers and, for crops such as soybean (*Glycine max* L. Merr), may increase the use of protoporphyrinogen oxidase inhibitors for POST control.

Multiple resistance to glyphosate and ALS inhibitors in Palmer amaranth has become more common, with resistance to both herbicides sometimes occurring in the same population. In 2014 it was demonstrated that glyphosate resistance had moved from Palmer amaranth to a Palmer amaranth × spiny amaranth hybrid (Nandula et al. 2014). Continued work with these hybrids indicated that they were resistant to ALS inhibitors as well. This finding, the transfer of a second type of resistance by the same hybridization event highlights the need to control all weedy amaranths and to set a zero tolerance policy when more than one species is present in an area. Transfer of herbicide resistance, though perhaps the most important aspect of these hybridizations from a weed control standpoint, is not the only concern. In some cases, the hybrids have altered morphologies including stature like Palmer amaranth, spines like spiny amaranth, and monoecious flower stalks that may allow self-fertilization.

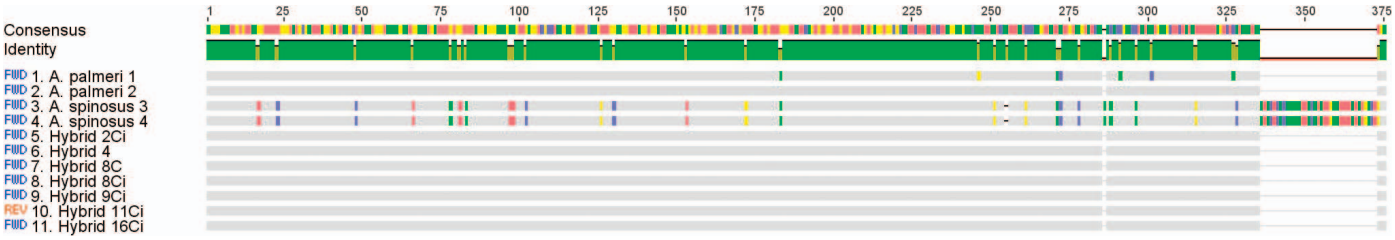

Figure 4. Alignment of sequence directly upstream of *als* for Palmer amaranth, spiny amaranth, and hybrids. Sequences are labeled according to species on the left. The grey bars represent sequence and the green bar at the top represents the quality of the alignment (brown indicates a position at which the sequence differs). Red, blue, green, and yellow colors on the grey bars indicate sequence differences. (Color for this figure is available in the online version of this article.)

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Altering the genetic background within the weed population may produce a more competitive weed and provide enhanced survival characteristics against new control measures.

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