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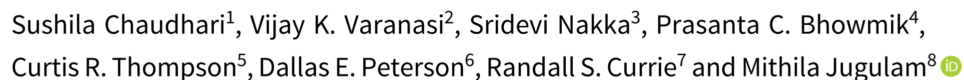
Evolution of target and non-target based multiple herbicide resistance in a single Palmer amaranth (*Amaranthus palmeri*) population from Kansas

Sushila Chaudhari[1], Vijay K. Varanasi[2], Sridevi Nakka[3], Prasanta C. Bhowmik[4], Curtis R. Thompson[5], Dallas E. Peterson[6], Randall S. Currie[7] and Mithila Jugulam[8]



[1]Assistant Professor, Department of Horticulture, Michigan State University, East Lansing, MI, USA; [2]Senior Biologist, Bayer Crop Science, Chesterfield, MO, USA; [3]Scientist, Heartland Plant Innovations, Manhattan, KS, USA; [4]Professor, Weed Science, Stockbridge School of Agriculture, University of Massachusetts, Amherst, MA, USA; [5]Extension Specialist and Professor Emeritus, Department of Agronomy, Kansas State University, Manhattan, KS, USA; [6]Professor, Department of Agronomy, Kansas State University, Manhattan, KS, USA; [7]Associate Professor, Kansas State University, Southwest Research–Extension Center, Garden City, KS, USA and [8]Associate Professor and Weed Scientist, Department of Agronomy, Kansas State University, Manhattan, KS, USA

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Author for correspondence:
Mithila Jugulam, Department of Agronomy, 2004 Throckmorton PSC, 1712 Claflin Road, Kansas State University, Manhattan, KS 66506-0110. Email: mithila@ksu.edu

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Abstract

The evolution of resistance to multiple herbicides in Palmer amaranth is a major challenge for its management. In this study, a Palmer amaranth population from Hutchinson, Kansas (HMR), was characterized for resistance to inhibitors of photosystem II (PSII) (e.g., atrazine), acetolactate synthase (ALS) (e.g., chlorsulfuron), and EPSP synthase (EPSPS) (e.g., glyphosate), and this resistance was investigated. About 100 HMR plants were treated with field-recommended doses (1×) of atrazine, chlorsulfuron, and glyphosate, separately along with Hutchinson multiple-herbicide (atrazine, chlorsulfuron, and glyphosate)–susceptible (HMS) Palmer amaranth as control. The mechanism of resistance to these herbicides was investigated by sequencing or amplifying the *psbA*, *ALS*, and *EPSPS* genes, the molecular targets of atrazine, chlorsulfuron, and glyphosate, respectively. Fifty-two percent of plants survived a 1× (2,240 g ai ha^{-1}) atrazine application with no known *psbA* gene mutation, indicating the predominance of a non–target site resistance mechanism to this herbicide. Forty-two percent of plants survived a 1× (18 g ai ha^{-1}) dose of chlorsulfuron with proline$_{197}$serine, proline$_{197}$threonine, proline$_{197}$alanine, and proline$_{197}$asparagine, or tryptophan$_{574}$leucine mutations in the *ALS* gene. About 40% of the plants survived a 1× (840 g ae ha^{-1}) dose of glyphosate with no known mutations in the *EPSPS* gene. Quantitative PCR results revealed increased *EPSPS* copy number (50 to 140) as the mechanism of glyphosate resistance in the survivors. The most important finding of this study was the evolution of resistance to at least two sites of action (SOAs) (~50% of plants) and to all three herbicides due to target site as well as non–target site mechanisms. The high incidence of individual plants with resistance to multiple SOAs poses a challenge for effective management of this weed.

Introduction

Palmer amaranth, one of the most troublesome weeds of the United States, is a summer-annual broadleaf weed native to the desert regions of the southwestern United States and northern Mexico (Sauer 1972; Steckel 2007). Palmer amaranth is an economically damaging weed causing extensive yield losses in crops such as soybean [*Glycine max* (L.) Merr.], corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), and grain sorghum (*Sorghum bicolor* L.). It is a dioecious weed, and a single female can produce 200,000 to 600,000 seeds per plant (Keeley et al. 1987). The availability of extensive genetic variability coupled with intense herbicide selection resulted in the evolution of resistance to herbicides with different sites of action (SOAs). Specifically, Palmer amaranth has evolved resistance to inhibitors of acetolactate synthase (ALS), microtubules, photosystem II (PSII), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), hydroxyphenylpyruvate dioxygenase (HPPD), and protoporphyrinogen (PPO), and more recently resistance to synthetic auxinic herbicides in the United States (Heap 2019). Resistance to ALS inhibitors in prostrate pigweed (*Amaranthus blitoides* S. Wats.) in Israel and multiple-herbicide resistance in Powell amaranth (*Amaranthus powellii* S. Wats.) in the Michigan nursery industry was also reported (Heap 2019). Importantly, evolution of resistance to multiple herbicides in a single population of Palmer amaranth is also widespread (Heap 2019). Multiple-herbicide-resistant weeds are of greater concern today, as they reduce options for herbicide rotation and increase in weed control costs.

Herbicides such as inhibitors of PSII, ALS, or EPSPS are commonly used to manage Palmer amaranth in several cropping systems. PSII inhibitors essentially inhibit photosynthesis by binding to the secondary quinone acceptor Q_B within the D1 protein encoded by the *psbA* gene and block the transport of the electrons to the plastoquinone (Hess 2000). The blockage of the electron transport chain by these herbicides results in depletion of ATP and NADPH synthesis, thereby leading to cellular damage by oxidative stress (Hess 2000). ALS inhibitors are among the most commonly used herbicides for controlling a wide spectrum of weeds in agronomic crops (Lamego et al. 2009). These herbicides inhibit the ALS enzyme, which catalyzes the biosynthesis of the branched-chain amino acids leucine, valine, and isoleucine (Devine and Eberlein 1997). Glyphosate, the most commonly used nonselective herbicide in agriculture, inhibits EPSPS, the key enzyme of the shikimate pathway catalyzing the biosynthesis of aromatic amino acids (Schönbrunn et al. 2001).

Both target site as well as non–target site based mechanisms of resistance to inhibitors of PSII, ALS, and EPSPS have been reported in weeds such as Palmer amaranth (Nakka et al. 2017a; Powles and Yu 2010). The target-site resistance involves amino acid substitutions in the target enzyme, preventing herbicide binding (Tranel and Wright 2002), or as a result of duplication, coupled with increased expression of the target gene (Sammons and Gaines 2014). Non–target site resistance, on the other hand, can evolve as a result of decreased herbicide penetration, translocation, and increased herbicide metabolism, or any one or a combination of these mechanisms that limit the amount of herbicide reaching a target site (Powles and Yu 2010; Yuan et al. 2007).

Palmer amaranth populations with multiple-herbicide resistance based on both target- and non–target site mechanisms have been identified previously (Nakka et al. 2017a, 2017b; Spaunhorst et al. 2019; Sosnoskie et al. 2011). However, there is a lack of information regarding the occurrence of evolution of resistance to one or multiple-herbicide SOA(s) in an individual Palmer amaranth plant. Therefore, the objectives of this study were to (1) confirm and characterize the incidence of resistance evolution to one or more herbicides (atrazine, chlorsulfuron, and glyphosate) in individual plants of a Hutchinson multiple-herbicide-resistant (HMR) Palmer amaranth population and (2) determine the mechanism of resistance (target site or non–target site based) to these herbicides.

Materials and Methods

Field History and Seed Collection

A population of Palmer amaranth (HMR), from a field in Hutchinson (Reno County), KS (37°93.16 N, 98°02.66 W, was found to be resistant to inhibitors of PSII (e.g., atrazine), ALS (e.g., chlorsulfuron), or EPSPS (e.g., glyphosate). The field had a history of repeated use of these herbicides over several years in corn, grown in rotation with soybean. The seeds of HMR, and a known Palmer amaranth biotype susceptible to atrazine, chlorsulfuron, and glyphosate (HMS), were used in this research. Both HMR and HMS seeds were germinated in small trays (25 by 15 by 2.5 cm) with a commercial potting mixture (Miracle-Gro®, Marysville, OH). Seedlings were transplanted into small pots (6 by 6 by 6.5 cm) when they reached 2 to 3 cm in height. All plants were grown in a greenhouse under a 16-h photoperiod, supplemented with an additional 250 µmol m^{-2} s^{-1} illumination provided with sodium vapor lamps and 25 C/20 C day/night temperature. Plants were watered and fertilized as needed regularly.

Assessment of Incidence of Resistance to Atrazine, Chlorsulfuron, and Glyphosate in HMR Palmer Amaranth

A set of 50 plants each of HMR and HMS Palmer amaranth (10 to 12 cm height), grown under greenhouse conditions (as above), were treated with the field-recommended dose (1×) of the following herbicides with label-recommended adjuvants: atrazine (AAtrex 4L; Syngenta Crop Protection, Greensboro, NC, 2,240 g ai ha^{-1}; 1% v/v crop oil concentrate), chlorsulfuron (Glean® XP; FMC Agriculture Solutions, Philadelphia, PA, 18 g ai ha^{-1}; 0.25% v/v non-ionic surfactant), and glyphosate (Roundup Weathermax; Bayer CropScience, St. Louis, MO, 840 g ae ha^{-1}; 2% w/v ammonium sulfate). Herbicide treatments were applied with a bench-type sprayer (Research Track Sprayer, Generation III; De Vries Manufacturing, Hollandale, MN) equipped with a flat-fan nozzle tip (80015LP TeeJet tip; Spraying Systems Co., Wheaton, IL) delivering 168 L ha^{-1} at 222 kPa in a single pass at 4.8 km h^{-1}. This experiment was repeated with another set of 50 plants for each of the above-mentioned herbicides.

Production of Vegetative Clones and Assessment of Their Response to Atrazine, Chlorsulfuron, and Glyphosate

To assess the occurrence of resistance to atrazine, chlorsulfuron, or glyphosate, in a single plant, vegetative clones of HMR (from at least 18 individual plants that survived a 1× dose of atrazine, chlorsulfuron, or glyphosate) and HMS (5 plants) Palmer amaranth were generated as described. When plants reached 15 to 20 cm tall, individual plants were multiplied via nodal cuttings. The nodal cuttings were treated with 0.10% indole 3-butyric acid powder (Bontone Rooting Powder; Bonide Products Inc., Oriskany, NY), transplanted in pots (6 by 6 by 6.5 cm), and covered with a plastic dome (Humidome Clear Plastic Propagation Domes; Hummert International, Topeka, KS) to maintain high humidity for root production. Herbicide applications were made when vegetative clones were established and when plants reached 10 to 12 cm tall. Three clones (replications) of each HMR (total of 54) and HMS (total 15) were separately treated with a 1× dose of each herbicide; that is, three clones produced from the same plant that initially survived atrazine treatment were treated with chlorsulfuron and glyphosate, respectively.

Genomic DNA (gDNA) Isolation and Target-Site Gene Sequencing

To test the presence of target-site resistance to atrazine, chlorsulfuron, or glyphosate, fresh leaf tissue was collected from HMR plants that survived herbicide applications and from nontreated HMS plants in the resistance assessment experiment. The collected tissue (100 mg) was frozen in liquid nitrogen until use. gDNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Inc., Germantown, MD), following the manufacturer's instructions. DNA was quantified using Nanodrop (Nanodrop 1000; Thermo Fisher Scientific, Waltham, MA), and quality was analyzed using 0.8% agarose gel electrophoresis. Based on the previously established protocols to confer resistance to inhibitors of PSII, ALS, or EPSPS in Palmer amaranth in our laboratory (Koo et al. 2018; Nakka et al. 2017a, 2017b), only gDNA was used in this experiment to determine any alternations in the herbicide target sites. The following primers and PCR conditions were used for testing the presence of any known mutations conferring resistance to atrazine, chlorsulfuron, or glyphosate. Forward (PsbAF: 5′- CTCCTGTTGCAGCTGCTACT-3′) and reverse (PsbAR: 5′-TAG AGGGAAGTTGTGAGC-3′) primers were used to amplify

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578 base pairs (bp) of the *psbA* gene to identify most of the known mutations. Each PCR reaction contained 80 to 100 ng of gDNA, 25 µL of Taq 2× PCR master mixture (Promega, Madison, WI), 0.5 µM of forward and reverse primers each, and the final volume made to 50 µL with nuclease-free water. The PCR cycling program was set to the initial denaturation at 95 C for 6 min, followed by 32 cycles of denaturation at 94 C for 30 s, at an annealing temperature of 55 C for 30 s, and extension at 72 C for 1 min, followed by a final extension of 72 C for 7 min. Primers used to amplify the *ALS* gene of ~2,000 bp in length (GenBank Population U55852; Whaley et al. 2007) were as follows: Forward (ALSF: 5′-CTGCAATCATCCATTTACGCTATC-3′) and reverse (ALSR: 5′-TCCAACCAACTAATAAGCCCTTC-3′). Each PCR reaction contained 80 to 100 ng of gDNA, 0.5 µM of forward and reverse primers each, and 25 µL PCR master mix, and the final volume made to 50 µL with nuclease-free water. The PCR conditions included initial denaturation at 94 C for 5 min, followed by 35 cycles of denaturation at 94 C for 30 s, annealing at 54 C for 30 s and extension at 72 C for 45 s, and a final extension at 72 C for 7 min. Primers used to amplify the *EPSPS* gene, ~204 bp in length (Gaines, et al. 2010), were as follows: Forward (EPSPSF: 5′ATGTTGGACGCTCTCAGAACT-3′) and reverse (EPSPSR: 5′TGAATTTCCTCCAGCAACGGC-3′′). The PCR reaction consisted of 50 ng of gDNA, 25 µL 2× PCR master mix, 0.5 µL of both forward and reverse primers, and the final volume made to 50 µL with nuclease-free water. The PCR was performed with the following conditions: initial denaturation at 95 C for 3 min, followed by 40 cycles of denaturation at 95 C for 30 s, annealing at 54 C for 45 s, and a final extension at 72 C for 7 min. The PCR products of the *psbA*, *ALS*, and *EPSPS* genes were purified using GENEjet PCR purification kit (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's instructions. The PCR products were sequenced by GENEWIZ (GENEWIZ Inc., South Plainfield, NJ), and the alignment of DNA sequences from HMR and HMS was performed using MultAlin software (Corpet 1988).

Quantitative Real-Time PCR (qPCR)

To determine the *EPSPS* copy number in HMR plants that survived glyphosate application, the genomic DNA was extracted from these plants as described above. Using the gDNA, qPCR was performed (CFX 96 Touch™ Real-Time PCR Detection System; Bio-Rad Inc., Hercules, CA) using β-tubulin as a reference gene. The following primer sequences were used to perform qPCR: EPSPSF: 5′-ATGTTGGACGCTCTCAGAACTCTTGGT-3′ and EPSPSR: 5′-TGAATTTCCTCCAGCAACGGCAA-3′ (Gaines et al. 2010); β-tubulin F: 5′- ATGTGGGATGCCAAGAACATGATGTG-3′ and β-tubulin R: 5′-TCCACTCCACAAAGTAGGAAGAGTTCT-3′ (Godar et al. 2015). The qPCR reaction mix consisted of 8 µL of SYBR Green master mix (Bio-Rad Inc., Hercules, CA), 2 µL each of forward and reverse primers (5 µM), and 2 µL of gDNA (15 ng µL^{-1}) to make the total reaction volume up to 14 µL. PCR conditions were 95 C for 15 min, and 40 cycles of 95 C for 30 s and 60 C for 1 min. A melt curve profile was included following the thermal cycling protocol to determine the specificity of the qPCR reaction. *EPSPS* gene copy number was measured with three technical replicates. Gene copy number was determined using the $2\Delta C_T$ method, where C_T is the threshold cycle, and ΔCT is $CT_{\text{target gene (EPSPS)}} - CT_{\text{reference gene (β-tubulin)}}$ (Godar et al. 2015).

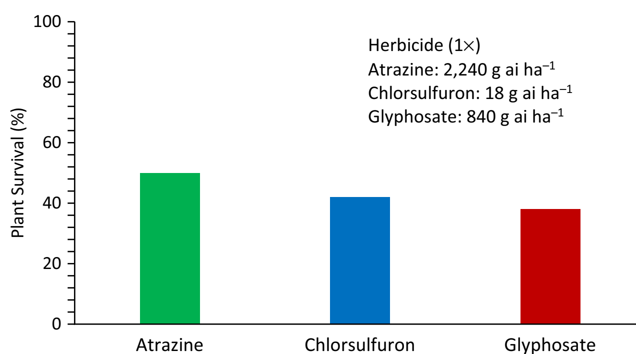

Figure 1. Percent survival of HMR Palmer amaranth (n = 100) in response to a 1× dose of atrazine, chlorsulfuron, and glyphosate. HMS Palmer amaranth plants did not survive the application of a 1× dose of these herbicides (data not shown). HMR, Hutchinson multiple-herbicide resistant; HMS, Hutchinson multiple-herbicide susceptible.

Results and Discussion

Confirmation of Resistance to Atrazine, Chlorsulfuron, and Glyphosate in HMR Palmer Amaranth

Approximately 52%, 42%, and 40% of HMR Palmer amaranth plants survived the field-recommended doses of atrazine, chlorsulfuron, or glyphosate, respectively (Figure 1), whereas all the HMS plants died (data not shown). These results suggest that the HMR population exhibits resistance at a 1× dose to these three herbicides most commonly used for Palmer amaranth management. Also, these data indicate that this population consists of a mixture of plants, some of which are either susceptible or resistant to the herbicides tested in this research. Recently, resistance to these herbicides in one or more populations of Palmer amaranth has been reported across the United States, including Kansas (Heap 2019). Although the evolution of resistance to PSII- and ALS-inhibiting herbicides in Kansas was reported as early as the 1990s (Horak and Peterson 1995), resistance to glyphosate was first reported in 2012 (Heap 2019). However, currently, the resistance to all these herbicides is widespread across Kansas.

Response of Vegetative Clones to Atrazine, Chlorsulfuron, and Glyphosate

The uniqueness of this research is the assessment of the incidence of resistance evolution to one or multiple SOAs (atrazine, chlorsulfuron, or glyphosate) in a single Palmer amaranth plant. These results suggested that 33% and 17% of the HMR plants were resistant to either atrazine or chlorsulfuron, respectively (Table 1). Nonetheless, 50% of HMR plants were found to be resistant to at least two herbicides––that is, either atrazine + chlorsulfuron (27%) or atrazine + glyphosate (6%), or chlorsulfuron + glyphosate (17%). Thus, these results confirm the presence of resistance to at least two herbicides in a single plant and to all three herbicides at the population level. This information is valuable to determine the evolutionary trajectory of multiple-herbicide resistance in Palmer amaranth. As indicated earlier, glyphosate resistance was first documented in 2012 in Kansas, much later than the resistance to PSII- and ALS-inhibiting herbicides. Hence, there is the predominance of resistance to atrazine or chlorsulfuron in the HMR population. Our data suggest that glyphosate-resistant plants are also resistant to either atrazine or chlorsulfuron (Table 1). The presence of resistance to only glyphosate in a single plant was not found, at least with the sample size that we

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Table 1. Assessment of percentage of a single Palmer amaranth (HMR) plant with resistance to at least one or two herbicides (atrazine, chlorsulfuron, or glyphosate).[a]

Resistance to herbicide	Palmer amaranth plants surviving
	%
Only atrazine	33.3
Only chlorsulfuron	16.7
Only glyphosate	0
Atrazine + chlorsulfuron	27
Atrazine + glyphosate	6
Chlorsulfuron + glyphosate	17
At least one herbicide	49
At least two herbicides	50

[a]For each herbicide (or a combination) listed, 18 vegetative clones were tested.

used for producing vegetative clones (18 survivors). However, based on the assessment of the percentage of plants resistant to atrazine, chlorsulfuron, or glyphosate in the HMR population (Figure 1), >50% of plants were found to be susceptible to these three herbicides. Although less likely, it is possible that some individuals among the plants that were susceptible to either atrazine or chlorsulfuron may have been resistant to only glyphosate.

Mechanism of Resistance to Atrazine, Chlorsulfuron, and Glyphosate in HMR Population

Atrazine Resistance

Sequencing of a portion of the *psbA* gene from ~25 atrazine survivors of HMR plants had no mutations known to confer atrazine resistance, i.e., valine$_{219}$isoleucine or serine$_{264}$glycine (Figure 2). These results indicate a high likelihood of the presence of non–target site metabolism-based resistance to atrazine in the HMR population. Resistance to atrazine is widespread in *Amaranthus* weeds, including Palmer amaranth (Heap 2019). Although a high level of resistance (>200-fold) to atrazine has been reported in Palmer amaranth and common waterhemp (*Amaranthus rudis* Sauer) (Ma et al. 2013; Nakka et al. 2017a; Vennapusa et al. 2018), none of these reports found any mutations in the *psbA* gene conferring resistance.

Metabolism of atrazine via glutathione *S*-transferase (GST) activity providing crop tolerance has been known in crops such as corn and sorghum (Jachetta and Radosevich 1981; Timmerman 1989). Although target-site resistance to atrazine via mutations in the *psbA* gene has been reported in some weeds such as lambsquarters (*Chenopodium album* L.), wild radish (*Raphanus raphanistrum* L.), kochia (*Kochia scoparia* L.), and common purslane (*Portulaca oleracea* L.) (Bandeen and McLaren, 1976; Friesen and Powles 2007; Ryan 1970; Varanasi et al. 2015), atrazine resistance in Palmer amaranth and common waterhemp is primarily due to metabolism as result of GST conjugation (Nakka et al. 2017a; Vennapusa et al. 2018). Although the metabolism of atrazine in the HMR population was not tested in this study, the lack of any known mutations conferring resistance to atrazine in the *psbA* gene (Figure 2) suggests the possibility of non–target site resistance to atrazine.

Chlorsulfuron Resistance

The HMR population under investigation had ~40% chlorsulfuron-resistant individuals (Figure 1). Sequencing of a portion of the *ALS* gene of HMR and HMS Palmer amaranth, covering known mutations conferring target-site resistance to ALS-inhibiting herbicides revealed several amino acid substitutions at the proline$_{197}$ residue. Specifically, proline$_{197}$serine, proline$_{197}$threonine, proline$_{197}$asparagine, or proline$_{197}$alanine substitutions that confer resistance to sulfonylurea herbicides were found in several HMR plants (Figure 3). Previously in chlorsulfuron-resistant Palmer amaranth from Kansas, we reported the presence of only a proline$_{197}$serine mutation in the *ALS* gene (Nakka et al. 2017b). However, amino acid substitutions at the alanine$_{122}$ and alanine$_{205}$ positions conferring resistance to imidazolinones (IMI) were not present in HMR plants (Figure 3). Nonetheless, the tryptophan$_{574}$leucine mutation, which is known to provide cross-resistance to both sulfonylurea (SU) and IMI herbicides, was found in only one plant among 18 plants sequenced. Although cross-resistance of HMR Palmer amaranth to IMI herbicides was not tested in this population, based on the presence of the tryptophan$_{574}$leucine mutation, we believe that some plants of this population may also be resistant to IMIs.

A varying level of resistance to ALS inhibitors, ranging from 60- to 3,200-fold depending on the type of amino acid substitutions; e.g., substitutions at alanine$_{122}$threonine, aspartate$_{376}$glutamate, tryptophan$_{574}$leucine, or serine$_{653}$threonine have been reported in several *Amaranthus* species such as smooth pigweed and waterhemp (Patzoldt and Tranel 2007; Whaley et al. 2006, 2007).

Generally, mutations at proline$_{197}$ confer resistance to SU but not IMI herbicides (Yu and Powles 2014). The proline$_{197}$ position on the *ALS* gene exhibits the highest variability in amino acid substitutions contributing to SU resistance in weeds. Similar to what was found in this research, multiple substitutions of serine, threonine, asparagine, or alanine at the proline$_{197}$ locus in the *ALS* gene have also been reported in other several weeds (Heap 2019). So far, 11 substitutions (Thr, Ser, Arg, His, Leu, Gln, Ala, Ile, Asn, Tyr, and Glu) at the proline$_{197}$ position conferring SU resistance in various weed species have been documented (Heap 2019). The herbicide-binding site of the ALS enzyme is reported to have greater flexibility at several conserved amino acid positions especially, for the SU herbicides, such as chlorsulfuron, used in this research. Amino acid substitutions at this position were known to tolerate herbicide application without affecting the function of the ALS enzyme, suggesting that the herbicide binding site is different from the enzyme's active site (Tranel and Wright 2002).

Cross-resistance to several ALS-inhibiting herbicides as a result of point mutations in the conserved domains of the *ALS* gene resulting in substitution of amino acids is commonly found in several resistant weeds (Tranel and Wright 2002). The level of cross-resistance to other ALS family herbicides depends on the specific amino acid that is substituted at the proline$_{197}$ position in the ALS enzyme (Park et al. 2012). Similar to what was found in this study, a proline-to-serine substitution was reported in ALS inhibitor–resistant cheatgrass (*Bromus tectorum* L.) (Park and Mallory-Smith 2004) and proline to serine or threonine in the crown daisy (*Chrysanthemum coronarium* L.) (Tal and Rubin 2004). Furthermore, cross-resistance to SUs as well as to sulfonylaminocarbonyl-triazolinone (SCTs), both families of ALS-inhibiting herbicides, was found in wind bentgrass [*Apera spica-venti* (L.) P. Beauv.] as a result of the substitution of proline$_{197}$, either to serine or threonine (Krysiak et al. 2011). These studies suggest that ALS gene mutations resulting in the substitution of proline$_{197}$ with amino acids serine or threonine would lead to resistance to both SUs and SCTs in weed species. The cross-resistance of HMR Palmer amaranth to SCT herbicides such as propoxycarbazone-sodium still has to be determined. Overall, our results suggest several substitutions at proline$_{197}$

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HMR 1  ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMR 2  ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMR 3  ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMR 4  ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMR 5  ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT.........
HMR 6  ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMR 7  ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMR 8  ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMR 9  ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMR 10 ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMR 11 ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMR 12 ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMR 13 ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT..........
HMS 1  ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT...........
HMS 2  ..........TAGTGCTATG CATGGTTCCT TGGTAACTTC TAGTTTGATC................TGATCTTCCA ATATGCTAGT TTCAACAACT CTCGTTCTTT...........
                                    Val 219                                        Ser 264
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Figure 2. Nucleotide sequence alignment of the *psbA* gene fragment from several atrazine-susceptible (HMS) and atrazine-resistant (HMR) Palmer amaranth plants. Nucleotide/amino acid numbering refers to the *Arabidopsis thaliana psbA* gene sequence. Nucleotide polymorphism does not exist between resistant and susceptible Palmer amaranth.

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HMR 1 .........TCGCCATTAC TGGGCAAGTT CCCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMR 2 .........TCGCCATTAC TGGGCAAGTT CCCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMR 3 .........TCGCCATTAC TGGGCAAGTT CCCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMR 4 .........TCGCCATTAC TGGGCAAGTT CCCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMR 5 .........TCGCCATTAC TGGGCAAGTT CTCCGGCGTA TGATTGGTAC TGATGCTTTT........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMR 6 .........TCGCCATTAC TGGGCAAGTT TCCCGGCGTA TGATTGGTAC TGATGCTTTT........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMR 7 .........TCGCCATTAC TGGGCAAGTT GCCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMR 8 .........TCGCCATTAC TGGGCAAGTT CCCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATTGG AAGATCGATT TTACAAAGCT.......
HMR 9 .........TCGCCATTAC TGGGCAAGTT GCCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMR 10.........TCGCCATTAC TGGGCAAGTT CCCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMR 11.........TCGCCATTAC TGGGCAAGTT ACCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMR 12.........TCGCCATTAC TGGGCAAGTT ACCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMR 13.........TCGCCATTAC TGGGCAAGTT AACCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMS 1 .........TCGCCATTAC TGGGCAAGTT CCCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
HMS 2 .........TCGCCATTAC TGGGCAAGTT CCCCGGCGTA TGATTGGTAC TGATGCTTTC........AGGTATGGTT GTTCAATGGG AAGATCGATT TTACAAAGCT.......
                                 Pro197(CCC)                                                   Trp574(TGG)

                     CTC Leu, TCC Ser, GCC Ala, ACC Thr, AAC Asn, TTG Leu
```

Figure 3. Nucleic acid sequence alignment of a section of the *ALS* gene from four HMR (resistant) and one HMS (susceptible) Palmer amaranth plants. The proline$_{197}$serine, proline$_{197}$threonine, proline$_{197}$asparagine, or proline$_{197}$alanine, and tryptophan$_{574}$leucine mutations are highlighted in different colors. The other commonly known *ALS* mutations conferring resistance to ALS inhibitors are highlighted in yellow. The figure shows only the regions of the *ALS* gene sequence where point mutations have been reported in other weed species and are not the complete sequence.

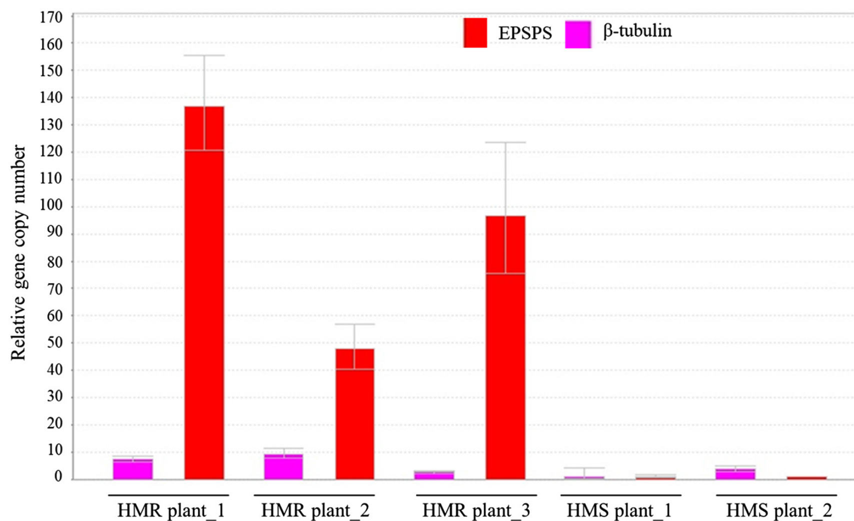

Figure 4. Estimation of *EPSPS* gene copy number for HMR Palmer amaranth plants that survived glyphosate treatment. The EPSPS copy number was measured relative to susceptible samples (HMS) of Palmer amaranth. Error bars represent ±SE from the mean (n = 3 technical replicates). The qPCR data were normalized using β-tubulin as a reference gene.

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and tryptophan$_{574}$ positions confer resistance to chlorsulfuron in HMR Palmer amaranth.

Glyphosate Resistance

The glyphosate-resistant plants in this population were also found to be resistant to either atrazine or chlorsulfuron (Table 1). No mutation in the *EPSPS* gene that was known to confer glyphosate resistance in weeds was found in HMR Palmer amaranth (data not shown). In several glyphosate-resistant Palmer amaranth populations across the United States, amplification of the *EPSPS* gene, the molecular target of glyphosate, was found to contribute resistance (Sammons and Gaines 2014). Also, more recently we reported that a massive number of extrachromosomal circular DNAs carry the amplified copies of *EPSPS* that are randomly distributed in the genome of glyphosate-resistant Palmer amaranth from Kansas (Koo et al. 2018). In this study, the *EPSPS* gene copy number was measured relative to the β-tubulin gene in the HMR population. The results suggested the amplification of the *EPSPS* gene as a mechanism of glyphosate resistance in HMR Palmer amaranth as well (Figure 4). The glyphosate-resistant plants had *EPSPS* copies ranging from 48 to 135 (Figure 4). Weed resistance to glyphosate has been shown to have evolved as a result of either non–target site mechanisms such as reduced absorption and translocation of glyphosate (Koger and Reddy 2005, Nandula et al. 2013), or because of mutation(s) in the *EPSPS* gene (Kaundun et al. 2008; Nandula et al. 2013; Yu et al. 2015). However, the most commonly found target-site resistance to glyphosate in *Amaranthus* species is due to amplification of the *EPSPS* gene (Chatham et al. 2015; Dillon et al. 2017; Gaines et al. 2010; Koo et al. 2018; Nandula et al. 2013). *EPSPS* gene amplification–based glyphosate resistance has also been confirmed in other weeds, e.g., kochia (Varanasi et al. 2015; Wiersma et al. 2015), Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] (Salas et al. 2012, 2015), and brome grass (*Bromus diandrus* Roth) (Malone et al. 2016). However, the mechanism of amplification of the *EPSPS* gene appears to be different in different weeds (Jugulam and Gill 2017).

In conclusion, these results confirm the presence of resistance to at least two herbicides in a single plant and all three herbicides at the population level. The resistance to atrazine, chlorsulfuron, and glyphosate in a single population (HMR) of Palmer amaranth was a result of non–target site and target-site mechanisms. Analysis of the *psbA* gene did not reveal any known mutations responsible for resistance to this herbicide (Figure 2), suggesting rapid metabolism as a mechanism for atrazine resistance in HMR. Therefore, it is likely that the atrazine resistance in this population is not as a result of alteratins in the target site. However, the resistance to chlorsulfuron and glyphosate is conferred by target-site alterations in this population. Although mutations at the proline$_{197}$ and tryptophan$_{574}$ positions on the *ALS* gene resulting in several amino acid substitutions were found in ALS inhibitor–resistant HMR plants, the glyphosate-resistant plants showed amplification of the *EPSPS* gene without any mutation in the *EPSPS* gene. The dioecious nature of Palmer amaranth, combined with high seed production and efficient pollen and seed distribution (Steckel 2007), may have facilitated the evolution of resistance to multiple herbicides. In particular, the presence of non–target based resistance in weed species poses a serious challenge, because such resistance mechanisms may predispose weeds to evolve resistance to other unknown chemistries. The evolution of herbicide-resistant species has increased steadily in agronomic cropping systems over the years. However, the incidence of resistant species in turfgrass, ornamental, and nursery crops has been slow. Careful design of strategies combining herbicide and non-herbicide methods (integrated) is crucial for the management of multiple-herbicide resistance in Palmer amaranth.

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