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Nontarget-Site Resistance to ALS Inhibitors in Waterhemp (*Amaranthus tuberculatus*)

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A waterhemp population (MCR) previously characterized as resistant to 4-hydroxyphenylpyruvate dioxygenase and photosystem II inhibitors demonstrated both moderate and high levels of resistance to acetolactate synthase (ALS) inhibitors. Plants from the MCR population exhibiting high resistance to ALS inhibitors contained the commonly found Trp574Leu ALS amino acid substitution, whereas plants with only moderate resistance did not have this substitution. A subpopulation (JG11) was derived from the MCR population in which the moderate-resistance trait was isolated from the Trp574Leu mutation. Results from DNA sequencing and ALS enzyme assays demonstrated that resistance to ALS inhibitors in the JG11 population was not due to an altered site of action. This nontarget-site ALS-inhibitor resistance was characterized with whole-plant dose–response experiments using herbicides from each of the five commercialized families of ALS-inhibiting herbicides. Resistance ratios ranging from 3 to 90 were obtained from the seven herbicides evaluated. Nontarget-site resistance to ALS has been rarely documented in eudicot weeds, and adds to the growing list of resistance traits evolved in waterhemp.

Nomenclature: Waterhemp, *Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer) Costea and Tardif AMATU.

Key words: Herbicide metabolism, herbicide resistance, malathion, resistance mechanisms, common waterhemp, tall waterhemp.

Waterhemp is a problematic weed in the midwestern U.S. crop production region and is notorious for its high reproductive output, extended emergence period, and rapid herbicide-resistance evolution (Costea et al. 2005; Steckel 2007; Tranel et al. 2011). Waterhemp has evolved resistance to herbicides from six site-of-action groups, including ALS inhibitors (Heap 2014).

The ALS enzyme, or acetohydroxyacid synthase enzyme, is the target site of five commercialized families of herbicides: sulfonylureas (SUs), imidazolinones (IMIs), pyrimidinylthiobenzoates, sulfonylaminocarbonyltriazolinones, and triazolopyrimidines (TPs) (Heap 2014; Tranel and Wright 2002). Inhibition of ALS blocks biosynthesis of branched-chain amino acids and causes slow plant death, with characteristic symptoms including cessation of growth and chlorosis of meristems (Duggleby and Pang 2000). Although ALS inhibitors are very effective herbicides, they surpass all other herbicide groups in terms of the number of weed species that have evolved resistance to them (Heap 2014).

In the vast majority of cases reported, resistance to ALS inhibitors is due to single point mutations in the ALS gene that result in the encoded enzyme being less sensitive to herbicide inhibition (Tranel and Wright 2002). More than 100 unique weed species-by-ALS-mutation combinations have been reported (Tranel et al. 2014). In waterhemp, ALS gene mutations causing Trp574Leu, Ser653Asn, and Ser653Thr amino acid substitutions have been reported. The Trp574Leu substitution confers high-level resistance broadly across all families of ALS inhibitors, whereas the Ser653 substitutions confer resistance most notably to IMI herbicides (Patzoldt and Tranel 2007).

Another method whereby plants overcome ALS inhibition is enhanced metabolism that detoxifies the inhibitors rapidly after herbicide uptake. Such metabolic detoxification mimics the process that enables crop plants to be tolerant to ALS inhibitors (Yu and Powles 2014). Compared with target-site resistance, metabolism-based resistance to ALS inhibitors has been reported in only a few species, including rigid ryegrass (*Lolium rigidum* Gaud.), blackgrass (*Alopecurus myosuroides* Huds.), rigid brome (*Bromus rigidum* Roth), wild oat (*Avena fatua* L.), late watergrass [*Echinochloa phyllopogon*

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(Stapf.) Koss.], and wild mustard (*Sinapis arvensis* L.) (Yu and Powles 2014). The magnitude of metabolism-mediated resistance usually is modest, in the range of 10-fold, whereas target-site resistance to ALS inhibitors often is 100-fold or more (Tranel and Wright 2002; Yu and Powles 2014).

A waterhemp population, designated MCR, was identified in Illinois with resistances to 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors and to atrazine, a photosystem II (PSII) inhibitor (Hausman et al. 2011). Field experiments indicated that the population also was resistant to ALS inhibitors (Hausman et al. 2013). In preliminary greenhouse experiments (described herein), we observed that plants from the MCR population exhibited two distinct resistance responses to normally lethal doses of ALS inhibitors: some plants were essentially unaffected, whereas others were substantially injured but nevertheless survived. We hypothesized that two different mechanisms of resistance to ALS inhibitors were present in the population. Research therefore was conducted to further investigate resistance to ALS inhibitors in the MCR population and to identify the underlying mechanism(s).

Materials and Methods

Plant Material, Greenhouse Cultivation, and Herbicide Application. The MCR waterhemp population was the focus of this research and was originally described by Hausman et al. (2011). Seeds derived from waterhemp populations ACR and WCS, described by Patzoldt et al. (2005), were used as references. Population ACR contains the ALS Trp574Leu substitution and population WCS is sensitive to ALS inhibitors. Seeds for both ACR and WCS were obtained from controlled crosses in the greenhouse and are highly uniform for the presence and absence, respectively, of the Trp574Leu ALS substitution.

Seeds were stratified before planting to improve germination as described by Bell et al. (2013). Stratified seeds were sown in plastic inserts containing commercial potting mix (LC1 Professional Growing Mix, Sun Gro Horticulture, Inc., 110 110th Ave. NE, Suite 490, Bellevue, WA 98004). When seedlings displayed one or two true leaves they were transplanted to individual inserts containing the same potting mix. A second transplantation was done when the seedlings were 5 cm tall. For the second transplanting, plants were moved into 11-cm square pots filled with 3 : 1 : 1 : 1 mixture of the commercial potting mix : sand : soil : peat. A slow-release fertilizer (Osmocote 13-13-13 slow-release fertilizer or Osmocote Plus 15-9-12 water-soluble fertilizer, The Scotts Company, 14111 Scottlawn Rd., Marysville, OH 43041) was mixed in with the growing medium. All plants were grown in a greenhouse at the University of Illinois Urbana-Champaign campus, and the environmental conditions were set to 28/22 C day/night temperature and 16-h photoperiod. Sunlight was supplemented with halide and sodium vapor lights to maintain a minimum of 800 μmol m^{-2} s^{-1} during the diurnal period.

For whole-plant herbicide applications, plants were sprayed with a TeeJet flat-fan nozzle (TeeJet 80015EVS. TeeJet Technologies, P.O. Box 7900, Wheaton, IL 60187) in a spraying chamber (Generation III Research Sprayer, DeVries Manufacturing, 28081 870th Ave., Hollandale, MN 56045) calibrated to deliver 185 L ha^{-1} at 275 kPa. Herbicides applied are listed in Table 1. Herbicide solutions included 1% crop oil concentrate, (Herbimax, Loveland Products, Inc., 3005 Rocky Mountain Ave., Loveland, CO, 80538) and 2.5% ammonium sulfate (N-Pak AMS Liquid, Winfield Solutions LLC, P.O. Box 64589, St. Paul, MN 55164), except for pyrithiobac, which instead was applied with 0.25% nonionic surfactant (Activator 90, Loveland Products). Nontreated controls were sprayed with water and adjuvant only.

Isolation of MCR Subpopulation Lacking Trp574Leu Mutation. Plants from the MCR population were treated with chlorimuron, 46 g ha^{-1}, or imazethapyr, 70 g ha^{-1} (equivalent to 4× and 1× typical field use rates, respectively). Surviving plants were evaluated for the presence of the Trp574Leu ALS mutation using a polymerase chain reaction (PCR) restriction fragment length polymorphism essentially as described previously (Foes et al. 1998). Primers used for the PCR were AmALS-F2 and AmALS-R2 (Table 2). Two plants that lacked the mutation were crossed and the resulting progeny was designated JG11.

ALS Gene Sequencing. The portion of the ALS gene encoding the mature protein was sequenced from eight JG11 plants that survived at least 17 g ha^{-1} primisulfuron when 10 to 12 cm tall. PCR products were generated with primers shown in Table 2 and fragments were sequenced without cloning using previously published procedures

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Table 1. List of herbicides used.

Herbicide	Trade name	Chemical family	Manufacturer	Rate range for dose response
Imazethapyr	Pursuit	Imidazolinone	BASF Corporation Research Triangle Park, NC www.basf.com	2 to 6,500 g ae ha^{-1}
Imazapyr	Arsenal	Imidazolinone	BASF Corporation Research Triangle Park, NC www.basf.com	0.009 to 9 g ae ha^{-1}
Chlorimuron-ethyl	Classic	Sulfonylurea	Dupont Wilmington, DE www.dupont.com	Not used for dose response
Primisulfuron-methyl	Beacon	Sulfonylurea	Syngenta Greensboro, NC www.syngenta.com	0.004 to 400 g ai ha^{-1}
Sulfometuron-methyl	Oust	Sulfonylurea	Dupont Wilmington, DE www.dupont.com	0.105 to 105 g ai ha^{-1}
Pyrithiobac-sodium	Staple	Pyrimidinylthiobenzoate	Dupont Wilmington, DE www.dupont.com	1.6 to 4,970 g ai ha^{-1}
Propoxycarbazone-sodium	Olympus	Sulfonylaminocarbonyl-triazolinone	Bayer CropScience Research Triangle Park, NC www.bayercropscience.us.com	0.173 to 173 g ai ha^{-1}
Cloransulam-methyl	FirstRate	Triazolopyrimidine	Dow AgroSciences Indianapolis, IN www.dowagro.com	0.035 to 3,500 g ai ha^{-1}

(Riggins et al. 2010). At least one resequencing was performed when ambiguous or putative heterozygous nucleotides were identified. Sequence data were compared and aligned with FinchTV software v1.4 (Geospiza, Inc., 100 West Harrison, North Tower, Suite #330, Seattle, WA 98119). Waterhemp ALS gene sequences from WCS, ACR, and an IMI-resistant population, IR-101 (which contains a Ser653Thr mutation; Paltzoldt and Tranel 2007), were obtained from GenBank (accessions EF157818, EF157819, and EF157821, respectively) and used for comparison.

ALS in Vitro Assay. Leaf tissues of WCS, ACR, and JG11 waterhemp populations were collected from plants when they were 15 to 20 cm tall. New leaves from plant apices were selected, and about 6 g of fresh leaf tissue (pooled from 10 to 15 plants from each population) were frozen with liquid nitrogen. Protein was extracted and used for in vitro ALS enzyme assays essentially as described by Schmitzer et al. (1993). Protein concentrations of the extracts were determined with a Nanodrop 1000 spectrophotometer v3.7.1 using Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific, 81 Wyman St., Waltham, MA 02454). Protein concentrations ranged from 1 to 1.5 μg μl^{-1} and were diluted to 1 μg μl^{-1}. Corning 96-well, clear, flat-bottom plates (Corning, Inc., One Riverfront Plaza, Corning, NY 14831) were used for enzyme assays. Technical-grade imazethapyr (98.1%, BASF Corporation, 26 David Dr., Research Triangle

Table 2. Primers used for acetolactate synthase (ALS) gene amplification and sequencing.

Region amplified[a]	Forward primer	Reverse primer
5′ UTR[b] to codon 253	ALS5UTR-F (CTTCAATCTTCAACAATGGCG)	ALSr1 (TCAATCAAAACAGGTCCAGG)
Codon 51 to codon 253	Whals-F (CGCCCTCTTCAAATCTCATC)	ALSr1 (TCAATCAAAACAGGTCCAGG)
Codon 106 to codon 253	ALSf1 (AGCTCTTGAACGTGAAGGTG)	ALSr1 (TCAATCAAAACAGGTCCAGG)
Codon 106 to codon 540	ALSf1 (AGCTCTTGAACGTGAAGGTG)	ALS1603-R (AACTCCCATCCCCATCAATGTC)
Codon 475 to 3′ UTR	ALS1426-f (ACGAAGGGTGATGCGATTGT)	AmALS-R2 (CTAAACGAGAGAACGGCCAG)
Codon 510 to 3′ UTR	ALS1530-f (TTTGGGGGCTATGGGGTTTG)	AmALS-R2 (CTAAACGAGAGAACGGCCAG)
Codon 557 to 3′ UTR	AmALS-F2 (TCCCGGTTAAAATCATGCTC)	AmALS-R2 (CTAAACGAGAGAACGGCCAG)

[a] Codons are numbered on the basis of the *Arabidopsis thaliana* ALS sequence (Tranel and Wright 2002).
[b] Untranslated region.

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Park, NC 27709) in 9% dimethyl sulfoxide (DMSO) was added to reactions to obtain final concentrations in 10-fold increments from 0.033 to 330,000 nM imazethapyr, all with 3% DMSO. The no-herbicide control treatment also contained 3% DMSO. A spectrophotometer blank was obtained by adding H_2SO_4 to the reaction before adding protein (Schmitzer et al. 1993). Two separate extracts were obtained from each population for two independent assays, and each assay contained three replications of each treatment (treated statistically as subsamples). Enzyme dose–response data were fit to a four-parameter, nonlinear logistic model in the "drc" package of R version 3.0.0 (R Core Team 2013) as described by Knezevic et al. (2007).

Whole-Plant Dose Response. Uniform, 10- to 12-cm-tall plants were selected from JG11 and WCS populations and treated with a range of dosages of primisulfuron, sulfometuron, imazethapyr, imazapyr, pyrithiobac, propoxycarbazone, or cloransulam (Table 1). Plants from the ACR population were included only at the highest dosage of each herbicide. After herbicide application, plants were returned to the greenhouse and placed in a randomized complete block design. Each treatment had four replicate plants and each herbicide was evaluated in two separate runs.

At 21 d after treatment (DAT), plant injury was visually evaluated and recorded using a scale from 0 to 100, with 0 indicating a plant was dead and without any green tissue and 100 indicating no observed injury. Aboveground plant tissue was harvested and dried at 65 C for 4 to 7 d before collecting dry weight data. Dry weight data (m) and visual rating data (v) were combined to obtain an adjusted dry weight (y) using the function:

$$y = mv/100 \quad [1]$$

This adjustment to dry weight partially accounts for inherent plant-to-plant growth-rate differences in a highly variable species such as waterhemp. For example, a plant that was killed by the herbicide will have an adjusted dry weight of zero, regardless of its actual dry weight.

Adjusted dry weights were expressed relative to the mean of the corresponding population's no-herbicide control. Data from the two runs of each herbicide were pooled and, because Levene's test for homogeneity of variance was not significant, the combined data were fit to dose–response curves as done for the enzyme dose–response data.

ALS Inhibitors Combined with Malathion. Waterhemp plants from the WCS and JG11 population were treated with primisulfuron (1.3 g ha^{-1}), sulfometuron (1.1 g ha^{-1}), cloransulam (0.3 g ha^{-1}), imazethapyr (21 g ha^{-1}), or pyrithiobac (5 g ha^{-1}), each with or without the known cytochrome P450 inhibitor, malathion insecticide (Spectracide Malathion insect spray concentrate, Spectrum Group, Division of United Industries, P.O. Box 142642, St. Louis, MO 63114). Malathion was applied as previously described (Ma et al. 2013). Plants were treated when 10 to 12 cm tall and evaluated 14 DAT as described for the dose–response experiment. Each treatment included six replicate plants and the experiment was repeated in time.

Results and Discussion

In a preliminary greenhouse experiment, plants from the MCR population—a population initially described by Hausman et al. (2011) as resistant to HPPD inhibitors and atrazine—survived a normally lethal rate of either an SU (chlorimuron) or an IMI (imazethapyr) herbicide. However, two distinct resistance phenotypes to ALS inhibitors were observed: some plants appeared to be highly resistant, with no apparent herbicide injury, whereas other plants appeared to be only moderately resistant, displaying stunting and chlorosis (Figure 1). All the plants in Figure 1 were evaluated for the presence of the Trp574Leu ALS mutation, which is the most commonly known mechanism of resistance—and confers a very high level of resistance—to ALS inhibitors in waterhemp (Patzoldt and Tranel 2007). All plants that were highly resistant but none of the plants that were moderately resistant to ALS inhibitors contained the Trp574Leu mutation (data not shown). Two plants lacking the mutation were crossed. The resulting progeny, designated JG11, was used for all subsequent experiments.

ALS Gene Sequencing. ALS gene sequences were obtained from each of eight JG11 plants that survived primisulfuron at dosages lethal to WCS plants, and aligned to reference sequences (Figure 2). Relative to WCS, 29 nucleotide polymorphisms were observed in the JG11 population. The high ALS gene diversity was not surprising and previously has been reported in waterhemp (Tranel et al. 2004). Most of the diversity was due to synonymous polymorphisms, however, with only six resulting in amino acid polymorphisms.

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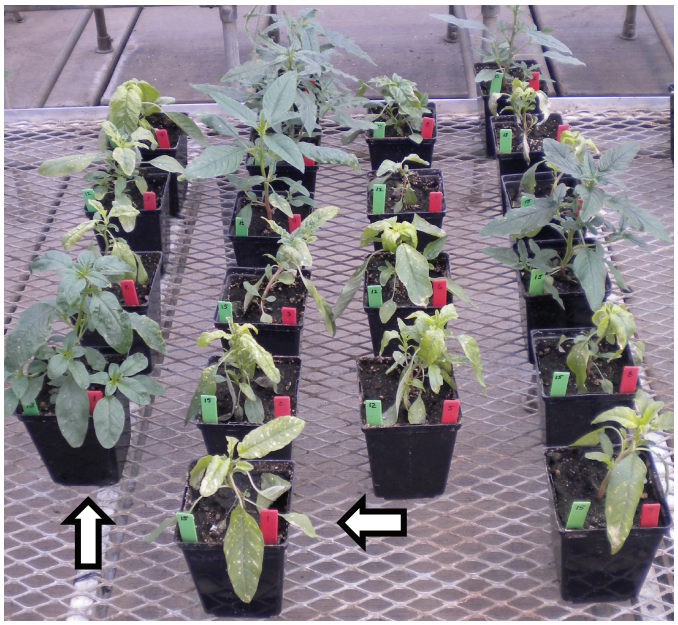

Figure 1. The MCR population contains two resistance phenotypes to acetolactate synthase (ALS) inhibitors. All plants shown survived after herbicide application, although some (e.g., plant above up-pointing arrow) displayed no herbicide injury and others (e.g., plant left of left-pointing arrow) displayed stunting and chlorosis.

Amino acid substitutions at neither Trp574 nor Ser653 were identified in the JG11 population. Substitutions at these two positions previously were identified in waterhemp resistant to ALS inhibitors, and are illustrated by the ACR and IR-101 biotypes. Amino acid substitutions also were not identified at any of the other six amino acid positions previously implicated in weeds resistant to ALS inhibitors (Tranel et al. 2014). The six amino acid substitutions identified in JG11 were shared among the plants sequenced (although the zygosity of each substitution was different among plants) but nevertheless are unlikely to be responsible for resistance: these same substitutions relative to WCS were identified in ACR and IR-101. In addition, these amino acid substitutions were observed in other weed species as well, in both resistant and susceptible biotypes, suggesting that they are in nonconserved regions of ALS and not associated with herbicide resistance (Diebold et al. 2003; McNaughton et al. 2005; Patzoldt and Tranel 2007).

ALS in Vitro Assay. To further investigate the role of possible target-site alterations on resistance in the JG11 population, herbicide dose–response curves for ALS enzyme activity were obtained (Figure 3). The response curves for WCS and JG11 were essentially identical, and the estimated 50% effective doses for WCS (1,642 nM imazethapyr) and JG11 (1,559 nM imazethapyr) were indistinguishable ($P = 0.92$). In contrast, ACR, which was included as a positive control, demonstrated target-site resistance with only a modest reduction in enzyme activity even at the highest imazethapyr concentration. Taken together, results from both ALS gene sequencing and the ALS enzyme assay lead to the conclusion that the observed whole-plant resistance to ALS inhibitors in JG11 is not target-site mediated.

Whole-Plant Dose Response. A series of dose–response experiments was performed to characterize whole-plant resistance of the JG11 population to ALS inhibitors. Injury symptoms typical of ALS inhibitors were observed on plants from both WCS and JG11 populations, whereas little or no injury was observed on ACR plants (data not shown). This

Codon position	198	232	261	283a	313	317	321	371	378	387	388	415	420	424	433	443	460	474	495	516	530	538	574	591	593	595	599	616	653	659
WCS	R	P	Q	T	S	L	K	G	V	S	R	V	R	N	V	E	D	L	F	G	A	D	W	P	N	S	P	T	S	D
ACR			**X**			**X**	**E**	**X**			**X**							**X**	**X**				**L**	**X**		**X**				
IR-101	**X**		**X**			**X**	**E**	**X**			**X**	**I**	**Q**	**K**	**L**	**X**		**X**	**X**						**K**				**T**	
JG11-1			X			X	E	X	X		**X**	I	Q	K	L	X	X	X		X	X	**X**			**K**			X	X	X
JG11-2		X		X	X	X	**E**	**X**	X	X	X	**I**	**Q**	**K**	**L**	**X**	X	**X**		X	X	**X**			**K**			X	X	X
JG11-3		X		X	X	X	**E**	**X**	X	X	X	**I**	**Q**	**K**	**L**	**X**	X	**X**			X	X		X	K	X	X		X	X
JG11-4		X		X	X	X	**E**	**X**	X	X	X	**I**	**Q**	**K**	**L**	**X**	X	**X**			X	X		X	K	X	X		X	X
JG11-5			X			X	E	X	X		**X**	I	Q	K	L	X	X	**X**		X	X	**X**			**K**			X	X	X
JG11-6			X			X	E	X	X		**X**	I	Q	K	L	X	X	X		X	X	**X**			**K**			X	X	X
JG11-7			X			X	E	X	X		**X**	I	Q	K	L	X	X	X	X	X	X	**X**			**K**			X	X	X
JG11-8			X			X	E	X	X		**X**	I	Q	K	L	X	X	X	X	X	X	**X**			**K**			X	X	X

Figure 2. Polymorphisms within the mature-protein-encoding portion of the acetolactate synthase (ALS) gene identified among eight plants of the experimental JG11 population. Polymorphisms are shown relative to the amino acid sequence of a herbicide-sensitive waterhemp biotype (WCS). Sequences of the ACR and IR-101 biotypes, which have resistance-conferring substitutions of Trp574Leu and Ser653Thr, respectively, are also included for comparison. All codons in which a polymorphism was identified are shown. Positions marked with "X" indicate a synonymous nucleotide polymorphism within the codon; nonsynonymous polymorphisms are shown as the amino acids corresponding to each codon. Polymorphisms found in the heterozygous or homozygous condition are shown in regular or bold font, respectively. Codon numbering is relative to the ALS sequence of *Arabidopsis thaliana* (Tranel and Wright 2002); codon "283a" refers to the first of four codon insertions of the waterhemp sequence between codons 283 and 284 of the *A. thaliana* sequence.

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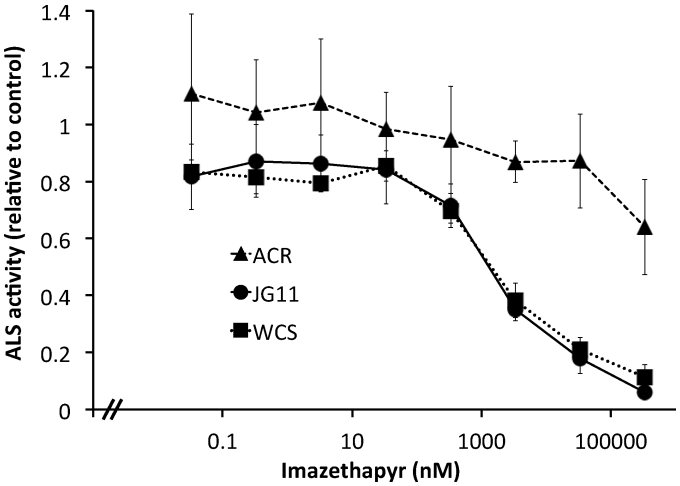

Figure 3. In vitro acetolactate synthase (ALS) enzyme activity dose responses to imazethapyr. JG11 is the experimental population and ACR and WCS are resistant and sensitive controls, respectively. Vertical error bars represent 95% confidence intervals around each mean.

was consistent with our previous observations (e.g., Figure 1). Despite the injury observed to JG11 plants, this population exhibited significant resistance ratios (relative to WCS) to all seven herbicides, which included representatives from each of the five commercialized ALS-inhibitor chemical families (Table 3 and Figure 4). Although resistance ratios for some herbicides were modest (e.g., only threefold for pyrithiobac and propoxycarbazone) and could represent natural variability in herbicide sensitivity among different waterhemp populations, resistance ratios for primisulfuron, imazethapyr, and cloransulam were substantial, ranging from 11- to 90-fold. These latter three herbicides included an SU, an IMI, and a TP. Thus, the JG11 population exhibits broad resistance across ALS-inhibiting herbicides.

One characteristic of metabolism-based herbicide resistance is that it sometimes confers unpredictable cross-resistance to herbicides with different sites of action (Délye 2013). For example, several weed populations with metabolism-based resistance to ALS inhibitors likely were selected by prior exposure to herbicides with different sites of action (Yu and Powles 2014). It is noteworthy that the field from which the original MCR population was obtained did not receive an application of an ALS-inhibiting herbicide in the seven growing seasons before its collection (Hausman et al. 2011). The presence of target-site resistance to ALS inhibitors in some MCR plants indicates prior selection in the field by ALS inhibitors, or immigration of resistance alleles from nearby fields. Whether the nontarget-site resistance arose the same way, or is a byproduct of selection by herbicides with other sites of action, is unknown.

Table 3. Whole-plant resistance to acetolactate synthase (ALS) inhibitors in the experimental JG11 waterhemp population relative to WCS, a sensitive control population.

Herbicide	GR_{50}[a] WCS	GR_{50}[a] JG11	R/S ratio[b]	P value[c]
	g ha^{-1}			
Imazapyr	0.046	0.41	8.9	0.02
Imazethapyr	0.54	10.5	19	< 0.01
Primisulfuron	0.016	0.17	11	0.01
Sulfometuron	0.17	0.97	5.8	< 0.01
Pyrithiobac	1.2	3.5	2.8	0.03
Propoxycarbazone	0.064	0.20	3.1	0.04
Cloransulam	0.08	7.2	90	0.04

[a] Herbicide dose causing a 50% growth reduction, which was determined using a combination of dry weights and visual observations of herbicide responses.
[b] GR_{50} of JG11 divided by GR_{50} of WCS.
[c] Probabilty that the WCS and JG11 GR_{50}s are different.

ALS Inhibitors Combined with Malathion. To determine whether the nontarget-site resistance to ALS inhibitors in the JG11 population might be due to cytochrome P450 monooxygenase activity, plant responses to herbicide with or without malathion were compared (Figure 5). Overall, the addition of malathion to herbicide resulted in greater injury to JG11 plants than that observed with herbicide alone. However, malathion alone resulted in substantial injury to both WCS and JG11 plants, which made ascertaining the synergistic herbicidal effects of malathion difficult. Nevertheless, for each of the five herbicides evaluated, herbicide plus malathion resulted in control of the JG11 population at levels similar to those obtained for WCS by herbicide alone. The observations obtained with the malathion experiment are consistent with resistance to ALS inhibitors being mediated by cytochrome P450s in the JG11 population. However, more research is needed to test this hypothesis.

Ma et al. (2013) concluded that resistance to HPPD inhibitors in the MCR population was due at least in part to elevated cytochrome P450 activity. Possibly the elevated cytochrome P450 activity was selected by HPPD inhibitors, and this activity confers cross-resistance to ALS inhibitors. Alternatively, prior exposure of the population to ALS inhibitors and other herbicides may have played a role in selection of the enhanced herbicide metabolic capabilities of the population. Ma et al. (2013)

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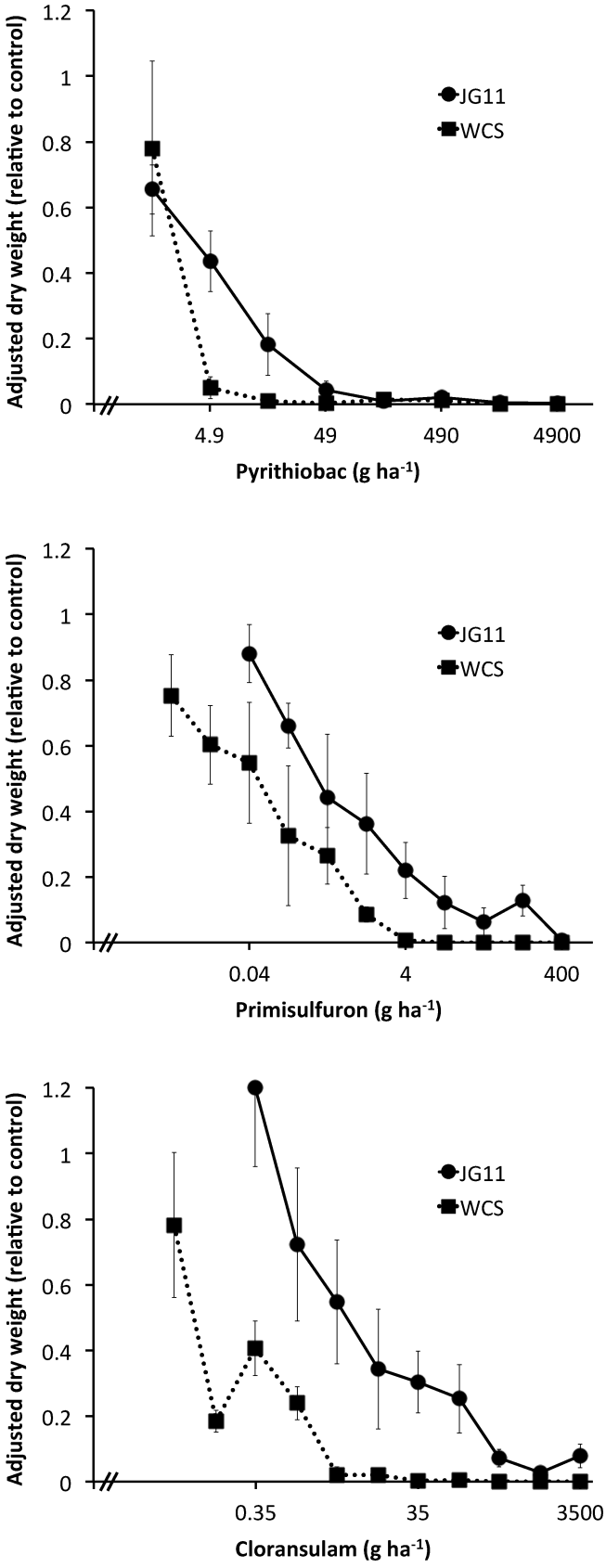

Figure 4. Whole-plant dose responses of the experimental population, JG11, compared with a herbicide-sensitive population, WCS. Responses, which were measured using a combination of dry weights and visual observations and are expressed relative to the nontreated control, are shown for three acetolactate synthase (ALS) inhibitors depicting the range in resistance levels observed (see Table 3). Vertical error bars represent 95% confidence intervals around each mean.

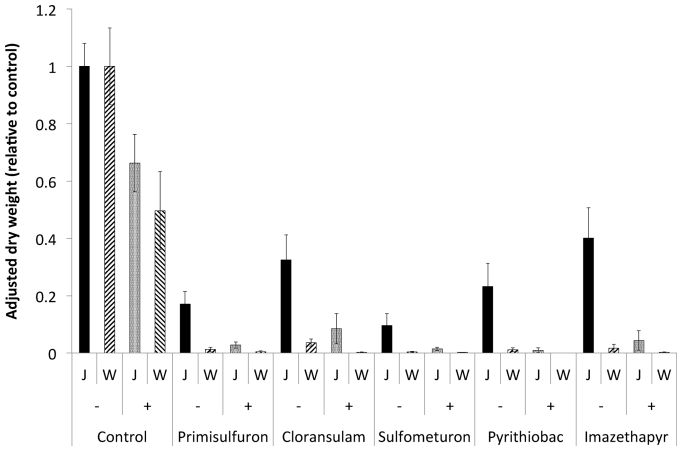

Figure 5. Comparison of whole-plant responses of the experimental population, JG11 (J), and a sensitive population, WCS (W), to acetolactate synthase (ALS)-inhibiting herbicides without (−) or with (+) malathion. Mean responses, which were measured using a combination of dry weights and visual observations and are expressed relative to the nontreated control, are shown with vertical error bars representing 95% confidence intervals.

also concluded that the MCR population has elevated rates of glutathione *S*-transferase activity, and this confers atrazine resistance in the population. Although it is far from clear as to how multiple resistance evolved in the MCR population, it is clear that this population contains multiple resistance mechanisms, and is resistant to inhibitors of PSII, HPPD, and ALS. The numbers of genes responsible for the various nontarget-site resistance mechanisms in the MCR population have not been reported. Genetic analysis, including cosegregation analysis of the different resistance traits, might provide insights on their evolutionary origins.

On the basis of results reported herein, we conclude that the original MCR population contained both target-site and nontarget-site resistance to ALS inhibitors. To date, nontarget-site resistance to ALS inhibitors has been identified in very few eudicot weeds, with wild mustard being the best-characterized example (Veldhuis et al. 2000). Unlike the broad resistance to ALS inhibitors observed in the JG11 population, resistance in the wild mustard population was specific to ethametsulfuron-methyl (Jeffers et al. 1996).

The relative rarity of characterized cases of nontarget-site resistance to ALS inhibitors, particularly in eudicots, likely downplays how frequently it

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actually occurs in weed populations (Délye 2013). For example, nontarget-site resistance to ALS inhibitors recently was inferred in populations of corn poppy (*Papaver rhoeas* L.), but was masked by the presence of target-site resistance within the same populations (Délye et al. 2011). Similarly, the MCR population contained high-level and broad cross-resistance to ALS inhibitors owing to the presence of the ALS Trp574Leu mutation. Only after the nontarget-site resistance in this population was isolated from the target-site resistance, as was done by generating the JG11 population, could it be characterized. Recent studies have begun to identify the genes that are involved with evolved metabolic herbicide resistance (Gaines et al. 2014; Iwakami et al. 2013, 2014). Increased awareness of nontarget-site herbicide resistance, along with candidate genes and next-generation sequencing technologies, promises rapid progress in our understanding of this significant weed management challenge.

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Literature Cited

Bell MS, Hager AG, Tranel PJ (2013) Multiple resistance to herbicides from four site-of-action groups in waterhemp (*Amaranthus tuberculatus*). Weed Sci 61:460–468

Costea M, Weaver SE, Tardif FJ (2005) The biology of invasive alien plants in Canada. 3. Amaranthus tuberculatus (Moq.) Sauer var. rudis (Sauer) Costea & Tardif. Can J Plant Sci 85:507–522

Délye C (2013) Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. Pest Manag Sci 69:176–187

Délye C, Pernin F, Scarabel L (2011) Evolution and diversity of the mechanisms endowing resistance to herbicides inhibiting acetolactate-synthase (ALS) in corn poppy (*Papaver rhoeas* L.). Plant Sci 180:333–342

Diebold RS, McNaughton KE, Lee EA, Tardif FJ (2003) Multiple resistance to imazethapyr and atrazine in Powell amaranth (*Amaranthus powellii*). Weed Sci 51:312–318

Duggleby RG, Pang SS (2000) Acetohydroxyacid synthase. J Biochem Mol Biol 33:1–36

Foes MJ, Liu L, Tranel PJ, Wax LM, Stoller EW (1998) A biotype of common waterhemp (*Amaranthus rudis*) resistant to triazine and ALS herbicides. Weed Sci 46:514–520

Gaines TA, Lorentz L, Figge A, Herrmann J, Maiwald F, Ott MC, Han H, Busi R, Yu Q, Powles SB, Beffa R (2014) RNA-Seq transcriptome analysis to identify genes involved in metabolism-based diclofop resistance in *Lolium rigidum*. Plant J 78:865–876

Hausman NE, Singh S, Tranel PJ, Riechers DE, Kaundun SS, Polge ND, Thomas DA, Hager AG (2011) Resistance to HPPD-inhibiting herbicides in a population of waterhemp (*Amaranthus tuberculatus*) from Illinois, United States. Pest Manag Sci 67:258–261

Hausman NE, Tranel PJ, Riechers DE, Maxwell DJ, Gonzini LC, Hager AG (2013) Responses of an HPPD inhibitor-resistant waterhemp (*Amaranthus tuberculatus*) population to soil-residual herbicides. Weed Technol 27:704–711

Heap I (2014) The International Survey of Herbicide Resistant Weeds. http://www.weedscience.org. Accessed June 11, 2014

Iwakami S, Endo M, Saika H, Okuno J, Nakamura N, Yokoyama M, Watanabe H, Toki S, Uchino A, Inamura T (2014) Cytochrome P450 CYP81A12 and CYP81A21 are associated with resistance to two acetolactate synthase inhibitors in *Echinochloa phyllopogon*. Plant Physiol 165: 618–629

Iwakami S, Uchino A, Kataoka Y, Shibaike H, Watanabe H, Inamura T (2013) Cytochrome P450 genes induced by bispyribac-sodium treatment in a multiple-herbicide-resistant biotype of *Echinochloa phyllopogon*. Pest Manag Sci 70: 549–558

Jeffers GM, O'Donovan JT, Hall LM (1996) Wild mustard (*Brassica kaber*) resistance to ethametsulfuron-methyl but not to other herbicides. Weed Technol 10:847–850

Knezevic SZ, Streibig JC, Ritz C (2007) Utilizing R software package for dose–response studies: the concept and data analysis. Weed Technol 21:840–848

Ma R, Kaundun SS, Tranel PJ, Riggins CW, McGinness DL, Hager AG, Hawkes T, McIndoe E, Riechers DE (2013) Distinct detoxification mechanisms confer resistance to mesotrione and atrazine in a population of waterhemp. Plant Physiol 163:363–377

McNaughton KE, Letarte J, Lee EA, Tardif FJ (2005) Mutations in ALS confer herbicide resistance in redroot pigweed (*Amaranthus retroflexus*) and Powell amaranth (*Amaranthus powellii*). Weed Sci 53:17–22

Patzoldt WL, Tranel PJ (2007) Multiple ALS mutations confer herbicide resistance in waterhemp (*Amaranthus tuberculatus*). Weed Sci 55:421–428

Patzoldt WL, Tranel PJ, Hager AG (2005) A waterhemp (*Amaranthus tuberculatus*) biotype with multiple resistance across three herbicide sites of action. Weed Sci 53:30–36

R Core Team (2013) A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/. Accessed April 15, 2013.

Riggins CW, Peng Y, Stewart CN Jr, Tranel PJ (2010) Characterization of *de novo* transcriptome for waterhemp (*Amaranthus tuberculatus*) using GS-FLX 454 pyrosequencing and its application for studies of herbicide target-site genes. Pest Manag Sci 66:1042–1052

Schmitzer PR, Eilers RJ, Cséke C (1993) Lack of cross-resistance of imazaquin-resistant *Xanthium strumarium* acetolactate synthase to flumetsulam and chlorimuron. Plant Physiol 103:281–283

Steckel LE (2007) The dioecious *Amaranthus* spp.: here to stay. Weed Technol 21:567–570

Tranel PJ, Jiang W, Patzoldt WL, Wright TR (2004) Intraspecific variability of the acetolactate synthase gene. Weed Sci 52:236–241

Tranel PJ, Riggins CW, Bell MS, Hager AG (2011) Herbicide resistances in *Amaranthus tuberculatus*: a call for new options. J Agric Food Chem 59:5808–5812

Tranel PJ, Wright TR (2002) Resistance of weeds to ALS-inhibiting herbicides: what have we learned? Weed Sci 50:700–712

Tranel PJ, Wright TR, Heap IM (2014) Mutations in Herbicide-Resistant Weeds to ALS Inhibitors. http://weedscience.org/mutations/mutationdisplayall.aspx. Accessed: June 11, 2014

Veldhuis LJ, Hall LM, O'Donovan JT, Dyer W, Hall JC (2000) Metabolism-based resistance of a wild mustard (*Sinapis arvensis* L.) biotype to ethametsulfuron-methyl. J Agric Food Chem 48:2986–2990

Yu Q, Powles SB (2014) Resistance to AHAS inhibitor herbicides: current understanding. Pest Manag Sci 70:1340–1350

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