Weed Science 2015 63:748–757

Acetolactate Synthase–Inhibiting, Herbicide-Resistant Rice Flatsedge (*Cyperus iria*): Cross-Resistance and Molecular Mechanism of Resistance

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Overuse of acetolactate synthase (ALS)–inhibiting herbicides in rice has led to the evolution of halosulfuron-resistant rice flatsedge in Arkansas and Mississippi. Resistant accessions were cross-resistant to labeled field rates of ALS-inhibiting herbicides from four different families, in comparison to a susceptible (SUS) biotype. Resistance index of Arkansas and Mississippi accessions based on an R/S ratio of the lethal dose required for 50% plant mortality (LD_{50}) to bispyribac-sodium, halosulfuron, imazamox, and penoxsulam was ≥ 21-fold. Control of Arkansas, Mississippi, and SUS accessions with labeled field rates of 2,4-D, bentazon, and propanil was ≥ 93%. An enzyme assay revealed that an R/S ratio for 50% inhibition (I_{50}) of ALS for halosulfuron was 2,600 and 200 in Arkansas and Mississippi, respectively. Malathion studies did not reveal enhanced herbicide metabolism in resistant plants. The ALS enzyme assay and cross-resistance studies point toward altered a target site as the potential mechanism of resistance. Trp_{574}–Leu amino acid substitution within the *ALS* gene was found in both Arkansas and Mississippi rice flatsedge accessions using the Illumina HiSeq platform, which corresponds to the mechanism of resistance found in many weed species. Field-rate applications of 2,4-D, bentazon, and propanil can be used to control these ALS-resistant rice flatsedge accessions.

Nomenclature: 2,4-D; acetolactate synthase; bentazon; bispyribac-sodium; halosulfuron; imazamox; malathion; propanil; rice flatsedge, *Cyperus iria* L; rice, *Oryza sativa* L.

Key words: ALS assay, ALS gene sequencing, cytochrome P450 monooxygenase–based resistance, herbicide-resistance mechanism, Illumina HiSeq, rice flatsedge.

Rice flatsedge, an annual sedge with a fibrous root system and a C_4 photosynthetic pathway, is a major agricultural weed in rice production systems (Bryson and Carter 2004). Its seedlings emerge shortly after rice is sown and are capable of flowering in 1 mo to produce a second generation in the same growing season (Galinato et al. 1999). Rice interference can reduce rice flatsedge shoot biomass and inflorescence production but cannot completely control these plants because they have evolved a mechanism of stem elongation to avoid shading (Chauhan and Johnson 2010).

Herbicide options for weed control in conventional, midsouthern U.S. rice are 2,4-D, bentazon, clomazone, cyhalofop, fenoxaprop, pendimethalin, propanil, quinclorac, thiobencarb, triclopyr, and acetolactate synthase (ALS)–inhibiting herbicides belonging to pyrimidinyl (thio) benzoate (PB) (bispyribac-sodium), sulfonylurea (SU) (bensulfuron-methyl, halosulfuron, and imazosulfuron), and triazolopyrimidine (TP) (penoxsulam) chemical families (Scott et al. 2012). Of these herbicides, clomazone, quinclorac, pendimethalin, and thiobencarb are applied PRE, delayed PRE, or POST, and the remaining herbicides are applied POST. Imazethapyr is applied PRE or POST in imidazolinone-resistant rice.

Timing of POST herbicide applications for prominent rice weeds, such as sedges (*Cyperus* spp.), barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.], and weedy rice (*Oryza sativa* L.) often overlap (Baltazar and Smith 1994). Clomazone applied PRE and graminicides, such as cyhalofop and fenoxaprop, applied POST can control barnyardgrass but have no activity on sedges (Jordan 1995). Propanil, a commonly used rice herbicide, can control both barnyardgrass and sedges when

DOI: 10.1614/WS-D-15-00014.1

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applied POST, but barnyardgrass in Arkansas and Mississippi has evolved resistance to propanil (Baltazar and Smith 1994) and quinclorac or both (Lovelace 2003). Another important sedge weed species of rice, smallflower umbrella sedge (*Cyperus difformis* L.) has evolved resistance to propanil in California (Valverde et al. 2014). Because of morphological and physiological similarities with rice, there are limited herbicide options for weedy rice control in dry-seeded conventional rice, but ALS-inhibiting herbicides of the imidazolinone (IMI) family (imazethapyr and imazamox) can be used to control weedy rice in IMI-resistant rice.

Because of propanil and quinclorac resistance evolution in barnyardgrass and few available herbicides for controlling weedy rice, the use of ALS-inhibiting herbicides has increased worldwide in conventional and IMI-resistant rice production systems (Sudianto et al. 2014). Imazethapyr, applied PRE followed by POST, controlled barnyardgrass and rice flatsedge ≥ 98% in IMI-resistant rice (Levy et al. 2006). A single application of clomazone at 448 g ai ha^{-1} applied at the rice pegging stage (4 to 7 d after planting) did not control rice flatsedge; however, a subsequent application of halosulfuron at 26 g ai ha^{-1} to rice at the three- to four-leaf stage provided ≥ 90% control of rice flatsedge (Mudge et al. 2005). Weedy rice and barnyardgrass control in IMI-resistant rice with imazethapyr-containing, POST-only programs was ≥ 90% (Webster et al. 2012). Overreliance on ALS-inhibiting herbicides, however, led to the evolution of ALS-resistant barnyardgrass (Panozzo et al. 2013; Riar et al. 2012, 2013) and weedy rice (Burgos et al. 2008; Norsworthy et al. 2013; Roso et al. 2010) worldwide. In 2010, halosulfuron failed to control rice flatsedge in some rice fields in Arkansas and Mississippi (Heap 2015).

Two prominent mechanisms of resistance in ALS-inhibiting, herbicide-resistant weed species are altered target site and enhanced herbicide metabolism by cytochrome P450 monooxygenases (CYP) (reviewed by Powles and Yu 2010; Siminszky 2006; Tranel and Wright 2002). Naturally occurring amino acid substitutions in the ALS enzyme that have imparted resistance to ALS-inhibiting herbicides in weed species are Ala_{122} to Thr, Tyr, or Val; Pro_{197} to Ala, Arg, Asn, Gln, His, Ile, Leu, Ser, or Thr; Ala_{205} to Val; Asp_{376} to Glu; Arg_{377} to His; Trp_{574} to Leu; Ser_{653} to Asn, Ile, or Thr; and Gly_{654} to Asp (Tranel et al. 2015). Multiple mechanisms of resistance have been found in some ALS-resistant barnyardgrass accessions from Arkansas and Mississippi (Riar et al. 2012). Mechanisms of resistance in ALS-resistant barnyardgrass include reduced herbicide translocation, which might be due to enhanced metabolism of the herbicide in the treated leaf, and an altered target site (Riar et al. 2013). These mechanisms of resistance have imparted variable levels of resistance to ALS-inhibiting herbicides belonging to different chemical families. Enhanced herbicide metabolism and not reduced ALS sensitivity was the mechanism of resistance in an ALS-resistant rice barnyardgrass [Echinochloa phyllopogon (Stapf) Koso-Pol] biotype from California (Fischer et al. 2000).

With evolution of resistance to halosulfuron in rice flatsedge, there was an imminent need to evaluate cross-resistance of rice flatsedge accessions from Arkansas and Mississippi to ALS-inhibiting herbicides from other chemical families and alternative herbicides for the control of ALS-resistant rice flatsedge. In addition, knowing the level of resistance and the mechanisms of resistance would be useful in developing programs for the management of resistant rice flatsedge accessions. Accordingly, experiments were conducted to (1) evaluate cross-resistance to the ALS-inhibiting herbicides (bispyribac-sodium, halosulfuron, imazamox, imazethapyr, and penoxsulam), (2) evaluate alternative rice herbicides (2,4-D, bentazon, propanil, quinclorac, and thiobencarb) for rice flatsedge control, (3) characterize the level of resistance to ALS-inhibiting herbicides, and (4) determine the mechanism of ALS-inhibiting herbicide resistance in rice flatsedge accessions from Arkansas and Mississippi.

Materials and Methods

Plant Material and Growth Conditions. Seeds of putative, halosulfuron-resistant rice flatsedge accessions were collected from fields under continuous IMI-resistant rice in Arkansas and Mississippi in 2009. Accessions from Arkansas and Mississippi were confirmed resistant to a labeled field rate of halosulfuron (53 g ai ha^{-1}) in comparison to a known susceptible rice flatsedge (SUS) accession collected from a field that was never treated with ALS-inhibiting herbicides. The greenhouse studies were conducted at the University of Arkansas (Fayetteville, AR). Plants from each accession were self-pollinated for two generations in a growth chamber under 30/20 ± 3 C day/night temperatures and 16-h photoperiod. Seeds of resistant and susceptible accessions were sown in the greenhouse under conditions similar to the growth chamber in

Table 1. Information about the herbicides used in cross-resistance and alternative herbicide study.

Common name	Trade name	Field rate	Manufacturer	
			Name	Address
		g ai ha^{-1}		
Bispyribac-sodium	Regiment	35	Valent U.S.A. Corp.	Walnut Creek, CA 94596
Halosulfuron	Permit	53	Gowan Company	Yuma, AZ 85364
Imazamox	Beyond	35	BASF Corp.	Research Triangle, NC 27709
Imazethapyr	Newpath	70	BASF Corp.	Research Triangle, NC 27709
Penoxsulam	Grasp	49	Dow AgroSciences	Indianapolis, IN 46268
Bentazon	Basagran	840	Winfield Solutions	St. Paul, MN 55164
Propanil	Riceshot	4480	RiceCo	Memphis, TN 38137
Quinclorac	Facet	560	BASF Corp.	Research Triangle, NC 27709
Thiobencarb	Bolero	4480	Valent U.S.A. Corp.	Walnut Creek, CA 94596
2,4-D	Weedar 64	1065	Nufarm Agricultural Products	Burr Ridge, IL 60527

individual 55.5- by 26.5- by 5.5-cm^3 plastic trays using commercial nonsterile potting mix (Sun Gro Horticulture Distribution Inc., Bellevue, WA). Plants of each accession were transplanted to 15-cm-diam pots at the one- to two-leaf stage for whole-plant bioassays (four plants per pot) and dose–response experiments (one plant per pot). Plants were watered daily and once weekly with a water-soluble fertilizer (Miracle-Gro® Water-Soluble All-Purpose Plant Food, Scotts Miracle-Gro Products, Inc., Marysville, OH).

Whole Plant Bioassays. The experiment was conducted in a randomized complete-block design with a 3 (accessions: Arkansas, Mississippi, and SUS) by 10 (herbicide treatments applied at three- to four-leaf stage) factorial arrangement of treatments and four replications (Table 1). Adjuvant was added to herbicide treatments in accordance with label recommendations. Nontreated control and solvent only (nonionic surfactant [NIS] at 0.25% v/v) treatments were also included. A single 15-cm-diam pot with four plants of the same accession represented one replication (16 plants per treatment with four replications). The experiment was repeated.

Herbicide treatments were applied using a compressed-air sprayer fitted with a boom containing two flat-fan 800067 nozzles (TeeJet® Technologies, Springfield, IL) calibrated to deliver 187 L ha^{-1} at 276 kPa. Visual assessments of plant response were taken 21 d after treatment (DAT) on a scale of 0 (no injury) to 100 (complete plant mortality). Data were arcsine square-root transformed before analyses to improve normality and were subjected to ANOVA using PROC MIXED in SAS (Version 9.1.3., SAS Institute Inc., Cary, NC) to evaluate the effect of different herbicides on control of rice flatsedge accessions. There were no treatment-by-experiment interactions; therefore, data from the two runs were pooled, and means were separated using Fisher's protected LSD at $\alpha = 0.05$. Data were also analyzed separately by herbicide to test for differences in response of each accession to these herbicides.

Dose–Response Experiments with ALS-Inhibiting Herbicides. Seedlings at the one- to two-leaf stage of each rice flatsedge accession were transplanted to separate 15-cm-diam pots. Plants of all three rice flatsedge accessions, at three- to four-leaf stage, were treated with eight doses of bispyribac-sodium, imazamox, and penoxsulam (the labeled field rate for bispyribac-sodium, imazamox, and penoxsulam was 35, 35, and 49 g ai ha^{-1}, respectively). The experiment was laid out in a completely randomized design with 20 replications (one plant per replication) per herbicide dose and was repeated. Rates of all three herbicides for SUS were 0, 1/64, 1/16, 1/8, 1/4, 1/2, 1, and 2 times the labeled field rate, and for Arkansas and Mississippi, the rates were 0, 1/2, 1, 2, 4, 8, 16, and 32 times the labeled field rate. Resistance to halosulfuron was also characterized using 0, 1/128, 1/64, 1/16, 1/8, 1/4, 1/2, 1, and 2 times the labeled field rate of halosulfuron for SUS and 0, 1/4, 1/2, 1, 2, 4, 8, 16, 32, and 64 times the field rate of halosulfuron for Arkansas and Mississippi accessions. Adjuvants were added to the herbicide treatments as mentioned above. Spraying solutions with adjuvants only were used as the nontreated controls. Treatment effect on plant mortality was recorded at 21 DAT. Data were subjected to probit analysis using PROC PROBIT in

SAS to determine the lethal dose needed to kill 50% (LD_{50}) of the treated plants of each accession.

ALS In Vitro Inhibition Assay. ALS inhibitor-resistant and -susceptible rice flatsedge plants were grown as in the cross-resistance study. ALS enzyme activity from plants at the three- to four-leaf stage was assayed in vitro using procedures similar to previous descriptions (Nandula and Messersmith 2000). Enzyme/protein was extracted from 4 g of fresh tissue, bulked from 10 to 15 plants, by grinding under liquid nitrogen. Each replication represented an independent extraction from a shoot sample. Herbicide concentrations used to inhibit ALS enzyme activity were 0, 0.001, 0.01, 0.1, 1, 10, 100, 1,000 μM for halosulfuron. This assay measured acetoin that was formed from acid decarboxylation of acetolactate. Background acetoin sources were included as controls. The experimental lay out was a completely randomized, factorial design with three replications per treatment (herbicide concentrations). The experiment was conducted three times. ANOVA was conducted using PROC GLM in SAS. Data from the three runs of the experiment were pooled because of no significant experimental effect (P = 0.8123). Nonlinear regression analysis was applied to define a three-parametric power equation of the following form:

$$y = y_0 + ax^b \quad [1]$$

to relate the effect of herbicide concentration (x) on ALS activity (y), where y_0 is an asymptote, a is a constant, and b is the slope of the curve. Equation parameters were computed using SigmaPlot (Version 12.5, Systat Software Inc., San Jose, CA).

CYP Inhibition by Malathion. The experiment was arranged in a randomized complete-block design with factorial arrangement of three biotypes (Arkansas, Mississippi, and SUS) and two herbicidal solutions (halosulfuron alone or in mixture with malathion at 1,000 g ai ha^{-1}). Ten plants at the three- to four-leaf stage were treated with herbicidal solutions, and spray adjuvants were added to all treatments as recommended on the label of these herbicides. The experiment was repeated. A treatment containing only malathion and a nontreated control for each accession was also included. Sprayer configuration and growth conditions were the same as for the cross-resistance experiments. Plant mortality was recorded at 21 DAT, and a chi-square test was performed with PROC FREQ in SAS to determine whether the addition of malathion to each herbicide increased mortality.

***ALS* Gene Amplification and Sequencing.** DNA was extracted from individual Arkansas and Mississippi plants surviving a field-rate application of halosulfuron and from a known susceptible plant, according to the methods of Murray and Thompson (1980). Primers for the rice flatsedge *ALS* gene were developed based on multiple alignments of the ALS coding-region sequences of rock bulrush [*Schoenoplectus juncoides* (Roxb.) Lye] (GenBank AB257441 and AB257443) and smallflower umbrella sedge (GenBank EF061294) using ClustalW2 multiple sequence alignment program (available online at http://www.ebi.ac.uk/Tools/msa/clustalw2/). Primers intended to amplify the partial-coding sequence of the rice flatsedge *ALS* gene were designed using the Oligo Calculator (Version 3.26 available online at http://www.basic.northwestern.edu/biotools/OligoCalc.html) and were synthesized by Eurofins MWG Operon (Eurofins MWG Operon, Huntsville, AL). Functional primers used to amplify coding and noncoding regions of the rice flatsedge *ALS* gene are listed in Figure 1 and Table 2. Polymerase chain reaction (PCR) amplification, amplified product purification, and DNA sequencing and analysis were performed using procedures described previously by Riar et al. (2013).

***ALS* Gene Assembly, Mapping, and Single-Nucleotide Polymorphism (SNP) Detection.** To verify the coding region of the *ALS* gene and to confirm SNPs detected via Sanger sequencing, the whole transcriptome of all three accession was sequenced via the parallelized sequencing platform using the Illumina HiSeq (Illumina, San Diego, CA; http://www.illumina.com/). Assembling and polymorphism detection was according to Brautigam and Gowik (2010) suggestions for a nonmodel organism with no resort transcriptome. RNA was extracted using a Qiagen (Valencia, CA) RNeasy Plant Mini Kit according to the manufacturer's protocol. RNA preparation and Illumina sequencing were conducted at the HudsonAlpha Institute for Biotechnology (Huntsville, AL). Sequencing reads were trimmed in CLC Genomic Workbench 6.0 (CLC Bio, Primset, Denmark; http://www.clcbio.com/) and assembled using Trinity RNASeq de novo assembler (http://trinityrnaseq.github.io/) (Brautigam and Gowik 2010; Haas et al. 2013). The *ALS* contigs were extracted from the three assemblies by searching against the ALS protein

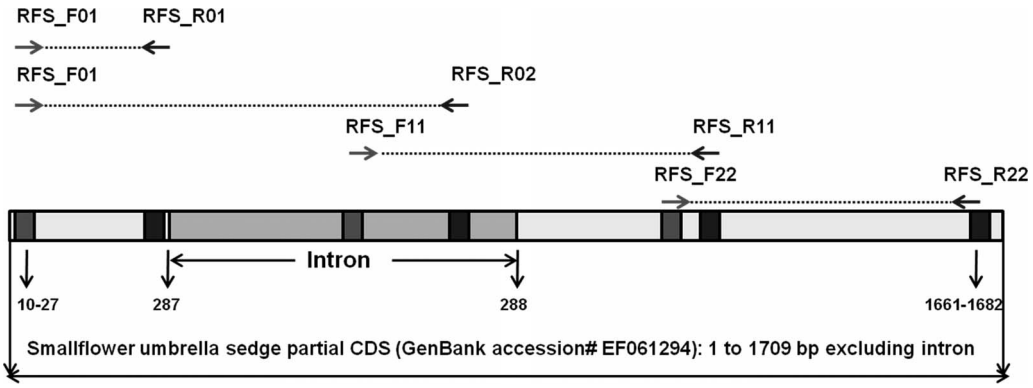

Figure 1. Putative locations of the primers with respect to smallflower umbrella sedge partial coding sequence of acetolactate synthase (*ALS*) gene used to amplify and sequence the *ALS* gene of ALS-susceptible (SUS) and ALS-resistant (Arkansas and Mississippi) rice flatsedge. Shaded region between nucleotide 287 and 288 of the smallflower umbrella sedge ALS sequence (GenBank EF061294) represents the corresponding location of the intron in the rice flatsedge ALS sequence.

sequence of *Schoenoplectiella wallichii* (Nees) Lye (AB492863) using tblastn within CLC Genomics Workbench. Subsequently, the *ALS* contigs were aligned using Clustal Omega (McWilliam et al. 2013). The trimmed reads were mapped to their corresponding *ALS* contigs using the Map Reads to Reference function in CLC Genomic Workbench, with a parameter set of mismatch cost = 3, insertion cost = 3, deletion cost = 3, length fraction = 0.9, and similarity fraction = 0.9.

Results and Discussion

Cross Resistance to ALS Inhibitors and Response to Alternate Herbicides. Control of susceptible rice flatsedge plants with the field rate of all ALS-inhibiting herbicides was ≥ 98% (Table 3). In contrast, control of both resistant accessions with the field rate of ALS-inhibiting herbicides was ≤ 6%, showing that they have evolved cross-resistance to all the tested ALS-inhibiting herbicides (i.e., bispyribac-sodium, halosulfuron, imazamox, imazethapyr, and penoxsulam belonging to PB, SU, IMI, IMI, and TP families, respectively). Among sedges, smallflower umbrella sedge in California has evolved cross-resistance to all five ALS-inhibiting herbicide families (Merotto et al. 2009) and rock bulrush in Japan has several accessions that have evolved resistance only to SU herbicides (Kohara et al. 1999) or cross-resistance to SU, IMI, and PB herbicides (Uchino et al. 2007).

Field-rate applications of 2,4-D, bentazon, and propanil controlled all the accessions ≥ 93%, whereas quinclorac and thiobencarb provided ≤ 52% control, with no differences among all three accessions (Table 3). However, 2,4-D cannot be used in some areas of Arkansas and Mississippi because of potential drift injury to sensitive crops, such as cotton (*Gossypium hirsutum* L.). The University of Arkansas currently recommends a combination of propanil + thiobencarb early POST followed by propanil + bentazon before flooding for control of ALS-resistant rice flatsedge. Propanil and bentazon provide good POST control, whereas thiobencarb provides good residual control, albeit, marginal POST control as seen in Table 3. In addition, as shown in Table 3, quinclorac is not an effective POST option nor does it provide effective residual control (residual activity is not shown in Table 3). Bentazon and propanil belong to photosystem II (PSII)–inhibitor group of herbi-

Table 2. Primers used for rice flatsedge *ALS* gene amplification and sequencing.

Primer	Sequence (5′–3′-')	Amplicon size	Annealing temperature
		pb	°C
RFS_F01	GTGGAGGCGCTCGAGAGA		60
RFS_R01	GTCCAGTGATTGCGACCAT	280	60
RFS_R02	TTGGTGATACCTTCGCTATCC	1,450	60
RFS_F11	CAGCAAGGCCTGGATTTTGTAT	1,130	60
RFS_R11	AGTTACCAAGACCCATCAAGGT		60
RFS_F22	TGTTCCAACTCAGGTGCAGA	1,050	60
RFS_R22	ATCATATCCTTGAATGCTCCTC		60

Table 3. Control of susceptible and resistant rice flatsedge accessions with recommended field rates of ALS-inhibiting and other herbicides labeled for rice at 21 d after treatment.[a]

Herbicide	Rate	Control					
		SUS		Arkansas		Mississippi	
	g ai ha^{-1}	%					
Bispyribac-sodium	35	100	aA	5	eB	5	dB
Halosulfuron	53	100	aA	3	eB	4	dB
Imazamox	35	98	aA	5	eB	5	dB
Imazethapyr	70	99	aA	5	eB	6	dB
Penoxsulam	49	100	aA	4	eB	5	dB
2,4-D	1,065	98	aA	96	abAB	94	aB
Bentazon	840	100	aA	100	aA	98	aA
Propanil	4,480	100	aA	93	bB	98	aA
Thiobencarb	4,480	39	bB	33	cB	52	bA
Quinclorac	560	24	cA	14	dB	22	cA

[a] Means for each accession within a column followed by the same lowercase letters and means for each herbicide within a row followed by the same uppercase letters are not significantly different according to Fisher's protected LSD test ($\alpha = 0.05$).

cides, to which other rice weeds, such as barnyardgrass (Baltazar and Smith 1994) and smallflower umbrella sedge (Valverde et al. 2014), have evolved resistance. Thus, prudent use of PSII-inhibiting herbicides is recommended for the management of ALS-resistant rice flatsedge. Rinskor™ active (ISO name applied for), a new arylpicolinate herbicide from Dow AgroSciences (Indianapolis, IN) can control key, midsouthern U.S. rice weeds and can be a possible management option for ALS-, PSII-, and quinclorac-resistant grass and sedge weeds, including barnyardgrass, rice flatsedge, smallflower umbrella sedge, and yellow nutsedge (*Cyperus esculentus* L.), in the future (Perry et al. 2015; Weimer et al. 2015; Yerkes et al. 2015).

Resistance Level to ALS-Inhibiting Herbicides. Resistant rice flatsedge accessions were cross-resistant to bispyribac-sodium, halosulfuron, imazamox, imazethapyr, and penoxsulam compared with the SUS biotype (Table 3). Even the highest dose of halosulfuron (3,368 g ha^{-1}) did not kill 50% of the Arkansas and Mississippi plants. In contrast, 6.9 g ha^{-1} of halosulfuron killed 50% of SUS plants, showing that resistant plants were > 483-fold more resistant to halosulfuron compared with SUS plants (Table 4). The Arkansas and Mississippi accessions were > 68-fold resistant to imazamox compared with SUS plants, with the LD_{50} of Arkansas and Mississippi plants being > 1,120 g ha^{-1} and that of the SUS plants being only 16 g ha^{-1}. Similarly, Arkansas and Mississippi plants were > 29-fold resistant to penoxsulam compared with SUS. Arkansas and Mississippi accessions showed varying level of resistance to bispyribac-sodium and were found to be > 49 and 21-fold more resistant, compared with SUS, respectively (Table 4). Bispyribac-sodium LD_{50} values of Arkansas, Mississippi, and SUS accessions were > 1,120, 473, and 23 g ha^{-1}, respectively. Similarly, variability in cross-resistance to ALS inhibitors has been documented in barnyardgrass accessions from Arkansas and Mississippi (Riar et al. 2012), rock bulrush accessions from Japan (Kohara et al. 1999), and smallflower umbrella sedge accessions from California (Merotto et al. 2009).

ALS Enzyme Assay. Results from the ALS enzyme assays revealed target-site resistance to halosulfuron in Arkansas and Mississippi (Table 5). The I_{50} values for Arkansas, Mississippi, and SUS plants were 65, 5, and 0.025 μM for halosulfuron, respectively, resulting in resistance index values (RI) of 2,600 and 200 for Arkansas and Mississippi, respectively. In general, a high level of resistance based on ALS enzyme inhibition by ALS-inhibiting herbicides indicates the presence of one or more point mutations resulting in amino acid substitutions in the *ALS* gene sequence of the resistant populations (Merotto et al. 2009; Nandula and Messersmith 2000; Riar et al. 2013).

***ALS* Gene Sequencing (Sanger Method).** The *ALS* gene of rice flatsedge has been sequenced for the first time and sequences have been submitted to GenBank as accession numbers: KF667362 (SUS), KF667363 (Arkansas), and KF667364 (Mississippi). The sequences of SUS (3,047 bp), Arkansas (3,036 bp), and Mississippi (3,036 bp) contained partial ALS coding sequences of 1,662, 1,651, and

Table 4. Dose of bispyribac-sodium, halosulfuron, imazamox, and penoxsulam required to kill 50% (LD_{50}) of rice flatsedge plants of the susceptible population (SUS) and the Arkansas and Mississippi accessions (with 95% CI in parenthesis).

Herbicide	Accession	LD_{50}[a]		RI[b]	
		g ai ha^{-1}		R : S	
Bispyribac	Arkansas	> 1120		49	(5–124)
	Mississippi	478	(383–621)	21	(1.8–69)
	SUS	23	(9.0–222)		
Halosulfuron	Arkansas	> 3368		> 483	(403–592)
	Mississippi	> 3368		> 483	(403–592)
	SUS	6. 9	(5.7–8.4)		
Imazamox	Arkansas	> 1120		> 68	(6.2–249)
	Mississippi	> 1120		> 68	(6.2–249)
	SUS	16	(4.5–182)		
Penoxsulam	Arkansas	> 1568		> 29	(5.7–57)
	Mississippi	> 1568		> 29	(5.7–57)
	SUS	53	(27–274)		

[a] LD_{50} was determined by conducting probit analysis in SAS.
[b] Resistance index (RI) was calculated by dividing the LD_{50} dose of the resistant accession by the LD_{50} of susceptible accession.

1,651 bp, respectively. The consensus sequences containing a 1,385 bp intron at the same position in all three accessions were identified and compared with smallflower umbrella sedge ALS sequence (GenBank EF061294).

Introns of Arkansas, Mississippi, and SUS *ALS* genes were identical, and no mutations were observed. The presence of intron within the *ALS* coding sequences has been reported in polyploid species e.g., rock bulrush (Uchino et al. 2007) and falsepimpernel (*Lindernia* All. spp.) (Uchino and Watanabe 2002), containing multiple copies of the genes. The presence of intronic and intronless *ALS* genes in ricefield bulrush [Schoenoplectus mucronatus (L.) Palla] represents gene isoforms in this species (Scarabel et al. 2010).

Alignment of assembled sequences of Arkansas, Mississippi, and SUS with *Arabidopsis thaliana* (L.) Heynh. The *ALS* coding sequence (GenBank NM114714), matched from the 104th amino acid (Val_{104}) to 653rd amino acid (Ser_{653}). These amino acid sequences spanned more than six highly conserved domains (domain C [amino acid 115 to 133], domain A [amino acid 191 to 203], domain D [amino acid 205 to 219], domain F [amino acid 365 to 380], domain B [amino acid 573 to 576], and domain E [amino acid 651 to 655]). Comparison of sequences revealed only eight SNPs in Arkansas and Mississippi, compared with the SUS (Glu_{147}, Ser_{152}, Thr_{158}, Val_{163}, Glu_{167}, Ser_{168}, Thr_{173}, and Val_{187}), and all were silent.

The reason for not finding the ALS herbicide-resistance mutations, i.e., Trp_{574}–Leu (see below), could be because errors occurred during PCR amplifications (Uchino et al. 2007) because intron sequences from all accessions contained a number of stop codons in all three reading frames. In addition, multiple *ALS* genes could be present in rice flatsedge, as observed in other sedge species, including smallflower umbrella sedge (Merotto et al. 2009) and ricefield bulrush (Scarabel et al. 2010). Multiple *ALS* genes in ricefield bulrush were

Table 5. Halosulfuron dose required for I_{50} of ALS enzyme of SUS, Arkansas, and Mississippi rice flatsedge accessions and corresponding regression equation parameters.[a]

Herbicide	Accession	Regression parameters[b]				I_{50}	RI[c]
		y_0	a	b	R^2		
						µM	R : S
Halosulfuron	Arkansas	99.8	−36.7	0.11	0.95	65	2,600
	Mississippi	99.3	−39.7	0.12	0.97	5	200
	SUS	100.2	−70.4	0.05	0.98	0.025	

[a] Abbreviations: ALS, acetolactate synthase; I_{50}, herbicide concentration required to cause a 50% inhibition of ALS enzyme activity in vitro; RI, resistance index; SUS, susceptible biotype.
[b] Regression equation parameters were generated by fitting a nonlinear regression equation of the form $y = y_0 + ax^b$ to the response of the ALS enzyme to the herbicide concentration. Details are provided in the text.
[c] RI was calculated by dividing the I_{50} values of the Arkansas and Mississippi accessions by the I_{50} of the SUS accession.

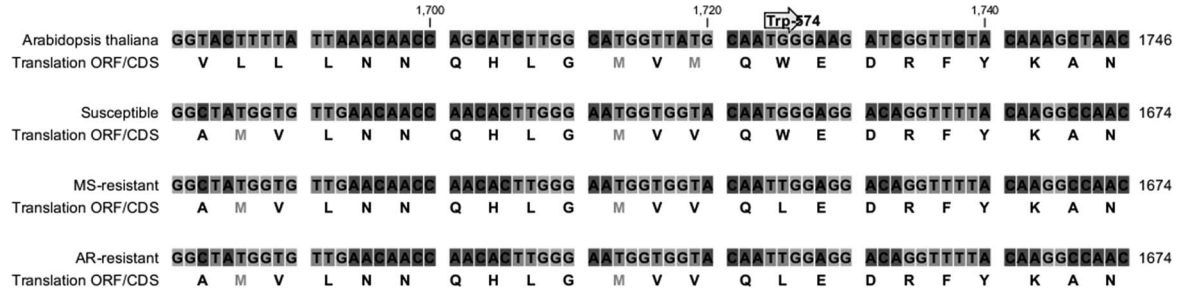

Figure 2. The alignment of the *ALS* transcripts from the susceptible and resistant rice flatsedge biotypes with *Arabidopsis thaliana* around the resistance-conferring codon Trp_{574}.

characterized by cytosine methylation, influencing the expression profile, and leading to detection of one gene with more frequency compared with the others (Scarabel et al. 2010). According to Merotto et al. (2009), in spite of differences in *ALS* gene activity between ALS-resistant and ALS-susceptible smallflower umbrella sedge, no amino acid substitution at the known sites of mutation were found in the partial *ALS* gene sequence of the ALS-resistant biotype. Point mutations endowing resistance e.g., Pro_{197}–His and Trp_{574}–Leu, were detected in *ALS1* of ricefield bulrush accessions, which had a higher detection frequency compared with other two *ALS* isoforms (Scarabel et al. 2010). Therefore, to elucidate the ALS-inhibiting herbicide-resistance mechanism of rice flatsedge accessions, the whole *ALS* gene was sequenced through next-generation sequencing technology.

Resistance-Endowing Mutation Detection. Complete coding sequences of ALS transcripts (1,941 bp) were assembled for the three rice flatsedge accessions. By aligning with the *Arabidopsis thaliana* *ALS* gene, a G to T mutation was identified to cause the Trp_{574}–Leu substitution in both Arkansas- and Mississippi-resistant biotypes (Figure 2). Mapping reads to the coding regions within the corresponding contigs revealed no polymorphism at the Trp_{574} codon, indicating that the resistance endowing mutations in the resistant biotypes were homozygous. Amino acid substitution of Trp_{574} to Leu has been detected in many ALS-resistant weed species, conferring high levels of resistance to multiple ALS-inhibiting herbicide families including SU, IMI, and PB (McElroy et al. 2013; Powles and Yu 2010).

CYP Inhibition by Malathion. Mortality of Arkansas, Mississippi, and SUS plants treated with a labeled field rate of tested ALS-inhibiting herbicides was similar when applied in mixtures with malathion at 1,000 g ai ha^{-1}, suggesting that the mechanism of resistance may be not associated with metabolism by CYP (data not shown). On the other hand, failure to observe plant growth reduction with POST application of the malathion/herbicide mixture could be due to the presence of CYP isozymes. According to Donaldson and Luster (1991), CYP hemoproteins can be inhibited selectively by various derivatives of triazole, imidazole, and pyrimidine, e.g., piperonyl butoxide. Therefore, ^{14}C-labeled herbicide studies need to be conducted to rule out metabolism as the mechanism of resistance. In spite of extensive search for vendors, we could not obtain ^{14}C-halosulfuron to carry out absorption/translocation/metabolism studies. Future ^{14}C studies focused on absorption, translocation, and metabolism of ALS-inhibiting herbicide will confirm whether herbicide translocation and metabolism are the mechanism(s) of resistance.

Based on our results in this research, it can be concluded that Trp_{574} to Leu amino acid substitution within the *ALS* gene of both resistant biotypes was the mechanism of halosulfuron resistance in rice flatsedge. Future research on identification of multiple *ALS* genes present in rice flatsedge could further clarify the genetics of resistance and the contribution of gene copies toward expression. Meanwhile, prudent use of 2,4-D, bentazon, and propanil is needed to decrease selection pressure for the evolution of ALS resistance in rice flatsedge accessions in midsouthern U.S. rice.

Acknowledgments

Authors are thankful to the Arkansas Rice Research and Promotion Board for funding this research. A part of this work was supported by the U.S. Department of Agriculture-Agricultural Research Service (USDA–ARS) project number 6402-21000-050-00D.

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Received January 26, 2015, and approved April 6, 2015.

Associate Editor for this paper: Christopher Preston, University of Adelaide.