Research Article

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First report of resistance to acetolactate-synthase-inhibiting herbicides in yellow nutsedge (*Cyperus esculentus*): confirmation and characterization

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Abstract

BACKGROUND: Yellow nutsedge is one of the most problematic sedges in Arkansas rice, requiring the frequent use of halosulfuron (sulfonylurea) for its control. In the summer of 2012, halosulfuron at 53 g ha^{-1} (labeled field rate) failed to control yellow nutsedge. The level of resistance to halosulfuron was determined in the putative resistant biotype, and its cross-resistance to other acetolactate synthase (ALS) inhibitors from four different herbicide families. ALS enzyme assays and analysis of the ALS gene were used to ascertain the resistance mechanism.

RESULTS: None of the resistant plants was killed by halosulfuron at a dose of 13 568 g ha^{-1} (256× the field dose), indicating a high level of resistance. Based on the whole-plant bioassay, the resistant biotype was not controlled by any of the ALS-inhibiting herbicides (imazamox, imazethapyr, penoxsulam, bispyribac, pyrithiobac-sodium, bensulfuron and halosulfuron) tested at the labeled field rate. The ALS enzyme from the resistant biotype was 2540 times less responsive to halosulfuron than the susceptible biotype, and a Trp_{574}-to-Leu substitution was detected by ALS gene sequencing using the Illumina HiSeq.

CONCLUSION: The results suggest a target-site alteration as the mechanism of resistance in yellow nutsedge, which accounts for the cross-resistance to other ALS-inhibiting herbicide families.

Keywords: ALS assay; herbicide resistance mechanism; ALS gene sequencing; Illumina HiSeq; yellow nutsedge; *Cyperus esculentus*

1 INTRODUCTION

Yellow nutsedge (*Cyperus esculentus* L.) is a perennial, herbaceous, tuber-forming member of the sedge family (Cyperaceae) and one of the prohibited noxious weeds in ten states of the United States.[1] It is one of the major monocot weeds in Arkansas rice,[2] and a problematic weed in irrigated row crops, polyethylene-mulched fruiting vegetables and cucurbits in the southern United States.[3]

Among the most commonly used herbicides for weed control in Arkansas rice, only few control yellow nutsedge effectively. Of the herbicides available in Arkansas rice, two acetolactate synthase (ALS) inhibitors, imazosulfuron applied pre-emergence (PRE) and halosulfuron applied post-emergence (POST), effectively control yellow nutsedge.[4] Halosulfuron is one of the most frequently applied POST herbicides of midsouthern United States rice because of its high level of efficacy on yellow nutsedge and other sedges.[2] Specifically, imazosulfuron applied PRE followed by (fb) an early post-emergence (EPOST) application of propanil + quinclorac fb thiobencarb + propanil preflood controlled ≥90% of yellow nutsedge in drill-seeded rice in the midsouthern United States.[5] In addition, various EPOST herbicide options for the control of yellow nutsedge in rice include halosulfuron, triclopyr + halosulfuron, imazethapyr fb imazethapyr/imazamox, propanil + halosulfuron and propanil + bensulfuron.[4]

ALS-inhibiting herbicides have been widely used over the last two decades for the control of weeds in rice.[6,7] Introduction of imidazolinone (IMI)-resistant rice and overreliance on ALS-inhibiting herbicides for several years has led to the evolution of ALS-resistant weeds in midsouthern United States rice.[8] Several reports of ALS-inhibiting herbicide-resistant weeds such as barnyardgrass (*Echinochloa crus-galli* L.),[9] smallflower umbrella sedge (*Cyperus*

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difformis L.)[10] and rice flatsedge (*Cyperus iria* L.)[11] have been documented in Arkansas rice, which demonstrates considerable adaptive evolution of these weed species to ALS inhibitors. In 2012, a rice grower in Arkansas failed to control yellow nutsedge with halosulfuron. Subsequently, the yellow nutsedge accession was confirmed to be resistant to halosulfuron in a herbicide screening program performed at the University of Arkansas in Fayetteville.

Possible mechanisms of resistance for weeds to herbicides include enhanced herbicide metabolism, differential absorption and/or translocation of herbicides and target-site mutations; amino acid substitutions caused by point mutations in the ALS gene are the most common means of resistance evolution to the ALS inhibitors. To date, eight spontaneous point mutations in the ALS gene sequence of different weed species have been reported that conferred resistance to ALS inhibitors. Mutations in the ALS gene have resulted in amino acid substitutions in the case of: Trp_{574} to Leu; Ser_{653} to Thr, Asn or Ile; Gly_{654} to Asp; Arg_{377} to His; Ala_{122} to Thr, Tyr or Val; Ala_{205} to Val; Pro_{197} to Ala, Arg, Asn, Gln, His, Ile, Leu, Ser or Thr; and Asp_{376} to Glu.[12] The above mutations result in the alteration of the binding sites on the ALS enzyme structure recognizable with ALS inhibitors, which often partially overlap across different classes of ALS-inhibiting herbicides.[13] Based on genetic inheritance studies, target-site ALS resistance alleles are attributed to a dominant nuclear gene in diploid species.[14] Nonetheless, in polyploidy the same resistance mechanism can be encoded by multiple copies of ALS gene at different expressional levels.[15]

The objectives of the present study are (1) to evaluate the cross-resistance pattern of halosulfuron-resistant and -susceptible biotypes to ALS-inhibiting herbicides across different herbicide families, (2) to establish the level of resistance to halosulfuron and (3) to determine the mechanism of resistance using ALS enzyme assays and gene sequence analysis.

2 MATERIALS AND METHODS

2.1 Plant regeneration and growth conditions

Tubers from surviving yellow nutsedge plants treated with halosulfuron were collected from a rice production field near Hoxie, Arkansas, following failure of the herbicide in 2012. Initial screenings for resistance of yellow nutsedge to halosulfuron (Permit, 53 g ha^{-1}; Gowan Company, Yuma, AZ) were conducted under greenhouse conditions in 2013 at the University of Arkansas in Fayetteville (data not shown). A biotype susceptible to halosulfuron collected from a farm near Stuttgart, Arkansas, was used for comparison. Tubers of yellow nutsedge susceptible (S) and resistant (R) biotypes were propagated in plastic trays (55.5 × 26.5 × 5.5 cm) filled with non-sterile potting mix (LC1Mix; Sun Gro Horticulture Distribution Inc., Bellevue, WA) and maintained at 34/25 °C day/night temperature under a 16 h photoperiod. Subsequently, individual young sprouts at the 2–3-leaf stage were transplanted into plastic pots (88 mm height × 100 mm diameter).

2.2 Whole-plant bioassay

2.2.1 Cross-resistance

An experiment with a randomized complete block (RCBD) factorial design with two biotypes and seven herbicide treatments was conducted for the investigation of cross-resistance. Five potted plants of both R and S biotypes were treated individually at the 4–5-leaf stage with a recommended field dose for each of the seven ALS-inhibiting herbicides as shown in Table 1. A research track sprayer fitted with a boom equipped with two flat-fan 80067 nozzle tips (Teejet® Technologies, Springfield, IL) was calibrated to deliver 190 L ha^{-1} of herbicide at 276 kPa. Above-ground biomass of all plants was harvested 28 days after treatment (DAT), then oven dried at 60 °C for 72 h, and dry weights were recorded. Dry weights were expressed as a percentage of the mean of the non-treated controls and were subjected to analysis of variance (ANOVA) using PROC MIXED in SAS v.9.3 (SAS Institute, Cary, NC). The experiment was repeated, and no significant differences were recorded between the two trials; therefore, pooled data from the two runs were analyzed, and the means were separated using Fisher's protected least significant difference test at $\alpha = 0.05$.

Table 1. Acetolactate-synthase-inhibiting herbicides used in whole-plant cross-resistance and dose–response experiments

Common name	Trade name	Herbicide family[a]	Field rate (g ha^{-1})	Adjuvant
Imazamox	Beyond	IMI	45	1% COC
Imazethapyr	Newpath	IMI	107	0.25% NIS
Bispyribac-sodium	Regiment	PB	36	0.75% NIS[b]
Pyrithiobac	Staple LX	PB	71	–
Halosulfuron	Permit	SU	53	1% COC
Bensulfuron	Londax	SU	79	1% COC
Penoxsulam	Grasp	TP	40	1% COC

[a] IMI: imidazolinones; PB: pyrimidinyl benzoate; SU: sulfonylurea; TP: triazolopyrimidine; NIS: non-ionic low-foam wetter/spreader adjuvant; COC: crop oil concentrate.
[b] Dyne-A-Pak: non-ionic spray adjuvant and deposition aid.

2.2.2 Dose response

Both yellow nutsedge biotypes were grown in the greenhouse as described above, and plants at the 4–5-leaf stage were treated with different rates of halosulfuron. S plants were treated with halosulfuron at 0, 0.0625, 0.125, 0.025, 0.5, 1, 2, 4 and 8 times the labeled field rate of 52.6 g ha^{-1}, whereas R plants were treated with 4, 8, 16, 32, 64, 128 and 256 times the labeled field rate. All halosulfuron applications contained 1% v/v of crop oil concentrate and were applied using the same spray chamber as in the previous experiment. Plant mortality was recorded 28 DAT, and dry weight was expressed as a percentage of the non-treated controls for all halosulfuron doses. The experimental design was an RCBD with 20 replications per dose and was repeated twice. Mortality data were subjected to probit analysis in SAS for determination of the lethal dose at 50% (LD_{50}) and 90% (LD_{90}) for each biotype. The level of resistance was determined by dividing LD_{50} of S by LD_{50} of R. The same calculation was conducted using LD_{90} values (Table 2). The dry weight biomass data of both biotypes were subjected to ANOVA using PROC MIXED in SAS. Data from two experiments were pooled, as the treatment by experiment interaction was not significant. To determine the herbicide dose needed to reduce S plant growth by 50% (GR_{50}), data were expressed as percentage of the mean of the non-treated controls, and a four-parameter logistic equation was fitted using SigmaPlot v.12.5 (Systat Software Inc., San Jose, CA):

$$y = y_0 + \frac{a}{1 + abs\left(x/x_0\right)^b} \quad (1)$$

where y is percentage of dry weight caused by halosulfuron dose x (g ha^{-1}), a is a constant, x_0 and y_0 are asymptotes and

Table 2. Halosulfuron dose required to kill 50 and 90% of yellow nutsedge biotypes

Biotype	LD_{50}[a] ($g\ ha^{-1}$)	LD_{50} (R/S)	LD_{90}[a] ($g\ ha^{-1}$)	LD_{90} (R/S)
Resistant	>13 568	>133	>13 568	>133
Susceptible	102 (81–129)[b]		2458 (1308–6418)[b]	

[a] LD_{50} and LD_{90}: dose of herbicide required to kill 50 and 90% of plants respectively.
[b] Values in parentheses indicate 95% confidence intervals.

b corresponds to the slope around GR_{50} of the dose response (Table 3).

2.3 ALS *in vitro* inhibition assay

Both yellow nutsedge biotypes were grown as described earlier. ALS enzyme activity from 4–5-leaf plants was assayed *in vitro* using procedures similar to previous descriptions.[8,16] Samples (3–4 g) of fresh shoot tissue (bulked from 5–8 individual plants) were measured, immediately frozen and ground in liquid nitrogen and stored at −80 °C until further analysis. The herbicide concentrations used were 0, 0.001, 0.01, 0.1, 1, 10, 100 and 1000 μM for halosulfuron. This assay measured the acetoin that was formed from acid decarboxylation of acetolactate. Background acetoin sources were included as controls. There were three replications per treatment, and each experiment was conducted 3 times. Each replication represents an independent enzyme/protein extraction from a shoot sample. All data were analyzed by ANOVA via the PROC GLM statement using SAS. No significant experiment effect was observed in repeated experiments; therefore, data from all experiments were pooled. Non-linear regression analysis was applied to define a three-parametric logistic equation of the form

$$y = \frac{a}{1 + \left(x/x_0\right)^b} \tag{2}$$

to relate the effect of herbicide concentration x on ALS activity y, where x_0 is an asymptote, a is a constant and b is the slope of the curve. Equation parameters were computed using SigmaPlot v.12.5 (Systat Software Inc., San Jose, CA).

2.4 ALS gene assembly, mapping and SNP detection

To identify potential target-site mutations, massively parallel sequencing using the Illumina HiSeq (Illumina, San Diego, CA; http://www.illumina.com/) platform was utilized in lieu of traditional short-read capillary sequencing using the polymerase chain reaction (PCR). Methodology for assembly and polymorphism detection in a non-model organism with no reference genome or transcriptome was based on suggestions by Brautigam and Gowik.[17] RNA was extracted using a standard RNA extraction kit (RNeasy Plant Mini kit; Qiagen, Venlo, The Netherlands; http://www.qiagen.com). Illumina sequencing, including all RNA preparation steps prior to sequencing, was conducted at the Hudson-Alpha Institute for Biotechnology (Huntsville, AL, USA; http://gsl.hudsonalpha.org/). Sequencing reads were processed using the Trinity *de novo* assembly pipeline (http://trinityrnaseq.sourceforge.net/).[18,19] Reads of the two biotypes were separately paired, trimmed and *de novo* assembled using the Trinity RNASeq *de novo* assembler. Contiguous assembled sequences (contigs) identified as ALS expressed genes were identified using a local BLAST search. To facilitate the BLAST search, full-length ALS protein sequences were downloaded from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). Local BLAST was conducted using tblastn within the CLC Genomics Workbench (CLC Bio, Primset, Denmark; http://www.clcbio.com/). The assemblies of the two biotypes were converted to BLAST databases, and NCBI protein sequences were searched against the assembly databases. Only one contig was identified and extracted from each assembly as similar to ALS proteins. Extracted putative ALS assembled contiguous sequences (contigs) were aligned and compared using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).[20] Sequence assemblies were submitted to the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) and are currently awaiting accession number assignment.

2.5 ALS gene amplification and sequencing

To verify the mutation observed in the assembled sequence of the resistant biotype, a primer pair was designed using NCBI/Primer-Blast (forward: 5′-AACCGCATGTGTCAATCTGC-3′ and reverse: 5′-CTAGTACACAGTCCGGCC-3′), based on the assembly to cover the region surrounding the Trp_{574} codon in susceptible and resistant biotypes. Primers were synthesized by Integrated DNA Technologies (Coralville, IA). RNA was extracted using RNeasy Plant Mini kit (Qiagen), and its quality and integrity were measured using Thermo Scientific (Waltham, MA) NanoDroop 2000c and denaturing gel electrophoresis respectively. Subsequently, cDNA was synthesized using Maxima H Minus double-stranded cDNA synthesis kit (Thermo Scientific). Each reaction for PCR amplification contained: 12.5 μL of GoTaq Green Master Mix (Promega, Madison, WI), 0.5 μL of each primer (18 μM), 2 μL of ∼16 ng DNA and 9.5 μL of nuclease-free water. A Bio-Rad (Hercules, CA) T100 thermal cycler was programed for 10 min initial denaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C (denaturation), 1 min at 57 °C (annealing) and 1 min at 72 °C (extension), and a final extension of 10 min at 72 °C. PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. Amplicons at ∼760 bp for both biotypes were purified using QIAquick PCR purification kit (Qiagen) and sequenced at the DNA Resource Facility, University of Arkansas. Sequences were analyzed for potential sequencing error using NCBI/blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and trimmed by the BioEdit sequence alignment editor. The sequence of the resistant biotype was submitted to NCBI (accession KM624613).

3 RESULTS AND DISCUSSION

3.1 Cross-resistance

Based on the percentage of shoot dry weight reduction, the S biotype was highly affected by all herbicide treatments. On the other hand, based on shoot dry weight reduction, the R biotype was not effectively controlled (Fig. 1). Growth reduction of S with imazamox, halosulfuron, bispyribac, penoxulam, imazethapyr, pyrithiobac and bensulfuron ranged from 72 to 97%. Halosulfuron and bispyribac treatments resulted in highest growth reduction (≥97%) in comparison with pyrithiobac (≥71%). In contrast, the average growth reduction of R ranged from 1 to 16%, showing that cross-resistance to ALS-inhibiting herbicides belonging to the sulfonylurea, imidazolinone, pyrimidinylthiobenzoate and triazolopyrimidine chemical families has evolved. This is the

Table 3. Parameters of the sigmoidal log-logistic equation [equation (1)] for the herbicide dose required to reduce growth of two yellow nutsedge biotypes by 50%

Biotype	*a*	*b*	x_0	y_0	R^2 [a]	*P*-value	GR_{50} [b] (g ha^{-1})	GR_{50} (R/S)[c]
Resistant	48.33	1.262	1854.8	52.32	0.97	0.0008	>13 568[d]	>2714
Susceptible	90.61	3.996	−4.838	9.631	0.99	<0.0001	5	

[a] $R^2 = 1 -$ (sums of squares of the residual/corrected total sums of squares).[31]
[b] GR_{50}: dose required to reduce yellow nutsedge biotype growth by 50%.
[c] Fold resistance: GR_{50} value of resistant yellow nutsedge divided by GR_{50} value of susceptible yellow nutsedge.
[d] The GR_{50} is greater than the maximum rate tested.

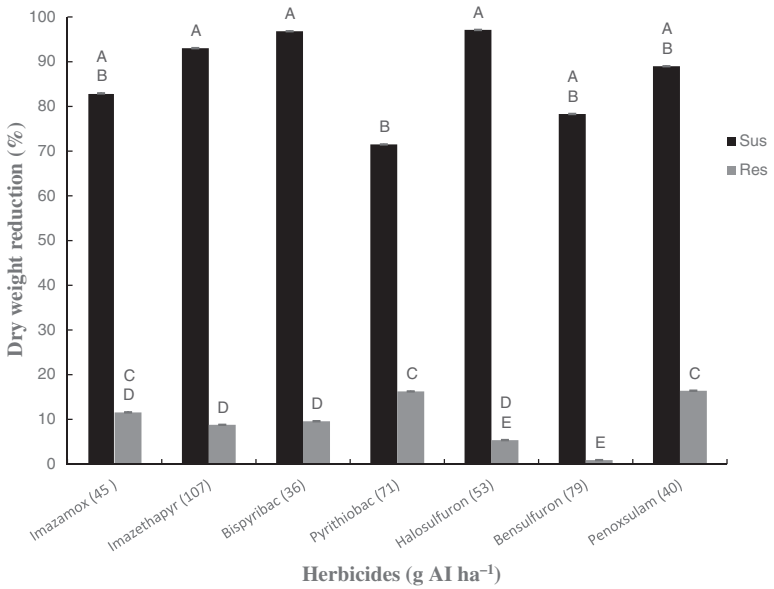

Figure 1. Percentage of above-ground biomass dry weight reduction of yellow nutsedge susceptible (S) and resistant (R) biotypes in response to ALS-inhibiting herbicides.

first report of yellow nutsedge evolving resistance to any herbicide worldwide. Among sedges (*Cyperus* spp.), cross-resistance to ALS-inhibiting herbicides has been reported in smallflower umbrella sedge and rice flatsedge within the United States.[21] In many cases, studies on ALS-resistant weed species such as wild radish (*Raphanus raphanistrum* L.),[22] Italian ryegrass (*Lolium multiflorum* Lam.)[23] and red rice (*Oryza sativa* L.)[24] showed variable levels of cross-resistance to ALS herbicide families, and in almost all cases the resistance was due to a target-site alteration.

3.2 Dose response

The halosulfuron LD_{50} values of S and R were 102 and >13 568 g ha^{-1} respectively (Table 2). The R/S ratio calculated from LD_{50} values showed that the R biotype is >133 times less sensitive to halosulfuron than the S biotype. The LD_{50} value of the S biotype was almost twice that of the labeled field rate of halosulfuron in Arkansas rice. Based on the LD_{90} values, 2458 g ha^{-1} of halosulfuron was needed to kill 90% of the S, but none of the resistant plants was killed at the highest tested rate of the herbicide. The size of the plants at application and the fact that plants were grown in potting mix partially contributed to the low control of the S biotype, because halosulfuron would only provide post-emergence activity rather than soil and foliar uptake of the herbicide as under field conditions. Furthermore, complete control is seldom achieved with a single application under field conditions.

Table 4. Halosulfuron concentration required for 50% inhibition (I_{50}) of acetolactate synthase (ALS) enzyme of resistant and susceptible yellow nutsedge populations and corresponding regression equation parameters[a]

	Regression parameters[b]					
Population	x_0	*a*	*b*	R^2	I_{50}(μM)	RI[c](R/S)
Resistant	94.7	101.7	0.3	0.99	203.00	2540
Susceptible	0.05	98.3	0.3	0.99	0.08	

[a] I_{50}: herbicide concentration required to cause a 50% inhibition of ALS enzyme activity *in vitro*; RI: resistance index.
[b] Regression equation parameters were generated by fitting a non-linear regression equation of the form $y = a/[1 + (x/x_0)^b]$ to the response of ALS enzyme to herbicide concentration. Details are provided in the text.
[c] RI was calculated by dividing the I_{50} values of the resistant population by the I_{50} of the susceptible population.

Reduction in dry weight reflects different responses of both biotypes to halosulfuron, which followed a trend similar to the LD_{50} values. The GR_{50} values of S and R biotypes were 5 and >13 568 g ha^{-1} respectively. Therefore, R was >2714-fold more resistant to

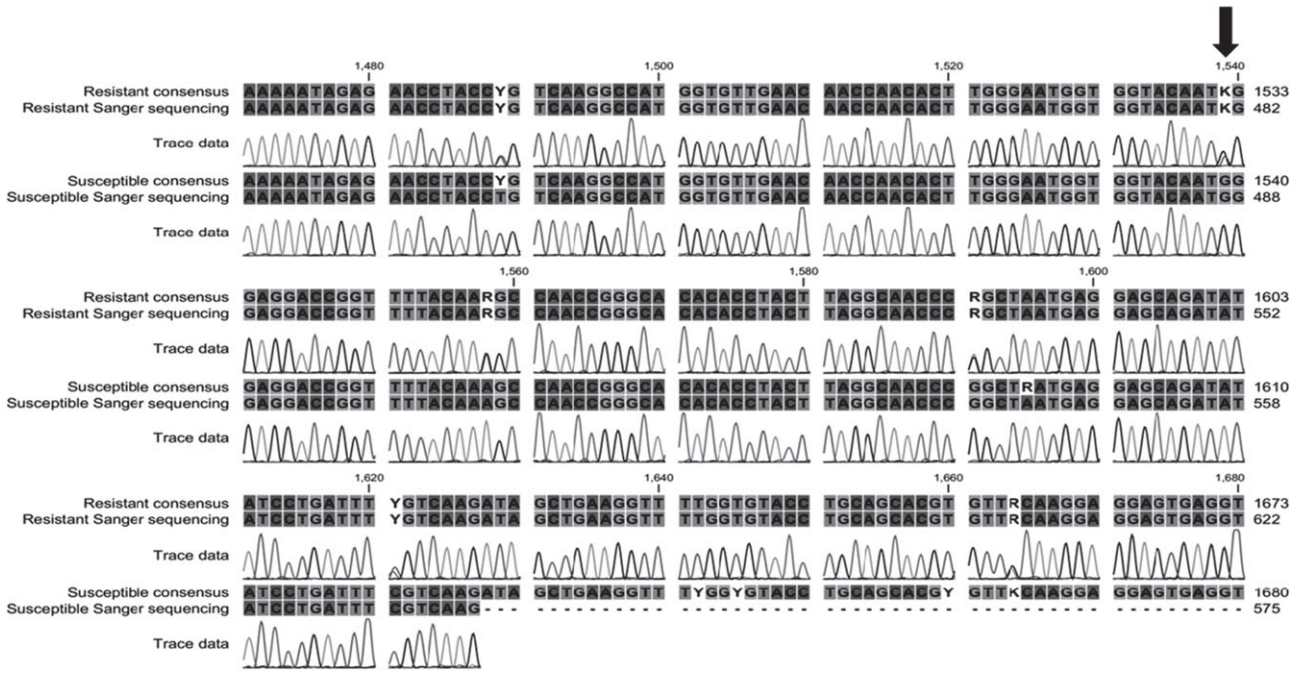

Figure 2. The alignment of ALS mapping consensuses and Sanger sequencing results for susceptible and resistant yellow nutsedge biotypes. The black arrow indicates the mutation that confers Trp_{574}-to-Leu substitution.

halosulfuron than the S biotype. The highest tested rate of halosulfuron (13 568 g ha^{-1}) did not kill any resistant plants; however, plant growth was suppressed by 44% relative to non-treated plants (data not shown). Once resistance to an ALS inhibitor evolves, the resistance is often at a high level and a result of a target-site alteration. For example, a tall waterhemp [*Amaranthus tuberculatus* (Moq.) JD Sauer] biotype in Illinois was >1350-fold less sensitive to halosulfuron than a sensitive biotype,[25] and a biotype of smallflower umbrella sedge in South Korea had a level of resistance to imazosulfuron of >1526-fold.[26]

3.3 ALS *in vitro* inhibition assay

The magnitude of inhibition of ALS in the resistant and susceptible yellow nutsedge biotypes by halosulfuron is presented in Table 4. The I_{50} values for the R and S biotypes were 203 and 0.08 μM of halosulfuron respectively, resulting in a resistance index value (ratio of resistant I_{50} to susceptible I_{50} values) of 2540 for the resistant population (Table 4). These results are similar to earlier findings of high levels of halosulfuron resistance based on enzyme assays in tall waterhemp (>540-fold)[25] and smallflower umbrella sedge (238-fold).[21] In addition, we have determined that ALS enzyme from biotypes of rice flatsedge in Arkansas and Mississippi was 200–2600-fold resistant to halosulfuron (unpublished data). Kuk *et al.*[26] reported high levels of resistance (416–3583-fold) at the ALS enzyme level in a smallflower umbrella sedge accession from Korea to several sulfonylurea herbicides, not including halosulfuron. In general, a high target-site resistance to ALS-inhibiting herbicides indicates the presence of one or more point mutations resulting in amino acid substitutions in the conserved region(s) of the ALS sequence in a resistant population. This will be more evident from the sequence analysis. RNA-seq technology can be used to analyze the whole transcriptome of the R and S biotypes to gain a better understanding of the findings from this research.

Table 5. Missense mutations in the susceptible and resistant yellow nutsedge biotypes as revealed by nucleotide read mapping and translation to amino acid sequence

Nucleotide position[a]	Reference	Polymorphism	Frequency[b]	Amino acid substitution[c]
Susceptible yellow nutsedge biotype				
578	A	C	41.7	Ile_{254} to Leu
1595	A	G	30.0	Asn_{593} to Asp
1664	G	T	37.5	Ala_{616} to Ser
Resistant yellow nutsedge biotype				
840	G	T	33.8	Glu_{343} to Asp
1532	G	T	48.4	Trp_{574} to Leu
1657	G	A	49.6	Ala_{616} to Thr

[a] Nucleotide position refers to the corresponding assembly of the ALS gene in susceptible and resistant biotypes.
[b] Frequency = number of mapped reads carrying the polymorphic nucleotide/number of mapped reads carrying the reference nucleotide.
[c] The number in the amino acid substitution corresponds to the position in the ALS amino acid sequence of *A. thaliana*.

3.4 ALS mutation

A single contig was identified as an ALS transcript from each of the R or S yellow nutsedge transcriptome assemblies. ALS genes were incompletely assembled owing to a lack of reads from the 5′-end containing the chloroplast transit peptide sequence. A total of 2052 and 2071 base pair sequences were assembled for R and

S biotypes respectively, and 1824 and 1830 bp of them were identified as ALS coding regions. By comparison, the complete coding region of ALS is 2013 bp in *Arabidopsis thaliana* (NCBI accession AY124092) and 1923 bp in *Lolium multiflorum* (NCBI accession AF310684). Comparison of initial assemblies revealed 59 nucleotide differences and a sequence identity of 97% between the two biotypes. Translation of sequences to amino acid revealed no amino acid difference between R and S biotypes.

Transcriptome assembly using Trinity does not account for potential polymorphic ambiguities caused by heterozygosity, gene duplication and homeologous genes.[27] To identify polymorphic ambiguities in the ALS coding sequence, reads were separately mapped to the assembled putative ALS contigs. Heterozygous events are revealed in read mapping as two nucleotides mapping to a single loci. Our minimum threshold for identification of polymorphic ambiguities was 30%, meaning that a minimum of 30% of a given nucleotide must map to a nucleotide position in order to identify a polymorphism. Read mapping identified 15 and 29 nucleotide polymorphisms in S and R biotypes. Of the identified nucleotide polymorphisms, only three in either S or R biotypes resulted in missense mutations (Table 5). Of the three separate polymorphisms in each biotype, ALA_{616} was polymorphic in both but resulted in Thr in R and Ser in S. The R biotype contained two other amino acid substitutions – Glu_{343} to Asp and Trp_{574} to Leu. Trp_{574} to Leu has been correlated in numerous species as target-site resistant amino acid substitution conferring resistance to multiple ALS-inhibiting herbicide families including sulfonylurea, imidazolinone and pyrimidinyl benzoic acids.[28,29] Considering that Glu_{343} is not one of the 18 amino acid in the ALS-inhibiting herbicide-binding region,[30] and Trp_{574} to Leu is historically correlated with target-site resistance, it can be concluded that an amino acid substitution to Leu_{574} is the molecular mechanism of resistance. The sequencing results were aligned with mapping consensus sequences of both biotypes. Double peaks were observed in the chromatograph and matched with the IUPAC code in the consensus sequences. A G/T double peak was found at the second base of the Trp_{574} codon in the resistant biotype, which confers the Trp_{574}-to-Leu substitution (Fig. 2). Double peaks in the ALS Trp_{574} to Leu are indicative of heterozygosity or homeologous differences in the polyploid species,[29] either of which confer resistance to ALS-inhibiting herbicides. Based on this evidence, we can clearly state that there is an ALS isoform containing a Trp_{574}-to-Leu substitution produced by the R biotype. However, what cannot be concluded at this time is whether the resistant ALS isoform is a result of heterozygosity or gene duplication. Analysis of the ALS gene copy number is beyond the scope of this research, as we have achieved our goal of identifying a polymorphism that can be correlated with resistance.

In summary, ALS enzyme activity assays and dose–response experiments revealed that halosulfuron resistance in the R biotype was due to target-site alteration. Eventually, a Trp_{574}-to-Leu substitution in the ALS gene of the R biotype confirmed the hypothesis that the target-site resistant amino acid substitution is the mechanism of ALS-inhibiting herbicide resistance in yellow nutsedge. Cross-resistance of the R biotype to all tested herbicides implied that ALS-inhibiting herbicides from different families would not control R. Therefore, alternative herbicide options within an integrated weed management program should be the approach going forward.

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REFERENCES

1 Anderson WP, Purple nutsedge (*Cyperus rotundus*) and yellow nutsedge (*Cyperus esculentus*), in *Perennial Weeds*. Iowa State University Press, Ames, IA, pp. 57–66 (1999).
2 Norsworthy JK, Bond J and Scott RC, Weed management practices and needs in Arkansas and Mississippi Rice. *Weed Technol* **27**:623–630 (2013).
3 Webster TM, Mulch type affects growth and tube production of yellow nutsedge (*Cyperus esculentus*) and purple nutsedge (*Cyperus rotundus*). *Weed Sci* **53**:834–838 (2005).
4 Scott RC, Boyd JW, Selden G, Norsworthy JK and Burgos N, Recommended chemicals for weed and brush control – MP44. University of Arkansas Extension Publication, Fayetteville, AR, p. 79 (2014).
5 Riar DS and Norsworthy JK, Use of imazosulfuron in herbicide programs for drill-seeded rice (*Oryza sativa*) in the Mid-south United States. *Weed Sci* **25**:548–555 (2011).
6 Saari LL, Cotterman JC and Thill DC, Resistance to acetolactate synthase inhibiting herbicides, in *Herbicide Resistance in Plants: Biology and Biochemistry*, ed. by Powles SB and Holtum JAM. Lewis Publishers Inc., Boca Raton, FL, pp. 83–140 (1994).
7 Busi R, Vidotto F, Fischer AJ, Osuna MD, De Prado R and Ferrero A, Patterns of resistance to ALS herbicides in smallflower umbrella sedge (*Cyperus difformis*) and ricefield bulrush (*Schoenoplectus mucronatus*). *Weed Technol* **20**:1004–1014 (2006).
8 Riar DS, Norsworthy JK, Srivastava V, Nandula V, Bond JA and Scott RC, Physiological and molecular basis of acetoacetate synthase-inhibiting herbicide resistance in barnyardgrass (*Echinochloa crus-galli*). *J Agric Food Chem* **61**:278–289 (2012).
9 Riar DS, Norsworthy JK, Bond JA, Bararpour MT, Wilson MJ and Scott RC, Resistance of barnyardgrass (*Echinochloa crus-galli*) populations to acetolactate synthase-inhibiting herbicides. *Int J Agron* **2012**: Article ID 893953, 8 p. (2012).
10 Norsworthy JK, Riar DS and Scott RC, Control options for ALS-resistant smallflower umbrella sedge in Arkansas rice, in *B.R. Wells Arkansas Rice Research Studies*, ed. by Norman RJ, Meullenet J-F and Moldenhauer KAK. University of Arkansas Agricultural Experiment Station Research Series 617, Fayetteville, AR, pp. 222–226 (2014).
11 Schrage BW, Norsworthy JK, Bond JA, Scott RC and Nandula V, Rice flatsedge resistance to acetolactate synthase inhibitors. *Proc Rice Technical Working Group*, Feb 2014, New Orleans, LA (2014).
12 Tranel PJ, Wright TR and Heap IM, *Mutation in Herbicide Resistant Weeds to ALS Inhibitors.* [Online]. Available: http://www.weedscience.com [24 September 2014].
13 Warwick SI, Sauder CA and Beckie HJ, Acetolactate synthase (ALS) target-site mutations in ALS inhibitor-resistant Russian thistle (*Salsola tragus*). *Weed Sci* **58**:244–251(2010).
14 Yu Q and Powles SB, Resistance to AHAS inhibitor herbicides: current understanding. *Pest Manag Sci* **70**:1340–1350 (2014).
15 Iwakami S, Uchino A, Watanabe H, Yamasue Y and Inamura T, Isolation and expression of genes for acetolactate synthase and acetyl-CoA carboxylase in *Echinochloa phyllopogon*, a polyploidy weed species. *Pest Manag Sci* **68**:1098–1106 (2012).
16 Nandula VK and Messersmith CG, Mechanism of wild oat (*Avena fatua* L.) resistance to imazamethabenz-methyl. *Pestic Biochem Physiol* **68**:148–155 (2000).
17 Brautigam A and Gowik U, What can next generation sequencing do for you? Next generation sequencing as a valuable tool in plant research*Plant Biol* **12**:831–841(2010).
18 Haas BJ, Papanicolaou A, Yassour M, Grabnerr M, Blood PD, Bowden J et al., *De novo* transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat Prot* **8**:1494–1512 (2013).
19 Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I et al., Full-length transcriptome assembly from RNA-seq data without a reference genome. *Nat Biotechnol* **29**:644–652 (2011).
20 McWilliam H, Li W, Uludag M, Squizzato S, Park YM, Buso N et al., Analysis tools web services from the EMBL-EBI. *Nucl Acids Res* **41**:597–600 (2013).
21 Heap I, *The International Survey of Herbicide Resistant Weeds.* [Online]. Available: http://www.weedscirnce.org [24 September 2014].

22 Tan MK and Medd RW, Characterisation of the acetolactate synthase (ALS) gene of *Raphanus raphanistrum* L. and the molecular assay of mutations associated with herbicide resistance. *Plant Sci* **163**:195–205 (2002).
23 Kuk YI and Burgos NR, Cross-resistance profile of mesosulfuron-methyl-resistant Italian ryegrass in Southern United States. *Pest Manag Sci* **63**:349–357 (2007).
24 Kaloumenos N, Capot N, Aguado A and Eleftherohorinos IG, Red rice (*Oryza sativa*) cross-resistance to imidazolinone herbicides used in resistant rice cultivars grown in northern Greece. *Pestic Biochem Physiol* **105**:177–183 (2013).
25 Sprague CL, Stoller EW and Wax LM, Response of an acetolactate synthase (ALS)-resistant biotype of *Amaranthus rudis* to selected ALS-inhibiting and alternative herbicides. *Weed Res* **37**:93–101 (1997).
26 Kuk YI, Kim KH, Kwon OD, Burgos NR, Jung S and Guh JO, Cross-resistance pattern and alternative herbicides for *Cyperus difformis* resistant to sulfonylurea herbicides in Korea. *Pest Manag Sci* **60**:85–94 (2004).
27 Vijay N, Poelstra JW, Kunstner A and Wolf JBW, Challenges and strategies in transcriptome assembly and differential gene expression quantification. *A comprehensive in silico assessment of RNA-seq experiments. Mol Ecol* **22**:620–634 (2013).
28 Powles SB and Yu Q, Evolution in action: plants resistant to herbicides. *Annu Rev Plant Biol* **61**:317–347 (2010).
29 McElroy JS, Flessner ML, Wang Z, Dane F, Walker RH and Wehtje GR, A Trp_{574} to Leu amino acid substitution in the ALS gene of annual bluegrass (*Poa annua*) is associated with resistance to ALS-inhibiting herbicides. *Weed Sci* **61**:21–25 (2013).
30 McCourt JA, Pang SS, King-Scott J, Guddat LW and Duggleby RG, Herbicide-binding sites revealed in the structure of plant acetohydroxyacid synthase. *Proc Natl Acad Sci USA* **103**:569–573 (2006).
31 Schabenberger O, Tharp BE, Kells JJ and Penner D, Statistical tests for hormesis and effective dosages in herbicide dose response. *Agron J* **91**:1–8 (1999).

