Independent Evolution of Acetolactate Synthase–inhibiting Herbicide Resistance in Weedy *Sorghum* Populations across Common Geographic Regions

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Traditional breeding has been used to develop grain sorghum germplasm that is tolerant to acetolactate synthase (ALS)-inhibiting herbicides (Inzen Technology, DuPont). Inzen sorghum carries a double mutation in the ALS gene ($Val_{560}Ile$ and $Trp_{574}Leu$), which confers high level of tolerance to ALS-inhibiting herbicides. Overreliance on ALS-inhibiting herbicides for weed control during the 1990s resulted in the evolution of ALS inhibitor–resistant shattercane populations in Nebraska. According to a survey conducted in 2013, ALS inhibitor–resistant weedy *Sorghum* populations persist in Nebraska. The objectives of this research were to determine whether the ALS mutations present in Inzen sorghum were present in the ALS inhibitor–resistant shattercane and johnsongrass populations detected in Nebraska and northern Kansas, and whether these populations evolved ALS resistance independently. Primers specific to the Val_{560} and Trp_{574} region of the ALS gene were used to screen the populations with PCR. The $Trp_{574}Leu$ mutation was present in one ALS inhibitor–resistant johnsongrass population. The $Val_{560}Ile$ was detected in three ALS inhibitor–resistant shattercane, one susceptible shattercane, one ALS inhibitor–resistant johnsongrass, and one susceptible johnsongrass population. Moreover, $Val_{560}Ile$ was present in resistant and/or susceptible individuals within johnsongrass and shattercane populations that were segregating for ALS resistance, indicating that by itself the $Val_{560}Ile$ mutation does not confer resistance to ALS-inhibiting herbicides. None of the populations presented both mutations simultaneously, as does Inzen sorghum. A shattercane population containing the $Ser_{653}Thr$ mutation was also detected. This research indicates that the ALS mutations present in Inzen sorghum already exist individually in weedy sorghum populations. Moreover, our results present strong evidence that ALS resistance in these populations evolved independently. Thus, widespread overreliance on ALS-inhibiting herbicides prior to adoption of glyphosate-tolerant crops in the 1990s exerted sufficient selective pressure on shattercane and johnsongrass populations for resistance to evolve multiple times in the Midwest. Finally, a survey of the 5′ portion of the ALS gene in more diverse wild and weedy *Sorghum* species was hampered by limited coverage in genomic resequencing surveys, suggesting that refined PCR-based methods will be needed to assess SNP variation in this gene region, which includes the Ala_{122}, Pro_{197}, and Ala_{205} codons known to confer ALS resistance in other species.

Nomenclature: Acetolactate synthase (ALS)-inhibiting herbicides, johnsongrass, *Sorghum halepense* (L.) Pers. SORHA, shattercane, *Sorghum bicolor* (L.) Moench ssp. *drummondii* (Nees ex Steud.) de Wet ex Davidse SORVU, sorghum, *Sorghum bicolor* (L.) Moench ssp. *Bicolor* SORVU

Key words: Fitness cost, gene flow, herbicide resistance evolution, herbicide-tolerant grain sorghum, mechanism of resistance.

Grain sorghum is an annual C_4 grass crop that is highly efficient in the use of solar energy and water and therefore a key component of cropping systems in the dry regions of the Great Plains (Staggenborg et al. 2008). In spite of its economic potential and food value, sorghum acres have declined in many parts of the United States (United States Department of Agriculture–National Agricultural Statistics Service 2016), due in part to the existence of few options for weed management. POST herbicides labeled for grain sorghum have limited activity on grasses. Consequently, PRE herbicides are the main option for annual grass control in grain sorghum (Hennigh et al. 2010). However, grain sorghum is often grown in dry environments under rainfed conditions, and the absence of adequate soil moisture often reduces the activation and efficacy of PRE herbicides (Hennigh et al. 2010).

In 2004 an acetolactate synthase (ALS)-resistant shattercane population was confirmed in Kansas. Using traditional breeding, the ALS-resistance gene was introgressed into grain sorghum (Tuinstra and Al-Khatib 2008). DuPont® has obtained the license of the ALS herbicide tolerance trait and has branded the technology as "Inzen". Inzen sorghum carries a double mutation in the ALS gene, $Val_{560}Ile$ and $Trp_{574}Leu$ (Tuinstra and Al-Khatib 2008), which confers high levels of tolerance to herbicides in the ALS sulfonylurea (SU) and imidazolinone (IMI) families (Werle et al. 2016a). The $Trp_{574}Leu$ mutation is one of the most common ALS amino acid substitutions, conferring high levels of resistance to ALS inhibitors in the SU and IMI families (Tranel et al. 2016). To our knowledge, the $Val_{560}Ile$ substitution has not been reported to confer resistance to ALS inhibitors in weed species. Nicosulfuron, an active ingredient in the ALS-SU family, is the herbicide to be labeled for POST grass control in Inzen sorghum. The Inzen technology will be commercially available in 2017 (DW Saunders and KL Carlson, personal communication) and has the potential to increase the use of grain sorghum in crop rotations and expand its production in environments where grain sorghum is better adapted than corn (*Zea mays* L.).

ALS-inhibiting herbicides are effective in small quantities (grams of active ingredient per hectare), have high safety margins on labeled crops, are effective on a broad spectrum of weed species, and have low mammalian toxicity (Tranel and Wright 2002). Owing to the versatility of these herbicides, the selective pressure applied by their use, and the number of genetic mutations in the ALS gene that can confer resistance, many weed species have evolved resistance to one or multiple ALS inhibitors (Tranel et al. 2016). There have been 159 reported ALS inhibitor–resistant weed species worldwide, and 50 are in the United States, including shattercane and johnsongrass (Heap 2016). The eight confirmed ALS enzyme sites that confer resistance to ALS-inhibiting herbicides are: Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Arg_{377}, Trp_{574}, Ser_{653}, and Gly_{654} (Tranel et al. 2016). At each site, multiple amino acid substitutions are possible. Substitutions at Pro_{197} and Trp_{574} have been the most common across several weed species. The specific amino acid substitution at each site may confer different types and levels of resistance to different ALS herbicide families (Tranel et al. 2016). For instance, substitution of Ala_{122} to Tyr results in resistance to both IMIs and SUs, whereas substitution of Ala_{122} to Thr

results in resistance to IMIs only. All nine confirmed substitutions in Pro_{197} confer resistance to the SUs but not necessarily to the IMIs and the other ALS herbicide families (Tranel et al. 2016).

Werle et al. (2016a) collected 190 shattercane and 59 johnsongrass populations from northern Kansas, northwestern Missouri, and Nebraska and evaluated their potential resistance to two ALS-inhibiting herbicides. Five and four shattercane populations were resistant to imazethapyr and nicosulfuron, respectively. Five and three johnsongrass populations were confirmed resistant to imazethapyr and nicosulfuron, respectively. Moreover, two shattercane and two johnsongrass populations appeared to be cross-resistant to nicosulfuron and imazethapyr. Given the variation in resistance to different ALS-inhibiting herbicides among these populations, we hypothesized that resistance is likely the result of different mutations in the ALS gene. Such a result would also suggest that resistance evolved independently in these populations; i.e., a single ALS-resistance allele was not transferred among populations by seed or pollen flow.

The objectives of this research were to determine whether the ALS mutations present in Inzen sorghum were present in any of the ALS inhibitor–resistant shattercane and johnsongrass populations detected in Nebraska and northern Kansas (Werle et al. 2016a), and whether these populations are likely to have evolved ALS resistance independently from each other. Since the double mutation ($Val_{560}Ile$ and $Trp_{574}Leu$) in the ALS gene present in Inzen sorghum originated from an ALS inhibitor–resistant shattercane population and the $Trp_{574}Leu$ has been commonly reported to confer ALS resistance in weeds, we expected that one or both of the mutations in Inzen may be present in some of the ALS inhibitor–resistant populations detected in the survey.

Materials and Methods

Greenhouse Study. All putative ALS inhibitor–resistant populations (populations with at least one individual surviving an application of imazethapyr and/or nicosulfuron applied at the labeled rate) detected in the field screening by Werle et al. (2016a) were included in this study with the objective of including a wide range of population phenotypic responses to ALS-inhibiting herbicides (12 shattercane and 12 johnsongrass populations; Table 1). Three susceptible shattercane (S-13,

S-125, and S-130), three susceptible johnsongrass (J-14, J-25, and J-52), and conventional (Pioneer 87P06) and Inzen (Pioneer YSA3527) sorghums were included in the experiment as our control populations. The susceptible shattercane and johnsongrass populations were selected from the field screening based on their high susceptibility to all herbicides tested and good germination rates (unpublished data). Plants used in the study were grown from seeds collected in the fall of 2013 (Werle et al. 2016a).

Glumes of shattercane and johnsongrass seeds were mechanically removed using a rub board, seeds were pregerminated on wet germination paper, and two seedlings with exposed radicles were transplanted to a 3.8-cm-wide by 21-cm-high cone-tainer filled with potting mix (Stuewe & Sons, Tangent, Oregon; Berger BM1 All-Purpose Mix, Berger Peat

Moss, Saint-Modeste, Quebec, Canada). Placement of seeds on wet germination paper was considered the starting point of the study (0 d after planting [DAP]; the study started on August 11, 2014). At 7 DAP, plants were thinned to one plant per cone-tainer. At 11 and 18 DAP, 30 ml of a nutrient solution containing 15 g of 20-20-20 fertilizer (Peters General Purpose Fertilizer, Scotts, Marysville, OH) diluted in 3.785 L of water was applied to each cone-tainer. A total of 46 plants from each population were grown. Greenhouse conditions during the study were set at 24/19 C day/night cycle with a 16 h photoperiod provided by metal-halide lamps to supplement ambient daylight. Plants were watered daily.

Two 1 cm leaf disks were collected from the newest expanded leaf from each plant using a hole puncher at 15 DAP. Leaf disks were placed in a

Table 1. Shattercane (S-) and johnsongrass (J-) populations evaluated in this study listed by state, county, latitude and longitude coordinates, and crop from which they were collected in the fall of 2013 by Werle et al. (2016a).

Population	State	County	Latitude (N)	Longitude (W)	Crop
Shattercane					
S-13	KS	Lincoln	39.126	98.178	Soybean
S-31	KS	Riley	39.335	96.796	Corn
S-46	NE	Adams	40.569	98.569	Corn
S-58	NE	Buffalo	40.919	99.141	Corn
S-63	NE	Buffalo	40.802	99.042	Soybean
S-105	NE	Jefferson	40.051	97.032	Corn
S-113	NE	Johnson	40.409	96.368	Corn
S-117	NE	Keith	41.109	101.832	Corn
S-125	NE	Lancaster	40.812	96.470	Corn
S-130	NE	Lincoln	41.183	101.051	Corn
S-134	NE	Nemaha	40.391	95.687	Soybean
S-136	NE	Nemaha	40.480	95.822	Corn
S-177	NE	Thayer	40.029	97.595	Corn
S-178	NE	Thayer	40.028	97.613	Corn
S-179	NE	Thayer	40.086	97.614	Corn
Johnsongrass					
J-10[a]	KS	Osborne	39.416	98.549	Railroad
J-12	KS	Pottawatomie	39.189	96.483	Corn
J-14[a]	KS	Pottawatomie	39.196	96.508	Roadside
J-15	KS	Pottawatomie	39.200	96.084	Soybean
J-18	KS	Riley	39.472	96.759	Sorghum
J-25[a]	MO	Caldwell	39.750	94.118	Roadside
J-35	NE	Buffalo	40.714	98.999	Corn
J-36	NE	Buffalo	40.859	99.046	Corn
J-37	NE	Buffalo	40.937	99.140	Corn
J-38	NE	Buffalo	41.013	99.109	Corn
J-40	NE	Gage	40.499	96.561	Corn
J-41	NE	Gage	40.509	96.572	Corn
J-44[a]	NE	Johnson	40.432	96.378	Roadside
J-52	NE	Nuckolls	40.043	98.159	Sorghum
J-55	NE	Red Willow	40.232	100.650	Forage sorghum

[a] Populations collected from non-crop areas.

1.2 ml tube, flash frozen in liquid nitrogen, and stored at −20 C until further use. Cone-tainers and tubes were labeled with matching codes identifying individual plants. Twenty-three plants from each population were treated with the field-labeled rate of imazethapyr (70 g ai ha^{-1}; WSSA group 2; IMI family) + crop oil concentrate (COC) at 1.25% v/v + 2,017 g ha^{-1} AMS; the remaining 23 plants were treated with nicosulfuron (35 g ai ha^{-1}; WSSA group 2; SU family) + COC at 1% v/v + 2,240 g ha^{-1} AMS. Herbicide treatments were applied at 21 DAP when sorghum, shattercane, and johnsongrass plants were at the 3- to 4-leaf stage (22 to 30 cm tall). Herbicide treatments were delivered using 140 L ha^{-1} carrier volume and a TP8001E flat-fan nozzle tip (TeeJet Technologies, Spraying Systems, Wheaton, IL) at 241 kPa in a spray chamber (Research Track Sprayer, DeVries, Hollandale, MN).

Visual evaluations of plant growth (VE; on a scale of 1 to 10, as suggested by Anderson et al. [1998], with 1 being dead and 10 being completely healthy) and plant mortality (% of individuals with VE ≤ 3) data were taken at 21 d after treatment (DAT). Plants with VE 1 to 3, 4 to 7, and 8 to 10 were considered susceptible (S; dead plants), moderately resistant (MR; stunted plants, with the main culm injured or dead but new tillers growing back), and resistant (R; light interveinal chlorosis and/or plant stunting to no detectable injury) to the labeled herbicide rate, respectively (Anderson et al. 1998). VE and mortality data were used to choose the populations and individuals to be used for ALS allele characterization.

Genomic DNA Extraction, PCR Amplification, and ALS Allele Determination. Three plants (2 MR [4 ≤ VE ≤ 7] or R [VE ≥ 8] and 1 S [VE ≤ 3]) from populations segregating for resistance to imazethapyr and/or nicosulfuron were randomly selected for DNA extraction and ALS allele determination. Two randomly selected plants from all aforementioned control populations were included. Frozen leaf samples were ground using a TissueLyser II (Qiagen, Hilden, Germany), and genomic DNA was extracted using the CTAB method (Folsom et al. 2014). DNA quantity and quality were assessed with a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) to ensure a minimum of 25 ng μl^{-1} sample^{-1} for use in PCR.

Three sets of primers were designed to amplify the ALS regions containing the amino acid substitutions confirmed in Inzen sorghum, based on published sorghum ALS sequence as a template (TA3960_4558; SEQ ID NO:1; Tuinstra and Al-Khatib 2008; Table 2). These primers were designed to cover the ALS gene sequence containing the Asp$_{376}$, Arg$_{377}$, Val$_{560}$, Trp$_{574}$, Ser$_{653}$, and Gly$_{654}$ sites (amino acid number standardized according to the *Arabidopsis thaliana* (L.) Heynh (GenBank X51514) sequence; Tranel et al. 2016). PCR conditions were as follows: a final volume of 25 μl containing 50 ng of genomic DNA, 0.20 μmol L^{-1} of each primer, 1.5 mmol L^{-1} MgCl$_2$, 0.2 mmol L^{-1} of each dNTP, 1X reaction buffer, and 1 U Phusion high-fidelity DNA polymerase (#F-530S, Thermo-Fisher Scientific, Waltham, MA) using a Mastercycler ep-gradient thermocycler (Eppendorf, Hamburg, Germany). Amplifications were performed for all sets of primers using the following program: denaturation step of 4 min at 94 C, followed by 35 cycles of a denaturation step at 94 C for 45 s, an annealing step at 60 C for 45 s, an extension step at 72 C for 1.5 min, and then a final extension step at 72 C for 5 min. Amplified sequences ranged from 400 to 500 bp (Table 2). PCR products were run on a 2% agarose gel (Benchmark Scientific, Edison, NJ) stained with SYBR Safe DNA Gel Stain (ThermoFisher

Table 2. Primers used for partial amplification of the acetolactate synthase (ALS) gene sequence from shattercane and johnsongrass populations.[a]

Primer	Sequence (5′ → 3′)	Amplicon size (bp)	Targeted mutation site[b]
ALS_1F	GGTTTGATGATCGTGTGACAGG	455	Asp376|Arg377
ALS_1R	TACGCCCCAAGACCAGCTGAAGA		
ALS_2F	GCAGTGGTTGTCTTCAGCTGGT	407	Val560 & Trp574
ALS_2R	GATCATAGGCAACACATGATCCT		
ALS_3R	TCCGTGTGACAAAGAAGAGCGAA	480	Ser653|Gly654
ALS_3R	GAGGCGTACAGAACCACTGCATAG		

[a] Primers were developed by M Gelli and I Dweikat (personal communication) for sequencing the ALS gene of grain sorghum and ALS inhibitor–resistant shattercane populations.
[b] Amino acid positions correspond to the amino acid sequence of ALS from *Arabidopsis thaliana* (L.) Heynh (GenBankX51514).

Werle et al.: ALS inhibitor resistance in weedy *Sorghum spp.* • 167

Scientific, Waltham, MA), and visualized using the BioDoc-IT Imaging system UV Transilluminator (UVP, Upland, CA) to confirm amplification and sizes of the products. Additional PCR assays were used to screen for Ala_{122}, Pro_{197}, and Ala_{205}, but attempts to design primers that would amplify the 5′ region of the ALS gene containing them were unsuccessful. Amplified PCR products were sequenced by Eurofins Genomics (Eurofins MWG Operon, Louisville, KY) in both forward and reverse directions. Nucleotide sequences from each sample were aligned to the sorghum reference genome using the BLASTN algorithm (Altschul et al. 1990). ALS nucleotide and amino acid sequences of interest were aligned using multiple sequence comparison by log-expectation software (MUSCLE; Edgar 2004) and compared with the ALS sequence of Inzen sorghum (Tuinstra and Al-Khatib 2008).

Comparative Analysis of ALS Sequence Variation in Sorghum and Other Panicoideae Species. To assess ALS variation in additional sorghum lines, the ALS gene was de novo assembled from Illumina paired-end sequencing data available in the Sequence Read Archive at National Center for Biotechnology Information, including *S. bicolor* ssp. *drummondii* (SRA accession number SRR998968), *S. bicolor* race 'guinea-margaritiferum' (SRR998976), *S. propinquum* (SRR999027), and *S. bicolor* ssp. *verticilliflorum* (SRR999023). Raw Illumina reads were assembled with Velvet, v. 1.2.03 (Zerbino and Birney 2008), essentially as described previously (Grewe et al. 2014; Zhu et al. 2014), using a range of Kmer values (33, 41, 51, 61) and expected coverage values (100, 50, 20, 10). ALS genes were identified in each Velvet assembly using the blastn search tool (Camacho et al. 2009) with default parameters. For *S. bicolor* ssp. *bicolor* 'BTx623', the ALS gene was initially taken from chromosome 4 of the reference genome (Paterson et al. 2009) available in GenBank (gene ID SORBI_004G155800 in GenBank accession number CM000763), but the sequence at the 5′ end of the gene did not align well with other species. Therefore, the ALS gene was also taken from a de novo assembly of Illumina read data (SRA accession number SRR1946846) derived from a 'BTx623-Rooney' line (Rizal et al. 2015), using the assembly procedure described above. The latter sequence aligned well with other species, including ALS genes from additional panicoid grasses acquired from GenBank, including johnsongrass biotype SJ (GenBank accession number KJ538785), corn (X63553), switchgrass (*Panicum virgatum* L.) (AC243242), foxtail millet [*Setaria italic*

(L.) Beauv.] (XM_004952503), and zoysiagrass (*Zoysia japonica* Steud.) (AB513331).

Results and Discussion

Greenhouse Screening. In previous dose–response studies, shattercane populations S-46, S-58, S-63, S-117, and S-179 were confirmed resistant to imazethapyr, and S-58, S-63, S-105, and S-178 to nicosulfuron (Werle et al. 2016a). Johnsongrass populations J-35, J-36, J-37, J-40, and J-44 were confirmed resistant to imazethapyr, and J-15, J-35, and J-36 to nicosulfuron. Populations S-58, S-63, J-35, and J-36 were cross-resistant to nicosulfuron and imazethapyr. The level of resistance to ALS-inhibiting herbicides varied from 2 to >1,000-fold across populations. Corroborating the dose–response results, in the current study, all confirmed resistant populations included individuals that were either MR ($4 \leq VE \leq 7$) or R ($VE \geq 8$) when treated with the labeled herbicide rate to which they had previously demonstrated resistance (Tables 3 and 4). Moreover, shattercane populations S-31, S-105, and S-113 and johnsongrass populations J-10, J-12, and J-18 had a few MR individuals to imazethapyr (≤ 2 individuals population^{-1} with $VE \leq 5$). Shattercane population S-134 had one MR individual to nicosulfuron ($VE = 5$). Even though it was not confirmed resistant in our previous studies, shattercane population S-113 had five MR individuals to nicosulfuron (VE 4 and 5), and johnsongrass population J-15 had two MR individuals to imazethapyr (VE 6 and 7). Thus, population J-15 had R individuals to nicosulfuron and also had a low frequency of individuals MR to imazethapyr (i.e., it was cross-resistant to the ALS chemistries). Since resistance alleles were not fixed in most populations (mortality $\neq 0\%$), these results reflect the baseline response of each population to common ALS chemistries ahead of commercialization of Inzen sorghum.

Shattercane populations S-58 and S-179 and johnsongrass populations J-36, J-37, J-40, and J-44 had highly R individuals to imazethapyr ($VE \leq 8$; Tables 3 and 4). Shattercane populations S-58 and S-63 and johnsongrass populations J-15, J-35, and J-36 had highly R individuals to nicosulfuron. Since the $Trp_{574}Leu$ mutation has been commonly reported to confer high levels of cross-resistance to IMI and SU herbicides, we suspected that R individuals from populations S-58 and J-36 might be carrying this mutation.

Table 3. Phenotypic response (visual evaluation [VE] and mortality [%]) of shattercane populations treated with a field-labeled rate of imazethapyr (70 g ai ha^{-1}) and nicosulfuron (35 g ai ha^{-1}).[a]

| | Imazethapyr | | | | Nicosulfuron | | | |
| | VE[b] | | | Mortality | VE | | | Mortality |
Population	Min.	Avg. ± SE	Max.	(%)	Min.	Avg. ± SE	Max.	(%)
Sorghum	1	1.3 ± 0.1	2	100.0	1	1.9 ± 0.2	3	100.0
Inzen	6	9.6 ± 0.2	10	0.0	6	9.7 ± 0.2	10	0.0
S-13	1	1.0 ± 0.0	1	100.0	1	1.0 ± 0.0	1	100.0
S-31	1	1.3 ± 0.2	5	95.7	1	1.0 ± 0.0	1	100.0
S-46	3	5.8 ± 0.3	7	13.0	1	1.0 ± 0.0	2	100.0
S-58	2	4.4 ± 0.4	9	47.8	9	9.7 ± 0.1	10	0.0
S-63	1	3.0 ± 0.3	6	82.6	1	6.8 ± 0.8	10	30.4
S-105	1	1.3 ± 0.1	4	95.7	2	3.3 ± 0.2	5	60.9
S-113	1	1.9 ± 0.2	5	91.3	1	2.9 ± 0.2	5	78.3
S-117	1	1.8 ± 0.3	6	87.0	1	1.0 ± 0.0	2	100.0
S-125	1	1.0 ± 0.0	2	100.0	1	1.0 ± 0.0	1	100.0
S-130	1	1.1 ± 0.1	2	100.0	1	1.0 ± 0.0	1	100.0
S-134	1	1.2 ± 0.1	2	100.0	1	1.6 ± 0.2	5	95.7
S-136	1	1.4 ± 0.1	3	100.0	1	1.0 ± 0.0	1	100.0
S-177	1	2.0 ± 0.2	3	100.0	1	1.8 ± 0.2	3	100.0
S-178	1	1.2 ± 0.1	2	100.0	1	2.5 ± 0.2	5	82.6
S-179	1	2.8 ± 0.7	9	78.3	1	1.0 ± 0.0	2	100.0

[a] Twenty-three shattercane plants from each population were exposed to imazethapyr, and 23 additional plants were exposed to nicosulfuron. Plants with VE ranging from 1 to 3, 4 to 7, and 8 to 10 were considered susceptible (dead), moderately resistant (stunted plants, with the main culm dead or injured but new tillers growing back), and resistant (light interveinal chlorosis and/or plant stunting to no detectable injury), respectively, when exposed to the labeled herbicide rate (adapted from Anderson et al. [1998]). Populations S-13, S-125, and S-130 were included as susceptible controls.
[b] Min., Avg., and Max. represent the minimum, average, and maximum VE observed for each population, respectively.

ALS Gene Sequencing. The amino acids encoded by ALS codons 376, 377, 560, 574, 653, and 654 in individuals from populations targeted for ALS allele determination are presented in Tables 5 and 6. The 5′ sequence of the ALS gene containing codons 122, 197, and 205 did not amplify in PCR in any species, even using protocols optimized for high GC%. Sequencing results indicated that the Trp$_{574}$Leu mutation present in Inzen sorghum was present only in johnsongrass population J-36 (Tables 6 and 7), which was highly resistant to imazethapyr and nicosulfuron. This mutation has been reported in resistant populations across multiple species highly resistant to IMI and SU herbicides (Tranel et al. 2016), including two ALS inhibitor–resistant johnsongrass populations detected in cornfields in Chile (Hernández et al. 2015). The Trp$_{574}$Leu mutation was not detected in all R individuals of J-36 (Table 6), indicating that multiple ALS-resistance mechanisms may be present within this population. Multiple mechanisms of ALS resistance within a population have been reported (Guo et al. 2015).

The Val$_{560}$Ile mutation, also present in Inzen sorghum, was detected in ALS-R individuals of shattercane populations S-105 and S-179 and in S individuals of S-63, S-105, S-125, and S-134 (Table 5). This mutation was also detected in ALS-R and S individuals of johnsongrass population J-35 and in S individuals of population J-52 (Table 6). From these results, we conclude that, by itself, the Val$_{560}$Ile amino acid substitution does not confer resistance to ALS-inhibiting herbicides. None of our populations presented both mutations simultaneously (Val$_{560}$Ile and Trp$_{574}$Leu), as does Inzen sorghum (Tables 5 and 6).

The double mutation present in Inzen sorghum was introgressed from an ALS inhibitor–resistant shattercane population detected in Kansas (Tuinstra and Al-Khatib 2008), and both amino acid substitutions were individually detected in ALS inhibitor–resistant weedy *Sorghum* species in the present study. We conclude that commercialization of Inzen technology will not transfer any novel/unprecedented ALS mutations to weedy sorghum relatives sympatric with grain sorghum production areas in the Midwest. However, pollen-mediated gene flow from Inzen sorghum to weedy relatives is expected to increase the frequency of ALS-resistance

Werle et al.: ALS inhibitor resistance in weedy *Sorghum spp.* • 169

Table 4. Phenotypic response (visual evaluation [VE] and mortality [%]) of johnsongrass populations treated with a field labeled rate of imazethapyr (70 g ai ha^{-1}) and nicosulfuron (35 g ai ha^{-1}).[a]

Population	Imazethapyr VE[b]			Imazethapyr Mortality	Nicosulfuron VE			Nicosulfuron Mortality
	Min.	Avg. ± SE	Max.	(%)	Min.	Avg. ± SE	Max.	(%)
Sorghum	1	1.3 ± 0.1	2	100.0	1	1.9 ± 0.2	3	100.0
Inzen	6	9.6 ± 0.2	10	0.0	6	9.7 ± 0.2	10	0.0
J-10	1	1.1 ± 0.1	4	95.7	1	1.0 ± 0.0	1	100.0
J-12	1	1.3 ± 0.1	4	95.7	1	1.0 ± 0.0	1	100.0
J-14	1	1.0 ± 0.0	1	100.0	1	1.0 ± 0.0	1	100.0
J-15	1	1.7 ± 0.3	7	91.3	1	1.6 ± 0.4	8	91.3
J-18	1	1.3 ± 0.2	4	91.3	1	1.0 ± 0.0	1	100.0
J-25	1	1.1 ± 0.1	3	100.0	1	1.0 ± 0.0	1	100.0
J-35	1	1.7 ± 0.3	5	87.0	1	2.9 ± 0.6	10	78.3
J-36	1	9.6 ± 0.4	10	4.3	1	8.9 ± 0.5	10	8.7
J-37	1	5.3 ± 0.6	10	26.1	1	1.0 ± 0.0	1	100.0
J-38	1	1.1 ± 0.1	2	100.0	1	1.0 ± 0.0	1	100.0
J-40	4	6.5 ± 0.2	8	0.0	1	1.0 ± 0.0	2	100.0
J-41	1	1.1 ± 0.1	2	100.0	1	1.0 ± 0.0	1	100.0
J-44	1	5.1 ± 0.5	8	30.4	1	1.0 ± 0.0	2	100.0
J-52	1	1.0 ± 0.0	1	100.0	1	1.0 ± 0.0	1	100.0
J-55	1	1.1 ± 0.1	2	100.0	1	1.0 ± 0.0	1	100.0

[a] Twenty-three johnsongrass plants from each population were exposed to imazethapyr, and 23 additional plants were exposed to nicosulfuron. Plants with VE ranging from 1 to 3, 4 to 7, and 8 to 10 were considered susceptible (dead), moderately resistant (stunted plants, with the main culm dead or injured but new tillers growing back), and resistant (light interveinal chlorosis and/or plant stunting to no detectable injury), respectively, when exposed to the labeled herbicide rate (adapted from Anderson et al. [1998]). Populations J-14, J-25, and J-52 were included as susceptible controls.
[b] Min., Avg., and Max. represent the minimum, average, and maximum VE observed for each population, respectively.

alleles in weedy populations, which will be selected by continued ALS-inhibitor use and result in rapid evolution of highly resistant populations. Effective weed resistance management strategies will be critical to maintain the efficacy of ALS inhibitors where Inzen sorghum is adopted (Werle et al. 2016b).

The Ser$_{653}$Thr mutation was present in individuals R to imazethapyr and absent in S individuals from shattercane population S-179 (Tables 5 and 7). Furthermore, this population was resistant to imazethapyr but susceptible to nicosulfuron (Table 3). This corroborates the findings of Patzoldt and Tranel (2007), who reported the Ser$_{653}$Thr mutation to confer resistance to IMI but not to SU herbicides in a common waterhemp [Amaranthus tuberculatus (Moq.) Sauer] population from Illinois. Beckie et al. (2012) also reported the Ser$_{653}$Thr mutation in an IMI-resistant wild oat (Avena fatua L.) population from western Canada. To our knowledge, this is the first time that the Ser$_{653}$Thr mutation has been reported in shattercane. No

mutations were detected at Asp$_{376}$, Arg$_{377}$, and Gly$_{654}$ in our populations; however, amino acid substitutions at these latter codons are reported to confer additional types and levels of ALS resistance in other weed species (Tranel et al. 2016). The current study focused on target-site mutations at Asp$_{376}$, Arg$_{377}$, Val$_{560}$, Trp$_{574}$, Ser$_{653}$, and Gly$_{654}$. Mutations at Ala$_{122}$, Pro$_{197}$, and Ala$_{205}$ (which have also been commonly reported; Tranel et al. 2016), unreported/novel mutations at different codons in the ALS gene, and non–target site resistance (i.e., a translocation-based mechanism) could be the mechanisms of resistance in the remaining ALS inhibitor–resistant populations detected in Nebraska and Kansas by Werle et al. (2016a). Non–target site ALS resistance has been reported in common waterhemp by Guo et al. (2015). Our future work includes development of high-throughput assays to characterize ALS mutations in shattercane and johnsongrass populations and to more thoroughly characterize putative structural variation at the 5′ end of the gene, where Ala$_{122}$, Pro$_{197}$,

Table 5. Phenotype and amino acid encoded by acetolactate synthase (ALS) codons 376, 377, 560, 574, 653, and 654 of selected individuals within ALS inhibitor–resistant and susceptible shattercane populations treated with labeled rate of imazethapyr (70 g ai ha^{-1}) and nicosulfuron (35 g ai ha^{-1}).

Population[a]	Herbicide	Plant phenotype[b]	376	377	560	574	653	654
			— ALS codon[c]					
Sorghum	Imazethapyr + nicosulfuron	S	Asp	Arg	Val	Trp	Ser	Gly
Inzen	Imazethapyr + nicosulfuron	R	Asp	Arg	**Ile**	**Leu**	Ser	Gly
S-13	Imazethapyr + nicosulfuron	S	Asp	Arg	Val	Trp	Ser	Gly
S-31[d]	Imazethapyr	MR	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
S-46	Imazethapyr	MR	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
S-58[e]	Imazethapyr + nicosulfuron	R	Asp	Arg	Val	Trp	Ser	Gly
	Imazethapyr	S	Asp	Arg	Val	Trp	Ser	Gly
S-63	Imazethapyr + nicosulfuron	MR & R	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	**Ile**	Trp	Ser	Gly
S-105[d]	Imazethapyr + nicosulfuron	MR	Asp	Arg	**Ile**	Trp	Ser	Gly
		S	Asp	Arg	**Ile**	Trp	Ser	Gly
S-113	Nicosulfuron	MR	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
S-117	Imazethapyr	MR	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
S-125	Imazethapyr + nicosulfuron	S	Asp	Arg	**Ile**	Trp	Ser	Gly
S-130	Imazethapyr + nicosulfuron	S	Asp	Arg	Val	Trp	Ser	Gly
S-134[f]	Nicosulfuron	MR	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	**Ile**	Trp	Ser	Gly
S-177	Imazethapyr + nicosulfuron	S	Asp	Arg	Val	Trp	Ser	Gly
S-178	Nicosulfuron	MR	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
S-179	Imazethapyr	R	Asp	Arg	**Ile**	Trp	**Thr**	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly

[a] Populations S-13, S-125, and S-130 were included as susceptible controls.
[b] Abbreviations: S, susceptible; MR, moderately resistant; R, resistant.
[c] Amino acid positions correspond to the amino acid sequence of ALS from *Arabidopsis thaliana* (L.) Heynh (GenBankX51514). Bold text denotes an amino acid substitution at the specified codon.
[d] Only one individual in the population survived the imazethapyr application.
[e] All plants sprayed with nicosulfuron were resistant.
[f] Only one individual in the population survived the nicosulfuron application.

and Ala$_{205}$ reside. All methods will be made publicly available for ongoing screening and monitoring of ALS inhibitor–resistant *Sorghum* species following commercialization of Inzen technology. We further intend to determine whether non–target site resistance is present in these populations.

Distribution of Resistance. Werle et al. (2016a) reported that populations from similar geographic regions had differential responses to two ALS-inhibitor chemistries. The current study correlated differential herbicide responses to known amino acid substitutions within the ALS gene sequence amplified from those populations. For instance, two shattercane populations from Thayer County, NE, S-178 and S-179, were 9 km apart and showed different types of resistance (Figure 1). S-178 was moderately resistant to nicosulfuron and did not contain any of the mutations evaluated in this study. S-179 was resistant to imazethapyr and contained two mutations, Val$_{560}$Ile and Ser$_{653}$Thr. Three johnsongrass populations from Buffalo County, NE, J-35, J-36, and J-37, were within a radius of 17 km and showed different types of resistance to ALS inhibitors (Figure 2). J-35 was moderately resistant to imazethapyr and resistant to nicosulfuron and contained the Val$_{560}$Ile mutation. J-36 was highly resistant to both imazethapyr and nicosulfuron and contained the Trp$_{574}$Leu mutation. J-37 was resistant only to imazethapyr and did not contain any mutations at the ALS codons evaluated in this study.

Werle et al.: ALS inhibitor resistance in weedy *Sorghum spp.* • 171

Table 6. Phenotype and amino acid encoded by acetolactate synthase (ALS) codons 376, 377, 560, 574, 653, and 654 of selected individuals within ALS inhibitor–resistant and susceptible johnsongrass populations treated with labeled rate of imazethapyr (70 g ai ha^{-1}) and nicosulfuron (35 g ai ha^{-1}).

			ALS codon[c]					
Population[a]	Herbicide	Plant phenotype[b]	376	377	560	574	653	654
Sorghum	Imazethapyr + nicosulfuron	S	Asp	Arg	Val	Trp	Ser	Gly
Inzen	Imazethapyr + nicosulfuron	R	Asp	Arg	**Ile**	**Leu**	Ser	Gly
J-10[d]	Imazethapyr	MR	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
J-12[d]	Imazethapyr	MR	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
J-14	Imazethapyr + nicosulfuron	S	Asp	Arg	Val	Trp	Ser	Gly
J-15	Imazethapyr + nicosulfuron	MR + R	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
J-18	Imazethapyr	MR	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
J-25	Imazethapyr + nicosulfuron	S	Asp	Arg	Val	Trp	Ser	Gly
J-35	Imazethapyr + nicosulfuron	MR + R	Asp	Arg	Val/**Ile**	Trp	Ser	Gly
		S	Asp	Arg	**Ile**	Trp	Ser	Gly
J-36[e]	Imazethapyr + nicosulfuron	R	Asp	Arg	Val	Trp/**Leu**	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
J-37	Imazethapyr	MR/R	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
J-40[f]	Imazethapyr	MR/R	Asp	Arg	Val	Trp	Ser	Gly
J-44	Imazethapyr	MR	Asp	Arg	Val	Trp	Ser	Gly
		S	Asp	Arg	Val	Trp	Ser	Gly
J-52	Imazethapyr + nicosulfuron	S	Asp	Arg	Val/**Ile**	Trp	Ser	Gly

[a] Populations J-14, J-25, and J-52 were included as our susceptible controls.
[b] Abbreviations: S, susceptible; MR, moderately resistant; R, resistant.
[c] Amino acid positions correspond to the amino acid sequence of ALS from *Arabidopsis thaliana* (L.) Heynh (GenBankX51514). Bold text denotes an amino acid substitution at the specified codon.
[d] Only one individual in the population survived the imazethapyr application.
[e] Not all resistant individuals screened presented the Trp$_{574}$Leu mutation. Four additional resistant individuals were screened, and three presented the Trp$_{574}$Leu mutation.
[f] All individuals in the population survived imazethapyr application.

Table 7. Acetolactate synthase (ALS) resistance–conferring mutations detected in one shattercane and one johnsongrass population.

	ALS codon[a]						Phenotype[b]	
Population	376	377	560	574	653	654	Imazethapyr	Nicosulfuron
Sorghum	gat (Asp)	cgt (Arg)	gtc (Val)	tgg (Trp)	agt (Ser)	ggt (Gly)	S	S
Inzen	gat (Asp)	cgt (Arg)	atc (Ile)	**ttg (Leu)**	agt (Ser)	ggt (Gly)	R	R
J-36	gat (Asp)	cgt (Arg)	gtc (Val)	**ttg (Leu)**	agt (Ser)	ggt (Gly)	R	R
S-179	gat (Asp)	cgt (Arg)	atc (Ile)	tgg (Trp)	**act (Thr)**	ggt (Gly)	R	S

[a] Nucleotide sequence and the encoded amino acid (in parenthesis) at each codon. Amino acid positions correspond to the amino acid sequence of ALS from *Arabidopsis thaliana* (L.) Heynh (GenBankX51514). Bold text denotes an amino acid substitution at the specified codon.
[b] Abbreviations: S, susceptible; R, resistant.

Together, these results support our initial hypothesis and present evidence that resistance in these populations evolved independently and was not due to a single source spread via seeds or pollen-mediated gene flow. However, the availability of high-throughput genotyping methods that could cost-effectively screen more individuals in the more than 200 populations we collected would be useful to confirm this; development of such technologies is a focus of our ongoing work.

Independent selection for resistance indicates overreliance on the same herbicide mode of action across a broad geographic region favored progeny of individuals containing naturally occurring mutations that originated at each field/site. Because there are multiple sites in the ALS gene where resistance-conferring mutations can occur (Tranel et al. 2016), resistance to ALS inhibitors is an ideal case study to research evolutionary mechanisms giving rise to herbicide-resistant weed populations and the spread of resistance alleles across the landscape. For example, if the same phenotypic response and ALS allele was encountered across populations from the same region, improper management at one or a few sites was likely the initial source of resistance alleles that colonized new fields via seed and/or pollen-mediated gene flow. Subsequent repeated applications of ALS inhibitors at these new sites would have favored plants carrying the migrated resistance alleles until they became established in the population. Using distribution and frequency of glyphosate resistance in common waterhemp populations from more than 100 farms across Illinois, Evans et al. (2016) presented strong evidence that the likelihood and frequency of resistant individuals within a population are inversely correlated to the number of herbicide modes of action used per application per season at each farm. Moreover, the likelihood and frequency of resistant individuals were not correlated to the distance from the nearest field infested with resistant weeds. These findings indicate that the use of a diversified approach (i.e., adopting multiple effective modes of action at each application) can significantly reduce selection pressure favoring herbicide resistance, even though neighboring properties may be infested with resistant weeds. Thus, the management program adopted at each farm will be the key driver for resistance evolution and management in situ.

Lee et al. (1999) reported ALS inhibitor–resistant shattercane populations in Buffalo, Webster, and Thayer counties of Nebraska. Resistance was still present in 2013 in two of these counties (Buffalo and Thayer). ALS inhibitor–resistant shattercane and johnsongrass were also detected in other areas of Nebraska and Kansas where ALS-inhibiting herbicides were commonly used prior to the introduction of glyphosate-tolerant crops (Figures 1 and 2). This is an indicator that target-site ALS resistance has little to no ecological fitness cost to weedy populations in the absence of selection by ALS-inhibiting herbicides (i.e., the amino acid
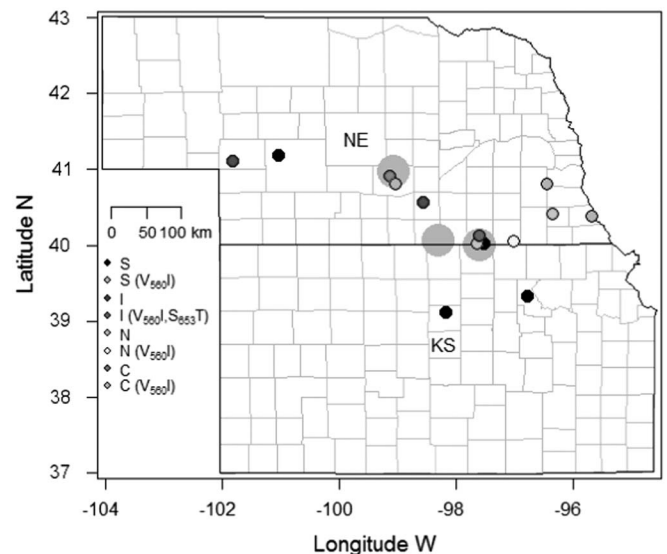

Figure 1. Distribution of shattercane populations from Kansas (KS) and Nebraska (NE) included in this study. Abbreviations: S, population susceptible to all herbicides tested; I, resistant to imazethapyr; N, resistant to nicosulfuron; C, cross-resistant to the ALS herbicides tested (nicosulfuron and imazethapyr); $V_{560}I$ and $S_{653}T$ indicate the populations that carried the $Val_{560}Ile$ and $Ser_{653}Thr$ mutations, respectively. The three gray circles represent the regions in Nebraska (Buffalo, Webster, and Thayer counties) where ALS inhibitor–resistant shattercane populations were detected by Lee et al. (1999).
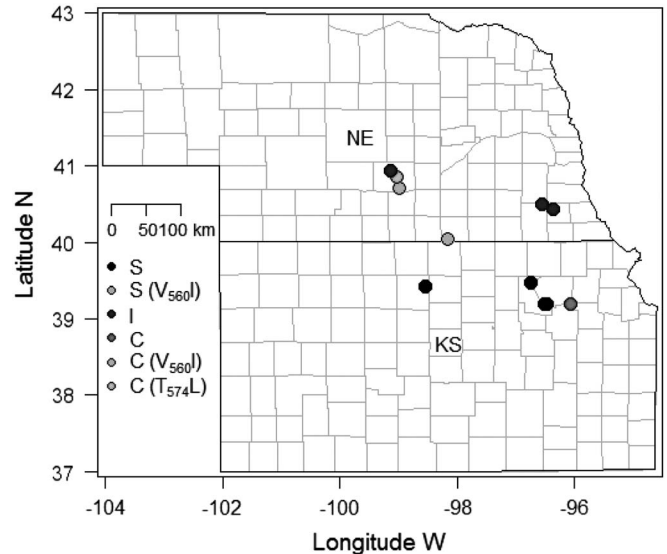

Figure 2. Distribution of johnsongrass populations from Kansas (KS) and Nebraska (NE) included in this study. S, population susceptible to all herbicides tested; I, resistant to imazethapyr; C, cross-resistant to the ALS herbicides tested (imazethapyr and nicosulfuron); $V_{560}I$ and $T_{574}L$ indicate the populations that carried the $Val_{560}Ile$ and $Trp_{574}Leu$ mutations, respectively.

substitutions identified herein are neutral mutations), corroborating observations of Davis et al. (2009), Park et al. (2004), and Sibony and

Werle et al.: ALS inhibitor resistance in weedy *Sorghum spp.* • 173

Rubin (2003). Cost-effective high-throughput genotyping is needed to facilitate more quantitative estimates of fitness costs associated with R alleles in these populations by directly monitoring changes in their frequencies over wide geographies and time.

The results of this research indicate that if growers observed ALS inhibitor–resistant shattercane and/or johnsongrass in their fields in the past, and these species are still present, they are likely to carry resistance alleles. Moreover, we found evidence that resistant populations across common geographical regions evolved resistance independently. Thus, if growers adopt Inzen technology, shattercane and johnsongrass should be properly managed prior to and following its adoption. Use of ALS-inhibiting herbicides in Inzen sorghum will favor weeds that are carrying an ALS-resistance allele. Under continuous use of ALS inhibitors, R individuals (heterozygous or homozygous for resistance alleles) will survive and reproduce, while most S individuals will be killed; hence, the frequency of R individuals in the population will increase until most are resistant, and the technology will rapidly lose value. Moreover, if surviving plants (either resistant individuals or escapes) are present at the end of the season, crop-to-weed gene flow may have occurred. If so, new resistance alleles will be introduced into the weedy sorghum population and the individuals carrying it would be expected to survive and reproduce following application of ALS inhibitors, as described earlier.

Modeling work in shattercane has indicated that crop rotation and proper management with effective herbicides during non-sorghum years (i.e., glyphosate and clethodim) will be key strategies to postpone fixation of the resistance alleles in shattercane and maintain its population density at low levels following deployment of Inzen technology (Werle et al. 2016b). During non-sorghum years, herbicides from multiple effective modes of action should be considered for shattercane and johnsongrass control and to reduce the likelihood of resistance evolution to alternative herbicide modes of action (Evans et al. 2016; Norsworthy et al. 2012). Alternative strategies such as interrow cultivation, rope wick herbicide application (using non-ALS herbicides), and/or rogueing surviving resistant or escape weeds before flowering may also play an important role in managing ALS resistance. These strategies can reduce both the likelihood of pollen-mediated gene flow from Inzen to weedy *Sorghum* species and seedbank replenishment

with resistance alleles (Goulart et al. 2012; Roeth et al. 1994).

Survey of ALS Variation in Wild *Sorghum* and Other Panicoid Grasses. To assess variation in the ALS gene more broadly, the gene sequence was aligned from an array of sorghum lines and more diverse panicoid grasses, including *S. bicolor* 'BTx623', *S. bicolor* ssp. *drummondii*, *S. bicolor* race 'guinea-margaritiferum', *S. propinquum*, *S. bicolor* ssp. *verticilliflorum*, johnsongrass, switchgrass, foxtail millet, corn, and zoysiagrass (Supplementary Figure 1). This alignment revealed a few unexpected results. First, the 5′ portion of the ALS gene from the *S. bicolor* 'BTx623' reference genome, which was used for primer design in PCR-based surveys of ALS codons, was found to be nonhomologous to the 5′ ALS sequences from other panicoid grasses or to the ALS gene that was de novo assembled in this study using the 'BTx623-Rooney' resequencing data generated by Rizal et al. (2015). In contrast, the 5′ portion of the ALS gene from the de novo assembled 'BTx623-Rooney' sequence matches well with other panicoid grasses. These results suggest that the ALS gene from the reference sequence available in GenBank for *S. bicolor* 'BTx623' may not be correct. The similarity of the ALS sequence from the 'BTx623-Rooney' data to other panicoid grasses suggests that this sequence is more accurate.

Second, the 5′ end of the ALS gene from most wild *Sorghum* species, was not recovered from the de novo assemblies (Figure 3). To rule out issues with assembly, the raw sequence reads generated from each of the wild *Sorghum* species resequencing projects were mapped to the 'BTx623-Rooney' and corn ALS gene sequences. No reads were detected that mapped to 5′ gene regions missing from the wild *Sorghum* species. This result suggests that the 5′ portion of the gene is truncated in the wild species but not in 'BTx623', indicating presence/absence variation among ALS genes in the genus and a lack of Ala_{122}, Pro_{197}, and Ala_{205} codons in shattercane and johnsongrass. Alternatively, the high GC% in the 5′ portion of the ALS gene may cause biased underrepresentation in the resequencing data. Regardless of the reason, Illumina-based resequencing approaches may not be reliable for detection of SNPs in the 5′ region of the ALS gene. PCR-based approaches, optimized for high GC% templates and using the 'BTx623-Rooney' template, will be needed to assess whether the 5′ portion of the ALS gene is truncated in wild
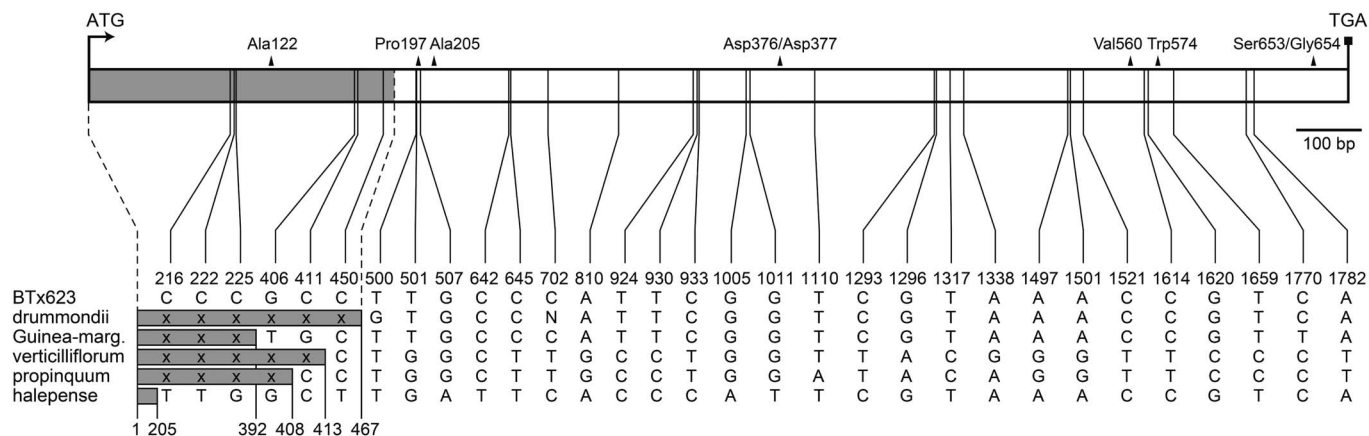
Figure 3. Sequence variation among ALS genes from cultivated, wild, and weedy *Sorghum*. Shown is a summary of the variation detected among *Sorghum* species from the ALS alignment in Supplementary Figure 1. Abbreviated names correspond to the full species names listed in "Materials and Methods." All positions exhibiting sequence variation in at least one *Sorghum* species are marked on the ALS gene map at top (drawn to scale), and the nucleotides found at that position for all *Sorghum* species are listed below. Position numbers correspond to the 'BTx623' gene sequence that was de novo assembled from the Illumina sequence data (see "Materials and Methods"). All other positions in the alignment were identical among the *Sorghum* species. The 5′ truncations in the ALS gene of most *Sorghum* species are shaded in gray, and the extent of each truncation is labeled with nucleotide positions. Positions of known resistance mutations are shown above the gene and marked with a triangle; these position numbers correspond to the *Arabidopsis thaliana* reference gene (Tranel et al. 2016).

Sorghum species and to score SNPs in this region if it is indeed present.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1614/WS-D-16-00095.1

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