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Bensulfuron-methyl resistant *Sagittaria trifolia* L.: multiple resistance, cross-resistance and molecular basis of resistance to acetolactate synthase-inhibiting herbicides

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Abstract: Acetolactate synthase (ALS)-inhibiting herbicides play an important role in controlling broad-leaved weeds. Populations of *Sagittaria trifolia* L. showed resistance to ALS-inhibiting sulfonylurea herbicides (e.g. bensulfuron-methyl) in paddy fields in the northeast of China. In our study, whole-plant bioassays were performed on eight suspected resistant *S. trifolia* populations that showed high levels of resistance to bensulfuron-methyl, with resistance indices from 31.06 to 120.35. The results of ALS-activity assays were consistent with the observed whole-plant dose-response data. This confirmed that resistant populations displayed significantly higher ALS activity than the sensitive population due to prevention of normal enzyme-herbicide interaction. The mutations Pro-197-Ser, Pro-197-His, Pro-197-Thr and Pro-197-Leu were identified in the ALS gene of resistant populations. Pro-197-His and Pro-197-Thr mutations conferring resistance to bensulfuron-methyl are reported for the first time in *S. trifolia*. All resistant populations were resistant to sulfonylurea (SU) herbicides, but not to imidazolinone (IMI) herbicides. HLJ-5 and JL-3 populations were resistant to bispyribac-sodium of the pyrimidinyl-thiobenozoate (PTB) class of ALS herbicides, JL-2 to penoxsulam of triazolopyrimidine (TP) class and JL-1 to pyribenzoxim, also of PTB class. The eight *S. trifolia* populations were susceptible to other herbicide modes of action tested.

Key words: acetolactate synthase; genetic analysis; herbicide resistance; target-site mutation; *Sagittaria trifolia* L.

INTRODUCTION

Herbicides are estimated to account for up to 50% of the global plant protection market. In particular, acetolactate synthase (ALS, also known as acetohydroxyacid synthase, AHAS)-inhibiting herbicides have gained increasing popularity among farmers since their appearance on the market and are among the most important chemical weed-control measures [1]. ALS is the first enzyme common to the biosynthesis of the branched-chain amino acids valine, leucine and isoleucine [2]. Five different chemical classes of herbicides act to inhibit ALS: sulfonylurea (SU) [3], imidazolinone (IMI) [4], triazolopyrimidine (TP) [5], pyrimidinyl-thiobenozoate (PTB) and sulfonylamino-carbonyl-triazolinone (SCT) [6]. These herbicides have been extensively used in the last decades due to their high efficacy at low concentrations, low impact on non-target organisms and good selectivity in several crops [7].

Global and persistent use of ALS-inhibiting herbicides has consequently resulted in the rapid evolution of many ALS herbicide-resistant weed populations since the first case reported in *Lolium rigidum* from Australia in 1982 [8]. Worldwide, 159 weed species have evolved ALS herbicide resistance [7]. Some prominent examples are *Monochoria korsakowii* (Korsakow's monochoria), *Myosoton aquaticum* L. (water chickweed) and *Descurainia sophia* L. (flaxseed) in China, all of which exhibit resistance to ALS herbicides [9-11]. Different mechanisms are known to endow herbicide resistance in plants, such as reduced sensitivity of the target site, reduced uptake or translocation, target-site amplification, decreased rate of herbicide activation, increased rate of herbicide detoxification, or sequestration of the herbicide away from the target site into the vacuole or the apoplast. In addition, reduced target-site sensitivity (based on a target-site mutation) due to point mutations occurring within the five separate highly conserved domains of the ALS gene is a common mechanism of resistance [12]. Eight mutation sites endowing target-site resistance to ALS inhibitors have been reported so far: Ala122, Pro197,

Ala205, Asp376, Arg377, Trp574, Ser653 and Gly654 (numbering standardized in accordance with the *Arabidopsis thaliana* L. sequence) [13-15]. Generally, ALS resistance mutations at Pro-197 confer SU and TP resistance, mutations at Ala-122, Ala-205, Ser-653 or Gly-654 confer IMI resistance, and mutations at Asp-376, Arg-377 or Trp-574 confer broad-spectrum resistance across ALS herbicides [16]. Different mutations occurring at these sites may confer various resistance and cross-resistance patterns in response to ALS herbicides. A sulfometuron methyl-resistant *Amaranthus retroflexus* L. (redroot amaranth) population evolved resistance to various herbicides of IMI, TP and PTB [17]. In addition, cross-resistance to ALS herbicides was confirmed in resistant populations of *Rotala indica*, *Lolium rigidum* and *Conyza canadensis* [18-20]. Multiple herbicide resistance is another problem for ALS inhibitors and has been reported in accession of *Echinochloa phyllopogon* Koss. and *Echinochloa oryzoides* Fritsch. involving four different herbicides of three chemical families with different modes of action [21].

Sagittaria trifolia L. is a widely-distributed perennial, emergent, aquatic plant that belongs to the Alismataceae family. It is mostly found in the shallow waters of marshes, ponds, stream margins and rice fields in Asia. It has sagittate leaf blades and produces numerous corms on branched rhizomes. It reproduces both sexually via achenes that are often called "seeds", and vegetatively via corms. *S. trifolia* is a common weed of rice paddies in northeast China [22]. SU herbicides (especially bensulfuron-methyl) have been widely and frequently used to control *S. trifolia* and, as a consequence, resistance is now widespread. Although many bensulfuron-methyl-resistant *S. trifolia* accessions have been reported [22, 23], cross-resistance and multiple resistance to herbicides of different modes of action were not tested. Our study was focused on three main objectives: (i) confirmation of resistance to bensulfuron-methyl by whole-plant bioassay and characterizing the level of resistance in an *S. trifolia* population using an enzyme assay; (ii) sequencing, screening and comparing the ALS gene sequence of *S. trifolia* from wild-type and resistant populations to document the target site that could be involved in resistance; and (iii) characterizing cross-resistance to ALS-inhibiting herbicides and multiple resistance to herbicides of varying mechanisms of action.

MATERIALS AND METHODS

Plant materials

Eight putative ALS herbicide-resistant (R) and one susceptible (S) *S. trifolia* samples were collected in May 2014 from paddy fields in the northeastern region of China (Table 1). The R populations were collected from Jilin and Heilongjiang provinces where bensulfuron-methyl had been used for more than 10 years. The S population was collected from a reserve of Liaoning Province where there had been no history of herbicide application. Seedlings of the R and S populations were planted in 25-cm diameter plastic pots (6-7 per pots) containing clay loam (3-5 cm water depth) and located in a glasshouse at 25:15°C day:night temperature with natural sunlight. The corms were dug out in the fall and stored at 4°C for >4 months [22].

Dose response experiments

Whole plant dose response assays were conducted to characterize the suspected resistant samples of *S. trifolia* compared with the susceptible standards. Corms of the R and S populations sprouted in Petri dishes in the light at 28°C for 5 days, and about 6-7 corms were

Table 1. Geographical origins and herbicide application histories of *Sagittaria trifolia* populations used in the study.

Population	Location	Co-ordinates	Estimated year of application
HLJ-1	Suihua	Lat. N45°49′30.36″ Long. E126°16′25.73″	>10 years
HLJ-2	Haerbin	Lat. N45°51′14.37″ Long. E126°48′30.88″	>10 years
HLJ-3	Jiamusi	Lat. N40°46′40.7″ Long. E130°18′59.17″	>10 years
HLJ-4	Shuangyashan	Lat. N46°18′42.91″ Long. E132°16′49.28″	>10 years
HLJ-5	Jixi	Lat. N45°42′09.45″ Long. E132°05′08.48″	>10 years
JL-1	Shulan	Lat. N44°23′16.94″ Long. E126°57′18.29″	>10 years
JL-2	Yushu	Lat. N44°33′30.29″ Long. E126°29′26.67″	>10 years
JL-3	Dehui	Lat. N44°38′01.85″ Long. E126°11′08.90″	>10 years
S	Shenyang	Lat. N41°56′34.08″ Long. E123°41′28.68″	No history of herbicides use

Lat. – latitude; Long. – longitude

planted in clay loam at a depth of 3 cm in 25-cm diameter plastic pots (3-5 cm water depth). Pots were kept in the glasshouse as described above. Ten days after planting, seedlings were thinned to 4 plants per pot before herbicide application. At the one-to-two-leaf stage, bensulfuron-methyl (recommended rate is 30 g a.i. ha^{-1}) was applied using a moving-boom cabinet sprayer with a flat fan nozzle with a water volume of 118 L ha^{-1} at a pressure of 0.29 MPa and 50 cm above the foliage. The resistant populations received bensulfuron-methyl at 0, 7.5, 30, 120, 480, 1920 and 2400 g a.i. ha^{-1}, while the susceptible standard received bensulfuron-methyl at 0, 0.048, 0.24, 1.2, 6, 30, 150 g a.i. ha^{-1} [22]. Harvesting of aboveground foliage (oven-dried at 70°C for 72 h) was performed 21 days after treatment. A total of 3 replicates with 4 plants per pot were included at each dose.

ALS extraction and enzyme activity assay

The S and R populations were grown until the three-to-four-leaf stage, at which point actively growing green leaf material was harvested, snap-frozen in liquid nitrogen and stored at -80°C. All extractions were performed using pH 7.5 extraction buffer [24] containing 0.1 M K_2HPO_4, 10 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM flavin adenine dinucleotide (FAD), 10% glycerol and 0.5% soluble PVP by volume. The frozen tissues (4 g) were loaded into mortars. Liquid nitrogen was poured into each mortar, and tissues were ground into a fine powder. Samples were then transferred to beakers containing a 3:1 ratio of cold extraction buffer. The homogenate was filtered through two layers of Mira cloth into 16-mL centrifuge tubes. Samples were centrifuged for 15 min at 4°C at 27000×g. Supernatants (6 mL) were transferred to beakers and 50% ammonium sulfate ($(NH_4)_2SO_4$) was added to saturation. The supernatant and ammonium sulfate mixtures were stirred at low speed for 10 min on ice to allow protein precipitation. Mixtures were again centrifuged at 4°C for 20 min at 27000×g. Supernatants were discarded and the pellet was redissolved in 4.5 mL of resuspension buffer (the same as the extraction buffer without PVP). Samples were desalted by gravity-flow passage through 2.5-mL PD-10 Sephadex G-25 columns. The column was previously washed with elution buffer of 50 mM *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 200 mM sodium pyruvate, 20 mM $MgCl_2$, 2 mM thiamine pyrophosphate and 20 μM flavin adenine dinucleotide, at pH 7.5, and was immediately used in the assay. The protein concentration of crude extract was measured by the Bradford method [16,25]. The reaction mixture contained 100 μL of enzyme extract and 100 μL of bensulfuron-methyl, and was incubated at 37°C for 60 min. Active ingredient concentrations of bensulfuron-methyl were 0, 10^{-2}, 10^{-1}, 1, 10, 10^2 and 10^3 μM for the eight R populations; and 0, 10^{-3}, 10^{-2}, 10^{-1}, 10 and 10^2 μM for the S population [22]. The enzymatic reaction was stopped by adding 40 μL of 6 N H_2SO_4 and incubated for 15 min at 60°C to convert acetolactate to acetoin [24]. Reaction mixtures that were acidified (40 μL of 6 N H_2SO_4) prior to the addition of enzyme were used as standard background controls for nonenzymatic formation of acetoin. Then, 190 μL of creatine solution (0.55%) and 190 μL of a-naphthol solution (5.5% in 5 N NaOH) were added and the mixture was incubated at 60°C for 15 min. Absorbance was determined at 530 nm using a UV spectrophotometer and the mean absorbance reading from blank wells was subtracted from each test well reading by measuring acetoin production. The total protein concentration in the reaction mixture was normalized to 320 μg for all samples. All assays were performed with four replicate samples per herbicide dose and were repeated three times for each population.

Molecular genetic analysis

DNA extraction

Genomic DNA was extracted from fresh leaf tissue using the plant Genomic DNA Kit (Sangon Biotech, China). Approximately 100 mg of plant material from a single plant was placed in a mortar and ground in liquid nitrogen to a fine powder. After adding extraction buffer (12 μL β-mercaptoethanol (BME), 600 μL Buffer PCB, pre-heated to 65°C), homogeneous oscillation samples were incubated at 65°C for 25 min. The suspension was gently mixed with one volume chloroform (mixed fully) and centrifuged for 5 min at 12000 rpm. One volume of Buffer BD and anhydrous alcohol were added to the aqueous phase, followed by 2 min of incubation at room temperature and centrifugation (1 min at 10000 rpm). Pellets were washed with PW

Table 2. Herbicides and rates used for ALS cross-resistance screening of Sagittaria trifolia populations.

Herbicide	Recommended dose (g a.i. ha^{-1})	Dose (g a.i. ha^{-1})	
		S	R
Pyrazosulfuron-ethyl (SU)	20	0, 0.31, 1.25, 5.00, 20, 80	0, 1.25, 5, 20, 80, 320
Ethoxysulfuron (SU)	13.5	0, 0.84, 3.38, 13.5, 54, 216	0, 3.38, 13.5, 54, 216, 864
Penoxsulam (TP)	30	0, 0.47, 1.88, 7.5, 30, 120	0, 0.47, 1.88, 7.5, 30, 120
Pyribenzoxim (PTB)	45	0, 0.7, 2.81, 11.25, 45, 180	0, 0.7, 2.81, 11.25, 45, 180
Bispyribac-sodium (PTB)	37.5	0, 0.59, 2.34, 9.38, 37.5, 150	0, 0.59, 2.34, 9.38, 37.5, 150
Imazethapyr (IMI)	6.5	0, 0.41, 1.63, 6.5, 26, 104	0, 1.63, 6.5, 26, 104, 416

S – Susceptible population; R – Resistant population

Solution and Wash Solution (twice), air-dried and dissolved in 50 μL TE Buffer.

Detection of mutant ALS alleles

PCR reactions were carried out using 2×PCR Master Mix Solution (iNtRON Biotechnology, Korea). Thermocycling was performed using a Geneamp PCR System 9700. The cycling program began with 4 min at 94°C, followed by 35 cycles, each consisting of 30 s at 94°C, 30 s at 63°C and 90 s at 72°C. The program ended with a final step of 10 min at 72°C. Each of the following primer pairs was used separately. Primer 1: F1, 5'-AGAGGGAGGGTGTCAA-AGACG-3' and R1, 5'-TTTCAGGTCGCCACAGATAGAG-3' yielded a 926-bp fragment of ALS gene; F2, 5'- TCTGTGGCGACCTGAAACTG-3' and R2, 5'-ACCTCCACTCGGAAT-CATCG-3' yielded a 734-bp fragment of the ALS gene.

PCR products were visualized by electrophoresis on 1% (wt/vol) agarose gel running in 0.5×TBE buffer and purified using the SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, China). The desired PCR bands were cloned with the pUT-T Cloning Kit (Sangon Biotech, China), and then the recombinant plasmids were introduced into DH5α-competent *Escherichia coli* (Sangon Biotech, China) according to the manufacturer's instructions. Positive clones were sequenced from both ends by Sangon Biotech (China). At least five clones from each individual plant were sequenced. Sequences data were aligned using DNAMAN 8.0 software.

Cross- and multiple resistance to other herbicides

Plants were grown in pots (4 seedlings per pot) under conditions similar to those described earlier. ALS herbicides were applied when R and S plants had reached the three-to-four-leaf stage [26]. The recommended doses of imazethapyr (IMI), ethoxysulfuron (SU), pyrazosulfuron-ethyl (SU), penoxsulam (TP), bispyribac-sodium (PTB) and pyribenzoxim (PTB) are 6.5, 13.5, 20, 30, 37.5 and 45 g a.i. ha^{-1}, respectively [4] (Table 2). Harvesting of aboveground foliage (oven-dried at 70°C for 72 h) was performed 21 days after herbicide application. Each treatment was replicated four times and the experiments conducted twice. Five herbicides with different modes of action other than ALS inhibition (bentazone (1500 g a.i. ha^{-1}); prometryn (900 g a.i. ha^{-1}); MCPA (800 g a.i. ha^{-1}); oxyfluorfen (180 g a.i. ha^{-1}); mesotrione (150 g a.i. ha^{-1})) were tested for efficacy on SU-resistant *S. trifolia*. The procedures were similar to those described in the previous section.

Statistical analysis of the data

Datasets from repeated experiments were analyzed by ANOVA with the software SPSS 21.0 and *t*-tests ($p<0.05$) to examine the significance of the regression parameters. When the variance between repeated experiments was not significant, data from each herbicide were pooled and used for subsequent analysis. The herbicide concentration causing 50% inhibition of growth (GR_{50}) or enzyme activity (I_{50}) was obtained by nonlinear regression analysis using Sigma Plot software [27]. The data were fitted to the three-parameter logistic curve model:

$$y = C + (D - C) / [1 + (x/ED_{50})^b],$$

where y represents the measured dry weight and enzyme activity, x is the herbicide concentration, C is the lower limit, D is the upper limit of the curve and *b* is proportional to the slope around GR_{50} (I_{50}), the herbicide dose or concentration at which 50% growth (enzyme activity) is inhibited. The levels of resistance for the plant dose-response and the ALS assays were calculated as the ratio of GR_{50} or I_{50} values of the R population relative to the S standard.

RESULTS

Whole-plant dose-response experiments

Dose-response experiments conducted on eight suspected *S. trifolia* populations confirmed resistance to bensulfuron-methyl. All populations showed strong resistance. At the recommended field rate of bensulfuron-methyl (30 g a.i. ha^{-1}), the dry weight of plants in the S population was reduced to 14% of the untreated control, whereas R populations were almost unaffected (Fig. 1). S plants had GR_{50} values of 1.6 g a.i. ha^{-1} for bensulfuron-methyl. The resistance ratios of HLJ-1, HLJ-5, JL-2 and JL-3 were 108.79, 99.45, 86.75 and 123.35 as compared with 40.47, 74.54, 76.48 and 31.06 for HLJ-2, HLJ-3, HLJ-4 and JL-1, respectively (Table 3). The levels of resistance for the R plants exhibited great differences.

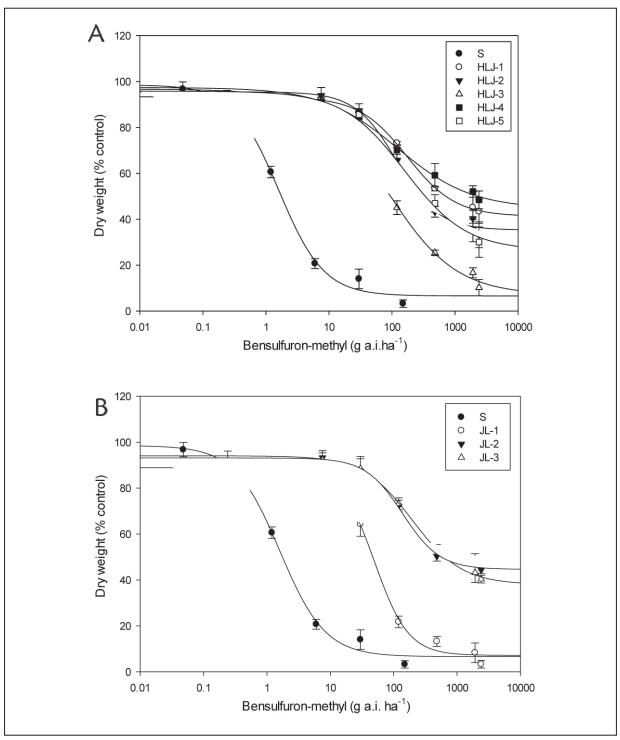

Fig. 1. Dose-response curve for aboveground dry weights of the susceptible population and resistant populations of *Sagittaria trifolia* treated with bensulfuron-methyl. (**A**) Susceptible population and resistant populations from Heilongjiang Province (HLJ-1, 2, 3, 4, 5). (**B**) Susceptible population and resistant populations from Jilin Province (JL-1, 2, 3). Each data point represents the mean±SE of three replicates.

ALS activity assay

Herbicide sensitivity of the acetolactate synthase enzyme isolated from R and S plants was determined using the SU herbicide bensulfuron-methyl. As shown in Table 4 and Fig. 2, wild-type ALS isolated from the S population was, as expected, strongly inhibited by bensulfuron-methyl, with an I_{50} value of 0.06 μM. However, the ALS isolated from the R populations were different, with I_{50} values 1.10- to 48.88-fold greater than that of the S population. The total ALS activity was similar for the S, HLJ-2 and JL-1 populations, while the other populations had significantly higher ALS activity than that of the S population. The result was consistent with the whole-plant dose-response data. It demonstrated that moderate levels of resistance to bensulfuron-methyl herbicides occur at the enzyme level.

Molecular genetic analysis

Two fragments of the *S. trifolia* ALS gene were directly sequenced spanning five highly conserved regions where mutations conferring resistance to ALS-inhibiting herbicides have been reported in other weed species. The first fragment, amplified by the primer pair F1/R1, resulted in a sequence approximately 926 bp in length covering domains C, A and D, while the second fragment, amplified by the primers F2/R2, resulted in a 734 bp sequence covering domains B and E. Based on a BLAST search using this sequence, a clear

Table 3. GR_{50} values and regression equation for whole-plant bioassays of susceptible (S) and resistant (HLJ-1, 2, 3, 4, 5 and JL-1, 2, 3) biotypes of *Sagittaria trifolia* to bensulfuron-methyl.

Population	$y = C + (D-C)\ [1+ (x/GR_{50})^b]$				RI
	C	D	GR_{50} (g a.i. ha^{-1})	b	
HLJ-1	41.23	93.36	174.06±14.15	1.1328	108.79
HLJ-2	9.29	87.78	64.75±12.29	1.5788	40.47
HLJ-3	35.26	95.52	119.26±25.60	1.3136	74.54
HLJ-4	44.55	97.42	122.36±38.59	0.7369	76.48
HLJ-5	26.45	95.83	159.12±54.76	0.8863	99.45
JL-1	7.14	88.91	49.70±9.29	1.6209	31.06
JL-2	44.60	93.15	138.81±17.28	1.4836	86.75
JL-3	37.97	94.07	197.36±19.35	1.1083	123.35
S	6.59	98.55	1.60±0.31	1.2149	1.00

GR_{50} – herbicide concentration at which 50% growth is inhibited;
RI – resistance index = $GR_{50}R/GR_{50}S$

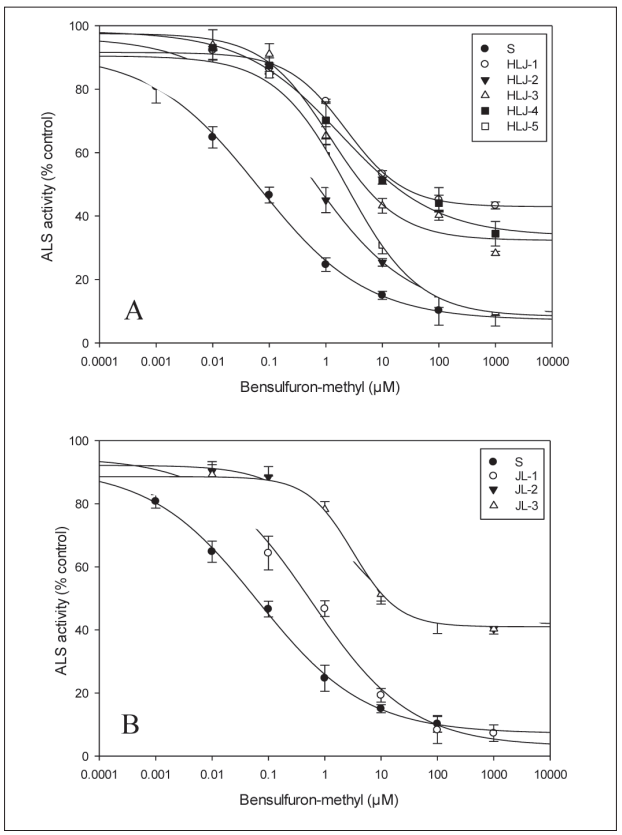

Fig. 2. Inhibition of ALS activity of the susceptible and resistant *Sagittaria trifolia* populations by bensulfuron-methyl. (**A**) Susceptible population and resistant populations from Heilongjiang Province (HLJ-1, 2, 3, 4, 5). (**B**) Susceptible population and resistant populations from Jilin Province (JL-1, 2, 3). Each data point represents the mean±SE of two extractions, each assayed in duplicate containing three replicates.

region in ALS gene from S and R plants showed more than 99.88% identities with documented ALS gene sequence of *S. trifolia* (Gene Bank accession number KC287227.1).

A single-nucleotide polymorphism (SNP) conferring a predicted amino acid substitution at position Pro197 was found to segregate with high-level resistance to bensulfuron-methyl in six out of the eight populations tested, but not in the susceptible population. These were Pro-197-Ser, Pro-197-His, Pro-197-Thr and Pro-197-Leu. All highly resistant individuals from the six populations had a Pro-197 substitution, with a SNP at the position of the Pro codon, whereas susceptible individuals were homozygous for Pro. A single nucleotide substitution in ALS of HLJ-3, HLJ-4 and JL-2 resulted in amino acid substitution of proline (CCC) to histidine (CAC), threonine (ACC) and leucine (CTC) at position 197, respectively. The mutation found in ALS of HLJ-1, HLJ-5 and JL-3 resulted in a substitution of proline (CCC) to serine (TCC) at position 197. The substitutions are commonly found to be associated with resistance to ALS inhibitors in other weed species [8, 22]. However, the nucleotide sequences of ALS from the R populations did not differ from that of the S population at positions 122, 205, 376, 377, 574, 653 and 654, where mutations have been reported in other weed species (Table 5). Further studies are required to determine the mechanism of resistance in those populations, where no polymorphisms were found to segregate with resistance across the five conserved domains of the ALS gene.

Cross- and multiple-resistance to other herbicides

To investigate ALS herbicide cross-resistance conferred by specific ALS resistance mutations, six herbicides with the same modes of action were tested. ALS herbicide cross-resistance screening for these populations identified differences in population responses both between and within ALS herbicide chemistries. All the R populations were resistant to SU herbicides, but not to IMI herbicides (Table 6). HLJ-5 and JL-3 populations were resistant to bispyribac-sodium of PTB herbicides, JL-2 to penoxsulam of TP herbicides and JL-1 to pyribenzoxim of PTB herbicides.

S. trifolia among the eight populations had no significant differences under different herbicides treatment. The results showed that it did not develop resistance against several herbicides. The control efficiency of oxyfluorfen against *S. trifolia* was below 50%, and not excellent. However, those of prometryn, bentazone and mesotrione were about 74%-88%. Although *S. trifolia* was not completely killed, its growth was suppressed. Among them, the control efficiency of MCPA against *S. trifolia* was the highest and reached 100% among the eight populations (Fig. 3).

DISCUSSION

Previous studies have reported that resistant *S. trifolia* populations have accumulated as a consequence of repeated applications of ALS-inhibiting herbicides [22]. Growers prefer to increase the dose of herbicides rather than apply alternatives with different modes of

Table 4. Total ALS activity without herbicide and I_{50} values of susceptible (S) and resistant (HLJ-1, 2, 3, 4, 5 and JL-1, 2, 3) populations of *Sagittaria trifolia* to bensulfuron-methyl.

Population	ALS activity without bensulfuron-methyl (nmol·mg^{-1}·min^{-1})	$y = C + (D-C)\ [1+ (x/I_{50})^b\ ]$				RI
		C	D	I_{50} (μM)	b	
HLJ-1	3.98±0.16 abc	42.93	91.58	2.29±0.48	0.8336	35.43
HLJ-2	3.76±0.14 bc	4.41	93.62	0.06±0.03	0.4943	1.10
HLJ-3	4.44±0.27 ab	32.33	97.58	1.18±0.71	0.7294	18.27
HLJ-4	4.59±0.26 a	33.63	98.28	1.77±0.90	0.5059	27.51
HLJ-5	3.93±0.15 abc	9.95	89.85	2.18±0.57	0.7038	33.86
JL-1	4.39±0.22 ab	1.54	89.36	0.10±0.04	0.7298	1.67
JL-2	4.94±0.28a	41.99	92.21	1.82±0.66	0.7501	28.26
JL-3	3.96±0.23 abc	40.98	88.61	3.15±0.43	1.0729	48.88
S	3.65±0.04 c	7.14	91.25	0.06±0.02	0.4558	1.00

I_{50} – herbicide concentration at which 50% enzyme activity is inhibited;
RI – resistance index = $I_{50}R/I_{50}S$

Table 5. A comparison of the regions of the ALS gene that contain mutations and the amino acid sequences from the resistant and susceptible *Sagittaria trifolia* populations. Letters in boxes represent mutations.

Population	The amino acid position, relative sequence of nucleotide and derived amino acid*							
	122	197	205	376	377	574	653	654
S	GCG Ala	CCC Pro	GCG Ala	GAT Asp	GAT Arg	TGG Trp	AGT Ser	GGA Gly
HLJ-1 (R)	GCG Ala	**TCC** **Ser**	GCG Ala	GAT Asp	GAT Arg	TGG Trp	AGT Ser	GGA Gly
HLJ-2 (R)	GCG Ala	CCC Pro	GCG Ala	GAT Asp	GAT Arg	TGG Trp	AGT Ser	GGA Gly
HLJ-3 (R)	GCG Ala	**CAC** **His**	GCG Ala	GAT Asp	GAT Arg	TGG Trp	AGT Ser	GGA Gly
HLJ-4 (R)	GCG Ala	**ACC** **Thr**	GCG Ala	GAT Asp	GAT Arg	TGG Trp	AGT Ser	GGA Gly
HLJ-5 (R)	GCG Ala	**TCC** **Ser**	GCG Ala	GAT Asp	GAT Arg	TGG Trp	AGT Ser	GGA Gly
JL-1 (R)	GCG Ala	CCC Pro	GCG Ala	GAT Asp	GAT Arg	TGG Trp	AGT Ser	GGA Gly
JL-2 (R)	GCG Ala	**CTC** **Leu**	GCG Ala	GAT Asp	GAT Arg	TGG Trp	AGT Ser	GGA Gly
JL-3 (R)	GCG Ala	**TCC** **Ser**	GCG Ala	GAT Asp	GAT Arg	TGG Trp	AGT Ser	GGA Gly

*Reference sequence for nucleotide and codon numbering is the coding sequence of *Arabidopsis thaliana* ALS gene (NM 114714.2)

Table 6. Cross-resistance pattern of resistant *Sagittaria trifolia* populations for the specific mutations relative to the susceptible population.

Population	Genotype Identified	SU		TP	PTB		IMI
		Pyrazosulfuron-ethyl	Ethoxysulfuron	Penoxsulam	Pyribenzoxim	Bispyribac-sodium	Imazethapyr
S	Wild type	S	S	S	S	S	S
HLJ-1	Pro/Ser-197	R	R	S	S	S	S
HLJ-2	---	R	R	S	S	S	S
HLJ-3	Pro/His-197	R	R	S	S	S	S
HLJ-4	Pro/Thr-197	R	R	S	S	S	S
HLJ-5	Pro/Ser-197	R	R	S	S	R	S
JL-1	---	R	R	S	R	S	S
JL-2	Pro/Leu-197	R	R	R	S	S	S
JL-3	Pro/Ser-197	R	R	S	S	R	S

SU – sulfonylurea; TP – triazolopyrimidine; PTB – pyrimidinyl-thiobenozoate; IMI – imidazolinone;
S – susceptible; R – resistant.

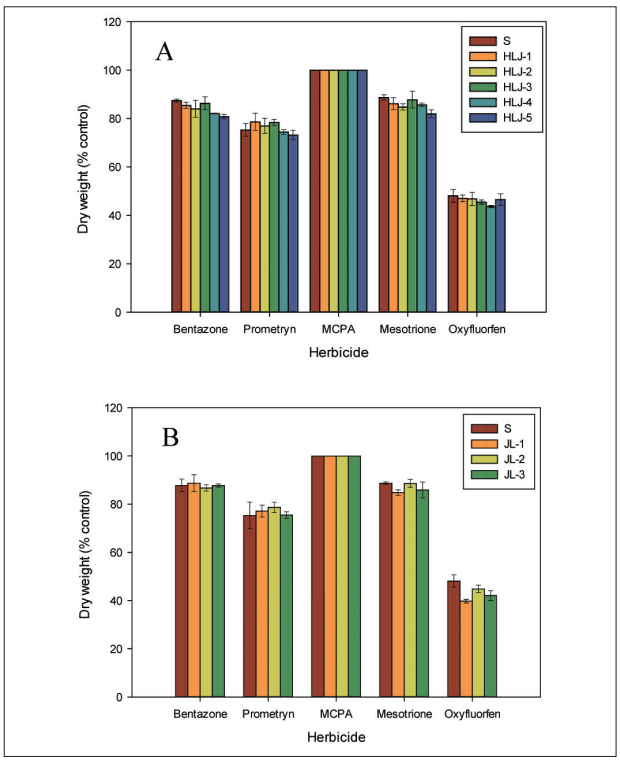

Fig. 3. Inhibitory effects of herbicide with different modes of action to *Sagittaria trifolia*. (**A**) Susceptible population and resistant populations from Heilongjiang Province (HLJ-1, 2, 3, 4, 5). (**B**) Susceptible population and resistant populations from Jilin Province (JL-1, 2, 3). Each data point represents the mean±SE of three replicates.

action. The confirmation of resistant *S. trifolia* populations has identified a serious threat to rice production in China. *S. trifolia* plants can become cross-resistant to other sulfonylurea or even to non-sulfonylurea ALS inhibiting herbicides. Although ALS inhibitors will continue to be the most popular and effective option for *S. trifolia* population control in many situations, the continued management of weed species where ALS resistance has evolved is dependent on alternative modes of action that can be used to control resistant populations.

Whole-plant dose-response assays demonstrated marked differences in response to the herbicide bensulfuron-methyl, with significant resistance detected in the HLJ-1, HLJ-5, JL-2 and JL-3 populations. The HLJ-2, HLJ-3, HLJ-4 and JL-1 populations showed no evidence of high-level bensulfuron-methyl resistance. Results from enzyme activity assays were consistent with whole-plant dose-response data and demonstrated that high and moderate levels of resistance to bensulfuron-methyl occur at the enzyme level. The HLJ-1, HLJ-5, JL-2 and JL-3 populations displayed significantly higher ALS activity than the S population, which is similar to previous results [24,28,29]. This indicates that this phenomenon is unlikely due to ALS gene overexpression. One of the possible explanations is that increased extractable ALS activity is due to increased ALS stability conferred by the specific resistance mutations [24]. It is well known that plant ALS is extremely labile and low in abundance, and therefore purification from plant tissues is difficult due to rapid loss of activity and sensitivity to amino acid feedback inhibition. However, it is much easier to isolate ALS from R plants with reproducible and higher activity levels, compared to ALS isolated from S plants [24]. This also requires further investigation.

Based on the sequence comparison, four mutations conferring resistance to ALS inhibitors through amino acid substitution at position 197 were found in the ALS gene. Pro-197-Ser and Pro-197-Leu have been previously reported in *S. trifolia* [22,23]. Pro-197-His and Pro-197-Thr mutations are reported for the first time in *S. trifolia*, but have been detected in many other weed species, such as *Descurainia Sophia* L. [30] and *Anthemis cotula* L. [31]. The mutation Pro-197-Ser in target-site resistance to ALS inhibitors showed a significant reduction in the inhibition of ALS activity (I_{50}) compared with the wild type. However, non-target-site-based resistance (NTSR) has been observed in *S. trifolia, Amaranthus hybridus* and *Papaver rhoeas* [23]. NTSR mechanisms can confer unpredictable resistance to herbicides with different chemistries or modes of action. High-level NTSR resistance was seen in the ALS-inhibitor resistance of *S. trifolia* [32, 33]. In this study, we did not examine herbicide metabolism so that the possibility of enhanced metabolism in the eight R populations of *S. trifolia* could not be ruled out. Penoxsulam and imazethapyr, belonging to systematic herbicides that can completely kill *S. trifolia* without weed regeneration, showed the best control efficiency against *S. trifolia* with low application dosage among six ALS inhibitors. Except for oxyfluorfen, other non-ALS inhibitors are also efficient herbicides of *S. trifolia*, among which MCPA has the best effect.

This work demonstrated resistance to bensulfuron-methyl in eight *S. trifolia* populations. Another

significant result is our observation that in six out of the eight R populations, the resistance to ALS inhibitors was most likely due to a target-site resistance mechanism due to substitution at Pro197. The Pro-197-His and Pro-197-Thr mutations were reported and characterized for the first time in *S. trifolia* populations at both the ALS and the whole-plant levels. This clearly needs further investigation. After analyzing the cross-resistance and multiple resistance of *S. trifolia*, it can be seen that the application of chemical herbicides whose irrational use can lead to the development of resistance should be addressed.

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Authors' contribution: Danni Fu, Bochui Zhao and Mingshan Ji conceived and designed the experiments; Songhong Wei contributed reagents, materials and analysis tools; Danni Fu and Bochui Zhao measured the growth parameters; Danni Fu collected and interpreted the data; Danni Fu and Xiuwei Li analyzed the data statistically; Danni Fu wrote the final version of the manuscript; Jamil Shafi and He Zhu revised the English language and drafting of the manuscript; all authors read and approved the final MS.

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