Herbicides cross resistance of a tribenuron-methyl resistant *Capsella bursa-pastoris* (L.) Medik. population in wheat field

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ABSTRACT

Shepherd's purse (*Capsella bursa-pastoris* [L.] Medik.) is a troublesome dicot weed that occurs in major wheat (*Triticum aestivum* L.) production areas in China. Tribenuron-methyl failed to control shepherd's purse in some fields in Runan County, Henan Province. This study aimed to establish the cross-resistance pattern of a resistant (R) population and explore the potential target-site and non-target-site based resistance mechanisms. *Acetohydroxyacid synthase* (*AHAS*) gene sequencing revealed a single nucleotide change of CCT to CGT resulting in the Pro to Arg substitution at amino acid position 197 in resistant individuals. Compared with the susceptible (S) population, R population displayed high level resistance to tribenuron-methyl. Cross resistance patterns showed that the R population was high resistant to flucarbazone-Na; moderately resistant to florasulam; low resistant to pyrithiobac sodium and pyroxsulam, and sensitive to imazethapyr. The pretreatment of malathion reduced the 50% growth reduction (GR_{50}) value of tribenuron-methyl by 29% in the R population, suggesting that target-site resistance and non-target-site resistance mechanisms were both present in tribenuron-methyl -resistance of shepherd's purse. This is the first report of the acetolactate synthase Pro197Arg mutation in shepherd's purse.

Key words: Acetolactate synthase, cytochrome P450, mutation, resistance, shepherd's purse.

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INTRODUCTION

Acetolactate synthase (ALS) (EC 4.1.3.18), also known as acetohydroxyacid synthase (AHAS), is a key enzyme for the biosynthesis of branched-chain amino acids (valine, leucine and isoleucine) (Ray, 1984; Duggleby et al., 2008). AHAS is a common target site of five commercially used herbicides, including sulfonylurea (SU) (Chaleff and Mauvais, 1984), pyrimidinyl thiobenzoate (PTB) (Stidham, 1991), triazolopyrimidine (TP) (Gerwick et al., 1990), sulfonylamino carbonyltriazolinone (SCT) (Sante et al., 1999) and imidazolinone (IMI) (Shaner et al., 1984). AHAS-inhibiting herbicides inhibit the AHAS enzyme, affecting the synthesis of branched-chain amino acids and causing plant death. AHAS inhibiting herbicides have been widely used worldwide for their low dose, high efficacy, low toxicity to mammals and broad-spectrum weed control. However, AHAS resistance has also evolved rapidly. Currently, 159 weed species are known to have evolved resistance to AHAS inhibitors worldwide, and the number is increasing (Heap, 2016).

Agricultural weeds cause large crop yield losses by competing for nutrients, water, light and space (Deng et al., 2015). Shepherd's purse (*Capsella bursa-pastoris* (Linn.) Medic) is an annual or biennial winter weed in the Brassicaceae (mustard family) (Michael, 2001). As to its competitive advantage and prolific seed, shepherd's purse has become one of the most troublesome dicot weeds in major wheat production areas of China. As an AHAS-inhibiting herbicide, tribenuron-methyl was introduced into China in 1988 and became the major herbicide rapidly to selectively control dicot weeds in wheat fields over the last two decades. Under intensive selection pressure, shepherd's purse has evolved different levels of resistance to tribenuron-methyl around China. Jin et al. (2011) reported one Pro197Ser substitution in a shepherd's purse population from Henan Province. Wang et al. (2011) and Cui et al. (2012) reported tribenuron-methyl-resistant shepherd's purse, resulting from the Pro197Thr, Pro197Ser, Pro197Leu and Pro197His substitutions respectively.

Previous studies have suggested that two main mechanisms are involved in resistance to AHAS inhibitors: target-site resistance (TSR) and/or non-target-site resistance (NTSR) (Yu and Powles, 2014a). Generally, TSR is mainly caused by single amino acid substitution in the target enzyme prohibiting herbicides from effective binding. To date, 26 amino acid substitutions endowing AHAS herbicide resistance have been identified: Ala122(3),

Pro197(12), Ala205(2), Asp376(1), Arg377(1), Trp574(3), Ser653(3) and Gly654(2) (the number in parentheses indicates the amount of different amino acid substitutions identified) (Heap, 2016).

Non-target-site based resistance (NTSR) to AHAS herbicides has been reported in many weed species, and enhanced metabolism is a powerful resistance mechanism in addition to target site resistance (Preston, 2004; Powles and Yu, 2010). NTSR is defined as "mechanisms that minimize the amount of active herbicide reaching the target site"; depending on the herbicide/environmental selection history and the genetic diversity of plants, different P450 and other genes may be involved in resistance to a given herbicide or herbicide class. Unlike TSR, metabolic resistance usually confers an unpredictable cross-resistance pattern (Yu and Powles, 2014b). Nevertheless, it still needs to prove whether or not cytochrome P450 monooxygenase plays a role in resistance of shepherd's purse to AHAS-inhibiting herbicides. As an organophosphate insecticide, malathion is one of a potent group of P450 inhibitors (Baerg et al., 1996; Preston, 2004). Therefore, this study used malathion as P450 inhibitor to explore whether P450 play a role in the resistant population.

In this article, we aimed to (1) identify target-site mutation(s) in *AHAS* gene; (2) conduct preliminary studies on the role of cytochrome P450 monooxygenase in resistant shepherd's purse population; and (3) evaluate the cross-resistance patterns to AHAS-inhibiting herbicides.

MATERIALS AND METHODS

Plant material and herbicides

All of the shepherd's purse seeds were collected in the summer of 2016. Seeds of the resistant (R) shepherd's purse population were collected from a wheat field in Runan County, Henan Province (114°8'54.6" N; 32°58'31.8" E), where methyl 2-[4-methoxy-6-methyl-1,3,5-triazin-2-yl(methyl)carbamoylsulfamoyl]benzoate (tribenuron-methyl) had been continuously used for over 20 yr. A susceptible (S) population collected from roadside ditches that had no history of herbicide use in Zhengzhou, Henan Province, was used as the control in this study. Seed samples were air-dried and stored in sealed paper bags at 4 °C until used.

The herbicides used in this study were: tribenuron-methyl (75% WG, DuPont, Shanghai, China); sodium 2-chloro-6-(4,6-dimethoxypyrimidin-2-ylthio)benzoate (pyrithiobac-sodium; 10% AS, Huayang, Shandong, China); *N*-(5,7-dimethoxy[1,2,4]triazolo[1,5-α]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)pyridine-3-sulfonamide (pyroxsulam; 7.5% WG, Dow AgroSciences, Shanghai, China); sodium [(4,5-dihydro-3-methoxy-4-methyl-5-oxo-1H-1,2,4-triazol-1-yl)carbonyl]{[2-(trifluoromethoxy) phenyl]sulfonyl}azanide (flucarbazone-Na; 70%, WG, Arysta, Shanghai, China); 2′,6′,8-trifluoro-5-methoxy[1,2,4] triazolo[1,5-c]pyrimidine-2-sulfonanilide (florasulam; 50 g L^{-1} SC, Lvba, Shandong, China); 5-ethyl-2-[(*RS*)-4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]nicotinic acid (imazethapyr; 10% AS, Binnong, Shandong, China); diethyl (dimethoxyphosphinothioylthio)succinate (malathion; 95%, Ningbo Sunjoy Cropscience, Ningbo, China).

AHAS gene sequencing

Approximately 100 mg young shoot tissue from individual R and S plants at the three to four leaf stage were harvested and used for genomic DNA extraction with the cetyltrimethylammonium bromide (CTAB) method. Based on the *AHAS* gene sequences of shepherd's purse (HQ880660.1), two primer pairs were designed using the Primer Premier 5.0 software (Biosoft, Palo Alto, California, USA) to amplify the *AHAS* gene fragment containing the five conserved regions, with domains A to E (Table 1) (synthetized by Sangon Biotech, Shanghai, China). The 25 μL polymerase chain reaction (PCR) mixture consisted of 1 μL genomic DNA (approximately 25 ng μL^{-1}), 1 μL each primer (10 μM), 2.5 μL 10×EasyTaq Buffer (TransGen Biotech, Beijing, China), 2 μL dNTPs (2.5 mM, TransGen Biotech, China), and 0.25 μL EasyTaq DNA Polymerase (5 U μL^{-1}, TransGen Biotech, China), with double-distilled H_2O added to achieve a final volume of 25 μL. PCR was conducted using T100 Thermal cycler (Bio-Rad, Hercules, California, USA), with the following reaction program: 5 min denaturation at 94 °C; 30 cycles of 30 s at 94 °C, 40 s at 55 °C, and 90 s at 72 °C; and a final step of 10 min at 72 °C. A positive clone was sequenced in forward and reverse directions by a commercial sequencing company (Sangon Biotech, Shanghai, China) to minimize sequencing errors.

Cross resistance to AHAS-inhibiting herbicides

The weed seeds were first immersed in a 0.3% gibberellin (SP) solution for 30 min and then rinsed thoroughly with distilled water before being germinated in Petri dishes. After 3 d, the germinated seedlings were transplanted into 12-cm diameter plastic pots containing loam soil. The pots were placed in a controlled greenhouse (25/15 °C, 14:10 h photoperiod, 75% RH). The shepherd's purse seedlings were thinned to 10 evenly sized plants per pot before herbicide application. The pots were watered every other day (80 to 100 mL water per pot).

Table 1. Primers designed for the shepherd's purse *AHAS* gene.

Primer	Sequence (5′-3′)	Amplicon size (bp)	Covering the confirmed point mutations
Forward 1	ATTCGTCTCCCGATTTGC	1196	Ala122, Pro197, Ala205
Reverse 1	GCCCGACACCAGTGCTTAT		
Forward 2	GGTATCCCTGTTGCGAGTA	1081	Asp376, Arg377, Trp574, Ser653, Gly654
Reverse 2	GCATACAAAGACCGTTTA		

AHAS: Acetohydroxyacid synthase.

The seedlings were treated with AHAS-inhibiting herbicides at the three-to four-leaf stage using a moving nozzle cabinet sprayer equipped with one TeeJet 903EVS flat-fan nozzle calibrated to deliver 450 L ha^{-1} at 280 kPa. The herbicide doses applied to the R and S populations were listed in Table 2. The aboveground parts of the plant were harvested 21 d after treatment (DAT). Subsequently, the aboveground parts were oven dried for 72 h at 80 °C and the dry-weight data were recorded. Each treatment had three replicates, and the experiment was conducted twice.

Tribenuron-methyl dose response with and without malathion pretreatment

The R population plants were treated with tribenuron-methyl, malathion, and malathion plus tribenuron-methyl at the 3- to 4-leaf stage as described above. Malathion was dissolved in acetone and then diluted to 1000 g ai ha^{-1} with water containing 0.1% Tween-80. The malathion was sprayed 30 min prior to the tribenuron application (Preston et al., 1996). Control plants were sprayed with aqueous solution of the solvent mixture. The doses of tribenuron-methyl were showed in Table 2, with or without malathion. Treated plants were harvested after 21 d, when dry weights of the above-ground part were assessed as described above (Guo et al., 2016).

Data analyses

The values from the two replicates of the dose-response experiment were subjected to ANOVA (SPSS version 22.0, IBM Analytics, Armonk, New York, USA), and data were combined as there was nonsignificant difference between the repeated experiments. All of the regression analyses were conducted with SigmaPlot (Version 12.5; Systat Software Inc., San Jose, California, USA). The combined data were fit to a four-parameter log-logistic curve for nonlinear regression analysis (Seefeldt et al., 1995):

$$y = c + (d-c)/\{1+exp[b(logx - logED_{50})]\}$$

where c is the lower limit, d is the upper limit, and b is the relative slope around the herbicide dose resulting in 50% growth inhibition (ED_{50}). The herbicide dose was the independent variable (x), and the growth response (percentage of the untreated control) was the dependent variable (y) in the regression equation.

This equation was used to estimate the dose causing a 50% dry-weight growth reduction (GR_{50}). The resistance index (RI) was calculated as the ratio of the GR_{50} of the R population to the GR_{50} of the S population (Guo et al., 2016).

RESULTS

AHAS gene sequencing

Two fragments of the *AHAS* gene, with a size of 1196 and 1081 bp were assembled into a single 1795 bp sequence and were compared with a known sequence (HQ880660.1) using Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information [NCBI], Bethesda, Maryland, USA). The results indicated that the sequences from all the populations showed 99% sequence identity with the documented *AHAS* gene from shepherd's purse.

The PCR fragment of the shepherd's purse covering the eight verified mutation sites was amplified from the R and S population. Point mutations were observed at the Pro197Arg (CCT to CGT) (corresponding to the *Arabidopsis thaliana* AHAS amino acid sequence) in the R population (Figure 1), and the S population did not have any documented *AHAS* gene mutation.

Sensitivity to AHAS-inhibiting herbicides

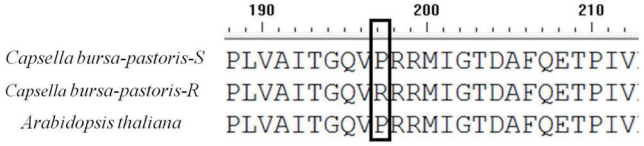
The whole-plant experiment demonstrated that the R population plants presented different level of resistance to all the tested AHAS inhibitors. The R population displayed high level resistance to tribenuron-methyl with a resistance index of 203.8 (Table 3). The GR_{50} values were 26.5 g ai ha^{-1}, which were much more than the field rates of tribenuron-methyl (Table 3). Compared with the S population, the R population showed high-level resistance to flucarbazone-Na, with a resistance index of 99.8; moderate-level resistance to florasulam, with a resistance

Table 2. The herbicide concentrations used for determining the sensitivity to AHAS-inhibiting herbicides in shepherd's purse.

Herbicide	Population	Dose						
		g ai ha^{-1}						
Tribenuron-methyl	S	0	0.0010	0.010	0.10	1.0	10.0	100.0
	R	0	0.0100	0.100	1.00	10.0	100.0	1000.0
Tribenuron-methyl+malathion	S	0	0.0010	0.010	0.10	1.0	10.0	100.0
	R	0	0.0100	0.100	1.00	10.0	100.0	1000.0
Pyrithiobac sodium	S	0	0.0720	0.360	1.80	9.0	45.0	225.0
	R	0	0.3600	1.800	9.00	45.0	225.0	1125.0
Florasulam	S	0	0.0120	0.060	0.30	1.5	7.5	37.5
	R	0	0.0600	0.300	1.50	7.5	37.5	187.5
Pyroxsulam	S	0	0.0192	0.096	0.48	2.4	12.0	60.0
	R	0	0.0960	0.480	2.40	12.0	60.0	300.0
Flucarbazone-Na	S	0	0.0010	0.010	0.10	1.0	10.0	100.0
	R	0	0.0100	0.100	1.00	10.0	100.0	1000.0
Imazethapyr	S	0	0.1600	0.800	4.00	20.0	100.0	500.0
	R	0	0.1600	0.800	4.00	20.0	100.0	500.0

AHAS: Acetohydroxyacid synthase.

Figure 1. Alignment of partial amino acid sequences of *AHAS* gene from resistant (R) and susceptible (S) shepherd's purse populations. The boxed codons indicate: P197R mutation in *AHAS* gene of R population.



AHAS: Acetohydroxyacid synthase.

index of 9.1; low-level resistance to pyrithiobac sodium and pyroxsulam with a resistance index of 3.0 and 4.7; and no resistance to imazethapyr, with a resistance index of 1.1 (Beckie and Tardif, 2012).

Tribenuron-methyl dose response with and without malathion pretreatment

When malathion was applied alone at 1000 g ai ha^{-1}, nonsignificant influence was observed on the survival or biomass (data not shown) of either the R or S populations. When tribenuron-methyl was applied with malathion, the GR_{50} value of tribenuron-methyl decreased 33% for the R population, from 26.5 ± 5.7 to 17.6 ± 3.8 g ai ha^{-1} (Table 3, Figure 2). In comparison, it decreased less than 8% for the S population, from 0.13 ± 0.05 to 0.12 ± 0.02 g ai ha^{-1} (Table 3, Figure 2).

DISCUSSION

Previous studies have confirmed that different mutations in the *AHAS* gene may confer various resistance and cross-resistance patterns to AHAS-inhibiting herbicides. The substitutions have been reported on the nuclear *AHAS* gene (Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, and Gly654) resulting in distinct patterns of resistance to different AHAS herbicides. Generally, Pro197 mutations confer resistance to sulfonylurea (SU) families and triazolopyrimidine (TP) families; Ala122, and Ala205 mutations confer resistance to imidazolinone (IMI) families; Ser653 evolves resistance to IMI and pyrimidinyl thiobenzoate (PTB) families; Gly654 contributes to resistance for the SU, IMI, and sulfonylamino carbonyltriazolinone (SCT) families; and Asp376 and Trp574 mutations confer broad-spectrum resistance to all five classes of AHAS inhibitors (Tranel and Wright, 2002; Powles and Yu, 2010). The herbicide binding site of plant AHAS revealed Pro197 at one end of an α-helix at the entrance to the active site access channel. The Pro197 residue always contacts the aromatic ring of the SUs, and therefore almost any Pro197 substitution will result in resistance to SUs (Liu et al., 2015).

The present study reported an amino acid mutation (Pro197Arg) in shepherd's purse *AHAS* gene, resulting in high-level SU (tribenuron-methyl) and SCT (flucarbazone-

Figure 2. Dose-response curves for dry weight (% of control) of the resistant (R) and susceptible (S) shepherd's purse populations treated with a range of tribenuron-methyl doses plus or minus malathion.

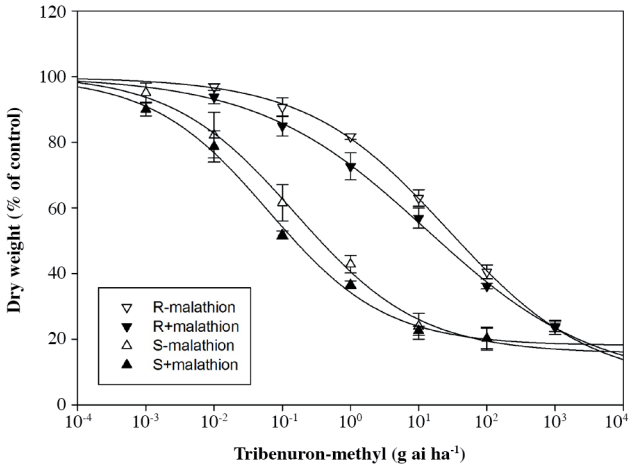


Table 3. Parameters of the four-parameter log-logistic equation[a] used to calculate the 50% growth reduction (GR_{50}) values of the purified resistant (R) and susceptible (S) populations of shepherd's purse. Standard errors are in parentheses.

Herbicide	Populations	Regression parameters					
		c	d	b	R^2	GR_{50}[b]	RI[c]
						g ai ha^{-1}	
Tribenuron-methyl	R	7.6(4.0)	99.3(1.4)	-0.43(0.03)	0.9998	26.50(5.7)	203.8
	S	14.1(4.8)	104.9(7.5)	-0.43(0.09)	0.9988	0.13(0.05)	
Tribenuron-methyl+ malathion	R	14.4(3.7)	94.8(1.6)	-0.54(0.06)	0.9996	17.60(3.8)	146.7
	S	14.8(2.1)	105.1(3.4)	-0.43(0.04)	0.9998	0.12(0.02)	
Pyrithiobac sodium	R	21.6(2.1)	100.1(3.0)	-0.69(0.07)	0.9996	12.10(1.7)	3.0
	S	7.8(1.1)	110.8(11.2)	-0.50(0.15)	0.9983	4.10(1.7)	
Florasulam	R	22.6(2.5)	95.1(2.5)	-1.27(0.21)	0.9985	3.20(0.5)	9.1
	S	19.4(1.1)	102.1(1.8)	-0.75(0.05)	0.9999	0.35(0.02)	
Pyroxsulam	R	19.2(2.1)	99.4(1.4)	-0.79(0.06)	0.9997	9.40(0.9)	4.7
	S	5.3(1.0)	100.4(6.8)	-0.51(0.13)	0.9989	2.00(0.7)	
Flucarbazone-Na	R	14.9(2.1)	95.1(0.7)	-0.72(0.04)	0.9998	45.90(4.9)	99.8
	S	21.1(1.4)	98.8(1.2)	-0.76(0.05)	0.9997	0.46(0.05)	
Imazethapyr	R	23.8(2.7)	96.2(5.1)	-1.31(0.33)	0.9971	2.70(0.6)	1.1
	S	26.3(3.4)	94.7(6.4)	-1.49(0.54)	0.9943	2.50(0.7)	

[a] $y = c + (d - c)/\{1+exp[b(logx-logED_{50})]\}$, where y is the percentage of the control, c and d are lower and upper asymptotic limits, b is the slope of the curve through GR_{50}.
[b] Data in the table are expressed as mean ± standard deviation.
[c] RI (Resistance index) was calculated as the ratio of GR_{50} values of the R and S populations.

Na) resistance, different levels of resistance across PTB (pyrithiobac sodium) and TPs (pyroxsulam and florasulam), and sensitive to IMI (imazethapyr). It is consistent with the results of the present study that the R population (with Pro197Arg mutation) of *Lamium amplexicaule* showed high-level resistance to SU and SCT, and no resistance to IMI (Varanasi et al., 2016). It is noteworthy that the Pro197Arg substitution has never been reported in shepherd's purse. Among 12 amino acid substitutions identified at Pro197 of *AHAS* gene, the frequency of Pro197Arg substitution is relatively low compared with other substitutions. The Pro197Arg substitution has only been reported in four weed species and all confers high level resistance to SU but low level resistance to other AHAS-inhibiting herbicides (Beckie and Tardif, 2012; Guo et al., 2016). However, we found the R population with Pro197Arg substitution conferring moderate resistance to TP (florasulam) herbicides in shepherd's purse. This demonstrates that the cross-resistance pattern to AHAS-inhibiting herbicides is dependent on not only the specific mutation (mutation site and amino acid) but also specific herbicides and weed species (Yu and Powles, 2014a). This also indicated us that the florasulam was no longer applicable for controlling the R population of shepherd's purse in the wheat field.

In the current study, pretreatment with malathion decreased the GR_{50} value of tribenuron-methyl by 29% in the R population while only 6% in the S population, suggesting that an enhanced metabolism mediated by cytochrome P450 monooxygenase may also play a role in tribenuron-methyl resistance of the R population. This was in accordance with former researches that NTSR often exists in populations containing target-site based resistance (Ahmad-Hamdani et al., 2012; Kaundun et al., 2013). Given the complexity of NTSR, further researches need to be done to investigate the NTSR in shepherd's purse.

CONCLUSION

In summary, the R population of shepherd's purse evolved high-level resistance to tribenuron-methyl and flucarbazone-Na, and showed different cross resistance levels to other AHAS-inhibiting herbicides. Target-site mutations in acetolactate synthase (Pro197Arg) and cytochrome P450 monooxygenase may both play a role in resistance to herbicides of shepherd's purse.

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