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Acetolactate synthase proline (197) mutations confer tribenuron-methyl resistance in *Capsella bursa-pastoris* populations from China

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ABSTRACT

The increasing use of AHAS-inhibiting herbicides has resulted in evolved resistance in key dicot weeds infesting cereal cropping systems worldwide. Shepherd's purse (*Capsella bursa-pastoris*) is a common dicot weed species in wheat in China with populations that have evolved resistance to the AHAS herbicide tribenuron-methyl. The seeds of eight resistant populations were collected from wheat fields and one susceptible population from road side in Hebei province of China. All eight populations showed high level resistance to tribenuron-methyl with resistance indices of over 100 fold based on whole plant dose response assays in the greenhouse. Comparison of the AHAS gene sequences of the susceptible and resistant populations with Arabidopsis revealed that proline at position 197 of the AHAS gene was substituted by threonine in population CAPBU-HB-2, serine in populations CAPBU-HB-3, CAPBU-HB-4, CAPBU-HB-5, and CAPBU-HB-6, leucine in population CAPBU-HB-7 and CAPBU-HB-8, histidine in population CAPBU-HB-9. The study confirmed tribenuron-methyl resistance in shepherd's purse in Hebei province of China due to target site mutations at AHAS codon position 197.

1. Introduction

Shepherd's purse is a member of the *Brassicaceae* family. It is weed that is widely distributed around the world but can also be served as vegetable [1]. Shepherd's purse is a strong competitor and a prolific seed producer. As such it is one of the most troublesome dicot weeds in major wheat producing areas [2–4], and can substantially reduce crop yield in winter wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and oilseed rape (*Brassica napus*).

Acetohydroxyacid synthase (AHAS, EC 2.2.1.6), also referred to as acetolactate synthase (ALS), is the first enzyme in the biosynthetic pathway for the branched-chain amino acids valine, leucine, and isoleucine. Many herbicide classes have been developed that inhibit AHAS. Plant death occurs due to sensitive plants shortage of branched-chain amino acids [5–9]. Due to their low use rates, high efficacy, broad spectrum weed control, multi-crop selectivity and favorable environmental profiles, AHAS-inhibiting herbicides have been widely used for weed control [10–14]. Tribenuron, an AHAS-inhibiting herbicide, was introduced in China in 1988 to selectively control dicot weeds in wheat fields [15].

AHAS inhibitor-resistant weeds are the fastest growing class of herbicide-resistant weeds, with 112 weed species reported worldwide [15–18]. As the result of intensive and continuous use of AHAS-inhibiting herbicides in wheat fields in China, flixweed (*Descurainia sophia*) populations in Shaanxi and Hebei province have first been reported to evolve resistance to tribenuron in 2005 [16]. Subsequently 21 flixweed populations from eight provinces in China were also found to express moderate to high levels of tribenuron resistance, which indicates that tribenuron resistant flixweed populations are increasing [19,20].

In most cases, resistance to AHAS inhibitors is due to amino acid substitutions in the AHAS enzyme. To date, twenty-two amino acid substitutions at eight conserved amino acid residues in the AHAS gene are known to confer AHAS-inhibitor resistance in field-selected weed populations. These consist of alanine 122 (Ala_{122}), proline 197 (Pro_{197}), alanine 205 (Ala_{205}), aspartate 376 (Asp_{376}), Arginine 377 (Arg_{377}), tryptophan 574 (Trp_{574}), serine 653 (Ser_{653}), and glycine 654 (Gly_{654}) [14,16]. Pro_{197} substitutions usually result in high sulfonylurea resistance and over ten substitutions have been reported for Pro_{197} (Ala, Arg, Asn, Gln, His, Ile, Leu, Lys, Met, Ser, Thr, or Trp) [21–30].

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Table 1
Primers used to amplify the Shepherd's purse AHAS gene.

Primers	Sequence (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)	Containing the confirmed point mutations
Forward 1	CTCCTCCAACGAAATCCAC	634	56	Ala122
Reverse 1	CCACCAACATACAAGACAGG			
Forward 2	CAAGGAGGTGTATTCGCAGC	783	60	Pro197, Ala205, Asp376, Arg377
Reverse 2	CTTATTCTTCCCAATCTCAGCCG			
Forward 3	ACGGGACGGTGTATGCGAAT	736	56	Asp376, Arg377, Trp574
Reverse 3	ATCTCGTTCTCCTGTGCTGGGT			
Forward 4	AAGGGATGAACAAGGTGCTT	765	57	Trp574, Ser653, Gly654
Reverse 4	TGTCTCTCAGTATTTCGTCCG			

Several cases of shepherd's purse resistance to AHAS herbicides have been reported. These include resistance to imazethapyr in 2000 in Israel, imazamox, imazethapyr, thifensulfuron-methyl, and tribenuron-methyl resistance in 2008 in Canada [16], and tribenuron-methyl resistance in Henan province of China in 2011 [31]. After about 20 y of application, tribenuron-methyl could not control shepherd's purse as effectively as before in Hebei province, China. The objective of this research was to elucidate the mechanism of the resistance in shepherd's purse by comparing the AHAS sequences between resistant and susceptible populations.

2. Materials and methods

2.1. Seed collection

The trials were conducted in the field to detect the sensitivities of some shepherd's purse populations to tribenuron-methyl. Seeds of 18 suspected resistant shepherd's purse populations to tribenuron-methyl were collected from separate wheat fields with over 10 y of repeated tribenuron applications, and eight of them expressed high level resistance to tribenuron-methyl by a whole plant dose response assay in the greenhouse [32]. Seeds of the eight populations were collected and prepared to elucidate the mechanism of the resistance in the molecular basis. One population from road side that had no history of AHAS-inhibiting herbicide use in Hebei province of China was used as susceptible control.

2.2. Genomic DNA extraction

All the populations were grown in the greenhouse and treated with tribenuron at 10 g ai ha^{-1} at the four-leaf growth stage except the susceptible population CAPBU-HB-1. Approximately 1 g of young shoot tissue of individual surviving plants from the above eight resistant populations and one susceptible population CAPBU-HB-1 were harvested and stored at −80 °C. DNA was extracted from 100 mg young shoot tissue of each plant using the CTAB method [33].

2.3. Oligonucleotide primers

Four pairs of forward and reverse overlapping primers (Table 1) were selected to amplify the highly conserved region of the AHAS encompass the eight point mutations affecting AHAS efficacies. The primers were designed based on the AHAS GenBank sequences from Arabidopsis (*Arabidopsis*, X51514) and Flixweed (*Descurainia sophia*, FJ715633).

2.4. DNA amplification and sequencing

The DNA engine Bio-RAD[1] was used to amplify AHAS gene fragments from Shepherd's purse genomic DNA. The polymerase chain reaction (PCR) was conducted in a 25 μl volume that consisted of 25 ng of genomic DNA, 12 pmol of each primers, 1 × PCR buffer (Mg^{2+} plus), 0.2 mM deoxynucleotide triphosphates (dNTPs) mixture, and 2.5 U *Taq* DNA polymerase (TaKaRa Taq Polymerase). PCR reactions were subjected to a 3-min denaturation at 94 °C; 25 cycles of 30 s at 94 °C, 30 s at *X* C, and 1 min at 72 °C; then 3 min at 72 °C, where *X* is the annealing temperature for each primer pairs used. Annealing temperatures were 56, 60, 56, and 57 °C for primer sets 1, 2, 3, and 4, respectively (Table 1).

The desired PCR products were cloned with the competent cell (JM109) and plasmid (pMD19-T) for sequencing. Each desired fragment was sequenced in forward and reverse directions, to minimize sequencing errors, by a commercial sequencing company.[2] The sequences of five plants of susceptible population were analyzed first and then compared with sequences from resistant populations to determine whether a nucleotide substitution occurred. A minimum of five plants was sequenced for each population examined. DNA Analyzer[3] with the common primers M13F (-47) (5′CGCCAGGGTTTTCCCAGTCACGAC3′) was used to obtain the complementary strand of the sequenced AHAS gene fragments. Sequences of Shepherd's purse and *Arabidopsis* were assembled and compared using DNAMAN software package (Version 5.2.2, Lynnon Biosoft, Canada).

3. Results and discussion

To identify the molecular basis for resistance, the AHAS gene from the above tribenuron-resistant populations were sequenced and compared. DNA was extracted from leaf tissue of individual plants of these populations. From Table 1, the length of the fragment amplified by the four pairs of primers was approximately 634, 783, 736, and 765 bp, respectively. The total length of the conserved AHAS gene was 1678 bp, encompassing the eight known point mutations for AHAS-inhibiting herbicide resistance.

AHAS gene fragments for the eight resistant shepherd's purse populations and one susceptible population were sequenced. Comparison of the AHAS gene sequences of the susceptible and resistant populations with Arabidopsis revealed that proline at position 197 of the AHAS gene was substituted by threonine in population CAPBU-HB-2, by serine in populations CAPBU-HB-3, CAPBU-HB-4, CAPBU-HB-5, and CAPBU-HB-6, leucine in population CAPBU-HB-7 and CAPBU-HB-8, histidine in population CAPBU-HB-9 (Table 2). The study firstly confirmed tribenuron-methyl resistance in shepherd's purse in Hebei province of China, with the resistance mechanism being conferred by specific AHAS point mutations at amino acid position 197, and it also provides further understanding of the molecular basis of resistance to AHAS-inhibiting herbicides in shepherd's purse.

Target site-based AHAS-inhibitor resistance is conferred by single amino acid substitutions of the AHAS gene enzyme [25,29,34–

[1] DNA engine, Bio-RAD, Hercules, CA.

[2] Sequencing service department, Beijing AuGCT Biotechnology Co., Ltd., Beijing, China.

[3] ABI 3730xl 96-capillary DNA Analyzer, Applied Biosystems, Los Angeles, CA.

Table 2
DNA and derived amino acid sequences of AHAS gene from susceptible (S) and resistant (R) populations of shepherd's purse. The bold nucleotide bases encode Pro-197 in S populations and the mutations in R populations with Arabidopsis.

Populations	The amino acid position, relative sequence of nucleotide and amino acid												
	191	192	193	194	195	196	197	198	199	200	201	202	203
Arabidopsis thaliana	GCA	ATC	ACA	GGA	CAA	GTC	**CCT**	CGT	CGT	ATG	ATT	GGT	ACA
	Ala	Ile	Thr	Gly	Gln	Val	**Pro**	Arg	Arg	Met	Ile	Gly	Thr
CAPBU-HB-1(S)	GCA	ATC	ACA	GGA	CAG	GTC	**CCT**	CGT	AGG	ATG	ATT	GGT	ACT
	Ala	Ile	Thr	Gly	Gln	Val	**Pro**	Arg	Arg	Met	Ile	Gly	Thr
CAPBU-HB-2(R)	GCA	ATC	ACA	GGA	CAG	GTC	**ACT**	CGT	AGG	ATG	ATT	GGT	ACT
							Thr						
CAPBU-HB-3(R)	GCA	ATC	ACA	GGA	CAG	GTC	**TCT**	CGT	AGG	ATG	ATT	GGT	ACT
							Ser						
CAPBU-HB-4(R)	GCA	ATC	ACA	GGA	CAG	GTC	**TCT**	CGT	AGG	ATG	ATT	GGT	ACT
							Ser						
CAPBU-HB-5(R)	GCA	ATC	ACA	GGA	CAG	GTC	**TCT**	CGT	AGG	ATG	ATT	GGT	ACT
							Ser						
CAPBU-HB-6(R)	GCA	ATC	ACA	GGA	CAG	GTC	**TCT**	CGT	AGG	ATG	ATT	GGT	ACT
							Ser						
CAPBU-HB-7(R)	GCA	ATC	ACA	GGA	CAG	GTC	**CTT**	CGT	AGG	ATG	ATT	GGT	ACT
							Leu						
CAPBU-HB-8(R)	GCA	ATC	ACA	GGA	CAG	GTC	**CTT**	CGT	AGG	ATG	ATT	GGT	ACT
							Leu						
CAPBU-HB-9(R)	GCA	ATC	ACA	GGA	CAG	GTC	**CAT**	CGT	AGG	ATG	ATT	GGT	ACT
							His						

35], which occur at multiple sites within the AHAS gene. Twenty-nine confirmed AHAS inhibiting herbicide resistant weed species were reported with various substitutions involved [14]. AHAS resistance endowing substitutions at proline 197 have been reported in many weed species. Pro197 in Domain A substituted to other amino acids, such as leucine and threonine, is known to confer AHAS-inhibitor resistance in 22 of the 107 AHAS inhibiting herbicide resistant weed species [14]. There are also so many Pro-197 AHAS resistance mutations in AHAS have evolved, and the Pro-197-Ser and the Trp-574-Leu AHAS resistance mutations are frequently found in many weed species [36]. It is clear that the mutation changes found in this study are similar to that reported for AHAS herbicide-resistant prickly lettuce (*Lactuca serriola*) populations due to the modification of proline 197 to threonine [37], wild radish (*Raphanus raphanistrum*) populations that also had point mutations of proline 197 to four different amino acid substitutions: histidine, threonine, alanine, and serine [26,30], and stinkweed populations that had a Pro (197) Leu mutation [21], and shepherd's purse population from Henan province that had a serine mutation (cytosine by thymine) at position 197 conferred the tribenuron-resistance [31]. From this research, proline at position 197 of the AHAS gene was substituted by serine in populations CAPBU-HB-3, CAPBU-HB-4, CAPBU-HB-5, and CAPBU-HB-6. However, the proline at position 197 of the AHAS gene substituted by threonine in population CAPBU-HB-2, leucine in population CAPBU-HB-7 and CAPBU-HB-8, histidine in population CAPBU-HB-9 were also determined, and it's first time to revealed in the Shepherd's purse in the world.

In China, farmers would like to increase the dosage of cheap tribenuron to realize high efficacy of weed control. They are reluctant to apply other herbicide modes of action at high prices. As we know, tribenuron has become a popular herbicide in wheat fields of China since 1988, and the consumption of tribenuron's formulation in the wheat field of China has reached 1492 tons in 2010. The populations CAPBU-HB-2 to CAPBU-HB-9 expressed high level resistance were collected from the cities where chemical herbicides were the main way of the weed control. The occurrences of tribenuron-resistant shepherd's purse may be due to continuous application of AHAS-inhibitors in wheat. The shepherd's purse populations CAPBU-HB-2 to CAPBU-HB-9 given the high levels of resistance (Rf > 100) were conferred by target site mutations at position 197, and it is likely that increasing rates of tribenuron-methyl would not be good way to control the resistant populations effectively. The effective weed control will only be achieved with herbicides with different modes of action.

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