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Molecular basis for resistance to tribenuron in shepherd's purse (*Capsella bursa-pastoris* (L.) Medik.)

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ABSTRACT

Capsella bursa-pastoris, a winter annual weed in the mustard family, can not be controlled by tribenuron after the herbicide has been continuously used for several years. The resistant biotype Lz-R was the generation of a population collected from Liangzhu, a place where tribenuron had been used for more than 15 consecutive years. To confirm and characterize the resistance of *C. bursa-pastoris* to tribenuron, whole-plant bioassays were conducted in the greenhouse. The results of whole-plant bioassays revealed that Lz-R was highly resistant to tribenuron with the resistance index (GR_{50} Lz-R)/(GR_{50} Lz-S) up to 236.6. To investigate the molecular basis of resistance in *C. bursa-pastoris*, the acetolactate synthase (ALS) genes were sequenced and compared between susceptible and resistant biotypes. Analysis of the nucleotide and deduced amino acid sequences between the biotypes indicated that one substitution had occurred in Domain A, cytosine by thymine (CCT to TCT) at position 197, that led to a change of the amino acid proline in the susceptible to serine in the Lz-R.

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1. Introduction

Acetolactate synthase (ALS) is the first enzyme common to the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine [1–3]. This enzyme is indispensable for plants and inhibited by five herbicide groups: sulfonylurea (SU) [4], imidazolinone (IMI) [5], triazolopyrimidine (TP) [6], pyrimidinylthiobenzoate (PTB) [7] and sulfonylamino-carbonyl-triazolinone (SCT) [8]. The aforementioned five groups inhibit ALS activity, block the biosynthesis of branched-chain amino acids, and finally lead to death of the plant.

Tribenuron, an ALS-inhibiting herbicide, was introduced into China to selectively control broadleaved weeds in wheat fields in 1988 [9]. Now, ALS-inhibiting herbicides have been widely used in China like elsewhere in the world because they unite the advantages of high activity at low use rates, low mammalian toxicity, broad-spectrum weed control, multi-crop selectivity and favorable environmental profile [10]. However, a major disadvantage of the ALS-inhibitors is the rapid development of weed biotypes resistant to these herbicides. To date, ALS-resistance has been described in 108 weed species, including monocots and dicots, all over the world, and this number is increasing at a linear rate [11]. This is generally due to repeated applications of ALS-inhibiting herbicides for more than 3 years [12].

Due to the ALS-inhibiting herbicides used widely and repeatedly, the ALS enzyme has received considerable attention in weed science. In many cases, the basis of resistance is due to a target site modification in ALS, which is able to reduce the sensitivity of ALS to the ALS-inhibiting herbicides. For example, a single nucleotide difference resulting in a substitution of an amino acid of the ALS enzyme was observed in at least one of five highly conserved regions of the ALS gene. To date, seven conserved amino acid substitutions, alanine 122 (Ala_{122}), proline 197 (Pro_{197}), alanine 205 (Ala_{205}), aspartate 376 (Asp_{376}), tryptophan 574 (Trp_{574}), serine 653 (Ser_{653}), and glycine 654 (Gly_{654}), numbered based on the ALS precursor in Arabidopsis, have been confirmed in target-site ALS inhibitor resistance in field-selected weed biotypes [13–28].

Weed management is an important factor in wheat production in China like elsewhere in the world. *Capsella bursa-pastoris*, a diploid and dicotyledonous weed [29], reproduces only by seed [30] and distributes widely in most of the major wheat-producing regions in China. It can tolerate cold and drought and has a wide range of adaptability and great vitality in the fields, therefore, it is a vigorous competitor of wheat. The first biotype of herbicide (simazine) resistant *C. bursa-pastoris* was reported in Poland in 1984 [31]. Subsequently, more *C. bursa-pastoris* biotypes were found to be resistant to different herbicides (Photosystem II inhibitors and ALS inhibitors) in Poland, Israel, USA, Canada and China [31–34]. Now herbicide resistant *C. bursa-pastoris* has became one of the predominating weeds in many wheat fields and orchards. After used for 3–15 years, tribenuron is incapable of controlling *C. bursa-pastoris* effectively at recommended use rate (11.25 g

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a.i. ha^{-1}). In this study, the resistant biotype was the descendant of a Liangzhu population collected from a wheat field, where tribenuron was used over 15 years; the susceptible biotype was the progeny of the seeds collected from a remote hill with no history of exposure to tribenuron. The aims of the study presented here were to (1) characterize the degree of resistance between resistant and susceptible biotypes and (2) determine if the resistance mechanism was due to modified ALS.

2. Materials and methods

2.1. Plant material

Resistant *C. bursa-pastoris* seeds were collected during the summer of 2009 from winter wheat fields in Henan province of China, which were treated the preceding years with tribenuron. The seeds were collected from each plant that survived from an annual treatment with tribenuron (Juxing, 75% a.i., Dupont, China) at 45.2 g a.i. ha^{-1}. That dosage had been applied for three successive years. Thirty bags of seeds were collected in total. In addition, 30 packages of sensitive *C. bursa-pastoris* seeds (S) from 30 plants were collected from a remote hill location that had not been treated previously with any herbicide. In the autumn of 2009, ten mature and intact seeds were chosen from each bag and planted. All of the resistant seedlings and half of the sensitive seedlings at the three to four leaves stage were treated with tribenuron (45.2 g a.i. ha^{-1} for R and 2.8 g a.i. ha^{-1} for S) with a compressed air, moving nozzle cabinet sprayer equipped with one Teejet XR 80031 flat fan nozzle and calibrated to deliver 400 L ha^{-1} at 0.3 MPa. All of the resistant plants survived but all of the sensitive plants died at 21 d after the treatment. In order to avoid outcrossing, each fertile tiller of each remanent plant was covered with one kraft bag prior to anthesis. The seeds from each plant were pooled in a bag. 150 packages of sensitive and resistant seeds (marked with "Lz-S" and "Lz-R", respectively) were collected, respectively in the summer of 2010.

2.2. Whole-plant pot experiments

Seeds of both biotypes were sterilized for 5 min in a 30% hypochlorite solution and then washed twice with sterile distilled water. Sterilized seeds were sown in 12-cm-diam pots containing moist loam soil. Pots were placed in growth chamber at 22/18 °C (day/night temperatures) with a 12 h/12 h day/night cycle, light quantum of 434.3 μmol m^{-2} s^{-1}, and 75% relative humidity. After emergence, the seedlings transferred to greenhouse were thinned to ten evenly sized plants per pot, watered and fertilized as required. Seedlings at the three to four leaves stage were treated with tribenuron. The herbicide doses for the Lz-S biotype were 0, 0.004, 0.02, 0.1, 0.5, 2.5 and 12.5 times as the recommended use rate. The recommended use rate is 11.25 g ai ha^{-1} for tribenuron. For the Lz-R biotype, the doses were 0, 0.1, 0.5, 2.5, 12.5, 62.5, and 312.5 times as the recommended use rate. At 21 d after treatment plants were cut at the soil surface and the fresh weights recorded. All treatments were replicated three times and the experiment was conducted twice.

Combined data over the two experimental runs was subjected to analysis of variance (ANOVA) and dose–response relationships were examined using the Probit model (Eq. (1)) of SPSS software (Version 13.0, SPSS Inc.):

$$Y = b + kx \quad (1)$$

where Y is probit, b is intercept, k is the regression coefficient and x is log 10 (dose). GR_{50} is the herbicide rate required for 50% growth reduction. The level of resistance for the Lz-R biotype was shown by the resistance ratio (GR_{50} Lz-R)/(GR_{50} Lz-S).

2.3. Extraction of RNA and cDNA synthesis

Approximately 1 g of young shoot tissue of single plant at four-leaf stage from biotypes Lz-R and Lz-S was harvested and temporarily stored at −80 °C. Total RNA were extracted from 100 mg young shoot tissue of each plant using the RNA simple Total RNA Kit (Tiangen Biotech, China) according to the manufacturer's instructions. Then cDNA was synthesized employing the QuantScript RT Kit (Tiangen Biotech, China) according to the manufacturer's instructions.

2.4. DNA amplification, cloning, and sequencing

Two pairs of primers (shown in Table 1) for middle region were designed using the software Primer Premier 5.0 to amplify the region containing the conserved domains A, B and D based on plant ALS sequences available in GenBank. The plant species used were common Arabidopsis (*Arabidopsis thaliana* L., X51514), Flixweed (*Descurainia sophia* L., EU520490) and Rape (*Brassica napus* L., M60068).

The DNA Engine BIO-RAD 2 was used to amplify ALS gene. The PCRs were performed in a final volume of 25 μl including 0.5 μl of cDNA (about 25 ng μl^{-1}), 1 μl of each primer (20 μM), 2.5 μl of 10× Taq Buffer (Mg^{2+}Plus, Tiangen Biotech, China), 2.5 μl of dNTP Mixture (2.5 mM, Tiangen Biotech, China), 0.5 μl Taq DNA Polymerase (2.5 U μl^{-1}, Tiangen Biotech, China). PCR reactions were subjected to a 5-min denaturation at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at

Table 1
Primers used to amplify the *C. bursa-pastoris* ALS gene.

Regions	Primers	Sequence (5′–3′)	Amplicon size (bp)
Middle region	OF[a]	TTAGAACGTCAAGGCGTAGAAA	–
	OR[b]	TGTTCTTTCTTTGTCACCCTC	
	IF[c]	TTAACTCGTTCCTCCTCAATC	1472
	IR[d]	TTGGAGCAGATTGTTAGGTTG	
3′ RACE	OF	CTGTCTTGTATGTTGGTGGTG	–
	OR	TACCGTCGTTCCACTAGTGATTT	
	IF	TGAACGGACAGAAACAGAAGT	882
	IR	CGCGGATCCTCCACTAGTGATTTCACTATAGG	
5′ RACE	OF	AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG	–
	OR	CCAATCATCCTACGAGAGACCTGTCCTGTG	
	IF	ATCAACGCAGAGTGGCCATTATG	584
	IR	ACGCATCGGCTAATCCGCTAA	

[a] Outer forward primer.
[b] Outer reverse primer.
[c] Inner forward primer.
[d] Inner reverse primer.

X °C, and *Y* s at 72 C; then 10 min at 72 °C, where *X* is the annealing temperature for each primer pairs used, and *Y* is the extending time. Annealing temperature was 55 and 53.7 °C for two primer sets, respectively. Extending time was 110 and 90 s, respectively.

The 5′ RACE and 3′ RACE gene-specific primers (shown in Table 1) were designed according to known regions of ALS cDNA. The cDNA ends of ALS were amplified using *TaKaRa* 3′-Full RACE Core Set Ver. 2.0 and 5′-Full RACE Kit (TaKaRa BioTech, China).

PCR products were detected by 1.0% agarose gels and extracted using the TIANgel Midi Purification Kit (Tiangen Biotech, China) based methods. The desired PCR bands were cloned with pMD18-T vector (TaKaRa BioTech, China) and then the recombinant plasmids were introduced into competent *Escherichia coli* (*Trans*1-T1 Phage Resistant Chemically Competent Cell, Trans BioTech, China) according to the manufacturer's introductions. Positive clones were sequenced on ABI PRISM 3730 DNA sequencer by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China). Three biological replicates of each biotype were used for ALS gene amplification. At least five clones for each biological replicate were sent for sequencing and used to construct the ALS consensus sequence.

Sequence data were aligned and compared using DNAMAN version 5.2.2 softwares.

2.5. Cloning a fragment of the C. bursa-pastoris ALS gene

After assembling and aligning the complete sequences of ALS of Lz-R and Lz-S bitypes, the two sequences were compared. One nonsynonymous mutation occurred in Domain A, cytosine by thymine (CCT to TCT) at position 197, that leads to a change of the amino acid proline in the susceptible to serine in the Lz-R. This mutation is a widespread, dominant gene of resistance to ALS-inhibiting herbicides. In order to prove this mutation was not accidental, the middle region of ALS gene was amplified for Lz-R and Lz-S biotypes (thirty seedlings per biotype) using the two pairs of primers.

Table 2
Whole-plant pot experiments. Averaged growth inhibition ratio for the fresh weight (% of the untreated) data against use rate for tribenuron.

Lz-S biotype		Lz-R biotype	
Dose (g a.i. ha^{-1})	IR[a] (%)	Dose (g a.i. ha^{-1})	IR (%)
0.045	29.9	1.125	25.1
0.225	46.6	5.625	33.6
1.125	72.4	28.125	44.4
5.625	89.6	140.625	59.0
28.125	94.4	703.125	67.9
140.625	98.3	3515.625	76.6

[a] Averaged growth inhibition ratio compared with the untreated control.

Table 3
Whole-plant pot experiments. Equations and coefficients of determination (R^2) of the fitted regression lines for the fresh weight (% of the untreated) data against use rate for tribenuron.

Biotype	Fitted equation	R^2	GR_{50}[a] (g a.i. ha^{-1})	R/S[b]
Lz-S	$Y = 0.518 + 0.798x$	0.991	0.224	236.6
Lz-R	$Y = -0.709 + 0.411x$	0.997	53.002	

[a] GR_{50}, the herbicide rate required for 50% growth reduction.
[b] R/S, calculated as the GR_{50} of the Lz-R biotype divided by the GR_{50} of the susceptible control (Lz-S) biotype.

3. Results

3.1. Whole-plant pot experiments

The ANOVA of pot experiments showed no significant difference between the two experiments, treatment means were averaged over the two experiments. Dose–response relationships examined using the Probit model (Eq. (1)) of SPSS software were shown in Table 3.

The Lz-R biotype was highly resistant to tribenuron while Lz-S biotype was very sensitive, as shown in Table 2. By comparing the GR_{50} values of the post-emergence treated Lz-R to Lz-S the estimated resistant ratio for Lz-R was 236.6 (Table 3).

3.2. Identification of C. bursa-pastoris ALS mutation

The dose–response experiments demonstrated that Lz-R biotype was highly resistant to tribenuron. To identify the molecular basis for resistance, the ALS genes from Lz-R and Lz-S were sequenced and compared. Total RNA were extracted from leaf tissue of individual plants of both biotypes. Three overlapping target fragments of ALS gene for the two biotypes were amplified by the primers (Table 1). The lengths of the amplified PCR product were approximately 1472 bp, 882 bp and 584 bp, respectively.

Sequencing results were assembled to obtain the full length cDNA sequences, which were translated into amino acid sequences for each biotype. The full length DNA sequence of ALS gene containes 2249 bp, which includes an open reading frame (ORF) of 1644 bp, 406 nucleotides of 5′ untranslated sequence (UTR) and 199 nucleotides of 3′ UTR. One nucleotide C was substituted by T at position 626 (Fig. 1) by comparing the sequences of the susceptible and the resistant biotype, which results in the amino acid changed at position 197 in Domain A, proline, was substituted by serine (Fig. 1). The ALS gene of the *C. bursa-pastoris* had 78.17% similarity to that of *A. thaliana*.

3.3. Comparison of the fragment of ALS gene from both biotypes

Analysis of the nucleotide and deduced amino acid sequences between the biotypes indicated that one substitution had occurred in Domain A, cytosine by thymine (CCT to TCT) at position 197, that leads to a change of the amino acid proline in the susceptible to serine in the Lz-R. In order to exclude that this was not an accidental mutation at position 197, the fragment (containing Domian A) of ALS gene was amplified from thirty seedlings of each biotype at random. After comparing these sequences, we found this location was Pro in all the Lz-S clones while in all the Lz-R clones it was Ser. Even though many methods [35–41] were developed to detect single-nucleotide polymorphism, the specific primers with a 3′ mismatch still amplified the fragments due to reasons for technology or the fidelity of the Taq DNA polymerase. However, the re-

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                                191                     197                     203
Capsella bursa-pastoris Lz-S: GCA ATC ACA GGA CAG GTC CCT CGT AGG ATG ATT GGT ACT
                               A   I   T   G   Q   V   P   R   R   M   I   G   T
Capsella bursa-pastoris Lz-R: GCA ATC ACA GGA CAG GTC TCT CGT AGG ATG ATT GGT ACT
                               A   I   T   G   Q   V   S   R   R   M   I   G   T
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Fig. 1. Domain A of ALS gene in both *Capsella bursa-pastoris* biotypes. Alignment of the nucleotide sequence of Domain A of the Lz-S and the Lz-R biotypes. Bold print in the amino acid sequence indicates the site confer resistance to ALS inhibitors, where CCT codon (**Pro**) in Lz-S biotype was substituted by a TCT codon (**Ser**) in Lz-R biotype.

sult gained through large number of experimental data revealed that Lz-R and Lz-S were homozygous and the mutant Ser197 ALS allele was associated with resistance to tribenuron in *C. bursa-pastoris*.

4. Discussion

Whole-plant Pot experiments have shown that Lz-R biotype is highly resistant to tribenuron compared with the susceptible biotype Lz-S. The emergence of the highly resistant weed population was attributed to lack of herbicide rotation as well as the strong selection pressure imposed by the long residual activity of the ALS-inhibiting herbicide [46]. Now, farmers are forced to use alternative herbicides in order to avoid yield losses in winter wheat fields in China. However, care should be taken to avoid the evolution of multiple herbicide resistance.

An altered target-site is the most common mechanism of ALS resistance, probably because it can be conferred by mutations that afford a high level of resistance to the herbicides while still maintaining normal ALS enzyme activity. Investigations of target site based ALS-inhibitor resistance in field-selected weed biotypes have revealed amino acid substitutions in several positions along the ALS gene: Ala122, Pro197, Ala205, Asp376, Trp574, Ser653 and Gly654 [19,24,27]. The mutation of ALS, Pro to Ser at position 197, confers resistance to sulfonylurea herbicides in several weedy species [20,43–45]. In the study, the Lz-R expressed highly resistant to tribenuron due to the changed target-site with the mutation (Pro to Ser) at position 197.

Target site-based resistance in many cases is due to single mutation in the amino acid sequence resulting in conformational changes to the herbicide binding site of the target enzyme [42]. And then the changed conformation of ALS enzyme maybe reduce sensitivity to the herbicide. Unpublished results in our laboratory have shown that the ALS enzyme of Lz-R biotype express lower sensitivity to tribenuron than that of Lz-S biotype.

This research indicated that ALS inhibitor resistance in biotype of *C. bursa-pastoris* is based on an altered target site conferred by a single-point mutation. For the first time, the molecular basis of tribenuron resistance is elucidated in *C. bursa-pastoris* in the world.

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