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Cross-Resistance of Eclipta (*Eclipta prostrata*) in China to ALS Inhibitors Due to a Pro-197-Ser Point Mutation

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Eclipta, widespread in tropical, subtropical, and temperate regions, is one of the main malignant broadleaf weeds and thrives in moist and dryland fields. Field rates of acetolactate synthase (ALS) inhibitors have failed to control eclipta in some farmlands in China. One ALS inhibitor–resistant population (R) collected from Jiangsu province in China was confirmed in the greenhouse in our preliminary work. Whole-plant assays revealed that this R population was highly resistant to four sulfonylureas (pyrazosulfuron-ethyl, 134-fold; bensulfuron-methyl, 172-fold; metsulfuron-methyl, 30-fold; and tribenuron-methyl, 195-fold), two triazolopyrimidines (pyroxsulam, 98-fold; penoxsulam, 30-fold), and one pyrimidinylthio-benzoate (bispyribac-sodium, 166-fold) and was moderately resistant to two imidazolinones (imazethapyr, 10-fold; imazapic, 19-fold). ALS enzyme-activity assays showed insensitivity of the ALS from the R population (resistance index values ranged from 12 to 293) to all of the above ALS inhibitors in vitro. Chromatograms from *ALS* gene sequence analysis detected a homozygous Pro-197-Ser amino acid substitution in the R population. These results confirmed that the Pro-197-Ser substitution results in broad-spectrum cross-resistance to ALS inhibitors in the eclipta R population. To our knowledge, this study is the first to report broad cross-resistance to ALS inhibitors in eclipta and to obtain the full-length *ALS* gene sequence.
Nomenclature: bensulfuron-methyl; bispyribac-sodium; imazapic; imazethapyr; metsulfuron-methyl; penoxsulam; pyrazosulfuron-ethyl; pyroxsulam; tribenuron-methyl; eclipta, *Eclipta prostrata* (L.) L.
Key words: ALS inhibitor resistance, mutation, target-site.

Acetolactate synthase (ALS, EC 2.2.1.6; also referred to as acetohydroxy acid synthase, EC 4.1.3.18) inhibitors have been widely employed in agriculture production since their first commercialization in the 1980s. The advantages of ALS inhibitors include being highly efficient at low rates, safe to mammals, and benign to the environment (Mazur and Falco 1989; Yu and Powles 2014). Resistance was first reported in a prickly lettuce (*Lactuca serriola* L.) biotype after chlorsulfuron was used for only 5 yr (Mallory-Smith et al. 1990). Worldwide, 159 weed species are ALS resistant (Heap 2016). Moreover, the number of ALS-resistant weeds is still increasing. ALS inhibitors inhibit the catalysis of the first process in the biosynthetic pathway of three branched-chain amino acids, valine, leucine, and isoleucine (Duggleby et al. 2008; Duggleby and Pang 2000; Singh 1999), resulting in plant death. ALS is the common target enzyme of five different chemical families of herbicides: sulfonylureas (SUs), imidazolinones (IMIs), sulfonylamino-carbonyltriazolinones (SCTs), triazolopyrimidines (TPs), and the pyrimidinylthio-benzoates (PTBs).

As a group of selective herbicides, ALS inhibitors are continuously prone to the evolution of resistance (Heap 2016). The less commonly observed non–target site resistance mechanism involves key enzymes such as glutathione *S*-transferases and cytochrome P450 monooxygenases (Iwakami et al. 2014; Park et al. 2004; Powles and Yu 2010; Veldhuis et al. 2000). Many resistance cases have resulted from the incidence of several known point mutations within the *ALS* gene (Liu et al. 2015a; Sada et al. 2013). To date, 27 different point mutations have been discovered in the *ALS* gene: Ala-122 (Riar et al. 2013), Pro-197 (Varanasi et al. 2015), Ala-205 (Brosnan et al. 2016), Asp-376 (Huang et al. 2015), Arg-377 (Massa et al. 2011), Trp-574 (Matzrafi et al. 2015), Ser-653 (Chen et al. 2015), and Gly-654 (Laplante et al. 2009), with the amino acid numbers standardized to the *ALS* gene sequences of mouse-ear cress [*Arabidopsis thaliana* (L.) Heynh.]. Superficial similarities were found between SUs and IMIs, suggesting that they have partially overlapping binding sites on the ALS (McCourt et al. 2006). Therefore, certain single point mutations may cause cross-resistance to two or more herbicide classes (Tranel and Wright 2002).

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Eclipta, distributed widely in the tropical, subtropical, and temperate regions and thriving in moist and dryland fields, is a common annual malignant broadleaf weed belonging to the *Asteraceae* family. It has gradually become a troublesome weed in several agronomic crops in many countries (Holm et al. 1977), including rice (*Oryza sativa* L.) (Smith 1988), cotton (*Gossypium hirsutum* L.) (Qiang et al. 2000), soybean [*Glycine max* (L.) Merr.] (Sharma and Amritphale 1988), and peanut (*Arachis hypogaea* L.) (Altom et al. 1995; Wilcut et al. 1991). It not only can directly cause growth and yield reduction but also can endanger crops indirectly by serving as the host of *Sclerotinia minor* (Melouk et al. 1992), *Amsacta moorei* (Galinato et al. 1999), *Alternanthera yellow vein virus* (He et al. 2008), and *Tomato leaf curl New Delhi virus* (Haider et al. 2006). When acting as the host of *S. minor*, it can reduce production in peanut fields by 25% to 50% (Melouk et al. 1992). In China, eclipta has widely infested paddy rice fields in Chongqing, Hunan, Hubei, and Jiangsu provinces, and the frequency of occurrence in many areas of the Jiangsu province can reach 100% (Li et al. 2009). When ALS inhibitors first appeared as commercial products, they could control this weed effectively. However, under intensive herbicide selection pressure, resistant individuals were repeatedly selected. Farmers have complained recently that the field rate of ALS inhibitors failed to control eclipta in some paddy fields. In our preliminary work in the greenhouse, one ALS inhibitor–resistant population (R) was confirmed.

The aims of this study were (1) to characterize the levels of cross-resistance to four chemical families (SUs, IMIs, TPs, and PTBs) of ALS inhibitors, (2) to determine the difference in ALS sensitivity between susceptible and resistant populations at the biochemical level, and (3) to sequence the *ALS* gene and detect the *ALS* mutations conferring resistance.

Materials and Methods

Plant Material. The seeds of two populations were collected in different areas of Jiangsu province in China: one R population identified in our preliminary study and one with known susceptibility (S). The R population seeds were collected in paddy fields with a history of repeated use of pyrazosulfuron-ethyl for more than 10 yr, and the S population seeds were collected in uncultivated areas with no history of herbicide application and used as a control in all biological and molecular assays.

Before planting, seeds were drenched in sterile distilled water at 4 C for 14 d to break dormancy. Then, the germinated seeds were sown into 10.5-cm-diameter pots containing loam soil (organic matter content ≥15%). Pots were placed in the greenhouse (temperature was maintained at 25 to 35 C) and watered and fertilized as required. The seedlings were thinned to 10 evenly sized plants per pot before herbicide application.

Cross-Resistance Whole-Plant Assays. The cross-resistance levels to ALS inhibitors were characterized by whole-plant assays. The herbicides were applied at the 4-leaf stage with a compressed-air, moving-nozzle cabinet sprayer (Compressed air cabinet sprayer 3WPSH-500D, Beijing Research Center for Information Technology in Agriculture, Beijing, China) equipped with one TeeJet® XR8002 flat fan nozzle and calibrated to deliver 367.5 L ha^{-1} at 0.275 MPa. The applied ALS inhibitors included four SUs (pyrazosulfuron-ethyl, bensulfuron-methyl, metsulfuron-methyl, and tribenuron-methyl), two TPs (pyroxsulam and penoxsulam), two IMIs (imazethapyr and imazapic), and one PTB (bispyribac-sodium); doses are listed in Table 1. A nonionic surfactant solution (a mixture of 78% polyoxyethylene dodecyl ether and 22% water) was added to bispyribac-sodium at 0.1% by volume, and the auxiliary was added to pyroxsulam at 0.1% by volume. The shoot biomass was harvested from ground level 21 d after treatment and dried at 80 C for 48 h, and dry weights were determined. All treatments had three replicates, and each experiment was conducted twice.

In Vitro ALS Activity Assays. Seeds of the eclipta S and R populations were germinated and cultivated under the appropriate conditions described earlier. Fresh plant tissue was harvested at the 4-leaf stage and stored at −80 C. ALS activity was measured based on the method described by Yu et al. (2004). Enzyme activity was determined colorimetrically (530 nm) by measuring the amount of acetoin formed. The protein concentration of the crude extract was measured by the Bradford method (1976). Herbicide doses used for ALS activity assays are listed in Table 2. Three subsamples from each extraction were assayed, and two extractions per population were used.

Statistical Analyses. Nonlinear log-logistic regression analysis was performed to evaluate the data of the aboveground dry biomass and ALS activity using

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Table 1. Basic information concerning ALS inhibitors used and doses applied to eclipta in cross-resistance whole-plant assays.

Herbicide	Content[a]	Trade name	Manufacturer	Address	Population[b]	Dose[c]
						g ai ha^{-1}
Pyrazosulfuron-ethyl	10% WP	PD20080594[d]	Jiangsu Changlnong Agrochemical	Taizhou, China; http://www.jschanglong.com	S	0, 0.0064, 0.032, 0.16, 0.8, 4, **20**
					R	0, 0.16, 0.8, 4, **20**, 100, 500
Bensulfuron-methyl	10% WP	Londax®	DuPont	Wilmington, DE; http://www.dupont.com	S	0, 0.0064, 0.032, 0.16, 0.8, 4, **20**, 100
					R	0, 0.16, 0.8, 4, **20**, 100, 500, 2500
Metsulfuron-methyl	60% WDG	PD20096008[d]	Repont	Nantong, China; http://www.repont.com/in.html	S	0, 0.0024, 0.012, 0.06, 0.3, 1.5, **7.5**
					R	0, 0.06, 0.3, 1.5, **7.5**, 37.5, 187.5
Tribenuron-methyl	75% WDG	PD20050099[d]	DuPont	Wilmington, DE; http://www.dupont.com	S	0, 0.024, 0.12, 0.6, 3, **15**, 75
					R	0, 0.12, 0.6, 3, **15**, 75, 375, 1875, 9375
Pyroxsulam	7.5% WDG	Priority®	Dow AgroSciences	Midland, MI; http://www.dow.com	S	0, 0.0032, 0.016, 0.08, 0.4, 2, **10**
					R	0, 0.08, 0.4, 2, **10**, 50, 250
Penoxsulam	25 g L^{-1} OD	Grasp®	Dow AgroSciences	Midland, MI; http://www.dow.com	S	0, 0.02, 0.1, 0.5, 2.5, **12.5**, 62.5
					R	0, 0.1, 0.5, 2.5, **12.5**, 62.5, 312.5
Bispyribac-sodium	100 g L^{-1} SC	Nominee®	Kumiai-chemical	Tokyo, Japan; http://www.kumiai-chem.co.jp/index.html	S	0, 0.00704, 0.0352, 0.176, 0.88, 4.4, **22**
					R	0, 0.176, 0.88, 4.4, **22**, 110, 550
Imazethapyr	50 g L^{-1} SL	Newpath®	BASF Corporation	Research Triangle, Germany, https://www.basf.com/en.html	S	0, 0.112, 0.56, 2.8, 14, **70**, 350
					R	0, 0.112, 0.56, 2.8, 14, **70**, 350, 1750
Imazapic	240 g L^{-1} SL	Plateau®	BASF Corporation	Research Triangle, Germany; https://www.basf.com/en.html	S	0, 0.02304, 0.1152, 0.576, 2.88, 14.4, **72**
					R	0, 0.02304, 0.1152, 0.576, 2.88, 14.4, **72**, 360

[a] WP, wettable powder; WDG, water-dispersible granule; OD, oil dispersion; SC, suspension concentrate; SL, soluble concentrate.
[b] S, susceptible population; R, resistant population.
[c] The number in bold represents the field rate (1X).
[d] PD, pesticide registration number.

Table 2. Concentration of ALS inhibitors for eclipta in ALS activity assays in vitro.

Herbicide	Population[a]	Concentration
		μM
Pyrazosulfuron-ethyl	S	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}, 10^{3}
	R	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}, 10^{3}
Bensulfuron-methyl	S	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}
	R	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}, 10^{3}
Metsulfuron-methyl	S	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}
	R	0, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}, 10^{3}
Tribenuron-methyl	S	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}
	R	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}, 10^{3}
Pyroxsulam	S	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}
	R	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}, 10^{3}
Penoxsulam	S	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}
	R	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}
Bispyribac-sodium	S	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}
	R	0, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}, 10^{3}, 10^{4}
Imazethapyr	S	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}
	R	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}, 10^{3}
Imazapic	S	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}
	R	0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}, 10^{3}

[a] S, susceptible population; R, resistant population.

the following model (Equation 1) (Seefeldt et al. 1995).

$$Y = C + \frac{D - C}{1 + (X/\mathrm{ED}_{50})^{b}} \quad [1]$$

In this equation, C is the lower limit, D is the upper limit, b is the slope at ED_{50} (effective dose producing 50% response). In this study, ED_{50} represents GR_{50} (the herbicide dose required for 50% growth reduction) and I_{50} (the herbicide concentration that inhibited ALS activity by 50%). Y is the response expressed as the percentage of the untreated control at herbicide dose or concentration X. The regression analyses were conducted using Sigma Plot v. 12.0 (Systat Software Inc., San Jose, CA). The resistance index (RI) was determined by dividing the GR_{50} or I_{50} of the R population by that of the S population.

ALS Gene Amplification and Sequencing

Genomic DNA Extraction. Fresh leaf tissue from one S population plant and 15 random R population plants was harvested, each sample was placed in plastic bags separately, and then stored at −80 C. Total DNA was isolated with the DNA secure Plant Kit (Tiangen Biotech, Beijing, China). Then, the DNA samples were used immediately for PCR.

Table 3. Primers designed to amplify the *ALS* gene.

Primer name[a]	Sequence (5′ → 3′)	Containing the confirmed point mutations	Annealing temperature
			C
1F	ATTTGTTGCTTGCGTTTG	W574	56.8
1R	AGGTATGAGCCCGATTCG		
2F	ATGGAGATCCACCAAGCT	P197, A205, D376, R377, W574	56.5
2R	CACATCCGAGAAACCTCC		
5′-SP1	ACAATTTGCTCTAACTGCCCATCAT	—	—
5′-SP2	ACCAATTGCTGCTGTATATCCTTCG	—	—
5′-SP3	GACCCGACGACGCAAGATAAAAAGC	—	—
3′-SP1	CACAATTCGCGTTGAAAATCTGCCT	—	—
3′-SP2	GCATTTGGGTATGGTGGTTCAGTGG	—	—
3′-SP3	GGAAATCCGTCGAACGAGACTGAAA	—	—
3F	CCCACCCTGCTTCATCCT	A122, P197, A205, D376, R377	57.8
3R	CCGAAACCCATCGCTCCT		
4F	GGAAGAATAAACAGCCCCAT	W574, S653, G654	53.3
4R	AGCCTGCTTACAGAACACAC		

[a] F, forward primer; R, reverse primer.

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Table 4. Dose–response parameters of eclipta populations in cross-resistance whole-plant assays.

Herbicide	S population[a]			R population[a]			RI[c]
	GR_{50} (SE)[b]	R^2	P	GR_{50} (SE)[b]	R^2	P	
	g ai ha^{-1}			g ai ha^{-1}			
Pyrazosulfuron-ethyl	0.21 (0.03)	0.99	<0.0001	28.05 (7.95)	0.99	<0.0001	133.57
Bensulfuron-methyl	0.13 (0.01)	0.99	<0.0001	22.37 (7.07)	0.99	<0.0001	172.08
Metsulfuron-methyl	0.07 (0.004)	0.99	<0.0001	2.12 (0.17)	0.99	<0.0001	30.29
Tribenuron-methyl	0.20 (0.02)	0.99	<0.0001	39.01 (11.73)	0.99	<0.0001	195.05
Pyroxsulam	0.40 (0.09)	0.99	<0.0001	39.02 (3.26)	0.99	<0.0001	97.55
Penoxsulam	0.22 (0.02)	0.99	<0.0001	6.68 (0.55)	0.99	<0.0001	30.36
Bispyribac-sodium	0.20 (0.02)	0.99	<0.0001	33.15 (5.36)	0.99	<0.0001	165.75
Imazethapyr	4.35 (0.35)	0.99	<0.0001	44.43 (2.40)	0.99	<0.0001	10.21
Imazapic	0.40 (0.06)	0.99	<0.0001	7.63 (1.65)	0.99	<0.0001	19.08

[a] S population, susceptible population; R population, resistant population.
[b] GR_{50}, herbicide dose required to decrease shoot dry weight by 50% compared with the untreated control; SE, standard error.
[c] Resistance index (RI) was determined by calculating the ratio of the GR_{50} values of resistant population to that of the susceptible population.

Partial ALS *Gene Amplification and Sequencing.* There are no *ALS* gene sequences of any *Eclipta* species available in the National Center for Biotechnology Information (NCBI) GenBank. Based on the sequences of common sunflower (*Helianthus annuus* L.) (GenBank accession AY541451.1), cocklebur

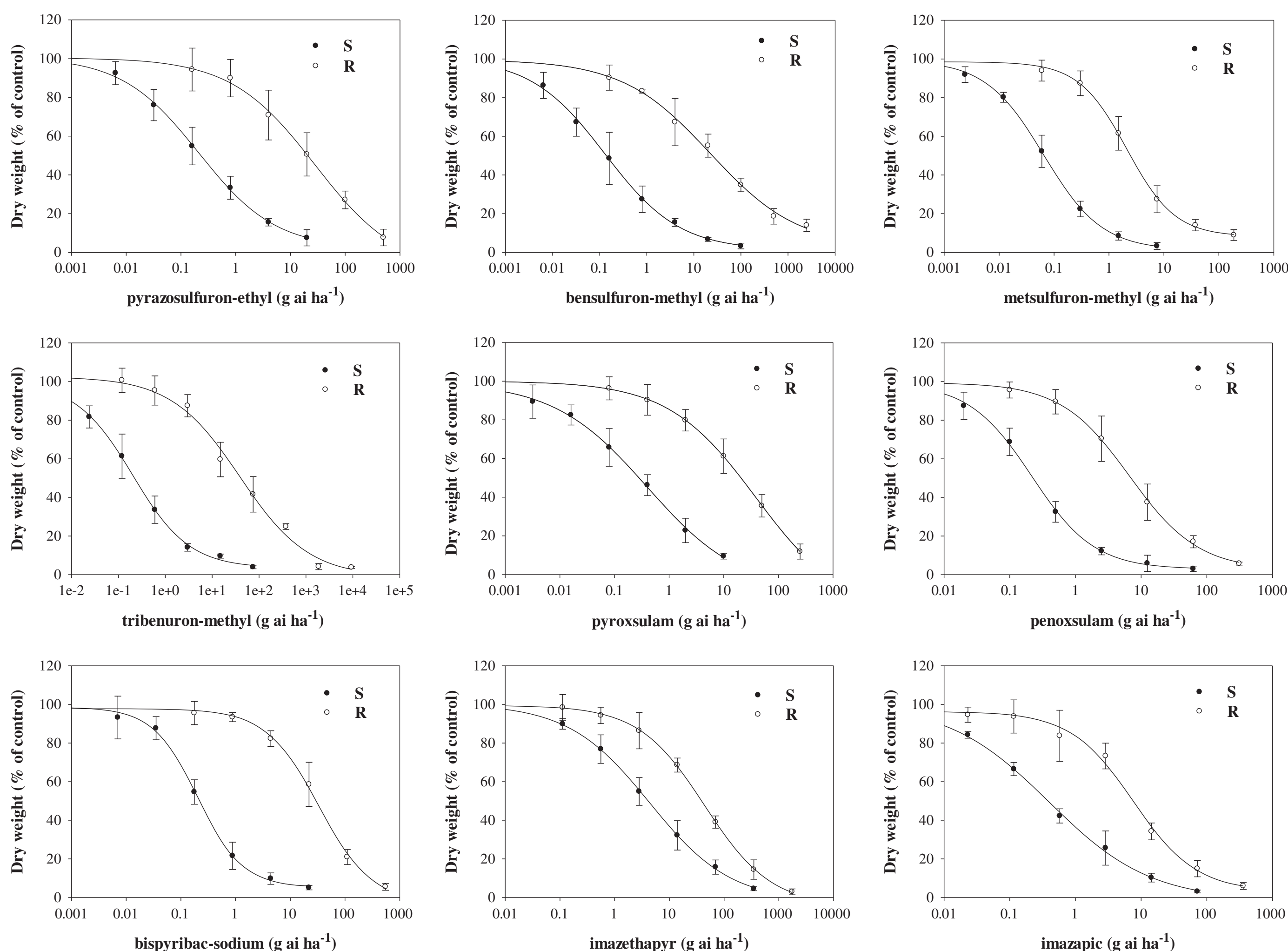

Figure 1. Dose–response curves of shoot dry weight of susceptible (S) and resistant (R) populations to different doses of ALS inhibitors. Error bars represented the SD.

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(*Xanthium* spp.) (GenBank accession U16280.1), and horseweed [*Conyza canadensis* (L.) Cronq.] (GenBank accession HM067014.1), two pairs of gene-specific primers (1F-1R and 2F-2R) (BGI·Tech, Beijing, China) were designed using Primer Premier v. 5.0 software (Premier Biosoft International, Palo Alto, CA) to amplify the *ALS* gene (Table 3).

The PCR was conducted in a 15-μl volume containing 0.5 μl genomic DNA (about 75 ng μl^{-1}), 0.3 μl of each primer (10 μM), 1.5 μl 10X PCR Buffer (Mg^{2+} plus), 1.0 μl dNTPs (2.5 mM), 0.3 μl rTaq DNA polymerase (5 U μl^{-1}) (TaKaRa Biotechnology, Dalian, China), and 11.1 μl ddH_2O. The amplification protocol was performed in an Eppendorf AG-22331 Hamburg automated thermal cycler (Eppendorf, Hamburg, Germany) as follows: 10 min denaturation at 94 C; 30 cycles of 0.5 min at 94 C, 0.5 min at annealing temperature, and 1 min at 72 C; then 10 min at 72 C (annealing temperatures for each primer pair are given in Table 3).

PCR products were sequenced commercially, and the sequencing results were aligned using DNAMAN v. 5.2.2 software (Lynnon LLC, San Ramon, CA). The spliced sequences contained domains A, D, F, and B, with high similarity to the *ALS* genes of other species, as determined by BLAST analysis (NCBI). The sequences were then used for the following assays.

ALS *Gene Walking PCR.* To amplify the full-length *ALS* gene, genome walking was used to amplify the 5′ end and 3′ ends of the *ALS* gene using three nested PCRs. According to the primer design principles of the Genome Walking Kit (TaKaRa Biotechnology, Dalian, China), six primers (5′-SP1, 5′-SP2, 5′-SP3, 3′-SP1, 3′-SP2, 3′-SP3) were designed (Table 3). After three rounds of amplification, the third PCR product was purified and cloned into the *pEASY*-T1 cloning vector and then transformed into *Escherichia coli Trans*1-T1 phage-resistant chemically competent cells using the *pEASY*-T1 Cloning Kit (Beijing TransGen Biotech, Beijing, China). Colonies were chosen, and positive recombinants were sequenced.

Cloning of the ALS *Gene Region.* After PCR amplification, the sequence of the full-length *ALS* gene from the S population was confirmed. Based on the obtained sequences, two gene-specific primer pairs: 5′-CCCACCCTGCTTCATCCT-3′ (3F) and 5′-CCGAAACCCATCGCTCCT-3′ (3R); 5′-GGAAGAATAAACAGCCCCAT-3′ (4F) and 5′-AGCCTGCTTACAGAACACAC-3′ (4R) were designed to clone the *ALS* gene containing eight resistance-endowing amino acid substitutions that have been reported in other resistant species. The PCR reaction volume and reaction condition were the same as that of 1F-1R (or 2F-2R). All PCR products were visualized on a 1% agarose gel with 2,000 bp markers to confirm amplicon size and then sequenced and analyzed.

Results and Discussion

Cross-Resistance Whole-Plant Assays. As expected, the results of whole-plant assay showed that the S population was very sensitive to all herbicides, whereas the R population was broadly resistant to all herbicides (the GR_{50} values of S and R populations to pyrazosulfuron-ethyl, bensulfuron-methyl, metsulfuron-methyl, tribenuron-methyl, pyroxsulam,

Table 5. Dose-response parameters of eclipta population in ALS activity assays in vitro.

Herbicide	S population[a]			R population[a]			
	I_{50} (SE)[b]	R^2	P	I_{50} (SE)[b]	R^2	P	RI[c]
	μM			μM			
Pyrazosulfuron-ethyl (SU)	0.13 (0.06)	0.99	<0.0001	15.18 (4.82)	0.99	<0.0001	116.77
Bensulfuron-methyl (SU)	0.08 (0.02)	0.99	<0.0001	14.21 (11.94)	0.97	<0.0001	177.63
Metsulfuron-methyl (SU)	0.04 (0.01)	0.99	<0.0001	2.54 (1.33)	0.99	<0.0001	63.50
Tribenuron-methyl (SU)	0.12 (0.04)	0.99	<0.0001	9.28 (5.79)	0.97	<0.0001	77.33
Pyroxsulam (TP)	0.08 (0.03)	0.99	<0.0001	23.46 (17.04)	0.99	<0.0001	293.25
Penoxsulam (TP)	0.16 (0.12)	0.99	<0.0001	4.45 (1.59)	0.99	<0.0001	27.81
Bispyribac-sodium (PTB)	0.17 (0.07)	0.99	<0.0001	33.40 (16.77)	0.99	<0.0001	196.47
Imazethapyr (IMI)	0.19 (0.12)	0.99	<0.0001	2.25 (1.72)	0.98	<0.0001	11.84
Imazapic (IMI)	0.19 (0.11)	0.99	<0.0001	3.47 (0.79)	0.99	<0.0001	18.26

[a] S population, susceptible population; R population, resistant population.
[b] I_{50}, herbicide dose required to inhibit the ALS activity by 50% compared with the untreated control; SE, standard error.
[c] Resistance index (RI) was determined by calculating the ratio of the I_{50} values of resistant population to that of the susceptible population.

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penoxsulam, bispyribac-sodium, imazethapyr, imazapic were 0.21 and 28.05, 0.13 and 22.37, 0.07 and 2.12, 0.20 and 39.01, 0.40 and 39.02, 0.22 and 6.68, 0.20 and 33.15, 4.35and 44.43, 0.40 and 7.63 g a.i. ha^{-1}, respectively) (Table 4). Obviously, in whole-plant assays, the GR_{50} values for the R population were higher than those for the S population for all herbicides. Moreover, the R population was highly resistant to four SUs (pyrazosulfuron-ethyl, 134-fold; bensulfuron-methyl, 172-fold; metsulfuron-methyl, 30-fold; and tribenuron-methyl, 195-fold), two TPs (pyroxsulam, 98-fold; and penoxsulam, 30-fold), and one PTB (bispyribac-sodium, 166-fold), and moderately resistant to two IMIs (imazethapyr, 10-fold; imazapic, 19-fold) (Figure 1; Table 4).

Continuous application of ALS inhibitors for several years has resulted in the rapid evolution of herbicide resistance (Heap 2016). Several weed species in paddy fields have been reported to be resistant to ALS inhibitors, including monochoria [*Monochoria vaginalis* (Burm. f.) Kunth] (Wang et al. 2004), *Monochoria korsakowii* Regel & Maack (Wu et al. 2007), barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.] (Yu et al. 2010), *Ammannia arenaria* Kunth (Wang et al. 2013), and threeleaf arrowhead (*Sagittaria trifolia* L.) (Liu et al. 2015b). So studying the resistance mechanism of an important malignant broadleaf weed such as the eclipta R population is essential to develop reasonable herbicide-use programs to delay or slow the evolution of resistance.

In Vitro ALS Activity Assays. The results of in vitro ALS activity assays indicated that ALS isolated from the R population was much less

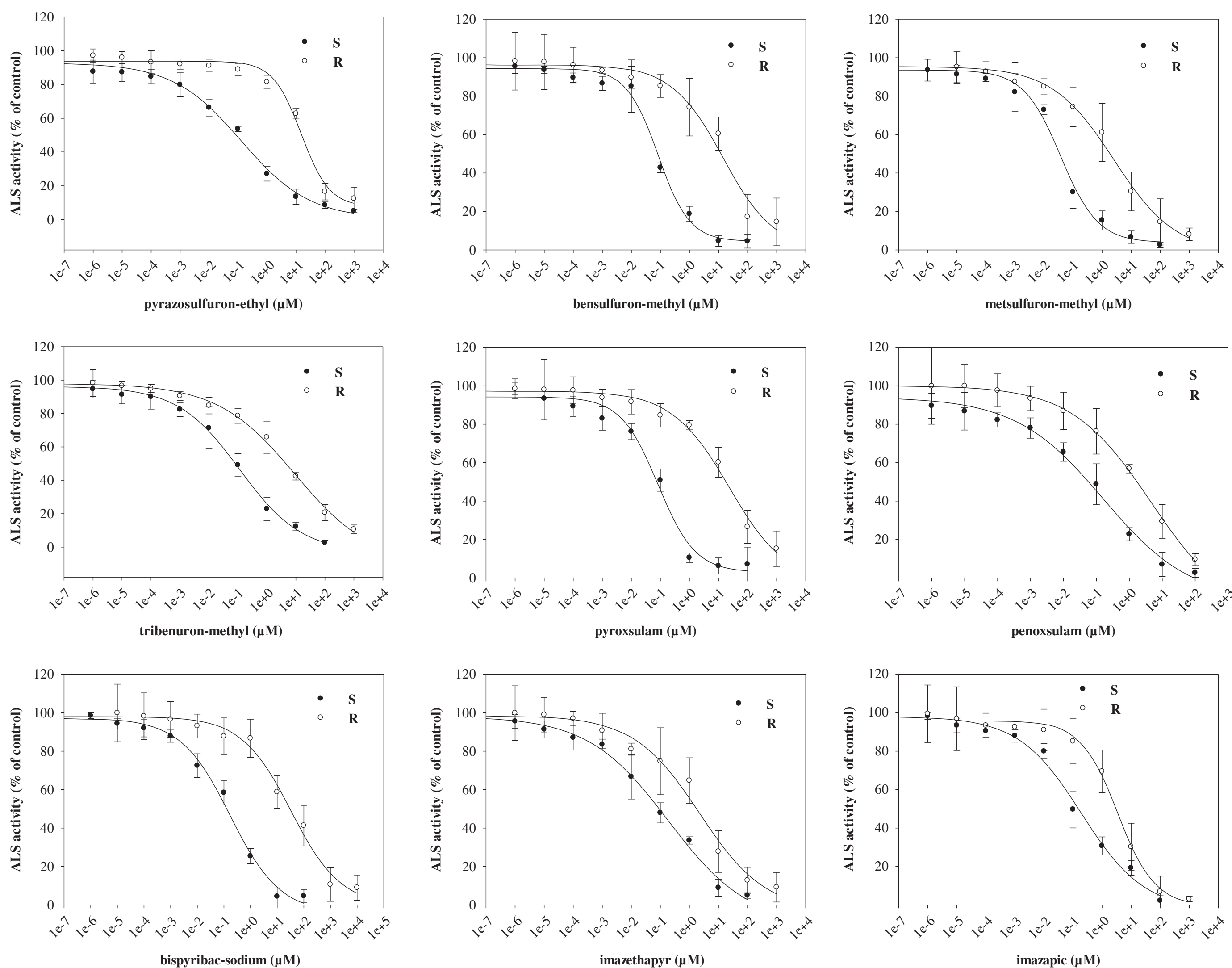

Figure 2. Dose–response curves of ALS activity of susceptible (S) and resistant (R) populations to different doses of ALS inhibitors. Error bars represented the SD.

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susceptible to all herbicides than that isolated from the S population. As shown in Table 5, the I_{50} values of S and R populations to respective pyrazosulfuron-ethyl, bensulfuron-methyl, metsulfuron-methyl, tribenuron-methyl, pyroxsulam, penoxsulam, bispyribac-sodium, imazethapyr, and imazapic were 0.13 and 15.18, 0.08 and 14.21, 0.04 and 2.54, 0.12 and 9.28, 0.08 and 23.46, 0.16 and 4.45, 0.17 and 33.40, 0.19 and 2.25, 0.19 and 3.47 µM, respectively, and the RI values ranged from 12 to 293 across all the above herbicides (Figure 2; Table 5). The results from the inhibition of ALS activity by ALS inhibitors were similar to the results of whole-plant assays in the greenhouse. More interestingly, the RI values of the R population for SUs, TPs, and PTBs were generally an order of magnitude more resistant than those for IMIs in whole-plant assays and in vitro ALS activity assays.

In most studies of resistance mechanisms, reduced susceptibility in vitro translates to herbicide resistance in the whole plant. In this study, the reduced susceptibility of ALS from the R population was in accordance with previous research of SU-R water starwort [*Myosoton aquaticum* (L.) Moench] (Liu et al. 2013) and ALS-R corn poppy (*Papaver rhoeas* L.) (Kaloumenos et al. 2011).

***ALS* Gene Amplification and Sequencing.** Full-length *ALS* gene sequences of eclipta without any introns were obtained, and their sequences were highly similar to the *ALS* genes of sunflower (87%), cocklebur (86%), horseweed (83%), and mayweed chamomile (*Anthemis cotula* L.) (80%). Using 3F-3R and 4F-4R primers and 1,416- and 847-base pair fragments that included the eight target sites, all fragments were amplified, respectively. Compared with the sequences of the S population, one missense mutation at position 197 was detected in the *ALS* gene of all plants tested in the R population. This single nucleotide replacement in the *ALS* gene of the R population resulted in an amino acid substitution of proline (CCC) to serine (TCC) at position 197 (Figure 3). The corresponding sequence chromatograms revealed no double peaks at that site in any resistant plant tested, indicating the mutation was homozygous and inherited steadily as a single dominant monogenic trait (Figure 3).

This is not the first instance of detection of the Pro-197-Ser substitution, and this substitution endowing herbicide cross-resistance has been reported in other weed species: prostrate pigweed (*Amaranthus blitoides* S. Wats.) (Sibony and Rubin 2003), wild radish (*Raphanus raphanistrum* L.) (Yu et al. 2012), corn poppy (Kaloumenos et al. 2011), downy brome

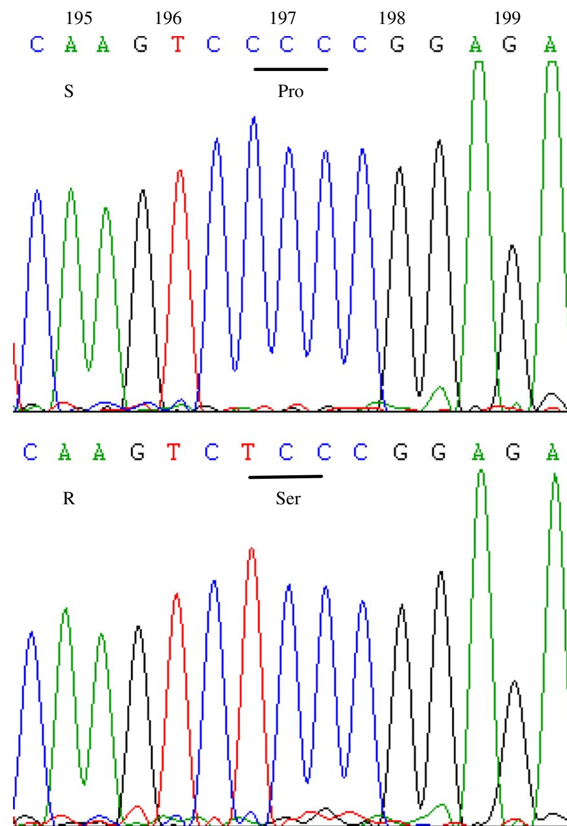

Figure 3. The sequence chromatograms of eclipta ALS gene sequence at position 197 in susceptible (S) and resistant (R) populations.

(*Bromus tectorum* L.) (Park and Mallory-Smith 2004), wild mustard (*Sinapis arvensis* L.) (Warwick et al. 2005), mayweed chamomile (Intanon et al. 2011), and water starwort (Liu et al. 2013). In these weed species, this amino acid substitution was correlated with broad cross-resistance to SUs, TPs, PTBs, or SCTs but low or negligible resistance to IMI herbicides. In this study, the eclipta R population had broad cross-resistance to SUs, TPs, and PTBs and moderate resistance to IMIs.

The Pro-197 position in the *ALS* gene exhibits the highest mutation frequency in amino acid mutations causing ALS resistance in weeds. Substitution types at position 197 occur frequently in cases of resistant weeds, and the Pro has been mutated to Thr, His, Arg, Leu, Gln, Ser, Ala, Ile, Asn, Glu, and Tyr (Heap 2016). Different cross-resistance patterns were determined by weed species, mutation types, ALS inhibitor classes, and certain herbicides of a given class (Yu and Powles 2014). The Pro-197-His substitution in smallflower umbrella sedge (*Cyperus difformis* L.) resulted in broad cross-resistance to SUs (halosulfuron, RI = 21), PTBs (bispyribac-sodium, RI = 71), IMIs (imazamox, RI = 12), and TPs (penoxsulam, RI = 22) (Tehranchian et al. 2015). The Pro-197-Arg

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substitution in henbit (*Lamium amplexicaule* L.) resulted in cross-resistance to SUs (chlorsulfuron, RI > 1000) and SCTs (propoxycarbazone-sodium, RI = 331) but also resulted in sensitivity to IMIs (imazamox) (Varanasi et al. 2015).

This study is the first to confirm the cross-resistance of eclipta to ALS inhibitors due to a Pro-197-Ser point mutation in Jiangsu province in China. Target site–based resistance was conferred by the Pro-197-Ser substitution in the *ALS* gene, resulting in the insensitivity of isolated ALS to ALS inhibitors. The eclipta populations resistant to ALS inhibitors are becoming a serious constraint in major rice production areas in China, so effective measures to manage the weed resistance are desperately needed.

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