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Mechanisms of Resistance to Pyroxsulam and ACCase Inhibitors in Japanese Foxtail (*Alopecurus japonicus*)

Yujuan Feng, Yuan Gao, Yong Zhang, Liyao Dong, and Jun Li*

Japanese foxtail is a predominant tetraploid grass weed in wheat and oilseed rape fields in eastern China. In China, pyroxsulam is mainly used to manage annual grass weeds, especially those resistant to acetyl coenzyme A carboxylase (ACCase)-inhibiting herbicides. Using dose–response studies, a pyroxsulam-resistant population, ACTC-1, was identified with a resistance index value of 58. Additionally, ACTC-1 was cross-resistant to sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinyl-benzoates, and sulfonylaminocarbonyl-triazolinones and multiresistant to ACCase and photosystem II inhibitors. Sequence analysis revealed four gene fragments encoding acetolactate synthase (ALS) from ACTC-1, and three from JNXW-1, a pyroxsulam-sensitive population. An Asp-376-Glu substitution was found in ALS1;2 and an Ile-2041-Asn in Acc1;1, which may be responsible for its resistance to pyroxsulam and ACCase inhibitors, respectively. In vitro assays of ALS activity revealed that in ACTC-1, the sensitivity of ALS to pyroxsulam was lower, and the basal ALS activity was twofold higher than that of sensitive population JNXW-1. Additionally, the combined application of pyroxsulam with malathion or piperonyl butoxide increased the sensitivity of ACTC-1 to pyroxsulam, although it could not completely overcome the resistance. It was inferred that both target-site-based resistance and nontarget-site-based resistance may be involved in the resistance to pyroxsulam.

Nomenclature: Malathion; piperonyl butoxide (PBO); pyroxsulam; Japanese foxtail, *Alopecurus japonicas* Steud; birdsrape mustard, *Brassica campestris* L.; wheat, *Triticum aestivum* L.

Key words: ACCase, ALS, nontarget-site-based resistance, target-site-based resistance.

Japanese foxtail, a tetraploid gramineous plant, is a major weed widely distributed in winter wheat fields in China, Japan, Turkey, and other Asian countries (Xu et al. 2014b). Japanese foxtail historically was managed by ACCase-inhibiting herbicides. Frequent use of ACCase-inhibiting herbicides has selected for resistance to ACCase-inhibiting herbicides in Japanese foxtail, and caused management failures in wheat fields in China (Xu et al. 2014a). Consequently, pyroxsulam was introduced into China in 2012 for the management of grass weeds, especially those resistant to ACCase inhibitors in wheat fields. Pyroxsulam, a new ALS-inhibiting herbicide and a triazolopyrimidine sulfonamide herbicide (Wells 2008), is typically applied at the dosage of 10 to 16 g ai ha^{-1}.

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* First, second, fourth, and fifth authors: Graduate Student, Graduate Student, Professor, and Associate Professor, College of Plant Protection, Key Laboratory of Integrated Pest Management on Crops in East China, Nanjing Agricultural University, Ministry of Agriculture, Nanjing 210095, China; third author: Associate Research Fellow, Institute of Plant Protection and Agro-products Safety, Anhui Academy of Agricultural Sciences, Hefei 230031, China. Corresponding author's E-mail: li_jun@njau.edu.cn

In plants, ALS (also called acetohydroxyacid synthase; EC 4.1.3.18) is a key enzyme that catalyzes the first step of the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine (Yu and Powles 2014). ALS is the target of five chemical herbicide families: sulfonylureas SUs, imidazolinones IMIs, triazolopyrimidines TPs, pyrimidinyl benzoates PBs, and sulfonylaminocarbonyl triazolinones SCTs (Beckie and Tardif 2012). ALS-inhibiting herbicides have been widely used worldwide for their high efficacy, low dose, low toxicity to mammals, and broad-spectrum weed control. However, ALS resistance has also evolved rapidly. Previous studies have suggested that two main mechanisms are involved in resistance to ALS inhibitors: target-site resistance (TSR) and nontarget-site resistance (NTSR) (Yu and Powles 2014).

TSR includes (1) the resistance endowed by alterations in the gene encoding the herbicide target protein that reduce the efficacy of the herbicide binding its target, (2) an increase in the gene expression, or (3) the increasing intrinsic activity of the herbicide target protein that compensates for the herbicide inhibitory action (Massa et al. 2011; Yu et al. 2010). To date, a total of 26 amino acid substitutions at eight conserved amino acid sites in ALS are reported to confer resistance to ALS

Table1. Sensitivity of different Japanese foxtail populations to pyroxsulam.

Populations	Location			ED_{50}[a] (SE)	RI	R or S
	Province	City	County	g ai ha^{-1}		
ACTC-1	Anhui	Chuzhou	Tianchang	92.77 (3.55)	57.98	R
ACTC-2	Anhui	Chuzhou	Tianchang	2.86 (0.13)	1.79	S
AHFD-1	Anhui	Hefei	Feidong	1.43 (0.04)	0.89	S
JCJT-1	Jiangsu	Changzhou	Jintan	1.29 (0.12)	0.81	S
JCWJ-1	Jiangsu	Changzhou	Wujin	1.59 (0.17)	0.99	S
JLGY-1	Jiangsu	Lianyungang	Ganyu	4.04 (0.29)	2.53	S
JNJP-1	Jiangsu	Nanjing	Jiangpu	4.36 (0.34)	2.73	S
JNXW-1	Jiangsu	Nanjing	Xuanwuqu	1.60 (0.18)	1.00	S
JYJD-1	Jiangsu	Yangzhou	Jiangdu	1.90 (0.12)	1.19	S

[a] Abbreviations: ED_{50}, the effective dose of herbicide causing 50% inhibition in fresh weight; RI, resistance index: the ratio between the ED_{50} values of the resistant (R) and sensitive (S) populations.

inhibitors in various weed species, which are Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, and Gly654 (numbered on the basis of the corresponding sequence of *Arabidopsis thaliana*) (Yu and Powles 2014). Substitutions at Pro197 and Trp574 have been reported frequently in field-evolved resistant weed populations. However, substitutions at Asp376 were rarely mentioned in grass weeds resistant to ALS inhibitors, as this substitution was first reported to confer resistance in smooth pigweed (*Amaranthus hybridus* L.) (Whaley et al. 2007).

NTSR encompasses a range of diverse mechanisms, including reduced penetration, impaired translocation, sequestration, and enhanced metabolism of the toxophores (Delye 2013). At present, NTSR is considered the predominant mechanism for resistance to ACCase and ALS inhibitors in many monocots (Delye et al. 2011). A variety of enzymes are involved in NTSR, of which cytochrome P450 monooxygenases (Cyt P450s) are the major ones (Yuan et al. 2007) and play an important role in the metabolic detoxification of herbicides (Kwon and Penner 1995). Piperonyl butoxide (PBO) and malathion, Cyt P450 inhibitors (Gressel 1990) have been used to detect resistance resulting from herbicide metabolism by Cyt P450s. They inhibit Cyt P450 activity toward several herbicides, including SUs, thereby overcoming resistance (Christopher et al. 1994; Kwon and Penner, 1995).

There have been several reports on the resistance to ACCase and ALS inhibitors in Japanes foxtail (Bi et al. 2015; Xu et al. 2014a). However, the involvement of TSR and NTSR simultaneously in resistance of Japanese foxtail to ALS inhibitors has been rarely addressed in previous studies. Therefore, the objectives of this study were to (1) determine the level of resistance to pyroxsulam in different populations, (2) explore the mechanism of resistance to pyroxsulam, and (3) characterize the cross-resistance and multiple-resistance patterns for the resistant population.

Materials and Methods

Plant Material. Seeds of eight Japanese foxtail populations (Table 1) were collected from winter wheat fields in the Jiangsu and Anhui provinces of China in 2012 and 2013. The putative resistant populations were collected from wheat fields in which pyroxsulam applied at the recommended dosage failed to control this weed. All the seeds from randomly selected plants were collected by hand, air-dried in shade, and stored in paper bags at 4 C until use. The reported sensitive population, JNXW-1 (Xu et al. 2013), was used as a wild-type population in all biological and molecular studies.

Sensitivity to Pyroxsulam. Seeds were soaked in water at 4 C for 7 d to break dormancy. Thirty pregerminated seeds were sown in a plastic pot (9 cm in diameter and 10 cm in height) and grown in incubators at 20 C/15 C (light/dark temperatures) with a 12-h light/12-h dark cycle, a light intensity of 8,000 lux, and a relative humidity of 85%. After emergence, the seedlings were thinned to 20 plants per pot. At the three- to four-leaf stage, herbicides were applied using a laboratory sprayer equipped with a flat-fan nozzle, delivering 280 L ha^{-1} at 230 kPa. Pyroxsulam at 0, 0.1875, 0.75, 3, 12, 48, and 192 g ai ha^{-1} and at 0, 0.75, 3, 12, 48, 192, and 768 g ha^{-1} were applied to the sensitive and

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Table 2. Herbicide rates applied for dose–response tests.

Group[a]	Herbicide[b]	Field dose	Application	
			ACTC-1	JNXW-1
		g ai ha^{-1}		
ALS inhibitors				
SU	Mesosulfuron-methyl	13.5	0, 3.2, 6.4, 12.8, 25.6, 51.2, 102.4, 204.8, 409.6	0, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2, 102.4
	Rimsulfuron	22.5	0, 5.625, 22.5, 90, 360, 1,440	0, 0.3516, 1.406, 5.625, 22.5, 90
	Nicosulfuron	40	0, 6.4, 12.8, 25.6, 51.2, 102.4, 204.8	0, 0.1, 0.4, 1.6, 6.4, 25.6
	Sulfosulfuron	30	0, 15, 60, 240, 960, 3,840	0, 0.46875, 1.875, 7.5, 30, 120, 480
IMI	Imazapic	96	0, 24, 96, 384, 1,536, 6,144	0, 1.5, 6, 24, 96, 384, 1,536
TP	Penoxsulam	18.75	0, 4.6875, 18.75, 75, 300, 1,200	0, 1.172, 4.6875, 18.75, 75, 300, 1,200
PB	Pyribenzoxim	26.25	0, 6.5625, 26.25, 105, 420, 1,680	0, 1.64, 6.5625, 26.25, 105, 420, 1,680
SCT	Flucarbazone-sodium	30	0, 120, 240, 480, 960, 1,920, 3,840	0, 1.875, 7.5, 30, 120, 480, 1,920
ACCase inhibitors				
APP	Fenoxaprop-p-ethyl	52	0, 52, 104, 208, 416, 832	0, 3.25, 6.5, 13, 26, 52, 104
	Clodinafop-propargyl	40	0, 45, 90, 180, 360, 720	0, 5.625, 11.25, 22.5, 45, 90, 180
DEN	Pinoxaden	75	0, 18.75, 75, 300, 1,200, 4,800	0, 4.6875, 18.75, 75, 300, 1,200
CHD	Clethodim	72	0, 9, 18, 36, 72, 144	0, 4.5, 9, 18, 36, 72
Photosystem II inhibitors				
Urea	Isoproturon	1,200	0, 300, 600, 1,200, 2,400, 4,800, 9,600	0, 18.75, 75, 300, 1,200, 4,800
Triazine	Prometryn	750	0, 93.75, 187.5, 375, 750, 1,500	0, 93.75, 187.5, 375, 750, 1,500, 3,000

[a] Abbreviations: SU, sulfonylurea; IMI, imidazolinone; TP, triazolopyrimidine; PB, pyrimidinyl-benzoate; SCT, sulfonylaminocarbonyl-triazolinone; APP, aryloxyphenoxypropionate; DEN, phenylpyrazoline; CHD, cyclohexanedione.

[b] Mesosulfuron-methyl mesosulfuron-methyl (30 g L^{-1} oil flowable [OF], Sigma, Bayer, Hangzhou, China), rimsulfuron (25% water-dispersible granules [WDG], Jiangsu Futian Agrochemical Co., Ltd., China), nicosulfuron (40 g L^{-1} wettable powder [WP], jiangsu Changqing Agrochemical Co., Ltd., China), sulfosulfuron (75% WDG, Jiangsu Institute of Ecomones Co., Ltd., China), imazapic (240 g L^{-1} aqueous suspension, Rotam, Suzhou, China), Penoxsulam (25% OF, Clipper, Dow AgroSciences, Beijing, China), pyribenzoxim (5% emulsifiable concentrate [EC], Korea's LG Life Science Co., Ltd., Shanghai, China), flucarbazone-sodium (70% WDG, Arysta LifeScience, Shanghai, China), fenoxaprop-p-ethyl (69 g L^{-1} emulsified in water, Puma, Bayer, Hangzhou, China), Clodinafop-propargyl (15% WP, Topic, Syngenta, Shanghai, China), pinoxaden (5% EC, Axial, Syngenta), clethodim (10% EC, Jiangsu Changqing Agrochemical), isoproturon (50% WP, Alon, Jiangsu Futian Agrochemical Co., Ltd., China), and prometryn (40% WP, Jiangsu Kuaida Agrochemical Co., Ltd., China).

putative resistant populations, respectively. Treated plants were put back into the incubators and cultured as described above. Three weeks after treatment, the fresh biomass of aboveground tissue was measured. The experiment was conducted twice in a completely randomized design with four replications.

Sensitivity to Other Herbicides. The cross-resistance and multiple resistance profiles of the pyroxsulam-resistant population ACTC-1 was determined via whole-plant pot bioassay as described above. The selected herbicides are used globally on arable crops, and include herbicides with three different modes of action. The application doses were selected on the basis of the results of a preliminary experiment (data not shown) and listed in Table 2.

Gene Cloning and Sequencing. Seeds of the pyroxsulam-resistant population ACTC-1 were pretreated with pyroxsulam at 90 g ai ha^{-1}, the effective dose causing 90% reduction in fresh weight (ED_{90}) of the sensitive population JNXW-1 in the seed bioassay (data not shown). The germinated seedlings were rinsed with water before transplanting into pots and then were grown as described above. Seeds of the nontreated sensitive

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Table 3. Primers used to amplify the acetolactate synthase (ALS) and acetyl coenzyme A carboxylase (ACCase) gene fragments of Japanese foxtail.

Primer name	Primers sequence (5′ to 3′)	Amplicon size	Annealing temperature	Usage	Containing the confirmed point mutations[a]
		base pairs	C		
ALS1-F	ACCACCGCTCGGGATCATAGGC	1,822	63	ALS fragment amplification	Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, Gly654
ALS1-R	CGTCGCCTTACCCAAACCTAC				
ACCp1F	GCAAACTCGGGTGCTCGGATTGGCA	553	61	ACCase fragment amplification	Ile1781
ACCp1R	GAACATAGCTGAGCCACCTCAATATATT				
ACCp2F	TGCATACAGCGTATTGACCAG	873	61	ACCase fragment amplification	Trp1999, Trp2027, Ile2041, Asp2078, Cys2088, Gly2096
ACCp2R	CTCTGACCTGAACTTGATCTC				

[a] Amino acid sequence positions in ALS fragment referring to the full-length sequence of ALS from *Arabidopsis thaliana* (GenBank accession no. NM_114714), amino acid sequence positions in ACCase fragment referring to the full-length sequence of plastid ACCase from *Alopecurus myosuroides* (GenBank accession no. AJ310767).

population JNXW-1 were grown in pots directly. Leaves of individual plants were harvested at the three-leaf stage and genomic DNA was isolated from 100 mg of young leaf tissues of each plant using the plant genomic DNA kit (Tiangen Biotech, China) according to the instructions.

One pair of primers (Table 3) amplifying a region encompassing all eight known mutation sites contributing to ALS resistance were designed by Primer Premier 5.0 on the basis of the ALS gene sequences of blackgrass (*Alopecurus myosuroides* Huds.) (AJ437300) and shortawn foxtail (*Alopecurus aequalis* Sobrol.) (JQ743908.1) in GenBank. The other two pairs of primers (Table 3) (Xu et al. 2014a) were used to amplify the ACCase gene fragments encoding the CT domain. The polymerase chain reaction (PCR) procedure was conducted as described in Xu et al. (2013). PCR products were purified using TaKaRa MiniBEST agarose gel DNA extraction kit (TaKaRa Biotechnology, Dalian, China) and then cloned into a pMD19-T vector (TaKaRa Biotechnology). The plasmids containing the fragment insertion were bidirectionally sequenced by Invitrogen Biotechnology Co. Ltd., Shanghai, China. Ten plants of ACTC-1 and JNXW-1 respectively were selected for gene cloning. At least eight transformed clones of each plant were sequenced to obtain the ALS and ACCase gene sequences. The sequences were aligned and compared using BioEdit Sequence Alignment Editor software.

ALS Activity Assay in Vitro. Seedlings at the three- to four-leaf stage from JNXW-1 and ACTC-1 were used for in vitro assays of ALS activity as described by Yu et al. (2004) with slight modifications as follows. Four grams of leaf blades without the petiole were harvested from each population, powdered in liquid nitrogen, and suspended in 4.5 ml of enzyme extraction buffer. Each dark reaction contained 100 µl of protein extract, 200 µl of the enzyme assay buffer (100 mM potassium phosphate buffer [pH 7.0], 200 mM pyruvate, 2 mM $MgCl_2$, 0.5 mM three-phase partitioning buffer, 10 µM flavin adenine dinucleotide, and 100 µl of ALS inhibitor, which was achieved by a series of concentrations of pyroxsulam at 0.003, 0.03, 0.3, 3, 30, 300, and 3,000 µM. A nontreated (no-herbicide) control was included for comparison. Acetoin was formed by incubating the mixture with 100 µl of creatine solution (0.55%) and 100 µl of α-naphthol solution (5.5% in 5 N NaOH) at 60 C for 30 min. ALS activity was monitored colorimetrically (530 nm) on a microplate photometer

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(Thermo Fisher) by measuring acetoin production (pure acetoin was used as the standard) and was expressed as a percentage of the nonherbicide control. The protein concentration extracted from the leaf blades was measured as described by Bradford (Bradford 1976), which uses bovine serum albumin as a standard. The herbicide concentrations required to inhibit 50% of the ALS activity (IC_{50}) were calculated from a nonlinear regression analysis. The assay was performed twice with independent extractions and each with three replications per herbicide concentration.

Synergistic Effect of Pyroxsulam and Cyt P450 Inhibitors on Weed Growth. Malathion and PBO are inhibitors of Cyt P450s. Previous studies showed that malathion and PBO had no adverse effects on the seedling growth (data not shown). Seeds from JNXW-1 and ACTC-1 were pregerminated and cultured as described above for determining sensitivity to pyroxsulam. The plants at the three- to four-leaf stage were sprayed with pyroxsulam at 0, 0.1875, 0.75, 3, 12, 48, 192, and 768 g ha^{-1}, with or without malathion or PBO. Malathion at 1,000 g ai ha^{-1} or PBO at 4,200 g ai ha^{-1} was applied 30 min before the application of pyroxsulam using the herbicide sprayer (Wang et al. 2013). The aboveground tissue was harvested 3 wk after treatment, and fresh biomass determined. The experiments were conducted twice using a completely randomized design with four replications.

Data Analysis. All dose–response data from two experiments were analyzed by ANOVA (SPSS 21, SPSS Inc.). There was no significant ($P > 0.05$) trial-by-treatment interaction from each experiment, so the data were pooled and fitted to a four-parameter nonlinear logistic-regression model (Equation 1) using SigmaPlot 10.0 statistical software, as the variance between repeated experiments was not significant. The effective dose of herbicide causing 50% inhibition in fresh weight (ED_{50}) was computed by Equation 1:

$$Y = c + (d - c)/\left[1 + (x/g)^{b}\right] \qquad [1]$$

where Y is the aboveground fresh weight expressed as a percentage of the nontreated control, x is the herbicide rate, b is the slope of the curve, c is the lower limit, d is the upper limit, and g is the herbicide rate at the point of inflection halfway between the upper and lower limits (Xu et al. 2014a). The same regression analyses were also used for the enzymatic experiment to calculate the IC_{50} value. Resistance indexes RIs were calculated via dividing the ED_{50} (or IC_{50}) of the resistant population by the ED_{50} (or IC_{50}) of the sensitive population.

Results and Discussion

Sensitivity to pyroxsulam. The ED_{50} value of ACTC-1 (93 g ha^{-1}) was much greater than the recommended field dose of pyroxsulam (10 to 16 g ha^{-1}), whereas that of JNXW-1 was only 1.6 g ha^{-1}, which was much lower than the recommended dose (Table 1). The RI of ACTC-1 was 58, whereas the RIs of the other populations were always <3, indicating that these populations were still sensitive to pyroxsulam.

Sensitivity to Other Herbicides. *Cross-Resistance to ALS Inhibitors.* The pyroxsulam-resistant population was also cross-resistant to representative herbicides from five classes of ALS-inhibiting herbicides (Table 4). ACTC-1 exhibited resistance to the SU herbicides mesosulfuron-methyl, rimsulfuron, nicosulfuron, and sulfosulfuron, the SCT herbicide flucarbazone-sodium, and the TP herbicide penoxsulam, with RIs of 72, 55, 5.2, 120, 18, and 14, respectively. This population also had high levels of resistance to the IMI herbicide imazapic, with a RI of 87, and was resistant to the PB herbicide pyribenzoxim, with a RI of 4.8.

Sensitivity to Other Herbicides. The response to other herbicides (ACCase and photosystem II inhibitors) varied in the pyroxsulam-resistant population (Table 4). High-level resistance was found in ACTC-1 to the aryloxyphenoxypropionates fenoxaprop-P-ethyl and clodinafop-propargyl, with RI values of 21 and 24, respectively. Additionally, ACTC-1 was moderately resistant to the pinoxaden (phenylpyrazolins) and the urea herbicide isoproturon, with RI values of 6.9 and 5.4, respectively. For the triazine herbicide prometryn and the cyclohexanedione herbicide clethodim, the RIs of ACTC-1 were only 2.7 and 3.3, respectively.

Gene Cloning and Sequencing. The target fragments of the ALS gene in Japanese foxtail individuals that encompassed all previously identified resistance mutation sites were amplified, cloned, and sequenced. Four partial ALS gene sequences of ACTC-1 and three of JNXW-1 were isolated, in which ALS1;1, ALS1;2, and ALS1;3 were found in both JNXW-1 and ACTC-1, whereas ALS1;4 only existed in ACTC-1. The frequencies of ALS1;1 and ALS1;2 isolated from both ACTC-1

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Table 4. Sensitivity of resistant and sensitive populations to other herbicides.

Herbicides[a]	Populations	ED_{50}[b] (SE)	RI
		g ai ha^{-1}	
Mesosulfuron-methyl	JNXW-1	0.39 (0.07)	1.00
	ACTC-1	27.99 (1.88)	71.77
Rimsulfuron	JNXW-1	9.48 (0.71)	1.00
	ACTC-1	521.55 (28.11)	55.02
Nicosulfuron	JNXW-1	12.05 (0.89)	1.00
	ACTC-1	62.54 (3.15)	5.19
Sulfosulfuron	JNXW-1	6.36 (0.17)	1.00
	ACTC-1	764.34 (49.64)	120.18
Imazapic	JNXW-1	37.87 (2.43)	1.00
	ACTC-1	3,311.99 (177.89)	87.46
Penoxsulam	JNXW-1	47.84 (1.85)	1.00
	ACTC-1	674.04 (22.68)	14.09
Pyribenzoxim	JNXW-1	309.29 (11.16)	1.00
	ACTC-1	1,481.74 (75.32)	4.80
Flucarbazone-sodium	JNXW-1	218.49 (12.09)	1.00
	ACTC-1	3,990.90 (289.05)	18.27
Fenoxaprop-p-ethyl	JNXW-1	9.57 (0.43)	1.00
	ACTC-1	199.25 (13.05)	20.82
Clodinafop-propargyl	JNXW-1	21.35 (0.74)	1.00
	ACTC-1	507.43 (9.22)	23.77
Pinoxaden	JNXW-1	93.38 (7.19)	1.00
	ACTC-1	644.92 (21.54)	6.91
Clethodim	JNXW-1	14.90 (0.87)	1.00
	ACTC-1	49.54 (3.43)	3.32
Isoproturon	JNXW-1	402.63 (26.14)	1.00
	ACTC-1	2,170.41 (111.07)	5.39
Prometryn	JNXW-1	325.25 (15.25)	1.00
	ACTC-1	861.71 (32.11)	2.65
Pyroxsulam	JNXW-1	1.60 (0.18)	1.00
	ACTC-1	92.77 (3.55)	57.98
Malathion + pyroxsulam	JNXW-1	3.13 (0.76)	1.96[c]
	ACTC-1	16.94 (0.53)	10.59[c]
PBO + pyroxsulam	JNXW-1	2.43 (0.64)	1.52[c]
	ACTC-1	19.61 (0.38)	12.26[c]

[a] Malathion + pyroxsulam, the effect of malathion on pyroxsulam resistance; PBO + pyroxsulam, the effect of piperonyl butoxide (PBO) on pyroxsulam resistance.

[b] Abbreviations: ED_{50}, the effective dose of herbicide causing 50% inhibition in fresh weight; RI, resistance index: the ratio between the ED_{50} values of the resistant and sensitive populations.

[c] The four RI values were calculated by dividing the ED_{50} of pyroxsulam-plus-malathion- or PBO-treated populations by the ED_{50} of pyroxsulam-treated-only population of JNXW-1.

and JNXW-1 were higher than ALS1;3. Only five ALS1;4 fragments were detected in 80 ALS gene clones of ACTC-1. The deduced Japanese foxtail gene sequences (all 1,822 base pairs in length) were subjected to Blast search in GenBank to be verified. The high scores of the search result showed that ALS1;1 was 100% identical to ALS of shortawn foxtail, ALS1;2 was 94%, ALS1;3 was 95%, and ALS1;4 was 99% identical to ALS of shortawn foxtail, respectively. One nucleotide mutation was detected in the ALS1;2 of ACTC-1 when compared with that of JNXW-1, which resulted in an amino acid substitution of aspartate (Asp) (GAT) to glutamate (Glu) (GAG) at position 376, as numbered relative to ALS of *Arabidopsis thaliana* (Figure 1). Another three amino acid substitutions were found in ALS1;4 when compared with the other ALS fragments in ACTC-1 and JNXW-1, which were Ala (GCC) to Thr (ACC) at codon 166, Lys (AAG) to Arg (AGG) at codon 282, and Glu (GAG) to Lys (AAG) at codon 473. Whether these substitutions were responsible for resistance needs to be verified.

The CT domain of ACCase in Japanese foxtail was also amplified, cloned, and sequenced. Two ACCase genes, Acc1;1 and Acc1;2, were obtained

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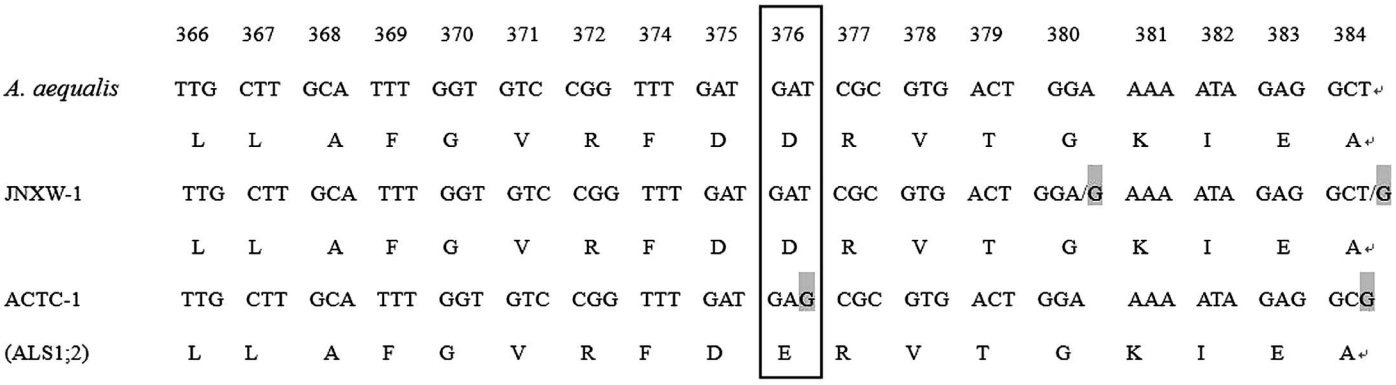

Figure 1. Partial DNA nucleotide and deduced amino acid sequences of acetolactate synthase (ALS) from Japanese foxtail. The boxed codon indicates a nonsynonymous mutation resulting in an Asp-376-Glu substitution in ALS1;2 of ALS gene in resistant population (ACTC-1).

from both the sensitive and resistant populations, which were identical to those reported by Xu et al. (2014a). By comparing the ACCase gene sequences of ACTC-1 and JNXW-1, an ATT-to-AAT mutation causing an Ile-to-Asn substitution at position 2041 was found in the Acc1;1 in ACTC-1 (Figure 2), which could explain its resistance to the selected ACCase inbihitors.

It has been reported that a Pro-197-Thr substitution in domain A and a Trp-574-Leu substitution in domain B of the ALS may be responsible for resistance to mesosulfuron-methyl in Japanese foxtail (Bi et al. 2013, 2015). In the present study, an Asp-376-Glu substitution, which occurs within a conserved region of five amino acids (RFDDR) in ALS, was detected in ACTC-1, indicating a novel mechanism of resistance different from previous reports. Three of 10 sequenced plants in ACTC-1 possessed the mutation, as ACTC-1 was a heterogenous population. The frequency of the mutation was about 0.4 in ALS1;2 across all three plants with mutation, indicating the heterozygous resistance. The Asp-376-Glu substitution has been reported to confer resistance to ALS inhibitors in smooth pigweed (Whaley et al. 2007), kochia [*Kochia scoparia* (L.) Schrad.] (Warwick et al. 2008), Powell amaranth (*Amaranthus powellii* S. Wats.) (Ashigh et al. 2009), monochoria [*Monochoria vaginalis* (Burm. F.) Kunth] (Imaizumi et al. 2008), horseweed [*Conyza canadensis* (L.) Cronq.] (Zheng et al. 2011), and wild radish (*Raphanus raphanistrum* L.) (Li et al. 2013), though it was thought to be a weak ALS resistance mutation in wild radish populations and easily missed in diagnostic studies (Yu and Powles 2014). In grass weed species, multiple copies of genes encoding ALS or ACCase were reported in several polyploid weed species (Panozzo et al. 2013; Xu et al. 2014a). It was reported that in wild oat (*Avena fatua* L.), any of the three Acc1 homoeologues (Acc1;1, Acc1;2, and Acc1;3) can host ACCase resistance mutations and confer resistance (Yu et al. 2013). So, although the Asp-376-Glu mutation was detected only in the ALS1;2 in tetraploid Japanese foxtail, the existence of multiple

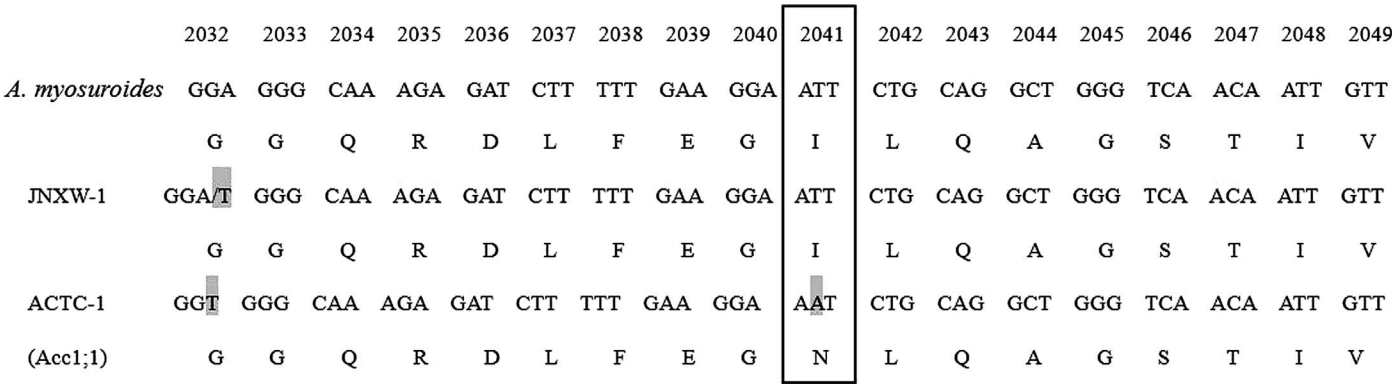

Figure 2. Partial DNA nucleotide and amino acid sequences of acetyl coenzyme A carboxylase (ACCase) from Japanese foxtail. The boxed codon indicates a nonsynonymous mutation resulting in an Ile-2041-Asn substitution in Acc1;1 of ACCase gene in resistant population (ACTC-1).

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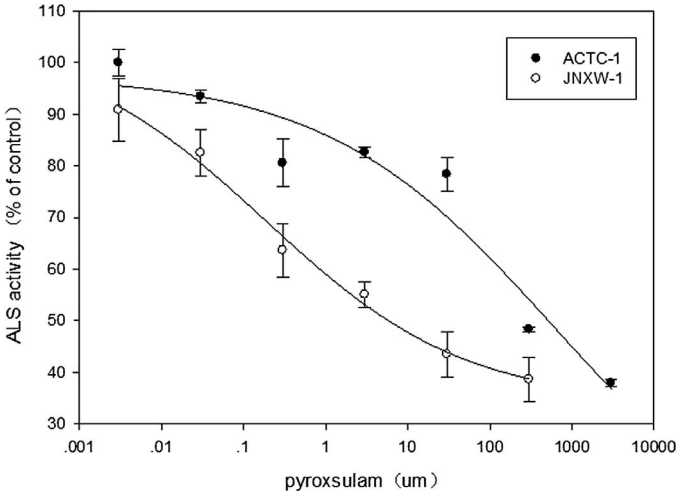

Figure 3. In vitro acetolactate synthase (ALS) activities of resistant population (ACTC-1) and sensitive population (JNXW-1) when treated by pyroxsulam. Vertical bars represent mean ± standard error.

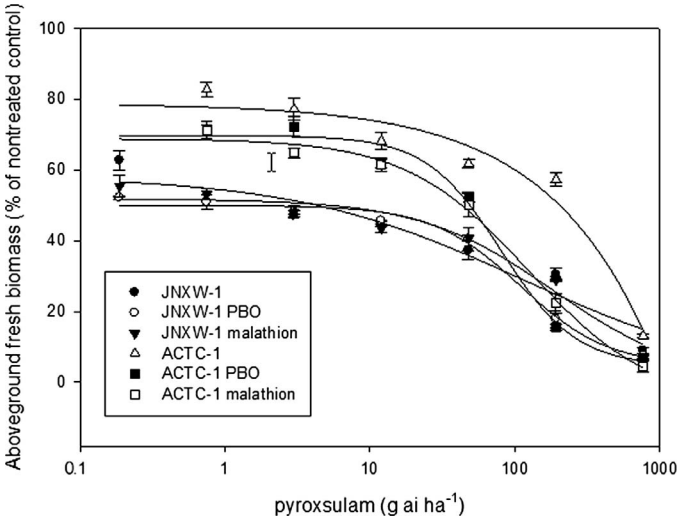

Figure 4. Dose response of resistant population (ACTC-1) and sensitive population (JNXW-1) to pyroxsulam with or without malathion (or piperonyl butoxide) pretreatment. Vertical bars represent mean ± standard error.

copies of ALS was inferred and needs to be confirmed.

Across a wide variety of weed species, different patterns of cross-resistance to ALS inhibitors within populations are documented because of the different substitutions at one of eight conserved amino acid residues (Han et al. 2012; Yu and Powles 2014), in which Glu_{376} and Trp_{574} bestow high resistance levels to all ALS inhibitors (Beckie and Tardif 2012; Whaley et al. 2007). In the present study, ACTC-1 exhibited cross-resistance to all five classes of ALS inhibitors, which might be due to the Asp-376-Glu substitution. Moreover, ACTC-1 was resistant to a novel herbicide pyribambenz-propyl (ZJ0273, used in oilseed rape fields) registered in China in 2003 (data not shown), which could inhibit the growth of plants similarly to that of the ALS inhibitors but had no inhibition on ALS in vitro (Chen et al. 2005).

ALS Activity Assay in Vitro. The ALS activities in vitro of resistant and sensitive populations were assayed and expressed as a percentage of control (Figure 3). The ALS activity in ACTC-1 was less inhibited by pyroxsulam, compared with that of JNXW-1. The IC_{50} of ACTC-1 was 600 μM, which was 52-fold greater than that for JNXW-1 (11.5 μM), which suggested that an insensitive ALS was responsible for conferring resistance to pyroxsulam in ACTC-1. Additionally, the basal ALS activity in vitro from ACTC-1 was two times higher than that from the JNXW-1. It has been reported in junglerice [*Echinochloa colona* (L.) Link] that enhanced basal 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity was considered as an additional target-site resistance mechanism, and in a lethal glyphosate treatment it could endow RL2B population that had no EPSPS mutation to maintain growth (Alarcon-Reverte et al. 2015). It was also reported that the ALS specific activity of wild radish plants with resistance alleles Tyr-122, Ser-197, or Glu-376 was 1.5-, 1.6-, or 1.9-fold greater than that of susceptible (S) subpopulations, respectively. However, in plants with resistance allele Leu-574, the ALS activity was about half of that determined for the ALS S subpopulations (Li et al. 2013). So the increased basal ALS activity of ACTC-1 might also contribute to the resistance to pyroxsulam, whereas the association between it and the Glu-376 substitution needs to be further confirmed.

Synergistic Effect of Pyroxsulam and Cyt P450 Inhibitors on Weed Growth. When malathion or PBO was applied separately at 1,000 g ai ha^{-1} or 4,200 g ha^{-1}, respectively, no significant influence was observed on the survival or biomass (data not shown) of either the ACTC-1 or JNXW-1 populations. When pyroxsulam was applied with malathion or PBO, no significant changes were found in the ED_{50} of JNXW-1, whereas the sensitivity to pyroxsulam of ACTC-1 was increased greatly, although not to the level of JNXW-1 (Figure 4). Therefore, a NTSR mediated by Cyt P450s might be involved in the pyroxsulam resistance of ACTC-1. However, the resistance was not fully overcome by Cyt P450 inhibitors

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(Table 4), which suggested that TSR played an important role in the resistance to pyroxsulam of the ACTC-1 population.

A comprehensive survey across a geographic range of blackgrass has indicated that NTSR to ACCase herbicides is more widespread than TSR (Delye et al. 2010). NTSR can endow unpredictable and complex resistance to herbicides with different modes of action or chemical structures and has been reported to have a widespread spectrum of resistance to glyphosate, ACCase, ALS, and photosystem II inhibitors (Cocker et al. 2001; Yu et al. 2009). In rice barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.], biotypes not previously exposed to ALS inhibitors exhibited resistance to ALS inhibitors; NTSR evolving through the selection pressure of ACCase inhibitors was considered to be responsible for this resistance (Iwakami et al. 2012). Therefore it could be deduced that the NTSR to pyroxulam in ACTC-1 might result from its resistance to ACCase inhibitor. In U.K. populations of perennial ryegrass (*Lolium Multiflorum* L.), resistance was noted from enhanced metabolism in response to isoproturon, which is a photosynthetic inhibitor (Cocker et al. 2001). In the present study, resistance to isoproturon in ACTC-1 may be caused by enhanced metabolism, as isoproturon had never been used in the fields where ACTC-1 was collected.

In conclusion, an Asp-376-Glu substitution in ALS of ACTC-1, which resulted in an insensitive ALS to pyroxsulam, may be a main cause for resistance to pyroxsulam and cross-resistance to all five different families of ALS inhibitors. The Ile-2041-Asn substitution in ACCase may confer resistance to ACCase inhibitors. Also, NTSR mediated by Cyt P450s might contribute to pyroxsulam resistance in ACTC-1 and be involved in ACTC-1′s cross-resistance to ALS inhibitors and multiple resistance to ACCase inhibitors and isoproturon. Japanese foxtail with multiple resistance poses a challenge to its management, notably in arable crops. Soil-applied herbicides, such as the amide herbicide flufenacet, displayed relatively good control of this resistant population (data not shown). It is noteworthy that Delye et al. (2011) pointed out that "Pre-selection" of NTSR to ALS inhibitors by ACCase inhibitors was expected to cause a faster failure of blackgrass control by ALS inhibitors than the sequential selection by ACCase and then ALS inhibitors. Therefore, the evolving mechanism of NTSR in pyroxsulam-resistant Japanese foxtail should be further explored.

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Literature Cited

Alarcon-Reverte R, Garcia A, Watson SB, Abdallah I, Sabate S, Hernandez MJ, Dayan FE, Fischer AJ (2015) Concerted action of target-site mutations and high EPSPS activity in glyphosate-resistant junglerice (*Echinochloa colona*) from California. Pest Manag Sci 71:996–1007

Ashigh J, Corbett C-AL, Smith PJ, Laplante J, Tardif FJ (2009) Characterization and diagnostic tests of resistance to acetohydroxyacid synthase inhibitors due to an Asp(376)Glu substitution in *Amaranthus powellii*. Pestic Biochem Physiol 95:38–46

Beckie HJ, Tardif FJ (2012) Herbicide cross resistance in weeds. Crop Prot 35:15–28

Bi Y, Liu W, Guo W, Li L, Yuan G, Du L, Wang J (2015) Molecular basis of multiple resistance to ACCase- and ALS-inhibiting herbicides in *Alopecurus japonicus* from China. Pestic Biochem Physiol 126:22–27

Bi Y-L, Liu W-T, Li L-X, Yuan G-H, Jin T, Wang J-X (2013) Molecular basis of resistance to mesosulfuron-methyl in Japanese foxtail, *Alopecurus japonicus*. J Pestic Sci 38:74–77

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–54

Chen J, Yuan J, Liu J, Fu Q, Wu J (2005) Mechanism of action of the novel herbicide ZJ0273. Acta Phytophy Sin 32:48–52

Christopher JT, Preston C, Powles SB (1994) Malathion antagonizes metabolism-based chlorsulfuron resistance in *Lolium rigidum*. Pestic Biochem Physiol 49:172–182

Cocker KM, Northcroft DS, Coleman JOD, Moss SR (2001) Resistance to ACCase-inhibiting herbicides and isoproturon in UK populations of *Lolium multiflorum*: mechanisms of resistance and implications for control. Pest Manag Sci 57:587–597

Delye C (2013) Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. Pest Manag Sci 69:176–187

Delye C, Gardin JAC, Boucansaud K, Chauvel B, Petit C (2011) Non-target-site-based resistance should be the centre of attention for herbicide resistance research: *Alopecurus myosuroides* as an illustration. Weed Res 51:433–437

Delye C, Michel S, Berard A, Chauvel B, Brunel D, Guillemin JP, Dessaint F, Le Corre V (2010) Geographical variation in resistance to acetyl-coenzyme A carboxylase-inhibiting herbicides across the range of the arable weed *Alopecurus myosuroides* (black-grass). New Phytol 186:1005–1017

Gressel J (1990) Synergizing herbicides. Rev Weed Sci 5:49–82

Han H, Yu Q, Purba E, Li M, Walsh M, Friesen S, Powles SB (2012) A novel amino acid substitution Ala-122-Tyr in ALS confers high-level and broad resistance across ALS-inhibiting herbicides. Pest Manag Sci 68:1164–1170
Imaizumi T, Wang GX, Ohsako T, Tominaga T (2008) Genetic diversity of sulfonylurea-resistant and -susceptible *Monochoria vaginalis* populations in Japan. Weed Res 48:187–196
Iwakami S, Uchino A, Watanabe H, Yamasue Y, Inamura T (2012) Isolation and expression of genes for acetolactate synthase and acetyl-CoA carboxylase in *Echinochloa phyllopogon*, a polyploid weed species. Pest Manag Sci 68:1098–1106
Kwon CS, Penner D (1995) Response of a chlorsulfuron-resistant biotype of *Kochia scoparia* to ALS inhibiting herbicides and piperonyl butoxide. Weed Sci 43:561–565
Li M, Yu Q, Han H, Vila-Aiub M, Powles SB (2013) ALS herbicide resistance mutations in *Raphanus raphanistrum*: evaluation of pleiotropic effects on vegetative growth and ALS activity. Pest Manag Sci 69:689–695
Massa D, Krenz B, Gerhards R (2011) Target-site resistance to ALS-inhibiting herbicides in *Apera spica-venti* populations is conferred by documented and previously unknown mutations. Weed Res 51:294–303
Panozzo S, Scarabel L, Tranel PJ, Sattin M (2013) Target-site resistance to ALS inhibitors in the polyploid species *Echinochloa crus-galli*. Pestic Biochem Physiol 105:93–101
Wang H, Li J, Lv B, Lou Y, Dong L (2013) The role of cytochrome P450 monooxygenase in the different responses to fenoxaprop-P-ethyl in annual bluegrass (*Poa annua* L.) and short awned foxtail (*Alopecurus aequalis* Sobol.). Pestic Biochem Physiol 107:334–342
Warwick SI, Xu R, Sauder C, Beckie HJ (2008) Acetolactate synthase target-site mutations and single nucleotide polymorphism genotyping in ALS-resistant kochia (*Kochia scoparia*). Weed Sci 56:797–806
Wells GS (2008) Pyroxsulam for broad-spectrum weed control in wheat. Pages 297–299 *in* Proceedings of the 16th Australian Weeds Conference. Queensland: Australian Weeds Conference
Whaley CM, Wilson HP, Westwood JH (2007) A new mutation in plant ALS confers resistance to five classes of ALS-inhibiting herbicides. Weed Sci 55:83–90
Xu H, Li J, Zhang D, Cheng Y, Jiang Y, Dong L (2014a) Mutations at codon position 1999 of acetyl-CoA carboxylase confer resistance to ACCase-inhibiting herbicides in Japanese foxtail (*Alopecurus japonicus*). Pest Manag Sci 70:1894–1901
Xu H, Zhang W, Zhang T, Li J, Wu X, Dong L (2014b) Determination of ploidy level and isolation of genes encoding acetyl-CoA carboxylase in Japanese foxtail (*Alopecurus japonicus*). Plos one 9:e114712
Xu H, Zhu X, Wang H, Li J, Dong L (2013) Mechanism of resistance to fenoxaprop in Japanese foxtail (*Alopecurus japonicus*) from China. Pestic Biochem Physiol 107:25–31
Yu Q, Abdallah I, Han H, Owen M, Powles S (2009) Distinct non-target site mechanisms endow resistance to glyphosate, ACCase and ALS-inhibiting herbicides in multiple herbicide-resistant *Lolium rigidum*. Planta 230:713–723
Yu Q, Ahmad-Hamdani MS, Han H, Christoffers MJ, Powles SB (2013) Herbicide resistance-endowing ACCase gene mutations in hexaploid wild oat (*Avena fatua*): insights into resistance evolution in a hexaploid species. Heredity 110:220–231
Yu Q, Han H, Vila-Aiub MM, Powles SB (2010) AHAS herbicide resistance endowing mutations: effect on AHAS functionality and plant growth. J Exp Bot 61:3925–3934
Yu Q, Powles SB (2014) Resistance to AHAS inhibitor herbicides: current understanding. Pest Manag Sci 70:1340–1350
Yu Q, Shane Friesen LJ, Zhang XQ, Powles SB (2004) Tolerance to acetolactate synthase and acetyl-coenzyme A carboxylase inhibiting herbicides in *Vulpia bromoides* is conferred by two co-existing resistance mechanisms. Pestic Biochem Physiol 78:21–30
Yuan JS, Tranel PJ, Stewart CN Jr (2007) Non-target-site herbicide resistance: a family business. Trends Plant Sci 12:6–13
Zheng D, Kruger GR, Singh S, Davis VM, Tranel PJ, Weller SC, Johnson WG (2011) Cross-resistance of horseweed (*Conyza canadensis*) populations with three different ALS mutations. Pest Manag Sci 67:1486–14 92

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