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Target-site and metabolic mechanisms of tolerance to penoxsulam in pond lovegrass (*Eragrostis japonica*)

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Research Article

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Abstract

The identification of herbicide tolerance is essential for effective chemical weed control. According to whole-plant dose–response assays, none of 29 pond lovegrass [*Eragrostis japonica* (Thunb.) Trin.] populations were sensitive to penoxsulam. The effective dose values of penoxsulam causing 50% inhibition of fresh weight (GR_{50}: 105.14 to 148.78 g ai ha^{-1}) in *E. japonica* populations were much higher than the label rate of penoxsulam (15 to 30 g ai ha^{-1}) in the field. This confirmed that *E. japonica* was tolerant to penoxsulam. *Eragrostis japonica* populations showed 52.83- to 74.76-fold higher tolerance to penoxsulam than susceptible barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.]. The mechanisms of tolerance to penoxsulam in *E. japonica* were also identified. In vitro activity assays revealed that the penoxsulam concentration required to inhibit 50% of the acetolactate synthase (ALS) activity (IC_{50}) was 12.27-fold higher in *E. japonica* than in *E. crus-galli*. However, differences in the *ALS* gene, previously found to endow target-site resistance in weeds, were not detected in the sequences obtained. Additionally, the expression level of genes encoding *ALS* in *E. japonica* was approximately 2-fold higher than in *E. crus-galli* after penoxsulam treatment. Furthermore, penoxsulam tolerance can be significantly reversed by three cytochrome P450 monooxygenase (CytP450) inhibitors (1-aminobenzotriazole, piperonyl butoxide, and malathion), and the activity of NADPH-dependent cytochrome P450 reductase toward penoxsulam in *E. japonica* increased significantly (approximately 7-fold higher) compared with that of treated *E. crus-galli*. Taken together, these results indicate that lower ALS sensitivity, relatively higher *ALS* expression levels, and stronger metabolism of CytP450s combined to bring about penoxsulam tolerance in *E. japonica*.

Introduction

With the rapid development of the direct-planting mode of rice, pond lovegrass [*Eragrostis japonica* (Thunb.) Trin.] has gradually become one of the most harmful weeds in addition to barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] and Chinese sprangletop [*Leptochloa chinensis* (L.) Nees] in rice fields in recent years, resulting in a significant loss of crop yield (Xu et al. 2020). *Eragrostis japonica* is distributed in Henan, Anhui, Jiangsu, Zhejiang, Jiangxi, Hubei, Fujian, Guangdong, Guangxi, Hainan, Taiwan, Sichuan, Guizhou, and Yunnan provinces in China, as well as in Africa, North America, and South America (Flora of China Editorial Committee 2016; USDA-NRCS 2016). Yet little research has been conducted on *E. japonica*, especially in terms of herbicide control.

Since acetolactate synthase (ALS)-inhibiting herbicides were popularized and used, they have been the leading agents for the control of annual weeds in rice fields. Penoxsulam, the ALS-inhibiting herbicide most commonly used in rice fields, has a wide herbicidal spectrum and good control effect on various weeds (Jabusch and Tjeerdema 2005). However, in some areas, penoxsulam has a poor control effect on *E. japonica* in rice fields (Xu et al. 2020). It has not been clearly reported or determined whether *E. japonica* has developed resistance to penoxsulam or whether it is naturally resistant (or tolerant) to penoxsulam. Herbicide resistance refers to the heritable ability of a plant biotype to survive and reproduce under the wild-type lethal-dose treatment due to the selective pressure of long-term and widespread use of herbicides or artificial genetic manipulation, also known as acquired resistance (Qiang et al. 2008). Herbicide tolerance refers to the heritable ability of a plant to naturally tolerate herbicide treatment and the ability to survive and reproduce after herbicide treatment without selection or genetic manipulation, also known as natural resistance (Price et al. 1983; Qiang et al. 2008). Herbicide tolerance usually

refers to the differences in herbicide susceptibility between species, while herbicide resistance usually refers to the development of a decreased response to a herbicide in a population within a species (Pantone et al. 1988). Notably, when weeds are tolerant to a herbicide, no weed biotypes are sensitive to the herbicide (Wang et al. 2013). It has thus become an important task for researchers to screen and monitor weeds for herbicide tolerance (or natural resistance) over time and to study the mechanisms of herbicide tolerance to guide growers to use herbicides rationally and control weeds scientifically.

The herbicide tolerance of weeds, similar to their herbicide resistance, is mainly caused by differences in herbicide target enzymes, the enhancement of metabolic capacity, and the isolation and shielding of herbicides (Wang et al. 2013, 2014). Previous studies have suggested that two main mechanisms are involved in resistance to ALS inhibitors: target-site resistance (TSR) and non–target site resistance (NTSR) (Yu and Powles 2014). TSR is conferred by (1) the increasing intrinsic activity of the herbicide target protein that compensates for the herbicide inhibitory action, (2) the change in herbicide target protein genes in the nucleotide sequence, or (3) an increase in gene expression (Massa et al. 2011; Yu et al. 2010). To date, researchers have identified 29 amino acid substitutions at nine conserved positions in the *ALS* gene (Fang et al. 2022). These positions are Ala-122, Pro-197, Ala-205, Phe-206, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654 (numbered on the basis of the corresponding sequence of *Arabidopsis thaliana*) (Fang et al. 2022; Yu and Powles 2014). In contrast, NTSR is less understood due to its complexity and unpredictability (Yu and Powles 2014). Reduced penetration, impaired translocation, and enhanced metabolism that reduce the dose of herbicide binding to the target protein are three mechanisms for NTSR, with metabolic resistance being the most important (Délye 2013). Enzymes related to metabolic resistance to herbicides have been identified, including cytochrome P450 monooxygenases (CytP450s), glutathione *S*-transferases (GSTs), glycosyltransferases, ATP-binding cassette transporters, oxidases, esterases, hydrolases, and peroxidases (Délye 2013). A variety of enzymes are involved in NTSR, of which CytP450s and GSTs play important roles in the metabolic detoxification of herbicides (Cai et al. 2022; Ma et al. 2016; Yuan et al. 2007). Piperonyl butoxide (PBO), malathion, 4-chloro-7-nitro-2,1,3-benzoxadiazole, and other metabolic enzyme inhibitors (Fang et al. 2019b; Feng et al. 2016; Ma et al. 2016; Wang et al. 2013; Zhang et al. 2017), all of which inhibit the activity of metabolic enzymes toward herbicides, thereby overcoming resistance, have been used to detect resistance resulting from herbicide metabolism. Currently, NTSR is considered the predominant mechanism for resistance to acetyl CoA carboxylase (ACCase) and ALS inhibitors in many monocots (Délye et al. 2011).

Both *E. japonica* and *E. crus-galli* are annual malignant Gramineae weeds in rice fields and are among the control objects registered on the label for penoxsulam (Fang et al. 2019b; Xu et al. 2020). Penoxsulam could effectively control *E. crus-galli* in practical application, but why was it unable to effectively control *E. japonica*? To answer this question, this study aimed to (1) identify whether *E. japonica* is tolerant to penoxsulam, (2) explore the target-site basis of this penoxsulam tolerance, and (3) confirm the role of metabolism in *E. japonica* in response to penoxsulam.

It should be noted that this was a comparative study of the tolerance mechanisms of *E. japonica* and *E. crus-galli.* Because little research has been done on herbicide tolerance, the general practice is that if there is no sensitive population in the same weed species that is tolerant to an herbicide, another weed species that is sensitive to the same herbicide and has a high herbicide target protein gene homology can be selected as a control (Wang et al. 2013, 2014; Yu et al. 2004).

Materials and Methods

Plant Materials and Herbicide

The weed species that were used are listed in Table 1. Partial *E. japonica* populations were collected from major rice-producing areas in China with regional representation, where the application of penoxsulam (Clipper 25 OD) at the recommended dose (15 to 30 g ai ha^{-1}) has failed to control this weed since 2015. The rest of *E. japonica* populations were collected from fallow fields where penoxsulam had never been applied. According to the pretest (data not shown), none of the *E. japonica* populations were sensitive to penoxsulam. Therefore, a sensitive *E. crus-galli* population JLGY-2019-S, whose herbicide target protein gene homology was as high as 91% compared with *E. japonica*, was selected as a control in all biological and molecular studies (Wang et al. 2013, 2014; Yu et al. 2004). All seeds were collected by hand, air-dried in the shade, and stored in paper bags at 4 C until use. Penoxsulam oil dispersion (Dow AgroSciences, Nantong, Jiangsu, China, 226000) of 25 g L^{-1} was used.

Whole-Plant Dose–Response Experiment with Penoxsulam

Greenhouse experiments were conducted to evaluate the penoxsulam sensitivity of *E. japonica* populations and an *E. crus-galli* population. Twenty seeds from each population were sown in plastic pots (9-cm diameter by 10-cm height) filled with a 2:1 (w/w) mixture of sand and pH 5.6 organic matter and grown in incubators at 30 /25 C (light/dark temperature) with a 12-h light/12-h dark cycle, a light intensity of 8,000 lux, and 85% relative humidity. The seedlings were thinned to 15 plants per pot before herbicide treatment. At the 3- to 4-leaf stage, penoxsulam was applied using a laboratory sprayer (machine model: 3WP-2000, Nanjing Research Institute for Agricultural Mechanization, National Ministry of Agriculture of China, Nanjing, China) equipped with a flat-fan nozzle delivering 280 L ha^{-1} at 230 kPa. Based on a preliminary experiment (data not shown), penoxsulam was applied at 0, 15, 30, 60, 120, and 240 g ha^{-1} to *E. japonica* and at 0, 0.94, 1.88, 3.75, 7.5, and 15 g ha^{-1} to *E. crus-galli*. The treated plants were returned to the incubators and cultured as described earlier. The fresh aboveground biomass was determined after 3 wk and expressed as a percentage of the untreated control (Feng et al. 2016; Gao et al. 2017). This experiment was conducted twice using a completely randomized design with four replicates.

ALS Activity Assay In Vitro

Plant materials for subsequent molecular experiments from a sensitive *E. crus-galli* population (JLGY-2019-S) and the most tolerant population of *E. japonica* (JHHY-2019-2) were prepared based on the results of the dose–response bioassay with penoxsulam (for convenience, the population name will be used hereafter). The response of ALS to penoxsulam was determined using crude enzyme extracts. The soil type and growth conditions were the same as those described earlier. Seedlings at the 3- to 4-leaf stage from JHHY-2019-2 and JLGY-2019-S were used for in vitro assays of ALS activity as described by Yu et al. (2004), with slight modifications as follow: 4 g of leaf blades without the petiole were

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Table 1. Sensitivity of 29 *Eragrostis japonica* populations and one *Echinochloa crus-galli* population to penoxsulam.

Weed	Population	Collection site	GR_{50} (SE)[a]	RI[b]
E. japonica	AHFD-2015-1	Feidong County, Hefei City, Anhui Province	116.17 ± 6.96	58.38
	JHJH-2017-1	Jinhu County, Huaian City, Jiangsu Province	125.58 ± 5.91	63.11
	AWWW-2017-1	Wuwei County, Wuhu City, Anhui Province	115.86 ± 4.43	58.22
	AWWW-2017-2	Wuwei County, Wuhu City, Anhui Province	120.82 ± 5.15	60.71
	AWWW-2017-3	Wuwei County, Wuhu City, Anhui Province	110.44 ± 8.83	55.50
	AWWW-2017-4	Wuwei County, Wuhu City, Anhui Province	112.26 ± 12.22	56.41
	AWWW-2017-5	Wuwei County, Wuhu City, Anhui Province	118.09 ± 6.86	59.34
	AWWW-2017-6	Wuwei County, Wuhu City, Anhui Province	140.15 ± 3.59	70.43
	AWWW-2017-7	Wuwei County, Wuhu City, Anhui Province	124.65 ± 3.78	62.64
	AWNL-2019-1	Nanling County, Wuhu City, Anhui Province	146.34 ± 3.60	73.54
	JHHY-2019-1	Huaiyin District, Huaian City, Jiangsu Province	116.32 ± 8.19	58.45
	JHHY-2019-2	Huaiyin District, Huaian City, Jiangsu Province	148.78 ± 4.49	74.76
	JHHY-2019-3	Huaiyin District, Huaian City, Jiangsu Province	141.45 ± 7.37	71.08
	JNLS-2019-1[c]	Lishui District, Nanjing City, Jiangsu Province	105.36 ± 6.90	52.94
	JNQH-2019-1[c]	Qinhuai District, Nanjing City, Jiangsu Province	105.14 ± 4.63	52.83
	JNLH-2019-1	Liuhe District, Nanjing City, Jiangsu Province	141.92 ± 6.51	71.32
	HZSP-2019-1	Suiping County, Zhumadian City, Henan Province	117.22 ± 4.13	58.90
	HXSH-2019-1	Shihe District, Xinyang City, Henan Province	109.34 ± 7.50	54.94
	HXSH-2019-2	Shihe District, Xinyang City, Henan Province	146.31 ± 4.59	73.52
	HHTE-2019-1	Tianerhe Town, Hanzhou City, Hubei Province	126.77 ± 12.00	63.70
	HHTE-2019-2	Tianerhe Town, Hanzhou City, Hubei Province	119.79 ± 11.70	60.20
	AWGJ-2019-1	Gejiang District, Wuhu City, Anhui Province	109.97 ± 12.78	55.26
	AWGJ-2019-2	Gejiang District, Wuhu City, Anhui Province	112.54 ± 12.36	56.55
	AWGJ-2019-3	Gejiang District, Wuhu City, Anhui Province	142.16 ± 5.05	71.44
	AWGJ-2019-4	Gejiang District, Wuhu City, Anhui Province	123.42 ± 10.79	62.02
	SHQP-2019-1	Qingpu District, Shanghai	116.62 ± 12.17	58.60
	SHQP-2019-2	Qingpu District, Shanghai	129.52 ± 7.50	65.09
	SHQP-2019-3	Qingpu District, Shanghai	117.99 ± 4.82	59.29
	SHQP-2019-4	Qingpu District, Shanghai	143.97 ± 4.11	72.35
E. crus-galli	JLGY-2019-S	Ganyu District, Lianyungang City, Jiangsu Province	1.99 ± 0.11	1.00

[a]GR_{50} refers to the effective dose of herbicide causing 50% inhibition of fresh weight and is indicated as grams of active ingredient per hectare (g ai ha^{-1}). Data are the means of two experiments.
[b]RI is the relative tolerance index: ratio of GR_{50} values relative to the susceptible *E. crus-galli* population (JLGY-2019-S). The recommended field dose of penoxsulam is 15–30 g ai ha^{-1}.
[c]Populations with no prior herbicide exposure.

harvested from each population, powdered in liquid nitrogen, and suspended in 4.5 ml of enzyme extraction buffer (10 mM sodium pyruvate, 1 mM $MgCl_2$, 1 mM thiamine pyrophosphate, and 10 µM flavin adenine dinucleotide [FAD] in 100 mM potassium phosphate buffer [pH 7.5]) to prepare crude enzyme extracts. The protein concentration extracted from the leaf blades was measured according to Bradford (1976) using bovine serum albumin (BSA) as a standard. Using enzyme assay buffer (100 mM potassium phosphate buffer [pH 7.5], 200 mM sodium pyruvate, 20 mM $MgCl_2$, 2 mM thiamine pyrophosphate, 20 µM FAD, and 1 mM dithiothreitol [DTT]), the concentration of the protein extracts was normalized to 0.30 mg ml^{-1}. Each dark reaction contained 100 µl of protein extract and 100 µl of ALS inhibitor, which was achieved using a series of concentrations of penoxsulam at 0.001, 0.01, 0.1, 1, 10, 100, 1,000, and 10,000 µM. A no-herbicide treatment was included for comparison (replacing penoxsulam with potassium phosphate buffer). Acetolactate was formed by incubating the mixtures at 37 C for 60 min. The reaction was stopped by the addition of 8 µl of 6 N H_2SO_4. The mixture was held at 60 C for 30 min to convert acetolactate to acetoin until the addition of 100 µl of 0.55% (w/w) creatine solution and 100 µl of 5.5% (v/v) α-naphthol in 5 N NaOH. Acetoin solution of a certain concentration was made with pure acetoin and H_2O, then an equal gradient dilution was performed, and in order to exclude the influence of creatine and α-naphthol, 100 µl of 0.55% (w/w) creatine solution and 100 µl of 5.5% (v/v) α-naphthol in 5 N NaOH were also added. Under the same total volume conditions as the above reaction solution, an acetoin standard curve was made with the acetoin concentration as the abscissa and the optical density at 530 nm (OD530) value as the ordinate. ALS activity was monitored colorimetrically (530 nm) on a microplate photometer (Thermo Fisher, Shanghai, China, 200000) by measuring acetoin production. According to the concentration of acetoin generated (convert OD530 to acetoin concentration based on the acetoin standard curve), ALS activity at a series of concentrations of penoxsulam was expressed as a percentage of the no-herbicide control treatment. The assay was performed twice using independent enzyme extractions, with three replicates per herbicide concentration.

ALS Gene Cloning and Sequencing

Young shoot tissues obtained from individual plants at the 3- to 4-leaf stage were used for DNA extraction using a Plant Genomic DNA kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Primers (Table 2) were designed using Primer Premier v. 5.0 to amplify DNA fragments encompassing all previously identified resistance mutation sites in the *ALS* gene. *ALS* gene fragments of the susceptible population JLGY-2019-S were amplified on the basis of previous study (Fang et al. 2019a). As information regarding the *ALS* gene of *E. japonica* was not available, three pairs of primers intended to amplify regions of the *ALS* gene in JHHY-2019-2 were designed based on the nucleotide sequences of ALS enzymes from the following species (respective GenBank nucleotide accession numbers included in parentheses): blackgrass (*Alopecurus myosuroides* Huds.) (AJ437300.2), *E. crus-galli* (MH013497), *E. crus-galli* var. *crus-galli* (LC006061.1), *E. crus-galli* var. *formosensis* (LC006063.1), rice barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.] (AB636580.1), cheatgrass (*Bromus tectorum* L.) (AF488771), and Italian ryegrass [*Lolium perenne* L. ssp.

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Table 2. Primers used to amplify the acetolactate synthase (*ALS*) gene fragments of *Echinochloa crus-galli* and *Eragrostis japonica*.

Primer name	Primer sequence (5′→3′)	Product size	Annealing temperature	Usage	Containing the confirmed point mutations[a]
		bp	C		
ALS0-F	TTGCCACCCTCCCCAAACCC	1,932	62	*E. crus-galli*	Ala-122, Pro-197, Ala-205, Phe-206, Asp-376, Arg-377, Trp-574, Ser-653, Gly-654
ALS0-R	GCACCACTCGCTGAAATCCG				
ALS1-F	GTGTTAGCCTACTCC	576	51	*E. japonica*	Ala-122, Pro-197, Ala-205, Phe-206
ALS1-R	ACCAACATAAAGAACAGG				
ALS2-F	GGCGTTCTTCCTCGCCTC	724	58	*E. japonica*	Asp-376, Arg-377
ALS2-R	TCCTTTAGTCAGCTCATC				
ALS3-F	TGATCCACTGTCTCTGCG	982	58	*E. japonica*	Trp-574, Ser-653, Gly-654
ALS3-R	CCTGCCATCACCTTCCAT				

[a]Amino acid sequence positions in the *ALS* fragment refer to the full-length sequence of *ALS* from *Arabidopsis thaliana* (GenBank accession no. NM_114714).

multiflorum (Lam.) Husnot] (AF310684) (National Center for Biotechnology Information, Bethesda, MD, http://www.ncbi.nlm.nih.gov).

A polymerase chain reaction (PCR) was performed as described by Xu et al. (2013). The PCR products were purified using a TaKaRa MiniBEST agarose gel DNA extraction kit (TaKaRa Biotechnology, Dalian, China) and then cloned into a pMD19-T vector (TaKaRa Biotechnology). Plasmids containing the fragment insertion were bidirectionally sequenced using GenScript Biotechnology (Nanjing, China). Ten plants each of JHHY-2019-2 and JLGY-2019-S were selected for gene cloning. At least 12 transformed clones from each plant were sequenced to obtain *ALS* gene sequences. The sequences were aligned and compared using the BioEdit Sequence Alignment Editor v. 7.2.5 (Tom Hall, Carlsbad, CA, USA). The Basic Local Alignment Search Tool (BLAST) procedure within the NCBI database was used to verify the accuracy of the obtained sequences.

Determination of ALS Expression by RT-qPCR

Real-time quantitative PCR (RT-qPCR) was used to measure the expression level of *ALS* gene relative to the housekeeping gene *β-actin* in JHHY-2019-2 and JLGY-2019-S. Plants were cultivated and treated with 7.5 g ha^{-1} penoxsulam, as previously described. Leaf tissue (0.1 g per population per time point) was harvested at 0, 1, 3, 5, and 7 d after treatment and flash-frozen in liquid nitrogen. RNA was extracted using an RNA Simple Total RNA Kit (Tiangen Biotech) according to the manufacturer's instructions. After RNA extraction, cDNAs were synthesized using HiScript II Q RT SuperMix for qPCR (+ gDNA wiper; Vazyme Biotech, Nanjing, China). Based on the *ALS* nucleotide sequences of JHHY-2019-2 and JLGY-2019-S obtained earlier, RT-qPCR primers (Table 3) were designed at the highly conserved region. The *β-actin* gene was selected as the endogenous housekeeping gene for RT-qPCR (Fang et al. 2022; Li et al. 2013), using its primers (Table 3). All primers were assessed for specific PCR amplification, and no PCR products were observed in the negative controls. RT-qPCR was performed according to a previously reported program (Fang et al. 2019b). Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). To exclude the influence of inherent differences between JHHY-2019-2 and JLGY-2019-S, *ALS* expression was displayed based on the Ct values relative to each population at 0 d (without penoxsulam treatment) (Fang et al. 2020, 2022). Three biological replicates were used, and the experiments were repeated at least twice.

Table 3. Information for primers used in the real-time quantitative PCR (RT-qPCR) study.

Primer name	Sequence (5′→3′)	Product size bp
β-actin-F	CACACTGGTGTCATGGTAGG	136
β-actin-R	AGAAAGTGTGATGCCAGAT	
QALS-F	AAGCAGGGCTAAAATTGTG	164
QALS-R	GCCATGTGCCAAAGTCAAAA	

Synergistic Effect of Penoxsulam and Metabolic Inhibitors on Weed Growth

Seeds from JHHY-2019-2 and JLGY-2019-S were cultured as described earlier. At the 3- to 4-leaf stage, weeds were treated with three CytP450 inhibitors (1-aminobenzotriazole [ABT], PBO, and malathion), one GST inhibitor (4-chloro-7-nitro-2,1,3-benzoxadiazole [NBD-Cl]), penoxsulam, ABT with penoxsulam, PBO with penoxsulam, malathion with penoxsulam, and NBD-Cl with penoxsulam. A pretest showed that these metabolic inhibitors had no adverse effects on seedling growth (data not shown). The applied doses and methods for ABT (1,000 g ai ha^{-1}) (Zhang et al. 2017), PBO (4,200 g ai ha^{-1}) (Wang et al. 2013), malathion (1,000 g ai ha^{-1}) (Wang et al. 2013), and NBD-Cl (270 g ai ha^{-1}) (Ma et al. 2016) have been previously reported. Malathion, ABT, and PBO were first applied 1 h before herbicide application, and NBD-Cl was first applied 48 h before herbicide application. Subsequently, penoxsulam was administered to JHHY-2019-2 and JLGY-2019-S at the doses described in the whole-plant dose–response experiment with penoxsulam. Fresh aboveground biomass was determined after 3 wk and expressed as a percentage of the no-herbicide treatment. The experiments were conducted twice using a completely randomized design with four replicates.

Determination of Metabolic Enzymes Activity In Vivo

Based on the results of the dose–response bioassay with metabolic inhibitors, the activities of NADPH-dependent cytochrome P450 reductase toward penoxsulam in JHHY-2019-2 and JLGY-2019-S were determined in vivo according to previous studies (Wang et al. 2013; Zimmerlin and Durst 1990). The seeds were planted in a plastic basin (5-cm radius, 10-cm height) filled with quartz sand, and the basin was wrapped with foil paper to ensure complete darkness, placed in a no-light incubator with a relative humidity of 85% at 25/30 C for 12 h/12 h, watered (no light exposure), and cultured for approximately 10 d. At the 2-leaf stage, a laboratory sprayer equipped with a flat-fan nozzle (machine model:

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3WP-2000, Nanjing Research Institute for Agricultural Mechanization), delivering 280 L ha^{-1} at 230 kPa and 291 mm s^{-1} with a 30-ml carrier volume, was used to spray the stems and leaves; the dose of penoxsulam sprayed was one-fourth the recommended label rate in the field: 7.5 g ha^{-1}. At 1 d after spraying, the aboveground parts of weeds were cut, 2 g was taken for each treatment, and untreated weeds were used as controls. After harvest, the aboveground tissue was soaked in 10 mM $Na_2S_2O_4$, dried with absorbent paper, placed in liquid nitrogen, and stored at −80 C until use. The activity of CytP450 declines as the age of seedling tissues increases (Frear et al. 1991). Consequently, for comparative purposes, all excised shoot tissues needed to be of the same or similar physiological age.

Aboveground tissue was then ground with liquid nitrogen; 0.10 g polyvinylpolypyrrolidone (PVPP) was added before grinding. The finely ground powder was transferred to a new precooled mortar, and 6.0 ml enzyme extraction buffer was added (0.10 M pH 7.5 phosphoric acid buffer, containing 10% glycerol [v/v], 1 g L^{-1} BSA, 5 mM DTT, 1 mM Li_2CO_3). After being ground evenly, it was kept at 4 C for 5 min and then filtered through gauze. Then, the liquid was transferred into a 10-ml centrifuge tube. After centrifugation at 10,000 × *g* for 20 min at 4 C, the supernatant was taken and added to a new centrifuge tube. After centrifugation at 100,000 × *g* for 80 min at 4 C, the supernatant was discarded and the precipitate was retained. The precipitate was resuspended in 1ml buffer (0.1 M pH 8.0 phosphoric acid buffer, containing 10% glycerol and 1.5 mM β-mercaptoethanol) and then stored in a refrigerator at −70 C for later use. All the above operations were carried out under temperatures of 0 to 4 C (Mcfadden et al. 1989; Yun et al. 2005).

The activity of NADPH-dependent cytochrome P450 reductase was determined by a modification of the method used by Feng et al. (1992) and Crankshaw et al. (1979). The reaction mix contained 50 µl NADPH (1.5 mg ml^{-1}), 1.5 ml of 0.1 M sodium phosphate buffer (pH 7.8, containing 50 µl cytochrome C [5 mg ml^{-1}] and 50 µl microsomal crude enzyme solution) and was mixed immediately to start the reaction. The reaction without enzyme solution was used as a control. The change value of OD550 at 550 nm was recorded for 180 s at room temperature. The amount of cytochrome C reduced was calculated based on OD550; the millimolar extinction coefficient of 21.1 mmol L^{-1} cm^{-1} and the protein content of the enzyme solution were calculated according to Feng et al. (1992). It can be used as a standard to measure the activity of NADPH-dependent cytochrome P450 reductase. The experiments were conducted twice with three replicates per treatment.

Data Analysis

After preliminary analysis, all data were subjected to a *t*-test analysis using SPSS v. 21.0 (SPSS, Chicago, IL, USA). The results showed no significant differences between assay repetitions (*t*-test, $P > 0.05$). The data from the whole-plant dose–response experiment with penoxsulam, ALS activity assay in vitro, and synergistic effect of penoxsulam and metabolic inhibitors on weed growth after preliminary analysis (percentage of the no-herbicide treatment) were then pooled and fitted to the four-parameter nonlinear log-logistic regression model (Equation 1) using SigmaPlot v. 14.0 (Systat Software, Chicago, IL, USA) (Cai et al. 2022; Fang et al. 2019b; Liu et al. 2019; Seefeldt et al. 1995):

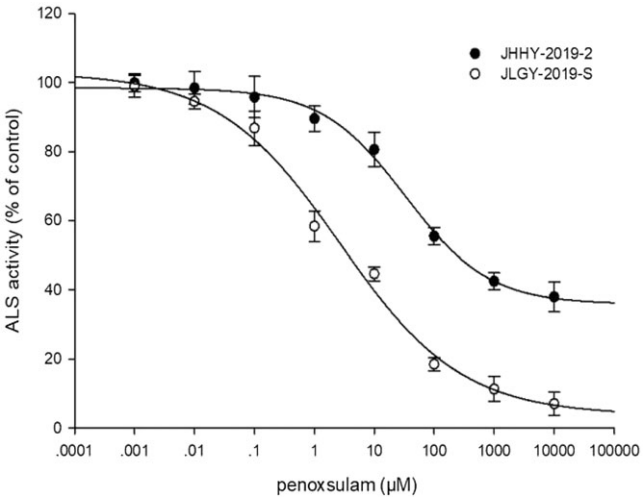

Figure 1. In vitro acetolactate synthase (ALS) activity of *Eragrostis japonica* (JHHY-2019-2) and *Echinochloa crus-galli* (JLGY-2019-S) populations when treated with penoxsulam. ALS activity of the no-herbicide control was set as 100%. Vertical bars represent the mean ± SE.

$$y = c + (d - c)/\{1 + \exp[b(\log x - \log x_0)]\} \quad [1]$$

where *y* denotes fresh weight of aboveground tissue, expressed as a percentage of the untreated control at herbicide dose *x*; *b* is the slope; *c* is the lower limit; *d* is the upper limit; and x_0 is the effective dose of herbicide causing 50% inhibition of fresh weight (GR_{50}) with or without metabolic inhibitors or the herbicide dose causing 50% inhibition of ALS activity (IC_{50}). Relative tolerance indices (RIs) were calculated by dividing the GR_{50} of the tolerant population by that of the susceptible population. Significant differences in the GR_{50} values and IC_{50} values, the expression levels, and the activities of NADPH-dependent cytochrome P450 reductase were also subjected to a *t*-test analysis using SPSS v. 21.0.

Results and Discussion

Sensitivity to Penoxsulam

The results of the whole-plant bioassay confirmed that *E. japonica* was tolerant to penoxsulam (Table 1). None of the *E. japonica* populations were sensitive to penoxsulam, even if there was no history of herbicide application. All 29 *E. japonica* populations displayed similar sensitivity levels to penoxsulam with significantly (*t*-test, $P < 0.01$) higher GR_{50} values (105.14 to 148.78 g ha^{-1}) than the recommended label rate in the field (15 to 30 g ha^{-1}). The GR_{50} values of *E. japonica* populations were approximately 52.83- to 74.76-fold significantly higher (*t*-test, $P < 0.01$) than those of the susceptible *E. crus-galli* population (1.99 g ha^{-1}). Thus, the results indicated a high tolerance to penoxsulam in *E. japonica*.

Growers typically prefer to use a higher dose of a single herbicide to improve weed control; however, for herbicide-tolerant weeds, control cannot be achieved only by increasing a certain dosage because of the plant's natural insensitivity (Wang et al. 2013). There is an urgent need to identify the tolerance of *E. japonica* to penoxsulam because of the increasing threat posed by this weed. Here, we determined the sensitivity levels of *E. japonica* populations to penoxsulam and confirmed that *E. japonica* was tolerant to penoxsulam (Table 1). To our best knowledge, this study is the first report of *E. japonica* being highly tolerant to penoxsulam.

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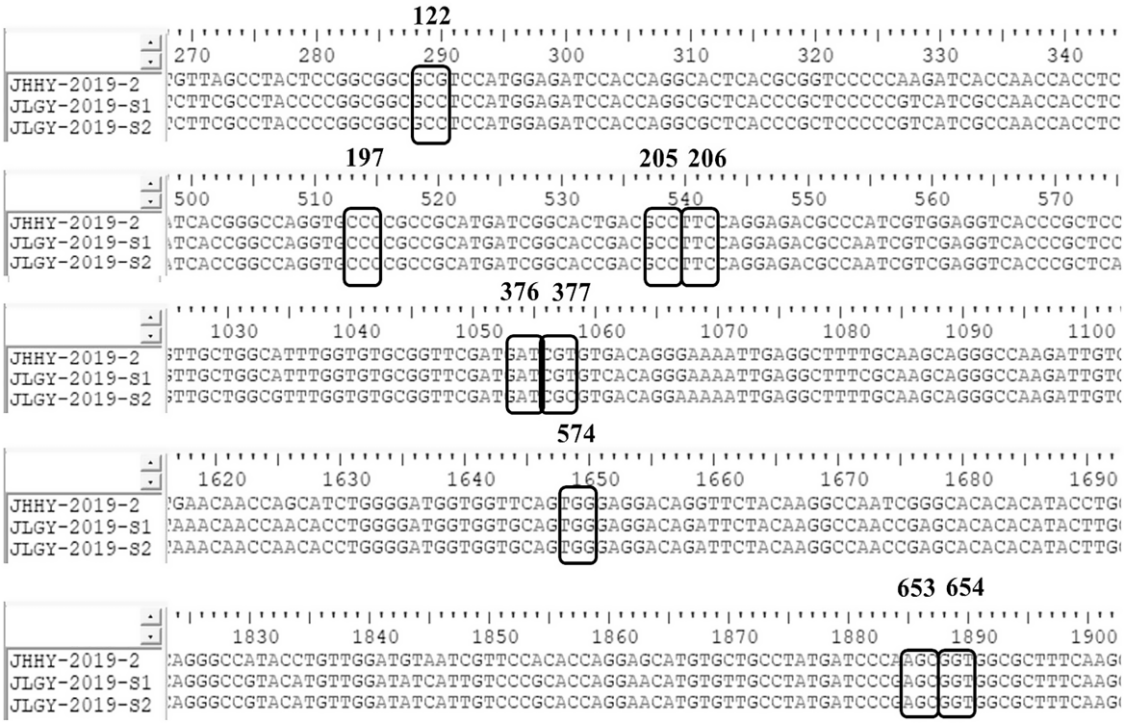

Figure 2. Sequence alignment of partial acetolactate synthase gene (*ALS*) from *Eragrostis japonica* (JHHY-2019-2) and *Echinochloa crus-galli* (JLGY-2019-S) populations. The boxed codons indicate amino acid sequence positions in the *ALS* fragment referring to the full-length sequence of *ALS* from *Arabidopsis thaliana* (GenBank accession no. NM_114714).

Target-Site Basis of Penoxsulam Tolerance

Lower ALS Sensitivity to Penoxsulam In Vitro

ALS activities in vitro were assayed and expressed as a percentage of the control (Figure 1). The ALS activities of JHHY-2019-2 and JLGY-2019-S were inhibited to different extents, and the inhibition was positively correlated with increasing concentrations of penoxsulam. The ALS of JLGY-2019-S was more sensitive to penoxsulam than that of JHHY-2019-2. At the same time, the penoxsulam concentrations causing 50% inhibition of the ALS activity (IC_{50}) were calculated: the IC_{50} value of JHHY-2019-2 was 32.88 ± 8.23 μM, which was 12.27-fold significantly higher (*t*-test, $P < 0.01$) than that of JLGY-2019-S (2.68 ± 1.21 μM), suggesting that the reduced sensitivity of ALS may be responsible for *E. japonica* tolerance to penoxsulam.

Target enzymes with reduced sensitivity to herbicides are a common TSR mechanism in weeds, especially for herbicides with clear targets, such as ALS inhibitors, ACCase inhibitors, and the very-long-chain fatty-acid elongase synthesis inhibitors (Cai et al. 2022; Fang et al. 2019b; Gao et al. 2017; Liu et al. 2019; Xu et al. 2013). In the current study, the sensitivity of ALS extracted from *E. japonica* was 12.27-fold lower than that of the susceptible weed *E. crus-galli* when treated with penoxsulam (Figure 1). The presence of a lower ALS sensitivity has also been documented in *E. phyllopogon* (Liu et al. 2019), Japanese foxtail (*Alopecurus japonicus* Steud.) (Feng et al. 2016), rigid ryegrass (*Lolium rigidum* Gaudin) (Yu et al. 2010), and flixweed [*Descurainia sophia* (L.) Webb ex Prantl] (Yang et al. 2018).

ALS Sequencing and Natural Mutation Identification

ALS gene fragments of JHHY-2019-2 that encompassed all previously identified resistance mutation sites were amplified, cloned, and sequenced. The assembled *ALS* gene fragment from JHHY-2019-2 was subjected to a BLAST search in GenBank for verification. The search result showed that it exhibited 91.62%, 91.32%, 86.39%, 85.98%, and 85.65% similarity to the *ALS* gene of *E. crus-galli* (MH013497), *E. crus-galli* var. *crus-galli* (LC006061.1), *B. tectorum* (AF488771), *A. myosuroides* (AJ437300.2), and *L. perenne* ssp. *multiflorum* (AF310684), respectively, indicating that we amplified the correct *ALS* sequence from *E. japonica*. The *ALS* gene sequence obtained in this study was the first DNA fragment of *E. japonica* in the GenBank database and was deposited in the GenBank database with accession no. ON652847 (see Supplementary Material). The *ALS* gene sequence also exhibited approximately 91% similarity between JHHY-2019-2 and JLGY-2019-S. However, mutations known to confer resistance to ALS inhibitors were not found (Figure 2). Thus, there were no natural mutations in JHHY-2019-2. It should be noted that we also sequenced the *ALS* gene of other *E. japonica* populations and obtained the same results (data not shown). Thus, tolerance to penoxsulam in *E. japonica* may not be related to target-site mutations in the *ALS* gene sequence.

Usually, amino acid mutations in the target protein reduce the binding affinity between the herbicide and target enzyme, resulting in reduced target enzyme sensitivity (Kukorelli et al. 2013; Mengistu et al. 2005; Thiel and Varrelmann 2014; Yu and Powles 2014). However, in our study, natural mutations in the *ALS* of *E. japonica* were not found (Figure 2). A similar situation has been reported for the target enzyme ALS in smallflower umbrella sedge (*Cyperus difformis* L.) (Merotto et al. 2009). It also has been reported in junglerice [*Echinochloa colona* (L.) Link] that lower basal 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) sensitivity can be considered an additional TSR mechanism, and in a lethal glyphosate treatment, this lower sensitivity

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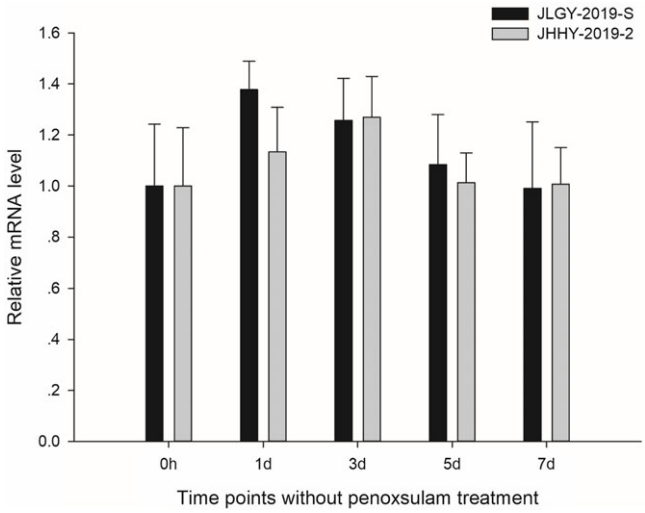

Figure 3. Relative mRNA level of *ALS* gene in *Eragrostis japonica* (JHHY-2019-2) and *Echinochloa crus-galli* (JLGY-2019-S) populations without penoxsulam treatment. Vertical bars represent the mean ± SE. No significant difference was found (*t*-test, $P > 0.05$).

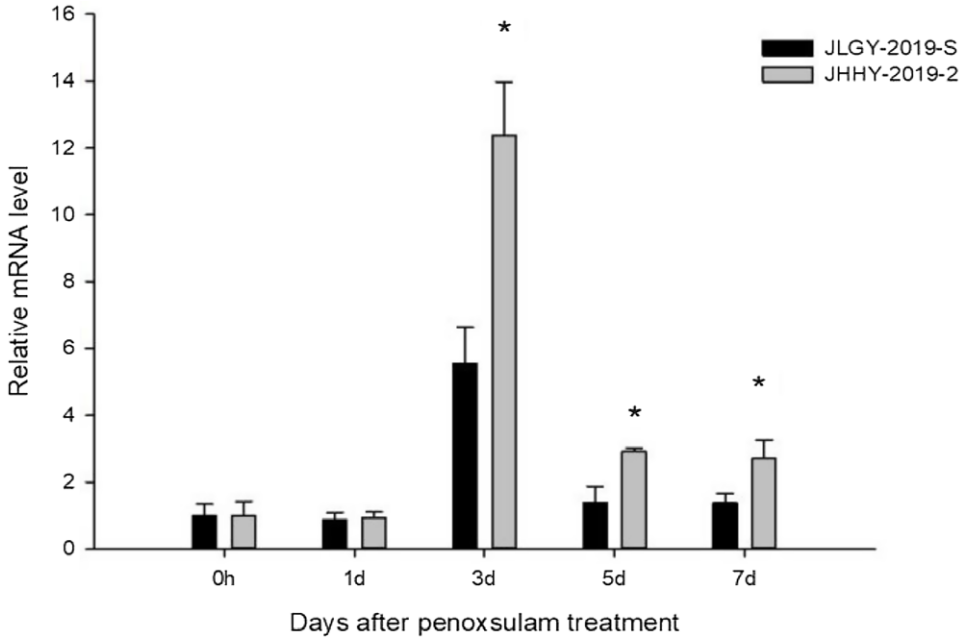

Figure 4. Relative mRNA level of *ALS* gene in *Eragrostis japonica* (JHHY-2019-2) and *Echinochloa crus-galli* (JLGY-2019-S) populations treated with penoxsulam. An asterisk (*) indicates significant difference (*t*-test, $P < 0.05$); standard errors of the mean are shown by vertical bars. Plants were treated with 7.5 g ai ha^{-1} penoxsulam.

enabled a population that had no *EPSPS* mutation to maintain normal growth (Alarcón-Reverte et al. 2015). It was also reported that no difference was found in target enzyme sensitivity between resistant and sensitive populations when there was a gene mutation in the resistant population (Boutsalis et al. 1999; Preston et al. 2006). Thus, the lower sensitivity of the herbicide target enzyme was not entirely consistent with the gene mutations.

Relatively Higher ALS Expression Level

The relative mRNA levels showed the trends in total *ALS* transcription. Without penoxsulam treatment, the relative *ALS* expression levels in JHHY-2019-2 and JLGY-2019-S were similar over five sampling time points (*t*-test, $P > 0.05$; Figure 3). After penoxsulam treatment, the relative *ALS* expression levels in JHHY-2019-2 were significantly upregulated (*t*-test, $P < 0.05$) by 2.23-, 2.10-, and 1.95-fold at 3, 5, and 7 d after penoxsulam treatment compared with levels in JLGY-2019-S, respectively (Figure 4). Therefore, the relatively higher *ALS* expression level may be one of the target enzyme mechanisms of penoxsulam tolerance in *E. japonica*.

Higher expression levels of target-site genes have also been found to confer herbicide resistance or herbicide tolerance, with reports mainly focusing on ACCase inhibitors, ALS inhibitors, and glyphosate (Fang et al. 2022; Laforest et al. 2017; Lorentz et al. 2014; Ngo et al. 2018; Wang et al. 2014; Zhang et al. 2018). In the present study, a higher level of *ALS* gene expression was found in *E. japonica* than in *E. crus-galli* after penoxsulam

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Table 4. Sensitivities of *Echinochloa crus-galli* (JLGY-2019-S) and *Eragrostis japonica* (JHHY-2019-2) populations to penoxsulam with/without four metabolic inhibitors.

Treatment	GR_{50}[a] (SE) of populations tested g ai ha^{-1}		RI[b]
	JLGY-2019-S	JHHY-2019-2	
Penoxsulam[c]	1.99 (0.11)	148.78 (4.49)	74.76
NBD-Cl[d] + penoxsulam	1.95 (0.13)	133.61 (2.86)	68.52
ABT[e] + penoxsulam	1.89 (0.09)	68.10 (3.19)*	36.03
PBO[f] + penoxsulam	2.11 (0.18)	59.91 (1.20)*	28.39
Malathion[g] + penoxsulam	2.07 (0.16)	66.31 (1.84)*	32.03

[a]GR_{50} refers to the effective dose of herbicide causing 50% inhibition of fresh weight and is indicated as grams of active ingredient per hectare (g ai ha^{-1}).
[b]RI is the relative tolerance index: ratio of GR_{50} values relative to the susceptible *E. crus-galli* population (JLGY-2019-S) at the same treatment. An asterisk (*) indicates significant difference (*t*-test, $P < 0.05$).
[c]Penoxsulam: applied at 0, 15, 30, 60, 120, and 240 g ai ha^{-1} to JHHY-2019-2 and at 0, 0.94, 1.88, 3.75, 7.5, and 15 g ai ha^{-1} to JLGY-2019-S.
[d]NBD-Cl (4-chloro-7-nitro-2,1,3-benzoxadiazole): 270 g ai ha^{-1}; applied 48 h before herbicide application.
[e]ABT (1-aminobenzotriazole): 1,000 g ai ha^{-1}; applied 1 h before herbicide application.
[f]PBO (piperonyl butoxide): 4,200 g ai ha^{-1}; applied 1 h before herbicide application.
[g]Malathion: 1,000 g ai ha^{-1}; applied 1 h before herbicide application.

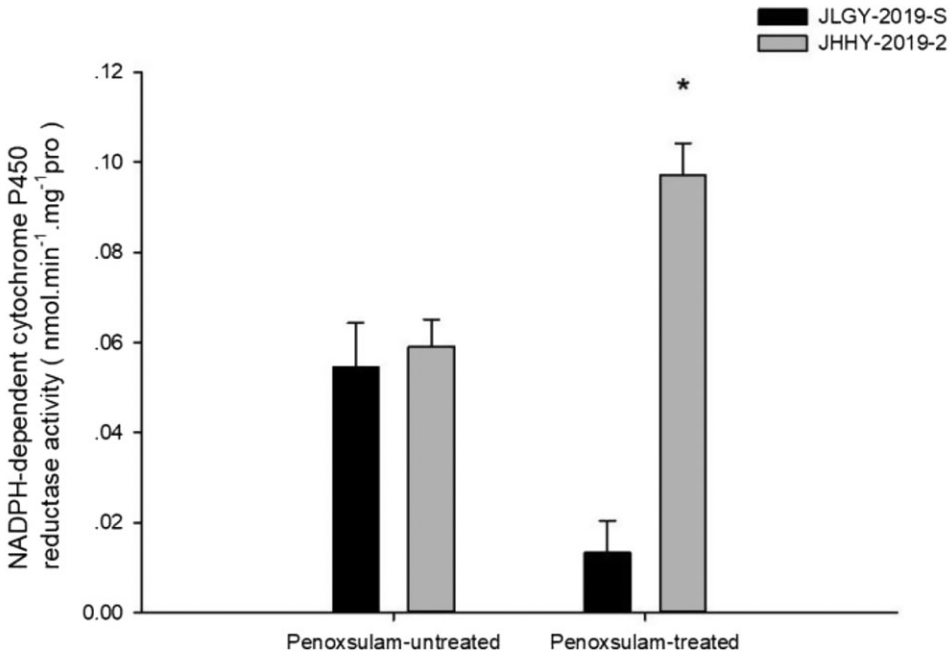

Figure 5. Comparison of the activities of NADPH-dependent cytochrome P450 reductase between *Echinochloa crus-galli* (JLGY-2019-S) and *Eragrostis japonica* (JHHY-2019-2) populations. An asterisk (*) indicates significant difference (*t*-test, $P < 0.01$); standard errors of the mean are shown by vertical bars. Plants were treated with 7.5 g ai ha^{-1} penoxsulam.

treatment (Figure 4). It was believed that this higher expression could lead to more protein production to normalize biochemical functions after herbicide treatment (Fang et al. 2020). This may partially explain why *E. japonica* has become tolerant to penoxsulam. Considering that the expression level of the *ALS* gene in *E. japonica* was not particularly high (up to 2.23-fold), the contribution of *ALS* gene overexpression to tolerance might be limited (Fang et al. 2020).

Metabolic Tolerance Mechanisms

Sensitivity Changes to Penoxsulam with CytP450 Inhibitors

As shown in Table 4, when penoxsulam was applied with NBD-Cl, no significant changes were found in the GR_{50} of JLGY-2019-S and JHHY-2019-2 compared with the penoxsulam-only treatment (*t*-test, $P > 0.05$), which suggests that GSTs are not involved in the penoxsulam tolerance of *E. japonica*; when penoxsulam was applied with ABT, PBO, or malathion, no significant changes were found in the GR_{50} of JLGY-2019-S (*t*-test, $P > 0.05$), whereas the sensitivity to penoxsulam of JHHY-2019-2 significantly increased by 54.23%, 59.73%, and 55.43%, respectively (*t*-test, $P < 0.05$). Therefore, NTSR mediated by CytP450s might be involved in the penoxsulam tolerance of *E. japonica*.

Higher Activity of NADPH-dependent Cytochrome P450 Reductase In Vivo

The results of studies on NADPH-dependent cytochrome P450 reductase activities of JLGY-2019-S and JHHY-2019-2 were analyzed (Figure 5). Before penoxsulam treatment, there was no significant difference in the NADPH-dependent cytochrome P450 reductase activity between JLGY-2019-S and JHHY-2019-2 (*t*-test, $P > 0.05$). After penoxsulam treatment, the NADPH-dependent

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cytochrome P450 reductase activity of JHHY-2019-2 was significantly higher (approximately 7-fold) than that of the sensitive population JLGY-2019-S (*t*-test, $P < 0.01$). This further indicated that CytP450s contributed to the metabolic tolerance to penoxsulam in *E. japonica*.

NTSR can endow unpredictable and complex resistance or tolerance to herbicides with different modes of action or chemical structures, such as ACCase inhibitors, ALS inhibitors, and photosystem II inhibitors (Cocker et al. 2001; Fang et al. 2019b; Feng et al. 2016; Iwakami et al. 2012; Wang et al. 2013; Zhang et al. 2017). It was reported that CytP450s may play an important role in tolerance to the ACCase inhibitor fenoxaprop-*P*-ethyl in annual bluegrass (*Poa annua* L.) (Wang et al. 2013). And high activity of NADPH-dependent cytochrome P450 reductase can enhance the metabolic capacity of CytP450s in plants (Wang et al. 2013; Zimmerlin and Durst 1990). In the present study, the tolerance to penoxsulam in *E. japonica* may also have been caused by the stronger metabolic effects of CytP450s, which were verified using both metabolic enzyme inhibitors (Table 4) and metabolic enzyme activities (Figure 5). However, the tolerance was not fully overcome by CytP450 inhibitors (Table 4), which suggested that NTSR played a partial role in penoxsulam tolerance.

In conclusion, the present study identified high tolerance to penoxsulam in *E. japonica*. This serves as a reminder that penoxsulam cannot be used to control *E. japonica*, even at high doses. Regarding tolerance mechanisms, lower ALS sensitivity, relatively higher *ALS* expression levels (target site–based tolerance), and CytP450-mediated metabolism may combine to confer penoxsulam tolerance in *E. japonica*. Additionally, other NTSR mechanisms not evaluated here (e.g., reduced absorption and translocation) may also contribute to the penoxsulam tolerance. More experiments are warranted to further clarify this point.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2022.63

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