Weed Science

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A Trp-574-Leu mutation in acetolactate synthase confers imazamox resistance in barnyardgrass (*Echinochloa crus-galli*) from China

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Research Article

Cite this article: Liu Y, Shi K, Gao H, Yin S, Dong L, Feng Z (2024). A Trp-574-Leu mutation in acetolactate synthase confers imazamox resistance in barnyardgrass (*Echinochloa crus-galli*) from China. Weed Sci. doi: 10.1017/wsc.2024.73

Received: 20 June 2024
Revised: 11 September 2024
Accepted: 28 September 2024

Associate Editor:
Vipan Kumar, Cornell University

Keywords:
ALS protein abundance; IMI-T rice fields; target-site resistance

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Abstract

Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] is increasingly infesting imidazolinone-tolerant (IMI-T) rice (*Oryza sativa* L.) fields in China, and *E. crus-galli* imazamox resistance has become the major concern for weed management in IMI-T rice fields. In this study, the susceptible population JLGY-3 (S) and the suspected resistant population JHXY-2 (R) collected from IMI-T rice fields were used as research subjects. When treated with imazamox, the JHXY-2 (R) population showed a high level of herbicide resistance with a resistance index of 31.2. JHXY-2 (R) was cross-resistant to all five acetolactate synthase (ALS) inhibitors from different chemical families, but sensitive to herbicides inhibiting acetyl-CoA carboxylase. To understand the reason why JHXY-2 (R) was resistant to imazamox, we performed experiments to characterize potential target-site resistance (TSR) and non–target site resistance (NTSR) mechanisms. A Trp-574-Leu amino acid mutation in ALS and low imazamox ALS sensitivity were the main mechanisms underlying imazamox resistance in this JHXY-2 (R) population. There was no significant difference in *ALS* gene expression and ALS protein abundance between R and S populations. High-performance liquid chromatography–tandem mass spectrometry analysis showed enhanced metabolism of imazamox in JHXY-2 (R), which was in contrast to the results of pretreatment with a metabolic enzyme inhibitor. Treatments with cytochrome P450 monooxygenase/glutathione *S*-transferase (P450/GST) inhibitors did not alter the resistance level of JHXY-2 (R) against imazamox. Transcriptome sequencing showed that there was almost no significant difference in the expression of P450 and GST metabolic enzyme genes between R and S populations, and only GSTU1 showed a significant upregulation in the R population, further clarifying the NTSR mechanism of JHXY-2 (R). In conclusion, amino acid mutation and higher enzyme activity of ALS are the main causes of imazamox resistance in JHXY-2 (R). However, given the differences in imazamox residues in the leaves of *E. crus-galli*, there may still be undetectable NTSR mechanisms that are causing imazamox resistance in the R population.

Introduction

Weed control has always been an important issue in agricultural production. If weeds are not controlled in time and effectively, grain yield will be seriously reduced (Singh et al. 2015). Weedy rice (*Oryza sativa* L.) is a harmful weed in rice fields and is extremely difficult to control with the herbicides registered in rice fields because of its botanical similarity to cultivated rice (De Leon et al. 2019). Weedy rice competes with cultivated rice for water and nutrients in paddy fields, resulting in serious loss of rice yield (Burgos et al. 2008). In response to this situation, the BASF Company initially developed the technology of planting rice tolerant to imidazolinone herbicides combined with the use of imazethapyr (Clearfield®) in the United States in 2003. Since then, Clearfield® rice has been rapidly popularized and planted in the United States and has brought considerable income to Clearfield® rice growers (Burgos et al. 2014). In recent years, this technology has also been developed in China, where farmers have begun to grow imidazolinone-tolerant (IMI-T) rice cultivars in combination with the use of imazamox to control weeds in paddy fields. Imazamox is one of the imidazolinone herbicides that can effectively control annual grasses and broadleaf weeds. In China, the main weeds in paddy fields include weedy rice, *Echinochloa* spp., Chinese sprangletop [*Leptochloa chinensis* (L.) Nees], large crabgrass [*Digitaria sanguinalis* (L.) Scop.], heusenkraut (*Ludwigia prostrata* Roxb.), and variable flatsedge (*Cyperus difformis* L.) (Liu et al. 2021). Most of the weeds, including *Echinochloa* spp., can be controlled by spraying imazamox in the IMI-T rice fields in the early years after introduction of the technology. However, after continuously planting IMI-T rice and using

imazamox for a few years, some farmers found that *Echinochloa* spp. became rampant in the rice fields. After observing the distribution of *Echinochloa* spp. in many places growing IMI-T rice, we found that *E. crus-galli* is the main species infesting IMI-T rice fields among the sites.

Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] is a very common malignant weed in the rice fields, and its control mainly depends on the use of various herbicides (Fang et al. 2019b). In specialized cultivation practices, such as the integration of IMI-T rice with imazamox, herbicides are applied repeatedly on an annual basis. Under this continuous selection pressure of a single herbicide, it is easy for *E. crus-galli* to evolve herbicide resistance (Chen et al. 2016).

Echinochloa crus-galli is an allopolyploid weed species and has a more intricate genomic architecture compared with diploid species (Panozzo et al. 2013, 2021). Due to its substantial genome size and the delayed initiation of genetic research on weeds, the genome sequence of *E. crus-galli* remained elusive until Guo et al.'s study (2017) unveiled the complexities of its genome. In polyploid species, the gene-copy number at each locus can vary (Panozzo et al. 2021); for instance, Fang et al. (2019a) reported differences in *ALS* gene-copy numbers across various populations of *E. crus-galli*, while Iwakami et al. (2017) observed similar variability in *ALS* gene-copy numbers within shortawn foxtail (*Alopecurus aequalis* Sobol.).

Imazamox is an acetolactate synthase (ALS) inhibitor, inhibiting the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine and eventually leading to arrested growth and plant death (Duggleby et al. 2008). At present, research on herbicide-resistance mechanisms is mainly concentrated on target-site resistance (TSR) or non–target site resistance (NTSR) (Yu and Powles 2014a). Research on TSR mainly focuses on the activity of the target enzyme (Fang et al. 2019b), the mutation of the target gene (Yu and Powles 2014b), and the change in target gene expression (Gao et al. 2017). Most studies have shown that the ALS activity of resistant weeds with *ALS* gene mutations is higher than that of susceptible weeds (Fang et al. 2019b; Gao et al. 2023; Li et al. 2017; Panozzo et al. 2013). Herbicide resistance mediated by target gene mutation has been investigated extensively. At present, a total of 31 amino acid substitutions at nine amino acid sites located in the *ALS* gene have been found in resistant weeds (Fang et al. 2022; Liu et al. 2021). The relationship between the target gene expression and weed resistance remains a mystery for researchers to unravel. The relationship between the accumulation of target proteins and herbicide resistance has been unclear, as well as whether the expression of target genes and the accumulation of target proteins are synchronized. Although antibodies can be used to determine protein accumulation, their application in herbicide-resistance research remains very limited (Chen et al. 2020; Lowe et al. 2024).

In comparison to TSR, NTSR presents greater management challenges, complicates investigative efforts, and necessitates the allocation of additional resources (Fang et al. 2019b). When weeds develop TSR to herbicides, farmers can effectively manage weed populations by employing herbicides with alternative modes of action. (Liu et al. 2021). However, when weeds develop NTSR to herbicides, the development of weed control strategies becomes more complex (Liu et al. 2021). For instance, research on a rice barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.] population with NTSR has demonstrated that this weed population has developed resistance to penoxsulam, cyhalofop-butyl, and florpyrauxifen-benzyl. A resistant *E. crus-galli* population has also been found to be resistant to penoxsulam, pinoxaden, and quinclorac. The study of NTSR mainly involves examination of three aspects: enhancement of herbicide metabolism (Délye 2013; Pan et al. 2022), decrease of herbicide penetration and transport (Riar et al. 2013), and alleviation of herbicide-induced oxidative stresses (Pan et al. 2021). Enhanced herbicide metabolism was identified as the most common NTSR mechanism (Yu and Powles 2014a). At present, the genes related to herbicide metabolism have been classified into eight main categories: cytochrome P450 monooxygenase (P450), glutathione *S*-transferase (GST), ATP-binding box transporter, glycosyltransferase, oxidase, esterase, peroxidase, and hydrolase (Délye 2013). Transcriptome sequencing is often used in the investigation of metabolic resistance. With this technique, researchers can quickly identify the metabolic enzyme genes that may play a key role in herbicide resistance (Pan et al. 2016; Zhao et al. 2022). The metabolic genes that have been characterized are mainly *P450* family genes and *GST* family genes. For example, it was found that CYP81A68 mediates herbicide resistance of *E. crus-galli* to penoxsulam and other herbicides (Pan et al. 2022); CYP81A12 and CYP81A21 mediates the resistance of *E. phyllopogon* to bensulfuron-methyl and penoxsulam (Iwakami et al. 2014); and GSTF1 mediates the resistance of blackgrass (*Alopecurus myosuroides* Huds.) to various herbicides, including chlorotoluron, fenoxaprop-*p*-ethyl, and clodinafop-propargyl (Cummins et al. 2013).

Therefore, this study aimed to (1) test whether the *E. crus-galli* population collected from IMI-T rice fields is resistant to imazamox, (2) determine the extent of resistance to other ALS inhibitors and acetyl-CoA carboxylase (ACCase)-inhibiting herbicides in this R population, and (3) understand the TSR and NTSR basis of this imazamox-resistant population. The results of this study may clarify the resistance mechanism of *E. crus-galli* to imazamox under this IMI-T rice-planting system while providing a new avenue for managing imazamox-resistant weeds.

Materials and Methods

Plant Materials

The JHXY-2 (R) population seeds were collected from IMI-T rice fields (33.01°N, 118.62°E) in the Jiangsu Province of China in 2021, where imazamox has been used for many years. Seeds of the susceptible population JLGY-3 (S) were collected from uncultivated land (34.83°N, 119.12°E) that had never been exposed to herbicides. All seeds were collected by hand, air-dried in the shade, and stored in paper bags at 4 C until use.

Sensitivity to Imazamox and Other Herbicides

Twenty seeds from each of the two populations were sown in plastic pots (9-cm diameter by 10-cm height), which were filled with a 2:1 (w/w) mixture of nutrient matrix and sandy soil. Seedlings were grown in a greenhouse and thinned to 10 plants per pot before herbicide treatment. At the 3- to 4-leaf stage, herbicides were applied using a laboratory sprayer equipped with a flat-fan nozzle, delivering 280 L ha^{-1} at 230 kPa. The commercial preparation of imazamox (Zhongqi Technology, NanJing, China) was used in this experiment. The subsequent use of imazamox was the same commercial preparation, diluted to the corresponding dose or concentration. Based on a preliminary experiment (data not shown), imazamox was applied at 0, 3.75, 7.5, 15, 30, 60, and 120 g ai ha^{-1} to the JLGY-3 (S) population. For the JHXY-2 (R) population, the dose of imazamox was set to 30, 60, 120, 240, 480,

https://doi.org/10.1017/wsc.2024.73 Published online by Cambridge University Press

and 960 g ai ha^{-1}. After 21 d of imazamox treatment, the aboveground parts of the *E. crus-galli* plants were collected and weighed. Sensitivity to other herbicides was also determined using the same method. The application doses were based on the results of a preliminary experiment (data not shown); detailed dose information is shown in Supplementary Table S1. This experiment was conducted twice in a completely randomized design, with three replications for every dose, and each replication contained 10 plants.

Effect of Metabolic Inhibitors on Imazamox Sensitivity

Two P450 inhibitors (PBO and malathion) and one GST inhibitor (NBD-Cl) were used to evaluate the effect of metabolic inhibitors on resistance level. The applied doses and methods of PBO (4,200 g ai ha^{-1}), malathion (1,000 g ai ha^{-1}), and NBD-Cl (270 g ai ha^{-1}) were previously reported (Gao et al. 2023). PBO and malathion were applied 1 h before herbicide application, and NBD-Cl was applied 48 h before herbicide application. The experiments were performed as described earlier.

ALS Enzyme Activity Assay

According to a method described by Yu et al. (2004), we assessed ALS enzyme responses of S and R populations to imazamox with slight modifications. The stems and leaves (3 g) of *E. crus-galli* were harvested from each population and powdered in liquid nitrogen. The ground plant tissue was collected into a 50-ml centrifuge tube, 8 ml of enzyme extraction buffer (100 mM potassium phosphate buffer, pH 7.5, 10 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM Thiamine Pyrophosphate (TPP), and 10 µM Flavin Adenine Dinucleotide (FAD)) was added, then shaken for 3 min and left on ice for 15 min. The plant homogenate was filtered with gauze mesh, and the filtrate was centrifuged and treated with ammonium sulfate to obtain protein precipitate. The protein precipitates of S and R populations were dissolved in 4-ml of enzyme assay buffer (100 mM potassium phosphate buffer, pH 7.5, 200 mM sodium pyruvate, 20 mM $MgCl_2$, 2 mM TPP, 20 µM FAD, and 50 mM HEPES) to obtain crude enzyme solution. Each reaction system consists of 100 µl of crude enzyme solution and 100 µl of ALS inhibitor (imazamox at 0.001, 0.01, 0.1, 1, 10, 100, and 1,000 µM; penoxsulam at 0.001, 0.01, 0.1, 1, 10, 100, and 1,000 µM). The reaction mixture was incubated at 37 C for 60 min. The reaction was terminated by adding 10 µl of 6 N H_2SO_4 at 60 C for 15 min. The color reaction was performed by adding 190 µl of creatine solution (0.55%) and 190 µl of α-naphthol solution (5.5% in 5 N NaOH) at 60C for 15 min. ALS activity was determined colorimetrically (530 nm) by measuring the acetoin production (pure acetoin was used as the standard; Aladdin, Shanghai, China) using a microplate photometer (Thermo Fisher, Waltham, MA). The assay was performed twice with independent extractions and three replicates per herbicide concentration, and each replication contained 100 µl of crude enzyme solution and 100 µl of ALS inhibitor.

Gene Cloning and Sequence Analysis

The DNA of *E. crus-galli* was extracted using the Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China). The *ALS* sequences ALS1 and ALS2 of the S population JLGY-3 (S) were described in previous studies (Fang et al. 2019a). Based on the conserved regions of these two sequences, we designed a primer pair (forward: TCTTCGCCTACCCCGGCG; reverse: TCAATACACGGTCCTGCCATCACC) with primer Premier v. 5.0 (Premier Biosoft International, Palo Alto, CA, USA); the amplified *ALS* gene fragment can cover the nine mutation sites (Ala-122, Pro-197, Ala-205, Phe-206, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654) reported to cause herbicide resistance (Fang et al. 2022; Liu et al. 2021). The polymerase chain reaction (PCR) mixture contained 20 ng of template DNA, 2 µl of each primer (10 µM), 25 µl of 2× Rapid Taq Master Mix (Vazyme Biotech, Nanjing, China), and ddH_2O to a final volume of 50 µl. The amplification reaction was set as follows: 3 min at 95 C for DNA denaturation; 35 cycles of 15 sec at 95 C for DNA denaturation, 15 sec at 63 C for annealing, and 1 min 10 sec at 72 C for DNA elongation; and a final elongation for 6 min at 72 C. *ALS* genes from two resistant *E. crus-galli* were used for gene cloning. Gene cloning was performed using the universal pMD19-T Rapid Cloning Kit (TaKaRa Biotech, Shiga, Japan). Briefly, the *ALS* gene from two resistant *E. crus-galli* was amplified utilizing the aforementioned method. The resulting amplified product was subsequently ligated to the pMD19-T cloning vector. The reaction mixture comprised 0.3 pmol of insert DNA, 1 µl of pMD19-T vector, and 5 µl of Solution I (ligase solution). This reaction was incubated at 16 C for 30 minutes before transforming 10 µl of the reaction product into *Escherichia coli* DH5α. Plates were then incubated overnight, followed by blue-white colony screening on the following day, during which white colonies were selected for further analysis. These bacteria were cultured at 37 C for an additional 6 h, after which bacterial liquid samples were collected for positive identification. Positive samples were subsequently sent for sequencing using universal primers M13F and M13R specific to the vector. At least 14 transformed clones from each plant were selected to obtain *ALS* sequences, which were aligned and compared using SnapGene v. 6.1 (GSL Biotech LLC, Boston, MA, USA).

Determination of ALS Gene Expression by Real-Time Quantitative Reverse Transcriptase PCR (RT-qPCR)

Plant tissues from S and R populations were collected at five time points (0 h, 12 h, 1 d, 3 d, and 5 d) after imazamox treatment, and three replicates were established for each time point, each containing 0.1 g of leaves. Total RNA of *E. crus-galli* was extracted using the RNA Simple Total RNA Kit (Tiangen Biotech, Nanjing, China) according to the manufacturer's instructions. All RNA samples were reverse transcribed into cDNA according to the instructions of the Reverse Transcription Kit (Vazyme Biotech, Nanjing, China). The β-actin gene of *E. crus-galli* (GenBank accession no.: HQ395760) was used as an internal reference gene, and the expression level of the *ALS* gene in JLGY-3 (S) and JHXY-2 (R) was determined by RT-qPCR. RT-qPCR analyses were performed on an ABI-7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using the ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech, Nanjing, China), following the manufacturer's instructions. The RT-qPCR primers were sourced from Fang et al. (2022), with the specific primer sequences provided in Supplementary Table S2. The fold changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method. Significant differences in expression levels were analyzed using Welch's *t*-test. The entire experiment was repeated twice.

Protein Extraction and Immunoblot Analysis

Samples from S and R populations were collected at two time points (0 h and 1 d) after imazamox treatment, with three replicates at each time point. Leaves of *E. crus-galli* were ground

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into powder using a mortar and pestle in liquid nitrogen and then transferred into a 2-ml plastic centrifuge tube. The plant samples were vortexed with 400 µl of protein extraction buffer (10% glycerol, 25 mM Tris, pH 7.5, 1 mM Ethylene Diamine Tetraacetic Acid (EDTA), 150 mM NaCl, 10 mM Dithiothreitol (DTT), 1× protein inhibitor cocktail, 2% Polyvinylpolypyrrolidone (PVPP)) for 2 min. Samples were centrifuged at 12,000 rpm for 5 min at 4 C, and the supernatant was collected. Protein loading buffer was added to the supernatant and the mixtures was boiled for 5 min. The protein samples were separated using 10% SDS-PAGE and then transferred to PVDF membrane. The primary ALS antibody against *E. crus-galli* ALS was prepared in our previous study (Liu et al. 2024b), and it was applied at a dilution of 1:5,000. Goat anti-mouse immunoglobulin G horseradish peroxidase was applied at a dilution of 1:10,000. The protein strips were exposed using enhanced chemiluminescence, and the gray scale of the bands was quantified using Image J software (NIH, Bethesda, MD, USA).

High-Performance Liquid Chromatography–Tandem Mass Spectrometry (HPLC-MS/MS) Analysis of the Imazamox Residue

When *E. crus-galli* reached the 3- to 4-leaf stage, each plant was treated with 4 µl of imazamox with a concentration of 0.03675 g ml^{-1} applied evenly as a liquid on the surfaces of fully opened leaves. The imazamox application dose on each *E. crus-galli* plant was equivalent to using 120 g ha^{-1} in the IMI-T rice fields. Samples from S and R populations were collected at three time points (12 h, 24 h, and 48 h) after imazamox treatment, with three replicates set at each time point, and each replicate containing five *E. crus-galli* plants. When collecting samples, we employed acetonitrile to cleanse the leaf surface, with the aim of eliminating as much of the remaining imazamox on the leaf surface. The samples were prepared with a modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) method according to Yasuor et al. (2009). Leaves of *E. crus-galli* were ground into powder using a mortar and pestle in liquid nitrogen and then transferred into a 50-ml plastic centrifuge tube. The plant samples were vortexed with 10 ml of acetonitrile for 5 min, then 2.0 g of NaCl and 4.0 g of $MgSO_4$ were added, with vortexing continued for 30 sec, and samples were then centrifuged at 4,000 rpm for 3 min. The extract (2 ml) was transferred to a purification tube containing 400 mg of C_{18} and 400 mg of $MgSO_4$. The tube was shaken for 5 min to complete the purification and centrifuged for 3 min at 12,000 rpm. One milliliter of supernatant was transferred with a syringe and then filtered using a 0.22-µm organic filter membrane. The imazamox amount was determined by HPLC-MS/MS (Agilent 6460 Triple Quad, Agilent, California, USA). Chromatography was performed on a Waters BEH C18 column (1.7 µm, 2.1 mm by 50 mm) (Waters, Milford, Delaware, USA). The injection volume was 1.0 µl. The mobile phase was an acetonitrile/water solution (0.01% formic acid + 5 mM of ammonium acetate). Gradient elution conditions are shown in Supplementary Table S3. Multiple reaction monitoring setting conditions are shown in Supplementary Table S4. The calibration curve of imazamox is provided in Supplementary Table S5. The residual imazamox concentration was determined by substituting the peak area of imazamox detected into the calibration curve. Significant differences in imazamox residue were analyzed using Welch's *t*-test.

Sample Preparation for RNA-seq

Echinochloa crus-galli plants were cultivated to the 3- to 4-leaf stage. Plant tissues from S and R populations were collected at 0 h, 12 h, and 24 h after treatment with imazamox (120 g ha^{-1}). The imazamox application method was the same as described earlier. Three biological replicates were set up for each time point, with each replicate containing 0.5 g of leaf tissue. After leaf samples were collected, they were immediately frozen in liquid nitrogen and stored at −80 C until use.

RNA Extraction, cDNA Library Preparation, Transcriptome Sequencing, and Bioinformatics Analysis

Total RNA was extracted from the R and S leaf material by using the RNA Simple Total RNA Kit (Tiangen Biotech) according to the manufacturer's instructions. cDNA library preparation and transcriptome sequencing were performed as previously reported (Liu et al. 2015; Zhong et al. 2011). Clean reads were mapped to *E. crus-galli* genome (https://ngdc.cncb.ac.cn/gwh/ncbi_assembly/56777/show) using TopHat2 software (Wu et al. 2022), and only unique mapping reads were retained for calculating gene expression. RNA-seq analysis was performed according to previous protocols (Trapnell et al. 2010, 2013). Contigs were selected based on statistical significance ($P < 0.05$), the magnitude of expression differences, and annotations related to known herbicide metabolism genes and signaling functions using the *E. crus-galli* genome. Differentially expressed genes were identified using the package (http://www.r-project.org) with an False Discovery Rate (FDR) < 0.05 and an absolute $\log_2$ ratio value ≥1.

RT-qPCR Validation

RNA samples returned from transcriptome sequencing were reverse transcribed into cDNA using HiScript III QRT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech); 14 genes from the metabolizing enzyme library (Pan et al. 2016) were selected to design primers for RT-qPCR, and the primer sequences are listed in Supplementary Table S6. RT-qPCR analyses were performed according to Determination of ALS Gene Expression by RT-qPCR. The *E. crus-galli* β-actin gene was used as the internal control gene. Total RNAs of S and R populations collected at the 0-h, 12-h, and 24-h time points were extracted using RNA Simple Total RNA Kit (Tiangen Biotech), following the manufacturer's instructions. The expression changes of 14 genes picked from the metabolizing enzyme library were verified by RT-qPCR.

Data Analysis

All whole-plant dose–response data were collected for ANOVA using SPSS v. 21.0 (SPSS Inc., Chicago, IL, USA). The results showed no significant difference between parallel experiments ($P > 0.05$). Data were processed using SigmaPlot v. 12.5 (Systat, San Jose, CA, USA). In simple terms, a four-parameter nonlinear logistic regression model was used to merge the data to determine the herbicide dose that resulted in a 50% fresh weight loss of weeds:

$$y = c + (d - c)/\left[1 + (x/g)^b\right] \quad [1]$$

where y is the aboveground fresh weight of weeds, x is the herbicide dose, b is the slope of the curve, c is the lower limit, d is the upper limit, and g is the herbicide dose at the point of inflection halfway between the upper and lower limits. The same methods were used

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Table 1. Sensitivities of the resistant and susceptible populations to imazamox with/without three metabolic inhibitors.

Herbicide treatment	GR_{50} (±SE) (g ai ha^{-1})		RI[a]
	JLGY-3 (S)	JHXY-2 (R)	
Imazamox	5.19 (±0.64)	166.71 (±1.59)	32.1
NBD-Cl + imazamox	6.4 (±2.0)	168.5 (±3.5)	26.3
PBO + imazamox	6.3 (±2.69)	214.6 (±3.08)	34
Malathion + imazamox	7.37 (±3.94)	257.91 (±9.42)	34.9

[a]RI, resistance index.

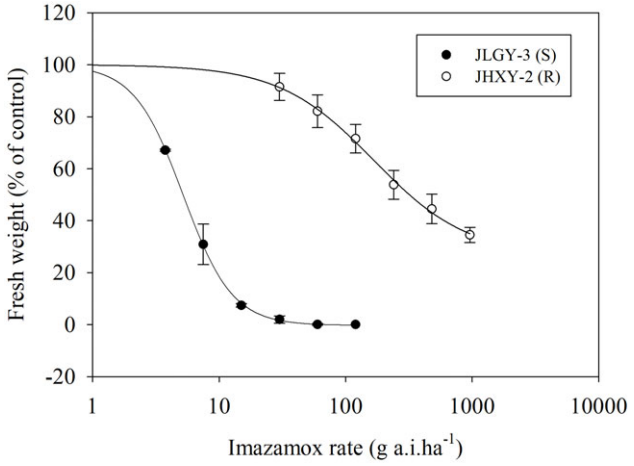

Figure 1. Fresh weight of *Echinochloa crus-galli* treated with imazamox. Vertical bars represent the mean ± SE.

to calculate the herbicide concentration that caused 50% inhibition of ALS activity (IC_{50}). Resistance indexes (RIs) were calculated by dividing the GR_{50} (or IC_{50}) of the R population by the GR_{50} (or IC_{50}) of the S population. The classification of resistance level refers to supporting information.

Results and Discussion

Sensitivity to Imazamox

The GR_{50} of the presumptive resistant population JHXY-2 (R) (166.71 g ha^{-1}) was higher than the recommended application dose (120 g ha^{-1}), while the GR_{50} of the susceptible population JLGY-3 (S) was only 5.19 g ha^{-1} (Table 1; Figure 1). Whole-plant bioassays showed that the JHXY-2 (R) population still survived tenaciously imazamox treatment at a much higher dose (960 g ha^{-1}). The RI of the JHXY-2 (R) population to imazamox was up to 32.1.

Sensitivity to Other Herbicides

After being treated with a variety of herbicides, it was observed that the R population had developed resistance to four ALS inhibitors from different chemical families in comparison to the S population. The RI for flucarbazone-sodium reached 8.5, indicating a medium level of resistance. The other three ALS inhibitors showed RI of 14.9 (penoxsulam), 21.8 (propyrisulfuron), and 18.5 (bispyribac-sodium), which indicated a high level of resistance. However, the R population JHXY-2 (R) did not show any resistance to ACCase inhibitors, with an RI of 1 for metamifop and 1.6 for cyhalofop-butyl (Table 2).

Sensitivity Change to Imazamox under Pretreatment with Three Metabolic Inhibitors

When three metabolic inhibitors (PBO, malathion, and NBD-Cl) were applied before imazamox treatment, the growth of the S population was hardly affected (Table 1). The GR_{50} value and RI of the R population increased under treatment with the two P450 inhibitors. Under treatment with the GST inhibitor (NBD-Cl), the GR_{50} value of the R population increased; however, the RI decreased by 18.06% (32.1 to 26.3).

In Vitro ALS Activity Assay

The sensitivity of the ALS enzyme to imazamox and penoxsulam was assessed in vitro, revealing that the extent of inhibition against the R population was comparatively lower than that observed in the S population (Figure 2). The IC_{50} value of JLGY-3 (S) was 0.8 μM, while the IC_{50} value of JHXY-2 (R) was 9.87 μM under treatment with imazamox (Figure 2A). There was a 12.3-fold difference in ALS activity between the two populations. The IC_{50} values for JLGY-3 (S) and JHXY-2 (R) were 0.61 μM and 4.03 μM, respectively, under treatment with penoxsulam, and there was also a 6.6-fold difference (Figure 2B).

Gene Cloning and Sequence Analysis

The JLGY-3 (S) population sequences have been identified in previous studies (Fang et al. 2019a). *Echinochloa crus-galli* is a hexaploid weed characterized by multiple copies of the *ALS* gene. Utilizing cloning sequencing, we acquired a substantial collection of *ALS* sequences from JHXY-2, subsequently translating these sequences into amino acid formats for comparative analysis. Through the analysis of this substantial number of sequences, we distinctly identified single-nucleotide polymorphisms at several conserved sites across different gene copies and established that the JHXY-2 population possesses *ALS* gene copies (Supplementary Table S7). Comparative analysis of codons at positions 217, 322, and 441 allows for the differentiation of ALS3 from ALS1 and ALS2. Furthermore, a distinction can be made between ALS1 and ALS2 by examining codons at positions 265 and 441 (Supplementary Table S7). We identified three copies of the *ALS* gene in the JHXY-2 (R) population, whereas, two copies of the *ALS* gene were observed in the JLGY-3 population. The observed variation in gene-copy number in the present study is consistent with that previously reported in *E. crus-galli*, *A. aequalis*, and Japanese foxtail (*Alopecurus japonicus* Steud.) (Fang et al. 2019a; Feng et al. 2016; Iwakami et al. 2017). Analysis of the *ALS* sequence in the R population revealed the same mutation (TGG to TTG) in all three *ALS* sequences, which resulted in the substitution of Trp with Leu at position 574 (Trp-574-Leu) (Figure 3A). Following our classification, we observed that the Trp-574-Leu mutation can manifest in two copies within an individual plant and may occur in either *ALS1* or *ALS3* or concurrently in *ALS2* and *ALS3*. In addition, we detected the *ALS* sequences of the R population in a total of 80 individual plants, of which 64 contained Trp-574-Leu mutations, accounting for 80% (Figure 3B).

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Table 2. Sensitivity of the two *Echinochloa crus-galli* populations to other herbicides[a].

Target	Group	Herbicide	GR_{50} (±SE) (g ai ha^{-1}) JLGY-3 (S)	JHXY-2 (R)	RI[b]
ALS	SCT	Flucarbazone-sodium	10.45 (±3.41)	89.61 (±4.58)	8.5
	TP	Penoxsulam	1.33 (±1.13)	19.88 (±3.6)	14.9
	SU	Propyrisulfuron	8.59 (±1.98)	187.81 (±3.42)	21.8
	PTB	Bispyribac-sodium	7.21 (±4.58)	133.5 (±6.05)	18.5
ACCase	APP	Metamifop	28.82 (±4.77)	28.82 (±3.17)	1
		Cyhalofop-butyl	17.54 (±7.39)	29.4 (±7.72)	1.6

[a]Abbreviations: ACCase, acetyl-CoA carboxylase; ALS, acetolactate synthase; APP, aryloxy phenoxy propionate; PTB, pyrimidinylthiobenzoate; SCT, sulfonylamino-carbonyl-triazolinone; SU, sulfonylurea; TP, triazolopyrimidine.
[b]RI, resistance index.

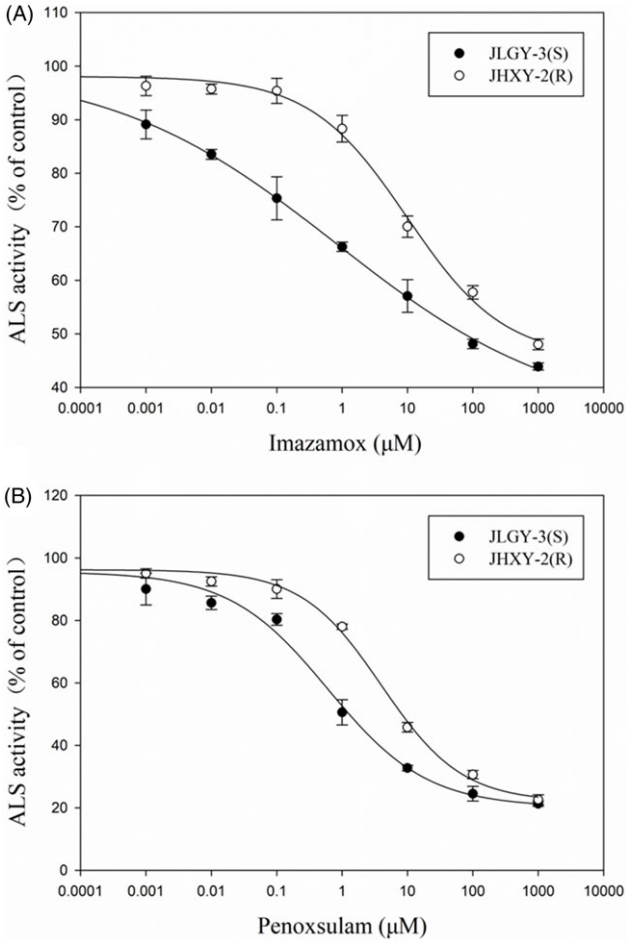

Figure 2. In vitro acetolactate synthase (ALS) activity of two *Echinochloa crus-galli* populations treated with (A) imazamox and (B) penoxsulam. Vertical bars represent the mean ± SE.

Gene Expression Level and Protein Abundance of ALS between R and S

The expression levels of *ALS* genes in the S and R populations were monitored at five time points (0 h, 12 h, 1 d, 3 d, and 5 d) after treatment with imazamox (120 g ha^{-1}) (Figure 4A). It was found that there was no significant difference in *ALS* gene expression between the two populations, and the variation trend of *ALS* gene expression in the two populations tended to be consistent within 5 d. In addition, anti-ALS immunoblotting was used to determine whether changes in ALS protein abundance were associated with imazamox resistance (Figure 4B). The results showed that there was no positive correlation among transcription expression, *ALS* Trp-574-Leu mutation, and ALS protein abundance. Imazamox resistance in JHXY-2 (R) population with the *ALS* Trp-574-Leu mutation did not relate with changes in ALS protein abundance.

HPLC-MS/MS Analysis of the Imazamox Residue

The residues of imazamox in stems and leaves of *E. crus-galli* were detected within 48 h after treatment with imazamox. In the S population, the residual amounts of imazamox at 12 h, 24 h, and 48 h were 84.4 μg, 99.8 μg, and 79 μg, respectively, while, the residual amounts were 72.8 μg, 81.6 μg, and 75 μg in the R population, respectively (Figure 5). We found that the amount of imazamox absorbed by *E. crus-galli* increases over time and may begin to decrease at 24 h. In addition, the imazamox residues of R population were consistently lower than those of the S population. A significant difference was found at 12-h and 24-h time points in the residual amounts of imazamox; thus the possibility of metabolic resistance was suspected.

RNA-seq Data

The sample in RNA-seq experiment is labeled S/R0/12/24-1/2/3. Eighteen cDNA samples were sequenced using the Illumina sequencing platform (Illumina, San Diego, California, USA), and each sample produced at least 5.4 G of clean data (Supplementary Table S8). The percentage of Q20 exceeded 98.13%, the percentage of Q30 exceeded 94.64%, and the GC (guanine / cytosine) content of each sample varied between 52.44% and 53.65% (Supplementary Table S8). Each population had a different number of single gene clean reads (36756570 to 58280754) (Supplementary Table S9), indicating a high degree of accuracy in sequencing. The read segment comparison tool in Bowtie 2 v. 2.2.8 (Johns Hopkins University, Washington, USA) was used to conduct a comparative analysis of high-quality clean read segments against the ribosomal sequences of the species. After the rRNA read segment was removed, the retained data were used for subsequent analysis.

Identification and Validation of Differentially Expressed P450 and GST Genes Using RNA-seq and RT-qPCR

Transcriptome sequencing was conducted on the two populations, resulting in a total of 110,617 sequences being identified using the *Paspalum* genome as a reference. Among these were 990 *P450* contig sequences and 283 *GST* contig sequences. Subsequently, genes exhibiting significant differences in expression levels as well as stably expressed genes were selected for validation (refer to

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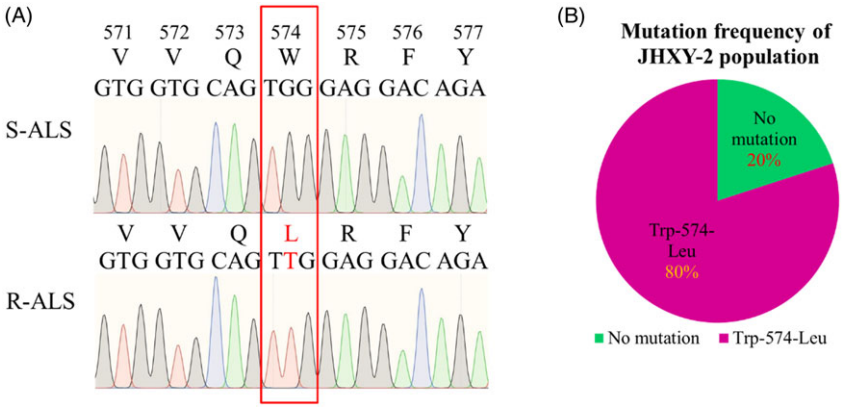

Figure 3. Sequence analysis of *ALS* gene in *Echinochloa crus-galli* resistant population JHXY-2 (R). (A) Nucleotide sequence alignment of *ALS* gene fragments in R and S populations. (B) Mutation frequency of the JHXY-2 (R) population.

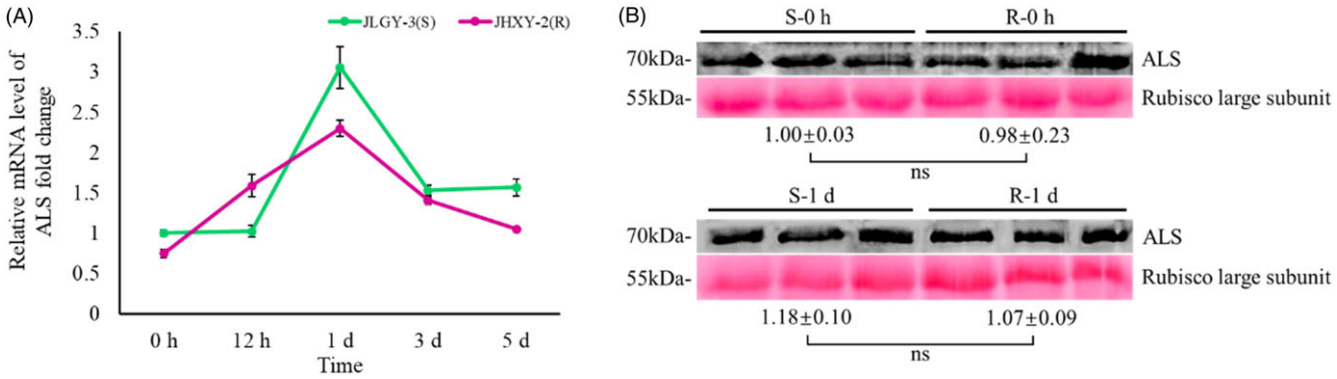

Figure 4. (A) Relative mRNA level of *ALS* gene in *Echinochloa crus-galli* JLGY-3 (S) and JHXY-2 (R) populations with imazamox treatment (120 g ha^{-1}). (B) The ALS (69 kDa) abundance in different populations determined by immunoblotting and the grayscale analysis of ALS protein. Protein levels are relative to S-0 h. Vertical bars represent the mean ± SE.

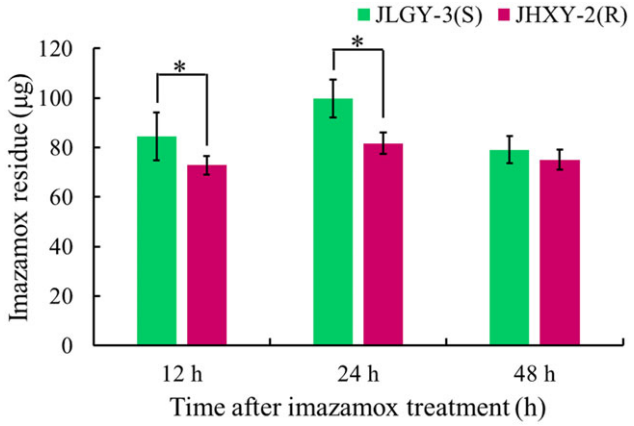

Figure 5. Imazamox residue in susceptible (S) and resistant (R) *Echinochloa crus-galli* at 12 h, 24 h, and 48 h after treatment. Means and SEs from three biological replicates are shown. An asterisk (*) indicates significant differences between R and S populations: P-value < 0.05.

Table 3). The findings revealed no significant difference in *P450* gene expression between the JLGY-3 (S) and JHXY-2 (R) populations; however, one gene (AH01.132, GSTU1) within the *GST* gene family exhibited statistically significant upregulation.

ALS inhibitors are the most commonly used herbicides in rice fields, but when weeds become resistant to one ALS inhibitor, growers may apply a different ALS inhibitor or herbicides with different modes of action (ACCase inhibitors, 4-hydroxphenyl-pyruvate dioxygenase inhibitors, or auxin mimic herbicides) (Liu et al. 2021). However, in China, farmers plant IMI-T rice and use imazamox to control weeds in rice fields, which leads to the continuous use of imazamox in IMI-T rice fields. This behavior undoubtedly accelerates the development of weed resistance. In a region of Jiangsu, China, we collected a suspected imazamox-resistant *E. crus-galli* population JHXY-2 (R) from IMI-T rice fields and determined its resistance to imazamox through whole-plant bioassays. The results showed that the population had a high level of resistance to imazamox, with an RI of 32.1. Based on our research findings, we do not advocate the use of alternative ALS inhibitors in IMI-T rice fields. Given that the weeds exhibiting the Trp-574-Leu mutation demonstrate resistance to all five classes of ALS inhibitors currently employed, it can be inferred that they may possess resistance to all ALS inhibitors. However, employing a rotation or mixture of herbicides with diverse modes of action may improve weed management efficiency.

The mechanisms of herbicide resistance in weeds are currently divided into TSR and NTSR (Yu and Powles 2014a). Many studies have shown that both TSR and NTSR coexist in resistant weeds, so studying either TSR or NTSR alone is not enough to fully understand the causes of herbicide resistance (Yu and Powles 2014a). Therefore, this study investigated both TSR and NTSR mechanisms of imazamox resistance in weeds from IMI-T rice

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Table 3. Identification of the upregulated genes potentially related with herbicide metabolism in *Echinochloa crus-galli* imazamox-resistant population via RNA-seq and real-time quantitative reverse transcriptase PCR (RT-qPCR).

			Relative expression change (R_T/R-CK)/(S_T/S-CK)[a]						
			RNA-seq		RT-qPCR RNA-seq samples		RT-qPCR parallel samples		
Definition	Gene ID	Function annotation	12 h	24 h	12 h	24 h	12 h	24 h	48 h
Cytochrome P450s	BH07.217	CYP	1.35	1.57	1.01	1.09	1.61	1.61	2.13
	AH01.4462	CYP74A2	0.49	1.47	0.33	3.01	0.57	2.32	2.85
	AH03.1780	CYP28	0.76	0.76	0.76	1.28	1.44	1.42	1.43
	BH01.1428	CYP71C4	1.27	0.87	0.19	0.70	0.25	1.98	0.66
	BH07.64	CYP74B2	1.22	0.99	0.98	1.16	1.01	2.15	0.89
	BH01.3861	CYP709B2-1	1.75	0.72	1.58	0.85	1.42	1.92	1.73
	CH01.4123	CYP709B2-2	0.79	3.71	0.76	0.96	0.48	0.84	0.36
Glutathione *S*-transferase	AH01.3113	GSTU6	1.34	1.60	0.73	1.54	1.68	1.52	0.72
	AH01.4114	GSTZ5	1.02	1.14	1.00	1.64	1.89	1.34	0.96
	AH05.456	GSTZ1	1.85	0.85	1.11	1.49	1.09	1.22	1.30
	AH01.132	GSTU1	3.96	4.07	1.98	2.53*	1.62	2.65*	1.55
	BH01.4420	GSTZ5-1	1.16	1.35	1.09	1.67	1.14	1.57	0.72
	BH07.3899	GSTZ1-1	1.26	0.63	1.09	1.10	1.94	1.15	0.89
	CH01.3708	GSTU6-1	0.75	0.95	0.84	1.36	0.87	1.75	0.62

[a]R_T means the R population was treated with imazamox. R_CK means the R population was not treated with imazamox. An asterisk (*) indicates that the gene expression level of the R population was significantly higher than that of the S population: P-value < 0.05.

fields. With respect to TSR, after determining the enzyme activity in vitro, we found that the ALS enzyme of the R population with the Trp-574-Leu mutation was less sensitive to imazamox and penoxsulam than that of the S population, resulting in relative resistance ratios of 12.33 and 6.6, which is basically consistent with the previous studies (Palma-Bautista et al. 2020; Panozzo et al. 2013). For example, it has been found that ALS in *E. crus-galli* is simultaneously less sensitive to penoxsulam and imazamox (Panozzo et al. 2013). Some studies have also found that the ALS of wild poinsettia (*Euphorbia heterophylla* L.) is less sensitive to imazamox (Palma-Bautista et al. 2020). In previous studies, a total of nine amino acid substitutions have been found to potentially cause herbicide resistance in weeds (Fang et al. 2022; Liu et al. 2021), and the frequency of the Trp-574-Leu substitution is much higher than that of other amino acid substitutions (Heap 2024). *E. crus-galli* is a hexaploid weed species possessing three copies of the *ALS* gene (Iwakami et al. 2015). Upon examining the *ALS* sequences in the R population, we indeed identified three distinct copies of the *ALS* gene. Furthermore, we observed variations in the number of *ALS* copies between susceptible and resistant populations. This observation of gene-copy number variation within a single species is not unprecedented; Iwakami et al. (2017) previously reported copy-number diversity among *A. aequalis* populations exhibiting resistance to thifensulfuron-methyl, with some populations harboring two copies while others contained four, but no differences in resistance levels were observed in these populations. In a polyploid species with multiple *ALS* copies, "a dilution effect" will occur if only some of the copies carry *ALS* mutations (Panozzo et al. 2021). However, this phenomenon does not seem to occur in resistant populations. Bioassay experiments indicated that the R population exhibited a higher RI compared with the S population under treatment with imazamox, and ALS enzyme activity assays corroborated this trend.

Changes in the expression level of target genes are often thought to be the cause of herbicide resistance in weeds (Laforest et al. 2017; Ngo et al. 2018). However, it was difficult to find a consistent change in the expression level of the *ALS* gene in weeds after herbicide treatment in many previous studies (Fang et al. 2020; Laforest et al. 2017; Liu et al. 2022; Ngo et al. 2018). To determine whether there is a difference in the expression of the *ALS* gene between the R and S populations after imazamox treatment, we monitored the expression of the *ALS* gene at five time points after imazamox treatment. The results showed that the expression of the *ALS* gene in the R population was only higher than that of the S population for a short period of time before and after 12 h. The expression of the *ALS* gene in both R and S populations increased significantly within 1 d after imazamox treatment, and the overall trend was almost the same. We hypothesize that after herbicide treatment, weeds will experience abiotic stresses; this phenomenon likely occurs in all weeds, and there is no difference between the resistant and sensitive populations. The changes in gene expression may not be the cause of herbicide resistance. Nevertheless, the primers employed in RT-qPCR were capable of amplifying all *ALS* genes from both populations; thus, it remained plausible that a single copy of *ALS* in the resistant population may exhibit higher expression level compared with others.

At the same time, we monitored the changes of ALS protein abundance with anti-ALS antibody in JHXY-2 (R) and JLGY-3 (S) populations after imazamox treatment, and no significant difference was found. Changes in ALS protein abundance were compared with the changes in *ALS* gene expression and amino acid mutation, and the results showed no direct correlation. Therefore, we hypothesized that the change in ALS protein abundance is not the cause of the resistance of JHXY-2 (R) to imazamox. Trp-574-Leu substitution did not affect ALS protein accumulation. However, it is still not ruled out that changes in ALS protein abundance in other resistant weeds can lead to resistance, and further studies are needed to clarify this phenomenon (Chen et al. 2020).

In NTSR studies, researchers often used some metabolic enzyme inhibitors to treat weeds, and the change in the RI was used to evaluate whether metabolic resistance was involved (Gao et al. 2023; Pan et al. 2022). A decreased RI may be interpreted as indicating metabolic resistance, and further research may be undertaken. However, there have been few studies on the metabolic resistance of weeds that show no significant change in RI under treatment with metabolic enzyme inhibitors (Liu et al.

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2024a). In this study, when S and R populations were treated with malathion, PBO, or NBD-Cl, no significant RI changes were detected in either the S or R populations. Nevertheless, the possibility remains that NTSR may be present in JHXY-2 (R). The results of HPLC-MS/MS showed that the residual amount of imazamox in the leaves of the JHXY-2 (R) population was lower than that of the JLGY-3 (S) population at the 12-h, 24-h, and 48-h time points. We suspect that there may be a small number of metabolic genes playing roles in improving the survival ability of the JHXY-2 (R) population under imazamox application. Transcriptome sequencing is often used to examine weed metabolic resistance, and this technology can identify a large number of differentially expressed genes and metabolic pathways (Dimaano and Iwakami 2021; Pan et al. 2019; Wang et al. 2023). Through transcriptome sequencing, it was discovered that *P450* genes *CYP99A44* and *CYP704A177* are associated with meso-sulfuron-methyl resistance in American sloughgrass [*Beckmannia syzigachne* (Steud.) Fernald] (Bai et al. 2022), while *P450* gene *CYP81A68* is linked to penoxsulam resistance in *E. crus-galli* (Pan et al. 2022). Additionally, the *GST* gene *AmGSTF1* is associated with fenoxaprop-*p*-ethyl resistance in *A. myosuroides* (Cummins et al. 2013). A total of 990 *P450* sequences and 283 *GST* sequences were identified through transcriptome sequencing. The FPKM (fragments per kilobase of exon model per million mapped fragments) of *P450* and *GST* genes was compared between S and R populations, revealing only a few genes with an expression difference ratio greater than 2. After selecting some genes for expression verification, it was found that only the gene expression of *GSTU1* was significantly upregulated in the R population in comparison with S populations. However, considering the differences in residues of imazamox in the leaves of *E. crus-galli*, there may still be some unidentified metabolic enzyme genes that mediate the metabolism of imazamox by *E. crus-galli*. Alternatively, there may be differences in the uptake of imazamox between S and R populations, with the R population reducing its uptake of imazamox (Yu et al. 2013).

In conclusion, we found that *E. crus-galli* collected from IMI-T fields has evolved imazamox resistance. Our study revealed that a Trp-574-Leu mutation in the JHXY-2 (R) population and decreased sensitivity of ALS to imazamox in JHXY-2 (R) are the main resistance mechanisms. Importantly, we further provided key evidence that the Trp-574-Leu mutation did not lead to the change in ALS protein abundance. Our research on NTSR has revealed differences in the residues of imazamox between R and S populations. Identifying new genes that may mediate the metabolism of imazamox in *E. crus-galli*, and detecting the uptake of imazamox between populations between S and R are worth investigating in future studies to clarify the potential NTSR in resistant *E. crus-galli*.

Supplementary material. For supplementary material accompanying this paper visit https://doi.org/10.1017/wsc.2024.73

Data availability. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive in National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA017006) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa. GenBank accession nos. MH013497.1 (ALS1) and MH013498.1(ALS2).

Acknowledgments. The authors appreciate helpful discussion with Dr. Hao Wang and Ms. Ying Liu.

Funding statement. This research was supported by the Natural Key R&D Program of China (2023YFD1400503).

Competing interests. The authors declare no conflicts of interest.

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https://doi.org/10.1017/wsc.2024.73 Published online by Cambridge University Press