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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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Abstract:
Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] is increasingly infesting imidazolinone-tolerant (IMI-T) rice fields in China, imazamox resistance of *E. crus-galli* 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, JHXY-2 (R) population showed a high level of herbicide resistance with a resistance index (RI) 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 (ACCase). In order to understand the reason why JHXY-2 (R) was resistant to imazamox, we performed experiments to characterize potential TSR and NTSR mechanisms. A trp-574-leu amino acid mutation in ALS and low imazamox ALS sensitivity were the main mechanism 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 the P450/GST inhibitors did not alter the resistance level of JHXY-2 (R) against imazamox. To further clarify the NTSR mechanism of JHXY-2 (R), 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 GST-U1 showed a significant induction in R population. 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 the *E. crus-galli*, there may still be undetectable NTSR that are causing imazamox resistance in the R population.

Keywords: *Echinochloa crus-galli*, imazamox, IMI-T rice fields, target-site resistance, ALS protein abundance

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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, 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 IMI-T rice cultivars, combined 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 broad-leaved weeds. In China, the main weeds in paddy fields include weedy rice, *Echinochloa* species, Chinese sprangletop [*Leptochloa chinensis* (L.) Nees], large crabgrass [*Digitaria sanguinalis* (L.) Scop.], heusenkraut (*Ludwigia prostrata* Roxb.) and variable flatsedge (*Cyperus difformis* L.) etc (Liu et al. 2021). Most of the weeds, including *Echinochloa* species, can be controlled by spraying imazamox in the IMI-T rice fields in 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* species in the rice field became rampant. After observing the samples of *Echinochloa* species 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.

Echinochloa crus-galli is a very common malignant weed in the rice fields, and the control of *E. crus-galli* mainly depends on the use of various herbicides (Fang et

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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 which have a more intricate genomic architecture compared to 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 in 2017 unveiled the complexities of its genome (Guo et al. 2017). In polyploid species, gene copy number at each locus can vary (Panozzo et al. 2021); for instance, Fang et al. reported differences in ALS gene copy numbers across various populations of *E. crus-galli* (Fang et al. 2019a), while Iwakami et al. observed similar variability in ALS gene copy numbers within shortawn foxtail (*Alopecurus aequalis* Sobol.) (Iwakami et al. 2017).

Imazamox is an ALS inhibitor, which inhibit the biosynthesis of the branched-chain amino acids, valine, leucine, and isoleucine, and eventually leads to growth arrest and plant death (Duggleby et al. 2008). At present, the research on herbicide resistance mechanisms is mainly divided into target-site resistance (TSR) and non-target-site resistance (NTSR) (Yu and Powles 2014a). The progress on TSR mainly focuses on the activity of target enzyme (Fang et al. 2019b), the mutation of target gene (Yu and Powles 2014b) and the change of target gene expression (Gao et al. 2017). Most studies have shown that the acetolactate synthase (ALS) activity of resistant weeds with ALS gene mutations is higher than that of the susceptible (Fang et al. 2019b; Gao et al. 2023; Li et al. 2017; Panozzo et al. 2013). The research of target gene mutation is more clear. At present, a total of 30 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

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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 utilized 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 rice barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.] population with NTSR have demonstrated that this weed population have developed resistance to penoxsulam, cyhalofop-butyl and florpyrauxifen-benzyl. A resistant population of *E. crus-galli* has also been found to be resistant to penoxsulam, pinoxaden and quinclorac. The study of NTSR mainly involves three aspects: the enhancement of herbicide metabolism (Délye 2013; Pan et al. 2022), the decrease of herbicide penetration and transport (Riar et al. 2013), and the 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 is found that CYP81A68

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mediate 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 *Am*GSTF1 mediates the resistance of blackgrass (*Alopecurus myosuroides* Huds.) to various herbicides, including chlorotoluron, fenoxaprop-p-ethy and clodinafop-propargyl (Cummins et al. 2013).

Therefore, this study was 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 ACCase-inhibiting herbicides in this R population, (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

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 susceptible population JLGY-3 (S) were collected from a 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 × 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 three- to four-leaf stage, herbicides were applied using a laboratory sprayer equipped with a flatfan nozzle, delivering 280 L ha^{-1} at 230 kPa. The commercial preparation of imazamox is provided by Jiangsu

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Zhongqi Technology Co., LTD. The subsequent use of imazamox is the same commercial preparation, diluted to the corresponding dose or concentration when used. Based on a preliminary experiment (data not shown), imazamox was applied at 0, 3.75, 7.5, 15, 30, 60, and 120 g of active ingredient (a.i.) 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, and 960 g a.i. ha^{-1}. After 21 days of imazamox treatment, the above-ground parts of the *E. crus-galli* 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 Supporting 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 (4200 g a.i. ha^{-1}), malathion (1000 g a.i. ha^{-1}), and NBD-Cl (270 g a.i. 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 above.

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 TPP and 10 μM FAD) was added, then shaken for 3 min and left it on ice for 15 min. The plant homogenate was filtered with gauze mesh, and the filtrate was centrifuged and treated with ammonium sulfate

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to obtain protein precipitation. The protein precipitates of S and R populations were dissolved in 4 mL 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 crude enzyme solution and 100 μL ALS inhibitor (imazamox at 0.001, 0.01, 0.1, 1, 10, 100 and 1000 μM, Penoxsulam at 0.001, 0.01, 0.1, 1, 10, 100 and 1000 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 Creatine solution (0.55%) and 190 μL α-naphthol solution (5.5% in 5 N NaOH) at 60°C 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 crude enzyme solution and 100 μL 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. Amplification reaction was set as follows: 3 min at 95 °C for

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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, 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. This reaction was incubated at 16 °C for 30 minutes before transforming 10 μL of the reaction product into *E. 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 hours, 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 6.1.

Determination of ALS gene expression by 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 RNAs of *E. crus-galli* was extracted using the RNA Simple Total RNA Kit (Tiangen, Beijing, 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, Nanjing, China). The β-actin gene of *E. crus-galli* (GenBank accession number: 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

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Biosystems, Waltham, MA) using the ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., 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 Supporting Table S2. The fold changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method. Significant differences in expression levels were analyzed by 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 set at each time point. Leaves of *E. crus-galli* were ground 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 Protein extraction buffer (10% Glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 10 mM DTT, 1×Protein inhibitor cocktail, 2% PVPP) for 2 min. Centrifuge at 12, 000 rpm for 5 min at 4 ℃ and collect the supernatant. Add protein loading buffer and boil for 5 min. The protein samples were separated by 10% SDS-PAGE and then transferred to PVDF membrane. Primary ALS-antibody against *E. crus-galli* ALS have been prepared in our previous study (Liu et al. 2024b), and it was applied at a dilution of 1:5000. Goat Anti-Mouse IgG HRP was applied at a dilution of 1:10000. The protein strips were exposed by enhanced chemiluminescence, and the grayscale of the bands was quantified by Image J software.

HPLC-MS/MS analysis of the imazamox residue

When the *E. crus-galli* reached the 3- to 4-leaf stage, each grass plant was treated with 4 μL of imazamox with a concentration of 0.03675 g/mL at the leaf surface. Applied the liquid evenly on the fully opened leaves of the *E. crus-galli*. The application dose of imazamox on each *E. crus-galli* plant was equivalent to using 120 g a.i. 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

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replicates set at each time point, and each replicate containing five grass 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 Acetonitrile for 5 min, then 2.0 g NaCl and 4.0 g $MgSO_4$ were added, continued vortexing for 30 sec, and centrifuged at 4000 rpm for 3 min. The extract (2 ml) was transferred to a purification tube containing 400 mg C_{18} and 400 mg $MgSO_4$. The tube was shaken for 5 min to complete the purification and centrifuged for 3 min at 12,000 rpm. 1 mL of supernatant was transferred to the syringe and then filtered using a 0.22 μm organic filter membrane. imazamox amount was determined by HPLC-MS/MS (Agilent 6460 Triple Quad, Agilent). The chromatography was performed on waters BEH C18 column 1.7 μm, 2.1 mm X 50 mm. 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 Supporting Table S3. Multiple reaction monitoring (MRM) setting conditions are shown in Supporting Table S4. The calibration curve of imazamox is provided in Supporting 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 by using Welch's t-test.

Sample preparation for RNA-seq

Cultivate the plants to the three- to four-leaf stage, and then collect the leaf tissues of S and R population of *E. crus-galli* at 0, 12 h and 24 h after treatment with imazamox (120 g a.i. ha^{-1}). The application method of imazamox was the same as that described above. Three biological replicates are set up for each time point, with each replicate containing 0.5 g of leaf tissue. After collecting the leaf samples, immediately freeze

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them in liquid nitrogen and store 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 RNA Simple Total RNA Kit (Tiangen, Beijing, China), 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 FDR < 0.05 and an absolute log2 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 Co., Ltd., Nanjing, China), fourteen 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 Supporting Table S6. RT-qPCR analyses were performed according to section 2.5. The *E. crus-galli* β-actin gene was used as the internal control gene. Total RNAs of S and R populations collected at 0 h, 12 h and 24 h time points were extracted using RNA Simple Total RNA Kit (Tiangen, Beijing, China), following the manufacturer's instructions. The expression changes of fourteen genes picked from the metabolizing enzyme library were verified with the above-mentioned method.

Data analysis

All whole-plant dose response data was collected for analysis of variance (ANOVA)

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using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). The results showed that there was no significant difference between parallel experiments ($P > 0.05$). Process the data using SigmaPlot 12.5 (SigmaPlot Software Inc.,Chicago, IL, 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:

$$Y=c+(d-c)/[1+(x/g)^b]$$

where Y was the aboveground fresh weight of weeds, x was the herbicide dose, b was the slope of the curve, c was the lower limit, d was the upper limit, and g was the herbicide dose at the point of inflection halfway between the upper and lower limits. The same methods were used 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 a.i. ha^{-1}) was higher than the recommended application dose (120 g a.i. ha^{-1}), while the GR_{50} of the susceptible population JLGY-3 (S) was only 5.19 g a.i. ha^{-1} (Table 1 and Fig. 1). Whole-plant bioassays showed that the JHXY-2 (R) population still survived tenaciously under imazamox treatment at a much higher dose (960 g a.i. ha^{-1}). According to the calculation, the RI of the JHXY-2 (R) population to imazamox was up to 32.1.

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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 a.i.ha^{-1}		RI
	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

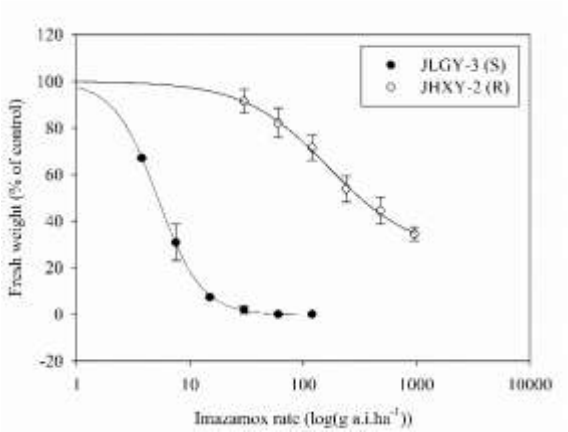

Fig. 1. Fresh weight of *E. crus-galli* treated with imazamox. Vertical bars represent the mean ±standard error.

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 RIs of 14.99 (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 RI of 1 for metamifop and 1.6 for cyhalofop-butyl.

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Table 2 Sensitivity of the two *E. crus-galli* populations to other herbicides

Target	Group [a]	Herbicide	GR_{50}(±SE)		RI[b]
			JLGY-3 (S)	JHXY-2 (R)	
acetolactate synthase	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
acetyl-CoA carboxylase		metamifop	28.82 (±4.77)	28.82 (±3.17)	1
	APP	cyhalofop-butyl	29.4 (±7.72)	17.54 (±7.39)	1.6

[a]Abbreviations: SCT, sulfonylamino-carbonyl-triazolinone; TP, triazolopyrimidine; SU, sulfonylurea; PTB, pyrimidinylthiobenzoate; APP, aryloxy phenoxy propionate.

[b]RI is the resistance index.

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 was increased under the treatment of the two P450 inhibitors. Under the treatment of GST inhibitor (NBD-Cl), the GR_{50} value of the R population was 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 (Fig. 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 the treatment of imazamox (Fig. 2A). There was a 12.3-fold difference in ALS activity

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between the two populations. The IC_{50} value of JLGY-3 (S) and JHXY-2 (R) was 0.61 μM and 4.03 μM under the treatment of penoxsulam, and there was also 6.6-fold difference (Fig. 2B).

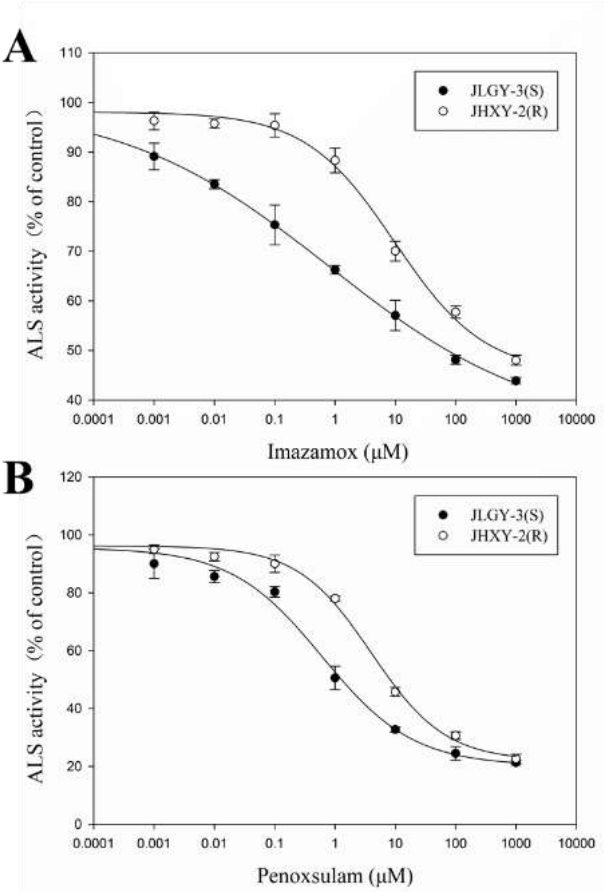

Fig. 2. *In vitro* ALS activity of two *E. crus-galli* populations. (A) Under the treatment of imazamox. (B) Under the treatment of penoxsulam. Vertical bars represent the mean ± standard error.

Gene cloning and sequence analysis

The sequences of JLGY-3 (S) population 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 a substantial number of sequences, we distinctly identified single nucleotide polymorphisms (SNP) at several conserved sites across different gene copies and

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established that the JHXY-2 population possesses ALS gene copies (Table S7). Comparative analysis of codons at positions 217, 322, and 441 allows for the differentiation of ALS3 from ALS1 and ALS2. Furthermore, by examining codons at positions 265 and 441, a distinction can be made between ALS1 and ALS2 (Table S7). In the JHXY-2 (R) population, we identified three copies of the ALS gene, whereas in the JLGY-3 population, two copies of the ALS gene were observed. The observed variation in gene copy number in the present study is consistent with that previously reported in *E. crus-galli*, *A. aequalis* and *A. japonicus* (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) (Fig. 3A). Following our classification, we observed that the trp-574-leu mutation can manifest in two copies within individual plant and may occur in either ALS1 or ALS3, or concurrently in both ALS2 and ALS3. In addition, we detected the ALS sequences of R population in a total of 80 individual plants, of which 64 contained trp-574-leu mutations, accounting for 80% (Fig. 3B).

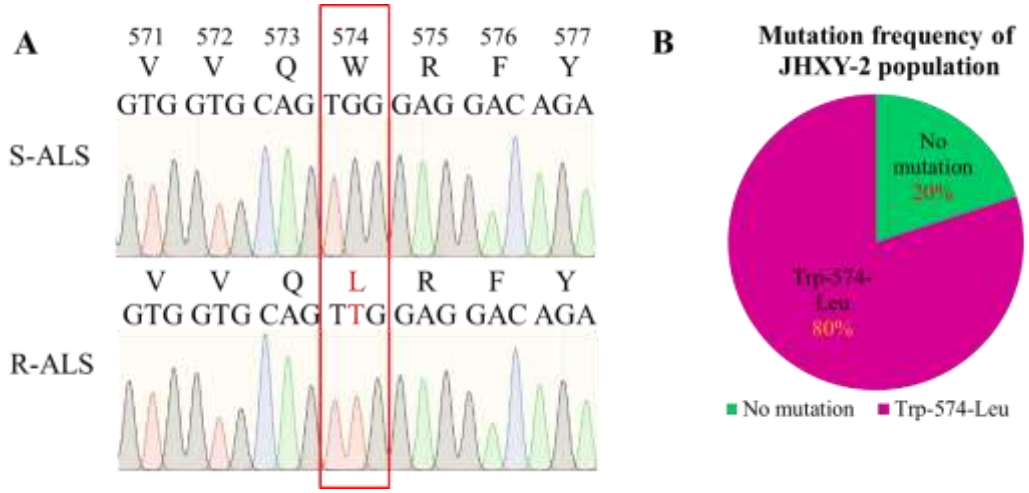

Fig. 3. Sequence analysis of acetolactate synthase gene in resistant population JHXY-2 (R). (A) Nucleotide sequence alignment of ALS gene fragments in R and S populations. (B) Mutation frequency of the JHXY-2 (R) population.

Gene expression level and protein abundance of ALS between R and S

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The expression levels of ALS genes in the S and R populations were monitored at five time points (0h、12h、1d、3d and 5d) after treatment with imazamox (120 g a.i. ha^{-1}) (Fig. 4A). It was found that there was no significant difference in ALS genes expression between the two populations, and the variation trend of ALS genes expression in the two populations tended to be consistent within 5 days. In addition, Anti-ALS immunoblotting was used to determine whether changes in ALS protein abundance were associated with imazamox resistance (Fig. 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 ALS trp-574-leu mutation did not relate with changes of ALS protein abundance.

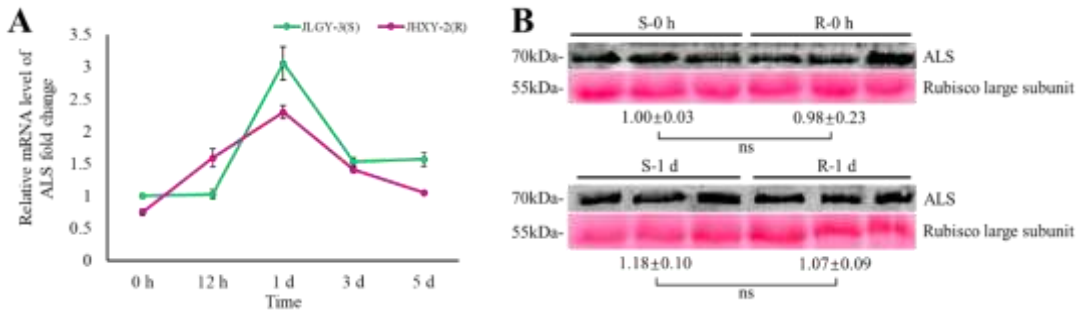

Fig. 4. (A) Relative mRNA level of ALS gene in JLGY-3 (S) and JHXY-2 (R) populations with imazamox treatment (120 g a.i. ha^{-1}). (B) The ALS (69 kDa) abundance in different populations determined by immunoblotting and the gray scale analysis of ALS protein. Protein levels are relative to S-0 h. Vertical bars represent the mean ± standard error.

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 (Fig. 5). We found that the amount of imazamox absorbed by *E. crus-galli* increased over time and may begin to decrease at 24 h. In addition, the imazamox residues of R population

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were consistently lower than those of S population. A significant difference was found between the 12-and 24-h treatments in the residual amounts of imazamox, thus the possibility of metabolic resistance was suspected.

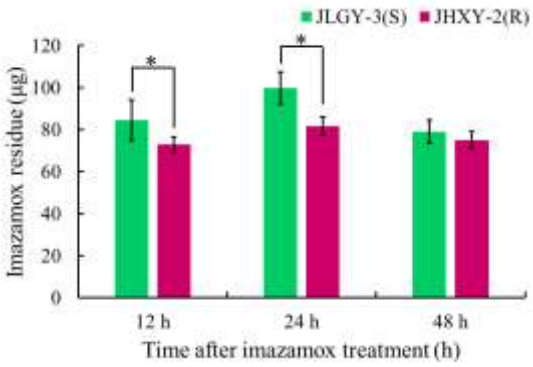

Fig. 5. Imazamox residue in susceptible (S) and resistant (R) after 12, 24 and 48 h treatment. Means and standard errors from three biological replicates are shown. *indicates significant differences between R and S populations, *P*-value < 0.05.

RNA-seq data

The sample in 2.8.1 is labeled S/R0/12/24-1/2/3. Eighteen cDNA samples were sequenced using the Illumina sequencing platform, and each sample produced at least 5.4 G of clean data (Table S8). The percentage of Q20 exceeded 98.13%, the percentage of Q30 exceeded 94.64%, and the GC content of each sample varied between 52.44% and 53.65% (Table S8). Each population had a different number of single gene clean reads (36756570-58280754) (Table S9), indicating a high degree of accuracy in sequencing. Utilize the read segment comparison tool Bowtie 2 (version 2.2.8) to conduct a comparative analysis of high-quality clean read segments against the ribosomal sequences of the species. After the rRNA read segment is removed, the retained data is 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, there were 990 P450 contig sequences and 283 GST contig sequences.

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Subsequently, genes exhibiting significant differences in expression levels as well as stably expressed genes were selected for validation (refer to Table 3). The findings revealed no significant difference in P450 gene expression between the JLGY-3 (S) and JHXY-2 (R) populations, however, only one gene (AH01.132, GSTU1) within the GST gene family exhibited a statistically significant upregulation.

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Table3 Identification of the up-regulated genes annotated to metabolism in *E. crus-galli* imazamox-resistant population via RNA-Seq and RT-qPCR

Definition	Gene ID	Function annotation	Relative expression change (R_T/R-CK)/(S_T/S-CK)[a]						
			RNA-seq		RT-qPCR RNA-Seq samples		RT-qPCR parallel samples		
			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
	AH01.1	GSTU1	3.9	4.0	1.98	2.53*	1.	2.6	1.

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32		6	7			62	5*	55
BH01.420	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 treatment with imazamox. R_CK means the R population was not treat with imazamox. *indicates that the gene expression level of the R population was significantly higher than that of the S population, *P*-value < 0.05.

ALS-inhibiting herbicides are the most commonly used herbicides in rice fields, and when weeds become resistant to ALS inhibitors, people usually choose different ALS inhibitors or different modes of action herbicides (ACCase inhibitors, HPPD inhibitors or Auxin mimic herbicides) to control weeds (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 a 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 trp-574-leu mutation demonstrates resistance to all five classes of ALS inhibitors currently employed, it can be inferred that it may possess resistance to all ALS inhibitors. However, employing a rotation or mixture of herbicides with diverse modes of action may improve the efficiency of weed management.

The mechanisms of herbicide resistance in weeds are currently divided into target-site resistance (TSR) and non-target-site resistance (NTSR) (Yu and Powles

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2014a). Many studies have shown that both TSR and NTSR co-exist 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 the mechanisms of imazamox resistance in weeds from IMI-T rice fields on both the TSR and NTSR sides. In the TSR aspect, after determining the enzyme activity *in vitro*, we found that the ALS enzyme of the R population with 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). Iwakami et al. proposed that *E. crus-galli* is a hexaploid weed species possessing three copies of the ALS gene. Upon examining the ALS sequences in 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. previously reported copy number diversity among shortawn foxtail populations exhibiting resistance to thifensul-furon-methyl, with some populations harboring two copies while others contained four, but no differences in resistance levels were observed in these populations (Iwakami et al. 2017). In apolyploid species with multiple ALS copies, “a dilution effect” will occur if only some of the copies carries ALS mutations (Panozzo et al. 2021). However, this phenomenon does not seem to occur in resistant populations. Bioassay experiments

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indicated that the R population exhibited a higher RI compared to the S population under the treatment of 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). In order 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 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 hours. The expression of the ALS gene in both R and S populations increased significantly within one day after imazamox treatment, and the overall trend was almost the same. We hypothesize that after herbicide treatment, weeds will experience abotic 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 to 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.

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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 the study of NTSR, people often used some metabolic enzyme inhibitors to treat weeds, and the change in the RI is used to evaluate whether metabolic resistance is involved (Gao et al. 2023; Pan et al. 2022). Usually, when the RI decreases, people will think that there may be metabolic resistance and continue with further research. 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. 2024a). In this study, when S and R populations were treated with Malathion, PBO or NBD-Cl, no significant RI changes were detected on either the S or R populations. Nevertheless, there still has the possibility that NTSR may still 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 still 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 in understanding 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 mesosulfuron-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 *Am*GSTF1 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 of P450 and GST genes was compared between S and R populations, revealing

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only a few genes with an expression difference ratio greater than 2 (FPKM: Fragments Per Kilobase of exon model per Million mapped fragments). After selecting some genes for expression verification, it was found that only the gene expression of GSTU1 was significantly up-regulated 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 which 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 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 mechanism. Importantly, we further provided the key evidence that trp-574-leu mutation did not lead to the change of ALS protein abundance. The 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 to investigate in future studies to clarify the potential NTSR in resistant *E. crus-galli*.

Supporting information

Table S1. Herbicide doses applied in whole-plant dose-response bioassays.

Table S2. Primers used to detect ALS expression in *Echinochloa crus-galli* by RT-qPCR.

Table S3. Gradient elution conditions with HPLC-MS/MS.

Table S4. Multiple Reaction Monitoring (MRM) setting conditions.

Table S5. The calibration curve of imazamox using QuEChERS and HPLC-MS/MS.

Table S6. Primers used for RT-qPCR.

Table S7. The single nucleotide polymorphisms in multiple sites of ALS sequences.

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Table S8. Base information statistics table before and after filtering of RNA-seq.

Table S9. Reads filter information statistics of RNA-seq.

Accession codes

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 numbers MH013497.1 (ALS1) and MH013498.1(ALS2).

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Competing interests

None

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