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A Cys-2088-Arg mutation in *ACCase* confers cross-resistance to ACCase-inhibiting herbicides in barnyard grass (*Echinochloa crus-galli*)

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Abstract

Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] is a dominant weed species occurring in rice fields across China. Metamifop, a common herbicide, is frequently applied to control *E. crus-galli* and other grassy weeds in rice fields. Herein, HS01, an *E. crus-galli* population suspected to be resistant (R) to metamifop, was collected from Hanshan County in Anhui Province. Whole-plant dose-response testing revealed that, compared with the susceptible (S) population FD03, HS01 had developed high-level resistance to metamifop with a resistance index (RI) of 11.76 and showed cross-resistance to cyhalofop-butyl (RI=9.33), fenoxaprop-*P*-ethyl (RI=5.80) and clethodim (RI=3.24). Gene sequencing revealed a Cys-2088-Arg mutation in *ACCase 1,5* allele of all the R plants, while *ACCase* gene overexpression was not involved in the resistance. Molecular docking indicated that the less negative binding energies might be the main reason for the resistance of HS01 to ACCase-inhibiting herbicides. A derived cleaved amplified polymorphic sequence (dCAPS) method was developed for the rapid identification of the Cys-to-Arg mutation in the *ACCase* gene at codon position 2088 in *E. crus-galli*. Additionally, pretreatment with the cytochrome P450 inhibitor piperonyl butoxide or the glutathione S-transferase inhibitor 4-chloro-7-nitrobenzoxa-diazole had no significant effects ($p > 0.05$) on the resistance of HS01 to metamifop. To our knowledge, this is the first report of a Cys-2088-Arg mutation in *E. crus-galli ACCase* that confers cross-resistance to ACCase-inhibiting herbicides.

Keywords: Cys-2088-Arg, metamifop, dCAPS, cross-resistance

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Introduction

Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] is one of the most harmful annual grasses and can coexist in rice (*Oryza sativa* L.), maize (*Zea mays* L.) and other summer crop filed (Akbarabadi et al. 2019). Owing to its strong adaptability, *E. crus-galli* is difficult to be managed, resulting in significant reductions in crop production (Panozzo et al. 2017).

In the last two decades, *E. crus-galli* has been controlled mainly with chemical herbicides, especially acetyl-CoA carboxylase (ACCase; EC.6.4.1.2)-inhibiting herbicides (Group 1, Herbicide Resistance Action Committee) (Cai et al. 2022). ACCase is a biotinylated carboxylase that is crucial in the initial step of fatty acid generation that converts acetyl-CoA into malonyl-CoA while consuming adenosine triphosphate (ATP) (Ye et al. 2020). Furthermore, ACCase is the most important target and can be inhibited by three major groups of synthetic herbicides, including aryloxyphenoxypropionates (APPs), cyclohexanediones (CHDs) and phenylpyrazolines (PPZs) (Wang et al. 2021; Jiang et al. 2024). Metamifop, an ACCase inhibitor, is used for the POST control of grassy weeds, especially *E. crus-galli*, in rice fields (Li et al. 2023). However, *E. crus-galli* has rapidly developed resistance to metamifop as a result of its extensive and continued application (Sun et al. 2023).

Resistance to herbicides occurs via two types of mechanisms: target-site resistance (TSR) and non-target-site resistance (NTSR) (Gaines et al. 2020). TSR involves mutations in the target gene that result in an amino acid substitution (AAS), which can affect the interaction of target enzymes with herbicide (Gaines et al. 2019). TSR is the most common mechanism of weed resistance in herbicides, and generally results in resistance to other herbicides with the same mode of action (MOA) (Kaundun 2014; Wang et al. 2024). To date, seven codon positions in *ACCase* (Ile-1781, Trp-1999, Trp-2027, Ile-2041, Asp-2078, Cys-2088 and Gly-2096) have been identified as conferring herbicide resistance in different weed species (Beckie and Tardif 2012; Zhang et al. 2024). Among them, the Cys-2088-Arg mutation has been identified in several species, including Chinese sprangletop (*Leptochloa chinensis* (L.) Nees), blackgrass (*Alopecurus myosuroides* Huds.), rigid ryegrass (*Lolium rigidum* Gaudin). and sterile wild oat (*Avena sterilis* L.) (Yu et al. 2007; Papapanagiotou et al. 2015; Lan et al. 2022; Liao et al. 2024). Moreover, the overexpression of target gene can also be considered as one of the herbicide-resistance mechanisms of TSR in large crabgrass [*Digitaria sanguinalis* (L.) Scop.] and *E. crus-galli* (Laforest et al. 2017; González-Torralva and Norsworthy 2023). In contrast, NTSR involves resistance mechanisms that are independent of the target enzyme, and is generally associated with altered absorption, translocation, excretion and sequestration of the herbicide and increased herbicide metabolism (metabolic resistance) (Délye 2013; Jiang et al. 2022). Conversely, NTSR mechanisms are complicated and unpredictable and usually confer resistance to herbicides with different MOAs (Yu and

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Powles 2014). Metabolic resistance caused by increased activity of cytochrome P450 monooxygenase (P450) and glutathione S-transferase (GST) activity is the most common NTSR mechanism (Cai et al. 2022; Zhao et al. 2022).

In this study, we obtained a suspected metamifop-resistant *E. crus-galli* population, HS01, from a rice field in Hanshan County, Anhui Province. To clarify its mechanisms of resistance and facilitate efficient control strategies, our aims were as follows: (i) determining the resistance level to metamifop in the HS01 population, (ii) exploring the potential TSR and/or NTSR mechanisms involved, and (iii) characterizing the cross- and multiple-resistance of HS01 to herbicides with different MOAs.

Materials and methods

Plant materials and growth conditions

Mature seeds from at least 40 individuals of the putatively resistant (R) population (HS01) were collected from Hanshan County, Anhui Province (117.95°E, 31.87°N), where metamifop has been applied for over a decade. Similarly, seeds of the susceptible (S) population (FD03) were collected from a fallow field in Feidong County, Anhui Province (117.53°E, 32.06°N), which has no history of herbicide application. All seeds were air-dried and stored at 4 ℃ until further use.

Seeds of both biotypes were chosen at random and germinated in Petri dishes containing distilled water. After germination, seedlings with 1-cm shoots were transplanted into plastic pots (12 cm in diameter, containing loam soil) and placed in a greenhouse under natural light. The temperature of the greenhouse was maintained at 15 °C/ 25 °C with approximately 75% relative humidity. The seedlings were thinned to six individuals per pot at the three- to four-leaf stage.

Herbicides and chemicals

A total of nine herbicides with different MOAs were used to evaluate the susceptibility of the R population (HS01) (Table 1). To explore the potential metabolic resistance of the R population to metamifop, a P450 inhibitor piperonyl butoxide (PBO, 95%) and a GST inhibitor 4-chloro-7-nitrobenzoxa-diazole (NBD-Cl, 97%) obtained from Aladdin (Shanghai, China) were used.

Dose-response experiments to metamifop in R and S populations

Whole-plant dose-response testing was carried out according to a previously established protocol (Zhao et al. 2022). A series of metamifop doses were applied to the R and S populations when the seedlings had grown to the three- to four-leaf stage (Table 1). Each treatment was applied using a laboratory cabinet sprayer (3WP-2000, Nanjing Mechanization Research Institute of the Ministry of Agriculture, Nanjing, China), which delivers 450 L ha^{-1} at 0.275 MPa and a speed of 0.5 m s^{-1}. Three weeks later, the above-ground biomass of the surviving plants in each pot was measured. The experimental design for all greenhouse

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studies was a completely randomized design with three replications per treatment, and the experiments were conducted twice.

Cytochrome P450 and GST inhibitors treatments

This experiment was conducted along with the whole-plant dose-response assays to identify the potential resistance of the R population to metamifop caused by increased metabolic enzyme activity. The R and S seedlings at the three- to four-leaf stage received foliar treatment with NBD-Cl, PBO, metamifop, NBD-Cl plus metamifop or PBO plus metamifop. PBO was applied 1 h prior to metamifop treatment at a rate of 4200 g a.i. ha^{-1}, NBD-Cl was applied 48 h prior to metamifop treatment at a rate of 270 g ha^{-1}, and metamifop was applied as previously described (Table 1). The above-ground fresh tissue material was harvested at 21 d after treatment (DAT). All the treatments had three replicates, and the experiment was repeated twice. In the dependent sample, a *t*-test was used to compare the differences in GR_{50} values between metamifop treatment and PBO plus metamifop, NBD-Cl plus metamifop treatments in each population studied.

Cross- and multiple-resistance to herbicides with different MOAs

Whole-plant dose-response experiments were conducted to ascertain the susceptibility of the R and S populations to herbicides with the same or different MOAs. Weed seedlings were cultivated to the three- to four-leaf stage and subjected to eight herbicide treatments (Table 1). The herbicides were applied according to the protocol as previously described, and the fresh weight of the above-ground tissue was recorded at 21 DAT. All the treatments had three replicates and the experiment was repeated twice.

ACCase gene sequencing

The plants from the R and S populations were cultivated to the three- to four-leaf stage. Fresh tissues were randomly sampled from at least 10 individual plants of each population for DNA extraction via a cetyltrimethylammonium bromide (CTAB)-based method (Porebski et al. 1997). Fragments of all six *ACCase* genes, including all known mutation sites, were amplified using gene-specific primers as described by Iwakami et al. (2015). Taq MasterMix (2× ES; CWBIO, Beijing, China) was used to perform polymerase chain reaction (PCR) according to the manufacturer's instructions. The PCR products were visualized on a 1.0% agarose gel in 1× TAE buffer and subsequently sequenced on both strands by Tsingke Biotech Co., Ltd (Nanjing, China). The sequences for the R and S biotypes were aligned and compared with DNAMAN v.6.0 software (Lynnon, Quebec, Canada). Thereafter, 10 DNA samples randomly selected from the HS01 and FD03 plants, respectively, were used for derived cleaved amplified polymorphic (dCAPS) marker analysis.

Computational analysis of ACCase-inhibiting herbicides binding to ACCase protein

To explore the structural alterations in the ACCase enzyme of *E. crus-galli* resulting from the Cy-2088-Arg mutation and its impact on herbicide binding affinity, the protein sequence of *E.*

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crus-galli wild-type (WT) ACCase was modeled by the SWISS-MODEL homology modeling server (https://swissmodel.ExPASy.org/). The crystal structure of *E. crus-galli* (UniProt: A0A291NFR1), with a sequence identity of 99.08%, was obtained from the AlphaFold database (https://alphafold.ebi.ac.uk/), and the herbicide ligands were acquired from PubChem (https:// pubchem.ncbi.nlm.nih.gov/). Protein ligand docking of the WT and Cys-2088-Arg mutant with metamifop and other ACCase-inhibiting herbicides was performed using PyMOL (DeLano Scientific, San Carlos, CA, USA). In addition, the binding affinities of the WT and Cys-2088-Arg mutant ACCase to the ACCase-inhibiting herbicides were evaluated. After docking, the 3D structures were visualized with PyMOL Viewer.

dCAPS assays to detect the Cys-2088-Arg mutation in E. crus-galli

A pair of primers (d2088-F: 5′-GGTGGTTGATAGCAAAATAAATCCAGACCGCATAGCG-3′ and d2088-R: 5′-GCTTTGCACCTTGGAGTTTT-3′) were designed to amplify the same fragment regions of the six *ACCase* genes in *E. crus-galli*. In the d2088-F primer sequence, the underlined "C" is the mismatch introduced to produce an *Hha*I restriction enzyme site (NEB, Beijing, China). The PCR system and reaction condition were prepared in accordance with the manufacturer's procedures using Taq MasterMix (2× ES; CWBIO). The PCR product, a 190-bp DNA fragment, was visualized on a 3.0% agarose gel in 1× TAE buffer. Following *Hha*I digestion, the mutant sequence (R) should produce two digested bands at 153 bp and 37 bp, respectively, whereas the wild-type sequence (S) should only produce an undigested band at 190 bp.

ACCase gene expression analysis

To investigate the potential difference in target gene expression between the R and S plants, the total *ACCase* expression levels were quantified via real-time quantitative PCR (RT–qPCR) according to the methods of Zhao et al. (2022). Metamifop was sprayed at the field-recommended rate (FRR) when the R and S plants reached the three- to four-leaf stage. Fresh tissue was collected from at least 10 plants at 0 h (untreated) and at 12 and 24 h following metamifop treatment. Total RNA was extracted from each sample using a TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA), and complementary DNA (cDNA) was synthesized using the HiFiScript gDNA Removal cDNA Synthesis Kit (CWBIO). RT–qPCR was conducted using the CFX96 Real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) according to instructions of the ChamQ SYBR qPCR Master Mix Kit (Vazyme, Nanjing, China). In accordance with previous studies by Li et al. (2023), *β-actin* was used as the internal control gene. The expression levels of *ACCase* relative to *β-actin* were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Each reaction contained three biological replicates and three technical replicates. The upregulation or downregulation of *ACCase* in the R plants compared with that in the S plants was determined based on the criteria of a two-fold or greater change in expression and a p value <0.05 according to Student's t-test.

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Data analysis

The datasets from repeated experiments with the same treatments were initially analyzed using analysis of variance (ANOVA) with SPSS v19.0 (IBM, Armonk, NY, USA). Laven's test was used to determine the homogeneity of the variance by performing an analysis of variance (ANOVA). No significant difference (p> 0.05) was observed among the replicates; therefore, the data from the same treatment were pooled and fit to a four-parameter logistic function (Eq. 1) using SigmaPlot v.14.0 (Systat Software, San Jose, CA, USA):

$$y = C + \frac{D - C}{1 + (\frac{x}{\mathrm{GR}_{50}})^{b}} \quad (1)$$

where y is the response at herbicide dose x, C is the lower limit of the response, D is the upper limit of the response, and b is the slope at the herbicide dose causing a 50% reduction in growth (GR_{50}). The resistance index (RI) was determined by dividing the GR_{50} of R population by that of S population. Population susceptibility was categorized according to the following criterion (Seefeldt et al. 1995): susceptible, RI< 2; low resistance, 2≤ RI< 5; moderate resistance, 5≤ RI< 10; and high resistance, RI≥ 10.

Independent-samples t tests (p< 0.05) were performed with SPSS v.19.0 software (IBM, Armonk, NY, USA).

Results and discussion

Dose–response to metamifop in R and S plants of E. crus-galli without or with P450 and GST inhibitions

To assess the susceptibility and confirm the GR_{50} values of the R and S populations to metamifop, whole-plant dose–response bioassays were performed. As expected, following herbicide application, the growth of the S plants was significantly suppressed, with a GR_{50} of 41.08 g ha^{-1}. In contrast, the R plants exhibited high-level resistance to metamifop, with a GR_{50} of 482.99 g ha^{-1} (Table 2; Figure 1). According to the GR_{50} values, the metamifop resistance of the R population was 11.76-fold greater than that of the S population. In addition, the effects of pretreatment with either the P450 inhibitor PBO or the GST inhibitor NBD-Cl on the resistance level of the R population to metamifop were determined. The growth of the R and S plants were unaffected by application of either PBO or NBD-Cl alone. Pretreatment with PBO or NBD-Cl did not significantly (p> 0.05) alter the susceptibility of the R population to metamifop, with the GR_{50} ranging from 482.99 to 510.92 g ha^{-1} (Table 2; Figure 1). These results indicated that P450s and GSTs may not be associated with metamifop resistance in the R population.

Echinochloa crus-galli, harboring six plastidic *ACCase* genes, is the most challenging weed to be effectively controlled and is the most significant threat to rice cultivation (Feng et

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al. 2024; Iwakami et al. 2024). Since 2011, metamifop, an ACCase-inhibiting herbicide, has been registered in China and exhibited excellent efficiency against *E. crus-galli* while also being safe for rice (Deng et al. 2023). However, the long-term and excessive use of such herbicides has let *E. crus-galli* develop high-level resistance (Yu et al. 2010; Délye et al. 2013). Recent studies revealed that *E. crus-galli* has developed resistance to herbicides with different MOAs, including the ACCase-inhibiting herbicide metamifop (Pan et al. 2022), the ALS-inhibiting herbicide penoxsulam (Gao et al. 2023), and the auxin mimics herbicides quinclorac and florpyrauxifen-benzyl (Zhang et al. 2023; Wang et al. 2024). In this work, the HS01 population was collected from a rice field in which metamifop has been used for more than 10 years. Whole-plant dose–response testing revealed that the HS01 population has developed a high-level of resistance to metamifop. This finding indicated that the continuous use of herbicides with the same MOA is one of the important factors contributing to herbicide resistance in weeds (Norsworthy et al. 2012).

Gene sequencing and expression analysis of ACCase in E. crus-galli

Partial fragments of all the six *ACCase* genes were amplified from *E. crus-galli*, and the sequences were compared between the R and S plants. Numerous single-nucleotide polymorphisms (SNPs) were identified, but most of these changes were not related to AAS. Notably, a TGT-to-CGT mutation, which leads to a Cys-to-Arg substitution, was identified at codon position 2088 of *ACCase 1,5* in all the R plants (Fig. S1). Also, direct sequencing of the PCR products consistently revealed sharp single peaks in chromatograms of mutant codon positions, indicating homozygous resistance (RR) at position 2088 of *ACCase 1,5* in the R plants (unpublished data). No AASs known to confer ACCase resistance was identified in the remaining codons of *ACCase 1,5* or in the other five *ACCase* genes (Fig. S2). In addition, the relative expression levels of total *ACCase* genes in the resistant (R, HS01) *versus* susceptible (S, FD03) *E. crus-galli* plants were compared before and after herbicide treatment. As shown in Figure 2, when the R and S plants were subjected to metamifop treatment at the FRR, the expression of *ACCase* decreased at 12 h and then increased at 24 h. However, no significant difference (fold change< 2, p> 0.05) in relative *ACCase* expression was observed in the R plants compared with the S plants with and without herbicide treatments.

Mutation of the target gene, resulting in an AAS is considered one of the primary mechanisms of herbicide resistance in various herbicide-resistant weeds (Murphy and Tranel 2019; Zou et al. 2023; Zhang et al. 2023). To date, seven AASs at codon positions 1781, 1999, 2027, 2041, 2078, 2088 and 2096 associated with herbicide resistance in weeds have been reported (Xu et al. 2014). In this study, a Cys-to-Arg substitution was detected at codon position 2088 in *ACCase 1,5* in the HS01 plants. According to previous research, Cys-2088-Arg substitution is well known to confer ACCase resistance in many grassy weeds, such as in *L. chinensis* (Liao et al. 2024), *A. myosuroides* (Lan et al. 2022), hedgehog dogtail

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(*Cynosurus echinatus* L.) (Fernandez et al. 2016) and Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] (Zhang et al. 2021). In addition, the overexpression of target genes can also contribute to resistance in different weeds (Sen et al. 2021). However, no significant difference ($p > 0.05$) was detected in the relative *ACCase* expression between the HS01 and FD03 plants both before and after herbicide treatments, indicating that the high-level metamifop resistance was not related to target gene overexpression.

dCAPS analysis of the Cys-2088-Arg mutation in E. crus-galli

To rapidly detect the Cys-2088-Arg mutation in *ACCase* genes of *E. crus-galli*, a set of dCAPS primers was designed based on the sequences around the Cys-2088-Arg AAS identified in the R population. Notably, the dCAPS primers designed could amplify the same fragments of all six *ACCase* genes from *E. crus-galli*, allowing the dCAPS marker to identify the Cys-2088-Arg mutation in any of the six *ACCase* genes. Following PCR and *Hha*I digestion, the S plants showed a single band at 190 bp in the gel, indicating they were homozygous sensitive (SS) at codon 2088 of all six *ACCase* genes. In contrast, all tested individuals from R plants presented two bands at 190 bp and 153 bp (and an invisible 37-bp band) (Figure 3), indicating the coexistence of both the Cys-2088-Arg mutant alleles and the wild-type Cys-2088 alleles. This is understandable because, in the *ACCase* gene sequencing, although all the R plants harbored a homozygous Cys-2088-Arg mutation in *ACCase 1,5*, they simultaneously carried the wild-type sequences of the other five *ACCase* genes. As reported, a polyploid genome may confound the ability of the molecular techniques to accurately distinguish true (allelic) heterozygotes from homoeoallelic heterozygotes (Warwick et al. 2010). Therefore, the heterozygosis showed by the dCAPS assay in this study is most likely to be a kind of homoeologous heterozygosity (R+S). The dCAPS assay result was completely identical with sequencing data for every plant used in the identification of target site mutation (data not shown). Similar results have been reported in other herbicide-resistant polyploid weed species, such as in tetraploid *Pseudosclerochloa kengiana* (Yuan et al. 2015) and hexaploid wild oat (*Avena fatua* L.) (Yu et al. 2013). Although the dCAPS marker developed here cannot distinguish the heterozygosity of mutation, it remains a powerful tool for identified the *ACCase* gene Cys-2088-Arg mutation in the hexaploid *E. crus-galli* species, thus aiding in its resistance monitoring.

Resistance patterns to different herbicides

The susceptibility of the metamifop-resistant HS01 population to other herbicides with the same or different MOAs was also investigated. The results showed that HS01 also developed moderate-level resistance to the APP herbicides cyhalofop-butyl (RI=9.33, Table 2, Figure S3(A)) and fenoxaprop-*P*-ethyl (RI=5.80, Table 2, Figure S3(B)), low-level resistance to the CHD herbicide clethodim (RI=3.24, Table 2, Figure S3(C)), but remained susceptible to imazamox (RI=0.77, Table 2, Figure S3(D)), penoxsulam (RI=0.94, Table 2, Figure S3(E)),

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bispyribac-sodium (RI=1.43, Table 2, Figure S3(F)), tripyrasulfone (RI=0.66, Table 2, Figure S3(G)) and florpyrauxifen-benzyl (RI=0.87, Table 2, Figure S3(H)).

The Cys-2088-Arg mutation in *L. chinensis* resulted in cross-resistance to herbicides with the same MOA, including APP (metamifop, fenoxaprop-*P*-ethyl, quizalofop-*P*-ethyl and clodinafop-propargyl) and CHD (clethodim) herbicides (Liao et al. 2024). A similar resistance pattern caused by the same *ACCase* mutation was also observed in *C. echinatus* (Fernandez et al. 2016) and *L. perenne* (Zhang et al. 2021). Here, the susceptibility of HS01 to different herbicides was also evaluated, and it exhibited cross- resistance to APP (cyhalofop-butyl and fenoxaprop-*P*-ethyl) and CHD (clethodim) herbicides but can be effectively controlled by imazamox, penoxsulam, bispyribac-sodium, tripyrasulfone and florpyrauxifen-benzyl.

Computational analysis of the effects of the Cys-2088-Arg mutation on the binding affinities of ACCase inhibitors with ACCase protein

To predict that the mechanism by which the Cys-2088-Arg mutation leads to resistance to ACCase-inhibiting herbicides, we used homology modeling to establish a 3D structure of the *E. crus-galli* ACCase protein. Furthermore, we calculated the binding affinities and evaluated the abilities of four ACCase-inhibiting herbicides, metamifop, cyhalofop-butyl, fenoxaprop-*P*-ethyl and clethodim, bind to the protein. Among them, the free interaction (binding) energy of the metamifop with the WT was −95.81 kcal mol^{-1}, whereas the binding energy of metamifop to the mutant was −39.64 kcal mol^{-1}, indicating that metamifop binds better to the WT. In addition, the binding energies of cyhalofop-butyl, fenoxaprop-*P*-ethyl and clethodim to the WT were −80.90, −71.24 and −108.93 kcal mol^{-1}, respectively, whereas those to the mutant were −41.29, −46.11 and −76.77 kcal mol^{-1} (Figure 4). These differences in binding energies were consistent with the findings of the cross-resistance patterns in the R population.

Molecular docking has been extensively used to predict binding sites and interaction mechanisms between target proteins and herbicides (Akbarabadi et al. 2019; Fang et al. 2022). Jiang et al. (2024) performed a molecular docking study and demonstrated that the binding energy of cyhalofop-butyl to the Trp-2027-Cys mutant in *L. chinensis* was lower than that to the WT. A lower binding energy/binding affinity value indicates a more efficient interaction between the ligand and the receptor (Akbarabadi et al. 2019). In the present study, the binding energies of four ACCase-inhibiting herbicides with the WT (Cys-2088) were lower than their binding energies to the mutant. The differences in the interactions and docking scores suggest that the Cys-2088-Arg mutation could reduce the binding affinity of these herbicides with ACCase, consequently conferring *E. crus-galli* resistance to the ACCase-inhibiting herbicides metamifop, cyhalofop-butyl, fenoxaprop-*P*-ethyl and clethodim. Therefore, the less negative binding energies may be one of the reasons for the resistance of R plants to ACCase-inhibiting herbicides.

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In summary, this study identified, for the first time, an *E. crus-galli* population (HS01) exhibiting high-level resistance to various ACCase-inhibiting herbicides due to a Cys-2088-Arg mutation in its *ACCase* genes. Molecular docking assays demonstrated that the less negative binding energies of ACCase-inhibiting herbicides to the ACCase mutant may account for the observed resistance. A dCAPS marker was developed to rapidly detect the TGT to CGT substitution resulting in the Cys-2088-Arg mutation of *ACCase* in *E. crus-galli*. Alternative herbicides, including the ALS inhibitors imazamox, penoxsulam, and bispyribac-sodium, the HPPD inhibitor tripyrasulfone, and the auxin mimic florpyrauxifen-benzyl, remained effective in controlling the resistant population. These herbicides could be ideal options for developing an improved herbicide rotation strategy to prevent or delay the evolution of resistance in *E. crus-galli*.

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Competing interests

The authors declare no conflicts of interest.

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Tables

Table 1. Details of the herbicide applications in the dose-response experimental tests

Group	Herbicide	Formulation	Supplier	Test of dose (g a.i. ha^{-1}) [a]	
				Resistant population	Susceptible population
APP	Metamifop	10% EC	FMC Corporation, Jiangsu	0, 40, **120**, 360, 1080, 3240, 9720	0, 2.2, 6.67, 20, 60, **120**, 360
	Cyhalofop-butyl	100 g L^{-1} EC	Dow AgroSciences, USA	0, 3.33, 10, 30, **90**, 270, 810	0, 1.11, 3.33, 10, 30, **90**, 270
	Fenoxaprop-*P*-ethyl	69 g L^{-1} EW	Bayer, Hangzhou	0, 2.3, 6.9, 20.7, **62.1**, 186.3, 504.9	0, 1.9, 3.9, 7.8, 15.5, 31.1, **62.1**
CHD	Clethodim	240 g L^{-1} EC	Aokun, Shandong	0, 4, 12, 36, **108**, 324, 972	0, 1.33, 4, 12, 36, **108,** 324
IMI	Imazamox	4% AS	Jiangsu Agrochem Laboratory	0, 0.59, 1.78, 5.3, 16, **48**, 144	0, 0.59, 1.78, 5.3, 16, **48**, 144
TP	Penoxsulam	25 g L^{-1} OD	Dow AgroSciences, USA	0, 0.37, 1.11, 3.33, 10, **30,** 90	0, 0.37, 1.11, 3.33, 10, **30,** 90
PTB	Bispyribac-sodium	10% SC	JiuYi, Anhui	0, 1.67, 5, 15, **45**, 135, 405	0, 1.67, 5, 15, **45**, 135, 405
PZ	Tripyrasulfone	6% OD	KingAgroot, Shandong	0, 1.7, 5, 15, 45, **135**, 405	0, 1.7, 5, 15, 45, **135**, 405
AM	Florpyrauxifen-benzyl	3% EC	Corteva, Shanghai	0, 1.13, 2.25, 4.5, 9, 18, **36**	0, 1.13, 2.25, 4.5, 9, 18, **36**

OD, oil dispersion; EW, emulsion in water; EC, emulsifiable concentrate; AS, aqueous solution; SC, suspension concentrate; APP, aryloxyphenoxypropionate; CHD, cyclohexanedione; IMI, imidazolinone; TP, triazolopyrimidine; PTB, pyrimidinylthio-benzoate; PZ, pyrazolone; AM, auxin mimic.

[a] Bold indicates the field-recommended rate (FRR).

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Table 2. Sensitivity of resistant population (HS01) and susceptible population (FD03) to different herbicides

Herbicide	Biotype [a]	GR_{50} (g a.i. ha^{-1}) (SE) [b]	RI [c]
Metamifop	R	482.99 (84.46)	11.76
	S	41.08 (8.85)	
PBO plus metamifop	R	493.37 (61.88) NS	12.01
	S	40.24 (8.06)	
NBD-Cl plus metamifop	R	510.92 (86.29) NS	12.44
	S	38.80 (8.07)	
Cyhalofop-butyl	R	142.63 (56.10)	9.33
	S	15.29 (10.75)	
Fenoxaprop-*P*-ethyl	R	59.15 (6.79)	5.80
	S	10.20 (2.34)	
Clethodim	R	43.38 (1.11)	3.24
	S	13.56 (2.74)	
Imazamox	R	15.23 (1.75)	0.77
	S	19.74 (3.54)	
Penoxsulam	R	8.22 (0.58)	0.94
	S	8.78 (1.10)	
Bispyribac-sodium	R	32.31 (5.83)	1.43
	S	22.55 (10.08)	
Tripyrasulfone	R	20.26 (3.96)	0.66
	S	30.72 (2.71)	
Florpyrauxifen-benzyl	R	2.44 (0.81)	0.87
	S	2.81 (0.67)	

[a] R, resistance population HS01; S, susceptible population FD03. [b] NS, no significant difference ($p < 0.05$) between the R and S populations with or without PBO and NBD-Cl pre-treatment. [c] RI, resistance index which was calculated the GR_{50} of the resistant population by the GR_{50} of susceptible population.

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Figure legends

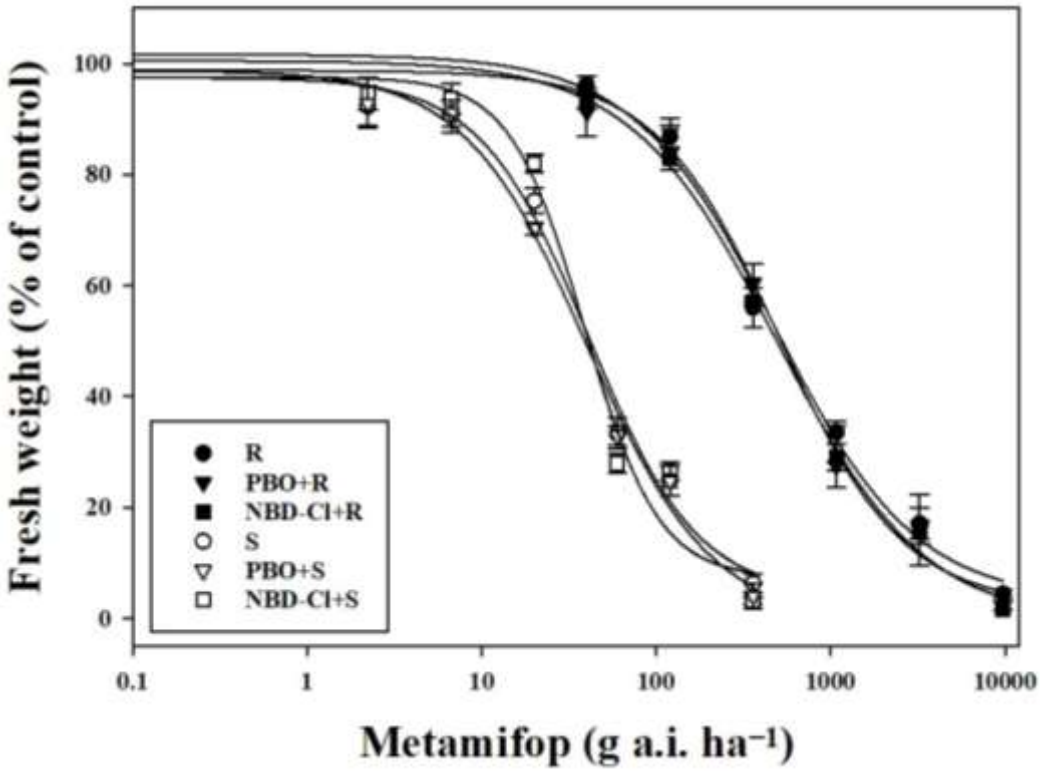

Figure 1. The dose-response curves for the R (HS01, ●) and S (FD03, ○) populations to metamifop or NBD-Cl plus metamifop (NBD-Cl+R, NBD-Cl+S) (■, □) or PBO plus metamifop (PBO+R, PBO+S) (▲, △). Vertical bars reflect the standard error of the mean.

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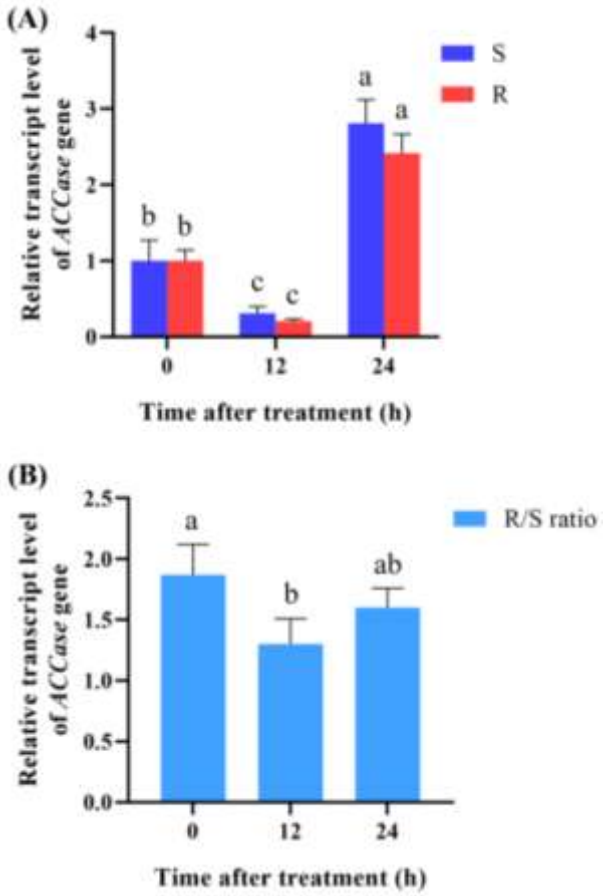

Figure 2. (A) Temporal *ACCase* expression in the R and S populations of *E. crus-galli* at 0 (untreated) and 12 and 24 (HAT) with metamifop. (B) Relative expression of *ACCase* in the resistant (R, HS01) versus susceptible (S, FD03) plants of *E. crus-galli* were compared at 0 (control) and 12 and 24 h after treatment with metamifop. Vertical bars represent SEs of the means. Different letters represent significant difference (p> 0.05).

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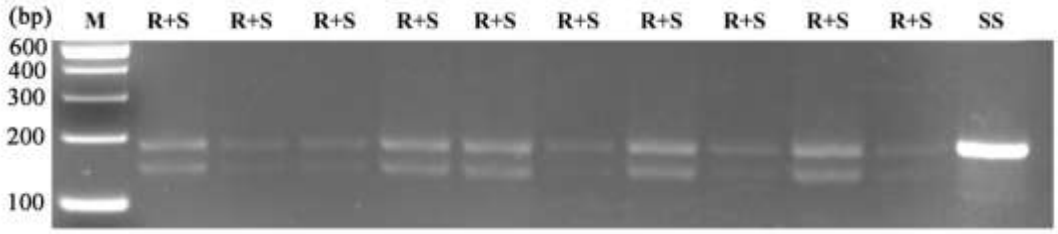

Figure 3. A dCAPS marker has been designed to detected the Cys-2088-Arg mutation in the R population. Following *Hha*I digestion, two restricted fragments (190- and 153-bp) correspond to resistant Cys-2088-Arg allele and an undigested 190-bp fragment correspond to sensitive Cys-2088 allele. M stands for marker. R+S, homoeologous heterozygosity at codon position 2088 of *ACCase*. SS, homozygous susceptible at codon position 2088 of *ACCase*.

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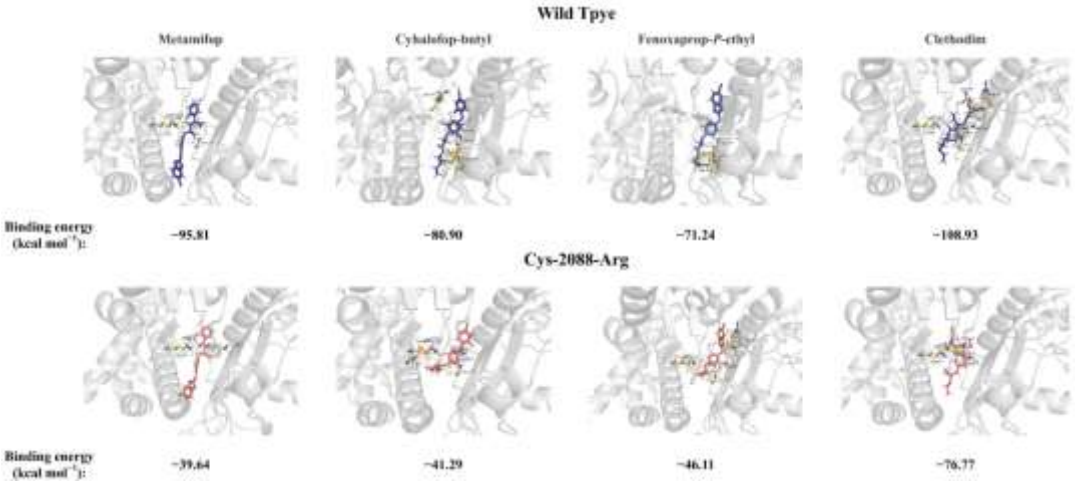

Figure 4. Molecular docking results of 2088-Arg protein and Cys-2088 protein to metamifop, cyhalofop-butyl, fenoxaprop-*P*-ethyl and clethodim. In the figure, the blue and red molecules represent herbicides, the length of the hydrogen bond is indicated by the numbers next to the yellow dashed lines. The numbers below the figure represent the binding energy.

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