

Research Article

Cite this article: Hu W, Li Y, Zhao C, Zhang L, Ma Y, Gao Q, Cao H, Liao M (2025). A Cys-2088-Arg mutation in ACCase confers cross-resistance to ACCase-inhibiting herbicides in barnyardgrass (*Echinochloa crus-galli*). *Weed Sci.* **73**(e34), 1–8. doi: [10.1017/wsc.2025.1](https://doi.org/10.1017/wsc.2025.1)

Received: 30 October 2024
Revised: 28 December 2024
Accepted: 10 January 2025

Associate Editor:

Christopher Preston, University of Adelaide


Keywords:

ACCase mutation; dCAPS; metamifop; herbicide resistance

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

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Abstract

Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] is a dominant weed species occurring in rice (*Oryza sativa* L.) 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, China. 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 the 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 acetyl-CoA carboxylase (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-nitrobenzoxadiazole 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.

Introduction

Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] is one of the most harmful annual grass weeds and can coexist in rice (*Oryza sativa* L.), maize (*Zea mays* L.), and other summer field crops (Akbarabadi et al. 2019). Owing to its strong adaptability, *E. crus-galli* is difficult to manage, 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 (Herbicide Resistance Action Committee Group 1) (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 ATP (Ye et al. 2020). Furthermore, ACCase is the most important target and can be inhibited by three major groups of synthetic herbicides, including aryloxyphenoxypyrone (APPs), cyclohexanediones (CHDs), and phenylpyrazolines (Jiang et al. 2024; Wang et al. 2021). Metamifop, an ACCase inhibitor, is used for postemergence 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 this herbicide's 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 herbicides (Gaines et al. 2019). TSR is the most common mechanism of weed

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Table 1. Details of the herbicide applications in the dose–response experimental tests.^a

Group ^a	Herbicide	Commercial name	Formulation ^b	Supplier	Test of dose ^c	
					Resistant population	Susceptible population
g ai ha ⁻¹						
APP	Metamifop	Hanqiuhao	10% EC	FMC Corporation, Jiangsu, China	0, 40, 120 , 360, 1080, 3240, 9720	0, 2.2, 6.67, 20, 60, 120 , 360
	Cyhalofop-butyl	Qianjin	100 g L ⁻¹ EC	Corteva Agriscience, Shanghai, China	0, 3.33, 10, 30, 90 , 270, 810	0, 1.11, 3.33, 10, 30, 90 , 270
	Fenoxaprop- <i>P</i> -ethyl	Puma	69 g L ⁻¹ EW	Bayer, Hangzhou, China	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	Jiujiuxi	240 g L ⁻¹ EC	Aokun, Shandong, China	0, 4, 12, 36, 108 , 324, 972	0, 1.33, 4, 12, 36, 108 , 324
IMI	Imazamox	Wocaotong	4% AS	Jiangsu Agrochem Laboratory, Jiangsu, China	0, 0.59, 1.78, 5.3, 16, 48 , 144	0, 0.59, 1.78, 5.3, 16, 48 , 144
TP	Penoxsulam	Daojie	25 g L ⁻¹ OD	Corteva Agriscience, Shanghai, China	0, 0.37, 1.11, 3.33, 10, 30 , 90	0, 0.37, 1.11, 3.33, 10, 30 , 90
PTB	Bispyribac-sodium	Guangshuang	10% SC	JiuYi, Anhui, China	0, 1.67, 5, 15, 45 , 135, 405	0, 1.67, 5, 15, 45 , 135, 405
PZ	Tripyrasulfone	Daoyu	6% OD	KingAgroot, Shandong, China	0, 1.7, 5, 15, 45, 135 , 405	0, 1.7, 5, 15, 45, 135 , 405
AM	Florpyrauxifen-benzyl	Lingske	3% EC	Corteva Agriscience, Shanghai, China	0, 1.13, 2.25, 4.5, 9, 18, 36	0, 1.13, 2.25, 4.5, 9, 18, 36

^aAM, auxin mimic; APP, aryloxyphenoxypropionate; CHD, cyclohexanedione; IMI, imidazolinone; PTB, pyrimidinylthio-benzoate; PZ, pyrazolone; TP, triazolopyrimidine.

^bAS, aqueous solution; EC, emulsifiable concentrate; EW, emulsion in water; OD, oil dispersion; SC, suspension concentrate.

^cBold indicates the field-recommended rate (FRR).

resistance to herbicides and generally results in resistance to other herbicides with the same mode of action (MOA) (Kaundun 2014; H Wang et al. 2024a). 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.) (Lan et al. 2022; Liao et al. 2024; Papapanagiotou et al. 2015; Yu et al. 2007). Moreover, over-expression of the target gene can also be considered one of the herbicide-resistance mechanisms of TSR in large crabgrass [*Digitaria sanguinalis* (L.) Scop.] and *E. crus-galli* (González-Torralva and Norsworthy 2023; Laforest et al. 2017). 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; ZL Wang et al. 2024). Conversely, NTSR mechanisms are complicated and unpredictable and usually confer resistance to herbicides with different MOAs (Yu and 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. 2022a).

In this study, we obtained a suspected metamifop-resistant *E. crus-galli* population, HS01, from a rice field in Hanshan County, Anhui Province, China. To clarify its mechanisms of resistance and facilitate efficient control strategies, our aims were as follows: (1) determining the resistance level to metamifop in the HS01 population, (2) exploring the potential TSR and/or NTSR mechanisms involved, and (3) 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, China (117.95°E, 31.87°N), where metamifop has been applied for more than a decade. Similarly, seeds of the susceptible (S) population (FD03) were collected from a fallow field in Feidong County, Anhui Province, China (117.53°E, 32.06°N), which has no history of herbicide application. All seeds were air-dried and stored at 4 °C 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 diameter) containing loam soil and placed in a greenhouse under natural light. The temperature of the greenhouse was maintained at 15/25 °C with approximately 75% relative humidity. The seedlings were thinned to 6 individuals per pot at the 3- to 4-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-nitrobenzoxadiazole (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. 2022a). A series of metamifop doses were applied to the R and S populations when the seedlings had grown to the 3- to 4-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 at a speed of 0.5 m s⁻¹. Three weeks later, the aboveground biomass of the surviving plants in each pot was measured. The experimental design for all greenhouse studies was a completely randomized design with three replications per treatment, and the experiments were conducted twice.

Cytochrome P450 and GST Inhibitor Treatments

This experiment was conducted alongside 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 3- to 4-leaf stage received foliar treatment with NBD-Cl, PBO, metamifop, NBD-Cl plus metamifop, or PBO plus metamifop. PBO was applied 1 h before metamifop treatment at a rate of 4,200 g ai ha⁻¹, NBD-Cl was applied 48 h before metamifop treatment at a rate of 270 g ha⁻¹, and metamifop was applied as previously described (Table 1). The aboveground fresh tissue 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 different GR₅₀ values of metamifop, PBO plus metamifop and 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 3- to 4-leaf stage and subjected to 8 herbicide treatments (Table 1). The herbicides were applied according to the previously described protocol, and the fresh weight of the aboveground 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 3- to 4-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 (Nanjing, China). The sequences for the R and S biotypes were aligned and compared with DNAMAN v. 6.0 software (Lynnon, QC, Canada). Thereafter, 10 DNA samples randomly selected from each population of the R and S plants, respectively, were used for derived cleaved amplified polymorphic sequence (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 Cys-2088-Arg mutation and its impact

on herbicide binding affinity, the protein sequence of *E. crus-galli* wild-type (WT) ACCase was modeled using 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 *Echinochloa 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 *HhaI* 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 *HhaI* digestion, the mutant sequence (R) should produce two digested bands at 153 bp and 37 bp, respectively, whereas the WT 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. (2022b). Metamifop was sprayed at the field-recommended rate (FRR) when the R and S plants reached the 3- to 4-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 ChamQ SYBR qPCR Master Mix Kit instructions (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^{-ΔΔC_t} 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 2-fold or greater change in expression and a P-value < 0.05 according to Student's *t*-test.

Data Analysis

The datasets from repeated experiments with the same treatments were initially analyzed using ANOVA with SPSS v. 19.0 (IBM,

Armonk, NY, USA). Levene's test was used to determine the homogeneity of the variance by performing an 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 (Equation 1) using SigmaPlot v. 14.0 (Systat Software, San Jose, CA, USA):

$$y = C + \frac{D - C}{1 + \left(\frac{x}{GR_{50}}\right)^b} \quad (1)$$

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 the R population by that of the S population. Population susceptibility was categorized according to the following criterion (Seefeldt et al. 1995): susceptible, $RI < 2$; low resistance, $2 \leq RI < 5$; moderate resistance, $5 \leq RI < 10$; and high resistance, $RI \geq 10$.

Independent-samples t -tests ($P < 0.05$) were performed with SPSS v. 19.0 software (IBM).

Results and Discussion

Dose-Response to Metamifop in R and S Plants of *Echinochloa crus-galli* with or without P450 and GST Inhibitors

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 was 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 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 led to *E. crus-galli* developing high-level resistance (Délye et al. 2013; Yu et al. 2010). 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 mimic herbicides quinclorac and florpyrauxifen-benzyl (H Wang et al. 2024b; LL Zhang et al. 2023). In this work, the HS01 population was collected from a rice field in which metamifop has been used for more than 10 yr. Whole-plant dose-response testing revealed that the HS01 population has

Table 2. *Echinochloa crus-galli* resistant population (HS01) and susceptible population (FD03) sensitivity to different herbicides

Herbicide ^a	Biotype ^b	GR_{50} (SE) ^c	RI ^d
		g ai ha ⁻¹	
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)	

^aNBD-Cl, 4-chloro-7-nitrobenzoxadiazole; PBO, piperonyl butoxide.

^bR, resistant population HS01; S, susceptible population FD03.

^cNS, no significant difference ($P < 0.05$) between the R and S populations with or without PBO and NBD-Cl pretreatment.

^dRI, resistance index, determined by dividing the GR_{50} of the R population by that of the S population.

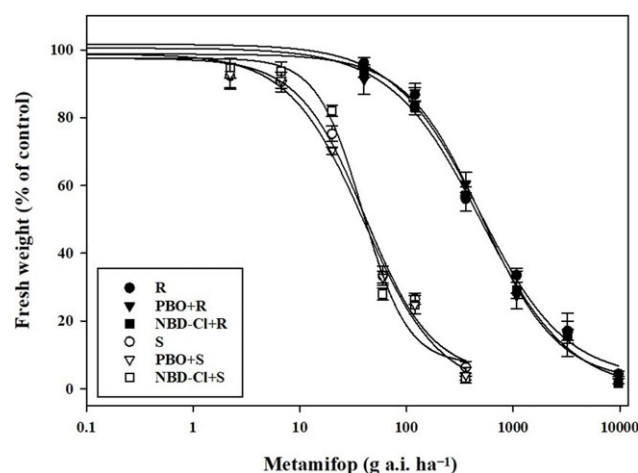


Figure 1. Dose-response curves of *Echinochloa crus-galli* resistant (R, HS01) and susceptible (S, FD03) populations for metamifop or 4-chloro-7-nitrobenzoxadiazole (NBD-Cl) plus metamifop (NBD-Cl+R, NBD-Cl+S) or piperonyl butoxide (PBO) plus metamifop (PBO+R, PBO+S). Vertical bars reflect the standard error of the mean.

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 *Echinochloa crus-galli*

Partial fragments of all six ACCase genes were amplified from *E. crus-galli*, and the sequences were compared between the R and S

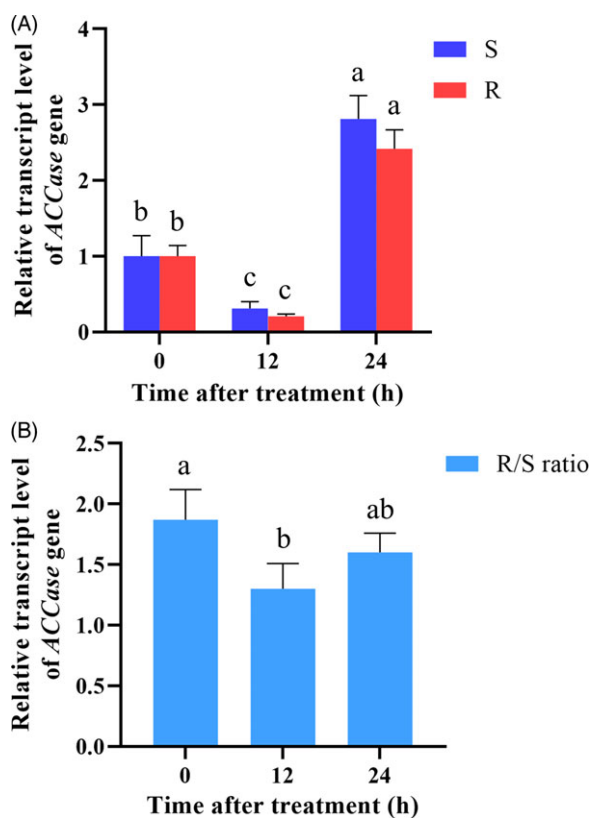


Figure 2. (A) Temporal *ACCase* expression in the *Echinochloa crus-galli* resistant (R, HS01) and susceptible (S, FD03) populations at 0 (untreated) and 12 and 24 h after treatment (HAT) with metamifop. (B) Relative expression of *ACCase* in the *E. crus-galli* R vs. S plants was compared at 0 (control) and 12 and 24 HAT with metamifop. Vertical bars reflect the standard error of the mean. Different letters represent significant difference ($P > 0.05$).

plants. Numerous single-nucleotide polymorphisms 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 (Supplementary Figure 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 (Supplementary Figure 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; LY Zhang et al. 2023; Zou 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, the 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 (*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 *Echinochloa 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 *HhaI* 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 WT 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 WT sequences of the

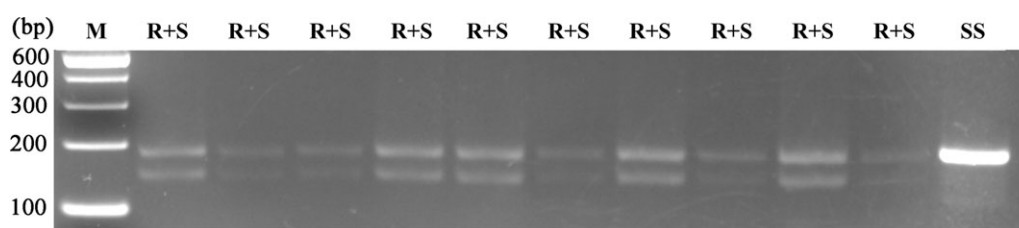


Figure 3. A derived cleaved amplified polymorphic sequence (dCAPS) marker was designed to detect the Cys-2088-Arg mutation in the *Echinochloa crus-galli* resistant (R) population. Following *HhaI* digestion, two restricted fragments (190- and 153-bp) correspond to the resistant Cys-2088-Arg allele, and an undigested 190-bp fragment corresponds to the sensitive Cys-2088 allele. M, marker; R+S, homozygous heterozygosity at codon position 2088 of *ACCase*; SS, homozygous susceptible at codon position 2088 of *ACCase*.

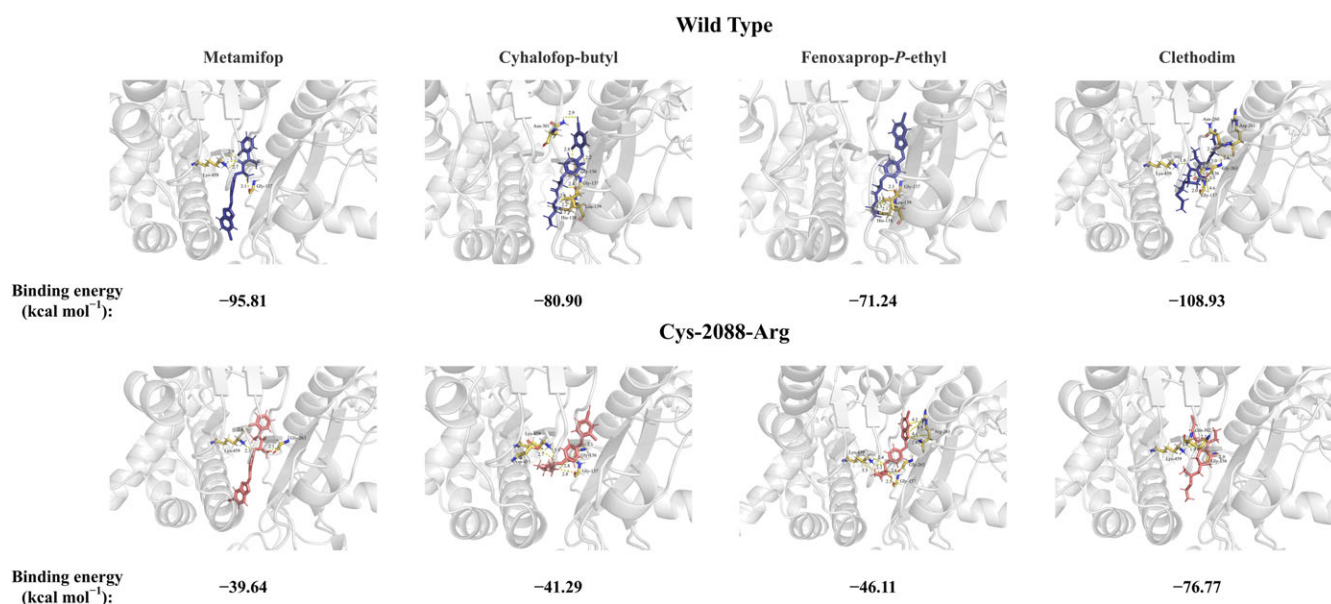


Figure 4. Molecular docking results of Cys-2088-Arg protein and wild type (WT) protein of *E. crus-galli* to metamifop, cyhalofop-butyl, fenoxaprop-*P*-ethyl, and clethodim. In the figure, the blue and red molecules represent herbicides, the length of the hydrogen bond is indicated by the numbers next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

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 shown with 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 Keng stiffgrass [*Pseudosclerochloa kengiana* (Ohwi) Tzvelev] (Yuan et al. 2015) and hexaploid wild oat (*Avena fatua* L.) (Yu et al. 2013). Although the dCAPS marker developed here cannot distinguish true (allelic) heterozygotes from homoeoallelic heterozygotes, it remains a powerful tool for identifying 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; Supplementary Figure S3A) and fenoxaprop-*P*-ethyl (RI = 5.80; Table 2; Supplementary Figure S3B) and low-level resistance to the CHD herbicide clethodim (RI = 3.24; Table 2; Supplementary Figure S3C), but remained susceptible to imazamox (RI = 0.77; Table 2; Supplementary Figure S3D), penoxsulam (RI = 0.94; Table 2; Supplementary Figure S3E), bispyribac-sodium (RI = 1.43; Table 2; Supplementary Figure S3F), tripyrasulfone (RI = 0.66; Table 2; Supplementary Figure S3G), and florpyrauxifen-benzyl (RI = 0.87; Table 2; Supplementary Figure S3H).

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 could 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, to bind to the protein. Among them, the free interaction (binding) energy of the metamifop with the WT was $-95.81 \text{ kcal mol}^{-1}$, whereas the binding energy of metamifop to the mutant was $-39.64 \text{ kcal mol}^{-1}$, 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 $-108.93 \text{ kcal mol}^{-1}$, respectively, whereas those to the mutant were -41.29 , -46.11 and $-76.77 \text{ kcal mol}^{-1}$ (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 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 were lower with the WT (Cys-2088) than with 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 R plants' resistance to ACCase-inhibiting herbicides.

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 ACCase-inhibiting herbicides' less-negative binding energies 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 4-hydroxyphenylpyruvate dioxygenase inhibitor tripyrasulfone, and the auxin mimic florypyrauxifen-benzyl, remained effective in controlling the R 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*.

Supplementary material. To view supplementary material for this article, please visit <https://doi.org/10.1017/wsc.2025.1>

Funding statement. This work was funded by the Major Natural Science Research Project of Anhui Universities (2023AH040143), the Science and Technology Major Project of Anhui Province (2022e03020005), and the College Students' Innovation Training Project in Anhui Agricultural University (X202210364248).

Competing interests. The authors declare no conflicts of interest.

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