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Target site–resistance mechanisms to imazamox in imidazolinone herbicide-resistant weedy rice (*Oryza sativa* f. *spontanea*) in China

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Research Article

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Abstract

Weedy rice (*Oryza sativa* f. *spontanea* Roshev.), a widespread and troublesome weed in rice (*Oryza sativa* L.) fields, is typically controlled using imazamox in imidazolinone-tolerant rice fields. However, suspected resistance to imazamox has emerged in weedy rice populations in Jiangsu Province, China. This study aimed to evaluate the degree of resistance and investigate the resistance mechanisms. A whole-plant bioassay was performed on 35 weedy rice populations, demonstrating that 26 populations developed resistance to imazamox. The effective dose values causing 50% inhibition of growth reduction (GR_{50}) in resistant (R) populations ranged from 129.2 to 280.2 g ai ha^{-1}, exceeding the recommended application rate of imazamox (120 g ai ha^{-1}) in imazamox-tolerant rice fields. R populations displayed cross-resistance to other acetolactate synthase (ALS)-inhibiting herbicides, except for certain sulfonylurea herbicides. Sequencing of the *ALS* gene identified a Ser-653-Asn substitution in resistant populations. A novel derived cleaved amplified polymorphic sequence (dCAPS) method was developed for the rapid and efficient detection of the Ser-653-Asn mutation in *O. sativa* f. *spontanea*. In vitro ALS activity assays revealed that the imazamox concentration required to inhibit 50% (IC_{50}) of ALS activity was 80.0- to 88.3-fold higher in R populations compared with a susceptible (S) population. After imazamox treatment, the *ALS* expression levels in both the S and R populations of weedy rice increased. Resistance was not reversed by cytochrome P450 oxidase system (CYP450) or glutathione *S*-transferase (GST) inhibitors, suggesting that metabolic resistance mechanisms were not involved. In conclusion, weedy rice developed a different resistance level to imazamox, and the Ser-653-Asn mutation in the target *ALS* was the main reason. To the best of our knowledge, this study is the first to reveal the mechanism of resistance to imazamox in weedy rice in China.

Introduction

Rice (*Oryza sativa* L.) is a globally significant food crop. In southern China in particular, it serves as the dominant food crop, accounting for one-third of the country's total grain output (Chauhan 2013). Weedy rice (*Oryza sativa* f. *spontanea* Roshev.) is one of the most notorious weeds distributed worldwide (H Wang et al. 2023). In recent years, weedy rice has rapidly infested many rice-planting areas in China, particularly in Jiangsu and Hainan provinces (Qiu et al. 2014; Wang et al. 2023). Weedy rice competes with cultivated rice in paddy fields for sunlight, water, and nutrients, interfering with rice growth and leading to yield decline. At the same time, high levels of weed contamination adversely affect rice quality (Li et al. 2023). Due to weedy and cultivated rice belonging to the same species, their biological characteristics are highly similar, making it impossible to apply postemergence herbicides in rice fields to control weedy rice (Cao et al. 2006; Hsu et al., 2022). Manual weeding has been the primary control method, but it is time-consuming, labor-intensive, and prone to accidental removal of cultivated rice plants. In 2017, many farmers in Jiangsu Province began to cultivate imidazolinone (IMI)-tolerant rice, a non-transgenic herbicide-tolerant rice cultivar, in combination with application of the imidazolinone herbicide imazamox. The infestation level of weedy rice decreased, indicating that the joint use of IMI-tolerant rice and imazamox could serve as an effective strategy for managing weedy rice.

Imidazolinones control weeds by inhibiting acetolactate synthase (ALS). ALS, also referred to as acetohydroxy acid synthase, is a critical enzyme in the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine (Fang et al. 2022). ALS serves as a common target site for five major classes of ALS-inhibiting herbicides, including sulfonylurea (SU), IMI, triazolopyrimidine (TP), pyrimidinyl oxybenzoates, and sulfonyl-aminocarbonyl triazolinone (Merriam et al. 2023; Sun et al. 2021). Selection pressure exerted by the persistent use of imazamox has resulted in the rapid evolution of many *O. sativa* f. *spontanea* populations. Weedy rice evolved resistance to imazamox in northern Greece in 2013 (Kaloumenos et al. 2013),

Colombia in 2018 (Hoyos et al. 2019), and Turkey in 2023 (Unan et al. 2024). In 2021, during field surveys of weedy rice conducted by our laboratory, insensitivity of weedy rice to imazamox was observed in Jiangsu Province, with suspected resistance development, rendering herbicide control increasingly difficult to achieve.

The main mechanisms of herbicide resistance include target-site resistance (TSR) and non–target site resistance (NTSR) (Gaines et al. 2020; Sun et al. 2021). TSR is due to (1) an increase in enzyme activity, (2) a modification of the nucleotide sequence in the herbicide target protein gene conferring an amino acid change that reduces herbicide binding, and (3) overexpression of a target enzyme (Fang et al. 2022; Gaines et al. 2020; L Liu et al. 2024). Previous studies have suggested that resistance to ALS-inhibiting herbicides is mainly due to single point mutations in the target *ALS* gene, thereby reducing ALS sensitivity. The *ALS* gene is easily mutated; 30 *ALS* resistance–endowing gene mutations have occurred to date in amino acids at nine conserved positions (Ala-122, Pro-197, Ala-205, Phe-206, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654, referring to the corresponding sequence of *Arabidopsis thaliana*) (Fang et al. 2022; L Liu et al. 2024). Point mutations in *ALS* at the Ala-122, Ser-653, Gly-654, and Val-669 codons have been reported to confer resistance to IMI herbicides in weedy rice throughout the world (Dilipkumar et al. 2018; Rajguru et al. 2005; Roso et al. 2010; Sales et al. 2008; Singh et al. 2017). The predominant mechanism of resistance in weedy rice is the Gly-654-Glu substitution in the ALS enzyme; however, Ser-653-Asn and Ala-122-Thr substitutions have also been observed (Busconi et al. 2012; Menezes et al. 2009; Roso et al. 2010; Sales et al. 2008; Scarabel et al. 2012; Unan et al. 2024). Alternately, the Val-669-Met substitution confers little to no resistance (Shivrain et al. 2010). Derived cleaved amplified polymorphic sequence (dCAPS) analysis, a straightforward and efficient method for the genetic analysis of single-nucleotide polymorphisms (SNPs), serves as a robust tool for the precise detection of SNPs (Neff et al. 1998).

NTSR impairs herbicide translocation, reduces absorption and penetration, and improves metabolism or the herbicide metabolic rate (Li et al., 2023; Rojano-Delgado et al. 2019). This reduces the dose of herbicide binding to the target protein and usually involves enzymes such as cytochrome P450 monooxygenases (CYP450s), glutathione *S*-transferases (GSTs), glycosyltransferases, peroxidases, and ATP-binding cassette transporters (Jugulam et al. 2019). NTSR to imazamox has been described in wheat (*Triticum aestivum* L.) and wild poinsettia (*Euphorbia heterophylla* L.), where the main identified metabolites were imazamox-OH and imazamox-glucose and the high root exudation of imazamox (Dominguez-Mendez et al. 2017; Rojano-Delgado et al. 2019).

The aims of this study were to (1) identify whether *O. sativa* f. *spontanea* is resistant to imazamox; (2) determine the resistance level of *O. sativa* f. *spontanea* to imazamox and assess cross-resistance to other ALS-inhibiting herbicides; (3) clarify the TSR mechanisms related to imazamox and, especially, the possible existence of point mutations responsible for resistance; and (4) establish the derived cleaved amplified polymorphic sequence (dCAPS) method in *O. sativa* f. *spontanea*.

Materials and Methods

Plant Materials

The weedy rice species used in this study are listed in Table 1. All *O. sativa* f. *spontanea* populations were collected from IMI-tolerant rice ('JinJing 818') fields in Jiangsu Province, where the application of imazamox at the recommend applied dose (120 g ai ha^{-1}) has failed to control most weedy rice populations since 2021. These areas have been consecutively treated with imazamox for more than 5 yr as a weedy rice control measure. Seeds of the susceptible population were collected from conventional rice lands without imazamox application in Funing District, Yancheng, Jiangsu Province, China. A total of 35 weedy rice biotypes were identified. All seeds were randomly collected by hand, threshed manually, and air-dried in the shade. Finally, the seeds were labeled and then stored in paper bags at 4 C until use. Dormancy was not observed in the collected weedy rice seeds.

Table 1. Location of collection sites and sensitivity to imazamox for 35 *Oryza sativa* f. *spontanea* populations used in this study.

Populations	Collection site in China	GR_{50} ± SE[a] g ai ha^{-1}	RI[b]
JSSH-2021-1	Sihong County, Suqian City	250.0 ± 46.9	46.9
JSSH-2022-1	Sihong County, Suqian City	174.5 ± 6.6	36.4
JSSH-2022-2	Sihong County, Suqian City	180.4 ± 5.5	37.7
JSSH-2022-3	Sihong County, Suqian City	258.7 ± 16.9	54.0
JSSH-2022-4	Sihong County, Suqian City	280.2 ± 62.3	58.5
JSSH-2022-5	Sihong County, Suqian City	255.2 ± 16.9	53.3
JSSH-2022-6	Sihong County, Suqian City	160.6 ± 11.9	33.5
JSSH-2022-7	Sihong County, Suqian City	204.0 ± 11.9	42.6
JSSH-2022-8	Sihong County, Suqian City	168.2 ± 1.5	35.1
JSSH-2022-9	Sihong County, Suqian City	185.4 ± 9.9	38.7
JSSH-2023-1	Sihong County, Suqian City	244.5 ± 8.3	51.1
JSSH-2023-2	Sihong County, Suqian City	173.6 ± 9.5	36.3
JSSH-2023-3	Sihong County, Suqian City	164.3 ± 16.7	34.3
JSSH-2023-4	Sihong County, Suqian City	202.5 ± 16.4	42.3
JSSH-2023-5	Sihong County, Suqian City	147.2 ± 34.41	30.7
JSSH-2023-6	Sihong County, Suqian City	130.0 ± 18.10	27.2
JSSH-2023-7	Sihong County, Suqian City	194.8 ± 10.7	40.7
JSSH-2023-8	Sihong County, Suqian City	155.5 ± 13.8	32.5
JLGY-2014-1[c]	Ganyu District, Lianyungang City	7.7 ± 1.2	1.6
JLGY-2014-2[c]	Ganyu District, Lianyungang City	7.7 ± 0.6	1.6
JLGY-2023-1	Ganyu District, Lianyungang City	196.1 ± 51.7	41.0
JLGY-2023-2	Ganyu District, Lianyungang City	145.5 ± 9.0	30.4
JYJD-2021-1	Jiangdu District, Yangzhou City	8.5 ± 0.4	1.8
JYJD-2022-1	Jiangdu District, Yangzhou City	133.4 ± 13.0	27.9
JYJD-2022-2	Jiangdu District, Yangzhou City	17.0 ± 6.2	3.5
JYJD-2022-3	Jiangdu District, Yangzhou City	152.4 ± 2.9	31.8
JYJD-2022-4	Jiangdu District, Yangzhou City	6.4 ± 0.3	1.3
JYJD-2023-1	Jiangdu District, Yangzhou City	6.1 ± 0.2	1.3
JYJD-2023-2	Jiangdu District, Yangzhou City	5.8 ± 1.0	1.2
JYFN-2023-1	Funing County, Yancheng City	4.8 ± 0.7	1.0
JTXH-2023-1	Xinghua City, Taizhou City	205.3 ± 8.2	42.9
JYJH-2023-1	Jianhu Village, Yangcheng City	167.1 ± 17.7	34.9
JYTH-2023-1	Tinghu District, Yancheng City	14.0 ± 4.3	2.9
JYTH-2023-2	Tinghu District, Yancheng City	129.2 ± 20.9	27.0
JYTH-2023-3	Tinghu District, Yancheng City	195.4 ± 14.2	40.8

[a]GR_{50} is the effective dose of herbicide causing 50% inhibition of fresh weight and is expressed as grams of active ingredient per hectare (g ai ha^{-1}). Data are the means of two experiments.
[b]RI is the relative resistance index, ratio of GR_{50} values relative to the susceptible *O. sativa* f. *spontanea* population (JYFN-2023-1). The recommended field dose of imazamox is 120 g ai ha^{-1}.
[c]Population with no prior herbicide exposure.

Whole-Plant Dose–Response Bioassay

Sensitivity to Imazamox

To evaluate the resistance levels of weedy rice populations, a whole-plant dose–response experiment was conducted in a greenhouse at Nanjing Agricultural University from June to August 2023. Twenty seeds from each population were sown in plastic pots containing a sand–organic matter mixture (1:2, pH 5.6) and placed in the greenhouse under a day/night temperature regime of 35 to 38/25 to 28 C with a 15/9-h photoperiod.

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Table 2. Recommended and applied doses of other acetolactate synthase (ALS)-inhibiting herbicides used in this study.

			Recommend field dose	Applied dose	
				S population	R populations
Group[a]	Herbicide	Crop[b]	g ai ha^{-1}		
IMI[a]	Imazethapyr	Soybean field	105	0, 13.13, 26.25, 52.5, 105, 210	0, 52.5, 105, 210, 420, 840
	Imazapic	Peanut field	108	0, 13.5, 27 54, 108, 216	0, 54, 108, 216, 432, 864
SCT[b]	Flucarbazone-sodium	Wheat field	31.5	0, 3.94, 7.88, 15.75, 31.5, 63	0, 15.75, 31.5, 63, 126, 252
PTB[c]	Pyribenzoxim	Rice field	45	0, 45, 90, 180, 360	
TP[d]	Pyroxsulam	Wheat field	15	0, 1.88, 3.75, 7.5, 15, 30	0, 7.5, 15, 30, 60, 120
	Penoxsulam	Rice field	30	0, 30, 60, 120, 240	
SU[e]	Nicosulfuron	Corn field	18	0,200, 300, 450, 675, 1,012.5	
	Mesosulfuron-methyl	Wheat field	15.75	3.94, 7.88, 15.75, 31.5, 63	0.98, 1.97, 3.94, 7.88, 15.75

[a]IMI, imidazolinone; PTB, pyrimidine thiobenzoates; SCT, sulfonamide carbonyl triazolinones; SU, sulfonylureas; TP, triazopyrimidines.
[b]Corn (*Zea mays* L.); peanut, (*Arachis hypogaea* L.); rice, (*Oryza sativa* L.); soybean, [*Glycine max* (L.) Merr.]; wheat (*Triticum aestivum* L.).

Table 3. Primers used to amplify *ALS* gene sequencing of *Oryza sativa* f. *spontanea* and primers used in real-time quantitative reverse transcriptase PCR (RT-qPCR) and derived cleaved amplified polymorphic sequence (dCAPS) study.

		Product size	Annealing temperature	
Primers	Sequence	bp	C	Usage
ALS-F1	CAAACCCAGAAACCCTCGC	2,153	58.6	Sequencing for *ALS*
ALS-R1	AGGATTACCATGCCAAGC			
QALS-F1	TACAAGGCGAATAGGGCGCA	273	60	*ALS* gene in RT-qPCR
QALS-R1	CACAGTCCTGCCATCACCAT			
Actin-F	CAACACCCCTGCTATGTACG	341	60	Reference gene in RT-qPCR
Actin-R	CATCACCAGAGTCCAACACAA			
*Nde*I-F	TCCCGCACCAGGAGCATGTGCTGCCTATGATCCCA**T**[a]	189	65.6	Detection for Ser-653-Asn in dCAPS
dCAPS-R	GGCATACCACTCTTTATGGG			

[a]Nucleotide in bold is a mismatched nucleotide base in the primer.

At BBCH 1.02 (second leaf fully expanded), seedlings were thinned to 10 plants per pot before herbicide treatment. Imazamox (4% AS, Zhongqi Technology, NanJing, China) was applied at BBCH 1.02 (second leaf fully expanded), using a laboratory walking spray tower (3WP-2000, Institute of Agricultural Mechanization, NanJing, China) with a flat-fan nozzle, delivering 280 L ha^{-1} at 230 kPa. Based on preliminary experiments, dose gradients were set as 0, 3.75, 7.5, 15, 30, 60, and 120 g ai ha^{-1} for the S population and 0, 120, 180, 270, 405, and 607.5 g ha^{-1} for the R population. Treated plants were maintained under the same greenhouse conditions and irrigated with clear water at 24 h after imazamox application. Aboveground fresh biomass was measured at 21 d after treatment (DAT). The experiment was repeated twice in a completely randomized design with four replicates per dose.

Sensitivity to Other ALS-inhibiting Herbicides

Based on preliminary experiments, two resistant weedy rice populations—JSSH-2021-1 (R1, the earliest identified resistant population in Jiangsu Province) and JSSH-2022-4 (R2, the most resistant population)—along with the sensitive population JYFN-2023-1 (S), were selected for subsequent analyses (population abbreviations used hereafter). A whole-plant dose–response assay was conducted to assess sensitivity to additional ALS-inhibiting herbicides; field-applied herbicides and dosages are detailed in Table 2. The experiment was repeated twice in a completely randomized design with four replicates per treatment.

Effect of Metabolic Inhibitors on Imazamox Sensitivity

At BBCH 1.02 (second leaf fully expanded), 10 plants per population were co-treated with imazamox and metabolic inhibitors: two CYP450 inhibitors (malathion, PBO) and one GST inhibitor (NBD-Cl). Malathion (1,000 g ai ha^{-1}) and PBO (4,200 g ai ha^{-1}) were applied 1 h before herbicide treatment, while NBD-Cl (270 g ai ha^{-1}) was applied 48 h before imazamox application, following previously reported protocols (Fang et al. 2019). The experiment was replicated twice with a completely randomized design (four biological replicates per treatment).

ALS Gene Sequencing

For each population, the plants were cultivated under previously described experimental conditions. Young leaf samples (approximately 100 mg) were obtained from the R population (R1, R2) and S population at BBCH 1.02 (second leaf fully expanded) and stored at −80 C until used. A Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) was used for DNA extraction, following the manufacturer's instructions. The primer pair (Table 3) was designed using Primer Premier v. 5.0 (Premier Biosoft International, California, USA) to amplify the *ALS* coding sequence of *O. sativa* f. *spontanea*, including all previously identified resistance mutation sites in *ALS*. *Oryza sativa* Japonica Group (NCBI accession no. AB049822) was the *ALS* gene reference sequence retrieved from the NCBI GenBank database and was included in the alignment. A polymerase chain reaction (PCR) was then performed. The PCR reaction contained template DNA (10 ng), 10 μM each primer (2 μl), 2× Phanta Max Master Mix (25 μl) (Vazyme Biotech, Nanjing, China), and ddH_2O (up to 50 μl). Amplification was conducted as follows: 3 min at 95 C for DNA denaturation; 35 cycles of 30 s at 95 C for DNA denaturation, 30 s at 58.6 C for annealing, and 90 s at 72 C for DNA elongation;

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and a final elongation for 5 min at 72 C. All products were separated on a 1% agarose gel and sequenced (QingKe Biotech, Beijing, China). The sequences were aligned and compared using the BioEdit Sequence Alignment Editor v. 7.2.5 (Tom Hall, Carlsbad, CA, USA).

ALS Activity Assay In Vitro

The plant materials R1, R2, and S were prepared for subsequent molecular experiments. The response of ALS to imazamox was determined using the crude enzyme extract. The methods were as described by Yu et al. (2004) with slight modifications. Briefly, leaf blade samples (3 g) of BBCH 1.02 (second leaf fully expanded) from each population were collected and immediately frozen in liquid nitrogen. Then, the samples (3 g) were macerated in a mortar using an enzyme extraction buffer (8 ml) composed of 10 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP), and 10 µM flavin adenine dinucleotide (FAD), as well as 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, and 100 mM potassium phosphate buffer (pH = 7.5). The homogenate was filtered through two layers of cheesecloth and centrifuged (27,000 × *g*, 4 C) for 15 min. The supernatant (7 ml) was extracted and mixed with $(NH_4)_2SO_4$ (1:1 v/v), and then slowly agitated for 10 min. The mixed solution was centrifuged (27,000 × *g*, 4 C) for 15 min to acquire precipitate. Then, an enzyme assay buffer composed of 20 mM $MgCl_2$, 2 mM TPP, 20 µM FAD, 200 mM sodium pyruvate, and 100 mM potassium phosphate buffer (pH = 7.5) was used to dissolve the extracted protein precipitate. The protein concentration was measured according to the Bradford method using bovine serum albumin as a standard. Each reaction system consists of 100 µl of protein extract and 100 µl of the ALS inhibitor (imazamox) at one concentration: 0.001, 0.01, 0.1, 1, 10, 100 or 1,000 µM). A non–ALS inhibitor control (imazamox was replaced with potassium phosphate buffer) was included in each assay for comparison.Acetolactate was converted to acetoin by incubating the mixtures at 37 C for 60 min in darkness. The reaction was stopped by the addition of 6 N H_2SO_4 (8 µl), and the mixture was maintained at 60 C for 30 min. Finally, a freshly prepared solution of 0.55% (w/w) creatine in water (190 µl) and a freshly prepared solution of 5.5% (v/v) α-naphthol in 5 M NaOH (190 µl) were added. The solution was again incubated at 60 C for 15 min. ALS activity was monitored by measuring the acetoin production. Acetoin absorbance was monitored at 530 nm using a microplate photometer (Thermo Fisher Scientific, Waltham, MA, USA). Under the same conditions used for the previous reaction solution, an acetoin standard curve was constructed with the acetoin concentration as the abscissa and the optical density at 530 nm (OD_{530}) as the ordinate. The assay was performed twice using independent enzyme extractions, with three replicates per herbicide concentration.

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

Plants were cultivated and treated with 120 g ha^{-1} imazamox, as previously described. Leaf samples (0.15 g per population per time point) were harvested at 0, 6, 12, 24, 48 and 72 h after treatment and immediately frozen in liquid nitrogen. Samples were stored at −80 C until use. Based on the *ALS* nucleotide sequences of R1, R2, and S obtained earlier, RT-qPCR primers (Table 3) were designed for highly conserved regions using Primer3 Plus (http://www.primer3plus.com/). The *O. sativa* actin (LOC_Os03g50885) gene was selected as the reference gene for RT-qPCR (W Wang et al. 2023). Total RNA was isolated individually from these samples using the RNA Simple Total RNA Kit (Tiangen Biotech), following the manufacturer's instructions. After RNA extraction, RNA (900 ng) was reverse-transcribed to cDNA using HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech). RT-qPCR was carried out using 2× chamQ SYBR qPCR Master Mix (10 µl, Vazyme Biotech, Nanjing, China), each primer (0.4 µl), 50× ROX Reference Dye 1 (0.4 µl, Vazyme Biotech, Nanjing, China), cDNA (1,000 ng), and ddH_2O (6.8 µl), with three biological replicates for each gene. The PCR program was conducted as follows: a pre-denaturation step (30 s at 95 C), followed by 40 cycles of 95 C for 10 s, 60 C for 30 s, and the final step of the melt curve was used for analysis to confirm the PCR product specificity using the default settings. The relative expression level fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated at least twice.

dCAPS Markers for Genotype

To detect the Ser-653-Asn mutation rapidly and accurately, a dCAPS method based on an ALS CT domain sequence was developed. Both primers and restriction enzymes were designed using dCAPS Finder software (http://helix.wustl.edu/dcaps/dcaps.html) (Neff et al. 2002). The forward primer (Table 3) introduced a mismatch to create restriction sites for *Nde*I at the end of a 169-bp fragment to be amplified from the *ALS* gene. The primers were designed such that only the mutant amplicons were digested with *Nde*I, not the wild-type ones.

The PCR reaction system refers to the system described in the "*ALS Gene Sequencing*" section of this study. The PCR product (17 µl) from each population was mixed with excess *Nde*I enzyme (1 µl) and rCut Buffer (2 µl, New England Biolabs, MA, USA). The reaction mixture was incubated at 37 C for 4 h, and the resulting products were separated on a 3% agarose gel stained with ethidium bromide for analysis.

Data Analysis

After preliminary analysis, all data were subjected to a *t*-test using SPSS v. 21.0 (SPSS, Chicago, IL, USA). The results showed no significant differences between assay repetitions (*t*-test, $P > 0.05$). The whole-plant dose–response data were fit to a four-parameter nonlinear log-logistic regression model (Equation 1) and calculated using SigmaPlot v. 12.5 (Systat, San Jose, CA, USA) to determine the herbicide doses causing 50% fresh weight inhibition (GR_{50}) in the whole-plant dose–response experiment with imazamox or in the synergistic effect experiment with other ALS herbicides and metabolic inhibitors and/or inhibiting 50% of the ALS activity (IC_{50}) in the enzyme assays.

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

where *Y* is the fresh weight of the aboveground tissue expressed as a percentage of the untreated control at herbicide dose *x*; *b* is the slope; *c* is the lower limit; *d* is the upper limit; and *g* is the GR_{50} or IC_{50}. Resistance index (RI) was calculated by dividing the GR_{50} (or IC_{50}) of the R population by that of GR_{50} (or IC_{50}) of the S population.

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Table 4. Sensitivity of the three *Oryza sativa* f. *spontanea* populations to other acetolactate synthase (ALS)-inhibiting herbicides.

Group[a]	Herbicide	Recommended field dose g ai ha^{-1}	Populations	GR_{50} ± SE g ai ha^{-1}	RI[b]
IMI	Imazethapyr	105	JYFN-2023-1(S)	18.2 ± 9.2	1.0
			JSSH-2021-1(R1)	413.0 ± 1.9	22.8
			JSSH-2022-4(R2)	399.5 ± 4.7	22.0
	Imazapic	108	JYFN-2023-1(S)	11.5 ± 1.8	1.0
			JSSH-2021-1(R1)	268.9 ± 1.0	23.4
			JSSH-2022-4(R2)	154.2 ± 16.2	13.4
SCT	Flucarbazone-sodium	31.5	JYFN-2023-1(S)	5.9 ± 0.1	1.0
			JSSH-2021-1(R1)	77.2 ± 23.2	13.0
			JSSH-2022-4(R2)	70.4 ± 6.6	11.9
PTB	Pyribenzoxim	45	JYFN-2023-1(S)	98.1 ± 1.0	1.0
			JSSH-2021-1(R1)	850.8 ± 24.2	8.7
			JSSH-2022-4(R2)	4087.3 ± 12.2	41.7
TP	Pyroxsulam	15	JYFN-2023-1(S)	3.9 ± 8.8	1.0
			JSSH-2021-1(R1)	16.1 ± 0.2	4.2
			JSSH-2022-4(R2)	18.8 ± 5.2	4.9
	Penoxsulam	30	JYFN-2023-1(S)	59.0 ± 1.0	1.0
			JSSH-2021-1(R1)	937.3 ± 32.1	15.9
			JSSH-2022-4(R2)	910.0 ± 5.2	15.4
SU	Nicosulfuron	18	JYFN-2023-1(S)	214.7 ± 3.2	1.0
			JSSH-2021-1(R1)	227.0 ± 15.1	1.1
			JSSH-2022-4(R2)	290.3 ± 9.1	1.4
	Mesosulfuron-methyl	15.75	JYFN-2023-1(S)	4.6 ± 0.8	1.0
			JSSH-2021-1(R1)	9.8 ± 2.8	2.1
			JSSH-2022-4(R2)	7.2 ± 22.0	1.6

[a]IMI, imidazolinone; PTB, pyrimidinyl thiobenzoate; SCT, sulfonylamino-carbonyl-triazolinone; SU, sulfonylurea; TP, triazolinones.
[b]RI, resistance index.

Results and Discussion

Whole-Plant Dose–Response Bioassay

Sensitivity to Imazamox

Oryza sativa f. *spontanea* resistance to imazamox was confirmed via whole-plant bioassay. As shown in Table 1, GR_{50} values for 28 *O. sativa* f. *spontanea* populations significantly exceeded ($P < 0.01$) the recommended field dose of 120 g ha^{-1}. Among these, 23 exhibited high resistance ($30 < RI \leq 150$), 3 showed intermediate resistance ($10 < RI \leq 30$), 2 displayed reduced sensitivity ($2 < RI \leq 4$), and 7 remained susceptible ($RI \leq 2$). Five years after the application of IMI-tolerant herbicides, resistant populations accounted for 80% of all tested *O. sativa* f. *spontanea* populations, indicating imazamox resistance is widespread in Jiangsu Province, China.

Similar resistance cases have also been reported abroad. For example, in Italy, IMI-tolerant rice was introduced in 2006, and 4 yr later, researchers detected resistant weedy rice in fields planted with this rice variety (Scarabel et al. 2012). Similarly, in Arkansas, USA, five years after the introduction of IMI-tolerant rice, a decline in the control efficacy of IMI herbicides against weedy rice was observed in corresponding fields (Burgos et al. 2008). These findings are consistent with our research results: resistance to imazamox in weedy rice emerged 4 to 5 yr after the combined use of imazamox and IMI-tolerant rice, reducing the effectiveness of weedy rice control in the field.

Sensitivity to Other ALS-inhibiting Herbicides

Whole-plant bioassays revealed varying RI values for the R1 and R2 populations against ALS-inhibiting herbicides relative to the S population (Table 4). Weedy rice exhibited intermediate resistance ($10 < RI \leq 30$) to imazethapyr and imazapic, which belong to the same IMI herbicide class as imazamox. Similarly, intermediate resistance ($10 < RI \leq 30$) was observed against flucarbazone-sodium (a sulfonylamino-carbonyl-triazolinone herbicide) and penoxsulam (a triazopyrimidine herbicide). Weedy rice exhibited susceptibility ($RI \leq 2$) only to the sulfonylurea (SU) herbicides nicosulfuron and mesosulfuron-methyl.

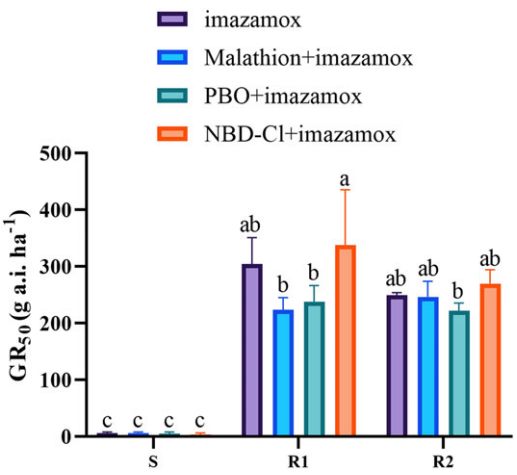

Figure 1. Sensitivities of *Oryza sativa* f. *spontanea* populations JYFN-2023-1(S), JSSH-2021-1(R1), and JSSH-2022-4(R2) to imazamox in the absence or presence of three metabolic inhibitors: malathion, PBO (piperonyl butoxide), NBD-Cl (4-chloro-7-nitro-2,1,3-benzoxadiazole). S, susceptible; R, resistant.

These findings align with prior research demonstrating that ALS enzymes carrying the Ser-653-Asn mutation confer resistance to IMI, but not to SU herbicides such as chlorsulfuron (Chen et al. 2015; Gaines et al. 2020; Piao et al. 2018). Cross-resistance to other IMI herbicides in imazamox-resistant weedy rice has been widely reported, including imazapic and imazapyr in Malaysia (Dilipkumar et al. 2018), imazethapyr in the United States (Burgos et al. 2014), and imazethapyr and imazapyr in northern Greece (Kaloumenos et al. 2013).

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Table 5. Sensitivities of *Oryza sativa* f. *spontanea* (JYFN-2023-1-S, JSSH-2021-1, JSSH-2022-4) populations to imazamox with/without three metabolic inhibitors.

Treatment	GR_{50} ± SE[a] of tested populations			RI[b]	
	JYFN-2023-1(S)	JSSH-2021-1(R1)	JSSH-2022-4(R2)	JSSH-2021-1(R1)	JSSH-2022-4(R2)
	g ai ha^{-1}				
Imazamox[c]	6.1 ± 1.2	307.3 ± 26.9	242.5 ± 2.6	50.4	39.8
Malathion[d]+imazamox	6.3 ± 1.0	222.2 ± 12.5	242.7 ± 15.8	36.5	39.8
PBO[e]+imazamox	4.4 ± 1.7	233.9 ± 16.4	222.8 ± 8.1	38.4	36.6
NBD-Cl[f]+imazamox	3.2 ± 1.6	318.9 ± 56.5	267.0 ± 14.2	52.3	43.8

[a]GR_{50} is the effective dose of herbicide causing 50% inhibition of fresh weight and is expressed as grams of active ingredient per hectare (g ai ha^{-1}). Data are the means of two experiments.
[b]RI is the relative resistance index, ratio of GR_{50} values relative to the susceptible *Oryza sativa* f. *spontanea* population (JYFN-2023-1). The applied field dose of imazamox is 120 g ai ha^{-1}.
[c]Imazamox applied at 0, 3.75, 7.5, 15, 30, 60, and 120 g ai ha^{-1} to JYFN-2023-1; and at 0, 120, 180, 270, 405, 607.5 g ai ha^{-1} to JSSH-2021-1 and JSSH-2022-4.
[d]Malathion: 1,000 g ai ha^{-1}; applied 1 h before herbicide application.
[e]PBO (piperonyl butoxide): 4,200 g ai ha^{-1}; applied 1 h before herbicide application.
[f]NBD-Cl (4-chloro-7-nitro-2,1,3-benzoxadiazole): 270 g ai ha^{-1}; applied 48 h before herbicide application.

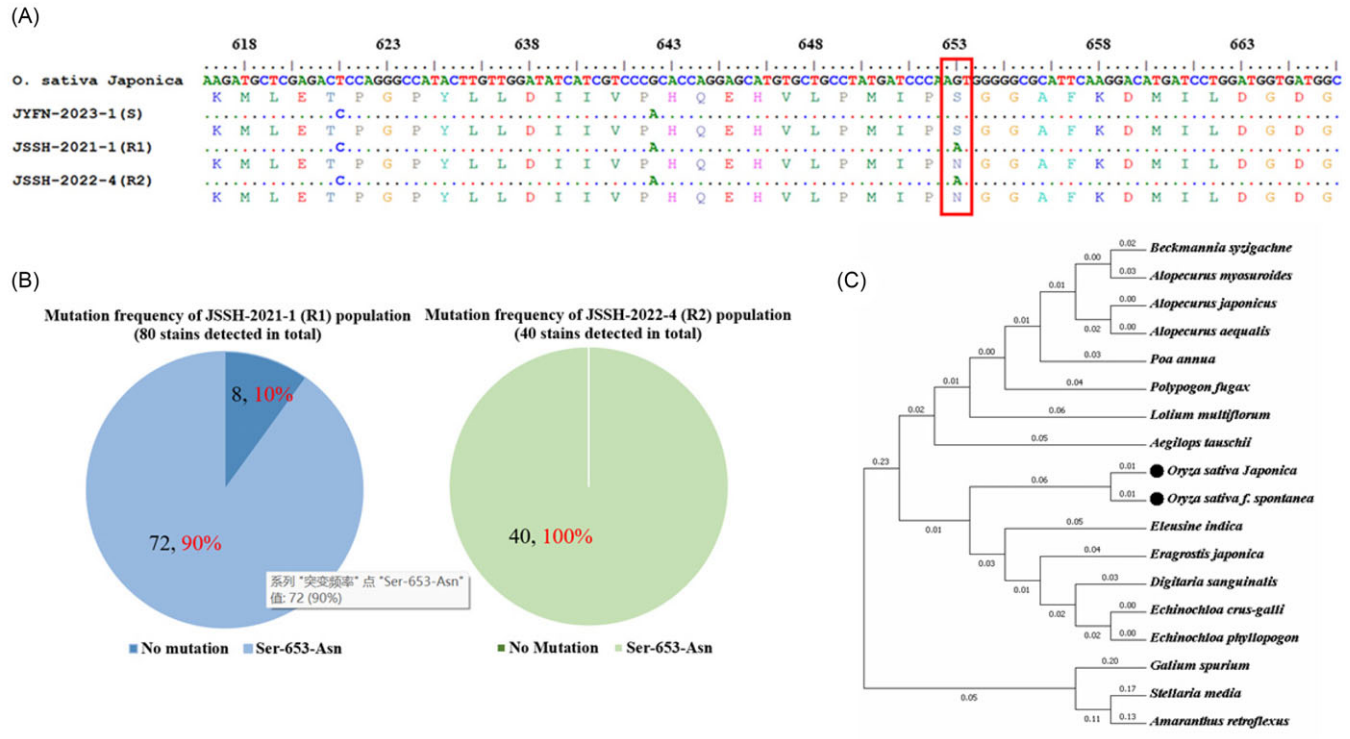

Figure 2. Nucleotide sequence alignment of *ALS* gene fragments from *Oryza sativa* f. *spontanea* (JYFN-2023-1 S, JSSH-2021-1 R1, JSSH-2022-4 R2) populations. S, susceptible; R, resistant. (A) Nucleotide substitution (G→A) was detected at position 653 of *ALS* gene and caused Ser-653-Asn amino acid mutation in R population. Serine (Ser, S), Asparagine (Asn, N). The boxed codons indicate 653 amino acid sequence positions in the *ALS* fragment referring to the full-length sequence of *ALS* from *Arabidopsis thaliana*. (B) Mutation frequencies of the R1 population (JSSH-2021-1) and R2 populations (JSSH-2022-4). (C) Phylogenetic tree related to *O. sativa* f. *spontanea*. *Beckmannia syzigachne* (Steud.) Fernald (accession: KR809881.1); *Alopecurus myosuroides* Huds. (AJ437300.2); *Alopecurus japonicus* (KR534607.1); *Alopecurus aequalis* Sobol. (JQ743908.1); *Poa annua* L. (KM388812.1); *Polypogon fugax* Nees (MN101598.1); *Lolium multiflorum* (AF310684.2); *Aegilops tauschii* Host (FJ997631. 1); *Oryza sativa japonica* S. Kato (AB049822.1); *Eleusine indica* (L.) Gaertn. (KU720629.1); *Eragrostis japonica* (Thunb.) Trin. (ON652847.1); *Digitaria sanguinalis* (L.) Scop. (OR640488.1); *Echinochloa crus-galli* (L.) P. Beauv. (KY071206.1); *Echinochloa phyllopogon* (Stapf) Koso-Pol. (AB636580.1); *Galium spurium* L. (HM006705.1); *Stellaria media* (HE998774.1); *Amaranthus retroflexus* L. (AF363369.1).

Sensitivity to Imazamox with Metabolic Inhibitors

NTSR is also known to play a role in the development of herbicide resistance in weeds and can be achieved by enhanced rates of herbicide metabolism involving CYP450 and GST enzymes (Yu et al. 2014). However, in the present study, the GR_{50} values estimated for R populations treated with the CYP450 inhibitors (malathion and PBO) and the GST inhibitor (NBD-Cl) were similar to those observed for plants treated only with imazamox (Figure 1). The GR_{50} and RI values were similar in both populations, ranging from 222 to 318 g ai ha^{-1} of imazamox and from 36- to 52-fold (Table 5). Therefore, the GR_{50} values were not significantly reversed in the R and S populations after the application of the three CYP450 and GST inhibitors, suggesting that the metabolic enzymes CYP450 and GST may be not the main reason for imazamox resistance in *O. sativa* f. *spontanea*, but further verification is still required.

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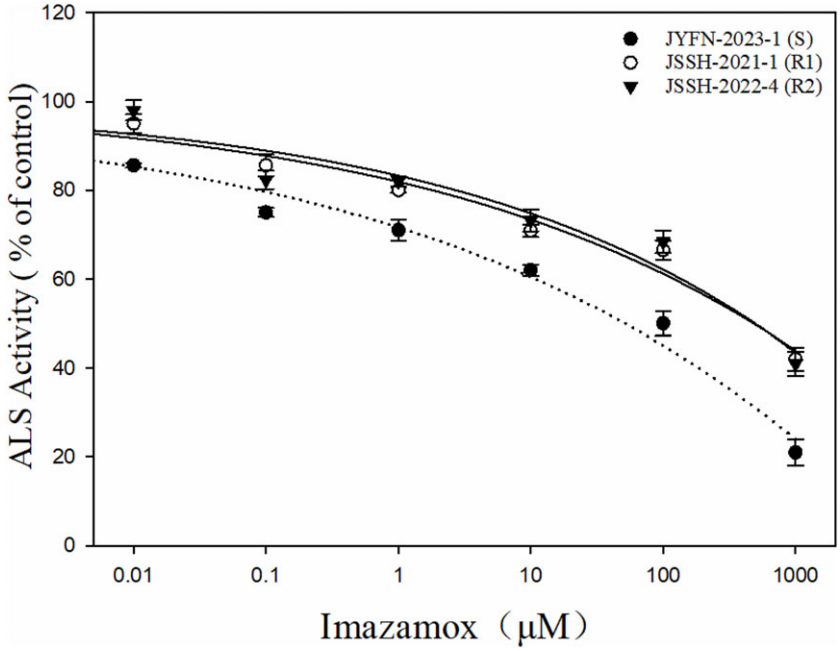

Figure 3. Dose-response curve of in vitro ALS activity of *Oryza sativa* f. *spontanea* populations (JYFN-2023-1-S, JSSH-2021-1, JSSH-2022-4) when treated with imazamox (0.001, 0.01, 0.1, 1, 10, 100 or 1,000 μM). S, susceptible; R, resistant. Vertical bars represent the mean ± SE.

Target-Site Basis of Imazamox Resistance

An ALS Ser-653-Asn Mutation

BLAST analysis of the amplified *ALS* gene sequence showed high similarity (97% to 99%) with *O. sativa* (GenBank accession no. AB049822), indicating that the correct *ALS* sequence was amplified. After nucleotide and predicted amino acid analyses, only one nucleotide mutation (AGT to AAT) in the R population of the *ALS* sequence was detected (Figure 2), resulting in the substitution of Ser to Asn at position 653 (the position is numbered relative to *Arabidopsis thaliana ALS*). No *ALS* mutations were detected in samples from the S population. In addition, the *ALS* sequences of individual plant samples from 80 resistant populations (R1) were detected, of which 72 contained the Ser-653-Asn mutation, accounting for 90%, and 8 (10%) contained no mutations. Similarly, all 40 R2 samples contained the Ser-653-Asn mutation in the *ALS* sequences, accounting for 100%. In many cases, weeds have been reported to evolve resistance to ALS-inhibiting herbicides due to the TSR mechanism (Ferreira et al. 2023; Wang et al. 2024). There were some *ALS* mutations reported in resistant *O. sativa* f. *spontanea* populations, such as Ser-653-Thr/Asn (Piao et al. 2018; Ruzmi et al. 2020; Unan et al. 2024). Numerous studies have shown that the substitution of Ser-653 in weedy rice confers resistance to IMI herbicides (Kaloumenos et al. 2013; Scarabel et al. 2012; Unan et al. 2024). In our study, the Ser-653-Asn mutation was also discovered in the *ALS* of *O. sativa* f. *spontanea* in China (Figure 2). Notably, two common mutations linked to IMI herbicide resistance, Ala-122 and Trp-574, were not found in the populations examined in this study (Wedger et al. 2022). Our results showed that *O. sativa* f. *spontanea* had resistance to imazamox because of the Ser-653-Asn mutation.

Lower ALS Sensitivity to Imazamox In Vitro

Many studies have indicated that the intensive targeting of herbicides by enzymes is a common TSR mechanism in weeds, especially for herbicides with specific targets (Fang et al. 2019; Gao et al. 2017; Li et al., 2023). The ALS activities of R1, R2, and S were inhibited to different extents, and this inhibition was positively correlated with the increasing concentrations of imazamox (Figure 3). The IC_{50} value of the S population was 10.8 μM; the IC_{50} values of the resistant populations R1 and R2 were 865.1 and 954.9 μM, respectively. Previous studies reported the ALS activity of *O. sativa* f. *spontanea* populations containing Ser-653-Asn mutation was higher compared with susceptible populations (Ruzmi et al. 2020). In this study, the RI of the R populations was 80.0, 88.3-fold higher than that of the S population, suggesting that the insensitive target ALS may be responsible for the resistance of *O. sativa* f. *spontanea* to imazamox. The in vitro ALS sensitivity assay results demonstrated that an insensitive target, ALS, may contribute to the resistance of *O. sativa* f. *spontanea* to imazamox.

ALS Gene Expression

TSR can also be due to overexpression of the target-site gene, with more enzyme produced than can be substantially inhibited by typical herbicide application rates (Gaines et al. 2020), but many studies have shown that there is no significant difference in the expression levels of target genes between resistant and susceptible populations. While some resistant populations showed high-level resistance to tribenuron-methyl, their *ALS* expression levels remained comparable to those in susceptible populations, indicating complex relationships between target enzyme expression and ALS-inhibiting herbicide-resistance development. In our study, without the treatment of imazamox, the expression level of the *ALS* gene in the S population was higher than that in the R1 and R2 populations. After the treatment with imazamox, the expression level of the *ALS* gene in the S population increased at 6, 12, 24, and 48 h after application by 5.4, 4.2, 1.3, and 3.1 times, respectively; in the R1 population, the expression level of *ALS* gene increased at 24, 48, and 72 h after application by 0.4, 1.3, and 0.9 times, respectively, compared with the untreated S population; in

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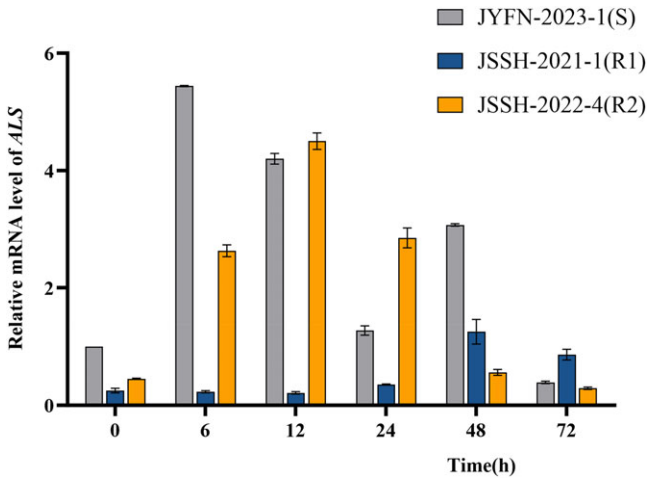

Figure 4. Relative mRNA level of *ALS* gene in *Oryza sativa* f. *spontanea* populations (JYFN-2023-1-S, JSSH-2021-1, JSSH-2022-4) treated with imazamox (120 g ai ha^{-1}). S, susceptible; R, resistant. Vertical bars represent the mean ± SE.

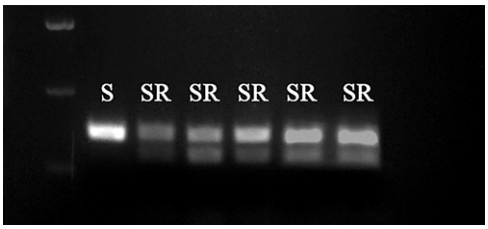

Figure 5. The derived cleaved polymorphic amplified sequence technique (dCAPS) method developed for detecting Ser-653-Asn mutations. Heterozygous resistant (SR) and homozygous sensitive (SS) genotypes are shown.

the R2 population, the expression level of *ALS* gene increased at 6, 12, 24, and 48 h after application by 2.6, 4.5, 2.9, and 0.6 times, respectively, compared with the untreated S population (Figure 4). The *ALS* expression level in the S population increased most significantly, reaching 5.44 times; while the expression levels in the R1 and R2 populations increased by 1.25 times and 4.5 times, respectively, both lower than in the S population. Therefore, we conclude that the changes in the expression levels of the *ALS* gene may not be the target enzyme mechanism for imazamox resistance in *O. sativa* f. *spontanea*.

dCAPS Method for the Ser-653-Asn Mutation in Oryza sativa f. spontanea

After restriction digestion with *Nde*I, all R populations detected were cut into two fragments of 135 and 34 bp, whereas the S populations were not cut. The results showed that all homozygous alleles (SS) were detected in the S populations, and resistant alleles (RS) were detected in the R populations (Figure 5). At the same time, the DNA sequence analysis results were consistent with the results of the dCAPS method, confirming that the dCAPS method was very accurate and effective in detecting the Ser-653-Asn mutation in *O. sativa* f. *spontanea*.

In conclusion, our study identified *O. sativa* f. *spontanea* resistance to imazamox and cross-resistance to the most commonly used ALS-inhibiting herbicides in Jiangsu Province. The TSR mechanism contributes to the resistance of *O. sativa* f. *spontanea* to imazamox, including an insensitive ALS target enzyme, the Ser-653-Asn mutation in *ALS*, and the overexpression of the *ALS* gene.

Data availability. Data will be made available on request.

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Competing interests. The authors declare no conflicts of interest and no competing financial interests.

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