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Molecular Basis of Resistance to Bensulfuron-Methyl in a Smallflower Umbrella Sedge (*Cyperus difformis* L.) Population from China

Shanshan Yin [1,†], Wei Hu [1,2,3,†], Yin Chai [1], Minghao Jiang [1,2,3], Jingxu Zhang [1,2,3], Haiqun Cao [1,2,3], Ning Zhao [1,2,3] and Min Liao [1,2,3,*]

[1] School of Plant Protection, Anhui Agricultural University, Hefei 230036, China
[2] Anhui Province Key Laboratory of Integrated Pest Management on Crops, School of Plant Protection, Anhui Agricultural University, Hefei 230036, China
[3] Anhui Province Engineering Laboratory for Green Pesticide Development and Application, School of Plant Protection, Anhui Agricultural University, Hefei 230036, China
* Correspondence: liaomin3119@126.com
† These authors contributed equally to this work.

Abstract: Smallflower umbrella sedge (*Cyperus difformis* L.) is an invasive weed, and infestations of *C. difformis* are increasing in rice (*Oryza sativa* L.) fields in China. Bensulfuron-methyl is a widely used sulfonylurea herbicide that inhibits the acetolactate synthase (ALS) enzyme and has been used in recent years for effectively controlling annual weeds in the Cyperaceae family. In this study, a suspected resistant population of *C. difformis* (BBHY1) was collected from a rice field in Huaiyuan County, Anhui Province, China, that survived treatment with bensulfuron-methyl at the field-recommended rate (FRR). Single-dose tests and whole-plant bioassays confirmed that the BBHY1 population was resistant to bensulfuron-methyl and had evolved a high level of resistance, with a resistance index (RI) of 12.87. Sequencing of the *ALS* gene revealed a CCT to CAT point mutation at codon 197, which caused a P-to-H substitution in the resistant plants. Analysis of the relative expression of *ALS* revealed no significant differences between the resistant and susceptible populations. Inhibiting the activity of cytochrome P450s (P450s) or glutathione S-transferases (GSTs) had no significant effect on bensulfuron-methyl resistance. The BBHY1 population exhibited cross-resistance to pyrazosulfuron-ethyl, penoxsulam, and bispyribac-sodium, with RIs ranging from 5.48 to 20.63, but remained susceptible to MCPA sodium, florpyrauxifen-benzyl, and bentazon, with RIs of <1.00. These herbicides could be potentially used as alternatives for controlling resistant populations and managing herbicide resistance in other aggressive weeds in rice fields.

Keywords: *Cyperus difformis*; bensulfuron-methyl; target-site resistance; cross-resistance

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1. Introduction

Rice (*Oryza sativa* L.) is one of the three major crops in the world and is also the main source of food for the global population [1]. Smallflower umbrella sedge (*Cyperus difformis* L.) is a problematic annual weed that grows in rice fields and is widely distributed throughout the tropical and warm temperate regions of the world [2]. Previous studies have reported that *C. difformis* has variable ploidy levels (diploidy and tetraploidy) and chromosomal deletions [3]. The weed has a strong tillering capacity that enhances its ability to compete with rice seedlings, which, subsequently, reduces crop growth and results in large yield losses. The traditional methods for controlling *C. difformis* in the past few decades primarily relied on the use of herbicides, especially those inhibiting the acetolactate synthase (ALS; EC 2.2.1.6) enzyme.

The ALS enzyme of plants catalyzes the initial step in the production of branched-chain amino acids, including valine, leucine, and isoleucine [4]. This enzyme is the principal

target of six types of herbicides, including imidazolinones (IMIs), pyrimidinyl benzoates (PYBs), sulfonylureas (SUs), sulfonanilides, sulfonylamino-carbonyl-triazolinones (SCTs), and triazolopyrimidines (TPs) [5]. Inhibitors of the ALS enzyme have been continually used for controlling weeds in crop fields for over 40 years. Unfortunately, their widespread application has resulted in the development of herbicide resistance in an increasing number of weeds (171 species at present) [6], which is seriously affecting the yield of various crop species.

There are two primary mechanisms of herbicide resistance, namely, target-site resistance (TSR) and nontarget-site resistance (NTSR) [7]. Recent studies have reported that mutations at nine sites, namely, A122, P197, A205, F206, D376, R377, W574, S653, and G654 (numbering standardized according to the sequence of *Arabidopsis thaliana* (L.)), induce TSR to ALS inhibitors [8,9]. The presence of mutations at different sites, including P197G/A/L/S/H, D376G, and W574L, has been confirmed in ALS-resistant *C. difformis* by molecular analysis. Resistance to ALS-inhibiting herbicides can also be mediated via other mechanisms, such as the overexpression of *ALS* in shortawn foxtail (*Alopecurus aequalis* sobol.) [10]. Unlike those of TSR, the mechanisms underlying NTSR reduce the number of active herbicide molecules reaching the target site [11]. Enhanced herbicide metabolism is the most common mechanism of NTSR [12] and requires the involvement of multiple metabolic enzymes, including cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), and ATP-binding cassette (ABC) transporters [13,14]. TSR and NTSR may coexist in herbicide-resistant weed populations in certain instances.

ALS inhibitors such as bensulfuron-methyl have been frequently applied for controlling *C. difformis* in rice fields since the 1980s, and there have been some reports on the resistance of *C. difformis* to ALS inhibitors in recent years. For instance, Merotto et al. reported an IR population from Italy that was resistant to five ALS inhibitors [15]. Another study by Ntoanidou et al. reported that the insufficient control of *C. difformis* following the application of ALS inhibitors in rice fields located in northern Greece was attributed to the evolution of TSR to these herbicides [16]. To date, there are few reports on the resistance of *C. difformis* to ALS inhibitors in China. In this study, a suspected resistant population of *C. difformis* (BBHY1) was collected from a rice field in Huaiyuan County, Anhui Province, China, that survived treatment with bensulfuron-methyl at the field-recommended rate (FRR). The study aimed to first determine the level of resistance of this population to bensulfuron-methyl and identify any cross-resistance or multiple resistance to different herbicides. The study also aimed to investigate the mechanisms underlying the resistance of the BBHY1 population of *C. difformis*.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Seeds of the susceptible (S) HFLJ1 population and the suspected resistant (R) BBHY1 population were randomly harvested from fallow land in Lujiang County (31.48° N, 117.23° E) and a rice field in Huaiyuan County (32.94° N, 117.14° E), Anhui Province, China, respectively. Mature seeds were collected from ≥300 individual plants of each population from a rice field treated with bensulfuron-methyl. The seeds were air-dried and stored in paper bags at 4 °C until further experimentation.

Plastic pots (9 × 9 × 7 cm) were filled with the cultivated soil, which comprised a mixture of equal volumes of nutrient soil and vermiculite. The seeds were germinated in Petri dishes covered with moist filter paper in an incubator at a constant temperature of 25 °C under a 12 h photoperiod. After seven days, 12 seedlings were transferred to pots and grown in a controlled greenhouse at a relative humidity of ~75% and a temperature of 35 and 25 °C during the day and night, respectively. The seedlings were thinned to six plants per pot when they had reached the two- to three-leaf stage.

2.2. Herbicides and Chemicals

Whole-plant dose-response experiments were performed to test the seven herbicides with three distinct modes of action (MOAs; Table 1). Malathion (M, 99%, Sigma-Aldrich, Shanghai, China) and 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl, 98%, Aladdin, Shanghai, China) were used as inhibitors of P450 and GST, respectively.

Table 1. Herbicides and rates used in whole-plant dose-response experiments.

Group	Herbicides	Formulation	Commercial Name	Supplier	Test Doses (g a.i. ha^{-1})	
					Susceptible Population	Resistant Population
SU	Bensulfuron-methyl	60% WDG	Shinong	Jisu, Jiangsu	0, 0.19, 0.56, 1.67, 5, 15, 45	0, 1.67, 5, 15, 45, 135, 405
SU	Pyrazosulfuron-ethyl	15% OD	Jiangxing	Shangheworld, Anhui	0, 0.09, 0.28, 0.83, 2.50, 7.50, 22.50	0, 0.83, 2.50, 7.50, 22.50, 67.50, 202.50
TP	Penoxsulam	25 g L^{-1} OD	Daojie	Corteva AgroSciences, Shanghai	0, 0.12, 0.37, 1.11, 3.33, 10, 30	0, 1.11, 3.33, 10, 30, 90, 270
PYB	Bispyribac-sodium	100 g L^{-1} SC	Bugao	Zhongshan, Zhejiang	0, 0.12, 0.37, 1.11, 3.33, 10, 30	0, 1.11, 3.33, 10, 30, 90, 270
AM	MCPA sodium	13% AS	Caojiang	Huaxing, Anhui	0, 4, 12, 36, 108, 324, 972	0, 4, 12, 36, 108, 324, 972
AM	Florpyrauxifen-benzyl	3% EC	Lingsko	Corteva AgroSciences, Shanghai	0, 1.13, 2.25, 4.50, 9, 18, 36	0, 1.13, 2.25, 4.50, 9, 18, 36
PS II inhibitor	Bentazon	480 g L^{-1} SL	Kuaijing	Ruibang, Jiangsu	0, 5.93, 17.78, 53.33, 160, 480, 1440	0, 5.93, 17.78, 53.33, 160, 480, 1440

SU, sulfonylurea; TP, triazolopyrimidine; PYB, pyrimidinyl benzoates; AM, auxin mimics; PS II inhibitor, photosystem II; WDG, water dispersible granule; OD, oil dispersion; SC, suspension concentrate; AS, aqueous solution; EC, emulsifiable concentrate; SL, soluble concentrate.

2.3. Single-Dose Experiments

The single-dose experiments were performed according to the methodology described by Zhao et al. [17]. Briefly, the seedlings were treated with bensulfuron-methyl at the FRR (corresponding to 45 g active ingredient (a.i.) ha^{-1}) at the three- to four-leaf stage, while the control group was only treated with water. The herbicides and water were applied using a laboratory cabinet sprayer (3WP-2000, Nanjing Mechanization Research Institute of the Ministry of Agriculture, Nanjing, China), which delivered 450 L ha^{-1} of the liquid at a pressure of 0.275 MPa. The growth status of the seedlings was visually checked after three weeks of treatment. A plant was considered to be "alive" if it exhibited visible regeneration; otherwise, it was regarded as "dead". Fresh leaves were collected from each of the control and surviving plants and frozen at −80 °C until further use.

2.4. Susceptibility to Bensulfuron-Methyl and Cross- and Multiple-Resistance to Different Herbicides

In order to investigate the level of susceptibility of the R population to bensulfuron-methyl and six other herbicides, including three ALS inhibitors (pyrazosulfuron-ethyl, penoxsulam, and bispyribac-sodium), two auxin mimics (MCPA sodium and florpyrauxifen-benzyl), and one photosystem II (PS II) inhibitor (bentazon), the seedlings of the R population were treated with these herbicides at the three- to four-leaf stages and the herbicide resistance was determined by whole-plant dose-response bioassays (Table 1). The resistance to a series of doses of the different herbicides was determined based on preliminary tests. The aerial parts of the plants were excised 21 days after treatment (DAT), and their fresh weights were recorded. Each treatment had three replicates, and the experiment was conducted twice.

2.5. Identification of Mutations in the ALS Gene

Fresh leaves were randomly harvested from the S and R plants that survived the single-dose experiments. The *ALS* gene was amplified using the genomic DNA extracted using the standard cetyltrimethylammonium bromide (CTAB) procedure [18]. The *ALS* gene was amplified by polymerase chain reaction (PCR) using the primers (Table 2) reported by Huang et al. [19] for amplifying fragments of the *ALS* gene containing all nine known resistant mutation sites. PCR amplification was performed using 2× Super Pfx MasterMix (CWBIO, Beijing, China) according to the manufacturer's instructions. The conditions of PCR were as follows: initial denaturation at 98 °C for 3 min, 35 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 10 min.

Table 2. The corresponding primers used to amplify and expression levels of *ALS* gene in *C. difformis*.

Primers	Sequence (5′-3′) [a]	Product Size (bp)	Annealing Temperature (°C)	Usage
ALS-F	ATCCAAGCACTCCAAACCCTCCT	1934	55	Sequencing for *ALS*
ALS-R	AGCCTACCATCAGAAAGTTCAA			
qALS-F	CCTAGCAATGATGAGCTGTCTCT	109	60	Expression level for *ALS*
qALS-R	CAAATCTCACCCCAAAGGCTAAC			
qACT-F	TGGTATTGTGCTTGACTCTGG	184	60	
qACT-R	TCTCACAATTTCCCGCTCG			

[a] DNA sequences from 5′ to 3′ and 5′ and 3′ mean "five prime" and "three prime", which indicate the carbon numbers in the DNA's sugar backbone.

The amplified PCR products were purified using a FastPure® Gel DNA Extraction Mini Kit (Vazyme, Nanjing, China) and directly sequenced by Tsingke Biotech (Beijing, China). The *ALS* genes of *C. difformis* were aligned with the *ALS* gene of *C. difformis* (GenBank accession number: EF061294.2) using the DNAMAN v 6.03 software (Lynnon, Quebec, Canada). The nine known sites of resistant mutation in the *ALS* gene were analyzed for detecting genetic mutations.

2.6. Analysis of the Expression of the ALS Gene

When the weed seedlings reached the two- to three-leaf stages, six individual plants were randomly selected from the S and R populations treated with bensulfuron-methyl, and approximately 50 mg of fresh leaf tissues was collected from each plant at 0, 12, and 24 h following herbicide treatment. The total RNA was extracted from each sample using a TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA) and stored in the refrigerator at −80 °C until further use. The first strand of cDNA was synthesized according to the instructions provided with the TransScript One-step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). The PCR cycle and reaction apparatus were selected based on the manufacturer's recommendations. In ALS-inhibiting herbicide resistance research, β-actin was constantly used as the internal control gene under ALS inhibitor treatment in weeds [20]. The primers [21] reported by Huang et al. were used for gene expression analyses (Table 2), and the expression of the ALS gene was determined relative to that of β-actin using the $2^{-\Delta\Delta Ct}$ method. Each of the experiments had six biological replicates, and each biological replicate comprised three technical replicates. The fold change (two-fold) and Student's *t*-test ($p < 0.05$) were used to determine whether the ALS gene was upregulated or downregulated in the R plants compared to the S plants.

2.7. Susceptibility to Bensulfuron-Methyl after P450 or GST Inhibition

The bioassay used for determining the susceptibility to bensulfuron-methyl following the inhibition of P450 and GST enzymes was performed in parallel to the bioassays for determining cross- and multiple-resistance (refer to Section 2.4). Briefly, the S and R plants were treated with malathion, malathion plus bensulfuron-methyl, NBD-Cl, or NBD-Cl plus

bensulfuron-methyl at the three- to four-leaf stages. Malathion was applied at a dose of 1000 g a.i. ha^{-1}, 1 h prior to the application of bensulfuron-methyl, while NBD-Cl was applied at a dose of 270 g a.i. ha^{-1}, 48 h before the application of bensulfuron-methyl. The malathion and NBD-Cl was first dissolved in DMSO (Dimethyl sulfoxide) until it was just dissolved completely, then it was diluted to constant volume at corresponding concentration with pure water. Bensulfuron-methyl was subsequently applied at a series of doses, which are enlisted in Table 1. The aerial parts of the plants in the respective pots were excised at 21 DAT, and their fresh weights were recorded.

2.8. Data Analyses

All the whole-plant dose-response experiments were performed twice (two runs). The results of ANOVA revealed no significant differences ($p > 0.05$) between the repeated experiments. Data from the same trial were, therefore, pooled across runs and fitted to a four-parameter log-logistic curve (Equation (1)) using the SigmaPlot v. 14.0 software (Systat Software, San Jose, CA, USA). The following equation was obtained by curve fitting:

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

where C represents the lower limit, D represents the upper limit, x is the dose of herbicide applied, and b is the slope at which the dose caused a 50% growth reduction (GR_{50}). The resistance index (RI) was calculated based on the GR_{50} value for estimating the level of herbicide resistance of the R population relative to that of the S population.

3. Results

3.1. Single-Dose Testing

The S and the suspected R populations were treated with bensulfuron-methyl at the FRR. The symptoms of herbicide damage began to appear in the S population, and the heart leaves became pale and the green coloration faded at 7 DAT. The growth of the aboveground parts of the plants in the S population was significantly inhibited at 14 DAT, and the growth of the new leaves was inhibited compared to that of the untreated control plants. As expected, all the plants in the S population died following treatment with bensulfuron-methyl, while all the plants in the R population exhibited visible regrowth at 21 DAT (Figure 1). The findings revealed that the BBHY1 population had decreased resilience to the ALS-inhibiting herbicide, bensulfuron-methyl.

3.2. Level of Susceptibility to Bensulfuron-Methyl and Cross- and Multiple-Resistance to Different Herbicides

The resistance of the R population to bensulfuron-methyl and six other common herbicides was determined based on the responses of the plants. As expected, the plants in the S population died following exposure to the herbicides applied at the FRR. The results demonstrated that the R population had evolved high levels of bensulfuron-methyl resistance (RI = 12.87, Table 3, Figure 2A) and cross-resistant to the other ALS-inhibiting herbicides, including pyrazosulfuron-ethyl (RI = 20.63, Table 3, Figure 2B), penoxsulam (RI = 7.76, Table 3, Figure 2C), and bispyribac-sodium (RI = 5.48, Table 3, Figure 2D). Nevertheless, the R population could be controlled by herbicides targeting other sites, including auxin mimics (MCPA sodium and florpyrauxifen-benzyl) and the PS II inhibitor (bentazon), similar to the S population (Table 3, Figure 2E–G).

Figure 1. The R (BBHY1) and S (HFLJ1) biotype plants 21 days after FRR doses of bensulfuron-methyl treatments, showing untreated (CK) and bensulfuron-methyl treatment (T).

Table 3. Parameters of the log-logistic equation (1) [a] for whole-plant dose-response of S and R populations for the seven herbicides tested.

Herbicide	Biotype [b]	Regression Parameters			GR_{50} (g a.i. ha^{-1}) (SEM)	RI [c]
		C (SEM)	*D* (SEM)	b (SEM)		
Bensulfuron-methyl	R	1.55 (5.43)	102.11 (4.71)	1.28 (0.25)	30.51 (4.53)	12.87
	S	3.89 (5.80)	104.41 (6.87)	1.43 (0.37)	2.37 (0.44)	
Pyrazosulfuron-ethyl	R	−7.52 (4.04)	101.43 (2.62)	0.95 (0.09)	19.80 (1.77)	20.63
	S	4.74 (2.13)	101.49 (2.84)	1.65 (0.19)	0.96 (0.07)	
Penoxsulam	R	3.71 (2.06)	96.20 (2.13)	1.47 (0.14)	16.60 (1.12)	7.76
	S	3.54 (1.70)	100.66 (1.55)	1.41 (0.10)	2.14 (0.11)	
Bispyribac-sodium	R	−3.48 (5.79)	100.83 (4.97)	1.49 (0.33)	21.11 (3.21)	5.48
	S	−22.17 (31.23)	102.57 (15.39)	0.71 (0.35)	3.85 (2.45)	
MCPA sodium	R	2.62 (3.57)	102.39 (3.72)	1.54 (0.24)	58.94 (6.41)	0.85
	S	−1.62 (3.48)	99.45 (3.18)	1.39 (0.19)	69.62 (6.80)	
Florpyrauxifen-benzyl	R	3.29 (0.88)	98.00 (1.02)	3.91 (0.26)	4.98 (0.08)	0.87
	S	1.81 (1.57)	95.57 (1.62)	4.15 (0.38)	5.71 (0.17)	
Bentazon	R	2.34 (1.05)	99.63 (1.45)	2.41 (0.22)	53.10 (1.57)	0.93
	S	1.58 (1.99)	95.24 (2.61)	2.21 (0.33)	56.94 (3.45)	

[a] Equation (1): $y = C + (D - C)/[1 + (x/GR_{50})^b]$, *C* is the lower limit, *D* is the upper limit, and b is the slope of the curve at the herbicide dose resulting in a GR_{50}. [b] R, resistant population BBHY1; S, susceptible population HFLJ1. [c] RI, resistance index. RI was calculated based on the GR_{50} values of the resistant versus susceptible population.

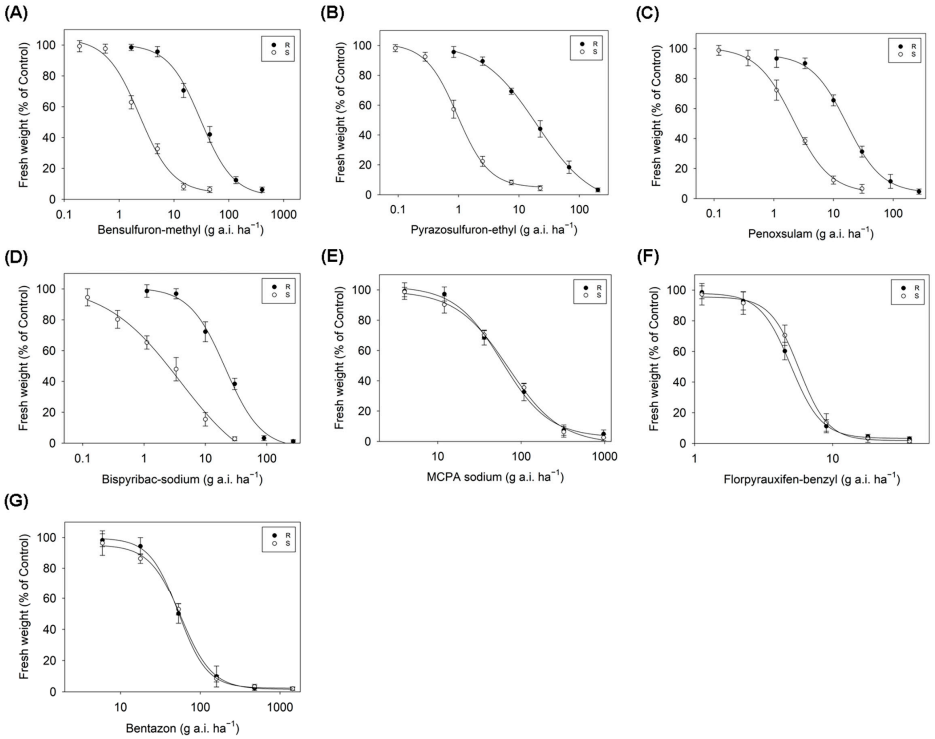

Figure 2. Different herbicide susceptibilities of the S (HFLJ1, ○) and R (BBHY1, ●) populations of *C. difformis*, including (**A**) bensulfuron-methyl, (**B**) pyrazosulfuron-ethyl, (**C**) penoxsulam, (**D**) bispyribac-sodium, (**E**) MCPA sodium, (**F**) florpyrauxifen-benzyl, and (**G**) bentazon. Vertical bars represent the standard errors of the means (SEMs).

3.3. Sequencing and Analysis of the Expression of the ALS Gene

In order to investigate whether the target-site mutations contributed to the development of the resistance phenotypes of *C. difformis*, a 1934 bp fragment spanning all nine known mutation sites in the *ALS* gene was obtained from each of the individual plants. The *ALS* genes shared 99.70% sequence identity with the *ALS* gene of *C. difformis* (GenBank accession number: EF061294.2), which implied that the target genes could be amplified. Notably, all the resistant plants contained a single-nucleotide mutation from CCT to CAT in codon 197 of the *ALS* gene, which resulted in a P-to-H substitution in the ALS enzyme (Figure 3A,B).

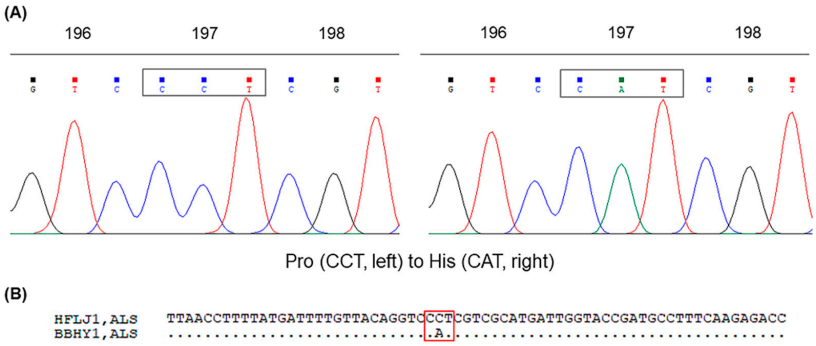

Figure 3. Gene sequencing of *ALS* from BBHY1 and HFLJ1 populations. (**A**) A P197H substitution of *ALS* in the R plants (BBHY1, right) compared with the S plants (HFLJ1, left). (**B**) Partial fragments of *ALS* genes used to sequence were derived from the S (HFLJ1) and R (BBHY1) plants. The region with a square represents the mutation at codon position 197 of *ALS*.

The expression levels of the *ALS* gene in the six plants obtained from the S and R populations were characterized. The expression of the *ALS* gene in S and R plants was normalized to that of *β-actin*. In the R population, the expression of the *ALS* gene relative to that of *β-actin* was not significantly different (fold change < 2, $p > 0.05$) from that of the S plants at the three time points; it ranged from 1.35- to 1.6-fold (Figure 4).

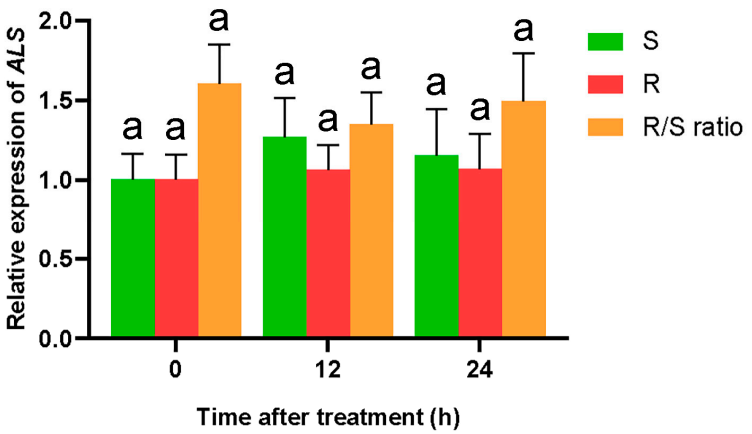

Figure 4. *ALS* gene expression levels relative to control at 0, 12, and 24 h after bensulfuron-methyl administration in *C. difformis* plants. Different letters represent a significant difference ($p < 0.05$). No obvious difference (fold change < 2, $p > 0.05$) was detected in the *ALS* expression between the R (BBHY1) and S (HFLJ1) plants.

3.4. Effects of Malathion and Pretreatment with NBD-Cl on Bensulfuron-Methyl Resistance

Analysis of the whole-plant response of the R and S populations treated with malathion or NBD-Cl alone revealed that there was no obvious difference in plant mortality relative to that of the untreated control. The results demonstrated that there was no significant difference in the GR_{50} of the R and S populations following treatment with bensulfuron-methyl plus malathion or pretreatment with NBD-Cl. The findings indicated that bensulfuron-methyl resistance in the R population was not attributed to metabolic enhancement mediated via the CYP450 or GST enzymes (Table 4, Figure 5).

Table 4. Parameters of the log-logistic equation (1) [a] for the whole-plant dose-response of the cytochrome P450 inhibitor (malathion) and GST inhibitor (NBD-Cl) on *C. difformis* resistance to bensulfuron-methyl.

Herbicide	Biotype [b]	Regression Parameters			GR_{50} (g a.i. ha^{-1}) (SEM)	RI [c]
		C (SEM)	*D* (SEM)	b (SEM)		
Bensulfuron-methyl	R	1.55 (5.43)	102.11 (4.71)	1.28 (0.25)	30.51 (4.53)	12.87
	S	3.89 (5.80)	104.41 (6.87)	1.43 (0.37)	2.37 (0.44)	
Bensulfuron-methyl + malathion	R	1.75 (5.44)	100.56 (4.75)	1.46 (0.32)	30.88 (4.71)	14.30 NSD
	S	2.33 (6.62)	104.20 (8.49)	1.43 (0.44)	2.16 (0.47)	
Bensulfuron-methyl + NBD-Cl	R	3.20 (1.35)	95.86 (1.16)	1.82 (0.12)	33.70 (1.30)	13.92 NSD
	S	−0.57 (2.06)	98.75 (2.37)	1.49 (0.14)	2.42 (0.16)	

[a] Equation (1): $y = C + (D - C)/[1 + (x/GR_{50})^b]$, *C* is the lower limit, *D* is the upper limit, and b is the slope of the curve at the herbicide dose resulting in a GR_{50}. [b] R, resistant population BBHY1; S, susceptible population HFLJ1. [c] RI, resistance index. RI was calculated based on the GR_{50} values of the resistant versus susceptible population. NSD, not significantly different ($p > 0.05$) for the corresponding treatment groups in the R or S populations.

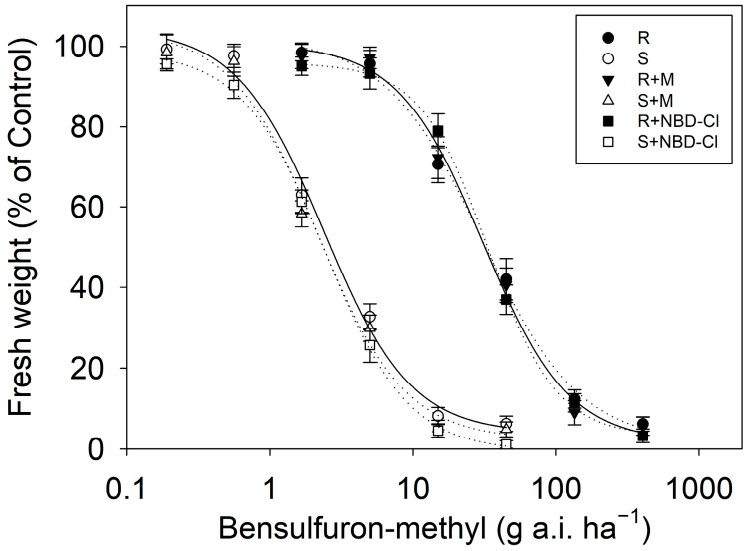

Figure 5. Dose–response curves for the fresh weights of S (HFLJ1, ○) and R (BBHY1, ●) populations of *C. difformis* treated with a range of bensulfuron-methyl doses plus or minus 1000 g a.i. ha^{-1} malathion (M) (▼, △) or 270 g a.i. ha^{-1} 4-chloro-7-nitrobenzoxadiazole (NBD-Cl) (■, □). Vertical bars represent the standard errors of the means (SEMs).

4. Discussion

Cyperus difformis is a malignant grass weed that massively invades the rice fields in China and has become a serious concern. Bensulfuron-methyl is a highly effective ALS-inhibiting herbicide that has been widely used for controlling *C. difformis* over the past decade. However, resistance to bensulfuron-methyl has been increasingly observed in *C. difformis* in recent years owing to the excessive use of this herbicide [19,21–23].

Several studies have reported that weeds can rapidly develop herbicide resistance if ALS inhibitors are continually used for three to five years [24,25]. The present study identified a BBHY1 population from rice fields in Anhui Province where ALS inhibitors had been continually applied for more than 10 years, and the population was found to have developed high levels of resistance to bensulfuron-methyl (RI > 10). The evolution of bensulfuron-methyl resistance in *C. difformis* is attributed to the selection pressure generated by herbicide stress, resulting from the historical application of ALS-inhibiting herbicides.

Mutations in the *ALS* gene that confer herbicide resistance constitute the primary mechanism underlying the evolution of resistance to ALS inhibitors in weeds [7]. Previous studies have identified nine mutation sites in the *ALS* gene that confer resistance to ALS inhibitors [26]. The substitution of P197 in the ALS enzyme to A, S, R, H, or L confers cross-resistance to ALS inhibitors in *C. difformis* [14,19,21,22]. Ntoanidou S et al. found that P197A or S mutation confer resistance to ALS-inhibiting herbicides in *C. difformis* [15]. Li et al. also reported a study on the ALS-inhibitor-resistant *C. difformis* population with a P197A mutation [22]. In this study, sequence analysis of the *ALS* gene revealed a P-to-H substitution at residue 197 (Figure 3A,B), which confirmed that this point mutation in the *ALS* gene is responsible for the herbicide resistance of the R population. A previous study reported that the P197H substitution conferred resistance to ALS inhibitors in a *C. difformis* population growing in the rice fields in Arkansas [27]. The present study is the first to identify resistant *C. difformis* from a rice field in China with a P197H mutation in the *ALS* gene. The P197H substitution has also been detected in several other species of weeds, including arrowhead plant (*Sagittaria trifolia* L.) [28], catchweed bedstraw (*Galium aparine* L.) [29], *Ammannia multiflora* Roxb. (Lythraceae) [30], rock bulrush (*Schoenoplectus juncoides* Roxb.) [31], and flixweed (*Descurainia sophia* L.) [32].

The overexpression or amplification of genes results in the increased abundance of target proteins and is regarded as another mechanism of TSR. Zhao et al. [10] reported that

the expression of the *ALS* gene in an R population of *A. aequalis* was upregulated by 21.0- and 15.5-fold at 2 and 3 DAT, respectively, compared to that of the S plants. Sen et al. [33] reported that the expression of the *ALS* gene of an R population of poverty brome (*Bromus sterilis* L.) increased by nearly two-fold compared to that of the S population, and the overexpression of the *ALS* gene, and not the copy number variation (CNV), contributed to pyroxsulam resistance. However, the present study revealed that there was no significant difference in the expression of the *ALS* gene between the R (fold change < 2, $p > 0.05$) and S populations at three time points, and the differences ranged from 1.35- to 1.6-fold (Figure 4). For S and R plants, we have found the expression level of *ALS* relative to untreated plants at 12 and 24 HAT, which indicated that long-term herbicide application could increase the target gene overexpression and confer resistance to the corresponding herbicide.

In this study, the P197H mutants of the *ALS* gene exhibited wide cross-resistance to ALS-inhibiting herbicides from three chemical families. The R plants exhibited variable responses to the ALS inhibitors in that they were highly resistant to the SU herbicide (pyrazosulfuron-ethyl; RI = 20.63) and moderately resistant to the TP (penoxsulam; RI = 7.76) and PYB (bispyribac-sodium; RI = 5.48) herbicides. Notably, the R plants exhibited moderate levels of resistance to penoxsulam and bispyribac-sodium; the GR_{50} values of the R plants to the two herbicides were much lower than the FRR. As previously reported, the P197H substitution results in broad-spectrum resistance to SU, TP, PYB, and IMI herbicides in *C. difformis* [27]. A previous study reported that the P197S substitution also confers cross-resistance to penoxsulam, imazapic, and bispyribac-sodium in *C. difformis* collected from Guangxi and Hunan Provinces in China [19]. Other mutations in the *ALS* gene, including D376G and W574L, can contribute to various degrees of cross-resistance to ALS inhibitors in *C. difformis* growing in China [22]. These findings indicated that the resistance of *C. difformis* populations has evolved in recent years, and further studies are necessary to determine the evolution of resistance to ALS inhibitors in the long term. In this study, the R population of *C. difformis* remained susceptible to MCPA sodium, florpyrauxifen-benzyl, and bentazon, which have different MOAs and can be used to control the R population. The results demonstrated that the populations that are resistant to herbicides can be effectively controlled by applying herbicides, and the development of herbicide resistance can be delayed by the biennial rotation of herbicides with different MOAs [34].

NTSR is another important mechanism of herbicide resistance in weeds [35,36], and it is primarily mediated via the enhanced metabolism of herbicides. Previous studies have confirmed that the resistance of several weeds to ALS inhibitors is driven by the enhanced metabolism of herbicides. The metabolic enzymes P450s, GSTs, and ABCs, are the most common detoxification enzymes in several weeds, including barnyard grass (*Echinochloa crusgalli* (L.) Beauv.) [37], American sloughgrass (*Beckmannia syzigachne* (Steud.) Fern.) [38], *D. sophia* [39], and *A. aequalis* [40]. Malathion and NBD-Cl typically inhibit the activities of P450 and GST enzymes, respectively, of plants, and previous studies have investigated their inhibitory effects on the metabolism of herbicides in different weeds [37–41]. However, the present study revealed that pretreatment with inhibitors of P450 or GST enzymes did not induce any difference in the sensitivity of the BBHY1 population to bensulfuron-methyl. This finding suggested that the resistance of *C. difformis* to bensulfuron-methyl was not attributed to its enhanced metabolism via P450 or GST enzymes.

5. Conclusions

Altogether, the study revealed that the evolution of resistance to ALS inhibitors is mediated via a P197H mutation in the ALS protein of *C. difformis*. The findings indicated that bensulfuron-methyl resistance was not attributed to the overexpression of the *ALS* gene and enhanced metabolism by the CYP450 or GST enzymes. The study revealed that three herbicides with different MOAs could effectively control the R population. The resistance was attributed to the long-term use of single-action chemical herbicides for weed control. The application of herbicides with different MOAs, combined with non-chemical weed management strategies, including the use of weeding robots, raising ducks or fishes

in rice fields, and cultivation of genetically modified herbicide-tolerant crops, can aid in controlling the noxious, resistant population of *C. difformis*.

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