

Article

Herbicide Resistance Status of Italian Ryegrass (*Lolium multiflorum* Lam.) and Alternative Herbicide Options for Its Effective Control in the Huang-Huai-Hai Plain of China

Wei Li ^{1,2,†}, Cuixia Wu ^{3,†}, Mali Wang ^{1,2}, Minghao Jiang ^{1,2}, Jingxu Zhang ^{1,2}, Min Liao ^{1,2}, Haiqun Cao ^{1,2,*} and Ning Zhao ^{1,2,*} 

¹ Anhui Province Key Laboratory of Integrated Pest Management on Crops, School of Plant Protection, Anhui Agricultural University, Hefei 230036, China

² Anhui Province Engineering Laboratory for Green Pesticide Development and Application, School of Plant Protection, Anhui Agricultural University, Hefei 230036, China

³ Tai'an Academy of Agricultural Sciences, Tai'an 271000, China

* Correspondence: caohq@ahau.edu.cn (H.C.); zhaon@ahau.edu.cn (N.Z.)

† These two authors contributed equally to this work.

Abstract: Italian ryegrass (*Lolium multiflorum* Lam.) was introduced into China as a kind of turfgrass and has invaded wheat fields of the Huang-Huai-Hai Plain, causing great losses to grain yield. The acetyl-CoA carboxylase (ACCase) inhibitor clodinafop-propargyl and the acetolactate synthase (ALS) inhibitor mesosulfuron-methyl are highly efficient herbicides that have been widely used for control of this species, which has also resulted in its resistance evolution. However, the resistance status of *L. multiflorum* in the Huang-Huai-Hai Plain of China remains unclear, which hinders the integrated management of this weed in winter wheat production systems. In the current study, a total of 37 *L. multiflorum* populations were collected from the wheat fields across the region, and their susceptibility to clodinafop-propargyl and mesosulfuron-methyl was assessed. Of these, 13 populations were resistant or evolving resistance to clodinafop-propargyl (R?, RR, and RRR) with resistance index (RI) ranging from 2.62 to 830.05, and 8 populations were resistant or evolving resistance to mesosulfuron-methyl (R? and RR) with RI ranging from 3.89 to 16.68. Seven populations showed multiple-resistance to both clodinafop-propargyl and mesosulfuron-methyl. Three ACCase (I1781L, I2041N, and D2078G) and four ALS (P197T, P197S, P197A, and W574L) resistance mutations were identified in the herbicide-resistant populations, and I1781L and P197T were predominant ACCase and ALS mutations, respectively. Real-time quantitative PCR assays showed that compared with the susceptible population, the ACCase expression was slightly upregulated in some of the clodinafop-propargyl-resistant populations (AH-01 and AH-05) following clodinafop-propargyl treatment, while the ALS expression in the mesosulfuron-methyl-resistant populations showed no significant change following mesosulfuron-methyl treatment. Whole-plant dose-response testing showed that the AH-01 population carrying an ACCase gene I2041N mutation exhibited cross-resistance to all the ACCase inhibitors tested and multiple-resistance to the ALS inhibitor bispyribac-sodium, the AH-05 population carrying an ACCase gene I1781L mutation and an ALS gene P197T mutation showed cross-resistance to all the ACCase and ALS inhibitors tested, and the HN-07 population carrying an ACCase gene D2078G mutation showed cross-resistance to all the ACCase inhibitors tested and multiple-resistance to some ALS inhibitors. All the resistant populations remained susceptible to the 5-enolpyruvylshikimate-3-phosphate inhibitor glyphosate and the photosystem II inhibitor isoproturon. This study has clarified the distributions of clodinafop-propargyl- and/or mesosulfuron-methyl-resistant *L. multiflorum* in the Huang-Huai-Hai Plain of China, and target gene mutation was one of the most common mechanisms responsible for the resistance.

Keywords: *Lolium multiflorum*; ACCase and ALS mutation; target gene expression; cross- and multiple-resistance; resistance distribution



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1. Introduction

Italian ryegrass (*Lolium multiflorum* Lam.) originated along the Mediterranean coast and was introduced into China as a lawn grass in the 1930s [1]. It is a diploid ($2n = 2x = 14$) or tetraploid ($2n = 4x = 28$), obligate outcrossing species that is widely planted as turf, pasture, and cover crop [2,3]. Currently, *L. multiflorum* is becoming a rampant grass severely threatening agricultural production due to the dispersal of its seeds in cultivated land and crop fields [2]. In 1987, the first case of diclofop-methyl-resistant *L. multiflorum* population was reported in Oregon, USA [4], and resistance evolved rapidly thereafter. In recent years, herbicide-resistant *L. multiflorum* has been found in wheat fields in Provinces of Hubei, Henan, and Jiangsu, China [5,6]. Resistant weeds have always been one of the major threats to agricultural production. Monitoring the distribution, spread, and evolution of resistant weeds in real time will help to develop effective weed management strategies.

Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) catalyzes the formation of malonyl-CoA and regulates de novo fatty acid biosynthesis and oxidation [7]. Two types of ACCase have been recognized: the heteromeric prokaryotic ACCase is composed of multiple subunits, whereas the homomeric eukaryotic ACCase is a large multidomain protein [8,9]. Plants have both the cytosolic and the plastidic ACCase [9]. In grasses, the plastidic ACCase is homomeric and is the target site for three classes of ACCase inhibitors [10–12]. ACCase inhibitors were first introduced into the market in 1978 with the appearance of diclofop-methyl, including aryloxyphenoxypropionate (APP), cyclohexanedione (CHD), and phenylpyrazoline (PPZ) chemical groups [7,13]. The multidomain type found in the cytosol of all plants and the multiple-subunit type found in plastids of dicots are insensitive to APPs and CHDs [7]. In contrast, the plastidic ACCase in grasses is herbicide-sensitive [7]. However, their repeated and extensive use for many years has led to the evolution of herbicide resistant populations in many important grass species, such as wild oat (*Avena fatua* L.) [14,15], annual ryegrass (*Lolium rigidum* Gaud.) [16,17], black-grass (*Alopecurus myosuroides* Huds.) [18,19], Asia minor bluegrass (*Polypogon fugax* Nees ex Steud.) [20], and Chinese sprangletop (*Leptochloa chinensis* (L.) Nees) [21]. Up to now, there have been reported 50 species with resistance to these herbicides [22].

Acetolactate synthase (ALS, EC 2.2.1.6), also referred to as acetohydroxyacid synthase (AHAS), is the first enzyme in the biosynthetic pathway for the branched-chain amino acids valine, leucine, and isoleucine [23]. ALS is the target site for a large number of herbicides across the dissimilar sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinyl-thiobenzoates (PTB), and sulfonylaminocarbonyl-triazolinones (SCT) herbicide chemistries [24]. ALS-inhibiting herbicides can control diverse weed species, have low mammalian toxicity, and are selective in major crops [25], which ensure their global and intensive use in different crops over huge areas. However, the evolution of herbicide-resistant populations rapidly followed, and a total of 170 ALS-resistant biotypes have now been reported [22].

The knowledge of resistance mechanisms is important for the design of effective weed control strategies to delay the onset of resistance to herbicides. Herbicide resistance includes target-site-based resistance (TSR) and non-TSR (NTSR) [26]. TSR is essentially caused by the changes of single amino acid, target enzyme activity, or the overexpression of a specific gene encoding the target protein [27]. In most cases, target-site mutations often lead to high-level resistance to herbicides with a same mode of action (MOA). Up to now, a total of seven and eight codon positions of ACCase and ALS can show a variety of mutations to confer ACCase and ALS resistance, respectively [13,28]. By contrast, NTSR can be due to any one or a combination of mechanisms that limit to a nonlethal dose the amount of herbicide reaching a target site [26], such as decreased herbicide penetration into the plant, decreased rates of herbicide translocation, and increased rates of herbicide sequestration/metabolism [26].

Today, resistance to herbicides with eight MOAs has been reported in *L. multiflorum* globally, with the most severe resistance occurring to ACCase and ALS inhibitors [22]. In China, Zhang et al. [29] first reported the ACCase-resistant *L. multiflorum* populations

collected from the Provinces of Henan and Jiangsu, with varying degrees of cross-resistance to haloxyfop-*R*-methyl, quizalofop-*P*-ethyl, and sethoxydim. In the past few years, farmers in the Huang-Huai-Hai Plain have found that an increasing number of *L. multiflorum* populations are becoming resistant to the commonly used herbicides clodinafop-propargyl and mesosulfuron-methyl. To determine the distribution of herbicide-resistant *L. multiflorum* and the extent of resistance and thus aid in developing effective weed management strategies, a total of 37 populations of *L. multiflorum* were collected from different sites in this region where the wheat fields were severely infested with this species. The objectives of this study were to (1) test the susceptibility of different populations to clodinafop-propargyl and mesosulfuron-methyl, (2) identify potential *ACCase* and *ALS* mutations from the resistant populations, (3) assess the effects of clodinafop-propargyl and mesosulfuron-methyl on the expression of *ACCase* and *ALS* genes, respectively, in different resistant populations, and (4) clarify their cross- and multiple-resistance patterns to different herbicides.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

During May 2013 to May 2021, mature seeds of 37 *L. multiflorum* populations were collected from the winter wheat fields with repeated clodinafop-propargyl and/or mesosulfuron-methyl use in Provinces of Shaanxi (SHX), Hubei (HB), Henan (HN), Anhui (AH), and Jiangsu (JS) across the Huang-Huai-Hai Plain of China (Table 1, Figure 1). The seeds of each population were randomly collected from more than 100 mature plants, after which they were dried and stored in paper bags at 4 °C until further use.

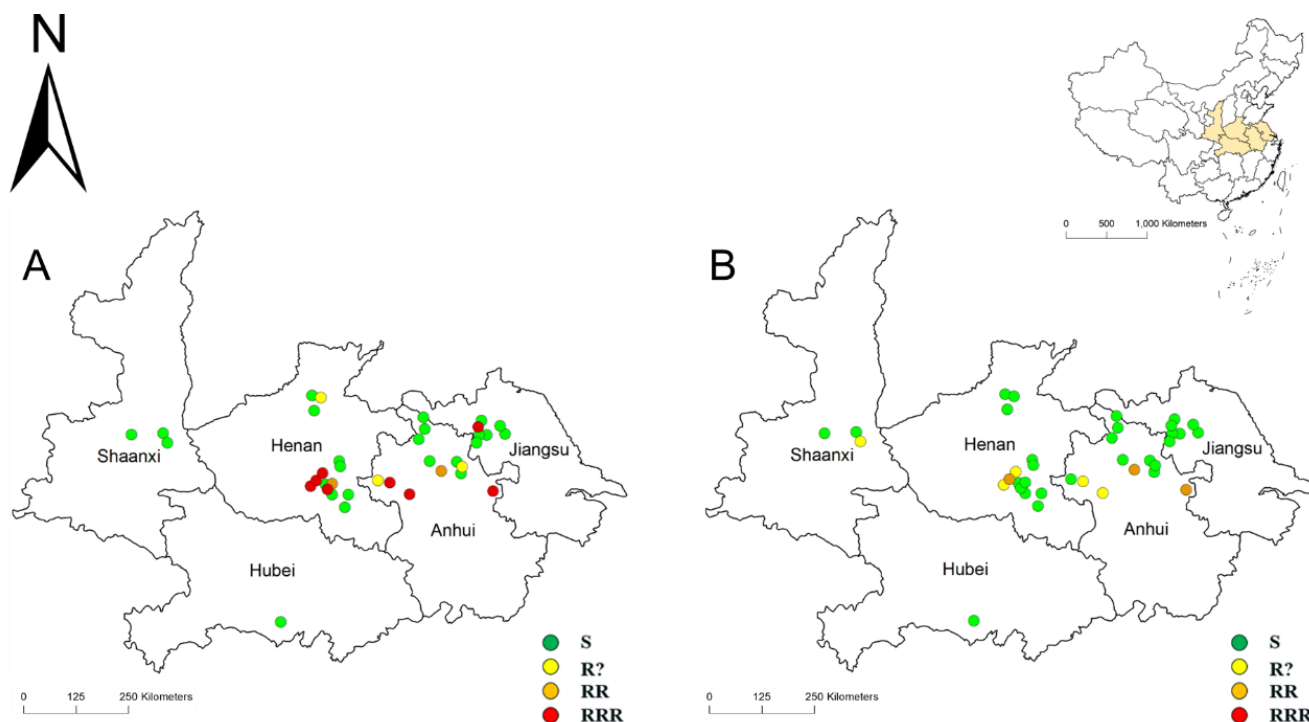


Figure 1. The distribution of 37 *L. multiflorum* populations collected from the Huang-Huai-Hai Plain, China. The susceptibility of different populations to clodinafop-propargyl (A) and mesosulfuron-methyl (B) was classified according the “R” rating system. RRR means resistance confirmed, highly likely to reduce herbicide performance; RR means resistance confirmed, probably reducing herbicide performance; R? means early indications that resistance may be developing, possibly reducing herbicide performance; and S means susceptible.

Table 1. Information on the *L. multiflorum* seeds collected in this study and the susceptibility of different *L. multiflorum* populations to clodinafop-propargyl and mesosulfuron-methyl.

Population	Location		Date (Year)	Clodinafop-Propargyl		Mesosulfuron-Methyl	
	Longitude °E	Latitude °N		Fresh Weight Inhibition (%)	Susceptibility	Fresh Weight Inhibition (%)	Susceptibility
AH-01	116.19	32.58	2021	5.21 (4.67)	RRR	87.08 (2.82)	R?
AH-02	117.65	32.87	2021	91.77 (3.58)	S	94.41 (2.52)	S
AH-03	117.58	33.15	2021	91.95 (0.95)	S	91.81 (1.61)	S
AH-04	116.86	34.26	2021	88.25 (6.28)	S	92.39 (2.13)	S
AH-05	118.42	32.36	2021	11.04 (1.37)	RRR	66.05 (6.55)	RR
AH-06	116.65	33.79	2021	92.18 (0.80)	S	89.03 (3.60)	S
AH-07	116.86	34.20	2021	96.44 (0.49)	S	98.28 (0.54)	S
AH-08	117.70	33.02	2021	85.84 (1.24)	R?	90.11 (3.34)	S
AH-09	116.87	34.27	2021	88.14 (2.71)	S	90.77 (1.76)	S
AH-10	115.71	32.91	2021	37.32 (0.59)	RRR	87.36 (0.45)	R?
AH-11	117.13	33.00	2021	57.12 (3.43)	RR	69.02 (4.79)	RR
AH-12	115.40	32.99	2021	85.08 (2.87)	R?	89.97 (1.05)	S
HN-01	113.98	32.95	2013	93.32 (1.93)	S	90.66 (3.42)	S
HN-02	114.13	32.90	2014	94.71 (0.35)	S	90.84 (2.81)	S
HN-03	113.58	32.95	2014	33.02 (2.14)	RRR	86.52 (4.19)	R?
HN-04	113.90	35.05	2014	89.86 (2.02)	S	91.34 (2.24)	S
HN-05	114.56	32.56	2014	93.60 (5.55)	S	91.58 (0.86)	S
HN-06	114.52	32.48	2014	91.32 (0.35)	S	89.37 (0.85)	S
HN-07	114.02	32.93	2015	10.76 (1.30)	RRR	88.87 (3.77)	S
HN-08	113.94	32.96	2015	21.16 (2.72)	RRR	74.58 (1.67)	RR
HN-09	114.16	32.95	2015	63.21 (1.09)	RR	91.87 (3.91)	S
HN-10	114.42	33.04	2015	90.69 (2.68)	S	92.58 (1.84)	S
HN-11	113.89	35.10	2016	92.71 (2.65)	S	92.36 (1.88)	S
HN-12	114.43	33.02	2016	93.97 (2.15)	S	95.19 (0.91)	S
HN-13	113.94	33.01	2015	24.62 (2.10)	RRR	85.38 (1.13)	R?
HN-14	114.13	35.03	2015	85.97 (0.38)	R?	91.35 (4.85)	S
HB-01	112.42	30.06	2013	92.70 (10.11)	S	90.73 (5.77)	S
JS-01	118.98	33.59	2014	91.39 (3.52)	S	90.94 (1.49)	S
JS-02	118.91	33.79	2014	96.00 (0.55)	S	97.21 (1.42)	S
JS-03	118.11	33.84	2014	88.56 (0.31)	S	91.38 (0.25)	S
JS-04	118.20	33.97	2015	93.88 (1.01)	S	94.00 (1.46)	S
JS-05	118.23	33.98	2015	94.71 (0.94)	S	96.04 (1.46)	S
JS-06	117.90	33.92	2016	87.58 (1.04)	S	89.10 (0.95)	S
JS-07	118.12	33.85	2021	5.19 (2.41)	RRR	88.57 (4.33)	S
SHX-01	108.80	34.54	2016	95.98 (1.26)	S	90.46 (2.44)	S
SHX-02	109.80	34.53	2016	93.71 (3.28)	S	96.07 (2.16)	S
SHX-03	109.78	34.51	2016	89.55 (4.45)	S	84.35 (0.20)	R?

Plump seeds were randomly selected from each population and placed into a 9 cm diameter Petri dish containing two pieces of filter paper moistened with 5 mL of distilled water. Then, the Petri dishes were transferred into an artificial growth chamber for incubation (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photonflux density, 12 h photoperiod, 25 °C constant temperature, with ~75% relative humidity). After germination, 10 seeds were sown into each plastic pot (10 cm \times 10 cm \times 8.5 cm) filled with loam soils and grown in a naturally lit glasshouse (25/15 °C day/night) with regular watering and fertilization. When the seedlings had reached the two- to three-leaf stage, they were thinned to eight uniformly sized seedlings per pot.

2.2. Initial Resistance Screening

Weed seedlings at the three- to four-leaf stage were treated with clodinafop-propargyl (56 g a.i. ha^{-1}) or mesosulfuron-methyl (11.25 g a.i. ha^{-1}) at the recommended field rate (RFR). An untreated control for each population was treated with water. Both herbicide

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 liquid at 0.275 MPa. At 21 d after treatment (DAT), the aboveground fresh weight per pot was recorded. Each treatment had three replicates, and the experiment was conducted twice.

According to the results of initial screening, the *L. multiflorum* populations were assigned into four resistance categories (RRR, RR, R?, and S) using the “R” rating system proposed by Moss et al. [30]. Of the four resistance categories, “S” means that the population is susceptible, and its fresh weight reduction ranged from 9*SS/10 to SS; “R?” means that the resistance may be developing, possibly reducing herbicide performance, and its fresh weight reduction ranged from 4*SS/5 to 9*SS/10; “RR” means that the resistance is confirmed, possibly reducing herbicide performance, and its fresh weight reduction ranged from 2*SS/5 to 4*SS/5; and “RRR” means that the resistance is confirmed, high likely reducing herbicide performance, and its fresh weight reduction ranged from 0 to 2*SS/5. In this current study, the standard susceptible (SS) population AH-07 was collected from a roadside without any herbicide use history, and a discriminating dose of clodinafop-propargyl at 56 g a.i. ha⁻¹ or mesosulfuron-methyl at 11.25 g a.i. ha⁻¹ caused a fresh weight reduction of 96.44% or 98.28%, respectively.

2.3. Whole-Plant Dose-Response Experiment

Based on the results of initial screening, the *L. multiflorum* population AH-07 susceptible to both clodinafop-propargyl and mesosulfuron-methyl was used as the susceptible (S) population. All the resistant populations (i.e., the R?, RR, and RRR populations) and the S population were then tested for herbicide susceptibility level by whole-plant dose-response experiments. Information on the herbicides used in the experiments is shown in Table 2. At 21 DAT, the aerial parts of the plants in each pot were cut off, and their fresh weight was recorded. Each treatment had three replicates, and the experiment was conducted twice.

Table 2. Herbicides and their doses used in the whole-plant dose-response experiments for different *L. multiflorum* populations.

Group ^a	Herbicide	Formulation	Manufacturer	Populations	Test Doses (g a.i. ha ⁻¹) ^b
APP	Clodinafop-propargyl	24% EC	Lantian, Anhui	AH-07	0, 0.08, 0.23, 0.69, 2.07, 6.22, 18.67
				AH-08, AH-12, HN-14	0, 2.07, 6.22, 18.67, 56 , 168, 504
				HN-09	0, 6.22, 18.67, 56 , 168, 504, 1512,
				AH-01, AH-05, AH-10, AH-11, HN-03, HN-07, HN-08, HN-13, JS-07	0, 18.67, 56 , 168, 504, 1512, 4536
SU	Mesosulfuron-methyl	30 g L ⁻¹ OD	Bayer, Beijing	AH-07	0, 0.05, 0.14, 0.42, 1.25, 3.75, 11.25
				AH-01, AH-10, HN-03, HN-13, SHX-03	0, 0.42, 1.25, 3.75, 11.25 , 33.75, 101.25
				AH-05, AH-11, HN-08	0, 1.25, 3.75, 11.25 , 33.75, 101.25 303.75

^a APP, aryloxyphenoxypropionate; SU, sulfonyleureas; EC, emulsifiable concentrate; OD, oil dispersion. ^b The recommended field rates of clodinafop-propargyl and mesosulfuron-methyl are shown in bold.

2.4. ACCase and ALS Sequencing

Fresh leaf tissues were collected from the individuals of the S plant (AH-07) and all the resistant plants (i.e., the R?, RR, and RRR populations) at their three- to four-leaf stage and stored at −20 °C. Genomic DNA was then extracted from the leaf tissues using the classical cetyltrimethylammonium bromide method [31]. To determine the frequency of plants with specific mutations in different resistant populations, at least 10 plants from each population were randomly selected. Primers used to amplify *ACCase* and *ALS* genes have been reported elsewhere (Table 3), and the amplified regions contained all the known

resistance mutation sites [29,32]. Polymerase chain reaction (PCR) was conducted in a 25 μ L volume that consisted of 1 μ L of genomic DNA, 1 μ L of each primer (10 μ M), 9.5 μ L of double-distilled H₂O, and 12.5 μ L of 2 \times Es Taq MasterMix (Dye). PCR cycling settings were as follows: 94 $^{\circ}$ C for 2 min, 35 cycles of 94 $^{\circ}$ C for 30 s, x $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s, followed by a final extension step of 5 min at 72 $^{\circ}$ C. The x refers to the respective annealing temperatures used for each primer pair (Table 3). The targeted PCR products were purified from agarose gels using a FastPure[®] Gel DNA Extraction Mini Kit (Vazyme, Nanjing, China) and were directly sequenced by Tsingke Biotech (Beijing, China) from both directions. The resulting sequences were aligned and compared using DNAMAN v 6.0 software (Lynnon, Quebec, Canada).

Table 3. Primers used for *ACCase* and *ALS* sequencing in different *L. multiflorum* populations.

Primer	Gene	Sequence (5'-3')	T _m ($^{\circ}$ C)	Product Size (bp)	Targeted Mutation Site
<i>ACCase</i> -F	<i>ACCase</i>	AATGGGTCGTGGGGCACTCC- TATAATTCC	61	1600	I1781, W1999, W2027, I2041, D2078, C2088, G2096
<i>ACCase</i> -R		CTCCCTGGAGTTGTGCTTTC			
<i>ALS</i> -F122	<i>ALS</i>	GGGCGCCGACATCCTCGTCG	57	491	A122, P197, A205
<i>ALS</i> -R197		ATCTGCTGCTGGATGTCCTT			
<i>ALS</i> -F376		ATTCTCTATGTTGGCGGTGG	55	440	E376, R377
<i>ALS</i> -R376		CTTTTCTGCTGCTCCAATC			
<i>ALS</i> -F574		TGGGCGGCTCAGTATTACAC	55	532	W574, S653, G654
<i>ALS</i> -R653		TCCTGCCATCACCTTCCATG			

T_m, annealing temperature.

2.5. *ACCase* and *ALS* Expression Analysis

Three clodinafop-propargyl-resistant populations (AH-01, AH-05, and HN-07) each carrying a specific *ACCase* resistance mutation and two mesosulfuron-methyl-resistant populations (AH-05 and HN-08) each carrying a specific *ALS* resistance mutation were chosen to detect the relative expression levels of *ACCase* and *ALS*, respectively. The population AH-07 susceptible to both herbicides was used as a control. Weed plants at the three- to four-leaf stage were treated with clodinafop-propargyl or mesosulfuron-methyl at their RFRs, respectively. Fresh leaf tissues were then sampled from their individual plant at 0 (CK), 2, 6, 12, 24, 48, 72, and 120 h after treatment (HAT) and immediately frozen in liquid nitrogen. RNA was then extracted from the leaf samples using the Trizol reagent (Ambion, life technologies, USA), and complementary DNA (cDNA) was obtained using a HiFiScript gDNA Removal and cDNA Synthesis Kit (CW BIO, Beijing, China).

In this current study, elongation factor 1-alpha (*EF1 α*) was used as the reference gene, and the primers for the reference gene and target genes were reported elsewhere [33,34]. The real-time quantitative PCR (RT-qPCR) was performed using the CFX96 Real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) with a ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China). The reaction system and cycling profile were prepared according to the manufacturer's instructions, and target gene expression relative to that of *EF1 α* was analyzed by the 2^{- $\Delta\Delta$ Ct} method [35]. Each experiment had six biological replicates (six individual plants), and each biological replicate had three technical replicates. Two threshold values including the fold-change (2-fold) and the Student's *t*-test ($p < 0.05$) were used to determine the up- or downregulation of target genes in the R relative to the S plants.

2.6. Cross- and Multiple-Resistance Patterns of Specific Populations to Other Herbicides

To determine the potential alternatives for control of the resistant *L. multiflorum* plants, three populations AH-01, AH-05, and HN-07 each carrying specific target-site mutations were selected to determine their cross- and multiple-resistance to different herbicides. Weed

plants of the S (AH-07) and the three resistant biotypes at the three- to four-leaf stage were treated with a series of doses of different herbicides (Table 4). An untreated control was established for each population and treated with water. At 21 DAT, the aboveground fresh weight of *L. multiflorum* was recorded. Each treatment had three replicates, and the experiment was conducted twice.

Table 4. Herbicides and their doses used in cross- and multiple- resistance experiments for different *L. multiflorum* populations.

Group ^a	Herbicide	Formulation	Manufacturer	Populations	Test Doses (g a.i. ha ⁻¹) ^b
APP	Fenoxaprop- <i>P</i> -ethyl	69 g L ⁻¹ EW	Bayer, Hangzhou	AH-07-S	0, 2.29, 6.87, 20.67, 62 , 186, 558
				AH-01-R AH-05-R	0, 20.67, 62 , 186, 558, 1674, 5022
				HN-07-R	0, 6.87, 20.67, 62 , 186, 558, 1674
	Haloxypop- <i>R</i> -methyl	108 g L ⁻¹ EC	Zhongqi, Jiangsu	AH-07-S	0, 0.21, 0.62, 1.85, 5.56, 16.67, 50
				AH-01-R	0, 16.67, 50 , 150, 450, 1350, 4050
				AH-05-R	0, 5.56, 16.67, 50 , 150, 450, 1350
				HN-07-R	0, 1.85, 5.56, 16.67, 50 , 150, 450
	Quizalofop- <i>P</i> -ethyl	15% EC	Fengshan, Yancheng	AH-07-S	0, 0.06, 0.19, 0.56, 1.67, 5, 15
				AH-01-R AH-05-R HN-07-R	0, 5, 15, 45 , 135, 405, 1215
				AH-07-S	0, 0.31, 0.93, 2.78, 8.33, 25, 75
	Cyhalofop-butyl	30% EC	Dow AgroSciences, Beijing	AH-01-R	0, 25, 75 , 225, 675, 2025, 4050
				AH-05-R HN-07-R	0, 8.33, 25, 75 , 225, 675, 2025
AH-07-S				0, 1.11, 3.33, 10, 30, 90 , 270	
Metamifop	10% EC	FMC, Suzhou	AH-01-R	0, 30, 90 , 270, 810, 2430, 7290	
			AH-05-R HN-07-R	0, 10, 30, 90 , 270, 810, 2430	
			AH-07-S	0, 0.40, 1.20, 3.59, 10.78, 32.33, 97	
CHD	Clethodim	240 g L ⁻¹ EC	Aokun, Shandong	AH-01-R AH-05-R	0, 1.20, 3.59, 10.78, 32.33, 97 , 291
				HN-07-R	0, 0.40, 1.20, 3.59, 10.78, 32.33, 97
				AH-07-S	0.21, 0.62, 1.85, 5.56, 16.67, 50
Sethoxydim	12.5% EC	Sinochem Lihua, Tianjin	AH-01-R	0, 1.85, 5.56, 16.67, 50, 150 , 450	
			AH-05-R	0, 16.67, 50, 150 , 450, 1350, 4050	
			HN-07-R	0, 5.56, 16.67, 50, 150 , 450, 1350	
PPZ	Pinoxaden	10% OD	Shanghewoda, Anhui	AH-07-S	0, 0.19, 0.56, 1.67, 5, 15, 45
				AH-01-R	0, 5, 15, 45 , 135, 405, 1215
				AH-05-R	0, 1.67, 5, 15, 45 , 135, 405
				HN-07-R	0, 0.56, 1.67, 5, 15, 45 , 135
TP	Pyroxsulam	7.5% WG	Corteva, Beijing	AH-07-S	0, 0.06, 0.17, 0.52, 1.56, 4.67, 14
				AH-01-R AH-05-R HN-07-R	0, 0.17, 0.52, 1.56, 4.67, 14 , 42

Table 4. Cont.

Group ^a	Herbicide	Formulation	Manufacturer	Populations	Test Doses (g a.i. ha ⁻¹) ^b
IMI	Imazamox	4% AS	Agricultural Hormone, Jiangsu	AH-07-S	0, 0.19, 0.56, 1.67, 5, 15, 45
				AH-01-R AH-05-R HN-07-R	0, 0.56, 1.67, 5, 15, 45 , 135
	Imazethapyr	5% AS	Jinling, Nantong	AH-07-S	0, 0.31, 0.93, 2.78, 8.33, 25, 75
				AH-01-R AH-05-R HN-07-R	0, 0.93, 2.78, 8.33, 25, 75 , 225
SCT	Flucarbazone-Na	5% OD	Luye, Anhui	AH-07-S AH-01-R	0, 0.12, 0.37, 1.11, 3.33, 10, 30
				AH-05-R HN-07-R	0, 1.11, 3.33, 10, 30 , 90, 270
PTB	Bispyribac-sodium	10% SC	Sida, Anhui	AH-07-S	0, 0.10, 0.30, 0.89, 2.67, 8, 24
				AH-01-R	0, 0.30, 0.89, 2.67, 8, 24 , 72
				AH-05-R	0, 2.67, 8, 24 , 72, 216, 648
				HN-07-R	0, 0.30, 0.89, 2.67, 8, 24 , 72
Urea	Isoproturon	50% WP	Guangxin, Anhui	AH-07-S	0, 12.35, 37.04, 111.11, 333.33, 1000 , 3000
				AH-01-R	
				AH-05-R	
				HN-07-R	
OP	Glyphosate	30% AS	Bayer, Beijing	AH-07-S	0, 4.94, 14.81, 44.44, 133.33, 400, 1200
				AH-01-R	
				AH-05-R	
				HN-07-R	

^a APP, aryloxyphenoxypropionate; CHD, cyclohexanedione; PPZ, phenylpyrazoline; TP, triazolopyrimidines; IMI, imidazolinones; SCT, sulfonylaminocarbonyl-triazolinones; PTB, pyrimidinyl-thiobenzoates; OP, organophosphates; EW, emulsion in water; EC, emulsifiable concentrate; OD, oil dispersion; WG, water-dispersible granule; AS, aqueous solution; SC, suspension concentrate; WP, wettable powder. ^b The recommended field rates of different herbicides are shown in bold.

2.7. Data Analysis

All the dose-response data from the two repeated experiments were compared and analyzed by variance (ANOVA) using the SPSS software (Version 22.0; IBM Corporation, Armonk, NY, USA). Since the two repeated experiments did not show significant difference ($p > 0.05$), the datasets were combined and fitted to a nonlinear logistic model using SigmaPlot software (Version 14.0; Systat Software Inc., CA, USA). The herbicide dose that reduced the aboveground fresh weight by 50% (GR₅₀) was calculated according to the following equation.

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

where y is the response at the herbicide dose x , C is the lower limit, D is the upper limit, and b is the relative slope at GR₅₀. Resistance index (RI) was calculated as the ratio of GR₅₀ values between the resistant and susceptible populations.

3. Results

3.1. Susceptibility of *L. multiflorum* to Clodinafop-Propargyl and Mesosulfuron-Methyl

Single-dose testing was first conducted to determine the susceptibility of different *L. multiflorum* populations to clodinafop-propargyl and mesosulfuron-methyl. Of the populations tested, 13 populations had evolved resistance to clodinafop-propargyl, and eight populations had evolved resistance to mesosulfuron-methyl (Table 1). Meanwhile, seven of the resistant populations showed multiple resistance to both clodinafop-propargyl

and mesosulfuron-methyl (Table 1). For the SS population AH-07, all plants died at the RFRs of clodinafop-propargyl and mesosulfuron-methyl, with the fresh weight inhibitions confirmed to be 96.44% and 98.28%, respectively (Table 1). Based on the “R” rating system, of the 13 clodinafop-propargyl-resistant populations, 10 were classified as “RRR” and “RR”, and three were classified as “R?”; of the eight mesosulfuron-methyl-resistant populations, three were classified as “RR”, and five were classified as “R?” (Table 1).

The resistance distributions of *L. multiflorum* to clodinafop-propargyl and mesosulfuron-methyl were then plotted by combining the geographical information and the results of single-dose testing (Figure 1). Based on the data, the herbicide-resistant *L. multiflorum* were mainly distributed in Provinces of Henan, Anhui, and Jiangsu.

3.2. Whole-Plant Dose-Response Experiment

The susceptibility of the S population AH-07 was confirmed by the whole-plant dose-response experiment. Clodinafop-propargyl applied at 1.61 g a.i. ha⁻¹ or mesosulfuron-methyl applied at 0.53 g a.i. ha⁻¹ provided a 50% growth reduction in the AH-07 population, which was much lower than their respective RFRs (Table 5). Compared with AH-07, the clodinafop-propargyl-resistant populations that classed as “R?, RR, and RRR” had GR₅₀ values ranging from 4.22 to 1336.38 g a.i. ha⁻¹, which were 2.62- and 830.05-fold more resistant (Table 5). Similarly, the mesosulfuron-methyl-resistant populations that classed as “R? and RR” had GR₅₀ values ranging from 2.06 to 8.84 g a.i. ha⁻¹, which were 3.89- to 16.68-fold more resistant than the AH-07 population (Table 5).

Table 5. The GR₅₀ values^a and RIs^b to clodinafop-propargyl and mesosulfuron-methyl in different resistant populations of *L. multiflorum*.

Population	Clodinafop-Propargyl		Mesosulfuron-Methyl	
	GR ₅₀ (g a.i. ha ⁻¹)	RI	GR ₅₀ (g a.i. ha ⁻¹)	RI
AH-01	1189.26 (113.26)	738.67	2.24 (0.53)	4.23
AH-05	862.34 (14.78)	535.61	8.84 (3.55)	16.68
AH-07	1.61 (0.18)	1.00	0.53 (0.16)	1.00
AH-08	4.26 (0.41)	2.65	-	-
AH-10	260.79 (62.16)	162.99	2.06 (0.30)	3.89
AH-11	125.98 (30.42)	78.25	8.07 (2.24)	15.23
AH-12	5.62 (0.34)	3.49	-	-
HN-03	292.87 (15)	181.91	2.38 (0.37)	4.49
HN-07	863.97 (43.07)	536.63	-	-
HN-08	430.23 (164.50)	267.22	6.43 (1.69)	12.13
HN-09	78.2 (42.74)	67.41	-	-
HN-13	384.45 (98.93)	238.79	2.78 (0.62)	5.25
HN-14	4.22 (0.44)	2.62	-	-
JS-07	1336.38 (5.49)	830.05	-	-
SHX-03	-	-	4.17 (0.32)	7.87

^a The clodinafop-propargyl or mesosulfuron-methyl dose causing 50% growth reduction (GR₅₀) was calculated according to a four-parameter log-logistic response equation: $y = C + (D - C) / [1 + (x / GR_{50})^b]$, where C is the lower limit, D is the upper limit, x is the herbicide application dose, b is the slope at GR₅₀, and y is the response at the herbicide dose x . ^b The resistance indexes (RIs) were the ratios between the GR₅₀ values of resistant and susceptible (AH-07) populations.

3.3. ACCase and ALS Resistance Mutations in *L. multiflorum*

Following PCR amplifications, the ACCase and ALS genes spanning all the known resistance codon positions were obtained from the ACCase- and ALS-resistant populations, respectively. Of the 13 clodinafop-propargyl-resistant populations (Table 1), 10 populations were detected for ACCase mutations, with I2041N detected in five populations, I1781L detected in four populations, and D2078G detected in one population (Table 6, Figure 2). No known resistance mutation was detected in the ACCase of three clodinafop-propargyl-“R?” populations (AH-08, AH-12, and HN-14). Similarly, of the eight mesosulfuron-methyl-resistant populations (Table 1), ALS mutations were detected in only three populations,

and a total of four mutations at codon positions 197 and 574 of *ALS* were identified (P197T, P197S, P197A, and W574L) (Table 6, Figure 2). In the HN-08 population, three mutations (P197S/A/T) were simultaneously identified in *ALS* of its individuals (Table 6). No known resistance mutation was detected in the *ALS* of five mesosulfuron-methyl-“R?” populations. Notably, mutations in *ACCase* and *ALS* were simultaneously detected in individuals from the AH-05, AH-11, or HN-08 population (Table 6).

Table 6. Mutations and their frequencies in different *L. multiflorum* populations.

Population	<i>ACCase</i> Gene		<i>ALS</i> Gene	
	Mutation	Frequency of Mutations (%)	Mutation	Frequency of Mutations (%)
AH-01	I2041N	90	Wild type	-
AH-05	I1781L	90	P197T	40
AH-08	Wild type	-	-	-
AH-10	I1781L	90	Wild type	-
AH-11	I1781L	30	W574L	10
AH-12	Wild type	-	-	-
HN-03	I2041N	60	Wild type	-
HN-07	D2078G	60	-	-
HN-08	I2041N	60	P197S/A/T	30
HN-09	I2041N	70	-	-
HN-13	I1781L	90	Wild type	-
HN-14	Wild type	-	-	-
JS-07	I2041N	100	-	-
SHX-03	-	-	Wild type	-

3.4. *ACCase* or *ALS* Expression Analysis

In this current study, three *ACCase*-resistant populations (AH-01, AH-05, and HN-07) and two *ALS*-resistant populations (AH-05 and HN-08) were selected for gene expression assays. The total expression of *ACCase* or *ALS* was normalized relative to that of *EF1 α* and compared between the R and S biotypes of *L. multiflorum* at 0 (control), 2, 6, 12, 24, 48, 72, and 120 HAT (Figure 3). According to the results, no significant difference was observed in the *ACCase* expression between the *ACCase*-resistant population HN-07 and the S population AH-07 at each time point (Figure 3A). However, the *ACCase* expression in the *ACCase*-resistant populations AH-01 and AH-05 was upregulated more than three-fold at 12 and 2 HAT when compared with the S population, respectively (Figure 3A). In addition, no significant difference in the *ALS* expression was revealed in the *ALS*-resistant populations AH-05 and HN-08 when compared with the S population before and after herbicide treatment (Figure 3B).

3.5. Cross- and Multiple-Resistance Patterns of *L. multiflorum* to Other Herbicides

In this current study, all the herbicides tested showed great control efficacies against the plants from the S population AH-07 (Table 7, Figure 4). In comparison, the AH-01 population carrying an *ACCase* I2041N mutation showed high resistance to haloxyfop-*R*-methyl, quizalofop-*P*-ethyl, and cyhalofop-butyl, moderate resistance to fenoxaprop-*P*-ethyl and metamifop, low resistance to clethodim, sethoxydim, pinoxaden, and bispyribac-sodium, and was susceptible to imazethapyr, imazamox, flucarbazone-Na, glyphosate, and isoproturon. The AH-05 population carrying both an *ACCase* gene I1781L mutation and an *ALS* gene P197T mutation showed high resistance to fenoxaprop-*P*-ethyl, haloxyfop-*R*-methyl, quizalofop-*P*-ethyl, cyhalofop-butyl, metamifop, sethoxydim, pinoxaden, and bispyribac-sodium, moderate resistance to imazethapyr, low resistance to clethodim, pyroxsulam, imazamox, and flucarbazone-Na, and was susceptible to glyphosate and isoproturon. The HN-07 population carrying an *ACCase* D2078G mutation showed high resistance to fenoxaprop-*P*-ethyl, quizalofop-*P*-ethyl, sethoxydim, moderate resistance to haloxyfop-*R*-methyl, metamifop, pyroxsulam, and bispyribac-sodium, low resistance level to clethodim,

imazamox, and imazethapyr, and was susceptible to flucarbazone-Na, glyphosate, and isoproturon (Table 7, Figure 4).

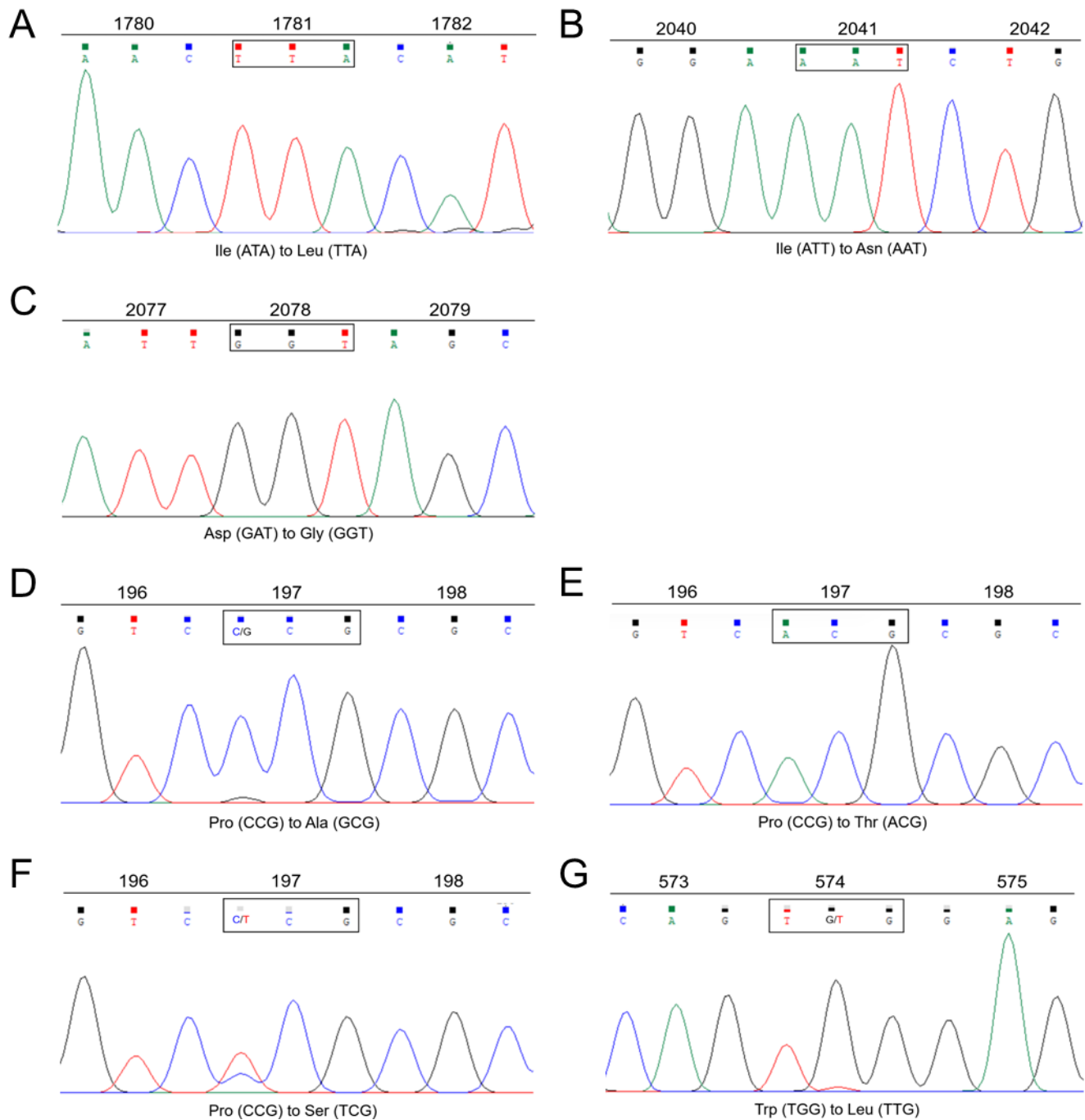


Figure 2. Partial *ACCase* and *ALS* gene sequences from different resistant populations of *L. multiflorum*. *ACCase* sequences showing (A) Ile-1781-Leu, (B) Ile-2041-Asn, and (C) Asp-2078-Gly mutations. *ALS* sequences showing (D) Pro-197-Ala, (E) Pro-197-Thr, (F) Pro-197-Ser, and (G) Trp-574-Leu mutations.

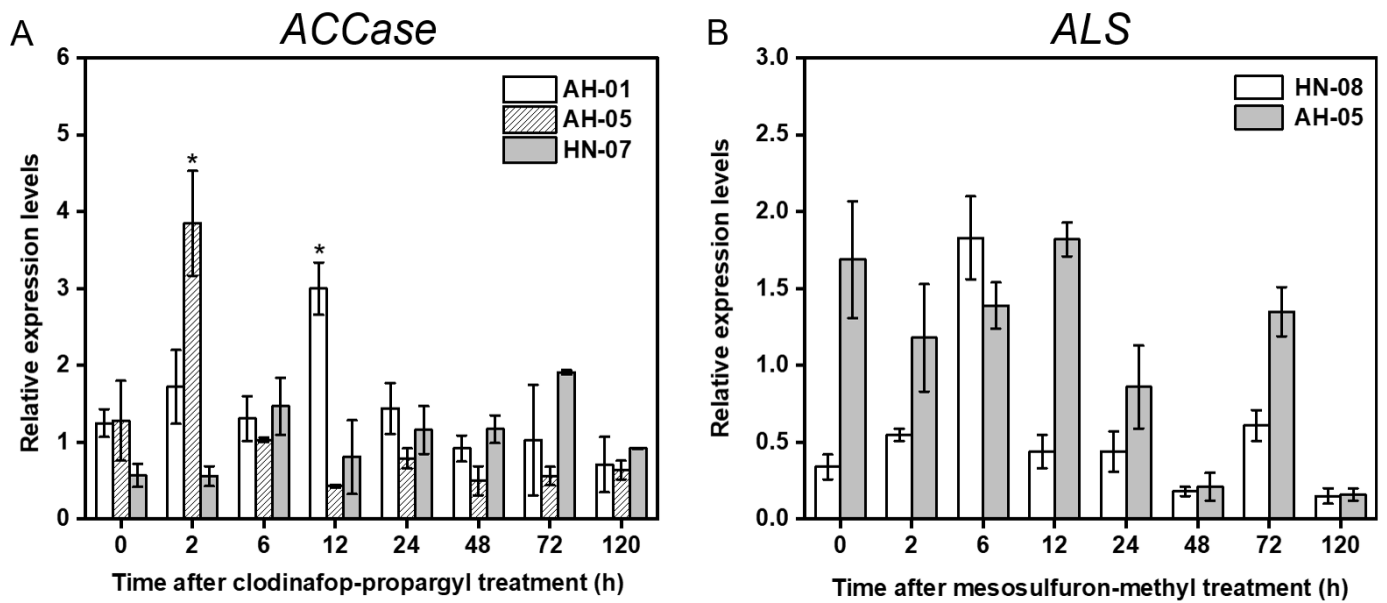


Figure 3. Relative expression levels of (A) *ACCase* and (B) *ALS* in *ACCase*- and *ALS*-resistant *L. multiflorum* plants at 0 (control), 2, 6, 12, 24, 48, 72, and 120 h after clodinafop-propargyl or mesosulfuron-methyl treatment, respectively. Asterisk indicates significant upregulation (fold change ≥ 2 and $p < 0.05$) of target gene in resistant plants compared with the susceptible plants (AH-07).

Table 7. The GR_{50} values ^a and RIs ^b of susceptible (AH-07) and resistant (AH-01, AH-05, and HN-07) populations of *L. multiflorum* to different herbicides.

Herbicide	Populations	GR_{50} (g a.i. ha ⁻¹)	RIs
Fenoxaprop- <i>P</i> -ethyl	AH-01 (R)	84.88 (49.99)	9.48
	AH-05 (R)	689.21 (72.08)	77.01
	HN-07 (R)	306.48 (2.52)	34.24
	AH-07 (S)	8.95 (1.70)	1.00
Haloxypop- <i>R</i> -methyl	AH-01 (R)	302.66 (22.60)	211.65
	AH-05 (R)	185.88 (34.65)	129.99
	HN-07 (R)	10.01 (1.58)	7.00
	AH-07 (S)	1.43 (0.20)	1.00
Quizalofop- <i>P</i> -ethyl	AH-01 (R)	21.59 (1.30)	69.65
	AH-05 (R)	100.28 (18.22)	323.48
	HN-07 (R)	39.12 (10.11)	126.19
	AH-07 (S)	0.31 (0)	1.00
Cyhalofop-butyl	AH-01 (R)	273.44 (16.13)	31.50
	AH-05 (R)	323.38 (1.12)	37.26
	HN-07 (R)	327.41 (1.60)	37.72
	AH-07 (S)	8.68 (0.80)	1.00
Metamifop	AH-01 (R)	651.00 (116.47)	9.78
	AH-05 (R)	728.21 (215.19)	10.94
	HN-07 (R)	579.42 (5.94)	8.71
	AH-07 (S)	66.55 (6.14)	1.00
Clethodim	AH-01 (R)	9.88 (5.89)	4.10
	AH-05 (R)	12.56 (4.96)	5.21
	HN-07 (R)	5.08 (0.23)	2.11
	AH-07 (S)	2.41 (0.15)	1.00

Table 7. Cont.

Herbicide	Populations	GR ₅₀ (g a.i. ha ⁻¹)	RI _s
Sethoxydim	AH-01 (R)	11.23 (0.44)	4.49
	AH-05 (R)	360.17 (69.31)	144.07
	HN-07 (R)	68.61 (29.50)	27.44
	AH-07 (S)	2.5 (0.41)	1.00
Pinoxaden	AH-01 (R)	6.03 (1.16)	4.82
	AH-05 (R)	18.04 (2.41)	14.43
	HN-07 (R)	14.64 (2.79)	11.71
	AH-07 (S)	1.25 (0.20)	1.00
Pyroxsulam	AH-01 (R)	0.97 (0.32)	0.80
	AH-05 (R)	4.07 (3.73)	3.63
	HN-07 (R)	6.66 (0.35)	5.50
	AH-07 (S)	1.21 (0.17)	1.00
Imazamox	AH-01 (R)	4.04 (4.15)	1.50
	AH-05 (R)	10.73 (0.57)	3.99
	HN-07 (R)	7.07 (1.71)	2.63
	AH-07 (S)	2.69 (0.27)	1.00
Imazethapyr	AH-01 (R)	5.13 (0.65)	1.22
	AH-05 (R)	34.50 (0.98)	8.18
	HN-07 (R)	18.25 (2.72)	4.32
	AH-07 (S)	4.22 (0.48)	1.00
Flucarbazone-Na	AH-01 (R)	1.99 (0.24)	0.29
	AH-05 (R)	23.05 (0.64)	3.33
	HN-07 (R)	8.04 (1.68)	1.16
	AH-07 (S)	6.92 (0.91)	1.00
Bispyribac-sodium	AH-01 (R)	7.12 (1.07)	4.88
	AH-05 (R)	133.28 (13.81)	91.29
	HN-07 (R)	10.38 (1.73)	7.11
	AH-07 (S)	1.46 (1.07)	1.00
Isoproturon	AH-01 (R)	398.68 (12.24)	0.62
	AH-05 (R)	93.45 (4.99)	0.15
	HN-07 (R)	275.21 (26.80)	0.43
	AH-07 (S)	642.39 (71.96)	1.00
Glyphosate	AH-01 (R)	90.40 (14.22)	0.87
	AH-05 (R)	61.79 (14.16)	0.60
	HN-07 (R)	42.38 (19.30)	0.41
	AH-07 (S)	103.35 (11.35)	1.00

^a The herbicide dose causing a 50% growth reduction (GR₅₀) was calculated according to a four-parameter log-logistic response equation: $y = C + (D - C) / [1 + (x/GR_{50})^b]$, where C is the lower limit, D is the upper limit, x is the herbicide application dose, b is the slope at GR₅₀, and y is the response at the herbicide dose x . ^b The resistance indexes (RI_s) were the ratios between the GR₅₀ values of the resistant and susceptible (AH-07) populations.

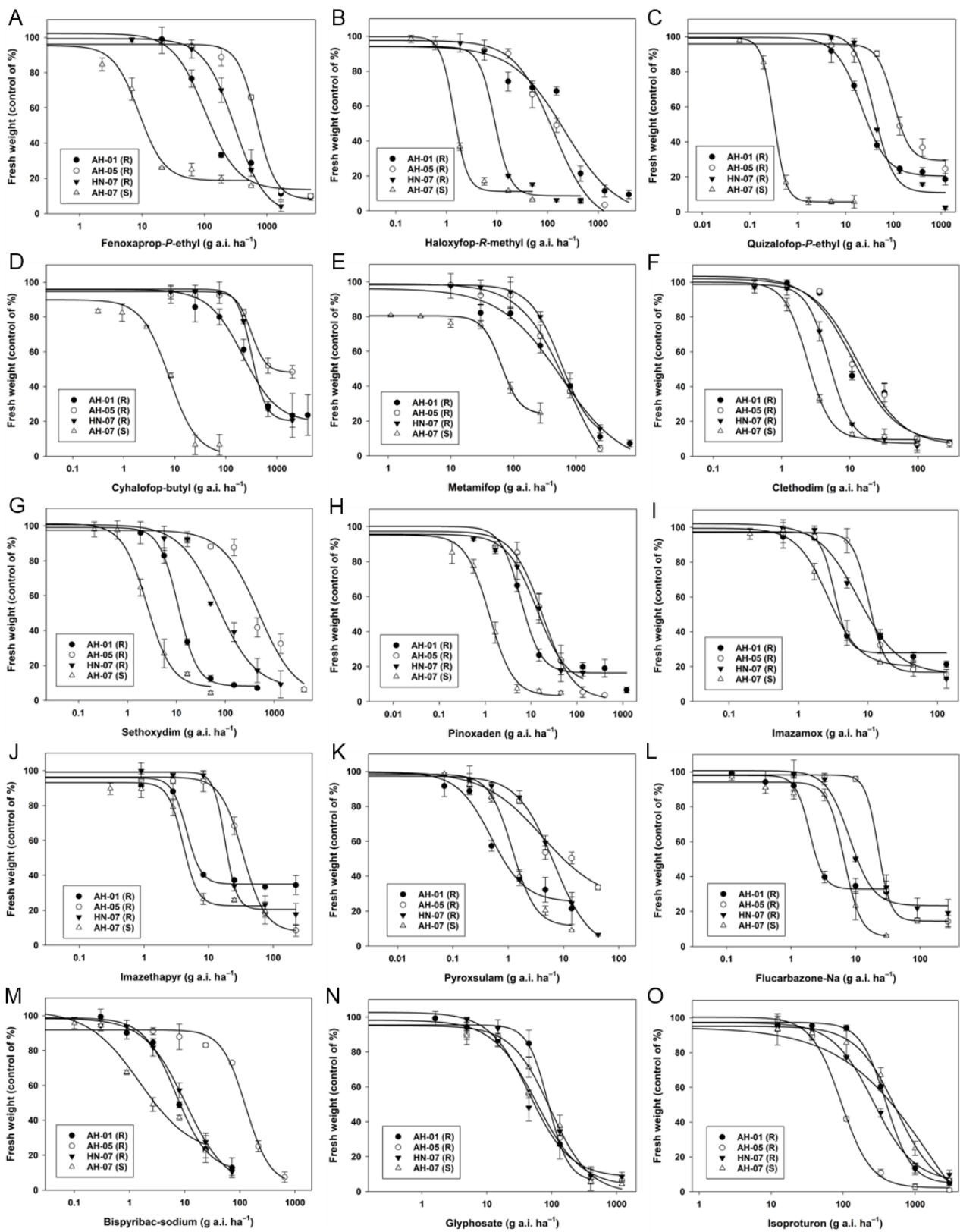


Figure 4. The doses-response curves of the aboveground fresh weights of the susceptible (AH-07) and different resistant (AH-01, AH-05, and HN-07) populations of *L. multiflorum* to (A) fenoxaprop-*P*-ethyl, (B) haloxyfop-*R*-methyl, (C) quizalofop-*P*-ethyl, (D) cyhalofop-butyl, (E) metamifop, (F) clethodim, (G) sethoxydim, (H) pinoxaden, (I) imazamox, (J) imazethapyr, (K) pyroxsulam, (L) flucarbazone-Na, (M) bispyribac-sodium, (N) glyphosate, and (O) isoproturon. Vertical bars represent the SE of the means.

4. Discussion

Lolium multiflorum, an invasive grass weed in wheat cropping systems in China, can occur even after application of the ACCase inhibitor clodinafop-propargyl and/or the ALS inhibitor mesosulfuron-methyl. Here, a total of 37 *L. multiflorum* populations were collected from the wheat fields across the Huang-Huai-Hai Plain, and 13 and 8 of the populations were confirmed to be resistant to clodinafop-propargyl and mesosulfuron-methyl, respectively (Figure 1). Most of the clodinafop-propargyl-resistant populations showed high- to moderate-level resistance, but the mesosulfuron-methyl-resistant populations mostly displayed low-level resistance. These results indicated that the herbicide resistance of *L. multiflorum* was prevalent in the Huang-Huai-Hai Plain, and the ACCase resistance seemed to be more severe than the ALS resistance.

In recent years, the infestation of *L. multiflorum* in wheat fields has been observed in some Provinces of China such as Henan and Shaanxi, and its effective control heavily relies on the chemical herbicides clodinafop-propargyl and mesosulfuron-methyl [5,6]. Zhang et al. [36] first reported that the *L. multiflorum* populations from Henan and Jiangsu had evolved resistance to fenoxaprop-*P*-ethyl and showed cross-resistance to different ACCase inhibitors. In addition, Wu et al. [37] recently found that a *L. multiflorum* population collected from Henan Province had evolved multiple herbicide resistance due to both a target-site mutation and the P450s-mediated enhanced metabolism. In this present study, different *L. multiflorum* populations were collected from the wheat fields across Provinces of Shaanxi, Henan, Hubei, Jiangsu, and Anhui, and their susceptibility to clodinafop-propargyl and mesosulfuron-methyl was determined by a single-dose herbicide-resistance testing and whole-plant dose-response experiments. The results showed that the herbicide-resistant populations mainly distributed in Henan, Jiangsu, and Anhui, and almost no resistant population was identified in Shaanxi and Hubei (Figure 1). This result may partially be due to the fact that only several populations of *L. multiflorum* were collected in the latter two Provinces, which may not be able to reflect the practical situation in most parts of the region. Moreover, the agricultural departments in Shaanxi Province have issued relevant warnings of the infestation of *L. multiflorum* in its wheat fields previously [5,6], suggesting that continuous attention should be paid to the possible resistance evolution in these regions. It is noteworthy that most populations exhibiting high-level clodinafop-propargyl resistance had simultaneously evolved mesosulfuron-methyl resistance (Table 5), which may be owing to the relevant herbicide application history. According to the local farmers, mesosulfuron-methyl has been commonly used as an alternative for control of the ACCase-resistant grass weeds in wheat fields, which may thus accelerate the evolution of multiple-herbicide-resistant weed biotypes.

In this study, three ACCase mutations including I1781L, I2041N, and D2078G were identified in 10 of the 13 clodinafop-propargyl-resistant populations (Table 6). Mutations at codon positions 1781, 2041, and 2078 of ACCase conferring ACCase resistance have been documented in diverse weed species [26]. Notably, among the 10 mutant populations, 5 were identified to have an I2041N mutation, 4 had an I1781L mutation, and only 1 had a D2078G mutation (Table 6). The rarity of the D2078G mutation may be partially due to its possible adverse pleiotropic effects on plant growth by changing enzyme functionality [38]. ACCase resistance conferred by the above three mutations has been identified in different weed species, such as *A. fatua* [14], shortawn foxtail (*Alopecurus aequalis* Sobol.) [39], and *A. myosuroides* [17]. In addition, four ALS mutations including P197A, P197S, P197T, and W574L were also identified in three of the eight mesosulfuron-methyl-resistant populations (Table 6). The codon positions 197 and 574 of ALS seem the most prone to mutation [22], and mutations at these two positions have been frequently identified in a variety of ALS-resistant weeds, such as wild radish (*Raphanus raphanistrum* L.) [24], *L. rigidum* [40], and *A. aequalis* [41]. Previous studies have found that P197S and W574L had no significant effect on ALS dynamics and plant vegetative growth, which may partially explain why these two mutations are often evolved under the selection of ALS herbicides [42]. In this study, no resistance mutation was identified in some ACCase- and ALS-resistant populations

that were classed as “R?” (Table 6), which indicates the potential presence of NTSR in these populations.

Target gene overexpression is also a TSR mechanism responsible for herbicide resistance, which has been frequently identified in the glyphosate-resistant weeds and rarely reported in the other herbicide-resistant weeds. Notably, Laforest et al. [43] found that transcription of *ACCase* was 3.4- to 9.3-times higher in the *ACCase*-resistant crabgrass (*Digitaria sanguinalis* (L.) Scop.) biotype than in the susceptible biotype, which was the first report of *ACCase* overexpression conferring *ACCase* resistance. Zhao et al. [44] also found that the *ALS* expression was significantly higher in the mesosulfuron-methyl-resistant than in the susceptible plants before and after mesosulfuron-methyl treatment. In the current study, the relative expression of *ACCase* or *ALS* in some herbicide-resistant populations of *L. multiflorum* was also determined (Figure 3). Compared with the susceptible population, the *ACCase* expression in the *ACCase*-resistant populations AH-01 and AH-05 was slightly but significantly upregulated at 12 and 2 HAT, respectively. However, the *ALS* expression in the *ALS*-resistant populations AH-05 and HN-08 showed no significant difference when compared with the susceptible population. These results indicated that the *ACCase* resistance in these populations may simultaneously involve the target-site mutations and the target-gene overexpression, while the *ALS* resistance was mainly due to the target-site mutations.

Cross- and multiple-resistance are defined as the expressions of genetically endowed mechanisms, including TSR and NTSR mechanisms, that can confer resistance to different herbicides of the same or different chemical groups [45]. Determining the resistance patterns of herbicide-resistant weed populations to the commonly used herbicides can help inform proactive or reactive resistant weed management by identifying less resistance-prone alternatives for growers. In this study, three target-site mutant populations, AH-01 with an *ACCase* I2041N mutation, AH-05 with both an *ACCase* I1781L mutation and an *ALS* P197T mutation, and HN-07 with an *ACCase* D2078G mutation, were selected for characterizing their cross- and multiple-resistance. Previously, the I1781L and D2078G mutations have been reported to confer resistance to all the three groups of *ACCase* inhibitors in canarygrass (*Phalaris minor* Retz.), *A. aequalis*, southern crabgrass (*Digitaria ciliaris* (Retz.) Koeler), and perennial ryegrass (*Lolium perenne* L.) [46–48], while the I2041N-mutant *A. myosuroides*, *A. aequalis*, and *A. fatua* showed high-level APP resistance [17,49,50]. In this current study, all the three *ACCase* mutations (I1781L, I2041N, and D2078G) conferred resistance to all the *ACCase* inhibitors tested, including APPs, CHDs, and PPZ. It is noteworthy that the AH-05 population with an *ACCase* I1781L mutation also carrying an *ALS* P197T mutation, which may be responsible for its resistance to the *ALS* inhibitors tested. Interestingly, two populations (AH-01 and HN-07) exhibiting moderate to low resistance to some *ALS* inhibitors had no *ALS* resistance mutations, indicating the possible participation of NTSR mechanisms.

In this study, all the mutant populations were confirmed susceptible to glyphosate and isoproturon. Isoproturon is a dominant herbicide widely used in the wheat field, which can be used as an alternative for control of the *ACCase*- and/or *ALS*-resistant *L. multiflorum*. However, we do not recommend its intensive and extensive use since the repeated use of the same-MOA herbicides is the greatest risk factor for fueling herbicide-resistance evolution [51]. Combining chemical control with other weed management methods may be wiser strategies to slow down the spread of resistance in these herbicide-resistant populations [21].

5. Conclusions

In summary, this study has clarified the resistance distributions and resistance levels of *L. multiflorum* to clodinafop-propargyl and mesosulfuron-methyl in wheat fields across the Huang-Huai-Hai Plain. Mutations at 1781, 2041, and 2078 codons of *ACCase* and at 197 and 574 codons of *ALS* were identified as the important TSR mechanisms to clodinafop-propargyl and mesosulfuron-methyl in *L. multiflorum*, respectively, and the

ACCase overexpression may also be involved in the ACCase resistance. The comprehensive cross- and multiple-resistance patterns were characterized for the resistance populations carrying different resistance mutations, and alternative herbicides were also identified for control of the resistant populations. Further studies are still required to figure out the exact mechanisms responsible for the resistance in the resistant populations without target-site mutations.

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References

- Zhang, X.; Li, Y.; Gou, W.; Zhang, R. Research development of Italian ryegrass. *Pratacultural Sci.* **2009**, *26*, 55–60.
- Brunharo, C.A.; Hanson, B.D. Multiple herbicide-Resistant Italian ryegrass [*Lolium perenne* L. spp. *multiflorum* (Lam.) Husnot] in California perennial crops: Characterization, mechanism of resistance, and chemical management. *Weed Sci.* **2018**, *66*, 696–701. [[CrossRef](#)]
- Bobadilla, L.K.; Hulting, A.G.; Berry, P.A.; Moretti, M.L.; Mallory-Smith, C. Frequency, distribution, and ploidy diversity of herbicide-resistant Italian ryegrass (*Lolium perenne* spp. *multiflorum*) populations of western Oregon. *Weed Sci.* **2021**, *69*, 177–185. [[CrossRef](#)]
- Stanger, C.E.; Appleby, A.P. Italian ryegrass (*Lolium multiflorum*) accessions tolerant to diclofop. *Weed Sci.* **1989**, *37*, 350–352. [[CrossRef](#)]
- Wang, H.; Song, Y.; Li, J.; Yang, G.; He, W.; Lou, Z. Precaution against malaria weed ryegrass in wheat field. *Plant Prot.* **2008**, *34*, 149–151.
- Hao, P.; Zhang, L. Highly alert to the spread of harmful ryegrass in weeds. *Shanxi J. Agric. Sci.* **2015**, *61*, 50–51.
- Liu, W.; Harrison, D.K.; Chalupska, D.; Gornicki, P.; O'donnell, C.C.; Adkins, S.W.; Williams, R.R. Single-site mutations in the carboxyltransferase domain of plastid acetyl-CoA carboxylase confer resistance to grass-specific herbicides. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 3627–3632. [[CrossRef](#)]
- Tate, T.M. Characterization of Acetyl Coenzyme an Inhibitor Resistance in Turfgrass and Grassy Weeds. Ph.D. Thesis, University of Georgia, Athens, GA, USA, 2012.
- Harwood, J.L. Fatty-acid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1988**, *39*, 101–138. [[CrossRef](#)]
- Takano, H.K.; Ovejero, R.F.L.; Belchior, G.G.; Maymone, G.P.L.; Dayan, F.E. ACCase-inhibiting herbicides: Mechanism of action, resistance evolution and stewardship. *Sci. Agric.* **2020**, *78*, e20190102. [[CrossRef](#)]
- Zhang, H.; Yang, Z.; Shen, Y.; Tong, L. Crystal structure of the carboxyltransferase domain of acetyl-coenzyme A carboxylase. *Science* **2003**, *299*, 2064–2067. [[CrossRef](#)]
- Egli, M.A.; Gengenbach, B.G.; Gronwald, J.W.; Somers, D.A.; Wyse, D.L. Characterization of maize acetyl-coenzyme A carboxylase. *Plant Physiol.* **1993**, *101*, 499–506. [[CrossRef](#)] [[PubMed](#)]
- Kaundun, S.S. Resistance to acetyl-CoA carboxylase-inhibiting herbicides. *Pest Manag. Science* **2014**, *70*, 1405–1417. [[CrossRef](#)] [[PubMed](#)]
- Yu, Q.; Ahmad-Hamdani, M.S.; Han, H.; Christoffers, M.J.; Powles, S.B. Herbicide resistance-endowing ACCase gene mutations in hexaploid wild oat (*Avena fatua*): Insights into resistance evolution in a hexaploid species. *Heredity* **2013**, *110*, 220–231. [[CrossRef](#)]
- Alwarnaidu Vijayarajan, V.B.; Forristal, P.D.; Cook, S.K.; Staples, J.; Schilder, D.; Hennessy, M.; Barth, S. First report on assessing the severity of herbicide resistance to ACCase inhibitors pinoxaden, propaquizafop and cycloxydim in six *Avena fatua* populations in Ireland. *Agronomy* **2020**, *10*, 1362. [[CrossRef](#)]

16. Yu, Q.; Cairns, A.; Powles, S. Glyphosate, paraquat and ACCase multiple herbicide resistance evolved in a *Lolium rigidum* biotype. *Planta* **2007**, *225*, 499–513. [[CrossRef](#)] [[PubMed](#)]
17. Yu, Q.; Abdallah, I.; Han, H.; Owen, M.; Powles, S. Distinct non-target site mechanisms endow resistance to glyphosate, ACCase and ALS-inhibiting herbicides in multiple herbicide-resistant *Lolium rigidum*. *Planta* **2009**, *230*, 713–723. [[CrossRef](#)]
18. Délye, C.; Matějček, A.; Michel, S. Cross-resistance patterns to ACCase-inhibiting herbicides conferred by mutant ACCase isoforms in *Alopecurus myosuroides* Huds. (black-grass), re-examined at the recommended herbicide field rate. *Pest Manag. Science* **2008**, *64*, 1179–1186. [[CrossRef](#)]
19. Bailly, G.C.; Dale, R.P.; Archer, S.A.; Wright, D.J.; Kaundun, S.S. Role of residual herbicides for the management of multiple herbicide resistance to ACCase and ALS inhibitors in a black-grass population. *Crop Prot.* **2012**, *34*, 96–103. [[CrossRef](#)]
20. Zhao, N.; Yang, J.; Jiang, M.; Liao, M.; Cao, H. Identification of essential genes involved in metabolism-based resistance mechanism to fenoxaprop-P-ethyl in *Polypogon fugax*. *Pest Manag. Sci.* **2022**, *78*, 1164–1175. [[CrossRef](#)]
21. Zhao, N.; Jiang, M.; Li, Q.; Gao, Q.; Zhang, J.; Liao, M.; Cao, H. Cyhalofop-butyl resistance conferred by a novel Trp-2027-Leu mutation of acetyl-CoA carboxylase and enhanced metabolism in *Leptochloa chinensis*. *Pest Manag. Sci.* **2022**, *78*, 1176–1186. [[CrossRef](#)]
22. Heap, I. International Herbicide Resistance Weed Database. Available online: www.weedscience.org (accessed on 24 June 2022).
23. Garcia, M.D.; Nouwens, A.; Lonhienne, T.G.; Guddat, L.W. Comprehensive understanding of acetohydroxyacid synthase inhibition by different herbicide families. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E1091–E1100. [[CrossRef](#)] [[PubMed](#)]
24. Yu, Q.; Han, H.; Li, M.; Purba, E.; Walsh, M.J.; Powles, S.B. Resistance evaluation for herbicide resistance—Endowing acetolactate synthase (ALS) gene mutations using *Raphanus raphanistrum* populations homozygous for specific ALS mutations. *Weed Res.* **2012**, *52*, 178–186. [[CrossRef](#)]
25. Shaner, D.L. Herbicide safety relative to common targets in plants and mammals. *Pest Manag. Sci.* **2004**, *60*, 17–24. [[CrossRef](#)] [[PubMed](#)]
26. Gaines, T.A.; Duke, S.O.; Morran, S.; Rigon, C.A.; Tranel, P.J.; Küpper, A.; Dayan, F.E. Mechanisms of evolved herbicide resistance. *J. Biol. Chem.* **2020**, *295*, 10307–10330. [[CrossRef](#)]
27. Powles, S.B.; Yu, Q. Evolution in action: Plants resistant to herbicides. *Annu. Rev. Plant Biol.* **2010**, *61*, 317–347. [[CrossRef](#)]
28. Yu, Q.; Powles, S.B. Resistance to AHAS inhibitor herbicides: Current understanding. *Pest Manag. Sci.* **2014**, *70*, 1340–1350. [[CrossRef](#)]
29. Zhang, P.; Wu, H.; Xu, H.; Gao, Y.; Zhang, W.; Dong, L. Mechanism of fenoxaprop-P-ethyl resistance in Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) from China. *Weed Sci.* **2017**, *65*, 710–717. [[CrossRef](#)]
30. Moss, S.R.; Perryman, S.A.; Tatnell, L.V. Managing herbicide-resistant blackgrass (*Alopecurus myosuroides*): Theory and practice. *Weed Technol.* **2007**, *21*, 300–309. [[CrossRef](#)]
31. Porebski, S.; Bailey, L.G.; Baum, B.R. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol. Biol. Report.* **1997**, *15*, 8–15. [[CrossRef](#)]
32. Yu, Q.; Han, H.; Powles, S.B. Mutations of the ALS gene endowing resistance to ALS-inhibiting herbicides in *Lolium rigidum* populations. *Pest Manag. Sci.* **2008**, *64*, 1229–1236. [[CrossRef](#)]
33. Zhang, P. Fenoxaprop-P-ethyl Resistance and the Control of *Lolium multiflorum* in Wheat Fields. Ph.D. Thesis, Nanjing Agricultural University, Nanjing, China, 2018.
34. Duhoux, A.; Delye, C. Reference genes to study herbicide stress response in *Lolium* sp.: Up-regulation of P450 genes in plants resistant to acetolactate-synthase inhibitors. *PLoS ONE* **2013**, *8*, e63576. [[CrossRef](#)] [[PubMed](#)]
35. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)] [[PubMed](#)]
36. Zhang, P.; Zhang, Y.; Chen, X.; Dong, L. Cross resistance patterns to acetyl-CoA carboxylase inhibiting herbicides associated with different mutations in Italian ryegrass from China. *Crop Prot.* **2021**, *143*, 105479. [[CrossRef](#)]
37. Wu, C.; Song, M.; Zhang, T.; Zhou, C.; Liu, W.; Jin, T.; Zhao, N. Target-site mutation and cytochrome P450s confer resistance to multiple herbicides in Italian ryegrass (*Lolium multiflorum* Lam.) from China. *Crop Prot.* **2022**, *161*, 106068. [[CrossRef](#)]
38. Zhao, N.; Yan, Y.; Du, L.; Zhang, X.; Liu, W.; Wang, J. Unravelling the effect of two herbicide resistance mutations on acetolactate synthase kinetics and growth traits. *J. Exp. Bot.* **2020**, *71*, 3535–3542. [[CrossRef](#)]
39. Guo, W.; Chi, Y.; Feng, L.; Tian, X.; Liu, W.; Wang, J. Fenoxaprop-P-ethyl and mesosulfuron-methyl resistance status of shortawn foxtail (*Alopecurus aequalis* Sobol.) in eastern China. *Pestic. Biochem. Physiol.* **2018**, *148*, 126–132. [[CrossRef](#)]
40. Tan, M.K.; Preston, C.; Wang, G.X. Molecular basis of multiple resistance to ACCase-inhibiting and ALS-inhibiting herbicides in *Lolium rigidum*. *Weed Res.* **2007**, *47*, 534–541. [[CrossRef](#)]
41. Marshall, R.; Moss, S.R. Characterisation and molecular basis of ALS inhibitor resistance in the grass weed *Alopecurus myosuroides*. *Weed Res.* **2008**, *48*, 439–447. [[CrossRef](#)]
42. Yu, Q.; Han, H.; Vila-Aiub, M.M.; Powles, S.B. AHAS herbicide resistance endowing mutations: Effect on AHAS functionality and plant growth. *J. Exp. Bot.* **2010**, *61*, 3925–3934. [[CrossRef](#)]
43. Laforest, M.; Soufiane, B.; Simard, M.J.; Obeid, K.; Page, E.; Nurse, R.E. Acetyl-CoA carboxylase overexpression in herbicide-resistant large crabgrass (*Digitaria sanguinalis*). *Pest Manag. Sci.* **2017**, *73*, 2227–2235. [[CrossRef](#)]

44. Zhao, N.; Yan, Y.; Wang, H.; Bai, S.; Wang, Q.; Liu, W.; Wang, J. Acetolactate synthase overexpression in mesosulfuron-methyl-resistant shortawn foxtail (*Alopecurus aequalis* Sobol.): Reference gene selection and herbicide target gene expression analysis. *J. Agric. Food Chem.* **2018**, *66*, 9624–9634. [[CrossRef](#)] [[PubMed](#)]
45. Powles, S.B.; Preston, C. Herbicide cross resistance and multiple resistance in plants. *Herbic. Resist. Action Comm. Monogr.* **1995**, *2*, 1–19.
46. Basak, S.; McElroy, J.S.; Brown, A.M.; Gonçalves, C.G.; Patel, J.D.; McCullough, P.E. Plastidic ACCase Ile-1781-Leu is present in pinoxaden-resistant southern crabgrass (*Digitaria ciliaris*). *Weed Sci.* **2020**, *68*, 41–50. [[CrossRef](#)]
47. Cruz-Hipolito, H.; Fernandez, P.; Alcantara, R.; Gherekhloo, J.; Osuna, M.D.; De Prado, R. Ile-1781-Leu and Asp-2078-Gly mutations in ACCase gene, endow cross-resistance to APP, CHD, and PPZ in *Phalaris minor* from Mexico. *Int. J. Mol. Sci.* **2015**, *16*, 21363–21377. [[CrossRef](#)]
48. Yannicari, M.; Gigón, R. Cross-resistance to acetyl-CoA carboxylase-inhibiting herbicides conferred by a target-site mutation in perennial ryegrass (*Lolium perenne*) from Argentina. *Weed Sci.* **2020**, *68*, 116–124. [[CrossRef](#)]
49. Zhao, N.; Yan, Y.; Ge, L.A.; Zhu, B.; Liu, W.; Wang, J. Target site mutations and cytochrome P450s confer resistance to fenoxaprop-P-ethyl and mesosulfuron-methyl in *Alopecurus aequalis*. *Pest Manag. Sci.* **2019**, *75*, 204–214. [[CrossRef](#)]
50. Cruz-Hipolito, H.; Osuna, M.D.; Dominguez-Valenzuela, J.A.; Espinoza, N.; De Prado, R. Mechanism of resistance to ACCase-inhibiting herbicides in wild oat (*Avena fatua*) from Latin America. *J. Agric. Food Chem.* **2011**, *59*, 7261–7267. [[CrossRef](#)]
51. Norsworthy, J.K.; Ward, S.M.; Shaw, D.R.; Llewellyn, R.S.; Nichols, R.L.; Webster, T.M.; Barrett, M. Reducing the risks of herbicide resistance: Best management practices and recommendations. *Weed Sci.* **2012**, *60* (Suppl. 1), 31–62. [[CrossRef](#)]