Investigating the resistance levels and mechanisms to penoxsulam and cyhalofop-butyl in barnyardgrass (*Echinochloa crus-galli*) from Ningxia Province, China

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Abstract

Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] is a noxious grass weed that infests rice fields and causes huge crop yield losses. In this study, we collected 12 *E. crus-galli* populations from rice fields of Ningxia Province in China and investigated the resistance levels to the acetolactate synthase (ALS) inhibitor penoxsulam and the acetyl-CoA carboxylase (ACCase) inhibitor cyhalofop-butyl. The results showed that eight populations exhibited resistance to penoxsulam and four populations evolved resistance to cyhalofop-butyl. Moreover, all four cyhalofop-butyl–resistant populations (NX3, NX4, NX6, and NX7) displayed multiple herbicide resistance to both penoxsulam and cyhalofop-butyl. The alternative herbicides bispyribac-sodium, metamifop, and fenoxaprop-*p*-ethyl cannot effectively control the multiple herbicide–resistant (MHR) plants. To characterize the molecular mechanisms of resistance, we amplified and sequenced the target site–encoding genes in resistant and susceptible populations. Partial sequences of three *ALS* genes and six *ACCase* genes were examined. A Trp-574-Leu mutation was detected in *EcALS1* and *EcALS3* in two high-level (65.84- and 59.30-fold) penoxsulam-resistant populations, NX2 and NX10, respectively. In addition, one copy (*EcACC4*) of ACCase genes encodes a truncated aberrant protein due to a frameshift mutation in *E. crus-galli* populations. None of the amino acid substitutions that are known to confer herbicide resistance were detected in ALS and ACCase genes of MHR populations. Our study reveals the wide spread of MHR *E. crus-galli* populations in Ningxia Province that exhibit resistance to several ALS and ACCase inhibitors. Non–target site based mechanisms are likely to be involved in *E. crus-galli* resistance to the herbicides, at least in four MHR populations.

Introduction

Rice (*Oryza sativa* L.) is one of the most important grain crops in the world, and it is the staple food for more than 3 billion people (Kuenzer and Knauer 2013). In China, weed infestation results in 10 billion kg of annual rice yield reduction, with an average loss rate of about 15%, and weeds are becoming the major causes of yield losses (Dong et al. 2018). Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.], an annual grass species, is one of the most problematic weeds that infest paddy fields, and it is considered a serious threat to rice production (Zhang et al. 2017).

Acetolactate synthase (ALS) and acetyl-CoA carboxylase (ACCase) inhibitors, represented in this study by penoxsulam and cyhalofop-butyl, respectively, are two groups of herbicides commonly used in rice fields (Dong et al. 2018). ALS inhibitors target the key enzyme in the biosynthesis of branched-chain amino acids and can be classified into sulfonylureas, triazolopyrimidines, imidazolinones, sulfonyl-aminocarbonyl-triazolinones, and pyrimidinylthiobenzoates (Tranel and Wright 2002). ACCase inhibitors prevent fatty-acid synthesis and fall into three chemical families, namely aryloxyphenoxypropionates, cyclohexanediones, and phenylpyraxoline (Sasaki et al. 1995). Both ALS and ACCase inhibitors have a high resistance risk in *E. crus-galli* (Bagavathiannan et al. 2014). Recurrent use of these herbicides has promoted resistance to ALS and ACCase inhibitors in 165 and 49 weed species, respectively (Heap 2021).

Both target site–based resistance (TSR) and non–target site based resistance (NTSR) are involved in resistance to ALS and ACCase inhibitors (Perotti et al. 2020; Powles and Yu 2010). Molecular mechanisms of TSR to ALS and ACCase herbicides in weeds have been well researched. Twenty-eight amino acid substitutions at the Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654 sites in ALS have been reported to confer resistance to ALS inhibitors (Heap 2021). Thirteen amino acid substitutions at seven sites (Ile-1781, Trp-1999, Trp-2027, Ile-2041, Asp-2078, Cys-2088, Gly-2096) in the carboxyltransferase domain of ACCase have been identified to be associated with resistance to ACCase inhibitors (Kaundun 2014). In the case of NTSR, enhanced herbicide metabolism is frequently reported (Ghanizadeh and Harrington 2017); however, the molecular mechanisms remain largely unknown (Délye 2013; Yu and Powles 2014).

Echinochloa spp. are the most troublesome weeds in rice fields and can compete very effectively with rice seedlings, causing huge crop yield losses (Dong et al. 2018). However, only a limited number of herbicides are registered and can be used in rice fields. Among them, the auxin herbicide quinclorac was used for nearly 30 yr in China, resulting in high resistance frequency (Gao et al. 2019). In addition, the ALS inhibitor bispyribac-sodium and ACCase inhibitor metamifop provided poor crop safety for some rice varieties, making them unpopular among rice growers in China. For these reasons, the foliar-applied herbicides penoxsulam and cyhalofop-butyl have been used widely for the control of *Echinochloa* spp. in rice. This overreliance on one or two herbicides is likely to result in the development of weed resistance. Penoxsulam-resistant *Echinochloa* spp. were reported in various rice-growing regions of China, including Jiangsu, Anhui, Hubei, Hunan, Jiangxi, and Heilongjiang provinces (Chen et al. 2016; Fang et al. 2019a; Liu et al. 2019; Yang et al. 2013). Xia et al. (2016) also reported *E. crus-galli* populations from Hubei Province that had developed resistance to the ACCase inhibitor metamifop.

Ningxia Province, located in the northwest of China, is one of the major rice-producing areas. Weed management in Ningxia rice fields mainly depends on chemical control. However, penoxsulam and/or cyhalofop-butyl cannot effectively control the occurrence of *E. crus-galli* in some regions of Ningxia Province due to overreliance on a limited number of herbicides. Here, we aimed to (1) investigate the levels of resistance to penoxsulam and cyhalofop-butyl in 12 *E. crus-galli* populations collected from Ningxia Province, (2) characterize the resistance mechanisms to these two herbicides, and (3) determine the cross-resistance patterns of resistant populations to other ALS and ACCase inhibitors.

Materials and Methods

Plant Materials and Growth Conditions

Seeds of 12 *E. crus-galli* populations were collected from Ningxia Province in 2017. A total of 11 putative herbicide-resistant populations (NX1 to NX11) were collected from rice fields where penoxsulam and cyhalofop-butyl failed to control the weeds. The known susceptible population (NX12) was collected from an area with no previous herbicide treatment (Figure 1). Seeds of each population were collected randomly from 15 to 20 mature plants and bulked.

Seeds were stratified in darkness for 3 d at 4 C before being transferred to a growth chamber (30/25 C, 14-h light/10-h dark). After germination, seedlings were transplanted to 7-cm-diameter plastic pots (12 seedlings per pot) containing a 1:1:1 (w/w/w) mixture
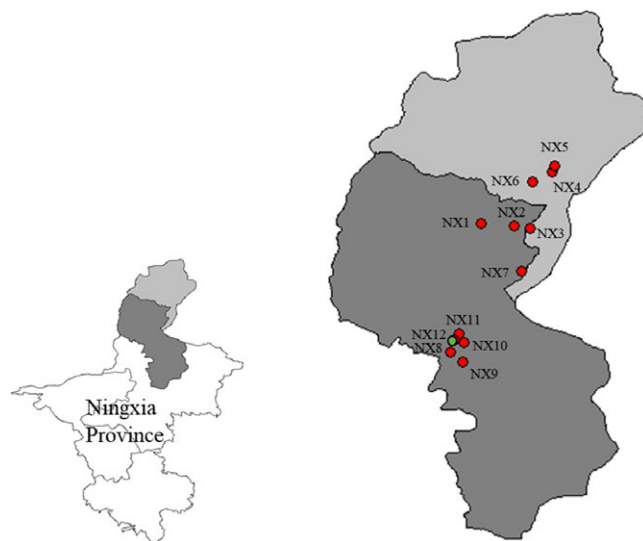

Figure 1. Locations of 12 *Echinochloa crus-galli* populations in this study.

of sand, vermiculite, and organic matter and grown in growth chamber under conditions of 30/25 C temperature, a 14-h light/10-h dark cycle, light intensity of 12,000 lx, and 75% relative humidity.

Whole-Plant Dose Response to Penoxsulam and Cyhalofop-Butyl

Before herbicide treatment, seedlings were thinned to 8 to 10 plants per pot. When the plants had grown to the 3-leaf stage, herbicides were applied using a laboratory sprayer (machine model: 3WPSH-500D, Nanjing Research Institute for Agricultural Mechanization, Nanjing, Ministry of Agriculture of China) equipped with a cone nozzle delivering 280 L ha^{-1} at 0.3 MPa. The penoxsulam doses were 0, 1/16×, 1/4×, 1×, 2×, 4×, and 8× the recommended field dose (30 g ai ha^{-1}) for putative resistant populations and 0, 1/64×, 1/16×, 1/4, 1×, and 2× the recommended rate for susceptible population. The cyhalofop-butyl doses were 0, 1/8×, 1/4×, 1/2×, 1×, 2×, 4×, and 8× the recommended field dose (120 g ai ha^{-1}) for putative resistant populations and 0, 1/16×, 1/8×, 1/4×, 1/2×, 1×, and 2× the recommended rate for susceptible population. The fresh weight of aboveground plants was recorded at 2 wk after herbicide treatment. Each herbicide dose had three replications, and each experiment was conducted twice.

Partial ALS and ACCase Gene Cloning and Sequencing

Primers used for the amplification of *E. crus-galli* ALS genes were designed based on the *ALS* (accession number LC006058, LC006059, and LC006061) sequences retrieved from the National Center for Biotechnology Information (NCBI) GenBank database (Table 1).

For *ACCase* genes, the primer set designed based on *ACCase* (accession numbers LC006070 to LC006075) sequences of *E. crus-galli* were used to amplify the carboxyltransferase domains of the orthologous genes *EcACC1*, *EcACC2*, *EcACC4*, and *EcACC6* in this study (Table 1). The other orthologous genes, *EcACC3* and *EcACC5*, were specifically amplified by primers described in Iwakami et al. (2015).

Survivors from dose–response treatments were selected for gene identification. Approximately 100 mg of shoot tissue from each individual plant was collected for further analyses. Genomic

Table 1. Primers used for amplification of *Echinochloa crus-galli ALS* and *ACCase* genes.

Gene	Forward primer	Reverse primer	Amplicon size	T_m[a]
			—bp—	—C—
EcALS	CCGCGACGTCTTCGCCTAC	TCAATACACGGTCCTGCCAT	1,678	58
EcACCase[b]	ATCTTGCTTCCGTGTTGGGT	CCTTGGAGTTTTGCTTTCAG	1,254	56
EcACC3[c]	TTAGGTGGATTATTGACTCTGTTGTG	GAGCAAATTCAAATGCCGAAT	2,274	53
EcACC5[c]	CAGCTGGATAGTGGGGAAAC	GGAAAGGCTAGTTTTACCTCTTTG	2,376	56

[a]T_m, melting temperature.
[b]*EcACCase*, amplify the carboxyltransferase domains of the orthologous *ACCase* genes *EcACC1*, *EcACC2*, *EcACC4*, and *EcACC6* of *E. crus-galli*.
[c]*EcACC3*, amplify *EcACC3*; *EcACC5*, amplify *EcACC5*.

Table 2. ALS- and ACCase-inhibiting herbicides used in this study.

Herbicide	Site of action	Formulation[a]	Recommended field dose	Company
			—g ai ha^{-1}—	
Penoxsulam	ALS	25 g L^{-1}OD	30	Dow AgroSciences, Indianapolis, IN, USA
Bispyribac-sodium	ALS	10% SC	45	Jiangsu Hormone Research Institute Co., Ltd., Jintan, Jiangsu, China
Cyhalofop-butyl	ACCase	10% EW	120	Jiangxi Zhongxun Agrochemical Co., Ltd., Nanchang, Jiangxi, China
Metamifop	ACCase	10% EC	120	Jiangsu Suzhou Fumeishi Plant Protective Agent Co., Ltd., Suzhou, Jiangsu, China
Fenoxaprop-*p*-ethyl	ACCase	69 g L^{-1}EW	62.1	Bayer CropScience China Co., Ltd., Hangzhou, Zhejiang, China

[a]OD, dispersible oil suspension; SC, suspension concentrate; EW, emulsion in water. EC, emulsifiable concentrate.

DNA was extracted from each sample using the Hi-DNAsecure Plant Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The polymerase chain reaction (PCR) mixture contained 2 µl of template DNA, 1 µl (10 µM) of each primer, 25 µl of 2×Taq PCR Master Mix (Tiangen Biotech), and ddH$_2$O to a final volume of 50 µl. The PCR program was set according to the following profile: 3 min at 95 C for DNA denaturation; followed by 35 cycles of 30-s denaturation at 95 C, 30-s annealing at each melting temperature (T_M), and 90-s elongation at 72 C; then a final 5 min at 72 C for further DNA elongation. The PCR products were purified using TIANgel Midi Purification Kit (Tiangen Biotech) and cloned into a pLB vector (Tiangen Biotech). The plasmids containing the fragment insertions were transformed into TOP10 chemically competent cells, and sequenced by Sangon Biotechnology (Shanghai, China) with pLB vector sequencing primers (forward: 5′-CGACTCACTATAGGGAGAG CGGC-3′; reverse: 5′-AAGAACATCGATTTTCCATGGCAG-3′). At least 10 transformed clones of each plant were sequenced to obtain partial *ALS* and *ACCase* gene sequences that were aligned and compared in DNAMAN v. 5.22 software (Lynnon Biosofe, Quebec, Canada).

Sensitivity to Other ALS- and ACCase-inhibiting Herbicides

Three ALS- and ACCase-inhibiting herbicides commonly used in rice fields were used to evaluate their effectiveness on all *E. crus-galli* populations. Bispyribac-sodium, metamifop, and fenoxaprop-*p*-ethyl at doses of 45, 120, and 20.7 g ai ha^{-1}, respectively were used to differentiate the resistant and susceptible populations. The doses were designed based on the primary experiments; the herbicides tested are listed in Table 2. When the plants grew to the 3-leaf stage, the herbicides were applied as described earlier with three replicates. At 2 wk after treatment, aboveground shoots were collected and their fresh weight recorded.

Statistical Analysis

The herbicide dose required for 50% growth reduction (GR$_{50}$) was evaluated using the following logistic-regression model (Equation 1) with SigmaPlot software (v. 12.0, Systat Software, San Jose, CA, USA) (Seefeldt et al. 1995).

$$y = C + (D - C)/[1 + (x/\text{GR}_{50})^b] \qquad [1]$$

In the equation, y is the fresh weight, expressed as percentage of the nontreated control at dose x of herbicide, b is the slope, C is the lower limit, and D is the upper limit. The resistance index (RI) was calculated as the GR$_{50}$ of the resistant population divided by that of susceptible population (NX12). The resistance levels of *E. crus-galli* populations to each herbicide were defined according to a standard (Beckie and Tardif 2012) and appropriately modified. The standard was as follows: no resistance (RI < 3), low resistance (3 ≤ RI < 5), moderate resistance (5 ≤ RI < 10), and high resistance (RI ≥ 10). The data for other ALS- and ACCase-inhibiting herbicide sensitivities of the 12 *E. crus-galli* populations were analyzed with one-way ANOVA (SPSS Statistics v. 20.0, IBM, Armonk, NY, USA).

Results and Discussion

Dose Response to Penoxsulam and Cyhalofop-Butyl

In this study, we surveyed and characterized penoxsulam and cyhalofop-butyl sensitivity of 12 *E. crus-galli* populations collected from rice fields in Ningxia Province, China. The results showed that eight *E. crus-galli* populations (NX1, NX2, NX3, NX4, NX6, NX7, NX9, and NX10) displayed resistance to the ALS inhibitor penoxsulam (Table 3; Figure 2A). The susceptible populations (NX5, NX8, NX11, and NX12) were completely killed by the recommended rate of penoxsulam, and the GR$_{50}$ values were 2.25, 1.71, 2.23, and 1.63 g ai ha^{-1}, respectively. The resistant populations (NX2, NX6, and NX10) were not injured by the recommended rate and exhibited more than 90% growth relative to untreated controls at that dose. The GR$_{50}$ values of NX2 and NX10 were similar to each other: 107.32 g ai ha^{-1} and 96.67 g ai ha^{-1}, respectively; and the resistance indexes were 65.84 and

Table 3. GR$_{50}$ and resistance index (RI) values to penoxsulam and cyhalofop-butyl in *Echinochloa crus-galli* populations.

Population[a]	Penoxsulam		Cyhalofop-butyl		
	GR$_{50}$ (SE)[b]	RI[c]	GR$_{50}$ (SE)[b]	RI[c]	Categories[d]
	—g ai ha^{-1}—		—g ai ha^{-1}—		
NX1	8.04 (1.04)	4.93	15.67 (1.65)	1.07	PR
NX2	107.32 (11.44)	65.84	26.08 (2.05)	1.78	PR
NX3	18.21 (1.74)	11.17	333.65 (17.73)	22.78	PRCR
NX4	7.87 (1.53)	4.83	91.05 (9.02)	6.21	PRCR
NX5	2.25 (0.24)	1.38	31.10 (1.74)	2.12	S
NX6	65.84 (11.05)	40.39	317.61 (13.81)	21.68	PRCR
NX7	12.25 (0.73)	7.52	182.89 (7.84)	12.48	PRCR
NX8	1.71 (0.20)	1.05	17.37 (2.06)	1.18	S
NX9	7.40 (1.88)	4.54	29.05 (2.96)	1.98	PR
NX10	96.67 (7.77)	59.30	33.48 (3.75)	2.29	PR
NX11	2.23 (0.18)	1.37	21.92 (2.39)	1.50	S
NX12	1.63 (0.19)	1.00	14.65 (1.66)	1.00	S

[a]NX12 was the known susceptible population.
[b]GR$_{50}$, herbicide dose causing 50% growth reduction; SE, standard error.
[c]RI, resistance index, which calculated with GR$_{50}$ value of resistant population divided by that of susceptible population.
[d]PR, *E. crus-galli* population resistant to penoxsulam; CR, population resistant to cyhalofop-butyl.
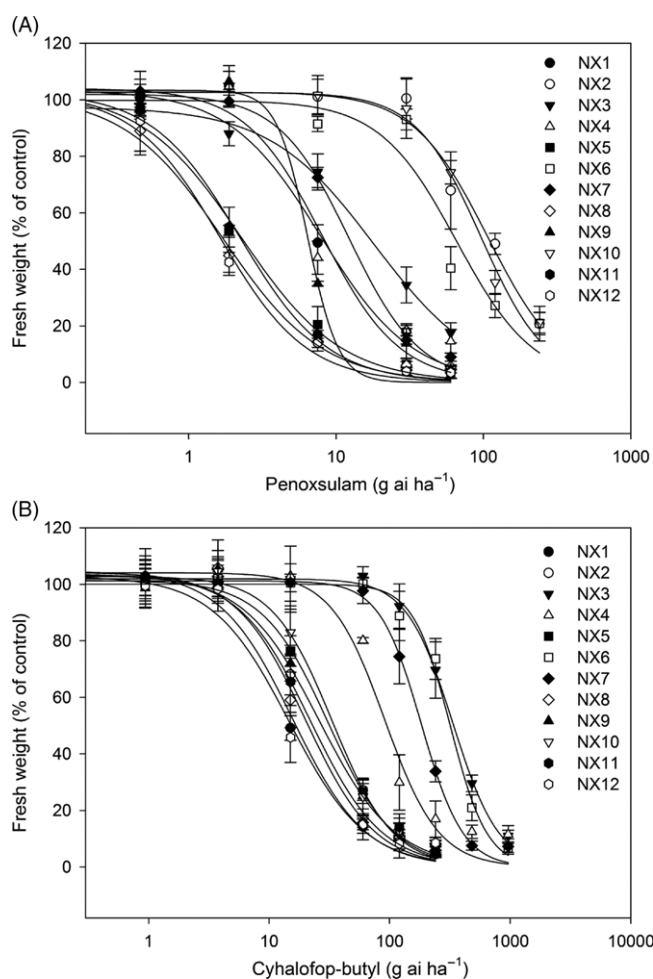

Figure 2. Dose-response curves of 12 *Echinochloa crus-galli* populations to penoxsulam (A) and cyhalofop-butyl (B). Shoot fresh weight is shown as percentages of the fresh weight of untreated controls. Bars indicate standard error ($n = 3$).

partially survived under the recommended rate application and exhibited approximately 20% growth relative to untreated controls. The GR$_{50}$ values of these populations were calculated to be 8.04, 18.21, 7.87, 12.25, and 7.40, respectively, showing at least moderate-level resistance to penoxsulam.

The results of the cyhalofop-butyl sensitivity assay showed that three (NX3, NX6, and NX7) out of 12 *E. crus-galli* populations had evolved high level resistance to cyhalofop-butyl, with 22.78, 21.68, and 12.48-fold resistance compared with the susceptible population (NX12) (Table 3; Figure 2B). The NX4 population demonstrated a moderate level of resistance, with an RI value of 6.21. The cyhalofop-butyl–susceptible populations (NX1, NX2, NX5, NX8, NX9, NX10, NX11, and NX12) were completely killed by the recommended application rate of cyhalofop-butyl, and the GR$_{50}$ ranged from 14.65 to 33.48 g ai ha^{-1}.

Interestingly, the four cyhalofop-butyl–resistant *E. crus-galli* populations (NX3, NX4, NX6, and NX7) also showed resistance to penoxsulam. The NX6 population exhibited especially high-level resistance to both penoxsulam and cyhalofop-butyl, with 40.39- and 21.68-fold resistance, respectively, relative to susceptible population (Table 3; Figure 2). The other three populations (NX3, NX4, and NX7) had evolved low- to moderate-level resistance to penoxsulam, and the RI values were 11.17, 4.83, and 7.52, respectively. The results indicate that at least two different types of herbicide resistance exist among *E. crus-galli* plants in Ningxia Province, and the resistance patterns are evolving from single herbicide resistance to multiple resistance. These findings revealed the widespread occurrence of ALS and/or ACCase inhibitor–resistant *E. crus-galli* populations in China, a situation that requires attention, especially the occurrence of MHR populations.

Cloning and Sequence Analysis of ALS and ACCase Genes

Three partial gene sequences of ALS that covered more than 85% of full-length sequences were isolated from each *E. crus-galli* population. The isolated region contains all known eight amino acid positions whose substitutions confer ALS-inhibitor resistance (Powles and Yu 2010) and exhibited highest homology with the *ALS* genes (accession number LC006058, LC006059, and LC006061) of *E. crus-galli* registered in the NCBI database. A Trp-574-Leu substitution caused by a nucleotide mutation (TGG to TTG) was identified in resistant *E. crus-galli* populations

59.30, respectively, compared with the susceptible population (NX12). The GR$_{50}$ of NX6 was 65.84 g ai ha^{-1}, with 40.39-fold higher resistance than the susceptible population. In addition, there were five populations (NX1, NX3, NX4, NX7, and NX9) that

Table 4. Target-site mutations in ALS genes of penoxsulam-resistant *Echinochloa crus-galli* populations.

Populations	RI[a]	*EcALS1*	*EcALS2*	*EcALS3*
NX1	Low	—	—	—
NX2	High	Trp-574-Leu	—	—
NX3	High	—	—	—
NX4	Low	—	—	—
NX6	High	—	—	—
NX7	Moderate	—	—	—
NX9	Low	—	—	—
NX10	High	—	—	Trp-574-Leu

[a]RI, resistance index: low, 3–5; moderate, 5–10; high, >10.
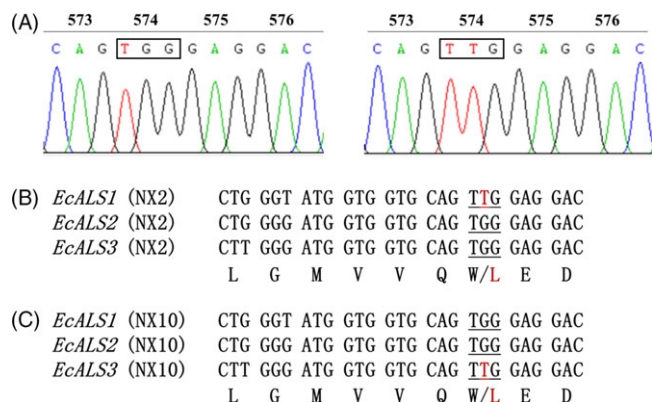
Figure 3. DNA sequencing results showing TGG for Trp of susceptible *Echinochloa crus-galli* plants (A, left), TTG for Leu of resistant plants (A, right). Sequence alignment identified a Trp-574-Leu substitution in *EcALS1* of the NX2 population (B) and in *EcALS3* of the NX10 population (C). The underlined bases indicate the amino acid mutation in codon position 574.

NX2 and NX10 (Table 4; Figure 3A). Moreover, the Trp-574-Leu substitution in the NX2 population occurred in *EcALS1* (Figure 3B), and in the NX10 population it occurred in *EcALS3* (Figure 3C). In addition, no known resistance-conferring mutations were found in the highly penoxsulam-resistant population NX6 and other resistant populations (Table 4). In these populations, the NTSR mechanism may be dominant, and further research is needed. The Trp-574-Leu mutation was mainly responsible for penoxsulam resistance in *E. crus-galli*, and the same substitution has been documented in other ALS inhibitor–resistant weeds, including catchweed bedstraw (*Galium aparine* L.) (Deng et al. 2019b), common gromwell [*Lithospermum arvense* (L.) I.M. Johnst.] common gromwell [*Lithospermum arvense* (L.) I.M. Johnst.] (Wang et al. 2019), johnsongrass [*Sorghum halepense* (L.) Pers.] (Hernandez et al. 2015), and annual bluegrass (*Poa annua* L.) (McElroy et al. 2013). Multiple *ALS* gene copies are a common phenomenon in weeds, especially in polyploid weeds. For example, there are two copies of *ALS* in flixweed (*Descurainia sophia* L.) (Deng et al. 2017) and shepherd's purse [*Capsella bursa-pastoris* (L.) Medik.] (Zhang et al. 2017). The variation in *ALS* copy number was also observed in shortawn foxtail (*Alopecurus aequalis* Sobol.) (Iwakami et al. 2017) and Japanese foxtail (*Alopecurus japonicus* Steudel) (Feng et al. 2017). Furthermore, it was demonstrated that any of the *ALS* gene copies could host the ALS-inhibitor resistance mutation (Deng et al. 2017; Zhang et al. 2017), which may also be the reason why these weeds are prone to develop resistance to ALS inhibitors. The Ala-122-Gly/Asn, Ala-205-Val, Trp-574-Leu, and Ser-653-Asn mutations in *ALS* have been reported to confer resistance to ALS-inhibiting herbicides in *E. crus-galli* (Fang et al. 2019a; Matzenbacher et al.

2015; Panozzo et al. 2013, 2017). In addition, the NTSR mechanism was reported in various ALS inhibitor–resistant weeds, such as *E. crus-galli*, *D. sophia*, and giant chickweed [*Myosoton aquaticum* (L.) Moench] (Bai et al. 2019; Fang et al. 2019b; Yang et al. 2016). Two homologous cytochrome P450 genes, CYP81A12 and CYP81A21, have been identified as conferring resistance to the ALS inhibitors bensulfuron-methyl and penoxsulam in rice barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.] (Iwakami et al. 2014). Therefore, the possible NTSR mechanism involved in the eight penoxsulam-resistant *E. crus-galli* populations, especially in NX6, could not be ruled out and requires further study.

Six partial sequences of *ACCase* genes were amplified and sequenced from the genomic DNA of each *E. crus-galli* population (Figure 4). All six *ACCase* genes showed highest sequence similarity to those registered in the NCBI database (accession numbers LC006070 to LC006075). However, none of the *ACCase* genes that we sequenced in the four cyhalofop-butyl–resistant populations of *E. crus-galli* encode proteins with any of the amino acid substitutions that have previously been reported to cause resistance to ACCase inhibitors. The sequencing results suggest that these populations have mechanisms of NTSR to the ACCase inhibitor cyhalofop-butyl. Our results were consistent with a previous report. Two *Echinochloa crus-galli* var. *formosensis* lines in Japan exhibited multiple resistance to cyhalofop-butyl and penoxsulam, and no amino acid substitutions conferring herbicide resistance were found in the *ACCase* and *ALS* sequences, suggesting an NTSR mechanism of herbicide resistance (Iwakami et al. 2015). It is noteworthy that two populations (NX3 and NX6) showed more than 20-fold resistance to cyhalofop-butyl. Generally, NTSR, particularly metabolic resistance, confers low to moderate levels of herbicide resistance (<10-fold) (Tranel and Wright 2002). Now, with the discovery of more NTSR cases in various weeds, it has been realized that NTSR could also endow high-level resistance compared with TSR. For example, an American sloughgrass [*Beckmannia syzigachne* (Steud.) Fernald] population with metabolic resistance was 63.5-fold resistance to fenoxaprop-pethyl (Bai et al. 2020). An NTSR mechanism in Chinese sprangletop [*Leptochloa chinensis* (L.) Nees] showed 66.7-fold resistance to cyhalofop-butyl (Deng et al. 2019a). In addition, it is worth noting that *EcACC4* has a base deletion resulting in an aberrant protein with a frameshift at Thr-1954, which is consistent with a previous report (Iwakami et al. 2015).

The copy numbers of target genes involved in TSR are negatively correlated with resistance evolution (Iwakami et al. 2015). Due to the "dilution effect," a single gene copy with a resistance-conferring mutation is diluted by more amounts of susceptible copies and will not necessarily confer strong resistance in plants with multiple copies of the target gene (Yu et al. 2013). For example, the hexaploid wild oat (*Avena fatua* L.) carrying a homozygous resistance mutation in one of the three *ACCase* gene copies shows relatively low-level (2- to 5-fold) resistance to ACCase-inhibiting herbicides compared with the resistance observed in a single-copy species (Yu et al. 2013); one polyploidy *E. crus-galli* population with a single Asp-2078-Glu mutant allele among the four *ACCase* gene copies only exhibited 1.7-, 5.7-, and 7.5-fold resistance to the ACCase inhibitors metamifop, cyhalofop-butyl, and fenoxaprop-*p*-ethyl, respectively (Fang et al. 2020). In such a case, additional mutations in other copies of the target gene and/or additive NTSR mechanisms may be required for the plant to develop resistance to herbicides (Iwakami et al. 2015). Therefore, the hexaploid *E. crus-galli* may be more likely to evolve NTSR than TSR to ACCase inhibitors, because six *ACCase* gene copies are present.
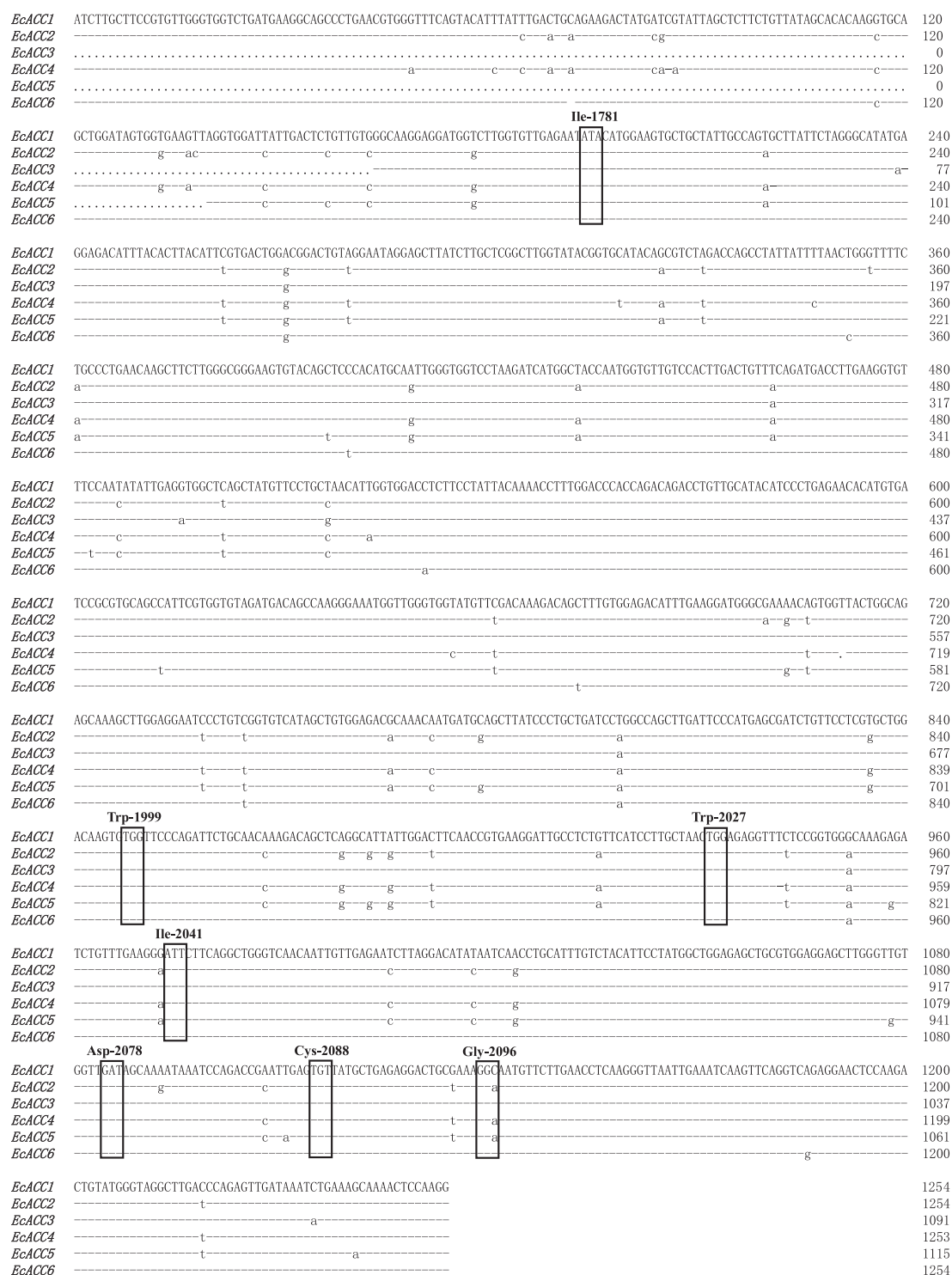
Figure 4. Partial sequences of six *ACCase* genes from *Echinochloa crus-galli*. The boxed regions represent seven amino acid positions whose substitutions are known to confer ACCase-inhibitor resistance.

This hypothesis may explain why the NX3, NX6, and NX7 populations without any *ACCase* gene mutations displayed high-level (>10-fold) resistance to cyhalofop-butyl.

Sensitivity to Other ALS and ACCase Inhibitors

Bispyribac-sodium, metamifop, and fenoxaprop-*p*-ethyl at doses of 1×, 1×, and 1/3× the field recommended application rate,

respectively, were used to examine the responses of *E. crus-galli* to other ALS- and ACCase-inhibiting herbicides. All susceptible populations (NX5, NX8, NX11, and NX12) were well suppressed by the application rates for three herbicides, with fresh weight less than 15% of that of untreated controls (Figure 5).

The single-dose assays indicated that six (NX1, NX2, NX3, NX4, NX6, and NX10) out of eight penoxsulam-resistant populations showed resistance to the ALS inhibitor bispyribac-sodium,
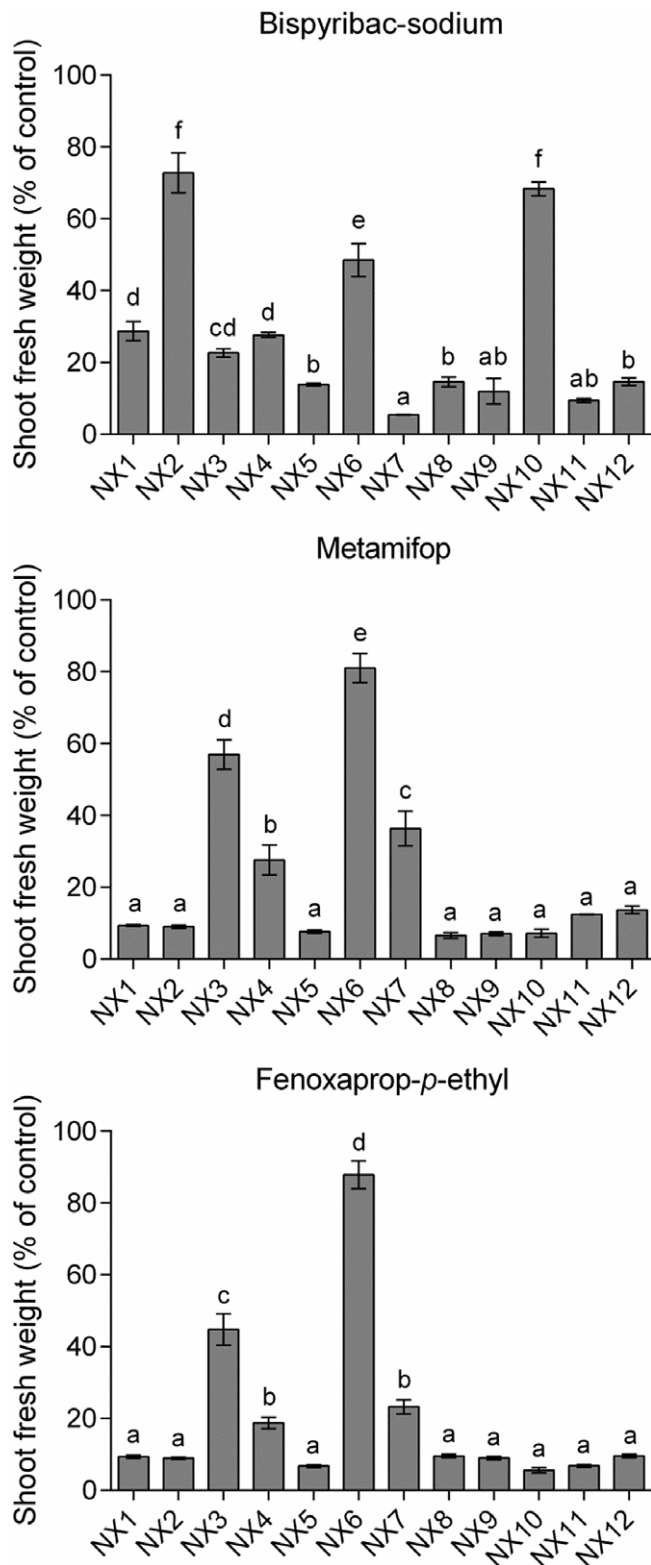
Bispyribac-sodium

Metamifop

Fenoxaprop-*p*-ethyl

Figure 5. Responses of 12 *Echinochloa crus-galli* populations to the ALS inhibitor bispyribac-sodium and ACCase inhibitors metamifop and fenoxaprop-*p*-ethyl. The herbicides were applied at 45, 120, and 20.7 g ai ha⁻¹, respectively. Shoot fresh weight is shown as percentage of untreated control. Bars indicate standard error ($n = 3$). One-way ANOVA was conducted for each treatment. Bars shown with the same letter are not significantly different ($p = 0.05$).

with 28.7%, 72.8%, 22.7%, 27.6%, 48.5%, and 68.4% shoot fresh weight of untreated control, respectively (Figure 5). Among them, the NX2 and NX10 populations with the Trp-574-Leu substitutions were the most resistant.

The four cyhalofop-butyl–resistant populations (NX3, NX4, NX6, and NX7) showed cross-resistance to the ACCase inhibitors metamifop and fenoxaprop-*p*-ethyl. Of these, NX6 showed the highest level of resistance to metamifop and fenoxaprop-*p*-ethyl, with more than 80% shoot weight compared with controls in both applications. NX3 exhibited 56.9% and 44.8% weight of controls for metamifop and fenoxaprop-*p*-ethyl treatments, respectively. In addition, NX4 and NX7 survived treatment with both herbicides, although growth was somewhat inhibited (fresh weight less than 40% of controls in both cases).

In conclusion, 4 of 12 *E. crus-galli* populations evolved multiple herbicide resistance to both penoxsulam and cyhalofop-butyl. Moreover, three alternative herbicides, bispyribac-sodium, metamifop, and fenoxaprop-*p*-ethyl, cannot effectively suppress the growth of MHR populations. Therefore, herbicides with other sites of action, rather than ALS and ACCase inhibitors, and efficient PRE herbicides should be considered as alternatives to control the *E. crus-galli* populations. In addition, in view of the rapid spread of MHR populations in *E. crus-galli* and the restricted development of new herbicides, diversified and sustainable weed management strategies, such as biological control, precision pesticide application, and automatic weeding technology should be encouraged to counteract resistance in the field.

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