JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2019, 67, 8085−8095

pubs.acs.org/JAFC

Target-Site and Metabolic Resistance Mechanisms to Penoxsulam in Barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv)

Jiapeng Fang,[†,‡] Yuhua Zhang,[†,‡] Tingting Liu,[†,‡] Bojun Yan,[†,‡] Jun Li,[†,‡] and Liyao Dong*[,†,‡]

[†]College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, People's Republic of China

[‡]State and Local Joint Engineering Research Center of Green Pesticide Invention and Application, Nanjing 210095, People's Republic of China

Supporting Information

ABSTRACT: Herbicide resistance identification is essential for effective chemical weed control. In this study, we quantified the differences in growth response between penoxsulam resistant (R) and sensitive (S) *Echinochloa crus-galli* populations, explored the changes in ALS, and performed genetic analyses to identify metabolic genes that are up-regulated by the application of penoxsulam and other common herbicides. The R population showed a 26.0-fold higher resistance to penoxsulam and varied resistance to most tested herbicides with indices ranging from 4.9 to 145.9. A Trp-574-Arg amino acid mutation in *ALS* and low penoxsulam ALS sensitivity were the main mechanisms underlying herbicide resistance. The penoxsulam resistance can be significantly reversed by two P450s inhibitors and one GST inhibitor. By RNA-Seq, thirty-six highly expressed contigs were selected, and 30 of them were up-regulated in the R population treated by penoxsulam. Many of these genes were significantly expressed when treated with pyroxsulam, metamifop, and quinclorac. These upregulated genes appear to be complementary for plant resistance to penoxsulam and other common herbicides.

KEYWORDS: *acetolactate synthase mutation, metabolic resistance, multiherbicide resistance, qRT-PCR*

1. INTRODUCTION

Weeds are a major threat in most rice planting systems, causing grain yield losses of up to 100%.[1] Barnyardgrass, or *Echinochloa crus-galli* (L.) P. Beauv, one of the most malignant weeds in rice fields, can generally be controlled only by herbicides.[2] Long-term herbicide applications, primarily of acetyl-CoA carboxylase (ACCase, EC.6.4.1.2)- and acetolactate synthase (ALS, EC 2.2.1.6)-inhibiting herbicides that act on a single target enzyme, have caused resistance to multiple-herbicides in *E. crus-galli* and other *Echinochloa* species.[3−9] ALS is the first enzyme in the biosynthesis pathway of three essential branched-chain amino acids, namely, leucine, isoleucine, and valine, and is the target of many commercial herbicides. These include sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinylthiobenzoates, and sulfonylaminocarbonyltriazolinones.[10] Penoxsulam, a typical TP ALS-inhibitor, was widely promoted in rice fields since being introduced to the herbicide market.[11] Penoxsulam has become the most important herbicide used for weed control in rice fields; however, resistance to this herbicide rapidly developed following annual application, especially in eastern China.[12]

Herbicide resistance mechanisms can be divided into target-site resistance (TSR) and nontarget-site resistance (NTSR).[13] TSR is conferred by the change of herbicide target protein genes in the nucleotide sequence and/or the expression level.[14,15] Twenty-eight types of amino acid substitutions at eight conserved positions (Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Arg_{377}, Trp_{574}, Ser_{653}, and Gly_{654}, numbered on the basis of the corresponding sequence of *Arabidopsis thaliana*) of ALS have been reported in various weed species in relation to ALS inhibitors.[16] Mutations of the ALS gene at the codon for Ala_{122}, Trp_{574}, and Ser_{653} confer resistance to ALS-herbicides in *E. crus-galli* and other *Echinochloa* species.[8,9,17,18] Recently, we also found that two mutations (Ala-205-Val and Ala-122-Gly) of *ALS* in *E. crus-galli* might be the target-site basis for penoxsulam.[19]

Compared with TSR, NTSR is less understood due to its complexity and unpredictability.[13] Reduced penetration, impaired translocation, and enhanced metabolism that reduces the dose of herbicide that binds with the target protein are the three mechanism categories for NTSR, and metabolic resistance is the most important of these.[20] Herbicide metabolism and resistance-related genes are gradually being identified and characterized and include cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), glycosyltransferases (GTs), ATP-binding cassette (ABC) transporters, oxidases, esterases, hydrolases, and peroxidases.[20] In most ALS-herbicide resistance cases, P450-mediated metabolism resistance has been identified. For example, 39 putative P450 gene fragments from bispyribac-resistant *E. phyllopogon* were isolated, and their nucleotide sequences and expression levels were compared; the amino acid polymorphisms and upregulated candidate gene expression were found.[21] Then, the overexpression of two P450s, *CYP81A12* and *CYP81A21*, were confirmed to confer resistance to bensulfuron-methyl and penoxsulam in *E. phyllopogon*.[22] Transcriptome sequencing (RNA-Seq) was performed in a quinclorac and penoxsulam-resistant *E. crus-galli* biotype, and the genes of four nontarget gene families were identified; however, the sequence and expression information were not reported.[23] Therefore, to

Received: March 13, 2019
Revised: June 19, 2019
Accepted: July 2, 2019
Published: July 2, 2019

DOI: 10.1021/acs.jafc.9b01641
J. Agric. Food Chem. 2019, 67, 8085−8095

completely understand the mechanism of action, unravelling the complete profile of metabolism resistance-related genes is required. To achieve this objective at the genetic level, transcriptome sequencing is currently the most effective tool for identifying metabolism-related genes with the gene sequences and expression profiles.[24] Reported cases using RNA-seq have primarily involved ALS-and ACCase-inhibitor herbicides, such as mesosulfuron-methyl,[25] fenoxaprop-*p*-ethyl,[26] tribenuron-methyl,[27] and pyroxsulam,[28] and other herbicides involved paraquat and glyphosate.[29−31] In these studies, many contigs were identified as major candidate genes for metabolic resistance; combining this information with a qRT-PCR validation study will improve our understanding of the metabolic resistance of herbicides.

Therefore, this study aimed to (1) determine the level of resistance to penoxsulam and other common herbicides in the R population of *E. crus-galli*, (2) explore the target-site basis of this penoxsulam resistance, (3) determine the effect of P450 and GST inhibitors on the R population resistance to penoxsulam, (4) confirm the information on RNA-seq by qRT-PCR in R and S populations, and then (5) explore whether the confirmed genes were overexpressed in the R population after other herbicides treatment. The information provided in this study could provide previously unavailable information regarding the precise genes and mechanisms of action involved in the herbicide resistance of an economically important weed, *Echinochloa crus-galli* (L.) P. Beauv.

2. MATERIALS AND METHODS

2.1. Plant Materials. AXXZ-6 (R) population seeds were collected from rice fields (30.78°N, 118.23°E) in the Anhui Province of China, where the application of penoxsulam at the recommended dose has failed to control this weed since 2012. The seeds of the JLGY-3 (S) population were collected from a leisure field (34.83°N, 119.12°E) in the Jiangsu Province of China that has never been treated with any herbicides. All seeds were collected by hand, air-dried in the shade, and stored in paper bags at 4 °C until use.

2.2. Whole-Plant Dose−Response Bioassay. *2.2.1. Penoxsulam Dose−Response Bioassay.* The whole-plant dose−response bioassay was identical to that in our previous report.[19] Twenty seeds from each of the three populations were sown in plastic pots (9 cm diameter × 10 cm height) and grown in incubators at 30 °C/25 °C (light/dark temperature) with a 12-h light/12-h dark cycle, a light intensity of 8000 lx, and 85% relative humidity. Prior to herbicide treatment, seedlings were thinned to 10 plants per pot. At the three- to four-leaf stage, herbicides were applied using a laboratory sprayer equipped with a flat-fan nozzle, delivering 280 L ha^{-1} at 230 kPa. Based on a preliminary experiment (data not shown), penoxsulam was applied at 0, 3.75, 7.5, 15, 30, and 60 g of active ingredient (a.i.) ha^{-1} to the R population and at 0, 0.94, 1.88, 3.75, 7.5, and 15 g a.i. ha^{-1} to the S population. Two weeks after penoxsulam application, the fresh aboveground biomass was determined. This experiment was conducted twice in a completely randomized design with four replications.

2.2.2. Multiple Herbicide Resistance Tests. Sensitivity to other herbicides was also determined via whole-plant bioassays, as described previously.[19] Application doses were based on the results of a preliminary experiment (data not shown), and detailed information was listed in Supplementary Table S1. The experiment was performed as described in Section 2.2.1.

2.2.3. Effect of Metabolic Inhibitors on Penoxsulam Sensitivity. This test was conducted at the same time as the penoxsulam dose−response bioassay. At the three- to four-leaf stage, two P450 inhibitors, PBO and malathion, and one GST inhibitor, NBD-Cl, were used to evaluate the effect of metabolic inhibitors. The applied doses and methods of PBO (4200 g a.i. ha^{-1}),[32] malathion (1000 g a.i. ha^{-1}),[32] and NBD-Cl (270 g a.i. ha^{-1})[33] were previously reported. Malathion and PBO were applied 1 h prior to herbicide application, and the NBD-Cl were applied 48 h prior to herbicide application. The experiment was performed as described in Section 2.2.1.

2.3. ALS Activity Assay. Analyses of the ALS enzyme response to penoxsulam were based on the methods of Yu et al.[34] with slight modifications and were the same as those in our previous report.[19] The assay was performed twice with independent extractions, each with three replications per herbicide concentration.

2.4. Gene Cloning and Sequencing. The DNA extraction, PCR procedures, and sequencing methods were the same as those described previously.[19]

2.5. RNA-Seq and Metabolic Profile Establishment. *2.5.1. Plant Treatment.* For each population, plants were cultivated to the three- to four-leaf stage under the experimental conditions described previously.[19] After penoxsulam treatment (7.5 g a.i. ha^{-1}), the aboveground tissues were harvested at 0, 6, 24, and 72 h. The plants at 0 h were used as the control plants, and leaves (0.1 g) from four or five plants per time point were pooled for each biological replicate. Leaf samples were harvested, immediately frozen in liquid nitrogen, and stored at −80 °C until use. Three replicates were collected for each time point and pooled together for the RNA extraction described in Section 2.5.2.

2.5.2. RNA Extraction, cDNA Library Preparation, Transcriptome Sequencing, and RNA Sequencing Analysis. Total RNA was extracted from each plant, including the base of the aboveground stem using RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. cDNA library preparation and transcriptome sequencing were performed as previously reported.[35,36] Clean reads were mapped to the *Echinochloa crus-galli* genome[37] (https://www.ncbi.nlm.nih.gov/bioproject/414998) using TopHat2 software,[38] and only unique mapping reads were retained for calculating gene expression. RNA-seq data analysis was performed according to previously published protocols.[39,40] Contigs were selected on the basis of statistical significance ($P < 0.05$), the magnitude of expression differences, and annotations related to known herbicide metabolism genes and signaling functions using the *Echinochloa crus-galli* genome. Differentially expressed genes were identified by the edge package (http://www. r-project.org/) with an FDR < 0.05 and an absolute log2 ratio value ≥ 1.

2.6. qRT-PCR Validation. Plants were cultivated, treated with penoxsulam, and harvested at 0, 6, 24, and 72 h under the experimental conditions described in Section 2.5.1. RNA extraction was conducted using the RNA simple Total RNA Kit (Tiangen, Beijing, China) following the manufacturer's instructions. cDNAs were synthesized by HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech Co., Ltd., Nanjing, China). The *E. crus-galli* β-actin gene (Genbank accession number: HQ395760) was used as a candidate reference gene, whose stability had been previously confirmed.[41−43] Thirty-six selected genes in the metabolizing enzyme library were used to design primers for qRT-PCR (Supplementary Table S2). All primers were assessed for a single specific PCR amplification, and no PCR amplification was detected on the negative control. qRT-PCR analyses were performed on an ABI-7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China) following the manufacturer's instructions. A dissociation curve was added to verify the primer specificity after the cycles, and the default settings were used. Gene expression level fold changes were calculated using the $2^{-\Delta\Delta CT}$ method.[44] Each experiment included three biological replicates and was repeated at least twice. Significant differences in the expression levels were analyzed using Welch's *t* test.[45] Two threshold values, a *t* test ($P < 0.05$), and a 2-fold change were used to determine up- or down-regulation.

2.7. Exploring Confirmed Genes Expression Patterns under Other Herbicide Treatments. Plants were cultivated, treated with three herbicides (pyroxsulam at 3.5 g a.i. ha^{-1}, metamifop at 30 g a.i. ha^{-1}, and quinclorac at 187.5 g a.i. ha^{-1}, respectively), and harvested at 0, 6, 24, and 72 h under the experimental conditions described in Section 2.5.1. According to the result of Section 2.6, 30 highly expressed contigs were used to explore the expression patterns of metabolic genes

DOI: 10.1021/acs.jafc.9b01641
J. Agric. Food Chem. 2019, 67, 8085−8095

under these herbicide treatments by qRT-PCR as described in Section 2.6.

2.8. Data Analysis. Whole-plant dose−response data were subjected to an analysis of variance (ANOVA) using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). The ANOVA results showed no significant differences between assay repetitions; thus, the repeated assay results were averaged. Data were then pooled and fitted to the four-parameter nonlinear logistic-regression model presented below that was calculated using SigmaPlot 10.0 (SigmaPlot Software Inc., Chicago, IL, USA) to determine the effective herbicide dose that caused 50% fresh weight inhibition (ED_{50}):

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

where Y denoted fresh weight, expressed as a percentage of the nontreated control at dose x of the herbicide; b was the slope; c was the lower limit; d was the upper limit; and g was the herbicide dose at the point of inflection, the halfway point between the upper and lower limits.[46]

The same analysis was used to calculate the herbicide concentrations required to inhibit 50% of ALS activity (IC_{50}) in enzymatic assays. Resistance indexes (RIs) were calculated by dividing the ED_{50} (or IC_{50}) of the R population by the ED_{50} (or IC_{50}) of the S population.

3. RESULTS

3.1. Whole-Plant Dose−Response. *3.1.1. Sensitivity to Penoxsulam.* The ED_{50} of the R population (50.96 g ha^{-1}) was considerably higher than the recommended application dose (15−30 g ha^{-1}), whereas that of the S population (1.96 g ha^{-1}) was lower than the recommended dose (Table 1 and Figure 1). The RI of the R population was 26.0, indicating a high resistance to penoxsulam, according to Beckie and Tardif.[47]

Table 1. Sensitivities of Penoxsulam Resistant and Sensitive Populations with/without Three Metabolic Inhibitors[a]

	ED_{50}[b] ± SE of tested populations (g a.i. ha^{-1})		
treatment	AXXZ-6	JLGY-3	resistance indexes
penoxsulam	50.96 ± 6.08 a	1.96 ± 0.57 c	26.0
PBO[c]+ penoxsulam	14.28 ± 3.14 b	2.00 ± 0.26 c	7.1
malathion + penoxsulam	12.23 ± 3.69 b	2.12 ± 0.14 c	5.8
NBD-Cl[d]+ penoxsulam	20.36 ± 3.35 b	2.21 ± 0.75 c	9.2

[a]The letters a, b, and c indicate ED_{50} with different letters that are significantly different at the $P = 0.05$ significance level. [b]ED_{50} refers to the effective dose of herbicide causing 50% inhibition of fresh weight and is indicated as grams of active ingredient per hectare (g a.i. ha^{-1}). Data were the means of two experiments. [c]PBO: piperomyl butoxide. [d]NBD-Cl: 4-chloro-7-nitro-2,1,3-benzoxadiazole.

3.1.2. Sensitivity to Other Herbicides. The R population also showed resistance to other ALS inhibitors, ACCase inhibitors, and synthetic auxins (Table 2). For ALS inhibitors, the R population was sensitive to imazapic (RI = 1.7) and showed moderate resistance to rest of the ALS inhibitors, with RIs varying from 6.4 to 9.1. For ACCase inhibitors, the R population showed low resistance to metamifop (RI = 4.9) and high resistance to cyhalop-butyl (RI = 15.2) and pinoxaden (RI = 18.3). For the two synthetic auxin herbicides, the R population conferred very high resistance to quinclorac (RI = 145.9) and moderate resistance to florpyrauxifen-benzyl (RI = 7.8). Notably, the plants of R population were strongly inhibited under the recommended dose of several herbicides (though the RIs values were all over 6.4), like propyrisulfuron, pinoxaden, and florpyrauxifen-benzyl, while the plants of S population could not survive.

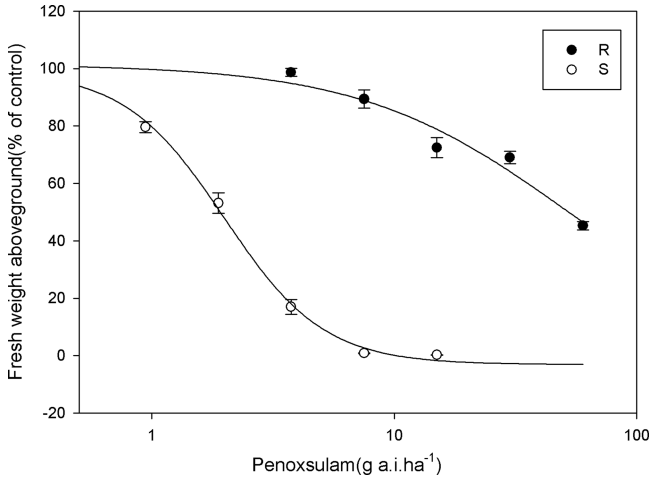

Figure 1. Fresh weight of the aboveground parts of *Echinochloa crus-galli* treated with penoxsulam. Vertical bars represent the mean ± standard error. R: AXXZ-6 population; S: JLGY-3 population (the following population names in Figures 2−5 are consistent with this).

Table 2. Sensitivity of the Two *Echinochloa crus-galli* Populations to Other Herbicides

herbicide	population[a]	ED_{50} (SE)[b]	RI[c]
pyribenzoxim	R	87.39(19.92)	9.1
	S	9.56(1.64)	
imazapic	R	13.15(2.79)	1.7
	S	7.58(1.18)	
flucarbazone-sodium	R	61.20(14.44)	9.0
	S	6.80(3.24)	
pyroxsulam	R	18.57(1.94)	7.1
	S	2.62(0.22)	
flucetosulfuron	R	23.55(2.21)	8.7
	S	2.71(0.63)	
propyrisulfuron	R	35.31(5.00)	6.4
	S	5.49(1.36)	
cyhalofop-butyl	R	156.45(14.45)	15.2
	S	10.30(0.89)	
metamifop	R	107.93(17.05)	4.9
	S	22.04(2.65)	
pinoxaden	R	26.75(3.88)	18.3
	S	1.46(0.52)	
quinclorac	R	3008(1356.52)	145.9
	S	20.62(6.02)	
florpyrauxifen-benzyl	R	10.24(2.33)	7.8
	S	1.32(0.25)	

[a]R: AXXZ-6 population; S: JLGY-3 population. [b]ED_{50} refers to the effective dose of herbicide causing 50% inhibition of fresh weight and is indicated as grams of active ingredient per hectare (g a.i. ha^{-1}). Data were the means of two experiments. SE: standard error. [c]RI is the resistance index. Herbicide resistance was classified into five groups: no resistance (RI < 2); low resistance (RI = 2−5); moderate resistance (RI = 6−10); high resistance (RI = 11−100); and very high resistance (RI > 100).

3.1.3. Sensitivity Change to Penoxsulam with Three Metabolic Inhibitors. When three metabolic inhibitors (PBO, malathion, and NBD-Cl) were applied before penoxsualm treatment, the ED_{50} of the R population significantly decreased by 72%, 78%, and 60%, respectively, compared to that of the

DOI: 10.1021/acs.jafc.9b01641
J. Agric. Food Chem. 2019, 67, 8085−8095

penoxsulam only treatment, while the ED_{50} of the S population rarely changed (Table 1). This indicated that P450 and GST metabolism might contribute to penoxsulam resistance.

3.2. Target-Site Basis of Penoxsulam Resistance. *3.2.1. Low ALS Sensitivity to Penoxsulam in Vitro.* The inhibitory effect of penoxsulam on ALS activity was lower in the R population than in the S population (Figure 2). After the

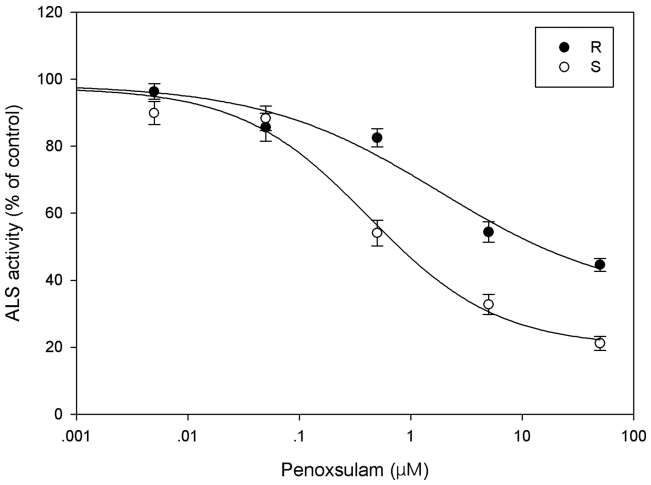

Figure 2. In vitro acetolactate synthase activity of three *Echinochloa crus-galli* populations when treated with penoxsulam. Vertical bars represent the mean ± standard error.

calculation, the R IC_{50} value was 12.31 μM, which was 8.4-fold that of the S population (1.46 μM), suggesting that low ALS sensitivity confers resistance to penoxsulam in *E. crus-galli* populations.

3.2.2. An ALS Trp-574-Arg Mutation. The R population possessed three copies of *ALS* sequences that were submitted to the NCBI database (GenBank accession numbers MH013489, MH013490, and MH013491 for *ALS1;3*, *ALS2;3*, and *ALS3;3*, respectively). After DNA and predicted amino acid analyses, a nucleotide mutation (TGG to CGG) was detected in the *ALS3;3* sequence of the R population, resulting in the substitution of Trp to Arg at position 574 (position is numbered relative to *A. thaliana ALS*). None of other mutations known to confer resistance to ALS inhibitors were detected in the current study.

3.3. Metabolic Resistance Mechanisms. *3.3.1. RNA-Seq Data.* cDNA samples were sequenced using the Illumina sequencing platform, and each one generated more than 6.7 G of clean data (Supplementary Table S3). After further filtering, each one produced more than 6.5 G of high quality clean data. The Q20 percentages all exceeded 98.85, and the Q30 percentages all exceeded 96.44%; the GC content of each sample varied from 54.15% to 57.53% (Supplementary Table S3). The number of unigene clean reads differed for each population (47792984−74113826), but the ratio (98.7%) of clean reads was consistent (Supplementary Table S4), indicating highly accurate sequencing. The read alignment tool bowtie2 (2.2.8) was used to compare high quality clean reads to the ribosome of the species. After removing the aligned rRNA reads, the retained data was used for subsequent analysis.

3.3.2. Penoxsulam-Resistance Metabolic Profile. A total of 108771 genes were annotated in the *Echinochloa crus-galli* genome, and 63643 (approximately 58%) were known genes. After the expression level comparison, the *E. crus-galli* genome was used to sequence the annotation. Approximately 850 metabolizing enzyme genes distributed in eight families[20,26] were acquired by transcriptome sequencing, and approximately 700 of these genes were significantly differentially expressed (Supplementary Table S5). The details of these genes were supplied as a supplementary excel file named the Penoxsulam Metabolic Profile.

3.3.3. Candidate Metabolic Resistance Contigs Selection and Validation. Given the important roles of metabolic enzymes in herbicide metabolism and resistance, the contigs that were up-regulated in at least one time point in the R samples and annotated as metabolic enzymes were selected as candidate metabolic resistance contigs. A total of 36 contigs were selected as candidate genes that could potentially confer penoxsulam resistance. Of these, 14 contigs were annotated to P450 families, 2 to esterase families, 6 to peroxidase families, 4 to oxidase families, 2 to hydrolase families, 3 to GST families, 2 to GT families, and 3 to ABC transporter families (Supplementary Table S6). The results (Table 3) showed that 30 candidate contigs exhibited significantly higher expression levels in the R samples compared to those of the S samples.

3.3.4. Confirmed Genes Expression Patterns under Other Herbicide Treatments. Among the 30 confirmed up-regulated genes, 18 were up-regulated in the R population after herbicide(s) treatment (Figures 3−5). In the R the population, the expression levels of remaining 12 genes were not significant higher than that in the S population (Supplementary Figure S1). Under the pyroxsulam treatment, 14 genes were overexpressed (4 P450s, 1 esterase, 5 peroxidases, 1 hydrolase, 2 GSTs, and 1 ABC transporter). Under the metamifop treatment, 11 genes were up-regulated (4 P450s, 1 esterase, 1 oxidase, 1 peroxidase, 1 hydrolase, 1 GST, and 2 ABC transporters). Under the quinclorac treatment, 5 genes were up-regulated (2 P450s, 1 esterase, 1 oxidase, and 1 hydrolase). Of note, two P450s, EC_v6. g088422 (Figure 3A) and EC_v6. g045480 (Figure 3C), 1 esterase, EC_v6. g099076 (Figure 3F), and 1 hydrolase EC_v6. g096321 (Figure 5B), were overexpressed in all three herbicide treatments.

4. DISCUSSION

In this study, the R population showed high penoxsulam resistance, and this resistance can be significantly reversed by three metabolic inhibitors (Table 1). Meanwhile, the R population also displayed different levels of resistance to other common herbicides (Table 2). Potential resistance mechanisms were explored in this multiherbicide resistant population.

4.1. TSR Mechanisms and Cross-Resistance. The TSR mechanism of penoxsulam resistance is well understood, since the target-site change is easy to detect. ALS amino acid substitutions and the lower sensitivity of ALS in vitro have been reported multiple times in relation to penoxsulam resistance. In the current study, the ALS sensitivity in vivo decreased 8.4-fold in the R population, and one relatively rare mutation (Trp-574-Arg) was found in the *ALS* gene of the R population. Trp-574-Arg was first reported in a kochia (*Kochia scoparia*) population that was resistant to two SU herbicides (thifensulfuron and tribenuron).[48] Recently, this mutation was also detected in crabgrass (*Digitaria sanguinalis*) and conferred broad resistance to ALS-inhibiting herbicides, such as nicosulfuron, flumetsulam, and imazethapyr (SU, TP, and IMI herbicides, respectively).[49] Characterization of the resistance to other ALS-inhibiting herbicides provided unique results and demonstrated that the R population also showed resistance to pyroxsulam (a TP),

DOI: 10.1021/acs.jafc.9b01641
J. Agric. Food Chem. 2019, 67, 8085−8095

Table 3. Identification of the Up-Regulated Genes Annotated to Metabolism in *Echinochloa crus-galli* Penoxsulam Resistance via RNA-Seq and qRT-PCR

			relative expression change (R/S)[a]					
			RNA-seq			qRT-PCR		
definition	gene ID	function annotation	6 h	24 h	72 h	6 h	24 h	72 h
cytochrome P450s	EC_v6.g024971	CYP74A2	0.84	1.98	2.42	9.07[b]	3.26	4.52
	EC_v6. g021245	CYP93A1	2.98	1.35	6.68	4.02[b]	1.38	1.26
	EC_v6.g088422	CYP74B2	1.21	1.20	2.08	3.45[b]	1.29	2.33[b]
	EC_v6. g024973	CYP74A2	0.54	1.37	3.22	4.81[b]	3.35	2.52
	EC_v6. g045480	CYP78A9	1.31	1.30	1.00	7.20[b]	4.35	13.8[b]
	EC_v6.g073605	CYP90A1	1.18	1.16	1.23	2.30[b]	2.52	2.82[b]
	EC_v6.g082320	CYP734A1	2.09	3.64	11.32	5.93[b]	5.15[b]	5.24[b]
	EC_v6.g091909	CYP93A1	2.83	0.95	4.52	4.88[b]	4.30[b]	5.87
	EC_v6.g105576	CYP98A1	9.66	1.25	11.72	4.06[b]	2.14[b]	11.0[b]
	EC_v6.g041677	CYP714B1	0.86	2.05	1.05	1.82	2.34[b]	2.16
	EC_v6.g010864	CYP72A15	1.01	1.64	0.28	2.70	4.93[b]	1.31
	EC_v6.g096099	CYP81E1	3.80	5.09	4.03	1.32	3.35	5.51[b]
esterase	EC_v6.g099076	palmitoyl-protein thioesterase 1	0.91	1.18	1.23	0.89	3.74[b]	2.60[b]
oxidases	EC_v6.g024163	1-aminocyclopropane-1-carboxylate oxidase	1.39	1.69	3.28	2.24[b]	2.25[b]	2.15[b]
	EC_v6.g073396	oxygen-dependent coproporphyrinogen-III	1.28	0.83	2.55	3.76[b]	2.12	2.44
	EC_v6.g100130	polyamine oxidase-like	4.82	6.08	2.37	4.24[b]	104[b]	8.71[b]
peroxidases	EC_v6.g098075	probable L-ascorbate peroxidase 6	2.93	2.89	2.35	8.00[b]	4.79	4.59[b]
	EC_v6.g107834	peroxidase A2-like	5.01	9.49	3.35	14.7[b]	6.07[b]	12.0[b]
	EC_v6.g107836	peroxidase 54 precursor	15.50	5.22	8.08	93.9[b]	25.93[b]	6.21[b]
	EC_v6.g021711	peroxidase 11	1.94	0.33	1.23	5.26[b]	44.5[b]	39.1[b]
	EC_v6.g065624	peroxidase A2-like	0.72	0.94	1.75	1.32	1.50	3.53[b]
	EC_v6.g002096	peroxidase 2-like	0.82	2.17	1.20	2.67[b]	5.77	4.72[b]
hydrolases	EC_v6.g096321	hydroxyacylglutathione hydrolase	0.59	2.19	9.19	2.82[b]	1.24	3.26[b]
	EC_v6.g007915	ubiquitin carboxyl-terminal hydrolase	0.57	1.56	1.09	4.64[b]	1.94	1.70
glutathione S-transferase	EC_v6.g020710	glutathione S-transferase 2	1.22	1.94	0.42	2.56	1.45	3.57[b]
	EC_v6.g027657	glutathione S-transferase T1	1.64	1.54	1.42	18.1[b]	2.93	2.15
	EC_v6.g052496	glutathione S-transferase F11-like	1.99	1.21	1.72	2.04[b]	4.32[b]	5.44[b]
ABC transporter	EC_v6.g050762	ABC transporter G family member 11-like	5.23	19.95	0.77	2.72	1.14[b]10[3b]	155[b]
	EC_v6.g018324	ABC transporter I family member 11	0.55	0.37	1.79	0.77	1.64	4.85[b]
	EC_v6.g030014	ABC transporter C family member 1	0.76	0.92	1.76	0.73	1.57	2.54[b]

[a]Relative expression change was calculated using the $2^{-\Delta\Delta CT}$ method. R/S values were calculated by dividing the expression level of the R population by that of the S population at a relative time point. [b]Indicated that the genes expression level of the R population was significantly higher than that of the S population (P-value < 0.05, SPSS analysis).

pyribenzoxim (a pyrimidinylthiobenzoate), flucarbazone-sodium (a sulfonylaminocarbonyltriazolinone), flucetosulfuron, and propyrisulfuron (two SUs). It was expected that the R population would show resistance to another TP herbicide pyroxsulam, since the two herbicides belong to the same chemical group and have a similar structure. Interestingly, the R population was still sensitive to imazapic (an IMI), possibly because this mutation does not inherently confer resistance to imazapic herbicides in *E. crus-galli*, which was different from previously reported findings.[49] This also indicated that cross-resistance cannot be judged by the response to one herbicide with a particular chemical structure. Additionally, the IMI herbicides demonstrated high effectiveness and have not yet been applied in Chinese rice fields. This indicates that the *E. crus-galli* samples had never been exposed to IMI herbicides and could be controlled by IMIs. As in our previous study, the two mutated AXXZ-2 (Ala-205-Val) and JNRG-2 (Ala-122-Gly) populations were also relatively sensitive to imazapic (RIs = 2.68 and 0.32, respectively).[19] The ED_{50} values to imizapic in these three populations were far lower than the recommended rate (108−144 g a.i. ha^{-1}). These findings indicate that these three mutations might not confer resistance to this IMI herbicide.

4.2. Multi-Herbicides Resistance. The R population also showed resistance to three ACCase-inhibiting herbicides and two synthetic auxin herbicides (Table 2). Among them, metamifop, cyhalop-butyl, and quinclorac have been widely used to control barnaryardgrass, while pinoxaden has only been used in wheat fields;[50] florpyrauxifen-benzyl is a new herbicide promoted for use in rice fields in 2018 in China. Thus, it is predicted that the R population may develop some mechanisms conferring herbicide resistance with different modes of action, especially on the induction of herbicides similar to those that have already been applied to this population. These mechanisms are often related to herbicide metabolism.[47,51] At present, metabolic resistance mediated by P450s have been well elucidated, and some genes have been clearly shown to confer metabolic resistance to some ALS and ACCase-inhibiting herbicides.[21,22,52,53] The sensitivity changes to penoxsulam in the R population with three metabolic inhibitors indicate that metabolism may be related to herbicide resistance.

DOI: 10.1021/acs.jafc.9b01641
J. Agric. Food Chem. 2019, 67, 8085−8095

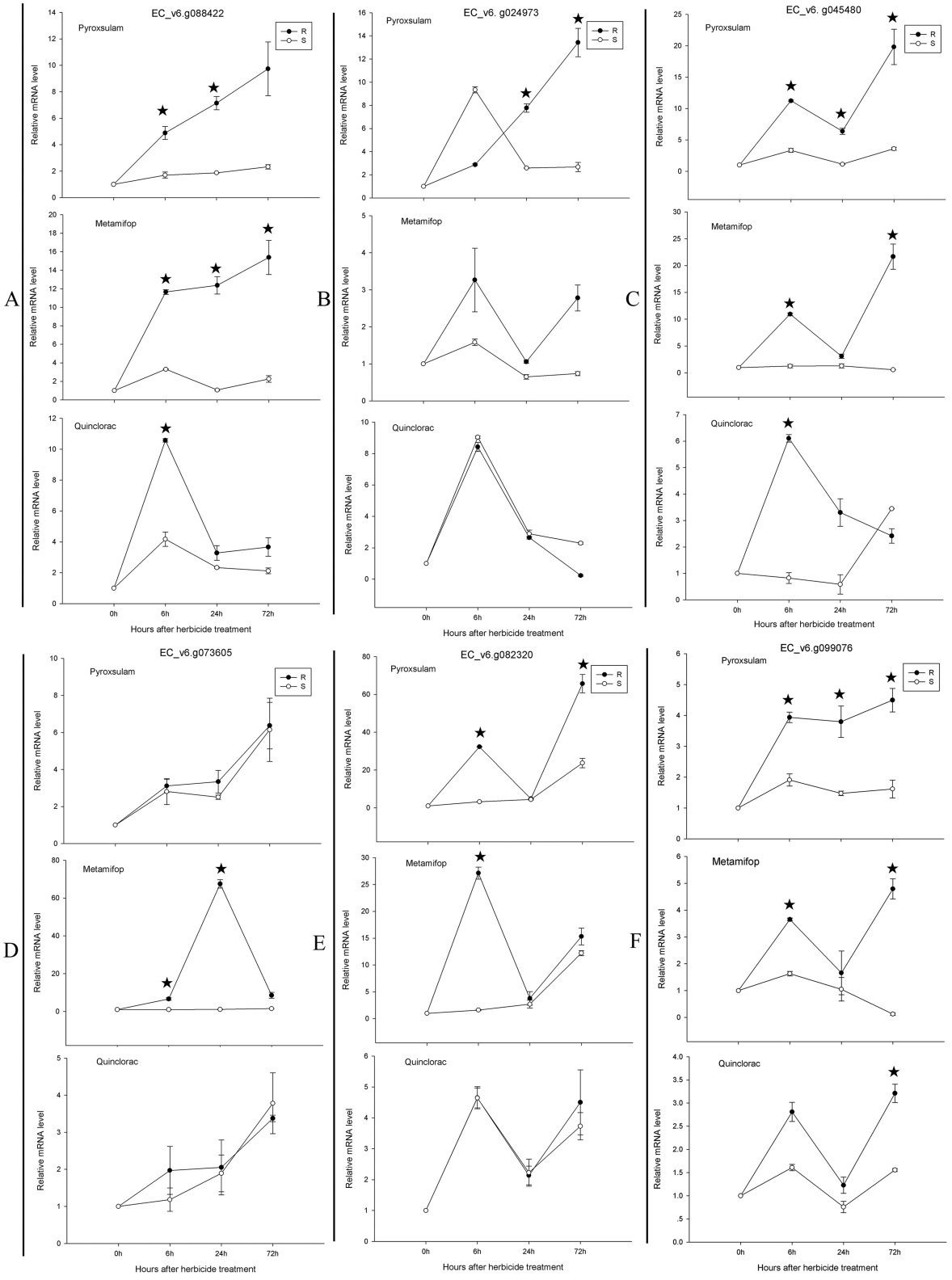

Figure 3. qRT-PCR validations of six genes annotated to cyt P450s and esterases families treated with three herbicides: (A) EC_v6.g088422, (B) EC_v6. g024973, (C) EC_v6. g045480, (D) EC_v6.g073605, (E) EC_v6.g082320, and (F) EC_v6.g099076. *Echinochloa crus-galli* β-actin was used as internal control genes; means and standard errors from three biological replicates are shown. An star symbol ★ indicated that the gene expression level of the R population was significantly higher than that of the S population; *P*-value < 0.05, SPSS analysis (this also applies to Figures 4, 5 and S1).

4.3. Potential Metabolic Resistance Mechanisms. RNA-seq was conducted to identify metabolic resistance-related genes and for a better understanding of the evolution of metabolic resistance in this weed species. The 30 genes identified in this study can be candidate genes that involve metabolic resistance driven by the expression patterns differences under the penoxsulam treatment. Previous studies have suggested that the P450 genes could play a key role (e.g.,

DOI: 10.1021/acs.jafc.9b01641
J. Agric. Food Chem. 2019, 67, 8085−8095

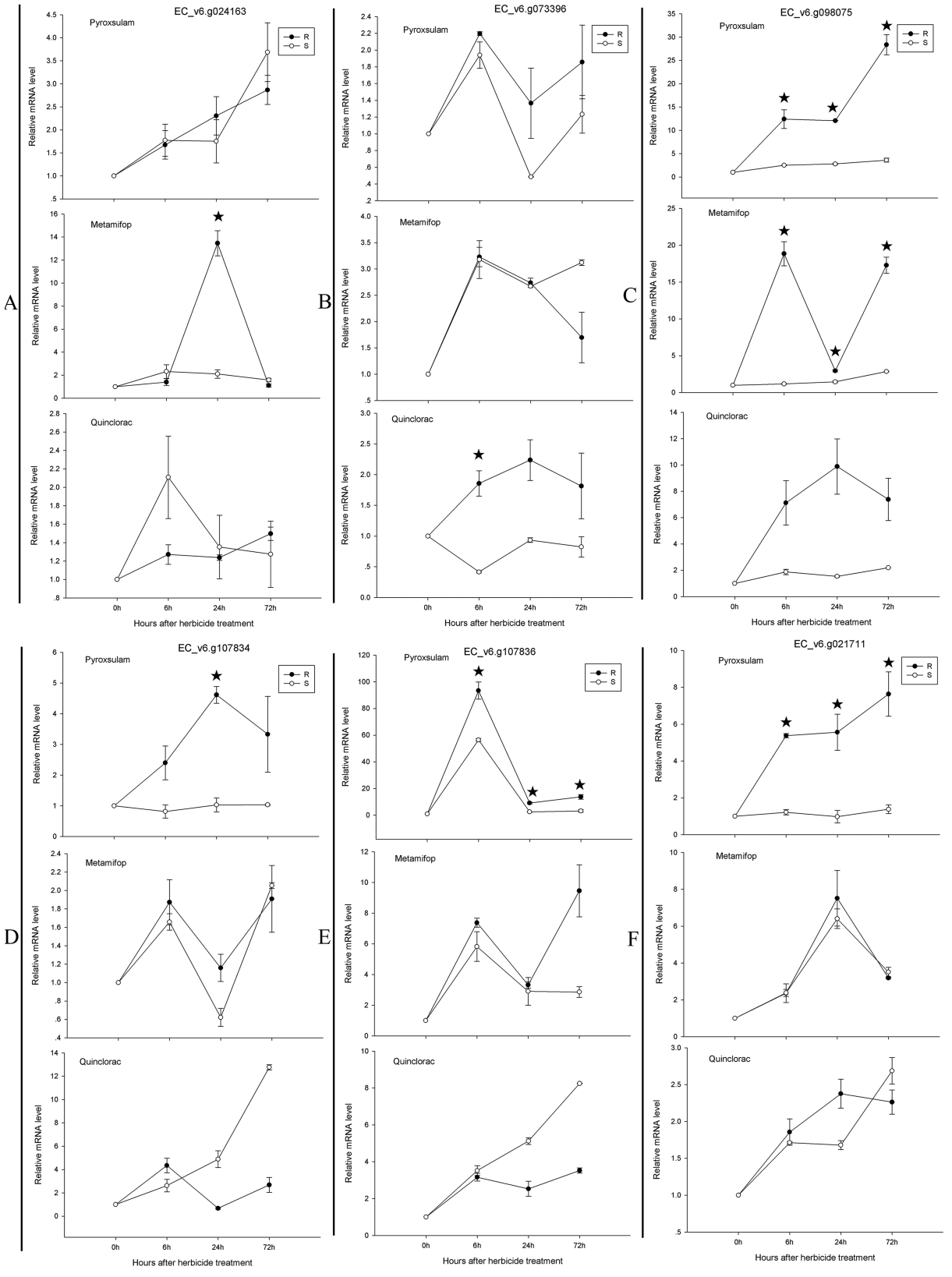

Figure 4. qRT-PCR validations of six genes annotated to oxidases and peroxidases families treated with three herbicides: (A) EC_v6.g024163, (B) EC_v6.g073396, (C) EC_v6.g098075, (D) EC_v6.g107834, (E) EC_v6.g107836, and (F) EC_v6.g021711. See Figure 3caption for additional information.

cleaving and oxidation) in phase I during the herbicide metabolism,[21−23,53] and 12 P450 contigs were validated among the seven families in this study. At present, CYP72A and CYP81A subfamilies have been confirmed to involve the

DOI: 10.1021/acs.jafc.9b01641
J. Agric. Food Chem. 2019, 67, 8085−8095

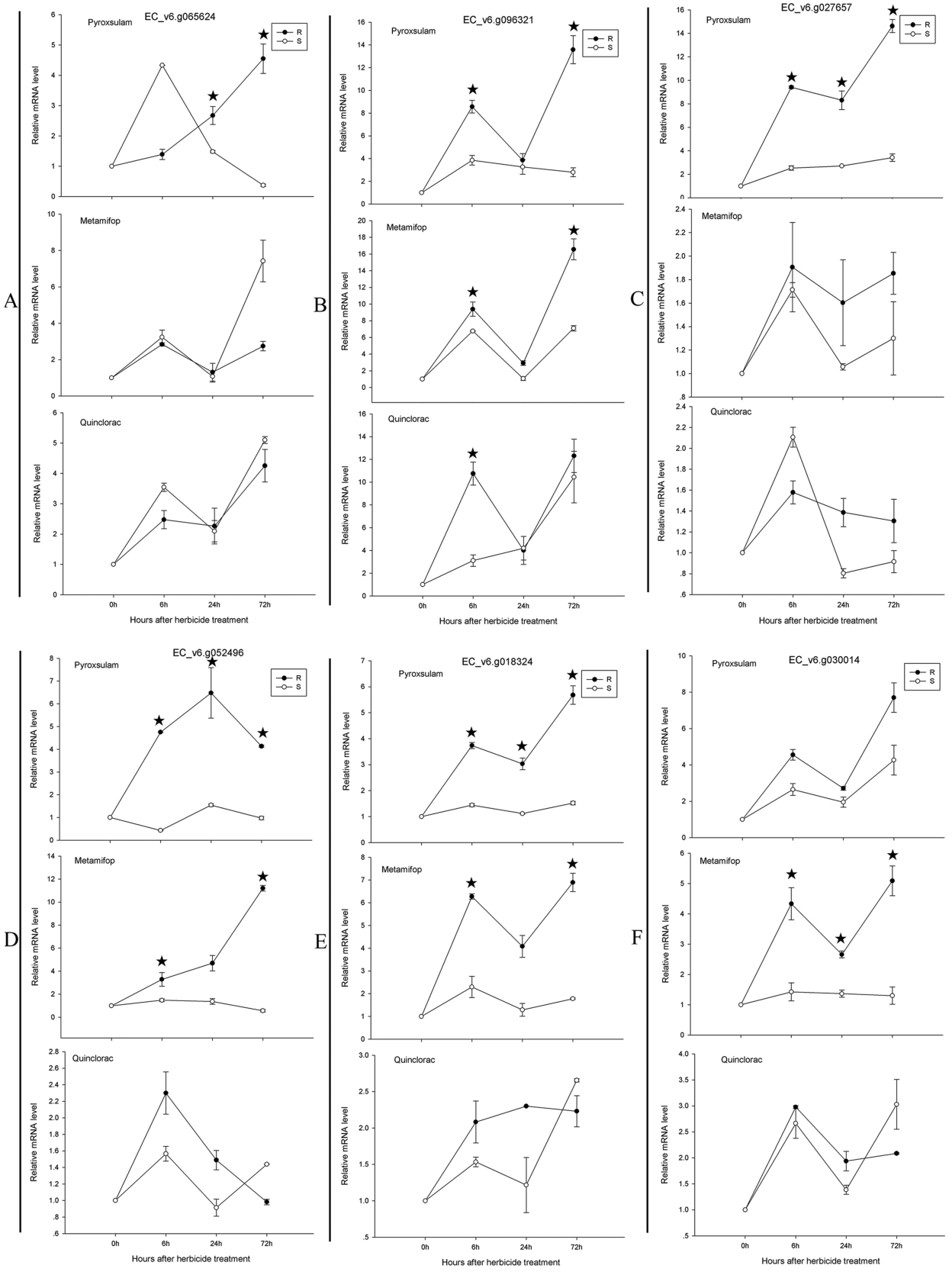

Figure 5. qRT-PCR validations of six genes annotated to hydrolases, glutathione S-transferases, and ATP-binding cassette transporters families treated with three herbicides: (A) EC_v6.g065624, (B) EC_v6.g096321, (C) EC_v6.g027657, (D) EC_v6.g052496, (E) EC_v6.g018324, and (F) EC_v6.g030014. See Figure 3caption for additional information.

resistance to ALS and ACCase herbicides resistance.[22,53,54] Similarly, a contig (EC_v6.g010864) from the CYP72A subfamily was identified in this research (Table 3) and might confer resistance to penoxsulam. In addition to the P450 genes,

DOI: 10.1021/acs.jafc.9b01641
J. Agric. Food Chem. 2019, 67, 8085−8095

six peroxidase genes, three oxidase genes, two hydrolase genes, and one esterase were identified under the penoxsulam treatment. In this phase, herbicides were modified to more hydrophilic metabolites; meanwhile, the oxidases and peroxidases may protect plants against oxidative stress.[20] In phase II, these molecules were conjugated to glutathione or sugar acceptors related to GSTs and GTs, respectively.[20] In the current study, three GST genes were overexpressed in the R population, while the two selected GTs were not confirmed by qRT-PCR. The four genes annotated to GTs occurred minimally among the eight families. The expression levels of the two selected GTs were relatively close as shown by RNA-seq (Supplementary Table S6), and therefore their expression levels did not differ significantly by qRT-PCR. These three GST genes may have played a more important role than those of GT genes in phase II during penoxsulam metabolism. After conjugation, metabolite(s) are exported from cytosol by ABC transporters in phase III.[20] Three ABC transporters were highly expressed in the R population and could play an important role in the transport of penoxsulam metabolites. Due to the complexity, the herbicides metabolism information was little known. Changes in the expression of these candidates could provide preliminary evidence to the understand of penoxsulam metabolic resistance. Functional characterization of these candidates needs to be further clarified.

Additionally, the expression pattern of these 30 genes under pyroxsulam, metamifop, and quinclorac treatments were documented by qRT-PCR. Metamifop and quinclorac are widely applied herbicides that initially control *E. crus-galli* and other *E.* species efficiently.[55] Pyroxsulam is a TP herbicide similar to penoxsulam in chemical structure that is widely used in wheat fields to control many weed species.[56] In the present study, the R population was found to be resistant to pyroxsulam, metamifop, and quinclorac (Table 2). It was interesting that the R population showed a 7.1-fold resistance to pyroxsulam, even though this herbicide had never been applied to that population. This might be caused by NTSR mechanisms, especially for metabolic resistance, as pyroxsulam is chemically similar to penoxsulam. Thus, the 30 confirmed genes in response to penoxsulam were selected as candidates to explore whether these genes were overexpressed under the pyroxsulam, metamifop, and quinclorac treatments. The results showed that a number of these 30 genes were still up-regulated in the R population in response to three herbicides and that these genes may be the candidates conferring potential resistance to multiple herbicides. Unsurprisingly, the highest number of genes (14) were detected under pyroxsulam treatment, considering that the chemical structures of penoxsulam and pyroxsulam are the most similar. The corresponding numbers for metamifop and quinclorac (11 and 5, respectively) were lower, since their chemical structures differ from penoxsulam. These overexpressed genes might take part in the metabolism of chemically similar herbicides. This is especially true for the two P450s, EC_v6. g088422 and EC_v6. g045480, one esterase, EC_v6. g098075, and one hydrolase, EC_v6. g096321, as these genes were overexpressed in pyroxsulam, metamifop, and quinclorac treatments, as well as in the penoxsulam treatment. It is possible that these four genes contribute to a common progressive herbicide metabolism and that their overexpression conferred resistance to the tested herbicides. To the best of knowledge, the present study is the first to identify metabolic genes that response to herbicides with three different modes of action. It is possible that this would eventually result in the invalidation of certain herbicides due to NTSR mechanisms, even in newly developed herbicides used for weed control. Using transcriptome-sequencing to identify NTSR-related genes provided necessary information about the molecular basis of herbicide resistance in *E. crus-galli.* This study might greatly improve our understanding of herbicide metabolism resistance mechanisms.

In conclusion, TSR and NTSR mechanisms coexist and cocontribute to herbicide resistance in *E. crus-galli*. The overexpressed metabolism-related genes identified in this study may confer resistance to multiple herbicides in the *E. crus-galli* population, and this hypothesis requires further gene function validation in a model plant species.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b01641.

Herbicide doses applied in dose−response tests, primer sequences used for the qRT-PCR, statistics information of RNA-seq and expression pattern of partial metabolic-related genes, and description of Figure S1 (PDF)

(Figure S1) 12 genes expression level that did not show a significant difference between the two populations treated with three herbicides in qRT-PCR validation (PDF)

Spreadsheet containing additional experimental details (XLSX)

Accession Codes

The raw Illumina sequence reads have been deposited in the NCBI Sequence Read Archive database, accession number SRP186893.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: dly@njau.edu. Tel.: (+86) 025-84395672. Fax: (+86) 025-84395672.

ORCID

Liyao Dong: 0000-0002-4842-713X

Funding

This research was supported by the National Natural Science Foundation of China (31871993).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Editage for their professional English language editing services.

■ REFERENCES

(1) Singh, M.; Bhullar, M. S.; Chauhan, B. S. Seed bank dynamics and emergence pattern of weeds as affected by tillage systems in dry direct-seeded rice. *Crop Prot.* **2015**, *67*, 168−177.

(2) Khedr, A.-h. A.; Serag, M. S.; Shaaban, H. E.; Abogadallah, G. M. Different Responses of *Echinochloa crus-galli* and *Echinochloa colona* to bisbyric-sodium (Nominee(tm)). *Egyptian Journal of Botany* **2018**, *58*, 109−118.

(3) Yasuor, H.; Milan, M.; Eckert, J. W.; Fischer, A. J. Quinclorac resistance: a concerted hormonal and enzymatic effort in *Echinochloa phyllopogon*. *Pest Manage. Sci.* **2012**, *68*, 108−115.

(4) Morran, S.; Moretti, M. L.; Brunharo, C. A.; Fischer, A. J.; Hanson, B. D. Multiple target site resistance to glyphosate in junglerice (*Echinochloa colona*) lines from California orchards. *Pest Manage. Sci.* **2018**, *74*, 2747−2753.

(5) Kaloumenos, N. S.; Chatzilazaridou, S. L.; Mylona, P. V.; Polidoros, A. N.; Eleftherohorinos, I. G. Target-site mutation associated with cross-resistance to ALS-inhibiting herbicides in late watergrass (*Echinochloa oryzicola* Vasing.). *Pest Manage. Sci.* **2013**, *69*, 865−873.
(6) Iwakami, S.; Uchino, A.; Watanabe, H.; Yamasue, Y.; Inamura, T. Isolation and expression of genes for acetolactate synthase and acetyl-CoA carboxylase in *Echinochloa phyllopogon*, a polyploid weed species. *Pest Manage. Sci.* **2012**, *68*, 1098−1106.
(7) Ruiz-Santaella, J. P.; De Prado, R.; Wagner, J.; Fischer, A. J.; Gerhards, R. Resistance mechanisms to cyhalofop-butyl in a biotype of *Echinochloa phyllopogon* (Stapf) Koss. from California. *Journal of Plant Diseases and Protection* **2006**, 95−100.
(8) Matzenbacher, F. O.; Bortoly, E. D.; Kalsing, A.; Merotto, A. Distribution and analysis of the mechanisms of resistance of barnyardgrass (*Echinochloa crus-galli*) to imidazolinone and quinclorac herbicides. *J. Agric. Sci.* **2015**, *153*, 1044−1058.
(9) Riar, D. S.; Norsworthy, J. K.; Srivastava, V.; Nandula, V.; Bond, J. A.; Scott, R. C. Physiological and molecular basis of acetolactate synthase-inhibiting herbicide resistance in barnyardgrass (*Echinochloa crus-galli*). *J. Agric. Food Chem.* **2013**, *61*, 278−289.
(10) Duggleby, R. G.; McCourt, J. A.; Guddat, L. W. Structure and mechanism of inhibition of plant acetohydroxyacid synthase. *Plant Physiol. Biochem.* **2008**, *46*, 309−324.
(11) Larelle, D.; Mann, R.; Cavanna, S.; Bernes, R.; Duriatti, A.; Mavrotas, C.; BCPC. Penoxsulam, a new broad spectrum rice herbicide for weed control in European Union paddies. *BCPC International Congress Crop Science and Technology*; The BCPC International Congress: 2003; pp 75−80.
(12) Chen, G. Q.; Wang, Q.; Yao, Z. W.; Zhu, L. F.; Dong, L. Y. Penoxsulam-resistant barnyardgrass (*Echinochloa crus-galli*) in rice fields in China. *Weed Biol. Manage.* **2016**, *16*, 16−23.
(13) Yu, Q.; Powles, S. Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. *Plant Physiol.* **2014**, *166*, 1106−1118.
(14) Powles, S. B.; Yu, Q. Evolution in action: plants resistant to herbicides. *Annu. Rev. Plant Biol.* **2010**, *61*, 317−347.
(15) Ngo, T. D.; Malone, J. M.; Boutsalis, P.; Gill, G.; Preston, C. *EPSPS* gene amplification conferring resistance to glyphosate in windmill grass (*Chloris truncata*) in Australia. *Pest Manage. Sci.* **2018**, *74*, 1101−1108.
(16) Heap, I. M. The International Survey of Herbicide Resistant Weeds. www.weedscience.org (accessed Jan. 8, 2019).
(17) Panozzo, S.; Scarabel, L.; Rosan, V.; Sattin, M. A New Ala-122-Asn Amino Acid Change Confers Decreased Fitness to ALS-Resistant *Echinochloa crus-galli*. *Front. Plant Sci.* **2017**, *8*, 1−13.
(18) Panozzo, S.; Scarabel, L.; Tranel, P. J.; Sattin, M. Target-site resistance to ALS inhibitors in the polyploid species *Echinochloa crus-galli*. *Pestic. Biochem. Physiol.* **2013**, *105*, 93−101.
(19) Fang, J.; Liu, T.; Zhang, Y.; Li, J.; Dong, L. Target site-based penoxsulam resistance in barnyardgrass (*Echinochloa crus-galli*) from China. *Weed science* **2019**, *67*, 281−287.
(20) Delye, C. Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. *Pest Manage. Sci.* **2013**, *69*, 176−187.
(21) Iwakami, S.; Uchino, A.; Kataoka, Y.; Shibaike, H.; Watanabe, H.; Inamura, T. Cytochrome P450 genes induced by bispyribac- sodium treatment in amultiple- herbicide- resistant biotype of *Echinochloa phyllopogon*. *Pest Manage. Sci.* **2014**, *70*, 549−558.
(22) Iwakami, S.; Endo, M.; Saika, H.; Okuno, J.; Nakamura, N.; Yokoyama, M.; Watanabe, H.; Toki, S.; Uchino, A.; Inamura, T. Cytochrome P450 *CYP81A12* and *CYP81A21* are associated with resistance to two acetolactate synthase inhibitors in *Echinochloa phyllopogon*. *Plant Physiol.* **2014**, *165*, 618−629.
(23) Yang, X.; Yu, X. Y.; Li, Y. F. De novo assembly and characterization of the Barnyardgrass (*Echinochloa crus-galli*) transcriptome using next-generation pyrosequencing. *PLoS One* **2013**, *8*, No. e69168.
(24) Martin, J. A.; Wang, Z. Next-generation transcriptome assembly. *Nat. Rev. Genet.* **2011**, *12*, 671−682.
(25) Zhao, N.; Li, W.; Bai, S.; Guo, W. L.; Yuan, G. H.; Wang, F.; Liu, W. T.; Wang, J. X. Transcriptome profiling to identify genes involved in mesosulfuron-methyl resistance in *Alopecurus aequalis*. *Front. Plant Sci.* **2017**, *8*, 16.
(26) Pan, L.; Gao, H.; Xia, W.; Zhang, T.; Dong, L. Establishing a herbicide-metabolizing enzyme library in *Beckmannia syzigachne* to identify genes associated with metabolic resistance. *J. Exp. Bot.* **2016**, *67*, 1745−1757.
(27) Yang, Q.; Deng, W.; Li, X.; Yu, Q.; Bai, L.; Zheng, M. Target-site and non-target-site based resistance to the herbicide tribenuron-methyl in flixweed (*Descurainia sophia* L.). *BMC Genomics* **2016**, *17*, 551.
(28) Duhoux, A.; Carrère, S.; Gouzy, J.; Bonin, L.; Délye, C. RNA-Seq analysis of rye-grass transcriptomic response to an herbicide inhibiting acetolactate-synthase identifies transcripts linked to non-target-site-based resistance. *Plant Mol. Biol.* **2015**, *87*, 473−487.
(29) An, J.; Shen, X. F.; Ma, Q. B.; Yang, C. Y.; Liu, S. M.; Chen, Y. Transcriptome profiling to discover putative genes associated with paraquat resistance in goosegrass (*Eleusine indica* L.). *PLoS One* **2014**, *9*, No. e99940.
(30) Chen, J. C.; Huang, H. J.; Wei, S. H.; Huang, Z. F.; Wang, X.; Zhang, C. X. Investigating the mechanisms of glyphosate resistance in goosegrass (*Eleusine indica* (L.) Gaertn.) by RNA sequencing technology. *Plant J.* **2017**, *89*, 407−415.
(31) Dogramaci, M.; Foley, M. E.; Horvath, D. P.; Hernandez, A. G.; Khetani, R. S.; Fields, C. J.; Keating, K. M.; Mikel, M. A.; Anderson, J. V. Glyphosate's impact on vegetative growth in leafy spurge identifies molecular processes and hormone cross-talk associated with increased branching. *BMC Genomics* **2015**, *16*, 22.
(32) Hongchun, W.; Jun, L.; Bo, L.; Yuanlai, L.; Liyao, D. The role of cytochrome P450 monooxygenase in the different responses to fenoxaprop-P-ethyl in annual bluegrass (*Poa annua* L.) and short awned foxtail (*Alopecurus aequalis* Sobol.). *Pestic. Biochem. Physiol.* **2013**, *107*, 334−342.
(33) Ma, R.; Evans, A. F.; Riechers, D. E. Differential responses to preemergence and postemergence atrazine in two atrazine-resistant waterhemp populations. *Agronomy Journal* **2016**, *108*, 1196−1202.
(34) Yu, Q.; Shane Friesen, L.J; Zhang, X. Q.; Powles, S. B Tolerance to acetolactate synthase and acetyl-coenzyme A carboxylase inhibiting herbicides in *Vulpia bromoides* is conferred by two co-existing resistance mechanisms. *Pestic. Biochem. Physiol.* **2004**, *78*, 21−30.
(35) Liu, H. J.; Yang, X. R.; Liao, X. H.; Zuo, T.; Qin, C.; Cao, S. L.; Dong, L.; Zhou, H. K.; Zhang, Y. Z.; Liu, S. S.; Shen, Y. O.; Lin, H. J.; Lubberstedt, T.; Zhang, Z. M.; Pan, G. T. Genome-wide comparative analysis of digital gene expression tag profiles during maize ear development. *Genomics* **2015**, *106*, 52−60.
(36) Zhong, S.; Joung, J.-G.; Zheng, Y.; Chen, Y.-r.; Liu, B.; Shao, Y.; Xiang, J. Z.; Fei, Z.; Giovannoni, J. J. High-throughput illumina strand-specific RNA sequencing library preparation. *Cold Spring Harbor protocols* **2011**, *2011*, 940−949.
(37) Guo, L. B.; Qiu, J.; Ye, C. Y.; Jin, G. L.; Mao, L. F.; et al. *Echinochloa crus-galli* genome analysis provides insight into its adaptation and invasiveness as a weed. *Nat. Commun.* **2017**, *8*, 1031.
(38) Kim, D.; Pertea, G.; Trapnell, C.; Pimentel, H.; Kelley, R.; Salzberg, S. L. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* **2013**, *14*, 13.
(39) Trapnell, C.; Williams, B. A.; Pertea, G.; Mortazavi, A.; Kwan, G.; van Baren, M. J.; Salzberg, S. L.; Wold, B. J.; Pachter, L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **2010**, *28*, 511−515.
(40) Trapnell, C.; Hendrickson, D. G.; Sauvageau, M.; Goff, L.; Rinn, J. L.; Pachter, L. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat. Biotechnol.* **2013**, *31*, 46−53.
(41) Gao, Y.; Pan, L.; Sun, Y.; Zhang, T.; Dong, L.; Li, J. Resistance to quinclorac caused by the enhanced ability to detoxify cyanide and its molecular mechanism in *Echinochloa crus-galli* var. zelayensis. *Pestic. Biochem. Physiol.* **2017**, *143*, 231−238.

DOI: 10.1021/acs.jafc.9b01641
J. Agric. Food Chem. 2019, 67, 8085−8095

(42) Li, G.; Wu, S.; Cai, L.; Wang, Q.; Zhao, X.; Wu, C. Identification and mRNA expression profile of glutamate receptor-like gene in quinclorac-resistant and susceptible *Echinochloa crus-galli*. *Gene* **2013**, *531*, 489−495.
(43) Li, G.; Wu, S. G.; Yu, R. X.; Cang, T.; Chen, L. P.; Zhao, X. P.; Cai, L. M.; Wu, C. X. Identification and expression pattern of a glutathione S-transferase in *Echinochloa crus-galli*. *Weed Res.* **2013**, *53*, 314−321.
(44) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* **2001**, *25*, 402−408.
(45) Zheng, L.; Diamond, J. M.; Denton, D. L. Evaluation of whole effluent toxicity 544 data characteristics and use of Welch's t-test in the test of significiant toxicity analysis. *Environ. Toxicol. Chem.* **2013**, *32*, 468−474.
(46) Xu, H. L.; Li, J.; Zhang, D.; Cheng, Y.; Jiang, Y.; Dong, L. Y. Mutations at codon position 1999 of acetyl-CoA carboxylase confer resistance to ACCase-inhibiting herbicides in Japanese foxtail (*Alopecurus japonicus*). *Pest Manage. Sci.* **2014**, *70*, 1894−1901.
(47) Beckie, H. J.; Tardif, F. J. Herbicide cross resistance in weeds. *Crop Prot.* **2012**, *35*, 15−28.
(48) Warwick, S. I.; Xu, R. L.; Sauder, C.; Beckie, H. J. Acetolactate synthase target-site mutations and single nucleotide polymorphism genotyping in ALS-resistant Kochia (*Kochia scoparia*). *Weed Sci.* **2008**, *56*, 797−806.
(49) Li, J.; Li, M.; Gao, X.; Fang, F. A novel amino acid substitution Trp574Arg in acetolactate synthase (ALS) confers broad resistance to ALS-inhibiting herbicides in crabgrass (*Digitaria sanguinalis*). *Pest Manage. Sci.* **2017**, *73*, 2538−2543.
(50) Muehlebach, M.; Cederbaum, F.; Cornes, D.; Friedmann, A. A.; Glock, J.; Hall, G.; Indolese, A. F.; Kloer, D. P.; Le Goupil, G.; Maetzke, T.; Meier, H.; Schneider, R.; Stoller, A.; Szczepanski, H.; Wendeborn, S.; Widmer, H. Aryldiones incorporating a 1,4,5 oxadiazepane ring. Part 2: Chemistry and biology of the cereal herbicide pinoxaden. *Pest Manage. Sci.* **2011**, *67*, 1499−1521.
(51) Yu, Q.; Powles, S. Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. *Plant Physiol.* **2014**, *166*, 1106−1118.
(52) Yun, M. S.; Yogo, Y.; Miura, R.; Yamasue, Y.; Fischer, A. J. Cytochrome P-450 monooxygenase activity in herbicide-resistant and -susceptible late watergrass (*Echinochloa phyllopogon*). *Pestic. Biochem. Physiol.* **2005**, *83*, 107−114.
(53) Iwakami, S.; Kamidate, Y.; Yamaguchi, T.; Ishizaka, M.; Endo, M.; Suda, H.; Nagai, K.; Sunohara, Y.; Toki, S.; Uchino, A.; Tominaga, T.; Matsumoto, H. CYP81A P450s are involved in concomitant cross-resistance to acetolactate synthase and acetyl-CoA carboxylase herbicides in *Echinochloa phyllopogon*. *New Phytol.* **2019**, *221*, 2112−2122.
(54) Saika, H.; Horita, J.; Taguchi-Shiobara, F.; Nonaka, S.; Nishizawa-Yokoi, A.; Iwakami, S.; Hori, K.; Matsumoto, T.; Tanaka, T.; Itoh, T.; Yano, M.; Kaku, K.; Shimizu, T.; Toki, S. A novel rice cytochrome P450 gene, *CYP72A31*, confers tolerance to acetolactate synthase-inhibiting herbicides in rice and Arabidopsis. *Plant Physiol.* **2014**, *166*, 1232−40.
(55) Endo, K.; Shirakura, S.; Nakamura, S.; Minegishi, N. Herbicide composition for paddy field. U.S. Patent US07855165, Dec 21, 2010.
(56) Feng, Y.; Gao, Y.; Zhang, Y.; Dong, L.; Li, J. Mechanisms of resistance to pyroxsulam and ACCase inhibitors in Japanese foxtail (*Alopecurus japonicus*). *Weed Sci.* **2016**, *64*, 695−704.

DOI: 10.1021/acs.jafc.9b01641
J. Agric. Food Chem. 2019, 67, 8085−8095