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Enhanced Metabolism Evolved High-Level Resistance to Fenoxaprop-P-Ethyl in *Alopecurus japonicus*

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Abstract: *Alopecurus japonicus* has been a serious weed across China and developed resistance to the acetyl-CoA carboxylase (ACCase)-inhibiting herbicide. The *A. japonicus* ACCase gene mutations accounting for target-site resistance (TSR) have been clarified, while non-target-site resistance (NTSR) is not distinct. Here, dose–response testing indicated that an *A. japonicus* population (R) was resistant to four ACCase-inhibiting herbicides fenoxaprop-P-ethyl, sethoxydim, clethodim, and pinoxaden. Compared with herbicide-susceptible *A. japonicus* (S), no known resistant mutations for TSR in ACCase were identified in the R population using sequencing. To investigate the NTSR mechanism, increased metabolism of fenoxaprop-P-ethyl was detected in the R population using high-performance liquid chromatography (HPLC) analysis. Notably, resistance cannot be reversed by P450 and GST inhibitors. RNA-seq was performed to further explore the resistance mechanisms, and eight candidate contigs (four glycosyl transferases (GT) and four ATP-binding cassette (ABC) transporters) were chosen and their expression patterns were validated using RT-qPCR. Three GT and three ABC transporter contigs were constitutively upregulated in the R population. In short, six contigs expressed highly in the R population causing enhanced fenoxaprop-P-ethyl metabolism appear to be involved in fenoxaprop-P-ethyl resistance.

Keywords: *Alopecurus japonicus*; fenoxaprop-P-ethyl; enhanced metabolism; glycosyltransferases; ABC transporter; gene expression

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1. Introduction

Excessive herbicide application for controlling weeds has consequently caused emergence and dispersal of herbicide resistance. Worldwide, 267 weed populations were found to have developed herbicide resistance [1]. Plant acetyl-CoA carboxylase (ACCase) is a key enzyme catalyzing the first part of fatty acid biosynthesis. Commercial ACCase-inhibiting herbicides, targeting weed-plastid ACCase, are categorized as three chemically different types: aryloxyphenoxypropionate (APP), phenylpyraxoline (DEN), and cyclohexanedione (CHD) [2]. Since they were applied to control weeds on a large scale, long-lasting usage of ACCase-inhibiting herbicides has caused the rapid increase in herbicide resistance. Up to now, 262 weed populations have evolved resistance to ACCase-inhibiting herbicides [1].

Herbicide-resistance mechanisms mainly contain the following: gene mutations causing target-site resistance (TSR) [3], non-target-site resistance (NTSR) involving enhanced metabolism, and reduced penetration and absorption [4]. Commonly, a considerable amount of NTSR research has centered on enhanced herbicide metabolism [5], and four gene families are regarded to be related to enhanced metabolism, including glutathione S-transferases (GSTs), cytochrome P450 monooxygenases (CYP450s), ATP-binding cassette (ABC) transporters, and glycosyl transferases (GTs) [4]. Among them, CYP450s can catalyze diverse reactions, including oxidation, dehydrogenation, epoxidation, and thus, detoxify toxic substances directly [5,6]. GSTs and GTs are related to conjugate herbicide or metabolic products, and ABC transporters can transport conjugates to the extracellular space or vacuoles [4,7]. Because of the great difficulty in the study of metabolic-enzyme

gene families and the absence of high-quality weed genomes, in weeds most metabolic resistance reports are based on herbicide metabolism in vivo studies or metabolic enzyme inhibitors research [5].

Evolved ACCase-inhibiting herbicide resistance was reported to be due to amino acid mutations of the weed ACCase enzyme in the following sites: 1781, 1999, 2027, 2041, 2078, 2088, and 2096 [3]. Enhanced ACCase-inhibiting herbicide metabolism has also been found to be related to herbicide resistance in several weeds [8–10]. In most cases, CYP450s-mediated metabolic resistance was confirmed [11–13]. CYP450 or GST inhibitors were extensively used to show a link between these metabolic enzymes and ACCase-inhibiting herbicide resistance [14,15]. Apart from CYP450s and GSTs, GTs and ABC transporters have also been reported to be involved with ACCase-inhibiting herbicide metabolic resistance [10,16].

Alopecurus Japonicus is a damaging weed widespread in Chinese cropping systems with wheat and rapeseed [17]. To date, herbicide control remains as the effective way to control *A. japonicus*. Nevertheless, large-scale herbicide use has led to a storm of herbicide resistance in *A. japonicus* [17–25]. Among them, ACCase-inhibiting herbicides were found to have high-level resistance in *A. japonicus* [17–19,21–25]. Up to now, the ACCase mutations involving I1781L, W1999C/L, W2027C, I2041N, and D2078G were found among ACCase-inhibiting herbicide-resistant *A. japonicus* [17–19,21,23]. Though ACCase-inhibiting herbicide metabolic resistance has been recognized in *A. japonicus*, identifying metabolic enzyme genes associated with resistance in *A. japonicus* is proceeding slowly [24], and only four CYP450 contigs were indicated to be related to this resistance mechanism in *A. japonicus* [24].

In the present study, we found an *A. japonicus* population resistant to fenoxaprop-P-ethyl. Therefore, we aimed to: (i) characterize the TSR mechanism involved in fenoxaprop-P-ethyl resistance; (ii) determine the impact of metabolic enzymes inhibitors on the resistance to fenoxaprop-P-ethyl; (iii) investigate specific gene expression in the resistant population; and (iv) confirm the cross-resistance to other tested ACCase-inhibiting herbicides in this resistant population.

2. Materials and Methods

2.1. Plant Material and Herbicide

The fenoxaprop-P-ethyl-resistant *A. japonicus* population (R) was gathered in Shandong province in May 2019, where fenoxaprop-P-ethyl has been repeatedly used for 6 years. The population susceptible to fenoxaprop-P-ethyl (S) was collected from a wasteland where no herbicide has been applied but close to the R population. Herbicide information used for sensitivity tests is listed in Table S1. Malathion (95%, CYP450-inhibitor) and 4-chloro-7-nitrobenzoxadiazole (NBD-Cl, 97%, GST-inhibitor) were purchased from Sigma.

2.2. Susceptibility Tests of Fenoxaprop-P-Ethyl and other ACCase-Inhibiting Herbicides

The fenoxaprop-P-ethyl susceptibility test was performed in *A. japonicus* as mentioned previously [19]. In total, 20 seeds pre-germinated were transplanted into each pot containing nutrition soil, and planted in the greenhouse with a controlled temperature (20 °C for daytime and 15 °C for nighttime) and 14 h of sunlight a day. Different fenoxaprop-P-ethyl concentrations (Table S1) were used to treat 3–4-leaf-stage seedlings. Plant overground parts in different populations were harvested after treatment for three weeks, and the fresh weights were measured. The whole experiment was performed two times and each contained three replicates in a random arrangement, and data analysis was performed as mentioned previously [21]. The cross-resistance pattern in the R *A. japonicus* was tested using single-dose testing with other ACCase-inhibiting herbicides (Table S1). If seedlings grew well after herbicide application they were regarded as resistant, and if seedlings suffered severe damage or death they were regarded as sensitive.

2.3. Analysis of ACCase-Resistance Mutations in *A. japonicus*

The Tiangen DNA extraction kit was used for the extraction of *A. japonicus* DNA with instructions. *A. japonicus* ACCase fragments involving all mutations that are known to be linked to resistance were amplified using published primers [21]. The PCR reaction was conducted as mentioned previously [7]. The PCR product was purified with a TransGen Biotech gel extraction kit and sequenced. In total, 15 seedlings surviving herbicide treatment in R *A. japonicus* and five seedlings in S *A. japonicus* were selected for sequencing. Changed nucleotide and amino acid sequences were compared using DNAMAN software.

2.4. Effect of CYP450 and GST Inhibitors on Fenoxaprop-P-Ethyl Resistance

Plant growth in the S and R populations was performed as mentioned above. Fenoxaprop-P-ethyl without and with CYP450 and GST inhibitors was applied using 3–4-leaf-stage seedlings [26]. Malathion (1000 g a.i. ha^{-1}) was treated 2 h before fenoxaprop-P-ethyl application and NBD-Cl (270 g a.i. ha^{-1}) was treated 48 h before fenoxaprop-P-ethyl application. Treatments contained malathion, NBD-CI, fenoxaprop-P-ethyl, malathion plus fenoxaprop-P-ethyl, and NBD-CI plus fenoxaprop-P-ethyl. Fenoxaprop-P-ethyl concentrations were applied as mentioned above. Plant overground parts in different populations were harvested after treatment for three weeks and the fresh weights were measured. The whole experiment was performed two times, each contained three replicates in a random arrangement, and data analysis was performed as mentioned previously [21].

2.5. Fenoxaprop-P-Ethyl Metabolism Analysis Using HPLC in *A. japonicus*

Fenoxaprop-P-ethyl (50 mg L^{-1}) was used to treat 3–4-leaf-stage R and S *A. japonicus* seedlings. After fenoxaprop-P-ethyl treatment for 1 and 5 days, overground parts of R and S seedlings were gathered. The remaining fenoxaprop-P-ethyl on the surface layer of *A. japonicus* leaf was rinsed off. Fenoxaprop-P-ethyl in the elution solution was quantified with the HPLC experiment as described [27]. The fenoxaprop-P-ethyl extraction and separation methods were performed to analyze metabolic differences between S and R *A. japonicus*. In short, liquid nitrogen was used to triturate 0.5 g leaf samples, and ultrasound (480 W, 40 kHz, 20 min) was used for the extraction. Then, the extracts were centrifuged (12,000 rpm, 5 min) after adding 1 g NaCl, and 4 mL supernatant was moved to a 10 mL centrifuge tube. The extracts were centrifuged (12,000 rpm, 5 min) again after adding graphitized carbon black, $MgSO_4$, and primary secondary amine. Finally, supernatants were filtered and HPLC was used to measure fenoxaprop-P-ethyl concentrations. The whole experiment was performed two times and each contained three replicates in a random arrangement.

2.6. RNA-Seq Analysis

R and S *A. japonicus* seedlings used for RNA-Seq were verified using vegetative cloning after fenoxaprop-P-ethyl application. In total, 30 uniform seedlings were selected and cloned when they reached the tillering stage in each population. RNA-extracted leaf samples were collected and liquid nitrogen was used to freeze them. The remaining plants were permitted to regrow for 10 days after cloning before the fenoxaprop-P-ethyl recommend dose treatment. Survival was confirmed after fenoxaprop-P-ethyl treatment for three weeks. A total of six characterized seedlings (3 R and 3 S) for RNA-Seq were confirmed.

The TaKaRa Biotech RNAiso Plus kit was used to extract total RNA from selected seedlings. The quantity and quality of total RNA were analyzed with a NanoPhotometer® spectrophotometer (IMPLEN, Los Angeles, CA, USA). RNA-Seq was performed with the Illumina platform and raw reads were used to filter clean reads to perform downstream analysis. Data from 3 R and 3 S seedlings were incorporated for generating a reference transcriptome using Trinity [28] and the readcount of each gene was obtained by mapping clean data to a reference transcriptome. A gene readcount for each transcript from each seedling was calculated using fragments per kilobase of exon per million fragments mapped

(FPKM), and RSEM was used to estimate gene expression levels [29]. A *T*-test at $p < 0.05$ was used to compare differential gene expression in R and S populations. Gene Ontology (GO) enrichment was analyzed with differentially expressed genes (DEGs) using GOseq R packages [30].

2.7. Differential Gene Expression of the Candidate Glycosyl Transferases (GT) and ABC Transporter Contigs from RNA-Seq

The candidate GT and ABC transporter contigs were chosen on account of the next selection standard: (1) upregulated >2-fold ($p < 0.05$) in R compared to S, (2) contig sequence length over 500 bp, and (3) annotated in relation to GT and ABC transporters. GT and ABC transporter candidate contigs were validated using quantitative real-time PCR (RT-qPCR) with RNA-Seq prepared samples. Fenoxaprop-P-ethyl was used to treat *A. japonicus* seedlings from each population and leaf seedlings tissues in each treatment were sampled to validate candidate contigs after treatment for 24 h. GT and ABC transporter contig primers (Table S2) were designed using software (sg.idtdna.com/scitools/Applications/RealTimePCR/, accessed on 9 June 2021). The ubiquitin protein (UBQ) gene with higher stability has been used as reference gene in *A. japonicus* [31]. RT-qPCR primers used for this study were amplified to acquire a single correct product and negative control without amplification. RT-qPCR experiments and data were analyzed as mentioned previously [32].

3. Results

3.1. Fenoxaprop-P-Ethyl Dose–Response and Resistance Testing to Other ACCase-Inhibiting Herbicides in A. japonicus

A dose–response experiment was used to confirm resistance level of fenoxaprop-P-ethyl in R *A. japonicus*. When the recommended fenoxaprop-P-ethyl dose of 62 g a.i. ha^{-1} significantly inhibited the S population with a 7.8 g a.i. ha^{-1} GR_{50} value, the R *A. japonicus* evolved high-level resistance to fenoxaprop-P-ethyl with a higher GR_{50} value (350.6 g a.i. ha^{-1}) (Figure 1). R *A. japonicus* evolved 44.8-fold fenoxaprop-P-ethyl resistance compared to S *A. japonicus* on account of the resistance index. Additionally, R *A. japonicus* remained susceptible to three tested APPs (haloxyfop-R-methyl, clodinafop-propargyl, and quizalofop-P-ethyl), and became resistant to two tested CHDs (sethoxydim, clethodim), and one tested DEN pinoxaden (Figure 2).

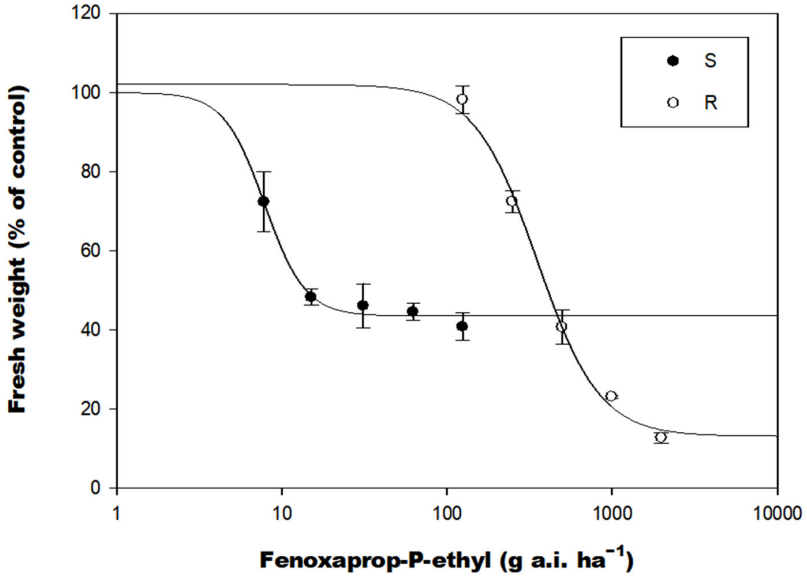

Figure 1. Dose-response of resistant (R) and susceptible (S) *Alopecurus japonicus* populations to fenoxaprop-P-ethyl. Data points are means ± SE ($n = 3$). X line is the fenoxaprop-P-ethyl application dose, Y line is the fresh weight at each fenoxaprop-P-ethyl dose.

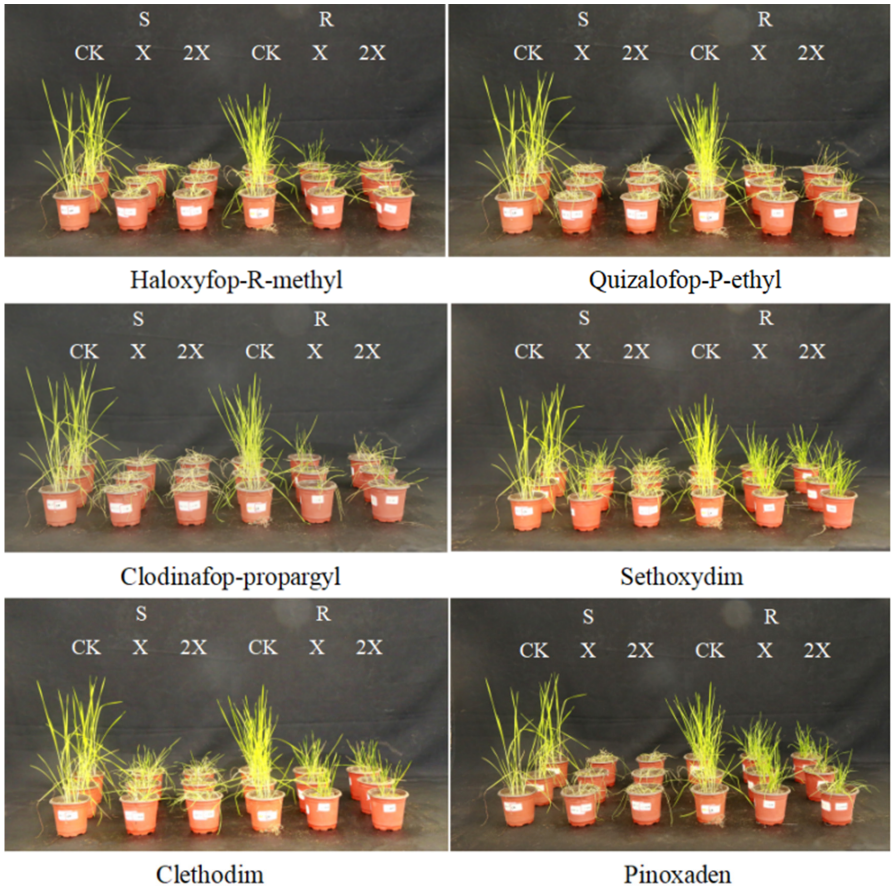

Figure 2. Growth response of resistant (R) and susceptible (S) *Alopecurus japonicus* populations to ACCase-inhibiting herbicides.

3.2. ACCase Gene Sequencing in A. japonicus

No ACCase mutations known to be linked to resistance were recognized in any individual R seedlings surviving after fenoxaprop-P-ethyl application (Figure 3). Therefore, fenoxaprop-P-ethyl resistance is not caused by TSR in the *A. japonicus* R population.

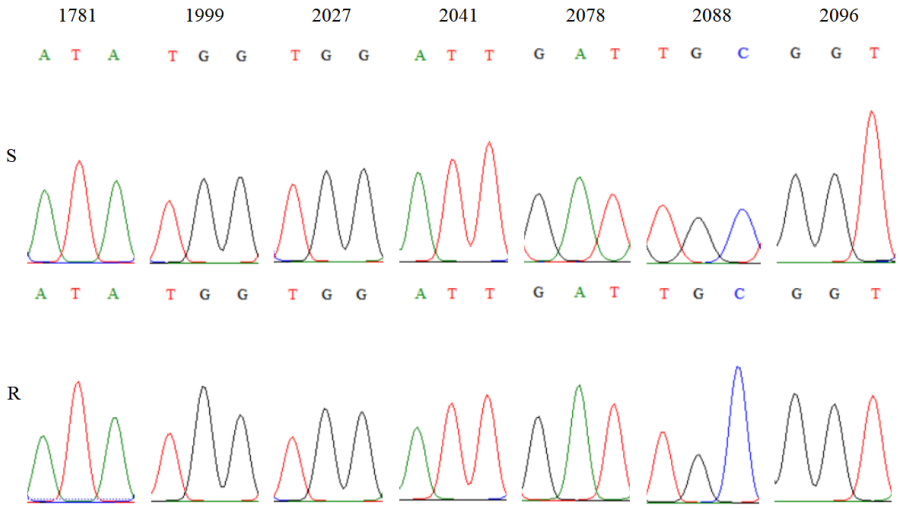

Figure 3. Acetyl coenzyme A carboxylase (ACCase) sequence in resistant (R) and susceptible (S) *Alopecurus japonicus* populations. Lines indicated alignment of partial amino acid sequences of plastidic ACCases from various weed species that are sensitive to ACCase herbicides.

3.3. Impact of CYP450 and GST Inhibitors on Fenoxaprop-P-Ethyl Resistance in *A. japonicus*

The NBD-CI (270 g a.i. ha^{-1}) and Malathion (1000 g a.i. ha^{-1}) treatments alone did not cause visual damage or growth reduction in *A. japonicus* seedlings. Additionally, applying malathion in the S and R populations cannot increase fenoxaprop-P-ethyl phototoxicity (Table 1). The NBD-CI pretreatment also made no difference to fenoxaprop-P-ethyl in R and S *A. japonicus* (Table 1). The implication of these results was that fenoxaprop-P-ethyl resistance in R *A. japonicus* did not result from CYP450s and GSTs.

Table 1. Effects of the cytochrome P450 inhibitor (malathion) and GST inhibitor (NBD-Cl) on *Alopecurus japonicus* dose response to fenoxaprop-P-ethyl.

Herbicide	Populations	Treatments	GR_{50} (g a.i.ha^{-1}) (SE) [a]	Resistance Index
Fenoxaprop-P-ethyl	R	NBD-CI+ Fenoxaprop-P-ethyl	322.7 (43)	41.2
		Malathion+ Fenoxaprop-P-ethyl	303.6 (36)	38.8
		Fenoxaprop-P-ethyl	350.6 (33)	44.8
	S	NBD-CI+ Fenoxaprop-P-ethyl	9.7 (1.4)	1.2
		Malathion+ Fenoxaprop-P-ethyl	7.6 (0.9)	1.0
		Fenoxaprop-P-ethyl	7.8 (0.5)	-

[a] SE, standard error.

3.4. Metabolic Rate Difference of Fenoxaprop-P-Ethyl in *A. japonicus*

An HPLC experiment was used to determine the fenoxaprop-P-ethyl concentration dynamic in *A. japonicus*. The fenoxaprop-P-ethyl metabolism in the R *A. japonicus* was markedly faster than S *A. japonicus* (Figure 4). Compared to S *A. japonicus*, the fenoxaprop-P-ethyl metabolic rate in R *A. japonicus* was 1.5-fold more rapid after herbicide application for 5 days (Figure 4), confirming that fenoxaprop-P-ethyl metabolism was faster in R *A. japonicus*. Combined with the results of the above experiment, fenoxaprop-P-ethyl resistance of the R *A. japonicus* is involved with metabolism unrelated to CYP450s or GSTs.

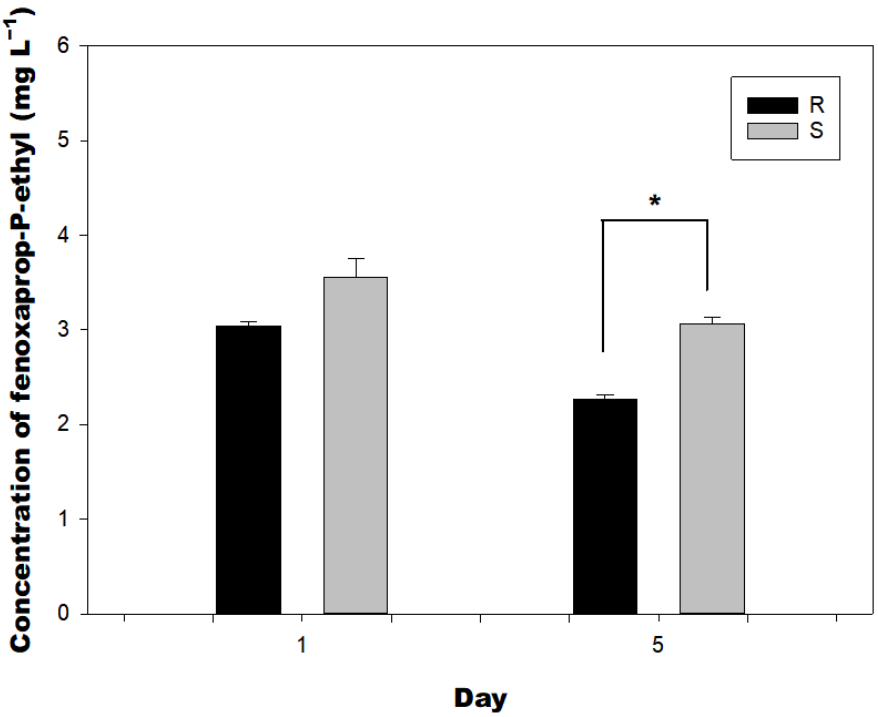

Figure 4. Concentration to fenoxaprop-P-ethyl of resistant (R) and susceptible (S) *Alopecurus japonicus* populations. Data are means ± SE (n = 3). Asterisk indicates significant differences by T-test ($p < 0.05$).

3.5. Identifying Differential Expression of GT and ABC Transporter Contigs in A. japonicus

Because of the *A. japonicus* genome deficiency, the de novo reference transcriptome was established by assembling R and S *A. japonicus* sequencing data. In total, 89951 putative genes with long contig N50 sizes (1326 bp) were identified using assembled transcripts (104361). The assembled sequences were also annotated using Nr, SwissProt, KO, PFAM, KOG, and GO databases (Table S3). Genes expressed differentially in S and R *A. japonicus* were recognized based on the FPKM *t*-test ($p < 0.05$) and 13802 differentially expressed genes were evaluated (Figure S1). DEG functions were evaluated using GO enrichment analysis and 5975 DEGs were appointed with metabolic processes most markedly abundant (Table S3 and Figure S2).

In all, 25 GT and 20 ABC transporter annotation contigs downregulated >2-fold ($p < 0.05$) in S than in R were found based on RNA-Seq data analysis. All these contigs were chosen to verify expression patterns using RT-qPCR with transcriptome sequencing samples and parallel materials, and 4 GT and 4 ABC transporter contigs were upregulated markedly and the fold-change results from RT-qPCR were no different to those from RNA-Seq (Table 2). After fenoxaprop-P-ethyl treatment, 3 GT (GT-86A, GT-91A, and GT-92) and 3 ABC transporter (ABCB11, ABCC10, and ABCE2) contigs in the R population were upregulated constitutively (Table 2). These 6 contigs expressed highly appear to be involved in fenoxaprop-P-ethyl resistance in R *A. japonicus*.

Table 2. Results of RNA-seq and relative transcript levels of glycosyl transferases and ABC transporters.

Gene	Family	log_2FC [a]	FC [b]	*t*-Test	FC [c]	*t*-Test
UDP-GT	71C	7.33	1.9	0.12	-	-
	73C	5.78	2.12	0.12	-	-
	75C	2.6	4.5	0.007	2.73	0.07
	80B	1.84	1.47	0.43	-	-
	83A	10.1	1.51	0.38	-	-
	86A	3.83	>8	-	>16	-
	88B	2.22	0.66	0.16	-	-
	89B	8.97	2.04	0.18	-	-
	91A	7.47	>8	-	>8	-
	92	6.98	9.42	0.02	6.35	0.24
ABC	B11	1.7	>16	-	>16	-
	B13	4.21	5.85	0.001	2.04	0.43
	B28	1.26	0.73	0.18	-	-
	C2	1.34	0.99	0.99	-	-
	C10	7.73	>32	-	>128	-
	D1	1.31	0.94	0.89	-	-
	E2	8.44	52.93	0.04	>64	-
	G53	2.55	1.26	0.16	-	-

[a] Results of RNA-seq. [b] Fold change of no fenoxaprop-P-ethyl treatment. [c] Fold change of fenoxaprop-P-ethyl field recommended dose treatment.

4. Discussion

4.1. NTSR Mechanism Can Confer High-Level ACCase-Inhibiting Herbicide Resistance

Here, an R *A. japonicus* population was confirmed to develop resistance to fenoxaprop-P-ethyl and ACCase target-site mutation was not the reason for resistance, indicating a possible correlation between resistance and NTSR mechanism. NTSR usually brings about herbicide resistance with a low or medium level (less than 10-fold). TSR often results in herbicide resistance at a higher level than NTSR, thus, NTSR is easily concealed. However, research on resistance mechanisms for herbicide-resistant cases has become deeper in recent years. NTSR are also highly resistant to ACCase-inhibiting herbicides, for example, NTSR in the *Lolium multiflorum* population was found to have a greater effect than TSR on ACCase-inhibiting herbicides (tepraloxydim and sethoxydim) [8]. Similarly, in this study, high-level resistance (44.8-fold) to ACCase-inhibiting herbicide fenoxaprop-P-ethyl resulting from NTSR was confirmed in the R *A. japonicus* (Figure 1 and Table 1). Therefore,

high- or low-level herbicide resistance caused by NTSR may result from weed species, the accumulative resistance evolution after a long period of herbicide application, or different metabolic enzyme types. As these findings show, NTSR is more complicated and further research to uncover the mechanism will increase our knowledge of NTSR.

4.2. Enhanced Metabolism Leads to a Specific Herbicide Resistance Spectrum in A. japonicus

Enhanced metabolism has represented, until now, the most studied type of NTSR [5,6]. Enhanced metabolism generally conferred wide-spectrum herbicide resistance with diverse target sites of herbicide action, including herbicides that never reached the market [5,33]. For example, *Echinochloa phyllopogon* P450 CYP81As have the ability to metabolize three chemically different types of ACCase-inhibiting herbicides [34]. In this study, the R *A. japonicus* population was demonstrated to evolve resistance to only one APP (fenoxaprop-P-ethyl), CHDs (sethoxydim and clethodim), and DEN pinoxaden (Figure 2), but no resistance to three other tested APPs (quizalofop-P-ethyl, clodinafop-propargyl, and haloxyfop-R-methyl). Reasons for this phenomenon may be due to the strong substrate specialization and high catalyst efficiency of the possible metabolic enzyme. Therefore, this NTSR mechanism can lead to a resistance spectrum to these herbicides tested. The commonly used wheat-field herbicide clodinafop-propargyl can control the R *A. japonicus* involving the specific NTSR mechanism effectively. This herbicide could be an ideal choice for developing a smarter strategy for rotating herbicides to block or delay resistance evolution of this specific NTSR mechanism [35]. Additionally, in comparison to relying merely on herbicides, other weed control methods should be promoted to control *A. japonicus* to avert the resistance evolution.

4.3. Specific Metabolic Enzymes Are Likely to Be Involved in Fenoxaprop-P-Ethyl Resistance in A. japonicus

Fenoxaprop-P-ethyl resistance was found in numerous weedy species, and target gene ACCase mutations have been the reason for this resistance on most occasions [23,36,37]. Research on the fenoxaprop-P-ethyl-resistant *A. japonicus* population pointed to the CYP450-mediated mechanism as the reason for metabolic resistance of this weed [24]. Most studies showed that the CYP450s-mediated mechanism enhanced metabolism existed in numerous ACCase-inhibiting herbicide-resistant weeds [7,10,38]. In our study, the R population metabolized fenoxaprop-P-ethyl faster than the S population in *A. japonicus*, and CYP450 and GST inhibitors made no difference for fenoxaprop-P-ethyl resistance (Table 1). Thus, we assumed that particular metabolic enzymes may precipitate in fenoxaprop-P-ethyl-resistant populations. GTs may play a role in enhanced herbicide metabolism by catalyzing saccharide moiety conjugation from glycosyldonor to xenobiotics [39]. Plant ABC transporters have also been shown to transport glutathione-conjugated chemicals [40]. The transport activity of the ABC transporter involved in herbicides or metabolites has been well-known in model and crop plants. However, limited research has connected GTs and ABC transporters to metabolic resistance in weeds. In this case, considering the high-level fenoxaprop-P-ethyl resistance and no reversion using CYP450 and GST inhibitors, resistance to fenoxaprop-P-ethyl in R *A. japonicus* was supposed to be caused by a specific metabolic enzyme (not CYP450 or GST) such as GTs or ABC transporters or a novel mechanism.

Recently, RNA-Seq has already succeed in investigating the NTSR mechanism in weed species. In this study, RNA-Seq was also used to identify the specific metabolic enzyme related to fenoxaprop-P-ethyl metabolism, and eight GT and ABC transporter gene expression levels in the R and S *A. japonicus* potentially catalyzing fenoxaprop-P-ethyl metabolism were compared. Between them, GT-86A, GT-91A, and GT-92 displayed constitutive overexpression and were upregulated significantly in R *A. japonicus* with fenoxaprop-P-ethyl application; moreover, ABCB11, ABCC10, and ABCE2 were also upregulated significantly in R *A. japonicus* with fenoxaprop-P-ethyl application (Table 2). GT enzymes were also found to be upregulated after herbicide treatment in diclofop-methyl-resistant *Lolium rigidum* [10] and fenoxaprop-P-methyl-resistant *Beckmannia syzigachne* [7]. Many reports

have proven that ABC transporters induce herbicide resistance [41]. These results suggest that the upregulation of GT and ABC transporter genes in the *A. japonicus* population play roles in fenoxaprop-*P*-ethyl resistance.

5. Conclusions

In conclusion, fenoxaprop-*P*-ethyl resistance involving enhanced metabolism in an *A. japonicus* population was found. Metabolic enzyme inhibitors cannot reverse resistance in this population. Instead, three GT and three ABC transporter contigs had constitutive and herbicide-induced upregulation in the R *A. japonicus* compared to S *A. japonicus*. Additionally, the R population exhibited a specific resistance pattern compared to other ACCase-inhibiting herbicides. This study offers evidence on particular detoxifying enzymes related to fenoxaprop-*P*-ethyl resistance. It is necessary to undertake further research to confirm these gene functions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12092172/s1, Figure S1: analysis of differentially expressed genes (DEGs) between resistant (R) and susceptible (S) *Alopecurus japonicus* populations; Figure S2: gene ontology (GO) classifications of *Alopecurus japonicus* and DEGs according to their involvement in the biological process, cellular component, and molecular function; Tabel S1: herbicides and their doses used in dose–response experiments for resistant (R) and susceptible (S) populations in *Alopecurus japonicus*; Tabel S2: primers used in the ACCase sequence and RT-qPCR analysis in *Alopecurus japonicus*; Tabel S3: sequence annotation of the *Alopecurus japonicus* transcriptome.

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Data Availability Statement: Not applicable.

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