agronomy

MDPI

Article

Nontarget Site-Based Resistance to Fenoxaprop-P-ethyl and Candidate Genes Involved in *Alopecurus japonicus*

Hongle Xu [1,†], Xiaofan Ye [2,†], Shaoqi Liang [3], Jingping Cheng [1], Qiuli Leng [1], Lanlan Sun [1], Wangcang Su [1], Fei Xue [1], Liyao Dong [3,*] and Renhai Wu [1,*]

[1] Henan Key Laboratory of Crop Pest Control, Institute of Plant Protection, Henan Academy of Agricultural Sciences, Zhengzhou 450002, China; xuhongle86@126.com (H.X.)
[2] College of Plant Protection, Hunan Agricultural University, Changsha 410128, China; leaf_comfort@163.com
[3] College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China
* Correspondence: dly@njau.edu.cn (L.D.); renhai.wu@163.com (R.W.)
† These authors contributed equally to this work.

Abstract: Nontarget-site resistance (NTSR) is a complex multigenic trait that is associated with the potential mechanisms of herbicide resistance which pose a serious threat to global crop protection. However, the NTSR mechanisms of *Alopecurus japonicus*, a malignant weed infesting wheat fields, are less characterized. In this study, we used RNA-sequencing transcriptome and enzyme activity detection to investigate the NTSR mechanisms and candidate genes involved in fenoxaprop-P-ethyl (FE) in a previously identified resistant population compared to the sensitive population of *A. japonicus*. Transcriptome analysis identified nine upregulated genes, which were constitutively overexpressed and upregulated by FE application in the resistant population, and the results were validated using quantitative real-time PCR. These genes including one cytochrome P450 monooxygenase (P450) gene (*CYP75B4*), one ATP-binding cassette (ABC) transporter gene *(ABCG36*), one laccase (LAC) gene (*LAC15*), one 9-cis-epoxycarotenoid dioxygenase (NCED) gene (*NCED5*), two purple acid phosphatase (PAP) genes (*PAP4*, *PAP15*), one sucrose phosphate synthase (SPS) gene (*SPS3*), one protein related to disease resistance gene (*RGA3*) and one immune protein gene (*R1B-17*). The activity assay of LAC, NCED, PAP and SPS revealed that the activities of these enzymes in the resistant population were significantly higher than those in the sensitive population at 0 h and after FE application at 12 h, 24 h and 72 h. Nevertheless, whether LAC, NCED, PAP and SPS genes were involved in herbicide metabolism needs to be further validated. Our results revealed that CYP, ABC transporter and LAC genes may participate in *A. japonicus* resistance. These genes identified in the present study provide new insights into the resistance mechanism of weeds in response to herbicide. Our study also implies the complexity of the NTSR mechanisms of weeds.

Keywords: herbicide; transcriptome; P450; laccase; resistance mechanism

Citation: Xu, H.; Ye, X.; Liang, S.; Cheng, J.; Leng, Q.; Sun, L.; Su, W.; Xue, F.; Dong, L.; Wu, R. Nontarget Site-Based Resistance to Fenoxaprop-P-ethyl and Candidate Genes Involved in *Alopecurus japonicus*. *Agronomy* **2023**, *13*, 1587. https://doi.org/10.3390/agronomy13061587

Academic Editor: Leslie A. Weston

Received: 16 May 2023
Revised: 6 June 2023
Accepted: 8 June 2023
Published: 12 June 2023

1. Introduction

Wheat is the second largest crop planted and the main winter crop in China [1]. Due to the large variety and wide area of weeds in wheat fields, nearly billions of kilograms of wheat is lost every year in China; therefore, herbicides are usually applied to control weeds and are important for wheat production [1]. In recent years, herbicide resistance among weeds in wheat fields has become an increasingly serious problem affecting wheat production [1]. Resistance to herbicides can be divided into target-site and nontarget-site resistance (TSR and NTSR, respectively) mechanisms [2]. The TSR mechanism results from specific amino acid substitution or the overexpression of the target enzymes of herbicides [3]. This mechanism of resistance has been clearly reported in many studies, and fifteen conserved amino acid mutations at seven amino acid sites led to the TSR of weeds to acetyl-CoA carboxylase (ACCase) inhibitors [4]. NTSR is linked to reduced uptake, translocation, and

enhanced detoxification and cytoprotective mechanism [5]. Compared with TSR, NTSR is more complex, and studies on it mostly focus on metabolic mechanisms [6]. It often involves cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), glucosyltransferases (GTs), and ATP-binding cassette (ABC) transporters [5–9]. The vast majority of studies have focused on these well-known metabolic enzymes, but less on other related enzymes.

Alopecurus japonicus is the main malignant grass weed in wheat fields and severely reduces wheat yield [10]. Fenoxaprop-P-ethyl (FE) is an ACCase inhibitor herbicide applied in wheat fields to control *A. japonicus*. Due to the extensive and continuous use of FE, resistance has developed in many *A. japonicus* populations [11–15]. At present, research on resistance to *A. japonicus* has mainly focused on TSR, and those mechanisms are relatively clear. TSR to FE is conferred mostly by mutations in a single gene encoding herbicide target protein site [11,13–15]. Bi et al. confirmed that the mutation of Ile-1781-Leu in the resistant population causes FE resistance in *A. japonicus* [11]. Xu et al. and Chen et al. reported that the Trp-1999-Cys or Leu, Trp-2027-Cys, Ile-2041-Asn and Asp-2078-Gly substitutions conferred resistance to FE [15–17]. However, NTSR to ACCase inhibitor studies were not a circumstance for TSR. Chen et al. found that FE resistance in *A. japonicus* was conferred by the upregulation of P450s in the *CYP72A* and *CYP81* subfamilies [12]. Most of the NTSR studies in *A. japonicus* focused on P450, while other related metabolic enzymes have rarely been reported.

RNA-sequencing (RNA-seq) technology is a powerful tool for investigating the genetic basis of abiotic stress response in plants. Currently, RNA-seq has been used to investigate abiotic stress in nonmodel species without genomic resources (including herbicide resistance) due to its development and popularity. Recently, using RNA-seq, several genes were identified in grass weeds that revealed the mechanism of NTSR to FE, such as Asia minor bluegrass (*Polypogon fugax*) [18], American sloughgrass (*Beckmannia syzigachne*) [19], black grass (*Alopecurus myosuroides*) [20], rigid ryegrass (*Lolium rigidum*) [21], and shortawn foxtail (*Alopecurus aequalis*) [10]. These studies revealed that the genes involved in the mechanism of NTSR can vary according to weed species. To date, there have been few studies on the NTSR of *A. japonicus*, and the mechanism of NTSR is still unclear.

In our study, RNA-seq transcriptome analysis and quantitative real-time (qRT)-PCR experiments were implemented to identify and validate the genes involved in the mechanism of NTSR in an FE-resistant *A. japonicus* population. In addition, we screened for some genes that might be associated with resistance to FE. Finally, we also selected laccase (LAC), 9-cis-epoxycarotenoid dioxygenase (NCED), purple acid phosphatase (PAP), and sucrose phosphate synthase (SPS), which differ from the metabolism-related enzymes previously used to measure their enzyme activities, to verify that they may be related to the resistance of *A. japonicus*. Through the means above, we aim to enrich and improve the understanding of NTSR to herbicides.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

A putative FE-resistant population (R, AHFD-3) and a reference sensitive population (S, JNXW-1) of *A. japonicus* were used in this study. The seeds of the R population were collected in 2011 from a wheat field in Feidong County, Anhui Province, in China. An Asp-2078-Gly substitution of ACCase and upregulation of cytochrome P450s in the *CYP72A* subfamily were identified in the R population to confer the resistance to FE [12]. The seeds of the S population were collected in 2011 on a river bank in Nanjing, Jiangsu Province, in China that had never been treated with herbicide.

Twelve seeds of R and S were sown in 12 L pots filled with a 2:1 (*wt*/*wt*) mixture of sand and soil for pre-germination. The pots were placed in an artificial climate chest at 20 °C/10 °C (12 h/12 h, day/night) with 15 μmol·m^{-2}·s^{-1}. After germination, the seedlings were returned and cultured in the climate chest. When the seedlings reached the 3- to 4-leaf stage, they were sprayed with FE/water using a track sprayer equipped with a

flat-fan nozzle to deliver 470 L·ha^{-1} at 0.8 MPa (3WP-2000, Nanjing Research Institute for Agricultural Mechanization, Nanjing, National Ministry of Agriculture of China). Technical grade FE was dissolved in 1 mL of dimethylbenzene and emulsified by 1% Tween-80 aqueous solution. R/S testing for FE was conducted at the recommended label rate (62.0 g a.i.·ha^{-1}), and the aboveground parts were taken as materials 24 h after spraying. Four treatments were considered: the R population treated with water (RCK), R population treated with FE (RT), S population treated with water (SCK) and S population treated with FE (ST). All treatments had five biological replicates and two technical replicates per replicate to ensure data reproducibility.

2.2. RNA Extraction, Library Construction, and Illumina Sequencing

Total RNA was extracted using the Plant RNA extraction kit (TaKaRa Co., Ltd., Tokyo, Japan). Subsequently, an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and a NanoDrop One microvolume UV-Vis spectrophotometer (Thermo Fisher, Waltham, MA, USA) were used to examine the concentration and purity of RNA. Illumina RNA-seq libraries were constructed using 10 µg of the total RNA from each sample using the NEBNext Ultra^{TM} RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). The library quality was measured using an Agilent 2100 (Agilent Technologies, CA, USA). Finally, the qualified libraries were sequenced with HiSeq X-ten sequencer (BioMarker Technologies, Beijing, China). The RNA samples from each treatment (RCK, RT, SCK and ST) were five biological samples, totaling 20 samples.

2.3. Identification of Differentially Expressed Genes (DEGs) and Functional Annotation

The adaptor and low-quality sequences were removed from the raw reads. Fragments per kilobase of transcript per million fragments mapped using StringTie was used to calculate the gene expression level. Differentially expressed genes (DEGs) were screened based on fold change $\geq$1.5 and false discovery rate <0.05 as the cutoff criteria using DESeq2 software. Based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, the function of the DEGs was determined.

2.4. Quantitative Real-Time PCR (qRT-PCR) Validation

The RNA samples used for the RNA sequencing and qRT-PCR analysis were the same. The RNA integrity was assessed using 1% agarose gel electrophoresis, while the purity and concentration were determined using a NanoDropTM 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Each RNA sample was converted to cDNA according to the PrimeScript^{TM} RT Reagent Kit (TaKaRa, Dalian, China). qRT-PCR was carried out in an Applied Biosystems QuantStudio^{TM} 6 Real-time PCR system (Thermo Fisher Scientific, Shanghai, China). Amplification was performed using TB Green Premix Ex Taq II BTli RNaseH Plus. The internal controls for qRT-PCR amplification were *Elongation factor-1 alpha* (*EF-1*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *18S ribosomal RNA* (*18S*) [22]. Three replicates were set for each reaction. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression levels of the tested genes. The primers which were designed using Primer Premier 5.0 for qRT-PCR and are shown in Table 1. The Pearson model (r) was used to reveal the correlations between the qRT-PCR and RNA-seq results.

2.5. Activity Assay of Related Metabolic Enzymes

Four speculated related metabolic enzyme indicators—LAC, NCED, PAP and SPS—were used to reflect the effects of FE treatment on the *A. japonicus*. The activities of LAC, NCED, PAP and SPS were determined using double antibody sandwich enzyme-linked immunosorbent assay (ELISA) with commercial kits. Leaf tissues of the S and R plants were harvested at the 3- to 4-leaf stage. Crude enzyme was ground and extracted from the leaf tissues (fresh weight: 50 mg) in 0.45 mL of 0.01 mol/L PBS buffer with a pH value of 7.4. Laccase activity detection was performed using a Plant LAC ELISA Kit (YJ298140, Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China), and the steps were,

briefly, as follows: 50 µL each of standard and sample dilution were added to the Microelisa stripplate. After sealing the plate with a closure plate membrane, it was incubated at 37 °C for 30 min. The closure plate membrane was removed and the liquid was discarded. The washing buffer was put into each well after drying, left still for 30 s, and then, the liquid was discarded, and this was repeated 5 times. A total of 50 µL of HRP-conjugate reagent was added to each well, except for the blank wells. The cells were incubated in the dark for 10 min at 37 °C after chromogen solution was added to each well. The reaction was stopped by adding 50 µL of stop solution to each well. Finally, the absorbance was read at 450 nm after adding the stop solution and within 15 min, taking the blank well as zero. A standard curve, which was calculated based on the OD value of the standard enzyme solutions, was used to determine the specific enzyme activity. Corresponding kits were used for NCED, PAP and SPS, and the methods used were those described above (YJ151873, YJ151873, YJ022832, Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China). The four metabolic enzyme indicators were measured at 0, 12 h, 24 h, 48 h and 72 h under FE treatment (62.0 g a.i.·ha^{-1}) in the R and S populations. Each treatment included five biological replications and two repeats.

Table 1. Primers used for qRT-PCR.

Gene	Gene Annotation	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)
c80456.graph_c1	Laccase-15, LAC15	TCAGGTGAGACAGTT GATGCTTTGG	GAGGCAATAGGCTC GTCAAGTTACC
c86897.graph_c0	Purple acid phosphatase 4, PAP4	AGCAGCAGC AGCAACACAGTAG	TCATAGCTCCACC AGTCCACATCC
c92899.graph_c0	Purple acid phosphatase 15, PAP15	GCTACTCCTGCTCA TTCGCCAAG	GGTCACTGATCTGT CCACCTTTGC
c97785.graph_c0	9-cis-epoxycarotenoid dioxygenase NCED5	CAGAGGTGGAAGC AGAAGCAGTC	CGACTTCGCCATC ACCGAGAAC
c97938.graph_c1	Sucrose-phosphate synthase 3, SPS3	TCCCGTGTGACATTT GCATTAGACC	AGCGTAGCGACTGG ACTCTCATC
c98820.graph_c0	Flavonoid 3′-monooxygenase CYP75B4	TGGTTACTTTGATGC GAGTGCTGAG	TGTGGCTTGATTTG ATGGTCTGGAG
c97281.graph_c2	ABC transporter G family member 36, ABCG36	AGCCATTCCATGT AGTCAAACGA	AAATTGCATTGG CTGCGTTG
c94207.graph_c0	Disease resistance protein RGA3	TGTGTCACCTTGTTT CAGTGGACTG	CAGCTCATGCCATCT GAACTCGTC
c93241.graph_c1	Late blight resistance protein homolog R1B-17	TCAGCAACGACAAA GAGGTGAAGAC	GCACTGTAATGGAG CCACGAAGG

3. Results

3.1. Transcriptome Sequencing and Assembly

The transcriptomic sequencing of the 20 samples was completed, and a total of 154.18 GB of clean data were obtained. The average GC content was above 53%, and the Q30 percentage (sequencing error rates < 0.1%) of 20 samples ranged from 95.00% to 95.99% (Table 2), indicating that the quality of the transcriptomic sequencing met the criteria for further analysis. We compared the treatments of two populations (SCK vs. ST, SCK vs. RCK, RCK vs. RT and ST vs. RT), and there were 378, 1543, 389, and 2354 DEGs, respectively (Figure 1).

Table 2. Summary of transcriptome sequencing data.

Sample	Read Number	GC Content	% ≥ Q30
RCK-1	24,623,730	55.79%	95.48%
RCK-2	27,571,212	56.03%	95.77%
RCK-3	25,166,323	55.38%	95.42%
RCK-4	24,346,668	55.16%	95.67%
RCK-5	24,336,167	55.33%	95.63%
SCK-1	27,500,747	56.57%	95.81%
SCK-2	26,315,594	54.43%	95.60%
SCK-3	26,531,889	54.79%	95.44%
SCK-4	24,984,973	56.16%	95.99%
SCK-5	26,884,101	55.70%	95.99%
RT-1	23,851,499	54.61%	95.78%
RT-2	27,098,152	54.26%	95.78%
RT-3	30,950,921	53.19%	95.00%
RT-4	23,823,583	56.63%	95.91%
RT-5	27,279,367	54.34%	95.56%
ST-1	30,872,907	55.31%	95.62%
ST-2	23,060,205	56.20%	95.93%
ST-3	25,114,713	56.29%	95.56%
ST-4	20,558,360	55.76%	95.59%
ST-5	24,928,922	55.83%	95.84%

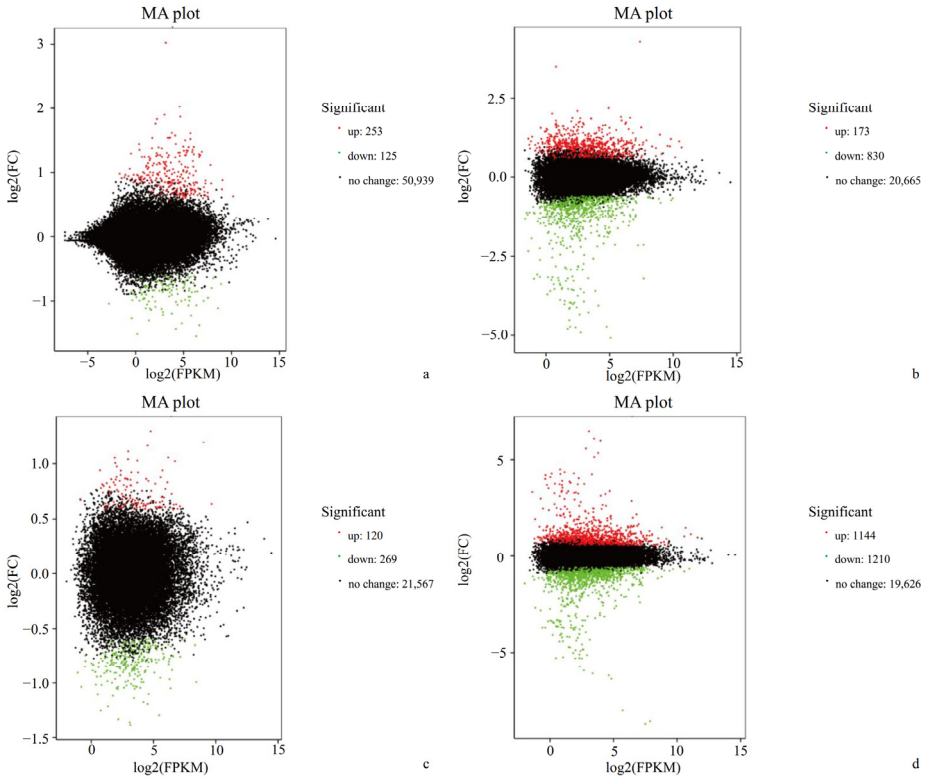

Figure 1. The volcano plot of differentially expressed genes in SCK vs. ST (**a**), SCK vs. RCK (**b**), RCK vs. RT (**c**) and ST vs. RT (**d**). The *x*-axis indicates the value of the log2(FC) difference between the two groups; the *y*-axis indicates the negative log10(FDR) of the two groups. Green dots represent down-regulated genes, red dots represent upregulated genes, while black dots represent nondifferentially expressed genes.

3.2. Differential Gene Expression and Functional Annotation

According to previous studies on the resistance of FE, the DEGs were focused on upregulated genes. A total of 1907 upregulated DEGs were screened by all four comparison groups. Based on the GO database, 120 DEGs (RCK vs. RT), 713 DEGs (SCK vs. RCK),

253 DEGs (SCK vs. ST) and 1144 DEGs (ST vs. RT) were annotated. The DEGs were assigned to 43 functional subgroups, including 16 for the biological process (BP), 15 for the cellular component (CC) and 12 for the molecular function (MF). Among the DEGs, the most enriched subgroups were "metabolic process" in BP, "cell" in CC and "binding" in MF (Figure 2).

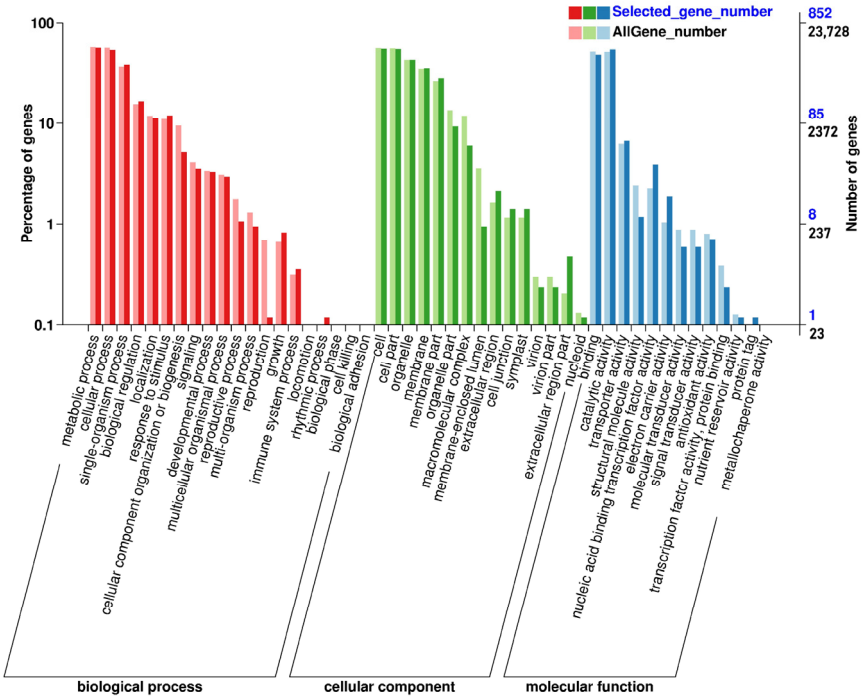

Figure 2. Gene Ontology (GO) annotation of upregulated DEGs in all four comparison groups. The DEGs were summarized in terms of biological process, cellular component and molecular function.

FE treatments induced fluctuates in transcript levels in the R and S populations of *A. japonicus*. We focused on 230 upregulated DEGs coexpressed by two treatments between the two populations (SCK vs. RCK and ST vs. RT) to study the resistance of FE (Figure 3). To further characterize the function of the identified DEGs, we performed GO and KEGG pathway enrichment analyses. In total, 230 DEGs were enriched in 28 GO subgroups (13BP, 10CC, 5MF). The three subgroups with greatest DEG enrichment were indicated for each aspect. These GO subgroups were "metabolic process", "cellular process" and "single-organism process" in BP; "cell", "cell part" and "organelle" in CC; and "binding", "catalytic activity" and "transporter activity" in MF (Table S1). Moreover, the DEGs were enriched in 25 KEGG pathways, with the plant metabolism pathway enriched in SCK vs. RCK and ST vs. RT (Figure 4).

3.3. Candidate NTSR Genes and qPT-PCR Validation

Given the important roles of metabolic enzymes in herbicide metabolism and resistance, the 230 coexpressed (SCK vs. RCK and ST vs. RT) upregulated DEGs annotated as metabolism-related enzymes were selected as the candidate NTSR genes. The gene expression of these candidate genes in R populations was higher than in S populations, which provided a basis for our subsequent analysis of resistance mechanisms. A total of nine genes were selected as candidate genes that may confer NTSR. These genes were also selected to confirm the reliability of the RNA-seq results by qRT-PCR. In our work, we divided the nine candidate genes into seven protein families related to herbicide transport and metabolism: one P450, one ABC transporter, one LAC, one NCED, two PAPs, one SPS and two proteins related to immune or disease resistance. The candidate genes may take part in FE resistance via secondary metabolite biosynthesis, transport and catabolism,

signal transduction mechanisms, as well as defense mechanisms (Table S2). The qRT-PCR results for these DEGs were all substantially compatible with the RNA-seq findings in terms of their expression patterns. The Pearson related coefficients demonstrated highly significant positive correlations between the RNA-seq and qRT-PCR data, confirming the RNA-seq data's dependability (r = 0.9038, $p < 0.001$; Figure 5).

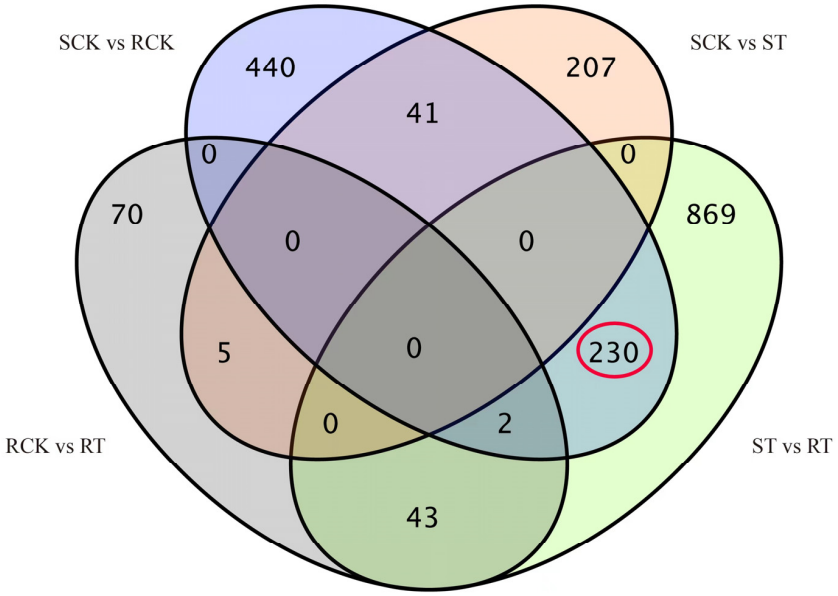

Figure 3. Venn diagram of upregulated DEGs after fenoxaprop-P-ethyl treatment in *A. japonicus* at 24 h. Red circle represents the 230 upregulated DEGs coexpressed by two treatments between the two populations.

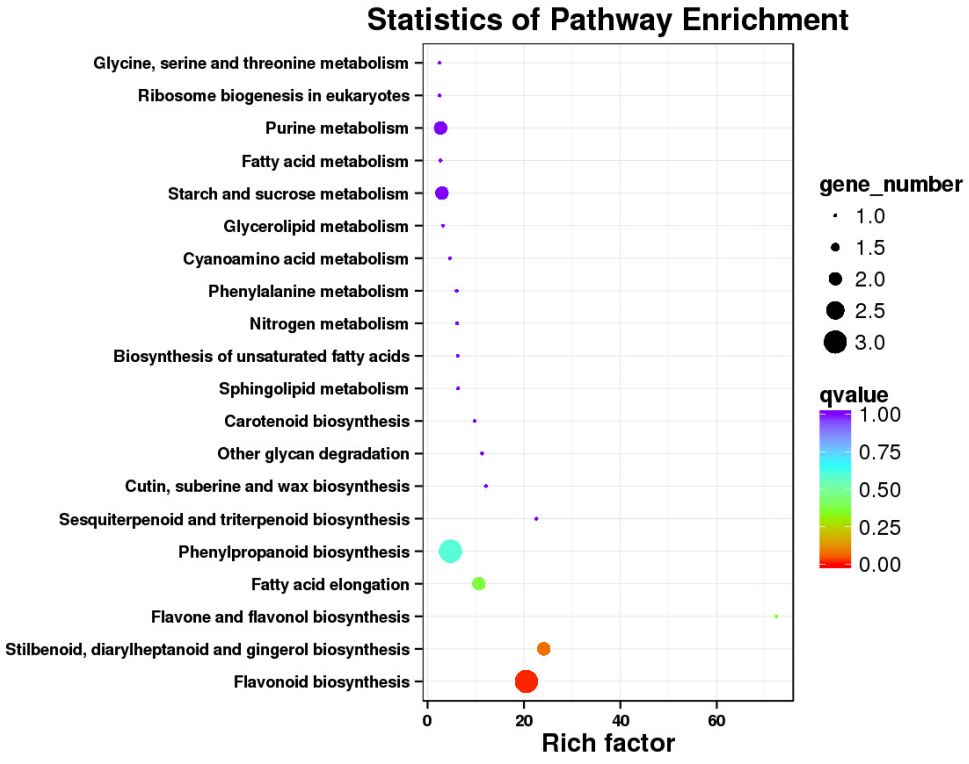

Figure 4. KEGG pathway enrichment bubble diagram of 230 upregulated DEGs after fenoxaprop-P-ethyl treatment in *A. japonicus*. Rich factor indicates the ratio of DEGs in this pathway to all genes in this pathway. The q-value indicates the corrected *p* value (range from 0 to 1).

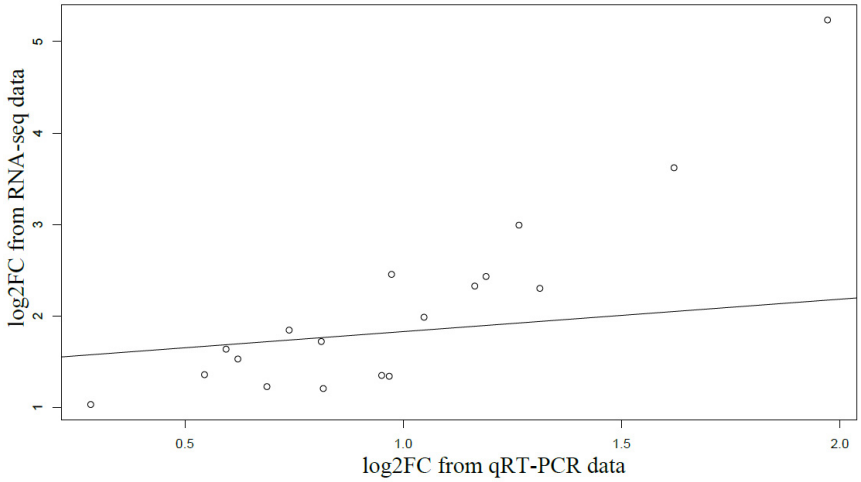

Figure 5. Linear correlation analysis using the Pearson correlation coefficient (r) of fold change (FC) data between the RNA-Seq and qRT-PCR validation.

3.4. Related Metabolic Enzyme Activity

LAC, SPS, PAP and NCED activities were assessed in both the R and S populations 0, 24, 48 and 72 h after FE treatments. Significantly, the activities of these enzymes in the R population were all significantly higher than those in the S population before FE treatment (0 h), which indicated intrinsically higher activities in the R population compared to the S population (Figure 6).

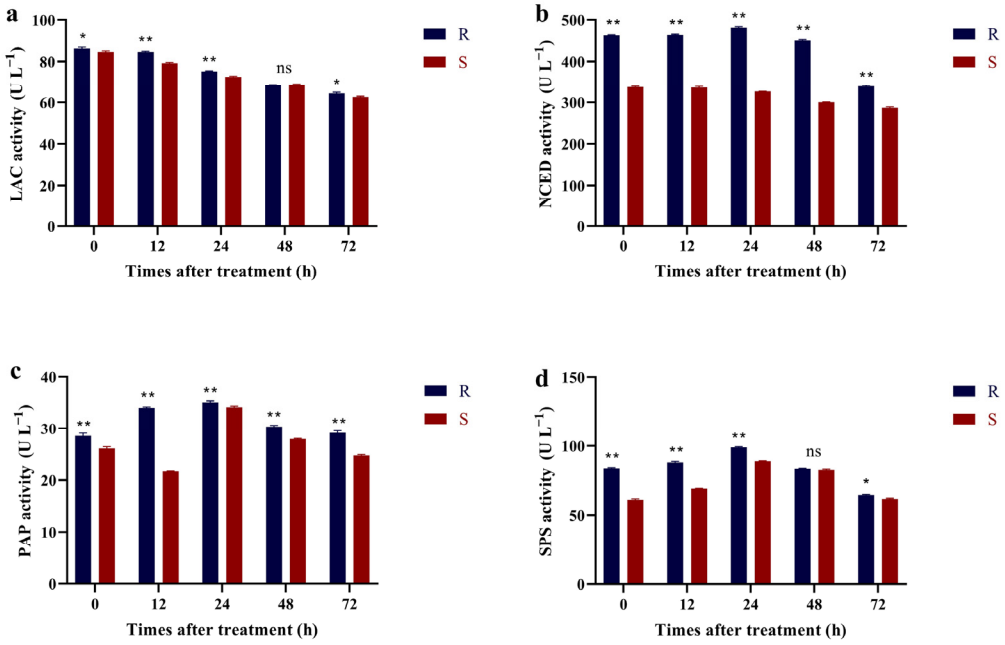

Figure 6. The activity of (**a**) LAC, (**b**) NCED, (**c**) PAP and (**d**) SPS in *A. japonicus* after 0 h, 12 h, 24 h, 48 h and 72 h of FE treatment. Values are means ± SEs based on 10 replicates pooled from two experimental repeats. Asterisks indicate statistically significant differences (Student's *t* test; * $p < 0.05$, ** $p < 0.01$, ns, no significant difference).

The FE treatment distinctly suppressed the activities of LAC in both R and S populations of A. japonicus. The LAC activity of the R and S populations reached a maximum of 86.50 U·L^{-1} and 84.63 U·L^{-1} at 0 h, respectively, and decreased the minimum of 64.49 U·L^{-1} and 62.58 U·L^{-1} at 72 h, respectively (Figure 6a). The activities of NCED in the R population were all significantly higher than those in the S population by 1.1~1.5-fold at

all time points (Figure 6b). After FE treatment, NCED activity increased in the R population and peaked at 24 h (481.50 $U \cdot L^{-1}$) and then decreased to 339.22 $U \cdot L^{-1}$ at 72 h. However, NCED activity in the S population was consistently decreased after FE treatment, ranging from 337.10 $U \cdot L^{-1}$ at 12 h to 286.76 $U \cdot L^{-1}$ at 72 h. The activities of PAP in the R population were all significantly higher than those in the S population at all time points tested (Figure 6c). PAP activity was increased in the R population and peaked at 24 h (35.08 $U \cdot L^{-1}$) and then decreased to 29.13 $U \cdot L^{-1}$ at 72 h. However, PAP activity in the S population first decreased at 12 h and then increased to 33.14 $U \cdot L^{-1}$ at 24 h and finally decreased to 24.76 $U \cdot L^{-1}$ at 72 h. A consistent trend was observed for SPS activities in the R and S populations (Figure 6d). After FE treatment, the activities of SPS in both populations of *A. japonicus* peaked at 24 h and then decreased to 29.14 $U \cdot L^{-1}$ in the R population and 24.76 $U \cdot L^{-1}$ of the S population at 72 h. In general, the activities of LAC, SPS, PAP and NCED in the R population were all significantly higher than those in the S population at 0 h and 24 h. These results are consistent with the gene expression patterns obtained from the RNA-seq data.

4. Discussion

NTSR has been reported to be the major type of resistance to ACCase-inhibiting herbicides in grass weeds [5]. These studies mostly focused on oxidase, reductase, P450, GST, GT, ABC transporter, etc. [6,7,12,18,21]. To uncover the NTSR-related genes in *A. japonicus* resistant to FE, we primarily focused on genes that were upregulated between the R and S populations both before and after treatment with FE, which implies that these genes were both constitutively and herbicide-induced upregulated DEGs. A total of 230 upregulated DEGs coexpressed were identified and annotated. Based on the knowledge of herbicide metabolic and resistance mechanisms, nine genes, including one P450 gene (*CYP75B4*), one ABC transporter gene (*ABCG36*), one LAC gene (*LAC15*), one NCED gene (*NCED5*), two PAP genes (*PAP4, PAP15*), one SPS gene (*SPS3*), one protein related to disease resistance gene (*RGA3*) and one immune protein gene (*R1B-17*), were finally selected as candidate genes and further validated by qPT-PCR.

One of the largest families of protein-coding genes involved in plant growth, development and biotic and abiotic stress tolerance is the plant P450 family [23]. Numerous studies have demonstrated that P450 is one of the most important enzyme systems participating in herbicide detoxification and resistance. *CYP75* genes are involved in the biosynthesis of the majority of plant secondary metabolites in the adaptation to biotic and abiotic stress [24]. In the previous study, Chen et al. (2018) demonstrated that FE resistance in AHFD-3 could be reduced using P450 inhibitors, and a significantly higher P450 content was observed in AHFD-3 compared to the control populations [12]. Furthermore, Chen et al. (2018) also found that *CYP72A* was related to resistance to FE in *A. japonicus* [12]. This result was slightly different from the P450 gene subfamily of *CYP75* identified in our results, which may be caused by the materials having different processing times and/or FE spraying rates. Nevertheless, Zhao et al. (2018) reported that the P450-based metabolism was important in conferring resistance to FE and mesosulfuron-methyl in *A. aequalis* [25]. *CYP71AH11* was induced by the application of 2,4-D-, and bromoxynil seemed to enhance herbicide tolerance and selectivity in tobacco [26]. Pan et al. (2022) found that the upregulation of *CYP81A68* gene in *Echinochloa crus-galli* endowed metabolic resistance to widely used ACCase-inhibiting herbicides in rice fields [27]. In general, studies of resistance in *A. japonicus* are consistent with the fact that P450-based metabolism is important in conferring resistance to ACCase-inhibiting herbicides [5,12,18,21,25].

Plant ABC transporters function in multiple physiological processes, such as the transport of hormones, lipids, metals, secondary metabolites, plant-microbe interactions, and the detoxification of xenobiotics, including herbicides [28]. Several ABC transporters have been documented to be involved in weed resistance to herbicides. In *Arabidopsis thaliana*, the overexpression of *AtPgp1* or *psNTP9*, which belong to the genes encoding members of the multidrug resistant family of ABC transporters, has been shown to confer resistance to

multiple herbicides [29]. *AtPDR11*, a member of the ABC transporter superfamily, is a potential transporter of paraquat [30]. Recently, Pan et al. found that the overexpression of the *ABCC8* transporter endows resistance to glyphosate in *Echinochloa colona* [8]. Furthermore, studies have shown that ABC transporter genes may play roles in conferring resistance in *A. aequalis* [31], *B. syzigachne* [19], *Myosoton aquaticum* [32] and *Capsella bursa-pastoris* [33]. In the present study, we identified an ABC transporter gene (*ABCG36*) that was constitutively overexpressed and upregulated in the R population with FE application. Therefore, it is likely that ABC transporters are involved in metabolic resistance to FE in *A. japonicus*.

In addition, we screened four infrequent metabolic-related enzyme genes, and their gene expression levels and enzyme activities in the R population were observably different from those in the S population. LAC is an oxidoreductase with application value in the oxidative degradation of carcinogenic compounds and toxics [34]. The *Verticillium wilt* resistance of transgenic *A. thaliana* was dramatically enhanced by the overexpression of *GhLAC15* [35]. The molecular mechanism of LAC has been clearly studied in pesticides. LAC was the target of the fungicide 4-chlorocinnamaldehyde thiosemicarbazide, which is an effective fungicide for preventing infections of the wheat root [36]. Huang et al. found that the laccase-coding genes could endow herbicides with degradation or detoxification, which indicated that laccase could be one of the most important enzymatic pathways involved in atrazine/isoproturon degradation and detoxification in rice [37]. However, in studies of resistance to FE in *B. syzigachne*, *bsy-Laccase* expression and laccase activity showed the opposite trend [38]. This result was inconsistent with our study. The enzyme complex contains multiple genes and different regulatory factors in the same family, and the regulatory mechanism between them can be divided into positive regulation and negative regulation. Therefore, we speculated that LAC may be a candidate gene involved in the mechanism of NTSR of *A. japonicus*.

As an important plant hormone, abscisic acid (ABA) not only participates in plant growth and development, seed dormancy and other physiological functions, but also regulates plant adaptability to abiotic stress as a signaling molecule, mainly including drought, high temperature, chilling and salinization [39]. In the ABA biosynthetic pathway, NCED is the key rate-limiting enzyme. It has been well established that NCED expression levels participate in the regulation of ABA biosynthesis during the plant's development (fruit ripening) and physiological changes (wilting) [40]. *RhNCED1* accelerates rose flower senescence, and *VvNCED1* genes are closely related to grape berry ripening [41,42]. By controlling ABA biosynthesis, *OsNCED3* regulates rice seed dormancy, foliage senescence, plant development, and abiotic stress tolerance [43]. *OsNCED3* mediates seed dormancy, abiotic stress tolerance, plant growth, and leaf wither by regulating ABA biosynthesis in rice. The *AhNCED1* gene was significantly upregulated in peanut under high salinity and dehydration but not under heat or cold stress [44]. It was demonstrated that the increase in *GaNCED1* gene expression in *Galium aparine* is an effect common to all auxin herbicides compared to the effects of auxin herbicides from different chemical classes, such as quinmerac, dicamba, and picloram [45]. Our results showed that the change in NCED activity was not visible with the extension of FE treatment time, but there was a significant difference between the R and S populations. In addition, the transcriptional sequencing results also showed that the expression level of the NCED gene in the R populations was much higher than in the S populations.

Plant PAP generally mediates phosphorus acquisition and redistribution [46]. A wide variety of phosphoric acids and anhydrides can be hydrolyzed by PAPs when they are functionally active at an acidic or neutral pH [47]. Increasing the expression level of PAPs, which hydrolyze organic phosphate into inorganic phosphate (Pi), plays a crucial role in the response to Pi starvation in plants. In comparison to Pi-sufficient circumstances, the overexpression of *PAP9* in *A. thaliana* enhanced more APase activity under Pi-deficient conditions [47]. Pi starvation upregulated *GmPAP7a* and *GmPAP7b*, and the overexpression these genes in the hairy roots of soybean significantly increased the root-associated APase activities and thus facilitated extracellular ATP utilization [48]. In addition, PAPs played

a defensive role against environmental stresses and conferred stress adaptation to the stress-tolerant plant. The overexpression of *GmPAP2.1* conferred soybean mosaic virus resistance in a susceptible soybean cultivar [49]. The expression of *PgPAP18* under abiotic stresses was examined in *Pennisetum glaucum*, wherein it revealed transcript upregulation in the presence of heat, salinization, drought and heavy metals [50].

Sucrose-phosphate synthase is a key enzyme in sucrose biosynthesis in both the photosynthetic and nonphotosynthetic tissues of plants [51]. Abiotic stress, including osmotic stress, low temperature, salinization and drought, can regulate SPS activity and thus affect sugarcane sugar synthesis and other biological functions. In rice under low-temperature stress, changes in the SPS phosphorylation process lead to changes in SPS activity and then affect glucose metabolism in plants, thus participating in the response to abiotic stress [52]. The SPS activity of wheat was significantly increased under low temperature and drought stress [53,54]. SPS activity tends to increase when plants are exposed to severe salt stress or drought, thereby increasing the contents of soluble sugars such as sucrose and altering the osmotic pressure of the cells to help the plants withstand the stressful environment [55]. *A. thaliana* cultivated in media with different osmotic potential suggested that *AtSPS2F* and *AtSPS4F* had a role in response to osmotic stress [56].

Currently reported PAP- and SPS-related studies mainly involve high or low temperature, drought, salinization, etc., all of which are common abiotic stresses. However, no studies have been reported on PAP and SPS in weeds against herbicide, which is also a lethal abiotic stress. The activity of these two enzymes peaked 24 h after FE treatment and then decreased, indicating that they responded strongly to herbicides at this stage. Combined with our transcriptome data, the gene expression of the R population was much higher than that of the S population. Nevertheless, further rigorous studies are required to assess direct evidence for the roles of these genes in the herbicide metabolism and resistance.

Plants have evolved a series of genes relevant to disease resistance and immunity to survive in the harsh living environment [57]. By triggering a series of early defense responses and upregulating relevant gene expression, disease resistance proteins may boost resistance [58]. Xie et al. demonstrated that PR1 proteins are rapidly and strongly induced during *Magnaporthe grisea* infection in rice and increase *PR1* gene expression involved in the defense pathway [59]. *RGA3* is associated with blast resistance in rice [60]. Kim et al. reported a method to engineer disease resistance in plants by means of an endogenous disease resistance gene, *RPS5*, from *A. thaliana* [61]. Disease resistance genes are regulated at the beginning of the plant response to biotic or abiotic stress. Although disease resistance and immune genes play an important role in resistance to various diseases, there have been few reports regarding the correlation between these genes and herbicide metabolism and resistance. Wang et al. investigated the multiple resistance mechanism in *B. syzigachne* and found that two immune protein genes (*R1B-16* and *R1A-10*) and four disease resistance protein genes (*At1g15890*, *At1g58602*, *RGA3* and two *RPP13s*) were upregulated between the R and S biotypes and that these genes may play an essential role in herbicide resistance [62]. Here, we identified one protein related to one disease resistance gene (*RGA3*) and one immune protein gene (*R1B-17*) that were constitutively overexpressed and were significantly upregulated in the R population with FE application. Nevertheless, whether these genes were involved in herbicide metabolism needs further validation.

5. Conclusions

In the present study, transcriptome analysis resulted in the identification of nine overlapping upregulated genes, which were constitutively overexpressed and upregulated by FE application in the R population. These genes included not only well-known CYP and ABC transporter genes, but also LAC, SPS, NCED and PAP genes, which are rarely reported in herbicide studies. The reliability of these genes was validated using qRT-PCR. Furthermore, the activity assay of LAC, NCED, PAP and SPS revealed that the activities of these enzymes in the R population were all significantly higher than those

in the S population at 0 h, 12 h, 24 h and 72 h. Our results showed that these genes may participate in resistance via secondary metabolite biosynthesis, signal transduction mechanisms, transport and catabolism and defense mechanisms. However, future work will involve a functional validation assay to validate the genes' participation in herbicide resistance. Accordingly, we conclude that CYP, ABC transporter and LAC genes may participate in *A. japonicus* resistance. These genes identified in the present study provide new insights into the resistance mechanism of weeds in response to herbicide. Overall, our study represents an important step forward in understanding NTSR and the resistance prevention and management of *A. japonicus* in China.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13061587/s1, Table S1: Gene Ontology (GO) analysis of the upregulated differentially expressed genes in *Alopecurus japonicus*; Table S2: Information on candidate genes.

Author Contributions: Conceptualization, H.X., X.Y. and R.W.; methodology, H.X., X.Y. and R.W.; software, X.Y., S.L., J.C., Q.L. and F.X.; validation, H.X., X.Y. and Q.L.; formal analysis, H.X., X.Y., S.L., J.C., Q.L., L.S., W.S., F.X., L.D. and R.W.; investigation, X.Y. and Q.L.; resources, H.X., L.D. and R.W.; data curation, H.X. and R.W.; writing—original draft preparation, H.X. and X.Y.; writing—review and editing, H.X., X.Y., S.L., J.C., Q.L., L.S., W.S., F.X., L.D. and R.W.; visualization, H.X. and X.Y.; supervision, R.W. and L.D.; project administration, R.W. and L.D.; funding acquisition, H.X. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Joint Fund for Scientific and Technological Research and Development of Henan (222301420110) and the Distinguished Young Scholars from the Henan Academy of Agricultural Sciences (2022JQ04).

Data Availability Statement: Not applicable.

Acknowledgments: The authors gratefully acknowledge the Joint Fund for Scientific and Technological Research and Development of Henan and the Distinguished Young Scholars from the Henan Academy of Agricultural Sciences for providing funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Zhu, J.W.; Wang, J.; DiTommaso, A.; Zhang, C.X.; Zheng, G.P.; Liang, W.; Islam, F.; Yang, C.; Chen, X.X.; Zhou, W.J. Weed research status, challenges, and opportunities in China. *Crop Prot.* **2020**, *134*, 104449. [CrossRef]
2. Gaines, T.A.; Duke, S.O.; Morran, S.; Rigon, C.A.G.; Tranel, P.J.; Küpper, A.; Dayan, F.E. Mechanisms of evolved herbicide resistance. *J. Biol. Chem.* **2020**, *295*, 10307–10330. [CrossRef]
3. Comont, D.; Lowe, C.; Hull, R.; Crook, L.; Hicks, H.L.; Onkokesung, N.; Beffa, R.; Childs, D.Z.; Edwards, R.; Freckleton, R.P.; et al. Evolution of generalist resistance to herbicide mixtures reveals a trade-off in resistance management. *Nat. Commun.* **2020**, *11*, 3086. [CrossRef] [PubMed]
4. Fang, J.P.; He, Z.Z.; Liu, T.T.; Li, J.; Dong, L.Y. A novel mutation Asp-2078-Glu in ACCase confers resistance to ACCase herbicides in barnyardgrass (*Echinochloa crus-galli*). *Pestic. Biochem. Physiol.* **2020**, *168*, 104634. [CrossRef]
5. Ghanizadeh, H.; Harrington, K.C. Non-target site mechanisms of resistance to herbicides. *Crit. Rev. Plant Sci.* **2017**, *36*, 24–34. [CrossRef]
6. 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. [CrossRef]
7. Chen, W.; Wu, L.M.; Wang, J.Z.; Yu, Q.; Bai, L.Y.; Pan, L. Quizalofop-p-ethyl resistance in *Polypogon fugax* involves glutathione S-transferases. *Pest Manag. Sci.* **2020**, *76*, 3800–3805. [CrossRef]
8. Pan, L.; Yu, Q.; Wang, J.Z.; Han, H.P.; Mao, L.F.; Nyporko, A.; Maguza, A.; Fan, L.J.; Bai, L.Y.; Powles, S. An ABCC-type transporter endowing glyphosate resistance in plants. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, 33846264. [CrossRef] [PubMed]
9. Von, R.U.; Hüttl, R.; Lottspeich, F.; Gierl, A.; Frey, M. Two glucosyltransferases are involved in detoxification of benzoxazinoids in maize. *Plant J.* **2001**, *28*, 633–642. [CrossRef]
10. Zhao, N.; Yan, Y.Y.; Wang, H.Z.; Bai, S.; Wang, Q.; Liu, W.T.; Wang, J.X. Acetolactate synthase overexpression in mesosulfuron-methyl-resistant shortawn foxtail (*Alopecurus aequalis Sobol.*): Reference gene selection and herbicide target gene expression analysis. *J. Agric. Food Chem.* **2018**, *66*, 9624–9634. [CrossRef]
11. Bi, Y.L.; Liu, W.T.; Guo, W.L.; Li, L.X.; Yuan, G.H.; Du, L.; Wang, J.X. Molecular basis of multiple resistance to ACCase- and ALS-inhibiting herbicides in *Alopecurus japonicus* from China. *Pestic. Biochem. Physiol.* **2016**, *126*, 22–27. [CrossRef] [PubMed]

12. Chen, G.Q.; Xu, H.L.; Zhang, T.; Bai, C.Q.; Dong, L.Y. Fenoxaprop-P-ethyl resistance conferred by cytochrome P450s and target site mutation in *Alopecurus japonicus*. *Pest Manag. Sci.* **2018**, *74*, 1694–1703. [CrossRef] [PubMed]
13. Mohamed, I.A.; Li, R.; You, Z.; Li, Z. Japanese foxtail (*Alopecurus japonicus*) resistance to fenoxaprop and pinoxaden in China. *Weed Sci.* **2012**, *60*, 167–171. [CrossRef]
14. Tang, H.; Li, J.; Dong, L.Y.; Dong, A.B.; Lü, B.; Zhu, X.D. Molecular bases for resistance to acetyl-coenzyme A carboxylase inhibitor in Japanese foxtail (*Alopecurus japonicus*). *Pest Manag. Sci.* **2012**, *68*, 1241–1247. [CrossRef]
15. Xu, H.L.; Zhu, X.D.; Wang, H.C.; Li, J.; Dong, L.Y. Mechanism of resistance to fenoxaprop in Japanese foxtail (*Alopecurus japonicus*) from China. *Pestic. Biochem. Physiol.* **2013**, *107*, 25–31. [CrossRef]
16. 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 Manag. Sci.* **2015**, *70*, 1894–1901. [CrossRef]
17. Chen, G.Q.; Wang, L.Y.; Xu, H.L.; Wu, X.B.; Pan, L.; Dong, L.Y. Cross-resistance Patterns to Acetyl-CoA Carboxylase Inhibitors Associated with Different Mutations in Japanese Foxtail (*Alopecurus japonicus*). *Weed Sci.* **2017**, *65*, 444–451. [CrossRef]
18. Yu, X.Y.; Tang, W.; Yang, Y.J.; Zhang, J.P.; Lu, Y.L. Comparative transcriptome analysis revealing the different germination process in aryloxyphenoxypropionate-resistant and APP-susceptible Asia minor bluegrass (*Polypogon fugax*). *Plants* **2020**, *9*, 1191. [CrossRef]
19. Pan, L.; Gao, H.; Xia, W.W.; Zhang, T.; Dong, L.Y. Establishing a herbicide-metabolizing enzyme library in Beckmannia syzigachne to identify genes associated with metabolic resistance. *J. Exp. Bot.* **2016**, *67*, 1745–1757. [CrossRef]
20. Franco, O.S.; Goldberg, C.A.; Walker, A.; Brazier, H.M.; Onkokesung, N.; Edwards, R. Non-target site herbicide resistance is conferred by two distinct mechanisms in black-grass (*Alopecurus myosuroides*). *Front. Plant Sci.* **2021**, *12*, 636652. [CrossRef]
21. Gaines, T.A.; Lorentz, L.; Figge, A.; Herrmann, J.; Maiwald, F.; Ott, M.; Han, H.P.; Busi, R.; Yu, Q.; Powles, S.B.; et al. RNA-Seq transcriptome analysis to identify genes involved in metabolism-based diclofop resistance in Lolium rigidum. *Plant J.* **2014**, *78*, 865–876. [CrossRef] [PubMed]
22. Xu, H.L.; Li, J.; Wu, R.H.; Su, W.C.; Wu, X.B.; Wang, L.Y.; Dong, L.Y. Identification of reference genes for studying herbicide resistance mechanisms in Japanese Foxtail (*Alopecurus japonicus*). *Weed Sci.* **2017**, *65*, 557–566. [CrossRef]
23. Tan, L.R.; Lu, Y.C.; Zhang, J.J.; Luo, F.; Yang, H. A collection of cytochrome P450 monooxygenase genes involved in modification and detoxification of herbicide atrazine in rice (*Oryza sativa*) plants. *Ecotoxicol. Environ. Saf.* **2015**, *119*, 25–34. [CrossRef]
24. Xiao, Y.; Wen, J.; Meng, R.; Meng, Y.; Zhou, Q.; Nie, Z.L. The expansion and diversity of the *CYP75* gene family in *Vitaceae*. *PeerJ* **2021**, *9*, e12174. [CrossRef]
25. Zhao, N.; Yan, Y.Y.; Ge, L.A.; Zhu, B.L.; Liu, W.T.; Wang, J.X. Target site mutations and cytochrome P450s confer resistance to fenoxaprop-P-ethyl and mesosulfuron-methyl in *Alopecurus aequalis*. *Pest Manag. Sci.* **2018**, *75*, 204–214. [CrossRef]
26. Ohkawa, H.; Inui, H. Metabolism of agrochemicals and related environmental chemicals based on cytochrome P450s in mammals and plants. *Pest Manag. Sci.* **2015**, *71*, 824–828. [CrossRef] [PubMed]
27. Pan, L.; Guo, Q.S.; Wang, J.Z.; Shi, L.; Yang, X.; Zhou, Y.Y.; Yu, Q.; Bai, L.Y. Cyp81a68 confers metabolic resistance to ALS and ACCase-inhibiting herbicides and its epigenetic regulation in echinochloa crus-galli. *J. Hazard. Mater.* **2022**, *428*, 128225. [CrossRef]
28. Zhang, X.D.; Zhao, K.X.; Yang, Z.M. Identification of genomic ATP binding cassette (ABC) transporter genes and Cd-responsive ABCs in *Brassica napus*. *Gene* **2018**, *664*, 139–151. [CrossRef] [PubMed]
29. Windsor, B.; Roux, S.J.; Lloyd, A. Multiherbicide tolerance conferred by *AtPgp1* and apyrase overexpression in *Arabidopsis thaliana*. *Nat. Biotechnol.* **2003**, *21*, 428–433. [CrossRef]
30. Xi, J.; Xu, P.; Xiang, C.B. Loss of *AtPDR11*, a plasma membrane-localized ABC transporter, confers paraquat tolerance in *Arabidopsis thaliana*. *Plant J.* **2012**, *69*, 782–791. [CrossRef]
31. 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*, 1391. [CrossRef]
32. Liu, W.T.; Bai, S.; Zhao, N.; Jia, S.S.; Li, W.; Zhang, L.L.; Wang, J.X. Non-target site-based resistance to tribenuron-methyl and essential involved genes in *Myosoton aquaticum* (L.). *BMC Plant Biol.* **2018**, *18*, 225. [CrossRef]
33. Zhang, X.L.; Wang, H.Z.; Bei, F.; Wu, C.X.; Zhang, L.L.; Jia, S.S.; Wang, J.X.; Liu, W.T. Investigating the mechanism of metabolic resistance to tribenuron-methyl in *Capsella bursa-pastoris* (L.) Medik. by full-length transcriptome assembly combined with RNA-Seq. *J. Agric. Food Chem.* **2021**, *69*, 3692–3701. [CrossRef] [PubMed]
34. Sharma, B.; Dangi, A.K.; Shukla, P. Contemporary enzyme based technologies for bioremediation: A review. *J. Environ. Manag.* **2018**, *210*, 10–22. [CrossRef]
35. Zhang, Y.; Wu, L.Z.; Wang, X.F.; Chen, B.; Zhao, J.; Cui, J.; Li, Z.K.; Yang, J.; Wu, L.Q.; Wu, J.H.; et al. The cotton laccase gene *GhLAC15* enhances Verticillium wilt resistance via an increase in defence-induced lignification and lignin components in the cell walls of plants. *Mol. Plant Pathol.* **2019**, *20*, 309–322. [CrossRef] [PubMed]
36. Wang, Z.W.; Peng, Q.; Gao, X.; Zhong, S.; Fang, Y.; Yang, X.L.; Ling, Y.; Liu, X.L. Novel fungicide 4-chlorocinnamaldehyde thiosemicarbazide (PMDD) inhibits laccase and controls the causal agent of take-all disease in wheat, Gaeumannomyces graminis var. tritici. *J. Agric. Food Chem.* **2020**, *68*, 5318–5326. [CrossRef] [PubMed]
37. Huang, M.T.; Lu, Y.C.; Zhang, S.; Luo, F.; Yang, H. Rice (*Oryza sativa*) laccases involved in modification and detoxification of herbicides atrazine and isoproturon residues in plants. *J. Agric. Food Chem.* **2016**, *64*, 6397–6406. [CrossRef]

38. Pan, L.; Zhao, H.W.; Yu, Q.; Bai, L.Y.; Dong, L.Y. miR397/Laccase gene mediated network improves tolerance to fenoxaprop-P-ethyl in *Beckmannia syzigachne* and *Oryza sativa*. *Front. Plant Sci.* **2017**, *8*, 879. [CrossRef] [PubMed]
39. Zhang, W.X.; Ruan, J.H.; Ho, T.H.D.; You, Y.; Yu, T.T.; Quatrano, R.S. Cis-regulatory element based targeted gene finding: Genome-wide identification of abscisic acid- and abiotic stress-responsive genes in *Arabidopsis thaliana*. *Bioinformatics* **2005**, *21*, 3074–3081. [CrossRef]
40. Rodrigo, M.J.; Alquezar, B.; Zacarías, L. Cloning and characterization of two 9-cis-epoxycarotenoid dioxygenase genes, differentially regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis L. Osbeck*). *J. Exp. Bot.* **2006**, *57*, 633–643. [CrossRef]
41. Dong, Y.H.; Guo, J.X. Transcriptional analysis of 9-cis-epoxycarotenoid dioxygenase, glucosyltransferase, 8′-hydroxylase and b-glucosidase genes that regulate abscisic acid homeostasis around the onset of grape berry ripening. *J. Agric. Sci. Technol. A* **2012**, *2*, 873–881. (In Chinese)
42. Luo, J.; Chen, S.J.; Cao, S.H.; Zhang, T.; Li, R.R.; Chan, Z.L.; Wang, C.Y. Rose (*Rosa hybrida*) ethylene responsive factor 3 promotes rose flower senescence via direct activation of the abscisic acid synthesis-related 9-cis-epoxycarotenoid dioxygenase gene. *Plant Cell Physiol.* **2021**, *62*, 1030–1043. [CrossRef]
43. Huang, Y.; Guo, Y.M.; Liu, Y.T.; Zhang, F.; Wang, Z.K.; Wang, H.Y.; Wang, F.; Li, D.P.; Mao, D.D.; Luan, S.; et al. 9-cis-epoxycarotenoid dioxygenase 3 regulates plant growth and enhances multi-abiotic stress tolerance in rice. *Front. Plant Sci.* **2018**, *9*, 162. [CrossRef]
44. Wan, X.R.; Li, L. Regulation of ABA level and water-stress tolerance of Arabidopsis by ectopic expression of a peanut 9-cis-epoxycarotenoid dioxygenase gene. *Biochem. Biophys. Res. Commun.* **2006**, *347*, 1030–1038. [CrossRef]
45. Kraft, M.; Kuglitsch, R.; Kwiatkowski, J.; Frank, M.; Grossmann, K. Indole-3-acetic acid and auxin herbicides up-regulate 9-cis-epoxycarotenoid dioxygenase gene expression and abscisic acid accumulation in cleavers (*Galium aparine*): Interaction with ethylene. *J. Exp. Bot.* **2007**, *58*, 1497–1503. [CrossRef] [PubMed]
46. Eliécer, E.M. The maize (*Zea mays ssp. mays var. B73*) genome encodes 33 members of the purple acid phosphatase gene family. Front. *Plant Sci.* **2015**, *6*, 341. [CrossRef]
47. Zhu, X.L.; Lee, S.Y.; Yang, W.T.; Lee, S.W.; Baek, D.W.; Li, M.S.; Kim, D.H. The burholderia pyrrocinia purple acid phosphatase Pap9 mediates phosphate acquisition in plants. *J. Exp. Biol.* **2019**, *62*, 342–350. [CrossRef]
48. Zhu, S.N.; Chen, M.H.; Liang, C.Y.; Xue, Y.B.; Lin, S.L.; Tian, J. Characterization of purple acid phosphatase family and functional analysis of *GmPAP7a/7b* involved in extracellular ATP utilization in Soybean. *Front. Plant Sci.* **2020**, *11*, 661. [CrossRef]
49. Widyasari, K.; Tran, P.T.; Shin, J.; Son, H.; Kim, K.H. Overexpression of a purple acid phosphatase *GmPAP2.1* confers soybean mosaic virus resistant in a susceptible soybean cultivar. *J. Exp. Bot.* **2021**, *73*, 1623–1642. [CrossRef]
50. Reddy, C.S.; Kim, K.M.; James, D.; Varakumar, P.; Reddy, M.K. PgPAP18, a heat-inducible novel purple acid phosphatase 18-like gene (PgPAP18-like) from *Pennisetum glaucum*, may play a crucial role in environmental stress adaptation. *Acta Physiol. Plant.* **2017**, *39*, 54. [CrossRef]
51. Taneja, D.; Das, N. Molecular cloning, sequence analyses, and expression studies of sucrose-phosphate synthase in the potato (*Solanum tuberosum* L.) cultivars. *Acta Physiol. Plant.* **2014**, *36*, 2253–2269. [CrossRef]
52. Almadanim, M.C.; Alexandre, B.M.; Rosa, M.T.G.; Sapeta, H.; Leitão, A.E.; Ramalho, J.C.; Lam, T.T.; Negrão, S.; Abreu, I.A.; Oliveira, M.M. Rice calcium-dependent protein kinase OsCPK17 targets plasma membrane intrinsic protein and sucrose-phosphate synthase and is required for a proper cold stress response. *Plant Cell Environ.* **2017**, *40*, 1197–1213. [CrossRef] [PubMed]
53. Nemati, F.; Ghanati, F.; Ahmadi Gavlighi, H.; Sharifi, M. Comparison of sucrose metabolism in wheat seedlings during drought stress and subsequent recovery. *Biol. Plant.* **2018**, *62*, 595–599. [CrossRef]
54. Zhang, W.J.; Wang, J.Q.; Huang, Z.L.; Mi, L.; Xu, K.F.; Wu, J.J.; Fan, Y.H.; Ma, S.Y.; Jiang, D.G. Effects of low temperature at booting stage on sucrose metabolism and endogenous hormone contents in winter wheat spikelet. *Front. Plant Sci.* **2019**, *10*, 498. [CrossRef] [PubMed]
55. María Gloria, S.G.; Gerardo, A.A.; José, L.B.; León Francisco, R.H.; Joel Edmundo, L.M.; Lenin, S.C.; Yazmín, C.A.; Miguel, M.T. *Arabidopsis thaliana* sucrose phosphate synthase (sps) genes are expressed differentially in organs and tissues, and their transcription is regulated by osmotic stress. *Gene Expression Patterns* **2017**, *25–26*, 92–101. [CrossRef]
56. Solís-Guzmán, M.G.; Argüello-Astorga, G.; López-Bucio, J.; Ruiz-Herrera, L.F.; López-Meza, J.E.; Sánchez-Calderón, L.; Carreón-Abud, Y.; Martínez-Trujillo, M. *Arabidopsis thaliana* sucrose phosphate synthase (sps) genes are expressed differentially in organs and tissues, and their transcription is regulated by osmotic stress. *Gene Expr. Patterns* **2017**, *25–26*, 92–101. [CrossRef] [PubMed]
57. Cai, Q.; Liang, C.; Wang, S.K.; Hou, Y.N.; Gao, L.; Liu, L.; He, W.R.; Ma, W.B.; Mo, B.X.; Chen, X.M. The disease resistance protein SNC1 represses the biogenesis of microRNAs and phased siRNAs. *Nat. Commun.* **2018**, *9*, 5080. [CrossRef] [PubMed]
58. Yan, B.W.; Liu, W.Z.; Yu, W.Q.; Li, P.; Zhao, C.J.; Yan, F.C.; Wang, G.J.; Zhang, L.G.; Xie, H.P.; Qiu, Z.M. Antitoxin EndoAI can induce disease resistance in tobacco as a protein elicitor. *Chem. Biol. Technol. Agric.* **2021**, *8*, 66. [CrossRef]
59. Xie, X.Z.; Xue, Y.J.; Zhou, J.J.; Zhang, B.; Chang, H.; Takano, M. Phytochromes regulate SA and JA signaling pathways in rice and are required for developmentally controlled resistance to *Magnaporthe grisea*. *Mol. Plant* **2011**, *4*, 688–696. [CrossRef]

60. Xu, X.; Hayashi, N.; Wang, C.T.; Fukuoka, S.; Kawasaki, S.; Takatsuji, H.; Jiang, C.J. Rice blast resistance gene Pikahei-1(t), a member of a resistance gene cluster on chromosome 4, encodes a nucleotide-binding site and leucine-rich repeat protein. *Mol. Breed.* **2014**, *34*, 691–700. [CrossRef]
61. Kim, S.H.; Qi, D.; Ashfield, T.; Helm, M.; Innes, R.W. Using decoys to expand the recognition specificity of a plant disease resistance protein. *Science* **2016**, *351*, 684–687. [CrossRef]
62. Wang, J.J.; Chen, J.C.; Li, X.J.; Li, D.; Li, Z.; Cui, H.L. Pro-197-Ser mutation in ALS and high-level GST activities: Multiple resistance to ALS and ACCase inhibitors in Beckmannia syzigachne. *Front. Plant Sci.* **2020**, *11*, 572610. [CrossRef] [PubMed]