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Candidate Genes Involved in Tolerance to Fenoxaprop-P-Ethyl in Rice Induced by Isoxadifen-Ethyl Hydrolysate

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Abstract: The metabolic resistance of plants to herbicides is similar to the herbicide metabolism process accelerated by safeners. The tolerance to fenoxaprop-P-ethyl (FE) is distinct among different varieties of rice in which phytotoxicity forms easily, resulting in the restricted use of FE in paddy. Safener effectively resolves this issue. This study showed that rice 9311 and Meixiangzhan No. 2 (MXZ) had different tolerance mechanisms to FE. Isoxadifen-ethyl hydrolysate (IH) alleviated FE the inhibition of rice growth. Transcriptome sequencing revealed numerous differentially expressed genes (DEGs) between the two varieties. A total of 31 metabolic enzyme genes related to herbicide detoxification were screened by analyzing the DEGs in different rice varieties or treatments. The results of the quantitative reverse transcription polymerase chain reaction indicated that 12 genes were potential metabolic genes resistant to FE in rice. Additionally, the enhanced expression of *GSTU6*, *DIMBOA UGT BX8*, and *ABCG39* was confirmed to be induced by safener. Taken together, our results demonstrated that the induced expression of these three genes might be crucial for resistance to herbicide phytotoxicity in crops. These results may help us to understand herbicide metabolism in crops and to develop novel strategies for the safe use of herbicides.

Keywords: metabolic resistance; metabolizing enzyme genes; non-target site resistance; *Oryza sativar*; safener

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1. Introduction

Weeds in agricultural fields seriously affect the growth of crops, resulting in reduced yields and lower quality cash crops [1]. Chemical herbicides are effective tools for weed control in the field [2]. Fenoxaprop-P-ethyl (FE) belongs to an aryloxyphenoxypropionate herbicide that effectively controls annual gramineous weeds in paddies such as *Digitaria sanguinalis*, *Echinochloa crus galli*, and *Leptochloa chinensis* [3]. However, there were distinctions in the tolerance of different varieties of rice to FE. For example, *japonica* (in northern China) is more tolerant of FE than *indica* (in southern China) [4]. The negative impact (e.g., growth inhibition, leaf necrosis, and lower yields) of growth and yields is produced when FE is applied to certain sensitive rice varieties. Thus, after treatment with herbicides, the multiple pathways within plants will be activated to resist the phytotoxicity caused by the herbicides.

The mechanisms of plant resistance to herbicides are complex and diverse, and these mechanisms are usually divided into target-site resistance (TSR) and non-target-site resistance (NTSR). TSR involves gene mutation at the target site [5], changes in target enzyme activity, and overexpression of the target enzyme gene [6]. Resistance mechanisms other than TSR are called NTSR, and include the weakening of herbicide penetration, differences in absorption and translocation, and enhancement of detoxification and metabolism [7]. Metabolic resistance is the most widely studied [8] and involves detoxifying enzymes and proteins such as cytochrome P450 enzymes (CYPs), glutathione-s-transferases (GSTs),

UDP-glucuronosyltransferase (UGTs), and ATP-binding cassette (ABC) transporters. Research has shown that the metabolic rates of FE are significantly faster in resistant *Polypogon fugax* than in susceptible plants, which may be governed by multiple genes, such as CYPs, GSTs, GTs, and ABC transporters [9]. Cutti et al. determined that the ability to metabolize herbicides of the resistance *E. crus galli* is superior to the susceptible biotype [10].

Herbicide safeners protect crops from potential injuries caused by herbicides, which is the most effective and cost-effective strategy for resolving the phytotoxicity of herbicides. Isoxadifen-ethyl (IE) is an isoxazole safener, and it is effective for multiple herbicides (with various modes of action) in multiple crops, such as rice to FE [11] and maize to nicosulfuron [12,13]. IE accelerates the metabolism of foramtsulfuron by inducing the activity of CYPs or UGTs in corn [14]. IE significantly reduced the phytotoxicity of nicosulfuron in maize and increased *GSTIV*, *GST6*, *GST31*, and *MRP1* gene expression in maize after the mixture of IE + niscosulfuron [13]. It has also been demonstrated that *GSTIV* and *GST31* are strongly induced by the safener dichlormid [15,16]. Therefore, *GSTIV*, *GST6*, and *GST31* may be the key genes in the metabolization process of herbicides. The effect of safener IE on FE metabolism was studied in *E. crus galli*. The resistant biotype overexpressed the *GST1* by nearly four-fold and the *GSTF1* nearly three-fold after FE + IE compared to the susceptible biotype [10]. The activity of key enzymes and/or gene expression at different stages of herbicide detoxification is induced by the safener, accelerating the detoxification process and protecting the crop from injury [17,18]. This process is similar to the mechanism of resistance to herbicides in crops.

The metabolism of herbicides in plants is a combination of multiple stages and enzyme systems. It is a common mechanism for the safener protection of crops and the nontarget-site resistance of plants to herbicides. The search for genes related to herbicide metabolism is a common baseline for determining the mechanism of action of safeners and the NTSR of plants. Given this consideration, this paper uses transcriptomic techniques to analyze the genes related to FE metabolism in different tolerance-level rice varieties. Then, these genes were validated using safeners, providing a new strategy to evaluate genes for plant metabolism of herbicides, which is of significant value for the breeding of herbicide-resistant crops.

2. Materials and Methods

2.1. Materials and Reagents

Oryza sativa var *indica* cv. 9311 and cv. Meixiangzhan No. 2 (MXZ), which were provided by the Institute of Food Crops, Henan Academy of Agricultural Sciences (Zhengzhou, China), were used in this study. Herbicide FE, chemicals, and reagents (pure or analytical grade) were purchased from Sigma Chemical Company (Shanghai, China). The safener isoxadifen-ethyl hydrolysate (IH) was described in previous work [11].

2.2. Seedling Treatment and Determination of Physiological Indices

Rice pre-germinated seeds were planted in the pots containing soil mixture. Ten seeds were planted in each pot. Then, seeds were cultured in a growth cabinet with a 16/8 h day/night photocycle at 28/25 °C. For the whole-plant bioactivity assay, the cultivation and treatment of seedlings were both performed as described by Zhao [19]. When the seedlings reached a one-leaf stage in the growth cabinet, FE and/or IH were sprayed using a track sprayer. The treatments are as follows: FE dose: 22.5 g of active ingredient (a.i.) applied per hectare (g a.i. ha^{-1}) (common dose in paddy), 45 g a.i. ha^{-1}; with/without IH dose: 180 g a.i. ha^{-1}. FE and IH were dissolved in dimethyl sulfoxide (DMSO), methyl oleate, and emulsifier formulation (2% v/v), and then diluted with distilled water. For control treatments (CK), the shoots were sprayed with only the solvent and emulsifier formulation. Then, the seedlings were cultivated under the conditions described above. After 7 days, the physiological parameters (shoot lengths and fresh weights) of the seedlings were measured. Three replicates from each treatment were used.

2.3. Transcriptome Profiling of Rice Seedlings in Different Treatments

2.3.1. Transcriptome Sequencing

Rice seedlings were collected 72 h after treatment (HAT) (i.e., RCK (the control of 9311), RT1 (FE 45 g a.i. ha^{-1} of 9311), RT2 (FE 45 g a.i. ha^{-1} +IH 180 g a.i. ha^{-1} of 9311), SCK (the control of MXZ)) to conduct the transcriptome analysis. There were four replicates for each treatment. In addition, rice seedlings were harvested 12, 24, 48, and 72 HATs to verify gene expression levels. The dosage was: RCK, RT1, RT2, SCK, and ST1 (FE 45 g a.i. ha^{-1} of MXZ). The collected samples were cryopreserved in liquid nitrogen immediately and then stored at −80 °C.

For transcriptome sequencing, the frozen leaf samples in liquid nitrogen were ground. Then, the total RNA was extracted using RNAiso Plus (Takara Co., Ltd., Tokyo, Japan), and the RNA concentrations were detected and quality-checked for integrity. The constructed sequences in the cDNA library were obtained via Biomarker Technologies Co., Ltd. (Beijing, China). The cDNA library was sequenced using the Illumina platform after successful construction and qualification. Ultimately, the clean reads were aligned with the rice reference genome (Oryza_sativa.IRGSP-1.0.45.gff3.gz, available online: http://ftp.ensemblgenomes.org/pub/plants/release-45/gff3/oryza_sativa/, accessed on 4 February 2021) after the adaptor and low-quality sequences were removed from the raw reads.

2.3.2. Differentially Expressed Gene (DEG) Analysis and Identification of FE Metabolism-Related Genes

To compare different samples, the expression of every single gene was normalized to fragments per kilobase transcript per million mapped reads (FPKM). Differential expression analysis of two groups was conducted using the DESeq2_1.20.0 R package. Fold Change (the ratio of expression between two samples/groups) $\geq$1.5 and p-value < 0.05 were used as screening criteria during the differentially expressed gene assay. The relative expression of DEGs was compared between the SCK and RCK treatment groups. The set of DEGs upregulated in the SCK vs. RCK comparison group was selected for Venn interaction with the set of DEGs upregulated in the RT1 vs. RT2 comparison group. The functions analysis of DEGs were determined based on the Gene Ontology (GO, http://geneontology.org/, accessed on 12 June 2022) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg, accessed on 17 June 2022) databases. Then, a comprehensive analysis of DEGs was performed to screen the genes related to herbicide metabolic resistance under the effect of safener.

2.3.3. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Validation

Based on the transcriptome sequencing results, 13 DEGs were randomly selected for qRT-PCR to validate the results of RNA-sequencing (RNA-Seq). The specific primers for qRT-PCR were designed using Primer Premier 5.0 and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Ubiquitin 5 (*UBQ5*, AK061988) was selected as an internal control gene. The primers sequences were listed in Table S1. The expression patterns of key genes in the samples collected at 12, 24, 48, and 72 HAT were then assessed via qRT-PCR. The total RNA in the samples was extracted using RNAiso Plus (Takara Co., Ltd., Tokyo, Japan). The PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time; Takara Co., Ltd., Tokyo, Japan) was used to perform reverse transcription. TB Green (code no. RR820A/B) was used to perform qRT-PCR in the Applied Biosystems QuantStudioTM 6 Real-time PCR system (Thermo Fisher Scientific, Shanghai, China). The following program was used: 30 s for 95 °C, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s, followed by melt curve analysis. The expression of each gene relative to that of the control was calculated using the $2^{-\Delta\Delta Ct}$ method [20]. Three biological replicates from each treatment and three technical replicates for each sample were used for validation.

2.4. Statistical Analysis

The methods of data analysis were performed as described by Zhao et al. [19]. The data were combined if there was no significant statistical difference between the repeated experiments. All data were analyzed via SPSS software v20.0 (IBM, Armonk, NY, USA) by using Duncan's test at a significance level of $p \leq 0.05$.

3. Results

3.1. Bioactivity of the Whole Plant Assay

In the present study, the whole-plant bioactivity method was used to determine the effect of IH on the phytotoxicity of FE in rice. At FE of 45 g a.i. ha^{-1}, the shoot length inhibition rates of 9311 and MXZ were 30.49% and 64.40%, respectively, and the fresh weight inhibition rates were 34.23% and 64.50%, respectively. When FE 45 g a.i. ha^{-1} was combined with safener IH 180 g a.i. ha^{-1}, the shoot length inhibition rates of 9311 and MXZ were 9.96% and 20.35%, respectively. The fresh weight inhibition rates were 12.61% and 26.27%, respectively, indicating that 9311 and MXZ had different resistance levels of FE, and the safener IH could significantly alleviate the phytotoxicity of FE in 9311 and MXZ (Figure 1).

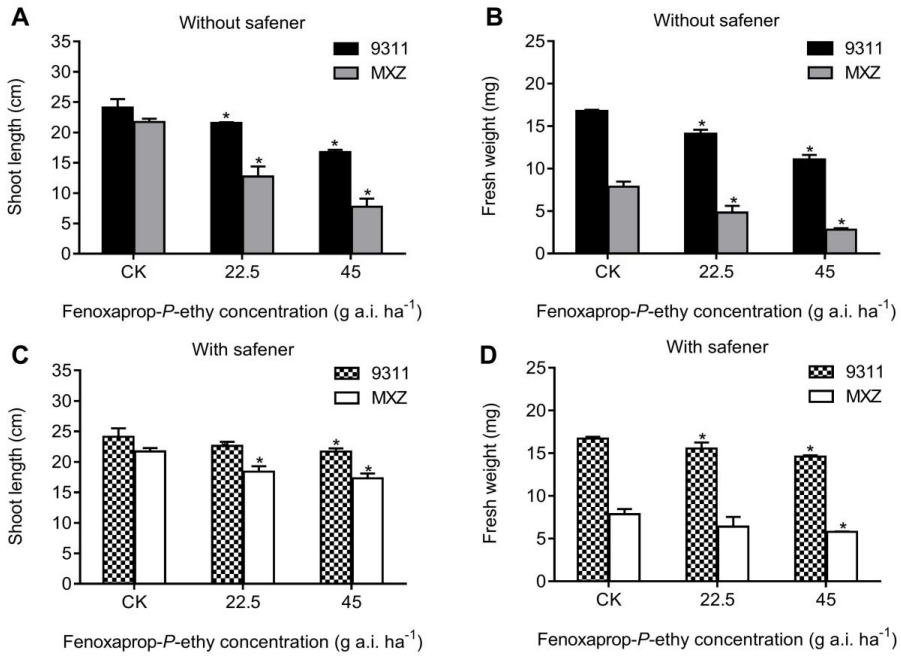

Figure 1. Shoot length and fresh weight in rice 9311 and MXZ exposed to fenoxaprop-P-ethyl alone or in combination with isoxadifen-ethyl hydrolysate 7 days after treatment. (**A**) Shoot length in rice exposed to fenoxaprop-P-ethyl alone; (**B**) Fresh weight in rice exposed to fenoxaprop-P-ethyl alone; (**C**) Shoot length in rice exposed to fenoxaprop-P-ethyl combination with isoxadifen-ethyl hydrolysate; (**D**) Fresh weight in rice exposed to fenoxaprop-P-ethyl combination with isoxadifen-ethyl hydrolysate. * indicates a significant difference between different concentrations of fenoxa-prop-P-ethyl and untreated control within each biotype according to Duncan's test ($p \leq 0.05$).

3.2. Transcriptome Sequencing and Analysis

Based on sequencing by synthesis technology, the cDNA library was sequenced using the Illumina high-throughput sequencing platform. All raw sequence read data were uploaded to the National Center for Biotechnology Information Sequence Read Archive (SRA, https://submit.ncbi.nlm.nih.gov/subs/sra/, accessed on 3 August 2022) under BioProject accession number PRJNA853152.

According to transcriptome data, most bases scored at or above Q30. A total of 114.49 Gb of clean data was obtained, with 6.21 Gb of clean data for each sample. The per-

centage of Q30 bases in all reads was 93.48% and above (Table S2). When the high-quality sequences obtained by RNA-Seq were compared with the reference genome, more than 92.22% of the sequences could be aligned to the reference genome, and 89.55–90.28% of the sequences could be aligned to the unique position of the genome. The transcriptome sequencing results showed a good correlation between four replicates within the sample group, with correlation coefficients R^2 greater than 0.95 (Figure 2). Thus, the sequencing results were of high quality and complied with the requirements of the subsequent transcriptome data analysis.

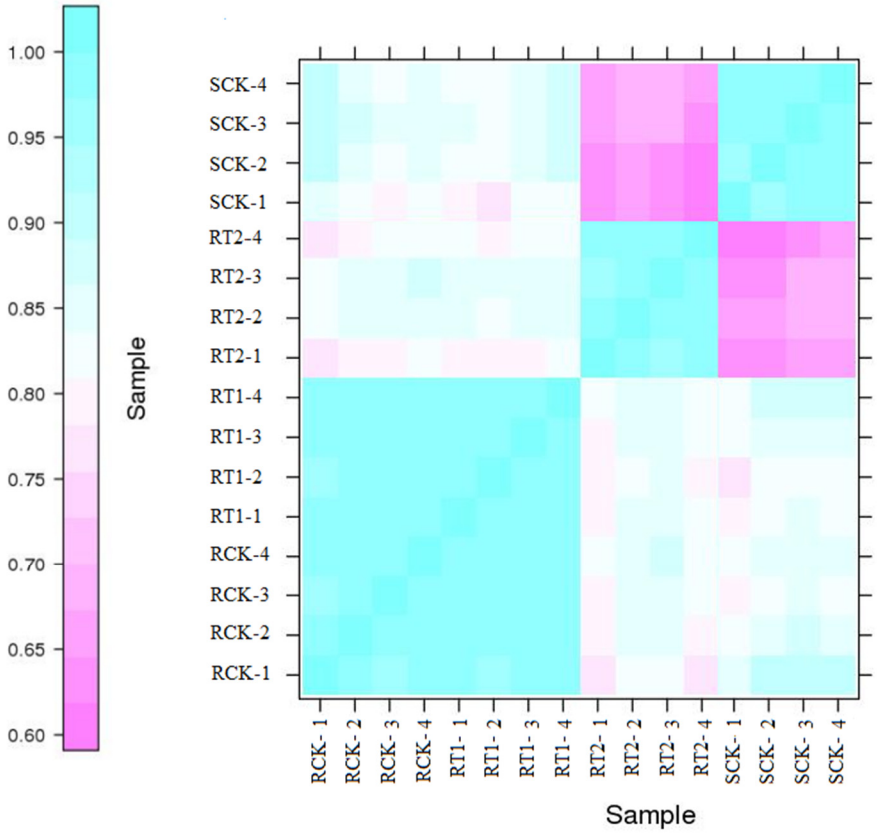

Figure 2. Heatmap of transcriptomic data correlation between different treatments. RCK: the untreated control of 9311; RT1: FE 45 g a.i. ha^{-1} of 9311; RT2: FE 45 g a.i. ha^{-1} +IH 180 g a.i. ha^{-1} of 9311; SCK: the untreated control of MXZ.

3.3. Functional Annotation of Differentially Expressed Genes (DEGs)

In the comparison group of SCK vs. RCK, a total of 6163 DEGs were analyzed for GO enrichment. The DEGs were significantly enriched in redox processes, defense reactions, flavonoid biosynthesis processes, dephosphorylation, and other biological processes. Using KEGG enrichment analysis, DEGs were found to be significantly enriched in photosynthesis-antenna proteins, starch and sucrose metabolism, phenylpropanoid biosynthesis, cyanoamino acid metabolism, and linoleic acid metabolic pathways. In the RT1 vs. RT2 comparison group, DEGs mainly enriched pathways containing photosynthesis-antenna proteins, cutin, suberin, and wax biosynthesis, glyoxylate and dicarboxylic acid metabolism, alpha-linolenic acid metabolism, carbon fixation in the photosynthetic organism, and amino sugar and nucleotide sugar metabolism (Figure 3).

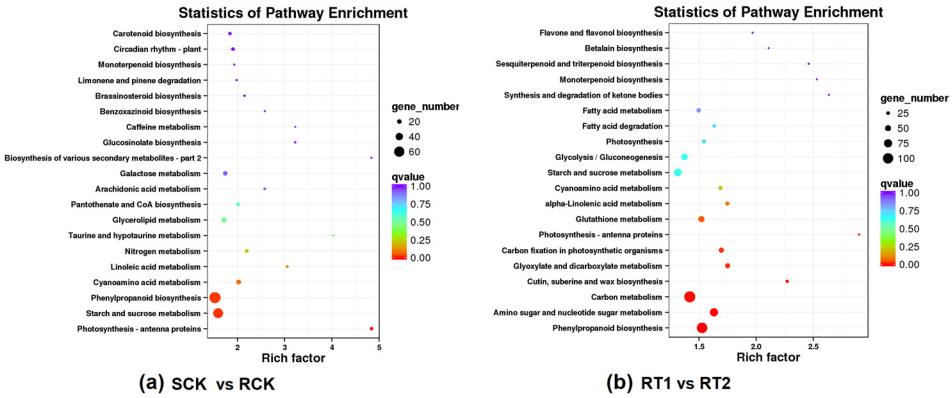

Figure 3. DEG enrichment of the KEGG pathway: (**a**,**b**).

3.4. qRT-PCR

The transcript abundance of 13 genes was measured via qRT-PCR to verify the reliability of RNA-Seq. The results were in accordance with the RNA-Seq expression trend, and the correlation coefficient between the two datasets was calculated using the CORREL formula in Excel with an r of 0.919 (Figure S1, data are mean ± SE). This indicates that the RNA-Seq used in this study was highly reliable.

3.5. Identification and Validation of Potential Fenoxaprop-P-ethyl Metabolic Genes

For transcriptome analysis, DEGs that were upregulated in both SCK vs. RCK and RT1 vs. RT2 comparison groups were Venn-interacted, with a total of 647 co-expressed DEGs (Figure 4a). Functional annotation of KEGG enrichment revealed that these DEGs were enriched in galactose metabolism, cyanoamino acid metabolism, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, and phenylpropanoid biosynthesis (Figure 4b).

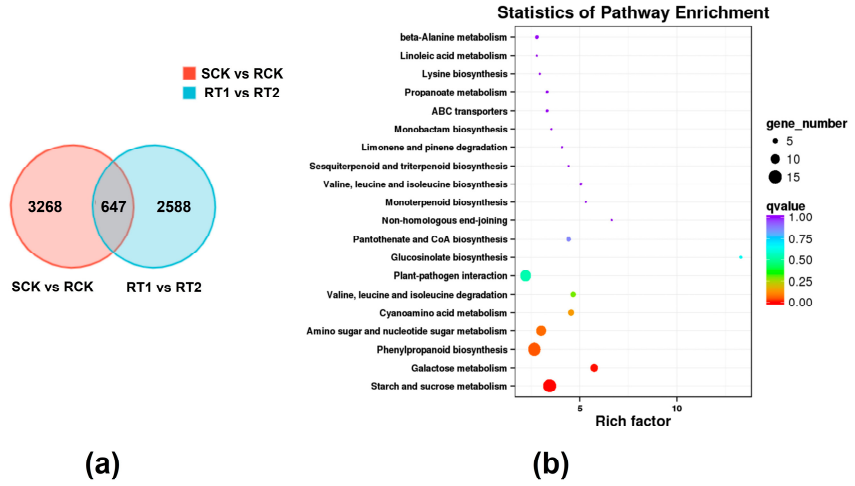

Figure 4. Screening for potential fenoxaprop-P-ethyl metabolic genes: (**a**) Venn interaction of different groups; (**b**) KEGG enrichment pathways of 647 genes.

Screening from 647 co-expressed DEGs encoding genes related to herbicide metabolic processes, including 9 genes encoding CYPs (*CYP704C1*, *CYP709B2*, *CYP71A1*, *CYP72A11*, *CYP72A15*, *CYP76M5*, *CYP78A6*, *CYP71D10*), 2 genes encoding GSTs (*GSTU6*), 4 genes encoding UGTs (*DIMBOA UGT BX8*, *UGT83A1*, *UGT79*, and *UGT85A2*), and 8 genes encoding ABC transporters (*ABCB11*, *ABCC10*, *ABCG11*, *ABCG39*, *ABCB4*, *MRP protein*). Additionally, 8 genes were screened from SCK vs. RCK, including 7 genes encoding serine carboxypeptidase (*SCP*; *SCP 34*, *SCP 1*, *SCP II-3*, *SCP 26*, *SCP 27*, *SCP II-2*, *Serine*

carboxypeptidase-like), and 1 gene encoding *Lactoylglutathione lyase* was also screened. The expression of these genes was upregulated in SCK vs. RCK treatment groups (Table 1).

Table 1. Potential fenoxaprop-P-ethyl metabolic genes in rice.

Gene ID	Accession ID	Description	GO Annotation
Os07g0635500	XM_015789232	*CYP709B2*	GO:0005506; GO:0016021; GO:0016709;
Os09g0530300	XM_015795881	*CYP71A1*	GO:0020037; GO:0044550; GO:0055114
Os10g0525000	XM_015759368	*CYP704C1*	GO:0004497; GO:0005506; GO:0009536; GO:0016021; GO:0016705; GO:0020037; GO:0022900
Os01g0628700	XM_015765608	*CYP72A11*	GO:0004497; GO:0005506; GO:0016021;
Os11g0635500	XM_015760069	*CYP71D10*	GO:0016705; GO:0020037; GO:0055114
Os01g0627500	XM_015760381	*CYP72A15*	GO:0004497; GO:0005506; GO:0016021; GO:0016705; GO:0020037; GO:0022900
Os08g0508000	XM_015792583	*CYP76M5*	GO:0005506; GO:0006952; GO:0016021; GO:0016709; GO:0020037; GO:0022900; GO:0051502
Os08g0465700	XM_015794639	*CYP76M5*	GO:0005506; GO:0016021; GO:0016709; GO:0020037; GO:0022900; GO:0044550
Os03g0134500	XM_015776235	*CYP78A6*	GO:0005506; GO:0016021; GO:0016709; GO:0020037; GO:0044550; GO:0055114
Os10g0527400	XM_015758264	*GSTU6*	GO:0004364; GO:0005739; GO:0006749; GO:0009407
Os10g0530900	XM_015758870.2	*GSTU6*	GO:0004364; GO:0005737; GO:0006749; GO:0009407
Os11g0441500	XM_015762250	*DIMBOA UGT BX8*	GO:0005488; GO:0005739; GO:0008194; GO:0009813; GO:0016758; GO:0052696
Os03g0757000	XM_015775386	*UGT83A1*	GO:0009813; GO:0043231; GO:0052696; GO:0080043; GO:0080044
Os04g0206700	XM_026024995.1	*UGT79*	
Os06g0220500	XM_015788488	*UGT85A2*	
Os01g0695800	XM_015766599	*ABCB11*	GO:0005524; GO:0016021; GO:0042626; GO:0055085
Os11g0155600	XM_026021048	*ABCC10*	GO:0005524; GO:0016021; GO:0042626; GO:0055085
Os09g0472100	XM_015757091	*ABCG11*	GO:0005524; GO:0005886; GO:0016021; GO:0042626; GO:0055085
Os02g0208300	XM_015770762	*ABCG39*	GO:0005524; GO:0005886; GO:0009699; GO:0016021; GO:0042349; GO:0042626; GO:0050790; GO:0055085
Os01g0696600	XM_015761603.2	*ABCB4*	
Os01g0696701	XM_015761603.2	*ABCB4*	GO:0005524; GO:0016021; GO:0042626;
Os02g0190000	XM_015769540	*MRP protein*	GO:0055085
Os02g0190300	XM_015769530	*MRP protein*	
Os02g0634700	XM_015769887	*SCP 34*	GO:0004185; GO:0005773; GO:0009505; GO:0051603
Os07g0479300	NM_001403059	*Serine carboxypeptidase*	GO:0004185; GO:0005773; GO:0005777; GO:0005789; GO:0005829; GO:0051603
Os12g0257000	XM_015762695	*SCP 1*	GO:0004185; GO:0005576; GO:0005773; GO:0005777; GO:0016747; GO:0019748; GO:0051603
Os07g0656900	XM_015791377	*SCP II-3*	GO:0004185; GO:0016021; GO:0051603
Os01g0332800	XM_015766668	*SCP26*	
Os01g0833500	XM_015795428	*SCP27*	GO:0004185; GO:0005773; GO:0051603
Os06g0186400	XM_015787453	*SCP II-2*	
Os08g0191700	NM_001403516	*Lactoylglutathione lyase-like*	GO:0004462; GO:0046872

3.6. The Expression Patterns of DEGs after Treated with Fenoxaprop-P-ethyl

Different varieties of rice were sprayed with FE and key gene expression patterns in seedlings were analyzed by time course (Figure 5). The qRT-PCR results showed that the expression of *CYP76M5*, *CYP71D10*, and *CYP71A11* in the seedlings of 9311 and MXZ increased and then decreased after FE spraying. The transcript abundance of these three genes in 12–24 HAT was significantly higher in RT1 than in ST1.

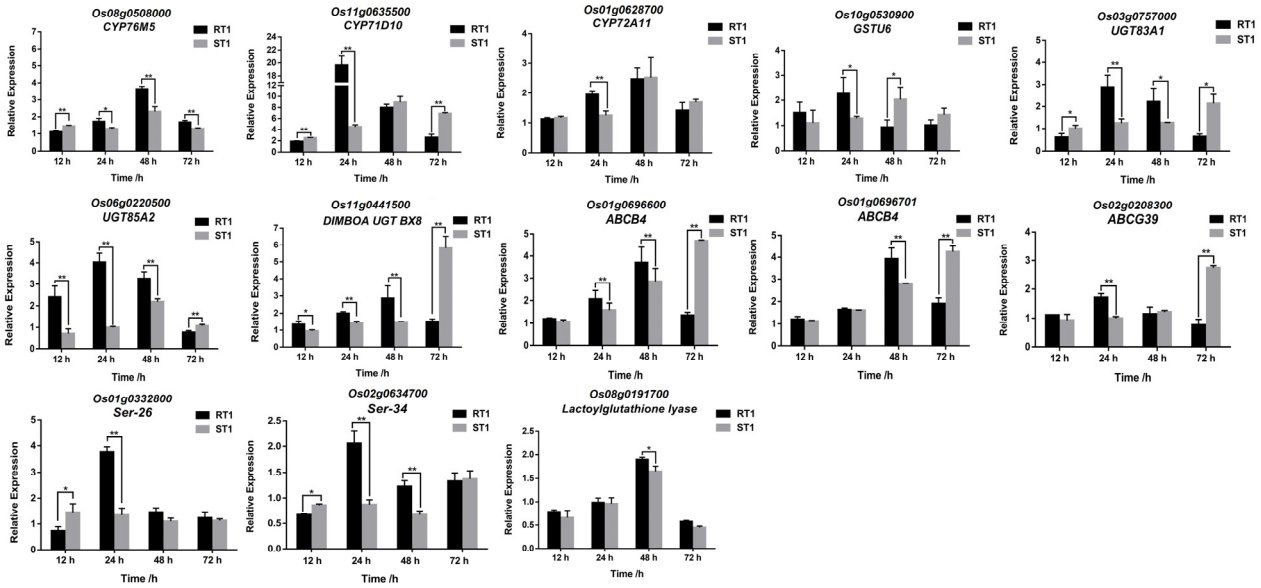

Figure 5. Expression patterns of key genes after FE treatment in 9311 and MXZ. The RT1 control was RCK, and the ST1 control was SCK. The gene expression levels were measured by using the $2^{-\Delta\Delta Ct}$ method. Values represent the averages of three replicated, and the error bars represent standard errors. Asterisks indicate statistically significant differences between the two treatments according to Duncan's test (*: $p \leq 0.05$; **: $p \leq 0.01$).

The relative expression of the gene encoding *GSTU6* was higher in RT1 than in ST1 at 12–24 HAT, and there was a significant difference at 24 HAT. The genes *UGT83A1* and *UGT85A2* were upregulated in different varieties of rice after spraying with FE. In RT1 treatment, the relative expression all peaked at 24 HAT. In addition, the relative expression of RT1 was 2.88- and 4.02-fold compared with RCK treatment, significantly higher than the expression of ST1 (compared with SCK). In ST1 treatment, the peaks of expression of *UGT83A1* and *UGT85A2* were reached at 72 HAT and 48 HAT, respectively, and were 2.14, 2.19 times higher than SCK. In addition, the relative expression of *DIMBOA UGT BX8* was significantly higher in RT1 than in ST1 at 12–48 HAT and gradually recovered to the control level at 72 HAT. Its relative expression peaked at 72 HAT, which was 5.82 times higher in ST1 than that of the control SCK.

In the RT1 treatment group, the expression of genes encoding ABC transporters (*ABCB4* and *ABCG39*) increased and then decreased. The transcript abundance of the genes peaked at 48, 48, and 24 HAT, respectively, which were strongly significantly higher than that of ST1. On the other hand, in ST1, these three genes all displayed a gradual increase and peaked at 72 HAT.

Both *SCP26* and *SCP34* are genes encoding serine carboxypeptidases involved in phase IV of herbicide metabolism. The qRT-PCR results indicated that the relative expressions of *SCP26* and *SCP34* in RT1 at 24 HAT were all significantly higher than in ST1. There was no significant change in the relative expression of *SCP26* in ST1 at different times after spraying. *Lactoylglutathione lyase* was also involved in phase IV of herbicide metabolism. The relative expression of *Lactoylglutathione lyase* was not significantly different from the control at 24 HAT. In addition, at 48 HAT the expression was significantly higher in RT1 than in ST1.

3.7. Revalidation of Potential Metabolic Genes by Safener

From the above analysis, the results of qRT-PCR showed that the relative expression of key enzyme genes encoding genes involved in various stages of herbicide metabolism was significantly different in 9311 and MXZ. In addition, the upregulation rate and difference-fold of relative expression in 9311 were significantly higher than in MXZ. Consequently,

the differences in the expression of these genes were compared in the FE vs. FE + IH (RT1 vs. RT2) treatment groups again.

qRT-PCR results showed that the expression trends of *GSTU6*, *DIMBOA UGT BX8*, and *ABCG39* in RT1 and RT2 at 72 HAT were consistent with RNA-Seq results (Figure 6). Then, the expression patterns of these three genes at different time points were analyzed by time course. The results showed that the relative expression of *GSTU6* and *DIMBOA UGT BX8* in the RT2 treatment group was higher than in RT1 from 12 to 72 HAT, and there were significant differences at 12, 24, and 72 HAT. At 24 HAT, the expression of *GSTU6* in the RT2 treatment group was 3.65 times higher than the expression in the RT1. The relative expression of *DIMBOA UGT BX8* increased first and then decreased in RT1 and RT2 treatment groups and reached its peak at 48 HAT. The relative expression of *ABCG39* in RT2 was significantly higher than that in the RT1 treatment group at 24, 48, and 72 HAT, and peaked at 24 HAT (Figure 6).

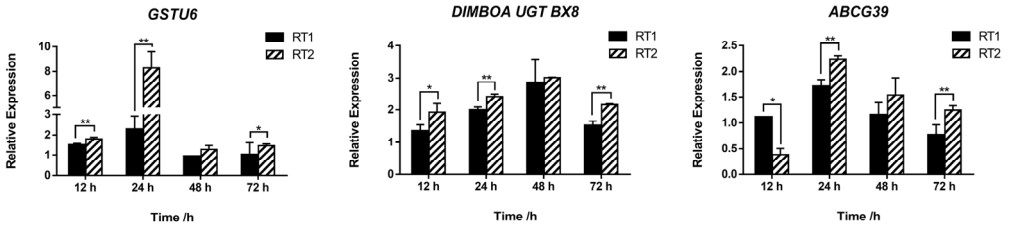

Figure 6. Effect of safener on the expression pattern of key genes. RT1: FE 45 g a.i. ha^{-1} of 9311, RT2: FE 45 g a.i. ha^{-1} +IH 180 g a.i. ha^{-1} of 9311, and the controls were all RCK. The gene expression levels were measured using the $2^{-\Delta\Delta Ct}$ method. Values represent the averages of three replication, and the error bars represent standard errors. Asterisks indicate statistically significant differences between the two treatments according to Duncan's test (*: $p \leq 0.05$; **: $p \leq 0.01$).

4. Discussion

Fenoxaprop-P-ethyl has a good control effect on common grassy weeds such as *E. crus galli* and *D. sanguinalis* in paddy, but it easily causes different degrees of injury to rice [21,22]. Therefore, some pesticide companies have registered the pesticide with a safener for use in paddy fields, while the single agent of FE has yet to be registered. In this study, the usual field dose of 22.5 g a.i. ha^{-1} and twice the amount of 45 g a.i. ha^{-1} of FE with/without 180 g a.i. ha^{-1} IH was used to explore its activity against different varieties of rice. Then, we found that there were significant differences in the sensitivity of 9311 and MXZ to FE. Consequently, 9311, which was more tolerant to FE, was evaluated as the resistant (R), and accordingly, the lesser tolerant MXZ was designated as a susceptible variety (S). The phytotoxicity in rice treated with IH in combination with FE was reduced, indicating that IH was able to improve the tolerance of rice with different levels of resistance to FE.

Currently, it is widely accepted that safeners accelerate the metabolism of herbicides in crops, inducing the expression of herbicide metabolism-related enzymes and transporters to accelerate the detoxification of herbicides [23–25]. Studies on the mechanism of action of safeners have mainly focused on the study of relevant metabolic enzyme activities at the enzymatic level, while the molecular mechanism has yet to be properly investigated [23,26]. In the model plant, *A. thaliana*, Behringer et al. systematically investigated the expression pattern of genes encoding enzymes and transporters involved in herbicide metabolism, including GSTs, UGTs, CYPs, and ABC transporters, under the effect of IE. They found that IE induced an increase in the expression of these genes [26] but did not protect *A. thaliana* from herbicide injury. The study was not fully validated.

The DEGs between different varieties of rice were searched based on their diverse susceptibility to FE. Then, Venn interaction was conducted for the screened genes with DEGs present between the FE vs. FE + IH comparison groups revealing 647 co-expressed genes, 31 of which encode genes for key enzymes of herbicide metabolic processes (eight of these were from the RCK vs. SCK comparison group). We verified that 12 genes exhibit

significant differences between R and S, and the expression of these genes was markedly higher in R than in S. Next, these 12 potential resistance genes were validated in rice from FE vs. FE + IH treatment groups.

The process of CYPs oxidation played a crucial role in the metabolism of herbicides [27], and safener can accelerate this process [28]. Research has shown that *CYP72A21* expression in rice is induced by various herbicides, such as acetochlor and esprocarb [29]. *CYP71D10* is an important gene involved in the metabolism of FE in *Beckmannia syzigachn* [30]. In this study, the relative expression levels of *CYP72A11*, *CYP71D10* at 24 HAT, and *CYP76M5* at 24–72 HAT were significantly higher in the RT1-treated rice shoots than in the ST1-treated shoots, but they were not induced by safener. These three genes encoding CYPs may be potential metabolic genes of FE, which accelerate the oxidation of FE and improve the crop's tolerance to herbicides.

GSTs, a vital detoxification enzyme in phase II of herbicide metabolism, can catalyze the conjugation of phase I products with GSH to form nontoxic S-glutathionylated substances [31,32]. For instance, Zou et al. observed that varieties with high GST activity in *Sagittaria trifolia* L. exhibited higher resistance to bensulfuron-methyl [33]. Tau-like GSTs have been shown to induce the expression by safeners [32,34]. *GSTU6* is a tau class GST, which may be a crucial gene in *B. syzigachne* to metabolic FE. [35]. In our study, the upregulated expression rate of *GSTU6* was higher in R than in S at 12–24 HAT of herbicide, followed by recovery towards the control level. The relative expression of *GSTU6* was induced by safener IH, and the expression was 3.65 times higher in the RT2 than the expression in the RT1-treated group at 24 HAT. The expression of *GSTU6* was significantly higher in R than the expression in S and was rapidly induced by the safener IH, suggesting that *GSTU6* is a crucial gene that accelerates the metabolism of FE.

Research has shown that UGTs inactivate toxic compounds via glycosylation modification of various functional groups in herbicide molecules to reduce their toxicity [36]. The reaction products produced in phase I are catalyzed by UGTs and combined with glucose to form *O*-glucoside, *N*-glucoside, or glucose esters. For example, UGTs increased the glucosylation activity towards sulcotrione, and overexpression of the *UGT91C1* gene enhances tolerance to sulcotrione in *A. thaliana* [37]. Glycosylation of herbicides in plants is regulated by safeners. Cloquintocet-mexyl selectively enhanced the metabolic activity of *O*-glucosyltransferase (OGT) to exogenous compounds in wheat plants [38]. GO annotation indicates that the molecular functions of *DIMBOA UGT BX8* include binding (GO:0005488), UDP-glycosyltransferase activity (GO:0008194), are involved in flavonoid biosynthesis processes (GO:0009813), flavonoid glucuronidation (GO:0052696), among other biological processes. The results of this study showed that the relative expression of *UGT83A1*, *UGT5A2*, and *DIMBOA UGT BX8* in rice was higher in R than in S at 24–48 HAT of herbicide. In addition, *DIMBOA UGT BX8* was significantly upregulated by IH, suggesting that *DIMBOA UGT BX8* may accelerate the glycosylation of metabolites during herbicide detoxification to generate nontoxic metabolic conjugates.

ABC transporters are associated with xenobiotic detoxification, which can translocate the herbicide and its metabolites [39,40]. *ABCG39* encodes the ABC transporter, and GO annotation indicates that the ABC transporter has ATPase activity (GO:0042626) and is also involved in biological processes such as the phenylpropanoid biosynthetic process (GO:0009699), transmembrane transport (GO:0055085) and regulation of catalytic activity (GO:0050790). Time-course results suggested that *ABCG39* could be activated by FE and respond to the induction of the safener IH. The expression of *ABCG39* increased to accelerate phase III of herbicide metabolism.

Behring et al. found that both serine carboxypeptidase and *lactoylglutathione lyase* are involved in the reprocessing of metabolites in phase IV of herbicide metabolism [26]. In this study, seven genes encoding SCP and one gene encoding *lactoylglutathione lyase* were screened from the SCK vs. RCK comparison group. GO annotation indicated that *SCP26* and *SCP34* both have serine-type carboxypeptidase activity (GO:0004185) and proteolysis involved in the cellular protein catabolic process (GO:0051603). *Lactoylglutathione lyase*

has *lactoylglutathione lyase* activity (GO:0004462). The qRT-PCR results indicated that the transcript abundance of *SCP26* and *SCP34* was significantly higher in R than in S at 24 HAT of FE. The transcript abundance of *lactoylglutathione lyase* in R was significantly higher than the transcript abundance in S at 48 HAT of herbicide. The results demonstrate indicate that the differential expression of these eight genes was not significant in the herbicide vs. herbicide+safener comparison groups. The time-course results also a lack of response to the safener IH, implying that the qRT-PCR results were consistent with the RNA-Seq results.

5. Conclusions

The present study showed that the tolerance of FE in different varieties of rice 9311 and MXZ was distinct. The FE tolerance was enhanced in rice as induced by the safener IH. Transcriptome analysis revealed numerous DEGs encoding detoxicating enzymes related to the herbicide metabolic pathway in 9311 vs. MXZ. Through the analysis of key DEGs, 31 potential FE resistance genes were screened. Among these genes, 12 genes were identified as resistance genes to FE within rice shoots, including three encoding CYPs, one GST, three UGTs, three ABC transporters, two SCP, and one lactoylglutathione lyase. Then, these 12 possible resistance genes were subjected to time-course analysis in rice shoots treated with the safener-herbicide mixture. The results indicated that three genes, *GSTU6*, *DIMBOA UGT BX8*, and *ABCG39*, were not only significantly more highly expressed in 9311 than in MXZ but also expressed by the safener IH and participated in the herbicide detoxification process. The results highlight the metabolic genes causing the increase in herbicide tolerance partially overlapping with the genes activated by safeners in crops. Thus, these three genes may be the major candidates for herbicide metabolic resistance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13010225/s1, Figure S1: Correlation between qRT-PCR and RNA Seq results; Table S1: Primers sequence used in qRT-PCR; Table S2: Summary of rice transcriptome sequencing, assembly, and comparison with the reference genome.

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