Effects of fenclorim on rice physiology, gene transcription and pretilachlor detoxification ability

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Abstract

Background Fenclorim (Fen) can effectively protect rice from pretilachlor (Pre) injury, but its effects on rice have not been formally evaluated; thus, the Fen mode of action for alleviating the phytotoxicity caused by Pre in rice is not clear. This study aimed to examine the biochemical and physiological effects of Fen on rice and to determine the changes induced by Fen at the transcriptome level. **Result** The chlorophyll content of the rice plants was significantly affected by Pre but not by Fen. The impacts of the activity of oxidative stress enzymes showed that Fen did not elicit any changes in oxidative stress; however, it reduced lipid peroxidation and oxidative damage induced by Pre. Fen did not affect the uptake of Pre but did affect its persistence in rice. In a transcriptome study, Fen upregulated genes in the detoxification pathway. Overall, 25 genes related to detoxification were identified, including P450, GST, and GT. Moreover, qRT-PCR analysis showed that four P450 genes, including CYP71Y83, CYP71K14, CYP734A2 and CYP71D55, and two GST genes, GSTU16 and GSTF1, were upregulated by Fen and/or Pre. **Conclusion** Our work indicates that Fen can act as an antioxidation defender in addition to enhancing the metabolism of herbicides in rice.

Background

It is well known that herbicides, which constitute the primary method of weed control in modern agriculture practices, have played a crucial role in controlling farmland weeds and subsequently reducing labor intensity. While herbicides can help increase crop yields, they can also pose risks to crops that are sensitive to them; for this reason, safeners were developed to increase crop selectivity [1]. Herbicides are biologically active xenobiotic compounds, and the over use of herbicides may disrupt the normal biochemical and physiological processes of plants. Each herbicide has at least one specific target site, and the inhibition of the target site is the main mode of action of many herbicides. In addition, many studies have shown that reactive oxygen species (ROS) can accumulate in plant cells as a secondary effect when exposed to herbicides. Excess ROS can damage plant cells by leading to membrane lipid peroxidation, protein oxidation, inhibition of enzyme activity, DNA and RNA damage, etc. and can even result in cell death [2-7].

Over the course of long-term evolution, plants have evolved the ability to minimize herbicide toxicity. One of their protective mechanisms is the antioxidant system, which is composed of a variety of enzymes, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and other enzymes for protecting against oxidative damage [5]. At present, antioxidant enzyme activity has been proven to be related to herbicide tolerance in a variety of plants [3, 4, 7, 8].

Another mechanism is the detoxification system, also known as the xenome, which removes complex exogenous compounds [9]. The detoxification system is composed of large families, such as P450s, GTs, GSTs, ABC transporters and other enzymes or transporters, and the process itself can be divided into four phases: phase I, hydrolysis or oxidation; phase II, conjugation; phase III, sequestration; and phase IV, catabolism [1, 10]. Scientists have devoted a large amount of work to the metabolic pathways of

exogenous compounds in crops in recent years; however, the relationships between the roles of the detoxification system in xenobiotic metabolism are still not clear [11]. Interestingly, the herbicide detoxification reactions of crops can be induced by herbicide safeners. Herbicide safeners are synthetic chemicals used to alleviate the phytotoxicity of herbicides to crops without reducing the efficiency of weed control [1, 12]. Herbicide use in combination with safeners represents approximately 30% of the market share of global herbicides [13]. The importance of safeners in agricultural production has attracted considerable research on their modes of action. The difference in the herbicide metabolic rate between crops and weeds was essentially one of the mechanisms of herbicide selectivity. What is unique and interesting about safeners is that they can obviously induce a series of key components of the detoxification pathway in plants at the same time, thus enhancing the metabolism, degradation and isolation of crops against herbicides and increasing the selectivity of weed control [1, 11, 14]. In the past several decades, the mechanisms by which safeners work have been the focus of attention for some scientists. However, until now, there have been no conclusions that have elucidated the modes of action of safeners, especially the molecular and genetic regulatory mechanisms and signaling pathways involved in safener action, which remain largely unclear.

The safener fenclorim (4, 6-dichloro-2-phenylpyrimidine, Fen) was developed to reduce the injury to rice (*Oryza sativa* L.) caused by chloroacetanilide herbicides. To ensure the safety of early stage rice, Fen is often formulated with pretilachlor [2-chloro-2', 6'-diethyl-N-(2-propoxyethyl) acetanilide, Pre], which is one of the most widely used herbicides in rice fields in southeast Asian countries, but Pre represents an existing phytotoxicity risk to rice [15]. It was reported that Fen protected rice from injury by Pre mainly due to the acceleration of the metabolism of Pre [16]. In the past, most studies have focused on the relationship between the induction of Fen and GST activity and the regulation of the expression of genes encoding GSTs [17-20]. Few studies have focused on detoxifying enzymes other than GST. As far as we know, only one study has investigated rice at the enzyme activity level, stating that Fen elevated the P450 content [21]. Moreover, many studies have been conducted on *Arabidopsis thaliana* [18, 22-25]. Little is known about the relationship between Fen induction and the genetic expression of other detoxification enzymes in rice.

Additionally, both safeners and herbicides are biologically active xenobiotic compounds, since herbicides can cause peroxidation in crops, there are still no clear results about the effects of safeners on ROS in rice. We now report on a systematic study to illuminate the effects of Fen on the physiology of rice, the ability of rice to affect the metabolism of Pre, and its effect on rice gene expression at the transcriptome level. Here, we aim to (1) study the ability of Fen to enhance the expression of GST activity and the metabolic rate of Pre, (2) investigate the activities of antioxidant enzymes of rice seedlings on the Pre substrate in the presence of Fen and (3) perform global transcriptome studies in rice treated with Fen to identify the associated genes that are closely related to the mode of action of Fen and Pre metabolic pathway.

Results

Effects of Pre and Fen on total chlorophyll

The total chlorophyll content of rice plants was decreased by 55.6% compared to the control under exposure to Pre. Fen alone did not decrease the total chlorophyll content, and there was no difference between the treatments of Pre alone and Fen combined with Pre (Fig. 1 A).

Effects of Pre and/or Fen on oxidative stress and antioxidant enzymes

As shown in Fig. 1 B, treatment with Pre alone caused a strong increase in the MDA contents of the rice shoots, reaching an MDA content 2.5-fold that of the control. Interestingly, Fen did not increase the MDA content; in contrast, it reversed the accumulation of MDA caused by Pre when it was used in combination with Pre. However, neither Pre nor Fen stimulated the MDA accumulation in the roots.

The activities of SOD, CAT and POD in the rice shoots and roots were variable when treated with Pre and/or Fen. In general, the SOD, CAT and POD activities were intrinsically higher in the roots than in the shoots (Fig. 2 A, B, C). The SOD, CAT and POD activities varied similarly in the rice shoots and roots under Pre and Fen exposure. Pre induced significant increases in the SOD, CAT and POD activities in both the shoots and roots. In the shoots, the SOD, CAT and POD activities increased by 32.7%, 59.6%, and 33.5%, respectively. In the roots, the increased SOD, CAT and POD values were 15.6%, 40.0% and 31.5%, respectively. Fen alone did not increase the SOD, CAT and POD activities in either tissue; in contrast, when Fen was used in combination with Pre, the SOD and CAT activities in the shoots were reduced by 4.9% and 80.5%, respectively, compared to treatment with Pre alone. The CAT and POD activities in the roots were reduced by 131.6% and 5.4%, respectively, compared to treatment with Pre alone. However, the POD activity in the shoots and the SOD activity in the roots did not decrease significantly when Fen was combined with Pre compared with Pre alone.

GST activity assay and effects of Pre and Fen

The intrinsic activity of GSTs in roots of rice was 2-fold that of the shoots of the control treatment. The Pre treatment significantly suppressed the activity of GSTs in the shoots, while the Fen treatment increased the activity by 35%. Fen treatment significantly increased the activity of GSTs in the roots compared to the control, and Pre did not elevate GST activity in the roots (Fig. 2 D).

Pre residue in rice shoots

Fen did not affect the accumulation of Pre in the rice shoots in the first 24 h. Starting at 48 h, the Pre content showed a significant difference between the Pre and Pre + Fen treatments. When Pre was applied alone, the amount of Pre in the shoots was slightly increased at 48 h, after which it degraded from 72 h to 120 h. When the Pre and Fen mixture was applied, the Fen reduced the Pre amount rapidly from 48 h to 120 h (Fig. 3).

Illumina sequencing, assembly, and Gene Ontology

Table 1 Summary of rice transcriptome sequencing, assembly and annotation

To investigate the gene expression profile involved in the Fen-mediated activation of the detoxification genes in rice, we compared the transcriptomes of rice plants 4 h and 24 h after safener treatment to that of the controls.

The 4 h and 24 h transcripts were sequenced for twelve samples. After quality control and data cleanup, we obtained 84.37 Gb of clean data ranging from 20,920,063 to 29,230,636 reads per sample, which were then used to map the sequence of the rice genome sequence (Table 1). A library was constructed after the sample was qualified. Finally, a total of 35,301 genes were obtained. Of these, 1000 nt $\geq$ 16,638 $\geq$ 300 nt, and 14,743 $\geq$ 1000 nt.

The 35,301 mapped genes were annotated with all seven databases (Swiss-Prot, KEGG, COG/KOG, NR, Pfam, eggnoG and GO databases). GO analysis assigned 28,662 genes into 54 functional categories: 17 for cellular components (CC), 17 for molecular functions (MF), and 20 for biological processes (BP) (Fig. S1). The most enriched subgroups in the CC were the “cell parts” (21,579 genes, 75.29%), followed by “cell” (21,555 genes, 75.20%) and “organelle” (19,691 genes, 68.70%). The most enriched subgroups in the MF category were “binding” (13,871 genes, 48.40%) and “catalytic activity” (12,970 genes, 45.25%). The most enriched subgroups in BP were “metabolic process” (15,693 genes, 54.75%), “cellular process” (13,512 genes, 47.14%) and “single-organism process” (11,396 genes, 39.76%). Additionally, KEGG analysis classified 7752 genes into 124 pathways.

Functional analysis of the DEGs

The transcript levels were calculated by FPKM. At 4 h, a total of 200 differentially expressed genes were found, among which 107 genes were upregulated and 93 were downregulated. At 24 h, a total of 81 differentially expressed genes were found, including 35 upregulated and 46 downregulated genes.

Based on the GO database, 168 differentially expressed genes (4 h) and 68 differentially expressed genes (24 h) were annotated. The DEGs at 4 h and 24 h were assigned to 54 functional subgroups, including 20 for “BP”, 17 for “CC”, and 17 for “MF” (Fig. S2). Among the DEGs, “catalytic activity” in the MF category and “metabolic process” in the BP category were significantly enriched in the Fen treatment relative to the CK.

Based on the known modes of action of herbicide safeners [1, 10, 26], we believe that the DEGs related to metabolism and signaling pathways were associated with herbicide metabolism, which can be strengthened by safeners. We selected the genes that were upregulated in the Fen treatment vs CK that had related functional annotations, including P450, GST, and UGT. The genes that were potentially involved in the herbicide detoxification process induced by Fen are listed in Table 2. There were sixteen detoxification genes belong to phase I and six belonging to phase II. In phase I, five genes coded for Cytochrome P450, four genes coded for oxygenases, and two genes coded for peroxidase. Some fenclorim-induced hydrolases (3 glycosyl hydrolases, 1 thioesterases, 1 lipases) may also be included in phase I. Two GSTs and five glycosyl transferases may contribute to phase II. Two GST genes belonging to the phi and tau classes were upregulated 24 h after Fen induction. Another important gene superfamily,

the glycosyl transferases, whose members can catalyze toxicants with sugars to form hydrophilic compounds ready for further catabolism, included four members that were also induced by the Fen treatments at 4 h [27].

Table 2 Fenclorim-induced detoxification genes in rice

Quantitative real-time RT-PCR (qRT-PCR) validation of metabolizing enzyme genes

To identify and validate the genes induced by Fen, the expression of 14 upregulated candidate genes were validated by qRT-PCR analysis under Fen treatment, and the expression profiles of these genes induced by Pre were also analyzed. Most of the selected genes had similar functional annotations, including P450 genes, GST genes, GT genes, POD genes, hydrolase genes, dehydrogenase genes and lipid transporters and metabolism genes (Fig. 4). Except for Os10g0565200, Os04g0688300, Os04g0556500 and Os04g0206500, 10 of the genes showed higher expression levels in shoots when exposed to Fen compared to the control; these 10 genes included four P450 (*CYP71Y83*, *CYP71K14*, *CYP734A2*, and *CYP71D55*) (Fig. 4 A, B, C, D), two GST (*GSTU16* and *GSTF1*) (Fig. 4 I, J), one hydrolase gene (Fig. 4 E), one POD gene (Fig. 4 H), one dehydrogenase gene (Fig. 4 M) and one lipid transporter and metabolism gene (Fig. 4N). Four of them, including two hydrolase genes (Os11g0701100 and Os10g0565200) (Fig. 4 E, F), one UGT gene (Os04g0556500) (Fig.4 K), and one lipid transporter and metabolism gene (Os08g0448050) (Fig. 4 N), had significantly greater expression levels in the roots treated with Fen. When treated with Pre, seven genes, including three P450 genes (Fig. 4 B, C, D), two GST genes (Fig. 4 I, J), one POD gene (Fig. 4 G), and one dehydrogenase gene (Fig. 4 M), had significantly greater expressions levels in the rice shoots, and five genes had significantly greater expression levels in the roots. Among them, the POD gene Os04g0688300 (Fig. 4 G) was significantly upregulated by Pre in both the shoots and roots, but was especially upregulated in shoots.

Discussion

Herbicide safeners can alleviate the injury caused by herbicides to crops. Although there has been some progress in the description of the mechanism by which safeners work, their mode of action has not been fully clarified. Fen is a safener specifically developed for Pre, and the use of Fen could significantly increase the tolerance of rice to Pre. However, most relevant previous studies have focused on the detoxifying enzyme GST [16-18, 20, 25, 28, 29], and the other effects of Fen on rice plants remain elusive. The present study determined the total chlorophyll content, lipid peroxidation, and transcriptome expression induced by Fen.

Our study has shown that Pre caused a loss of chlorophyll content. The addition of Fen did not alleviate the chlorophyll content, indicating that Pre can disrupt the biosynthesis of chlorophyll and that the safener Fen could not affect the biosynthesis of chlorophyll. This may be one of the characteristics of this particular compound. This observation is also in line with the results that Fen alone does not affect rice growth. Nevertheless, when Fen was used in combination with Pre, the chlorophyll content did not recover, perhaps because the recovery of chlorophyll biosynthesis requires a much longer time.

ROS have been linked to herbicide toxicity and cause the loss of membrane integrity via lipid peroxidation; lipid peroxidation is considered to be the most destructive process molecular process in all organisms [30]. Antioxidant systems play a key role in maintaining a balance between ROS production and scavenging in plants. The antioxidant response to herbicides in crops has been a recent subject of investigation, and it has been established that most herbicides can induce the formation of ROS, but there are very few studies about the effects of safeners on crop antioxidant systems. Because of this, another aim of our study was to clearly define the effect of Fen on lipid peroxidation and antioxidant systems in rice. In the present study, we determined the activity of SOD, CAT and POD, which are markers of oxidative stress in plant cells. Our study showed that Pre can generate high levels of ROS. The antioxidant activities of the enzymes SOD, CAT and POD were significantly increased by Pre, and Fen alone did not increase the SOD, CAT and POD activities in either the shoots or the roots. In contrast, Fen ameliorated the oxidative stress induced by Pre. In addition, Fen also reduced the accumulation of MDA increased by Pre to normal levels in rice shoots. Since Pre can cause serious harm to rice seedlings, it is not surprising that the rice seedlings suffered from severe oxidative stress caused by Pre, but the effect of Fen on the antioxidant systems in rice suggested that the antioxidative defending properties are also one mechanisms of Fen in protecting rice from injury caused by Pre. In a previous study related to the effects of chloroacetamides and safeners (mefenpyr and dichlormid) on human blood cells, it was reported that the safeners alone did not elicit any changes in oxidative stress but instead they reduced the lipid peroxidation induced by the chloroacetamide herbicides [31]. This result, together with ours, suggests a role of safeners as antioxidants both in human blood cells and in rice.

Based on previous studies, it can be concluded that enhancing the rate of metabolism and/or the sequestration of herbicides are the major mechanism of safeners in protecting plants from injury [1, 32]. With regard to the effects of Fen on the accumulation and persistence to Pre residues in rice shoots, we found that Fen did not interfere with the uptake of Pre, but it induced a rapid reduction in Pre persistence. These results were expected since it has come to light that Fen is able to protect rice from Pre injury by enhancing its metabolism [33, 34]. Furthermore, the detoxification of Pre in rice is highly correlated with GST activity. Deng *et al.* [17] and Wu *et al.* [20] observed that Fen significantly increased GST activity in rice. The results of this study also found that, Fen could significantly induce the activity of GST both in shoots and roots, which is consistent with previous studies. Our results ascertained that Fen can strengthen the metabolism of rice by increasing the presence of detoxification enzymes, such as GST.

The molecular mechanisms of safener action may involve complex interactions between multiple signals and detoxification pathways, which can then protect plants from herbicides and other exogenous compounds [1]. Several studies have investigated the mechanism of safeners in model plants and very few graminaceous crops at the transcriptome level, but the exact mechanism remains unclear. Our purpose of RNA-Seq was to identify new candidate genes involved in the Fen response in rice. In view of the time effectiveness of mRNA expression, we set two time points for the RNA-Seq approach. One hundred seven and 35 genes were upregulated at 4 h and 24 h, respectively. Among the 142 differentially upregulated genes, 26 genes potentially encoded detoxification enzymes related to herbicide metabolism, and most of them were upregulated at 24 h. This result is consistent with previous studies showing that

safeners induce a series of components of the detoxification system in *Arabidopsis thaliana* [10, 22]. Similar results were also reported in rice and sorghum. Most of the genes that encoded detoxification enzymes, including P450s, GSTs, and UGTs, were upregulated by fluxofenim in sorghum [32]. Brazier-Hicks *et al.* induced rice seedlings with Fen and found that many enzymes, such as CYP, GST and UGT, had upregulated expression [11]. In the current study, 5 CYP genes, 2 GST genes, 4 GT genes and several other detoxification genes were upregulated when treated with Fen in rice. We did not find overlap in the inducible genes that were identified between Brazier-Hicks's study and our results, and the number of inducible genes identified in this study was much less than that of Brazier-Hicks's study, which may be due to the differences in species and the lower dosage of Fen (10 μ M vs 100 μ M). Since it is inferred that only a small number of safener-induced enzymes possess high detoxifying activities towards xenobiotics [11], the small quantity of genes induced by Fen in this study are not a bad thing. Combined with later qRT-PCR validation, we identified at least four P450 genes, two GST genes and one UGT gene that appeared to be related to the Fen mode of action, some of which were also induced by Pre. In particular, the two GST genes (*GSTU16* and *GSTF1*) exhibited strong inducible expression. This result indicated that these genes may be involved in the metabolism of Pre, but Fen itself can also be metabolized by GSTs [24], the function of these enzymes in metabolizing Pre needs further study.

Conclusion

This study further confirmed that Fen can protect rice from the damage caused by Pre by enhancing the metabolism of Pre in rice. Moreover, we did not find any oxidative damage caused by Fen on rice; rather, it reduced lipid peroxidation and the ROS induced by Pre. These findings provides new insight into the antioxidant activity of Fen, and we suppose that antioxidative-defensive properties are another mode of action of Fen. The transcriptome analysis indicated that Fen induced many response genes related to the detoxification pathway, including P450, GST, and GT. qRT-PCR results showed that some of them were concurrently induced by Fen and Pre. We believe there is great value to be gained by the further study of these differentially expressed genes. These results should be useful for elucidating the mode of action of Fen.

Methods

Plant material and growth conditions

Rice seeds 9311 (the seeds were acquired from China National Hybrid Rice R&D Center and deposited in the Germplasm repository of Hunan Agricultural University complying with legislation of China) were sterilized in 3% sodium hypochlorite for 5 min, washed in distilled water three to four times, soaked in tap water for 24 h, and then germinated at 28 C in the dark for 48 h. The germinating seeds were transferred into a plastic case containing Hoagland nutrient solution under the conditions of a 14 h/10 h light/dark cycle at 28/25 $\pm$ 1 C (day/night). For studies of the residue dynamics of herbicides in rice, the rice plants were divided into four treatments 6 days later: one treatment was left as the control, the second treatment

was 10 μ M Pre, the third treatment was 10 μ M Fen, and the fourth treatment was Pre + Fen (10 μ M +10 μ M). For the chlorophyll, MDA and antioxidant activity analyses, rice seedlings were treated with herbicide and/or safener 3 days after the rice seeds were inoculated and were cultured continually for 5 days. For transcriptome analysis and qRT-PCR determination, rice seedlings were cultivated to the 3-leaf stage and were then treated the same as in the residue dynamics experiment. Rice shoots and roots were harvested after 4 and 24 h to extract the total RNA for the transcriptome analysis and qRT-PCR determination. All the chemicals, including Pre and Fen, that were involved in these experiments were dissolved in acetone and diluted with water containing 0.1% tween-80. The control treatments contained the same amount of acetone and tween-80. The shoots and roots of the rice seedlings were collected separately and stored at -70C before analysis.

Analysis of chlorophyll and lipid peroxidation

The chlorophyll extraction and the content assay were performed according to the protocols described by Porra *et al.* [35]. Fresh rice flag leaves (0.1 g) were weighed and cut into 1 mm filaments, added to 10 mL of 80% acetone and stored at 4 C for 72 h. The absorbance of the solution was then determined at 663.6 nm and 646.6 nm using an ultraviolet spectrophotometer, and the total chlorophyll content was calculated.

Lipid peroxidation was determined in terms of malondialdehyde (MDA) content according to the method described by Wang *et al.* [36]. Fresh tissue (0.5 g) was weighed and ground in liquid nitrogen and then extracted in 3 mL of 0.67% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 10,000 g for 30 min, and then 2 mL of the supernatant was mixed with 2 mL of 0.5% TCA (the solvent was 20% TCA). The mixture was bathed in boiling water for 30 min, cooled to room temperature and centrifuged at 15,000 g for 5 min. The absorbance of the supernatant was measured at 532 nm, and the nonspecific absorbance value at 600 nm was subtracted (0.5% thiobarbital acid (20% TCA in solvent) was blank).

GST and antioxidant activity analysis

The extraction of GST and the activity assay were performed according to the protocols described by Li *et al.* [37]. The activities of CAT, POD, SOD were determined with assay kits (Jiancheng, Nanjing, China) according the manufacturer's instructions. The protein content was determined by the Bradford method [38].

Determination of Pre in rice

The extraction of Pre from rice refers to the method described previously [34]. Shoot samples (2 g) were powdered in liquid nitrogen, extracted and homogenized with 20 mL methanol and water (4:1, V/V) for 3 min. The mixture was then centrifuged at 5000 g for 15 min, and the supernatant was extracted twice with 30 mL of CHCl_3 . The organic phase was evaporated to dryness, and the residual was rinsed with 1 mL of water/acetonitrile (3:7 v/v). The samples were qualitatively analyzed using an HPLC equipped with

a reversed-phase column of C₁₈. Pre was quantified by a UV detector at a wavelength of 210 nm with a mobile phase (water: acetonitrile, 3:7 v/v) at a flow rate of 1 mL/min.

Library preparation for transcriptome sequencing and bioinformatics analysis

Two shoot samples were collected, with and without Fen. Total RNA was extracted from each sample using RNAiso Plus (TaKaRa Biotech, Dalian, China) following the manufacturer's recommendations. The quality and quantity of the total RNA were analyzed using an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

The cDNA library construction, including mRNA isolation, mRNA fragmentation, cDNA synthesis, and PCR amplification, was performed at Beijing BioMarker Technologies (Beijing, China). In brief, sequencing libraries were generated using the NEB Next, UltraTM RNA Library Prep Kit for Illumina (Illumina, Inc., San Diego, CA, USA) according to standard methods. The quality of the cDNA library was evaluated by the Agilent Bioanalyzer 2100 system. Finally, paired-end reads were sequenced on an Illumina HiSeq 2500 platform.

The raw data were filtered to remove adaptor sequences and low quality reads, and high quality clean data were subsequently obtained. The mapped data were obtained as a sequence alignment between the clean data and the specified reference genome. Gene function was annotated using the following databases: NR (NCBI nonredundant protein sequences), NT (NCBI nonredundant nucleotide sequences), PFAM (Protein family), KOG/COG (Clusters of Orthologous Groups of proteins), Swiss-Prot (A manually annotated and reviewed protein sequence database), KO (KEGG Ortholog database), and GO (Gene Ontology).

Differentially expressed gene (DEG) analysis

The quantification of the gene transcript abundance was measured by FPKM [39]. The gene expression differences between the control and the Fen treatment were analyzed using the DESeq R package. The P value calculations and false discovery control were performed using Benjamini's approach [40]. Genes with an adjusted P value < 0.05 and |log₂ (fold change)| ≥ 1 were considered differentially expressed. The DEGs were further subjected to GO enrichment and KEGG pathway enrichment analysis.

Transcript analysis by qRT-PCR

To verify the expression of the DEGs, 14 candidate detoxification genes were selected and quantified by qRT-PCR. The total RNA was extracted from samples using Trizol. cDNA was synthesized using a TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen Biotech, Beijing, China) and following the instructions. The qRT-PCR mix (20 µl) contained 10 µl of 2×TransStart Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China), 0.4 µl of each primer (10 µM), 0.4 µl of Passive Reference Dye II, 2 µl of cDNA, and 6.8 µl of Nuclease-free water.

The primers were designed using the online software Primer3, and the sequences of the primers are listed in supplemental Table 1. *Actin* was used as a reference gene. qRT-PCR was performed by an ABI 7300 plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The qRT-PCR program consisted of 94 °C for 30 s, 40 cycles of 94 °C for 5 s and 60 °C for 34 s, and finally 60 °C for 15 s. The expression of each gene relative to the control was calculated in accordance with the $2^{-\Delta\Delta C_t}$ method. Each experiment consisted of three biological replicates.

Statistical analysis

Three biological replications were carried out independently in this study. The data are expressed as the means $\pm$ standard deviations of three replicates. The significance of the differences between the treatments was statistically analyzed by one-way analysis of variance (ANOVA) at the 5% level using SPSS software 20.0.

Abbreviations

ABC: ATP-binding cassette; ANOVA: one-way analysis of variance; APX: ascorbate peroxidase; BP: Biological process; CAT: catalase; CC: Cellular component; CYP: Cytochrome P450 monooxygenase; DEGs: Differentially expressed genes; Fen: Fenclorim; GO: Gene Ontology; GSTs: Glutathione-S-transferase; GTs: Glycosyltransferase; KEGG: Kyoto Encyclopedia of Genes and Genomes; KOG/COG: Clusters of Orthologous Groups of proteins; MDA: malondialdehyde; MF: Molecular function; NR: NCBI redundant protein sequences; NT: NCBI nonredundant nucleotide sequences; qRT-PCR: quantitative real-time PCR; POD: peroxidase; Pre: pretilachlor; ROS: oxygen species; RNA-Seq: RNA sequencing; RPKM: reads per kilobase million mapped reads; SOD: superoxide dismutase; TCA: trichloroacetic acid

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Authors' contributions

This study was designed by LFH and LYB, YY carried out qRT-PCR validation, RWC and KLL performed the rest of the biochemical and physiological analyses, LP analyzed the transcriptome data and identified the DGEs, LFH wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors have read and approved the manuscript for publication.

Competing interests

The authors declare no competing financial interests.

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Additional File Legend

Additional file 1: Table S1. Primer pairs used for qRT-PCR verification of gene expression in rice

Additional file 2: Fig. S1. GO classification and statistical results of all genes. The genes were summarized in biological process, cellular component and molecular function. 28,662 genes were categorized.

Additional file 3: Fig. S2. GO classification and statistical results of DEGs at 4 h (A) and 24 h (B) treatment. The genes were summarized in biological process, cellular component and molecular function. 168 differentially expressed genes at 4 h treatment and 68 differentially expressed genes at 24 h treatment were annotated.

Tables

Table 1 Summary of rice transcriptome sequencing, assembly and annotation

	Reference transcriptome
total clean reads	284,404,035
clean base	84.37 Gb
all reads (mapped genes)	35,301
annotation in COG	9912
annotation in GO	28662
annotation in KEGG	8112
annotation in KOG	32636
annotation in Pfam	24882
annotation in Swiss-Prot	20664
annotation in NR	35253
annotated in at least one database	35301

Table 2 Fenclorim-induced detoxification genes in rice

Phase/Gene ID	Fold	P value	Description	Induced time
Phase I				
P450				
Os12g0150200	2.4	4.38E-03	Cytochrome P450, <i>CYP94C2b</i>	4 h
Os02g0703600	2.0	5.52E-05	Cytochrome P450, <i>C7IY83</i>	4 h
Os02g0204700	2.6	5.41E-04	Cytochrome P450, <i>CYP734A2</i>	24 h
Os06g0497275	2.1	6.74E-03	Cytochrome P450, <i>CYP71K14</i>	24 h
Os06g0497200	3.1	7.37E-03	Cytochrome P450, <i>CYP71D55</i>	24 h
oxygenases				
Os03g0289850	1.4	2.09E-03	2OG-Fe(II) oxygenase superfamily	4 h
Os04g0497000	1.3	5.44E-05	oxidoreductase activity	
Os04g0372700	1.2	6.51E-03	Putative quinone-oxidoreductase homolog	4 h
Os07g0561500	1.3	9.64E-03	oxidoreductase activity	24 h
Peroxidase				
Os04g0688300	2.2	1.17E-03	Peroxidase	4 h
Os07g0676900	1.5	5.85E-04	Peroxidase	24 h
Glycosyl hydrolase				
Os10g0565200	2.5	7.92E-10	Glycosyl hydrolases family 14	4 h
Os04g0604300	2.1	4.69E-03	Glycosyl hydrolases family 16	4 h
Os11g0701100	3.1	5.43E-03	Glycosyl hydrolases family 18	4 h
Lipases/thioesterases				
Os01g0253900	1.1	5.31E-03	Lipase (class 3)	4 h
Os06g0143400	1.9	2.35E-05	Acyl-ACP thioesterase; Acyl-ATP thioesterase	24 h
Phase II				
GSTs				
Os10g0528651	1.3	5.10E-03	Glutathione S-transferase; <i>OsGSTU16</i>	24 h
Os01g0369800	1.7	5.42E-03	glutathione S-transferase; <i>OsGSTF5</i>	24 h
Glycosyl transferases				
Os04g0206500	2.6	2.56E-06	UDP-glucoronosyl and UDP-glucosyl transferase	4 h
Os01g0880200	1.1	1.66E-03	Glycosyl transferase family 8	4 h
Os10g0555100	1.4	5.30E-04	Glycosyl transferase family 8	4 h
Os04g0556500	1.3	7.04E-03	UDP-glucoronosyl and UDP-glucosyl transferase	4 h
Phase IV				
Os04g0516600	1.7	3.33E-05	Beta-eliminating lyase; DegT/DnrJ/EryC1/StrS aminotransferase family	24 h

Os04g0516701	1.7	1.26E-04	Beta-eliminating lyase; DegT/DnrJ/EryC1/StrS aminotransferase family	24 h
Others Os04g0543900	2.0	5.78E-03	Glutamate/Leucine/Phenylalanine/Valine dehydrogenase	4 h

Figures

Figure 1

Effect of Pre and Fen on the total chlorophyll content and MDA. Three-day-old rice seedlings were used in the following treatments: control (CK), Pre (10 μ M), Fen (10 μ M) and Pre+Fen (10 μ M +10 μ M) and grown for 5 days. The bars are the averages $\pm$ standard deviations of three replicates, Lowercases and capital letters indicate significant differences within each treatment in the shoots and roots of rice plants, respectively ($p < 0.05$).

Figure 2

Effects of Pre and Fen on the activities of SOD, CAT, and POD. Three-day-old rice seedlings were used in the following treatments: control (CK), Pre (10 μ M), Fen (10 μ M) and Pre + Fen (10 μ M + 10 μ M) and grown for 5 days. The bars are the averages $\pm$ standard deviations of three replicates, Lowercases and capital letters indicate significant differences within each treatment in the shoots and roots of rice plants, respectively ($p < 0.05$).

Figure 3

Residual dynamics of Pre in rice shoots. Six-day-old rice seedlings were used in the following treatments: Pre (10 µM) and Pre + Fen (10 µM + 10 µM). The data points are the averages $\pm$ standard deviations of three separate replicates.

Figure 4

qRT-PCR validation for fourteen upregulated genes induced by Fen and Pre in rice. The actin gene was used as the internal control. Lowercases and capital letters indicate significant differences within each treatment in the shoots and roots, respectively ($p < 0.05$). The means and standard deviations from three biological replicates are shown.

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