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Title: RNA-sequencing *Oryza sativa* transcriptome in response to herbicide isoprotruon and characterization of genes involved in IPU detoxification†

Running head: Characterization of transcriptome in rice exposed to isoproturon

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Abstract

The soil residue of isoproturon (IPU) has become one of the environmental contaminants due to its intensive use in crop production. But how plants respond to IPU and the mechanisms for IPU degradation and detoxification in plants are poorly understood. In this study, we used recent advances in RNA sequencing (RNA-Seq) technology to dissect novel re-programming of transcripts in IPU-exposed rice plants. Four libraries were constructed from shoots and roots with or without IPU exposure. Mapping the clean reads to rice genomic databases generated 31,009~32,118 annotated genes for a single library. Most of annotated genes were differentially expressed (DEGs) among the libraries. Gene Ontology (GO) and Kyoto Encylopedia of Genes and Genomes (KEGG) analyses of DEGs showed modified biological functions and metabolic pathways associated with the resistance to environmental stress, degradation of xenobiotics and molecular metabolism. Validation of gene expressions by qRT-PCR confirmed the RNA-Seq results. DEGs encoding proteins involved in xenobiotics metabolism, detoxification, transporters, and transcription factors were comprehensively investigated. Activities of several enzymes closely related to xenobiotic metabolism were determined. Notably, the specific *cis*-elements of degradation-associated DEGs were predicted, and their regulatory networks were analyzed. To evidence the IPU-metabolism in rice, 19 degradations and 5 conjugates were chemically characterized using UPLC-LTQ-MS/MS. Overall, the transcriptome data presented here provide a new insight into the molecular and chemical mechanisms of IPU-metabolism in rice.

Key words: *Oryza sativa*; isoproturon; transcriptome; detoxification; UPLC-LTQ-MS/MS

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Introduction

Isoproturon [3-(4-isopropylphenyl)-1, 1-dimethylurea] (IPU), a member of the phenylurea herbicide family, is widely used for controlling pre- and post-emergence weed in soils where graminaceous crops are cultivated.[1] IPU as a photosystem II (PSII) inhibitor interferes with the electron transport of PSII by competing with plastoquinone for binding to the D1 protein in the thylakoid membrane of plant plastids.[2] While in agronomic practice IPU is applied to crop field, not all administrated IPU is absorbed by its targets. Instead, the left herbicide (or residue) may accumulate in soils, crops or run-off into the adjacent ecosystems.[3] Due to its moderately hydrophobicity and weak soil absorption, IPU from crops-rotation soils is freely absorbed by monocotyledonous crops such as rice and wheat.[4–6] Overloaded residues of IPU tend to accumulate in plants, and consequently risks crop production and food safety.[7,8] Thus, it is of great importance to uncover the detoxified mechanism for plant tolerance to the herbicide.

Many plants or crops have developed sophisticated strategies to nullify adversary effects of herbicides for their survival.[9] Several mechanisms for catabolism and detoxification of herbicides have been proposed.[10] Phase I reaction (functionalization reactions) is responsible for the reduction of herbicide, which involves the addition or 'unmasking' of a polar functional group, typically a hydroxyl (–OH), carboxyl (–COOH) or amino ($–NH_2$) within the molecule; following the process, Phase II (conjugation reactions) is responsible for the conjugation of activated herbicides with polar donor molecules such as sugars, glutathione and amino acids; in Phase III, the conjugates into the vacuole are transported by a group of carriers such as multi-drug resistance-associated proteins.[9–11] During the entire phase period,

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several major genes encoding cytochrome P450 monooxygenases (P450s), glutathione *S*-transferases (GSTs) and glucosyltransferases (GTs) have been implicated in the important mechanisms for degradation of toxicants.[12–14] However, the molecular mechanism for catabolism and detoxification of herbicides in plants is largely unknown.

Rice (*Oryza sativa*) is one of the most important staple crops all over the world. As one of the best studied model plants, a wealth of knowledge about its genetics, molecular biology, genomic sequence and genetic transformation has been documented, all of which make rice an ideal plant to investigate genes and associated pathways that control phenotypes of economic importance, tolerance to environmental stress and detoxification of herbicide in plants.[15] To date, no report is available on how IPU is degraded and detoxified in rice plants. Also, little is known about the molecular mechanisms for regulation of plant tolerance to the herbicide. Recent genome-wide profiling of transcriptome has resulted in identification of many functional genes associated with herbicide accumulation and detoxification in plants.[14,16–18] A global analysis of transcriptome will help understand the regulatory processes for plant adaptive responses to xenobiotic stress. In this work, we employed recent advances in next-generation RNA sequencing technology to analyze transcripts in IPU-exposed rice. A large number of IPU-responsive genes have been identified between control (IPU-free) and IPU-treated rice plants. We further characterized IPU-derivatives and IPU-conjugated products by ultra-performance liquid chromatography-double mass spectrometer (UPLC-MS/MS) to investigate the pathway of IPU degradation. These data will broaden our understanding of the global IPU-responsive molecular events and figure out the mechanisms for regulating IPU detoxification and degradation in rice plants. Thus, the goal of this study is

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to: (1) utilize the RNA-Seq datasets to get hints on a larger scale for relevant changes in gene expression, that facilitates mining genes responsible for IPU detoxification or degradation, (2) investigate the networks of genes enriched for regulating rice degradation of IPU in plants, and (3) develop an effective way to characterize IPU-metabolized or IPU-degraded products catalyzed by metabolizing enzymes in the rice crop.

Materials and methods

Plant materials and treatment. Isoproturon was obtained from Academy of Agricultural Sciences in Jiang Su, Nanjing, China, with a purity of 96.9%. Seeds of rice (*Oryza sativa* L. japonica. cv. Nipponbare) were surface-sterilized, rinsed and germinated. To eliminate the fungal colonization that attenuated plant responses to herbicide, the germinating seedlings were hydroponically grown under the condition described previously.[13] At the stage of two real leaf growth, twenty seedlings were transplanted into each pot and treated with IPU. Studies were performed in triplicate. In oxidative stress experiments, seedlings were treated with IPU at 0, 2, 4, 6 and 8 mg L^{-1}, for 1, 2, 3 and 4 d, respectively. For other study, seedlings were usually treated with 2 mg L^{-1} IPU for 4 d.

Measurement of physiological responses of rice to IPU. The malondialdehyde (MDA) content was measured according to the method of Liu et al. (2012),[19] with slight modification. Frozen rice shoots and roots (0.5 g) samples for each treatment were homogenized in 10 mL of 0.1 % trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 × *g* for 5 min.

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One mL of supernatant was added to 4.0 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was heated to 95 °C, stood for 30 min and quickly cooled in an ice bath. After centrifugation at 10,000 × *g* for 10 min, the absorbance of the supernatant was monitored with a spectrophotometer at 532 nm and 600 nm, respectively, and calculated using the extinction coefficient of 155 $mmol^{-1}$ cm^{-1}. The following formula was used.

$$\text{MAD (nmol } mL^{-1}FW^{-1}) = \frac{(A_{532}-A_{600})}{155{,}000} \times 10^6 \quad (1)$$

Total chlorophyll content was assayed with 80% acetone and its content was expressed as mg g^{-1} fresh weight.[20] Plasma membrane permeability of tissues was determined according to the method of Belkhadi et al. (2010).[21] Briefly, leaf and root (1 g) were cut into small segments (2 cm in size) and immersed in tubes with 20 mL distilled water. The test tubes were vortexed for 5 s, and the solution was assayed for initial electrical conductivity (EC_0) with a conductivity meter (METTLER TOLEDO FE30-FiveEasy™). The tubes were immersed at 25 °C for 30 min and assayed for EC_1. After the solution was boiled for 20 min and cooled to room temperature, the conductivity of killed tissues (EC_2) was measured. The percent of tissues membrane permeability was calculated as follow:

$$\text{EC (\%)} = \frac{EC_1-EC_0}{EC_2-EC_0} \times 100\% \quad (2)$$

RNA extraction and library construction. Total RNA was extracted from shoots and roots using Trizol (Invitrogen, Carlsbad, CA), and the RNA quality was assayed with an absorbance at 260/280 nm between 1.8 and 2.0. mRNA was enriched and purified with oligo (dT)-rich magnetic beads and broken into short fragments. The cleaved mRNA fragments

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were taken as templates. The first and second strand cDNAs were synthesized. The resulting cDNAs were subjected to end-repair and phosphorylation using T4 DNA polymerase and Klenow DNA polymerase. After that, an 'A' base was inserted as an overhang at the 3' ends of the repaired cDNA fragments and Illumina paired-end solexa adaptors were subsequently ligated to these cDNA fragments to distinguish the different sequencing samples. To select a size range of templates for downstream enrichment, products of the ligation reaction were purified and selected on a 2% agarose gel. The PCR amplification was run to enrich the purified cDNA template. Finally, the four libraries (Shoot+IPU, Shoot-IPU, Root+IPU, Root-IPU) were sequenced using an Illumina HiSeq^{TM} 2000.

RNA-sequencing and data processing. The library products were subject to sequencing analysis via the Illumina sequencing platform (HiSeq 2000).[22] The original image data generated by the sequence providers were transferred into nucleotide sequences data by base calling, defined as raw reads and saved as 'fastq' files. Clean sequence reads were generated by filtering out the raw reads using three separate criteria, namely 1) removing reads with sequence adaptors; 2) removing reads in which unknown bases represent more than 10%, and 3) removing reads in which the percentage of low quality bases (quality value ≤ 5) represents more than 50% in the read. All subsequent analyses were performed on the high-quality clean read datasets according to the bioinformatics analysis approach summarized in ESI Tables S1 and S2.

Analysis of GO and KEGG pathways. All read-mapped genes were identified by Blastx

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searching against the Gene Ontology (GO) Consortium database (http://www.geneontology.org/) and Kyoto Encylopedia of Genes and Genomes (KEGG) databases (http://www.genome.jp/kegg/pathway.html). The GO enrichment analysis of functional significance was subject to the ultra-geometric test with Benjamini-Hochberg correction.[23] GO terms with corrected *p*-value < 0.05 were regarded as significant enrichment for the DEGs compared to the genome background. Pathways with Q value < 0.05 indicate significantly enriched in DEGs.

Prediction of specific *cis*-elements of detoxification-associated genes. The *cis*-acting regulatory DNA elements in the promoter regions (3 kb) of detoxification-associated genes were predicted and analyzed using the plant *cis*-acting regulatory DNA elements database (PLACE, http://www.dna.affrc.go.jp/htdocs/PLACE) and plant transcription factor database v3.0 (http://planttfdb.cbi.pku.edu.cn).[24]

Quantitative RT-PCR validation of genes by RNA-Seq. Twelve genes related to IPU metabolism were randomly selected for validation using qRT-PCR. Primers were designed with the Primer 5 software (ESI Table S3). A reaction mixture for each PCR run was prepared with the SYBR Green PCR Core Reagents (TaKaRa), with a total volume of 25 μL containing 2 μL of template cDNA, 12.5 μL of the 2×TransStartTM Top Green qPCR SuperMix (Beijing TransGen Biotech Co., Ltd.) and 200 nM primers. The thermal cycling conditions were 1 cycle of 94 ℃ for 30 s for denaturation and 40 cycles of 94 ℃ for 5 s and 60 ℃ for 30 s for annealing and extension. All reactions were run in triplicate by monitoring the dissociation

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curve to detect and eliminate the possible primer-dimer and nonspecific amplifications. The PCR efficiency was determined by a series of 5-fold dilutions of cDNA in RNase-free water. The calculated efficiency of all primers was 0.9-1.0. Relative expression levels were normalized with the internal standard ubiquitin (Os03t13170.1) gene and presented as $2^{-\Delta\Delta Ct}$ to simplify the presentation of data.

Measurement of enzymes activities. Rice leaves or roots (0.3 g) were treated with 0 mg L^{-1} (control) and 2 mg L^{-1} IPU for 2 d and separately homogenized in 1.5 mL ice-cold extraction buffer containing 50 mM Tris-HCl (pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA) and 1.5 percent (w/w) polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 × *g* at 4 °C for 20 min. The supernatant was used as crude extract for determination activities of antioxidant enzymes and detoxification enzymes according to the methods of Lu et al. (2015),[9] Tan et al. (2015)[14] and Zhang et al. (2014),[20] respectively.

Antioxidant enzymes included laccase (EC 1.10.3.2), guaiacol peroxidase (POD, EC 1.11.1.7), superoxide dismutase (SOD, EC 1.15.1.1) and ascorbate peroxidase (APX, EC 1.11.1.11). Detoxification enzymes included NADPH-cytochrome P450 reductase (CPR, EC 1.6.2.4), UDP-glucosyltransferases (GTs, EC 2.4.x.y), *O*-methyltransferases (*O*-MT, EC 2.1.1.68) and glutathione *S*-transferase (GST, EC 2.5.1.18). The details of determination methods were summarized in the ESI Data S1.

IPU quantification. Rice seedlings were cultured in 1/2 strength Hoagland nutrient solutions containing 0 (control) and 2 mg L^{-1} IPU for 1, 2, 3 and 4 d, respectively. Shoots and

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198 roots of plants were separately harvested after IPU treatment. Fresh shoots or roots (3.0 g)
199 were ground and extracted ultrasonically three times in 15 mL of acetone-water (3:1, *v/v*) for
200 30 min, followed by centrifugation at 5,000 × *g* for 15 min. The supernatant was concentrated
201 to remove acetone in a vacuum rotary evaporator at 40 °C. The residual water was loaded
202 onto an LC-C_{18} solid phase extraction column. The eluent was discarded. The column was
203 washed with 4 mL methanol, which was collected for analysis with HPLC. The spiked
204 recovery and relative standard deviation (RSD) of IPU extraction and detection limit from
205 rice tissues were showed in ESI Table S4.
206
207 **Analysis of IPU metabolites and conjugates in rice.** Fresh shoots or roots (5.0 g) were
208 ground with liquid nitrogen. The extraction and purification were run with the same analytical
209 method indicated above. The washing solution for the LC-18 column was collected for
210 analysis. Metabolites and conjugates of IPU in rice were analyzed using ultrahigh
211 performance liquid chromatography (UPLC) (Thermo, USA) coupled to a linear ion
212 trap-Orbitrap hybrid mass spectrometer (LTQ Orbitrap XL) equipped with a
213 heated-electrospray ionization probe. Instrument control was through Tune 2.6.0 and
214 Chromeleon programs. Separations were performed on a Hypersil gold C_{18} (100 mm × 2.10
215 mm, 3 μm particle size, Thermo Fisher Scientific). Mobile phase was composed of (A) water
216 + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. A linear gradient program was
217 performed in 36 min at a flow rate of 0.20 mL min^{-1} under the following conditions: 5% B for
218 1 min, 1-15min from 5% to 35% B, 15-25 min from 35% to 95% B, 95% B for 5 min, 30-31
219 min from 95% to 5% B, and 5% B for 5 min. Column oven and autosampler temperature were

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set at 35 and 10 °C, respectively. The injection volume was 10 μL.

The mass spectrometer was operated in positive mode. HESI-source parameters were as follows: capillary temperature 300 °C, the source voltage 4 kV, and auxiliary gas 25. Accurate mass spectra were recorded from 50 to 1000 *m/z*. For fragmentation study, a data dependent scan was performed by deploying collision-induced dissociation (CID). The product ions were generated by the LTQ ion trap at normalized collision energy of 35 % and *q*-activation of 0.25 using an isolation width of 2 Da. The external mass calibration of the Orbitrap was performed once a week to ensure a working mass accuracy ≤ 5 ppm. Data analysis was handled by a computer equipped with Xcalibur software, version 2.1. IPU and its metabolites were identified according to the corresponding spectral characteristics: accurate mass, mass spectra, and characteristic fragmentation.

Statistical evaluation. Statistical analysis was performed to identify DEGs between the libraries using a rigorous algorithm described previously.[25] The gene expression was normalized to transcripts per million clean reads. The statistical *t*-test was used to identify genes expressed between libraries. *p*-values were adjusted by the multiple testing procedures described by Benjamini and Yekutieli (2001),[23] by controlling false discovery rate (FDR). In this study, we used stringent value FDR < 0.001 and the absolute value of $Log_2Ratio \leq 1$ as the threshold to judge the significant difference of gene expression. The correlation of the detected count numbers between parallel libraries was statistically assessed by calculation of Pearson correlation coefficient. All the experiments were performed at least three repetitive treatments. The values were expressed as means ± standard deviation. ANOVA was conducted

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using the mixed model procedure in SPSS statistics 20. The significance of the differences among the means was calculated by Turkey's test. Statistical significance was set at $p<0.05$. Principal component analysis (PCA) was used to classify treatments according to four libraries using SIMCA-P 13.03. The network graph was laid out using Cytoscape 3.2.1.

Results and discussion

Physiological response to IPU

To investigate an effective IPU dose to treat rice tissue for RNA-Seq, an initial physiological response to IPU was assessed. MDA is the final product of lipid peroxidation and widely used to evaluate the damage degree of plants under toxicant exposure.[19] Overall, MDA contents in IPU-treated rice were remarkably higher than those in IPU-free rice (ESI Fig. S1A). The maximum accumulation in shoot (116.0 percent relative to control) and root (176.7 percent relative to control) was observed at 2 mg L^{-1} IPU for 4 d (ESI Fig. S1A). Then, the MDA contents experienced a gentle decline at 4-8 mg L^{-1} IPU (ESI Fig. S1A), indicating that the over-dosage of herbicide IPU may damage plant antioxidative system and lead to loss of cellular homeostasis of ROS formation.[19] Assessment of chlorophyll content, tissue elongation and membrane permeability was further made. The chlorophyll content, elongation and membrane permeability of shoots and roots were reduced under 2 mg L^{-1} IPU exposure (ESI Fig. S1B–D), indicating that IPU at 2 mg L^{-1} could effectively inhibit the rice growth and injure the integrity of plasma membrane.

Antioxidant enzymes are important for protecting plants from injury of pesticide-induced

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oxidative stress.[26,27] Compared to the controls, a significant increase was observed for the activities of laccase, superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) in rice tissues exposed to 2 mg L^{-1} IPU (ESI Fig. S1E–H). The laccase activities in shoot were dramatically enhanced 10-fold over the control. The SOD activities of shoot and root exposed to 2 mg L^{-1} IPU were 145.5% and 160.8% of the control (without IPU treatment), respectively. The changes of POD and APX activities showed the same trend as those of SOD. These results indicate that 2 mg L^{-1} of IPU could be the effective concentration that triggered the initial oxidative stress in rice tissues. Based on the observation, the effective dose of IPU at 2 mg L^{-1} was used for assessing the following transcriptome response.

Profiling of transcriptome in response to IPU by RNA-Seq

We sequenced four RNA libraries (Shoot+IPU, Shoot-IPU, Root+IPU and Root-IPU) taken from rice shoot and root at 0 and 2 mg L^{-1} IPU, respectively. Four libraries generated 22.2~26.1 million sequence reads of 99.25~99.56 bp in length (ESI Table S1). After removal of adaptor, duplicate, ambiguous and low-quality reads, 21.7~29.0 million high-quality clean reads (97.1~98.1% of the raw data) remained. The clean reads were mapped to the rice genome (Japonica cv. Nipponbare, http://rapdb.dna.affrc.go.jp/download/irgsp1.html) using soap 2.21, with no more than two base mismatches allowed in the alignment. Of the total clean reads, 94.25%~94.45% in shoots and 72.36%~77.34% in roots were perfectly matched (ESI Table S2).

Analysis of datasets by RNA-Seq and differential gene expression

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Variations of gene expression between two libraries give insights into the molecular events involved in plant response to IPU exposure. To understand each gene expression within the libraries, the transcript abundance of mapped genes was normalized using fragments per kb per million reads (FPKM). We then used the false discovery rates (FDRs) < 0.001 and absolute value of Log_2 foldchange ≥ 1 as a threshold to estimate the differential expression genes (DGEs).[28] As shown in Fig. 1A–D, a large set of genes were found differentially expressed between two libraries. Notably, the expression of genes between root and shoot was found to be more significant than that between the IPU-treatments and controls. This could be validated by the principal component analysis (PCA). Samples with IPU (Shoot/Root+IPU) were on the one side of PC2, while samples without IPU (Shoot/Root-IPU) were on the other side of PC2 (Fig. 1E), indicating that PC2 was markedly influenced by IPU exposure or a remarkable difference between the two treatments. Similarly, shoot samples (Shoot+/-IPU) were clearly distinguished from root samples (Root+/-IPU) in the two sides of PC1, indicating that the tissue-specific was the largest effect on the first principal component (Fig. 1E). In the box chart, the FPKM median line of Shoot+IPU was higher than other three samples, suggesting that more genes in shoots were induced by IPU (Fig. 1F). Moreover, the number of genes in the controls (32,118 in shoot and 31,549 in root) was more than that in the IPU-treatments (31,009 in shoot and 31,312 in root), indicating that fewer transcripts were found in IPU-treated tissues than the controls (IPU-free tissues) (Fig. 1F).

When we compared the IPU-treated transcripts with the controls, 5,255 DEGs (15.52% of all genes) were found to be upregulated and 6,672 DEGs (19.7% of all genes) were downregulated in shoot, while 5,785 DEGs (17.10% of all genes) were upregulated and 5,895

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DEGs (17.43% of all genes) were downregulated in root (ESI Fig. S2), indicating that upregulated genes were less than the downregulated genes in response to IPU, and the number of downregulated DEGs in shoot was more than that in root.

Venn diagrams revealed 33,864 and 33,827 enriched genes in root and shoot with IPU, respectively. These genes were differentially expressed in the presence of IPU. A certain proportion of them responded specifically to IPU exposure. For instance, in shoot 5,255 transcripts were upregulated and 6,672 downregulated under IPU exposure (Fig. 1G). Meanwhile, in root 5,785 transcripts were upregulated and 5,895 were downregulated. Only 1,108 and 1,148 transcripts existed in both uniform-regulated libraries. These results suggested that most of genes were transcriptionally reprogrammed by IPU exposure.

We next presented the 200 most DEGs ($|Log_2 foldchange| \geq 1$; mean FPKM ≥ 10) in two libraries. The relative abundance was expressed as a FPKM ratio of +IPU/-IPU responsive transcripts. As shown in ESI Tables S5 and S6, the top three upregulated genes in +IPU/-IPU shoot encode conserved hypothetical protein (Os01t0729900-03), alpha amylase isozyme (Os08t0473900-03) and ferripyochelin-binding protein-like (Os01t0283100-02), and the top three upregulated genes in +IPU/-IPU root encode the branched chain alpha-keto acid dehydrogenase E1 beta subunit (Os07t0170100-02), alcohol dehydrogenase (Os11t0210500-02) and isoform 2 of potassium transporter 1 (Os04t0401700-02). Notably, a plenty of upregulated DEGs in +IPU/-IPU concerned cytochrome P450 family protein, dehydrogenases, hydrolases, haem peroxidase, protein kinases and ABC transporters, which are most likely related to xenobiotics metabolism and detoxification. We also found several other interesting genes coding for glycosyltransferase (Os04t0660400-03), methyltransferase

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(Os04t0570800-01) and heat shock proteins (Os10t0575200-03; Os04t0107900-04), all of which are possibly involved in the secondary metabolism. Several other genes encoding transcription factors were strongly responding to IPU, such as zinc finger and NAC-domain proteins. These genes encode regulatory proteins essential for plant acclimation to IPU stress. Unexpectedly, some phytohormone-responsive genes like anxin (Os06t0335500-02) and gibberellin (Os11t0240600-02), were positively regulated in shoot, suggesting that the phytohormone may play important roles in signal transduction during the IPU stress.

Furthermore, the forty most abundantly DEGs in the libraries were presented (ESI Table S7). The top upregulated transcripts in four libraries were heat shock protein 70 (Os11t0703900-01 in Shoot+IPU), AMP-binding protein (Os03t0305100-01 in Root+IPU), light regulated Lir1 family protein (Os01t0102900-01 in Shoot-IPU) and pollen-specific desiccation-associated LLA23 protein (Os11t0167800-01 in Root-IPU). Transcripts such as dioxygenase (Os05t0171900-01, a bleomycin resistance protein) and sugar-starvation induced-protein (Os03t0701200-01) were found to be abundantly expressed in the two libraries and extremely upregulated by IPU. By contrast, the expression of two genes (Os07t0529600-01 and Os04t0678700-01) involved in chlorophyll biosynthesis was inhibited by IPU treatment.

Analysis of gene functional enrichment and pathway categories

Genes with similar expression patterns may be functionally and phenotypically correlated.[20] To better understand the functions of DEGs between IPU-free and IPU-treated plants, we carried out Gene Ontology (GO) category enrichment analysis using Fisher's test, with

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352 *p*-value ≤ 0.01 as a threshold.[29] By GO analysis, 1016 shoot (4.86%) and 867 root (4.15%)
353 DEGs were classified into 120 functional categories using the complete set of GO terms for
354 three main categories: biological process, cellular component and molecular function (ESI
355 Table S8). For Shoot+IPU/Shoot-IPU pair, we observed that the terms hydrolase,
356 oxidoreductase and transferase represented large proportions of metabolic process (Fig. 2A),
357 which were proved to play a vital role in IPU-metabolism in vivo.[30,31] Importantly, a high
358 percentage of DEGs was mapped to the groups of response to biotic, abiotic, chemical and
359 external stimulus (Fig. 2A). Besides, gene groups related to antioxidant, catalytic, transporter
360 and oxidation reduction processes were considerably enriched. Most of GO terms in
361 Root+IPU/Root-IPU pair were similar to those in shoot (Fig. 2B). Especially, some genes
362 encoding drug transporter in root were considerably stimulated by IPU, but those for carbon
363 utilization and development process were inhibited (Fig. 2B).

364 To examine the IPU-responsive DEGs in specific tissues, we further identified a group of
365 IPU-induced transcripts which also have higher expression than other tissues. For instance, A
366 total of 2,441 genes were shown to be upregulation under IPU treatment
367 (Log_2Shoot+IPU/Shoot-IPU), as well as higher-level expressions in shoot than those in root
368 (Log_2Shoot+IPU/Root+IPU; ESI Fig. S3A). Likewise, a similar result was illustrated in 2,448
369 genes of root (ESI Fig. S3B). These DEGs annotated to the GO terms are well-known for
370 their roles in oxidative stress response and antioxidant protection (*e.g.* cellular response to
371 xenobiotic stimulus, response to abiotic/chemical stimulus, hydrolase, oxidoreductase and
372 peroxidase) (ESI Fig. S3C). Interestingly, some GO terms were only found in shoot or root.
373 For instance, genes encoding drug transporter were considerably enriched only in root and the

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374 term vesicle only in shoot, suggesting that rice roots were primarily responsible for the IPU
375 translocation, while shoots for IPU accumulation.[32]

376 We further used KEGG ontology assignments to classify functional annotations of the
377 identified genes.[33] The KEGG pathway database records networks of molecular interactions
378 in cells, as well as their variants specific to particular organisms, that can help to understand
379 the biological functions of genes. We identified 46 genes of 107 DEGs (29 upregulated and 17
380 downregulated) and 45 genes of 142 DEGs (25 upregulated and 20 downregulated) for the
381 two library pairs (Shoot+IPU/Shoot-IPU and Root+IPU/Root-IPU), and each of them could
382 be further assigned to 16 and 23 pathways, respectively (ESI Table S9). Among the
383 assignments, DEGs (p-value $\leq$ 0.05) belonging to "protein processing in endoplasmic
384 reticulum" and "ubiquitin mediated proteolysis" were most abundantly presented in the two
385 library pairs. Each of them comprised 16 (14.95%) and 7 (4.93%) genes for
386 Shoot+IPU/Shoot-IPU and Root+IPU/Root-IPU libraries, respectively. Three upregulated
387 genes were sorted into phosphatidylinositol signaling system in Shoot+IPU/Shoot-IPU pair
388 (Osa04070). Additionally, 15 genes involved in amino acid, sugar and taurine metabolism
389 were enriched in Shoot+IPU/Shoot-IPU and Root+IPU/Root-IPU, suggesting that IPU
390 exposure modified the basic metabolisms. Obviously, the KEGG annotations provide
391 important clues for investigating specific biological process that can be influenced by the
392 expression of genes responding to IPU treatment.

393

394 **qRT-PCR validation of genes by RNA-Seq**

395 To confirm IPU-induced genes identified by Illumina RNA-Seq, we selected 12 genes for

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qRT-PCR analyses, including those Os08t0547300 coding for cytochrome P450 78A1, Os08t0152400 for P450, Os04t0206700 for UDP-glucuronosyl/UDP-glucosyltransferase, Os10t0555100 for glucosyltransferase like protein, Os12t0123200 for glutathione S-transferase GST7, Os07t0168300 for glutathione S-transferase GSTU6, Os09t0344500 for encoding *O*-methyltransferase ZRP4, Os05t0102000 for SAM dependent carboxyl methyltransferase family protein, Os03t0273200 for laccase, Os01t0850700 for laccase-7, Os01t0770500 for ABC transporter ATP-binding protein, and Os09t0472100 for ABC transporter (Fig. 3). It is shown that all genes analyzed by qRT-PCR had an expression pattern similar to RNA-Seq, indicating the accuracy of the results from RNA-Seq.

Identification of genes involved in IPU detoxification or degradation

To figure out whether IPU-responsive genes were associated with IPU detoxification or degradation, the functional genes encoding proteins or enzymes were identified and divided into four categories including metabolic enzymes, antioxidase, transporter and transcription factors ESI Table S10. The first group (also Phase I) is the cytochrome P450 monooxygenases (cytochrome P450s or P450s)-coding genes that play important roles in metabolizing or degrading herbicides.[11,14,34] A total of 221 genes coding to cytochromes P450 were identified. Of these, 41 genes expressed in shoot, 63 genes expressed only in root, and 117 genes expressed both in shoot and root. Thirty-four cytochrome P450s genes showed higher mRNA levels in IPU-treated rice (Fig. 4A). Os03t0760200-01(*CYP81A6*) which has been reported to resist bentazon and sulfonylurea herbicides in hybrid rice,[35] was significantly upregulated in shoot. Also, expression of Os03t0417700-01 (*GL3.2*) related to grain growth,[36] was induced

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418 by IPU exposure in root, suggesting its possible participation in the P450-mediated
419 detoxification of IPU. Oxidoreductase and monooxygenase represent two main branches of
420 P450 family genes and are correlated with oxidation of a variety of aromatic and recalcitrant
421 compounds such as herbicides.[37] Seven DEGs annotated as oxidoreductase and nine genes
422 annotated as monooxygenase were found highly expressed in IPU-treated rice (Fig. 4A).

423 The second group comprised several types of genes such as those encoding
424 glucosyltransferases (GTs), methyltransferases (MTs) and glutathione *S*-transferases (GSTs)
425 (ESI Table S10). Plants modify harmful low-molecular-mass compounds by the way of
426 glycosylation, methylation and glutathione conjugation to cope with many xenobiotics
427 toxicity in their environments.[10] Several DEGs related to phase II metabolism were
428 upregulated by IPU exposure, including 22 GTs, 12 MTs, and 2 GSTs genes (Fig. 4B). As a
429 key mediator of development and chemically-induced disease resistance, expression of
430 *OsSGT1* (Os09t0518200-01) was 4.53-fold increased in IPU-treated shoot compared to the
431 control. Os11t0256900-01 (*OsBISAMT1*) encoding S-adenosyl-L-methionine:salicylic acid
432 carboxyl methyltransferase, was differentially up-expressed in IPU exposed rice. It could be
433 induced by benzothiadiazole and salicylic acid.[38] Our recent study also showed that salicylic
434 acid plays a role in promoting degradation of IPU in wheat.[9] There are 55 DEGs encoding
435 GSTs (Fig. 4B; ESI Table S10). Of these, only 2 transcripts were moderately upregulated by
436 IPU treatment, suggesting that the transcripts might be sensitive to IPU. We identified one
437 transcript (Os02t0114400-00) encoding acyltransferases that was inducible under IPU
438 exposure in shoot (Fig. 4B). A previous report indicated that the glucose moieties of
439 glycosylation were modified by acylation via ATs in vivo.[39]

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Currently, some kinases, such as serine-threonine protein kinases (S/TPK), were reported as key players in plant signal transduction pathways for resistance metabolism.[40] Of 193 S/TPK-annotated DEGs, 25 genes were stimulated by IPU (ESI Table S10). *OsCIPK14* and *OsCIPK15* were proven to involve various MAMP-induced immune responses such as defense-related gene expression, phytoalexin biosynthesis and hypersensitive cell death (Fig. 4C).[41] We also identified other protein family genes for hydrolase in rice (ESI Table S10). Additionally, expression of *OsTPS1* relevant to abiotic stress tolerance was enhance by IPU treatment (Fig. 4C).[42]

The last group (Phase III) including the ABC transporter-coding genes responsible for transferring metabolites or degraded products,[11] was shown to be differentially expressed under IPU stress (Fig. 4D; ESI Table S10). The ATP-binding cassette (ABC) transporters and multidrug and toxic compound extrusion (MATE) play an essential role in efflux of xenobiotics in *Arabidopsis*, but their functions are rarely reported in rice.[43] Several DEGs encoding ABC transporters were highly expressed under IPU stress. For instance, Os01t0218700-02 was strongly induced by IPU stress specifically in shoot, Os05t0137200-03 specifically in root, and Os01t0770500-02 in all tissues (Fig. 4D; ESI Table S10). Sugar transporters are multiple transmembrane domain proteins located in the Golgi apparatus and the endoplasmic reticulum.[44] They play a critical role in providing the substrates for glucosyltransferase that have their catalytic sites facing the lumen of these organelles.[45] In this study, we identified some sugar and monosaccharide transporter (MST) genes. *OsMST3* and *OsMST6* that have transport activity for some monosaccharides in an energy-dependent H^+ co-transport manner,[46] were found to be highly expressed in shoot (Fig. 4D). Furthermore,

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several other peptide transporters whose substrates include glutathione, hormone-amino acid conjugates and peptide phytotoxins that play diverse roles in plant growth and resistance,[47] were also highly expressed in IPU-treated shoot and root (Fig. 4D). For example, *OsPTR7* and *OsPTR8* encoding peptide transporters were induced by abiotic stress.[48]

Recent studies have demonstrated that many transcription factors-coding genes participate in various biotic or abiotic stress responses.[49] In this study, three types of TFs genes MYB, WRKY and NAC TFs were identified to be induced by IPU, and some of them showed tissue-specific patterns. *OsSKIPa* and *MYBS3* were reported to positively regulate drought and cold stress responses.[50] Both were highly expressed in shoot (Fig. 4E). Meanwhile, we observed that *OsMYB2P-1*, *SRWD3* and *OsMYB3R-2* were highly expressed in root (Fig. 4E). Their functions were involved in the positive regulation of phosphate-starvation, salt and chilling responses in rice.[51–53] Most of WRKY genes were shown to be stimulated by IPU. Five genes (*OsGAmyb*, *OsWRKY45/47/62* and Os07t0416100-01) were positively expressed in shoot, three (*OsWRKY57/72/83*) in root, and two (*OsWRKY39/40*) in both tissues. NAC proteins are another type of plant-specific TFs. Some of them function in relation to abiotic stress responses.[54,55] Expression of *OsNAC10* was 69.6-fold higher in IPU-treated shoot than its control, suggesting that this gene has potential to regulate IPU stress response in rice (Fig. 4E).

Many genes related to reactive oxygen species (ROS) scavenging were differentially regulated in rice after IPU treatment, which likely contributed to the resistance to IPU. Six genes encoding laccase, monodehydroascorbate reductase, SOD, POD, CAT and APX were identified. DEGs analysis revealed that laccase (Os01t0850700-01), monodehydroascorbate

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reductase (Os02t0707100-02), SOD (Os01t0284500-01) and POD (Os04t0688300-01) (ESI Table S10) were upregulated under IPU exposure, in a good agreement with their enzymatic activities as described above (ESI Fig. S1E–H).

Analysis of enzyme activities involved in IPU detoxification and degradation

To investigate whether the induction of IPU stress-responsive genes was associated with their enzymatic activities, the activities of four main xenobiotic-metabolic enzymes (GTs, P450, GST and MTs) were assayed. Recently, glucosyltransferases with bifunctional *N*- and *O*-activity have been shown to involve xenobiotic metabolism.[56] In this study, the activities of *O*-glucosyltransferase (*O*-GTs) were increased by 1.61-fold in root and 2.50-fold in shoot, respectively (Fig. 5A and 5B). Similarly, *N*-glucosyltransferase (*N*-GT) activities in IPU-treatment seedlings were significantly changed by IPU treatment. We further selected IPU as subtract to determine the activities of GTs under IPU-exposure, and showed that the GTs activities in root was significantly induced by IPU but not induced in shoot (Fig. 5C). These results indicate that the activity was subject to the enzymes types and tissue specificity. Several *O*-MTs involves biosynthesis of melatonin that plays an important role in the resistance to chemical stress.[57,58] In shoot and root, treatments with IPU led to the higher activity of *O*-MTs compared to the controls (Fig. 5D).

NADPH-cytochrome P450 (CPR) plays a central role in cytochrome P450 action involved in metabolism-based insecticide resistance.[59] The CPR activities in IPU-exposed rice plants were significantly higher than the control, with the activities of CPR in root and shoot being increased by 1.61- and 2.05-fold, respectively (Fig. 5E). In contrast, the activity of GST

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was repressed by IPU (Fig. 5F), suggesting that both GST genes and proteins were sensitive to IPU (ESI Table S10), an observation consistent with the previous report.[60]

Prediction of *cis*-elements of metabolism-associated DEGs and gene network analysis

Because numerous genes responded to IPU, we were interested in identifying *cis*-elements of metabolism-associated DEGs. Promoter Analysis 2.0 was used to predict *cis*-acting elements in the upstream of 57 DEGs.[61] A total of 12 specific elements were identified (ESI Table S11). Each gene has more than one such site for TFs binding. Notably, the top three motifs in the upstream of DEGs can be bound by *WRKY71OS* (promoter II, a transcriptional repressors of the gibberellin signaling pathway), *MYBCORE* (promoter V, a plant MYB proteins that are responsive to abiotic stress) and *WBOXATNPR1* (promoter Xin promoter of *Arabidopsis thaliana* NPR1 gene recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins) and were enriched in Os01t0628700-01 and Os03t0594900-01 (encoding cytochrome P450 family proteins) and Os03t0182000-00 (encoding flavin-dependent monooxygenase 1), respectively (ESI Table S11).

Recent studies have shown that many genes may have a similar expression pattern under certain environmental stimuli, and in this case, they are usually working in a similar and specific pathway.[62] As the upstream of the DEGs shares the 12 common *cis*-elements, we assumed that these DEGs may co-express under IPU exposure. Five genes *OsWRKY24/71/72*, *OsMYB1* and *OsMYBS1* were identified as core (or guide) genes that can connect the 47 DEGs for gene-network generation (Fig. 6A). The regulatory network revealed several major subnetworks of gene interactions. Distinct modules were formed, reflecting expression

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patterns and a regulatory relationship between the TFs and their target genes. Of the 47 target genes, 9 genes were regulated by one category TF such as a MT gene (Os07t0206700-02), 37 genes by two categories TFs such as *CYP81A6*, and only 3 genes by three categories TFs such as *GL3.2*. We further analyzed the transcripts of the predicted TFs. *OsMYB1*, *OsMYBS1* and *OsWRKY72* were highly expressed in all tissues. Other genes like *OsWRKY71* and *OsWRKY24* were upregulated in shoot rather than in root (Fig. 6B), suggesting that the core and target genes were well connected.

Analysis of IPU accumulation and characterization of derivatives in rice tissues

Both tissues accumulated IPU progressively with the time of exposure (ESI Fig. S4). When plants were treated with 2 mg L^{-1} IPU for 4 d, the contents of IPU in shoot and root were 3.06 and 2.26 mg kg^{-1}, respectively.

It is well-known that cytochromes P450, UDP-glucosyltransferase and glutathione *S*-transferase are the most important enzymes for conjugation reactions of herbicide metabolism.[13,14] To confirm the importance of enzymes-mediated biotransformation and detoxification of IPU, we characterized degradation products and conjugates of IPU in the rice shoot and root using UPLC-LTQ-MS/MS. The accurate mass data (< 5 parts per million errors) by high resolution MS were applied to confirming elemental formula. A total of twenty degradation products via Phase I pathway and four glycosylated-IPU conjugates via Phase II pathway in IPU-exposed rice have been successfully characterized. Their mass spectrometric data were summarized in Table 1. All IPU-derivatives were detected in shoot. Of these, seven (metabolites 3#, 4#, 5#, 11#, 19# and conjugates 1#, 4#) were also detected in root.

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Importantly, 9 metabolites (1#, 2#, 6#, 8#, 13#, 14#, 16#, 17# and 19#) and 2 conjugates (1# and 5#) were reported here for the first time in plants (Table 1).

According to the extracted ion chromatograms by full-scan acquisition, signals of IPU and its metabolites were detected in IPU-treated rice samples but not in the control (IPU-free) (ESI Fig. S5). Based on our previous study of IPU metabolites in wheat,[9] we deduced the metabolic pathway of IPU in rice tissues and surprisingly found that the routes of IPU-metabolism were species-specific (Fig. 7). For example, *O*-methylated degradations were characterized in rice alone rather than in wheat, such as 2-methoxyl-IPU (metabolite 2#, *m/z* 237), 2-methylehanoic-demethyl-IPU (metabolite 5#, *m/z* 223), *N*-OH-demethyl-IPU (metabolite 8#, *m/z* 209) and 4-(1-methoxy-2-methyl-2-propanyl)-*N*-methylanline (metabolite 14#, *m/z* 180). The *O*-methylation reaction for the IPU-metabolism can be catalyzed by *O*-methyltransferase, which was reported to involve the detoxification of catechol drugs in mammals,[63] but rarely described in plants.

Apart from the two major degradations (OH-isopropyl-IPU and monodemethyl-IPU), more subsequent and smaller degradations which constituted via hydroxylation and/or dealkylation (Fig. 7) were detected in rice rather than in wheat. The demethylation and hydroxylation of phenylurea herbicide by cytochrome P450 were studied in detail.[64] Furthermore, isopropenyl-IPU (*m/z* 205) and its derivatives were supposed to arise as a spontaneous artifact from OH-isopropyl-IPU (*m/z* 223) in rice shoot because tertiary alcohols were easily eliminated a water molecule.[65] Isopropenyl-IPU was also detected in wheat and soybean cell cultures.[9,65]

The conjugation of xenobiotics with sugars is the most frequently observed Phase II

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572 biotransformation seen in plants.[56] In rice tissues, IPU was predominantly metabolized to the
573 *O*-glucoside. The similar phenomena of IPU conjugation to *O*-glucosyl and *N*-glucosyl
574 moieties were observed in wheat.[9] As a widespread enzyme in plant kingdom, GTs have the
575 potential to conjugate IPU and supply the substrates for other metabolic enzymes. Several
576 glycosylated derivatives and subsequently formed *O*-acetylglucoside were characterized in
577 rice (Fig. 7). Our recent study explored malonyl-glucosyl and *N*-acetyl-glucosyl
578 IPU-derivatives in wheat.[9] The conjugation of small molecules with acetic acid or malonic
579 acid in cytoplasm provides a signal for the export of the resulting acidic conjugates into the
580 vacuole and has been shown to be an important biotransformation step in the detoxification of
581 several xenobiotic.[66] The detailed description about the chemical structure analyses was
582 included in ESI Data S2.
583
584
585 **Conclusion**
586 The rice samples exposed to IPU were employed to generate the first large-scale
587 transcriptome sequencing data using Illumina platform. Our data show 11,927 DEGs (35.22%
588 of all genes) in shoot and 11,680 DEGs (34.54% of all genes) in root, indicating that
589 expression of a large number of genes was altered by IPU exposure. GO analysis of DEGs
590 revealed that the transcriptome alterations were highly related to stress responses, metabolic
591 enzyme activities, antioxidant and transporters. Notably, several members of metabolic
592 resistance genes, which are central to the biotransformation of xenobiotics, were differentially
593 expressed in tissues following IPU treatment. Furthermore, activities of enzymes

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corresponding to IPU-metabolic resistance were generally induced by IPU treatment, including NADPH-cytochrome P450, GTs, *O*-MTs and others. The *cis*-elements in the upstream of some DEGs in responses to IPU were predicted. Gene co-expression suggests that the genes were possibly connected with some specific core transcription factors such as *OsWRKY24/71/72*, *OsMYB1* and *OsMYBS1*. Using UPLC-MS/MS, we characterized 20 degraded products and 4 conjugates in specific tissues, and eleven IPU-derivatives in plants were reported for the first time. The IPU metabolic pathway in rice tissues has been inferred. Further study will focus on the specific functions of the genes that differentially expressed in IPU rice. Collectively, our study provides new information for understanding the molecular and chemical mechanisms involved in IPU absorption, transport, degradation or detoxification.

Conflicts of interest

The authors declare no competing financial interests.

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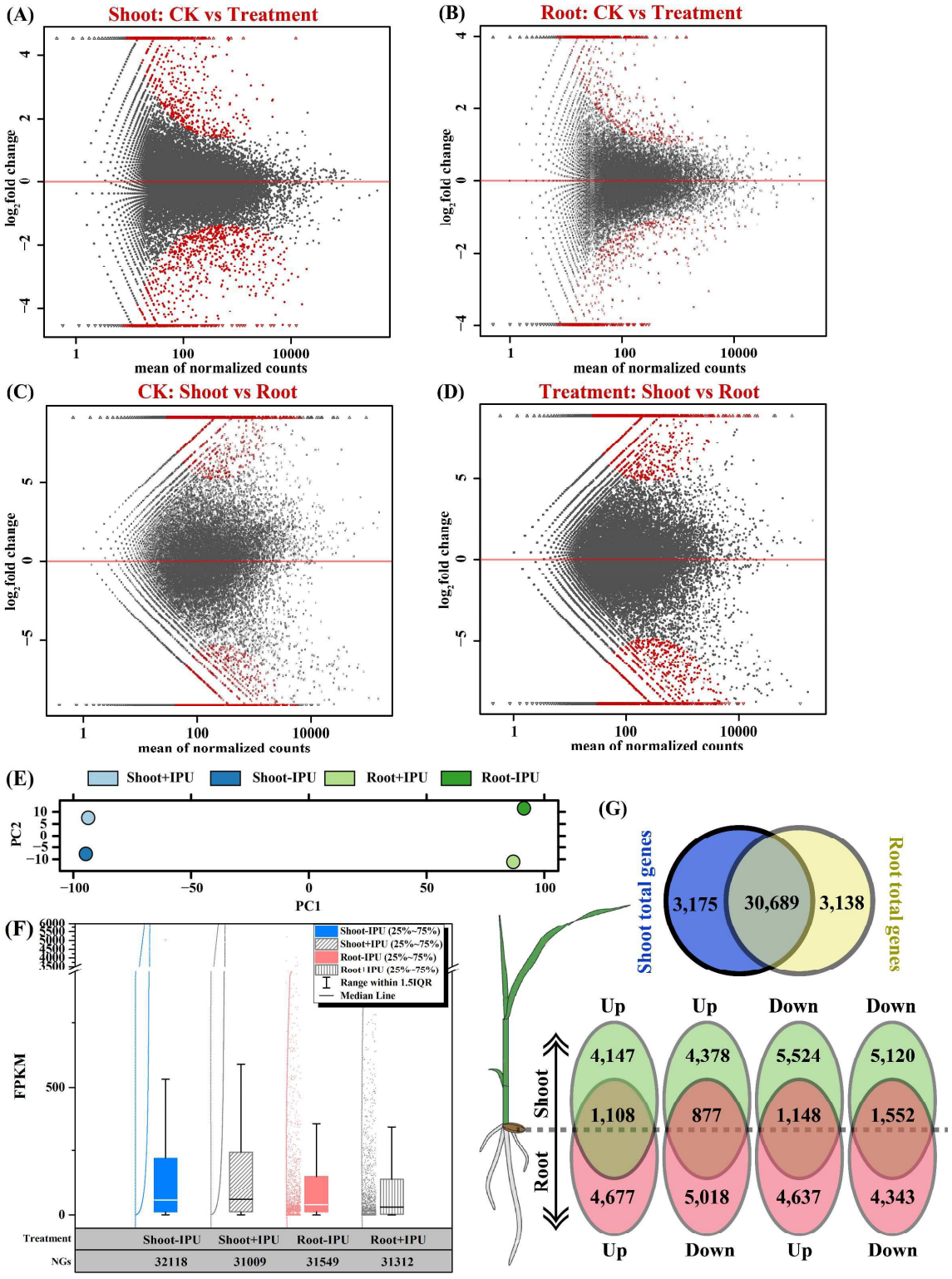

Figure 1. Summary of expressed genes of rice transcriptome. (A~D) Scatter plot analysis of

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four sample pairs (Shoot-CK (Control) vs Shoot-treatment, Root-CK vs Root-treatment, Shoot-CK vs Shoot-treatment, Root-CK vs Root-treatment) from rice. (E) Principal component analysis (PCA) of rice transcript profile of four samples (Shoot-IPU, Shoot+IPU, Root-IPU and Root+IPU). PCA was carried out on the correlation matrix of FPKM expression values measured for two conditions of IPU exposure and control condition. (F) Box chart of gene expression of four samples. Boxes, quartiles 25-75% black lines within boxes, median of the distribution (quartile 50%). Error bars, quartiles 1-25% (below) and 75-100% (above). NGs, the number of genes. (G) Venn diagram showing the genes expressed in each groups. Shoot total genes, the total number of genes in shoot transcripts; Root total genes, the total number of genes in root transcripts; Shoot-up/down, the number of up/down-regulated genes in shoot transcripts; Root-up/down, the number of up/down-regulated genes in root transcripts.

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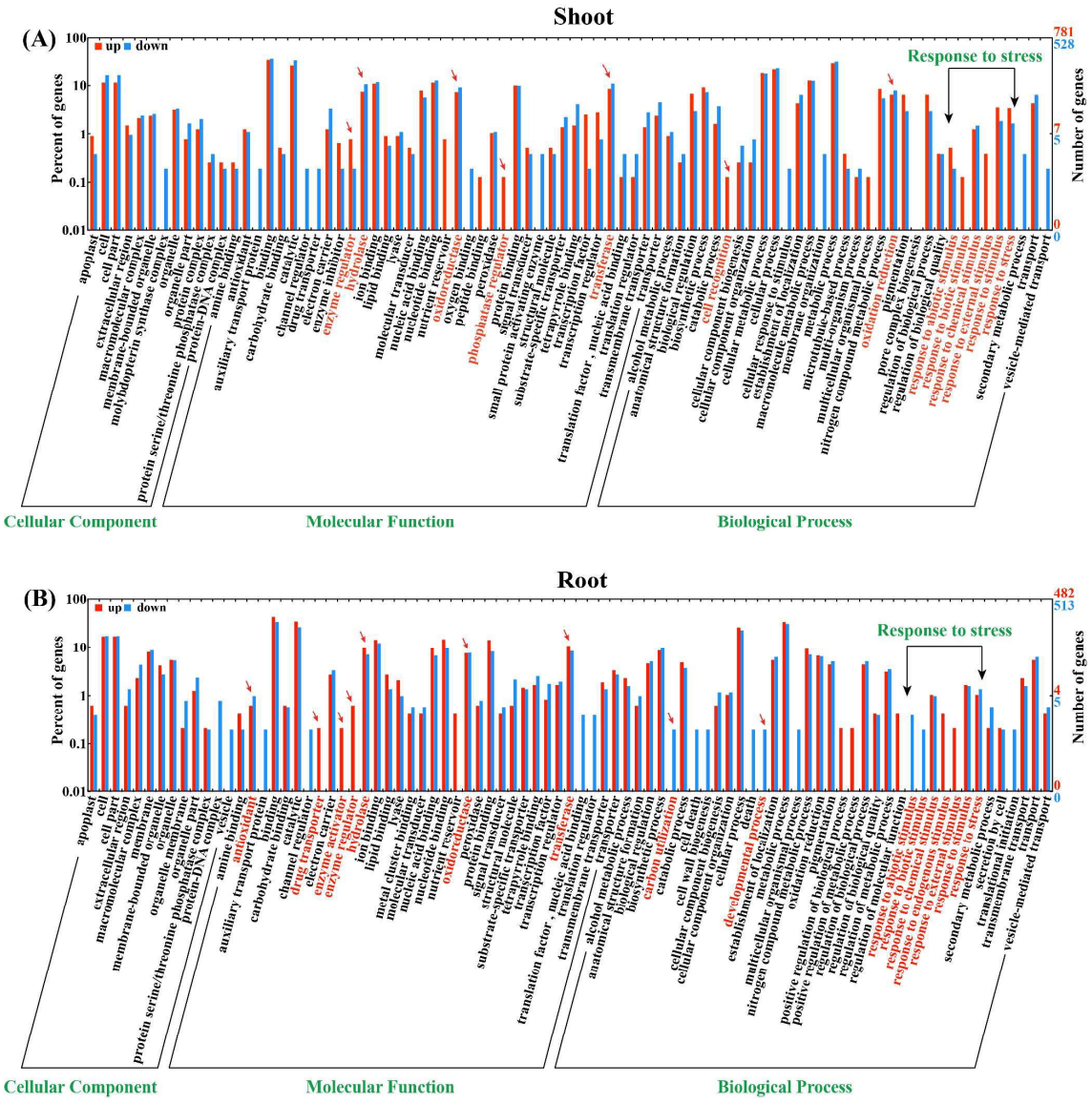

Figure 2. Gene Ontology (GO) term enrichment analyses of DEGs. Annotations are grouped by cellular component, molecular function or biological process based on the rice GO annotation information. (A) Overrepresented GO terms for unigenes that are up/down-regulated in shoot. (B) Overrepresented GO terms for unigenes that are up/down-regulated in root.

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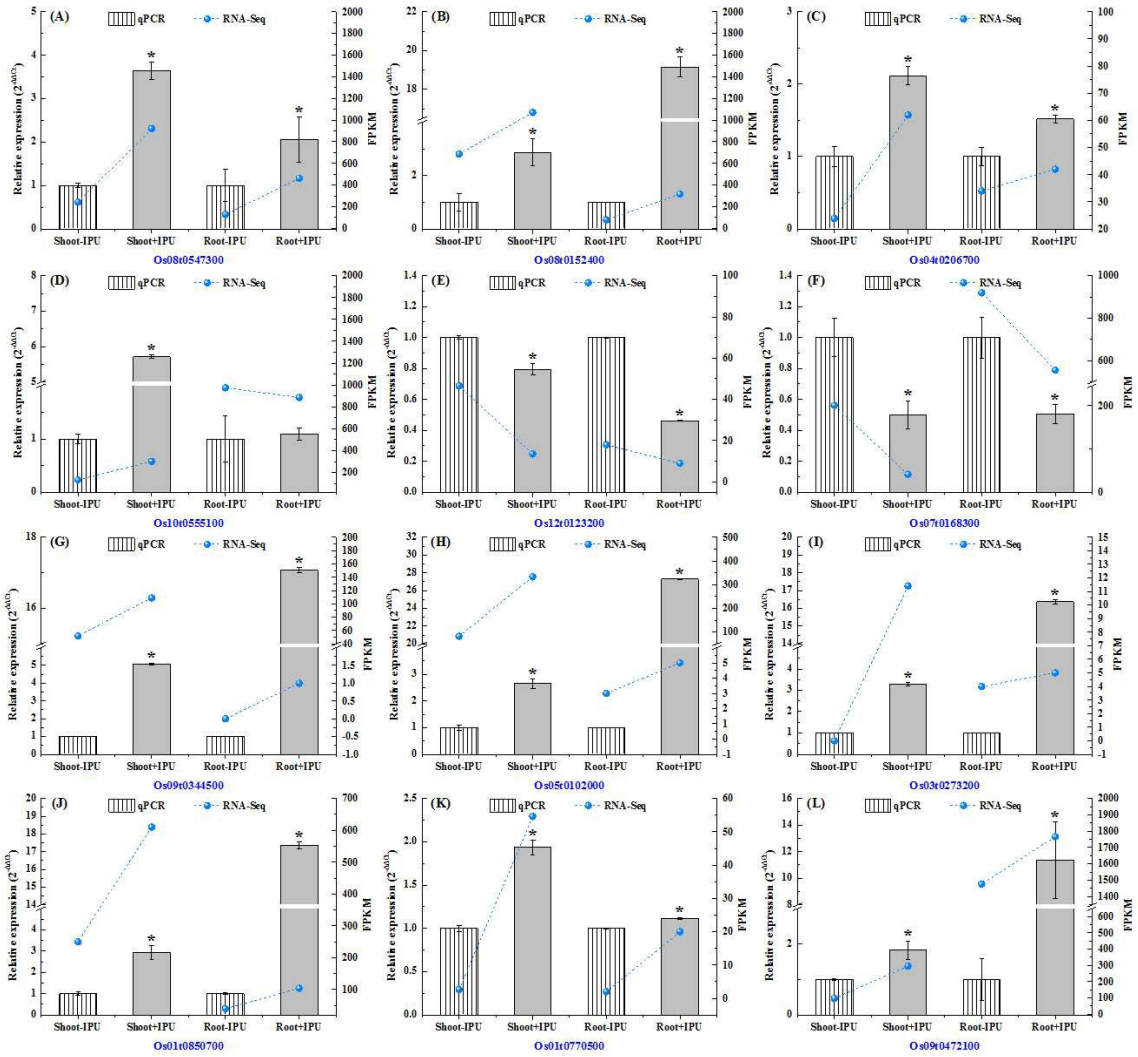

Figure 3. Quantitative PCR validation of genes ($|Log_2Ratio| > 1$) from the differentially expressed genes (DGEs) profiling. Seedlings were cultured in the 1/2 strength Hoagland nutrient solution containing 2 mg L^{-1} IPU for 4 d. Values are the means ± SD (n=3). Asterisks indicate significant differences between the treatments and control ($p < 0.05$). Bars, relative expression values measured by qRT-PCR; Blue lines, relative expression values computed from the FPKM counts.

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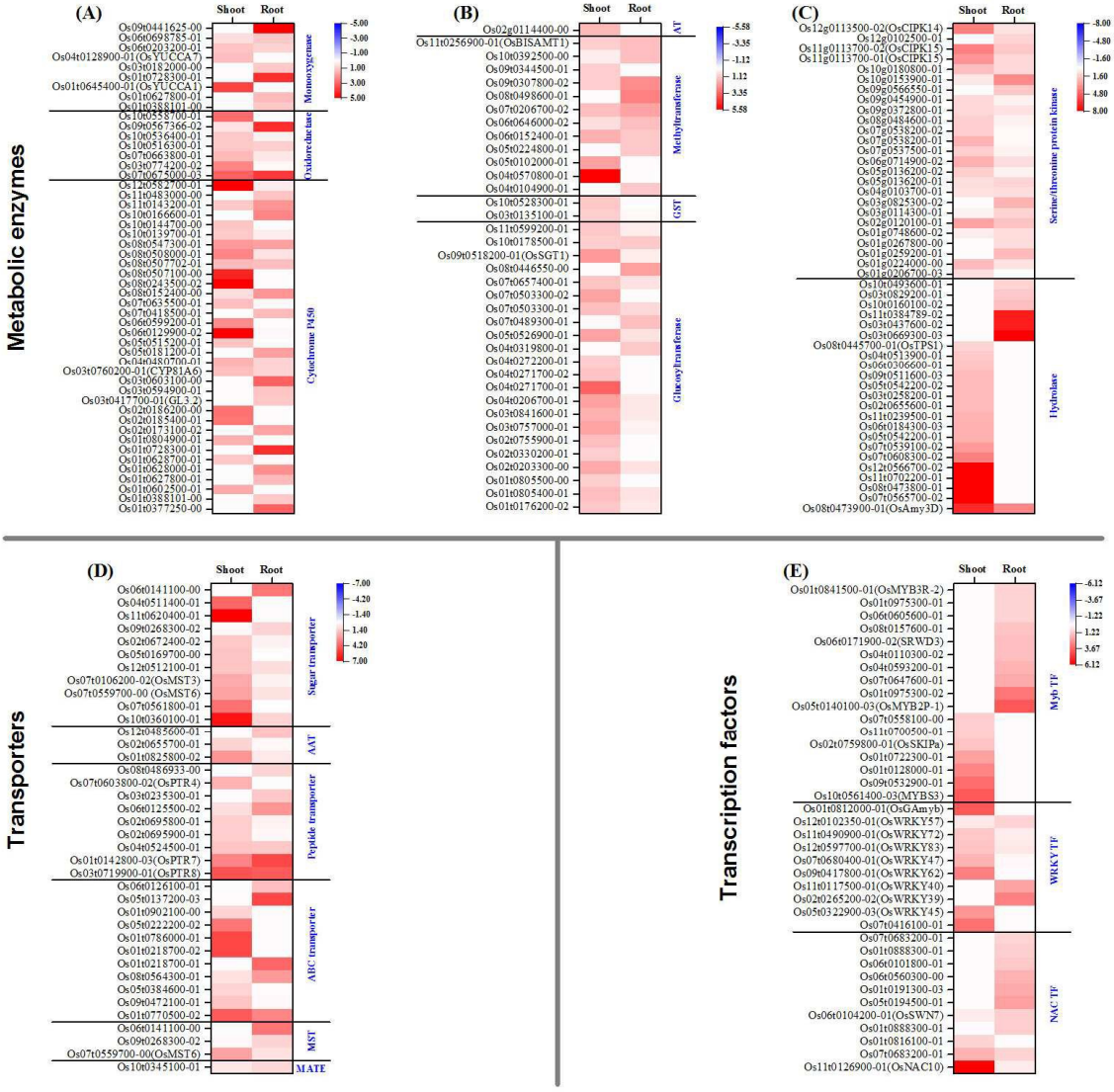

Figure 4. Expression profiles of upregulated DEGs encoding to metabolic enzymes, transporters and transcription factors in shoot and root of *Oryza sativa*. (A-C) Metabolic enzymes include cytochrome P450, oxidoreductase, monooxygenase, AT, glucosyltransferase, GST, methyltransferase, serine/threonine protein kinase and hydrolase. (D) Transporters include MATE, ABC transporter, sugar transporter, peptide transporter and AAT. (E) Transcription factors include WRKY family, Myb family and NAC family transcription

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factors. The gene-normalized signal intensities are shown in the heat maps using a Log_2foldchange. The detail information was summarized in ESI Table S8. AT, acyltransferase; GST, glutathione *S*-transferase; MATE, multi antimicrobial extrusion protein; AAT, amino acid transporter; and MST, monosaccharide transporter.

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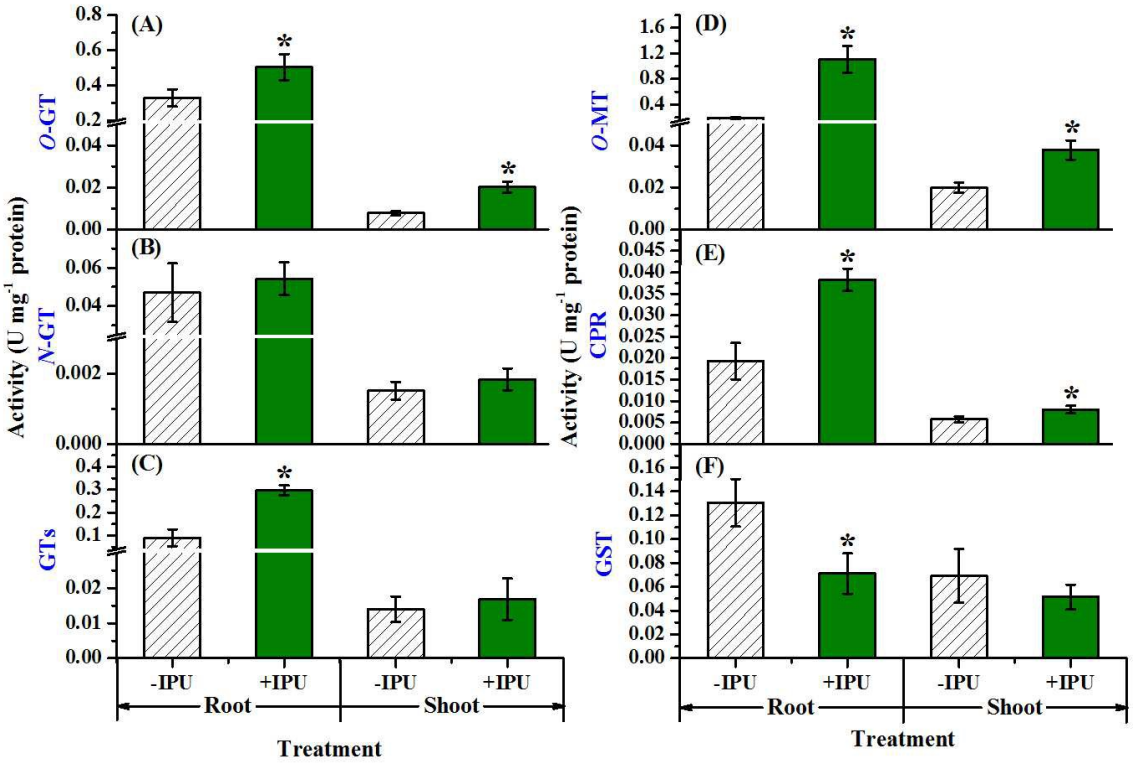

Figure 5. Effect of isoproturon on the activity of *O*-GT (A), *N*-GT (B), GTs (C), *O*-MT (D), CPR (E) and GST (F) in rice. Seedlings were cultured in the 1/2 strength Hoagland nutrient solution containing 2 mg L^{-1} IPU for 4 d. Values are the means ± SD (n = 3). Asterisks indicate significant differences between the treatments and control ($p < 0.05$).

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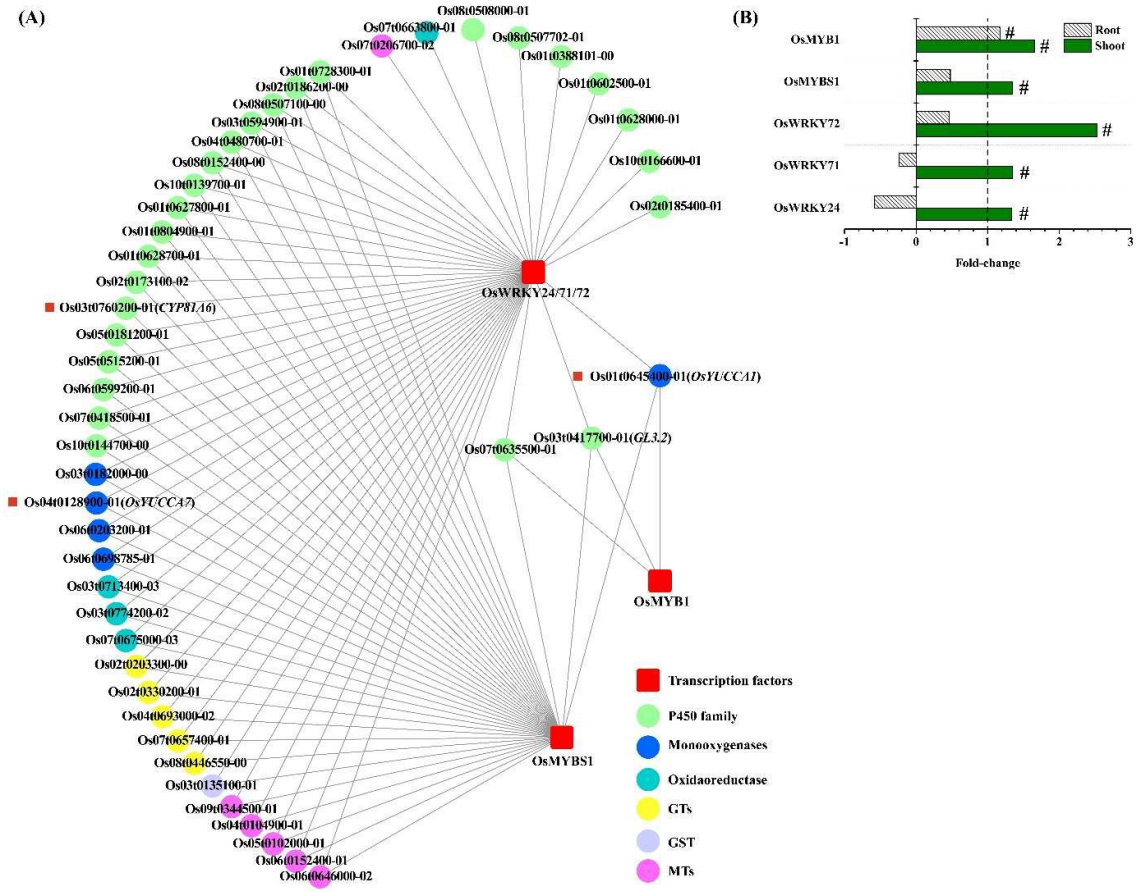

Figure 6. Gene co-expression network analysis. (A) The co-expression network was constructed based on the predicted transcription factors (TFs) and their targeted genes corresponding to IPU metabolism. The targeted genes under IPU exposure were upregulation which were more than 2-fold FPKM in shoot or root, compared to control. The names of the genes were shown in the present heat maps. (B) The fold-change values of TFs. In the network figure, the squares indicate TFs; the circles with different colors indicate different metabolism enzyme genes.

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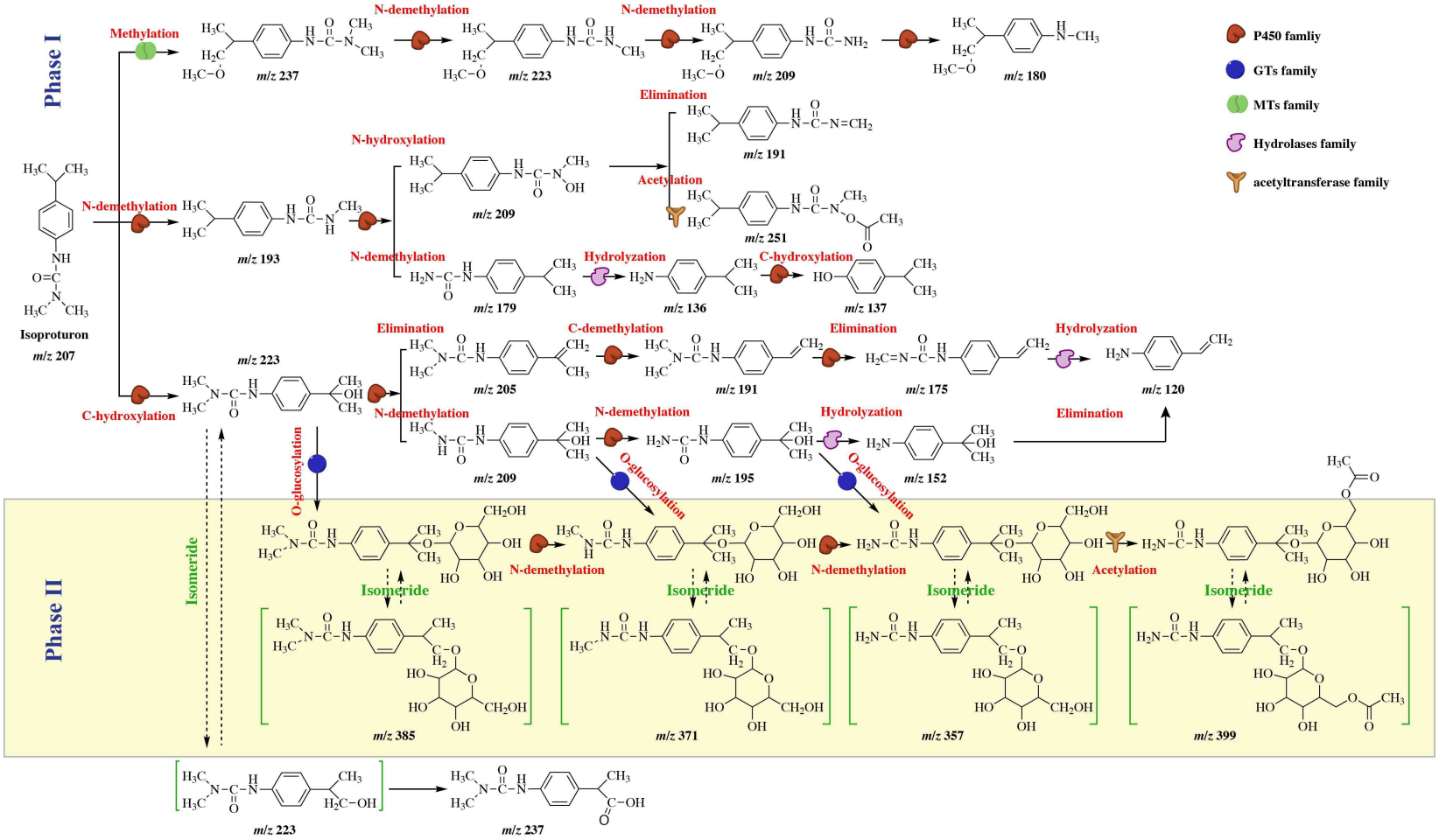

Figure 7. The proposed pathways of IPU-metabolism in shoot and root of *Oryza sativa*. The white region indicates the Phase I metabolism of IPU, and the yellow region indicates the Phase II metabolism of IPU.

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Table 1 Summary of all MS and MS^2 data for metabolites of IPU in rice

No.		Chemical formula	t_R[b]	Theor *m/z*	Exptl *m/z*	Delta	Location[c]	Fragments
Metabolites of IPU			(min)	$[M+H]^+$	$[M+H]^+$	(ppm)		MS^{2d}
1	IPU	$C_{12}H_{18}ON_2$	20.22	207.1492	207.1479	-1.29	S, R	164.8821, 134.0373, **71.6818**
2	2-methoxyl-IPU[a]	$C_{13}H_{20}O_2N_2$	15.71	237.1598	237.1575	-2.224	S	205.0577, **164.9981**
3	2-methylehanoic-IPU	$C_{12}H_{16}O_3N_2$	14.49	237.1234	237.1212	-2.199	S, R	219.0644, **177.0037**
4	1-OH-isopropyl-IPU (2-OH-isopropyl-IPU)	$C_{12}H_{19}O_2N_2$	10.66	223.1441	223.1423	-1.774	S, R	205.0757, **159.9032**, 133.8907
5	2-methylehanoic-demethyl-IPU[a]	$C_{12}H_{19}O_2N_2$	11.69	223.1441	223.1423	-1.774	S, R	205.0311, **164.9033**, 160.0845, 133.9805
6	*N*-OH-demethyl-IPU[a]	$C_{11}H_{16}O_2N_2$	10.18	209.1285	209.1276	-0.904	S	**191.0083**, 166.9824, 150.9500, 133.9364
7	1-OH-monodemethyl-IPU (2-OH-monodemethyl-IPU)	$C_{11}H_{16}O_2N_2$	9.65	209.1285	209.1276	-0.904	S	191.0025, 150.8973, **133.9591**
8	2-methoxyl-didemethyl-IPU[a]	$C_{11}H_{16}O_2N_2$	9.40	209.1285	209.1276	-0.904	S	191.0129, **135.9044**
9	Isopropenyl-IPU	$C_{12}H_{16}ON_2$	17.35	205.1335	205.1323	-1.26	S	**159.9131**
10	2-OH-didemethyl-IPU (2-OH-didemethyl-IPU)	$C_{10}H_{14}N_2O_2$	7.94	195.1128	195.114	-0.976	S	**176.9940**, 151.0396, 136.9683
11	Monodemethyl-IPU	$C_{11}H_{16}ON_2$	18.96	193.1335	193.1326	-0.91	S, R	**150.9291**, 135.9277
12	Methyleneimido-IPU[a]	$C_{11}H_{14}ON_2$	8.63	191.1179	191.1172	-0.73	S	173.0554, 160.0135, 145.9161, **133.8456**
13	Isopropenyl-monodemethyl-IPU	$C_{11}H_{14}ON_2$	17.88	191.1179	191.1172	-0.73	S	173.0217, **133.8720**
14	4-(1-methoxy-2-methyl-2-propanyl)-*N*-methylanline[a]	$C_{11}H_{17}ON$	17.59	180.1383	180.1371	-1.211	S	149.0315, **106.8587**
15	Didemethyl-IPU	$C_{10}H_{14}ON_2$	17.78	179.1179	179.1167	-1.22	S	**136.9184**
16	Isopropenyl-demethyl-methyleneimido-IPU[a]	$C_{10}H_{10}ON_2$	3.15	175.0866	175.0856	-1.02	S	160.0135, **145.1216**, 133.8456
17	1-(4-aminophenyl)2-propanol[a] (2-(4-aminophenyl)2-propanol)	$C_9H_{13}ON$	10.23	152.107	152.1061	-0.871	S	133.9105, 121.0020, **105.8634**, 93.8941
18	4-isopropylphenol	$C_9H_{13}N$	18.84	136.1121	136.1111	-0.996	S	**93.7707**
19	4-isopropylanline[a]	$C_9H_{10}O$	16.66	135.0804	135.0794	-1.052	S, R	116.8988, **106.8853**, 92.9868
20	4-vinylanline	C_8H_9N	2.74	120.0808	120.0802	-0.546	S	**103.0033**, 93.0137
Conjugates of IPU								
1	*N*-acetyloxy-monodemethyl-IPU[a]	$C_{13}H_{17}O_3N_2$	15.97	251.139	251.1369	-2.089	S, R	**233.1279**, 205.1077, 191.0454
2	1/2-OH-didemethyl-IPU-O-glucoside	$C_{16}H_{24}N_2O_7$	17.67	357.1656	357.1643	-1.089	S	**179.1167**, 137.0702
3	1/2-OH-monodemethyl-IPU-O-glucoside	$C_{17}H_{26}O_7N_2$	9.35	371.1813	371.1792	-2.108	S	353.1576, **209.0831**
4	1/2-OH-IPU-O-glucoside	$C_{18}H_{28}O_7N_2$	10.69	385.1969	385.1943	-2.658	S, R	**223.0465**
5	1/2-OH-didemethyl-IPU-O-acetylglucoside[a]	$C_{18}H_{26}O_8N_2$	10.46	399.1762	399.173	-3.222	S	**381.1250**, 340.2589, 179.1173

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[a] Compounds that have been reported for the first time in plants;

[b] Retention time;

[c] Location: Distribution of metabolites of atrazine in plant;

[d] MS^2 fragments: Base peak of MS^2 fragment ions are shown in bold;

S, shoot; R, root.

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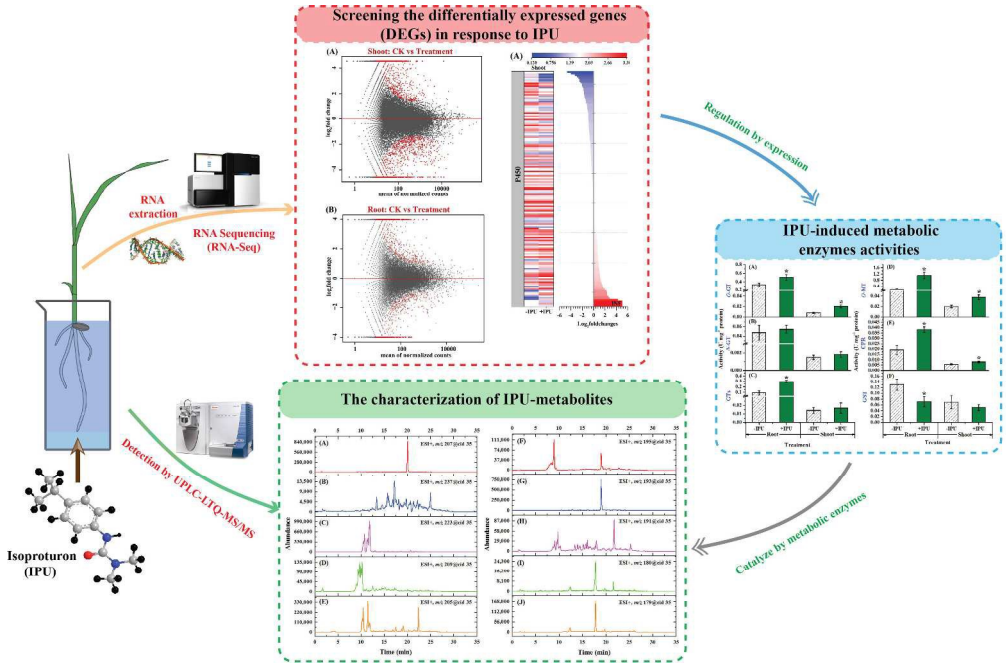

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