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Multiple Metabolism Pathways of Bentazone Potentially Regulated by Metabolic Enzymes in Rice

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ABSTRACT: Bentazone (BNTZ) is a selective and efficient herbicide used in crop production worldwide. However, the persistence of BNTZ residues in the environment has led to their increasing accumulation in farmland and crops, posing a high risk to human health. To evaluate its impact on crop growth and environmental safety, a comprehensive study was conducted on BNTZ toxicity, metabolic mechanism, and resultant pathways in rice. The rice growth was compromised to the treatment with BNTZ at 0.2−0.8 mg/L (529.95−1060.05 g a.i./ha), while the activities of enzymes including SOD, POD, CAT, GST, GT, and CYP450 were elevated under BNTZ stress. A genome-wide RNA-sequencing (RNA-Seq) was performed to dissect the variation of transcriptomes and metabolic mechanisms in rice exposed to BNTZ. The degradative pathways of BNTZ in rice are involved in glycosylation, hydrolysis, acetylation, and conjugation processes catalyzed by the enzymes. Our data provided evidence that helps understand the BNTZ metabolic and detoxic mechanisms.

KEYWORDS: *bentazone, rice, toxicity response, transcriptome, multiple metabolism*

INTRODUCTION

Bentazone (3,4-dihydro-3-isopropyl-1H-2,1,3-benzothiadiazin-4-one 2,2-dioxide, BNTZ) is a globally used selective herbicide for controlling broadleaf and multi-perennial weeds in the fields of rice, corn, wheat, soybean, alfalfa, or vegetables.[1,2] BNTZ is a member of the benzothiadiazole family. The major role of BNTZ in herbicidal plants is to prevent photosynthetic activity by blocking photosynthetic electron flow in chloroplasts. Detailed mechanisms include the competition with quinone B (QB) for the binding site in the photosystem II (PSII) reaction center, making plastoquinone A (QA) unable to re-oxidize.[2] With excessive usage, the BNTZ residue becomes a contaminant threatening environmental safety and human health.[3] The high solubility of BNTZ makes it migrate fast in runoff, and the leaching concentration of BNTZ in groundwater in some countries is as high as 120 μg/L, exceeding the limit of 0.1 mg/L specified by the European Union.[4] In China, the maximal residue limit (MRL) of BNTZ in food stuffs is also formulated. In rice, for example, the MRL of BNTZ is restricted to 0.1 mg/L (GB 2763-2019).[5] BNTZ is highly resistant to chemical hydrolysis in the environment.[6] Its residues in aquifers were predicted to persist for 20 years after the last application.[7] Ecotoxicological evaluation of BNTZ and understanding of its natural degradation mechanism are of great importance to make strategies for controlling BNTZ contaminants in environments and crops.

Plants are immobile species and unable to escape toxic chemicals in the environment, but their long history of adaptive evolution has allowed them to develop elaborate mechanisms to cope with xenobiotics.[1] Upon exposure to herbicides, plants may over-generate the reactive oxygen species (ROS) to oxidize toxic substances, while excessive ROS can also damage the plant cells.[8] To reduce oxidative injury and maintain survival of plants, the enzymatic and non-enzymatic antioxidant systems are activated.[9] SOD, for example, is involved in catalyzing superoxide radicals (O_2^-) to H_2O_2 and O_2, while catalase (CAT) and peroxidase (POD) can further remove excessive H_2O_2.[10] Furthermore, there are canonical processes to transform toxic compounds catalyzed by cytochrome P450 enzymes (CYP450s), oxidases, and hydrolases in phase I reaction, making the compounds more active in chemistry, high polarity, and water solubility. The transformed compounds through phase I reaction can be further modified through phase II reaction, in which the herbicide-modified intermediates are conjugated with sugars, amino acids, or hydroxyl and carboxyl groups by acetyltransferase (ACE), glycosyltransferases (GTs), or glutathione S-transferase (GST) to generate more hydrophilic and less toxic compounds.[11−14] Finally, these detoxified metabolites or conjugates are transported to vacuoles or other organelles through phase III reaction for deep catabolism.[15−17] Recent studies show that BNTZ to 6-OH- or 8-OH-BNTZ could be rapidly transformed in soybean, wheat, and maize, by which BNTZ interacts with glucose, leading to the reduced toxicity of BNTZ in the crops. However, the molecular basis for regulation of BNTZ degradation and pathway in plants is still poorly understood.

Rice (*Oryza sativa* L.) is one of the most important food crops sustainable for more than half of the world population. It is of great significance to evaluate the impact of BNTZ on rice growth

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and to understand the degradation mechanism of BNTZ residues. In this study, the toxic response, accumulation, and translocation of BNTZ in rice were investigated. A global RNA-sequencing (RNA-Seq) was conducted between the control (−BNTZ) and treated groups (+BNTZ) to get insights into the BNTZ detoxic pathways. The relevant enzymes for BNTZ metabolism and degradation were dissected by profiling the differentially expressed genes in response to BNTZ. Using the HRLC/Q-TOF-MS/MS technique, the BNTZ metabolites and conjugates were characterized. The aim of the study was to unveil the novel responsive genes involved in the mechanism for degradation of BNTZ in rice crops.

MATERIALS AND METHODS

Growth Condition and Treatment of Plants. Rice (cv. Japonica) seeds were sterilized with 75% alcohol and 10% sodium hypochlorite and placed in Petri dishes at 28 °C in darkness for 2 days. The germinating seeds were transferred to a half-strength Hoagland solution (pH 5.6−5.8) and grown in an incubator with a 25/30 °C (night, 10 h/day, 14 h) cycle and illumination of 200 μmol/m^2/s for 10 days.[18] The nutrient solution was changed every other day. Rice seedlings at the three-leaf stage were treated with 0−1.0 mg/L (529.95−1325.07 g a.i./ha) BNTZ under the same conditions for 6 days (the recommended dose of BNTZ in rice fields is 1152−1440 g a.i./ha for stem and leaf spray, http://www.chinapesticide.org.cn/hysj/index.jhtml), which was below the maximum recommended application rate. BNTZ was added into the half-strength Hoagland solution to simulate pesticide residues in the rice field. The rice was cultivated by hydroponics. The shoots and roots of rice seedlings were collected for measuring physiological parameters and enzyme activities. All experiments were performed in triplicate.

Determination of Rice Physiological Responses to BNTZ Exposure. Fresh shoots and roots of rice were collected and dried in an oven at 105 °C for 20 min and then at 70 °C for 72 h. The dry weight of the plants was measured. The electrolyte leakage of rice seedlings for cellular membrane damage was determined by immerging the samples in deionized water at 25 °C for 2 h (EC1) and treated with boiling deionized water for 30 min (EC2). The electrolyte leakage was calculated as the ratio of EC1/EC2 × 100%.[19] The content of thiobarbituric acid reactive substance (TBARS) in rice was determined according to the method of Song et al.[20] Fresh shoots (0.4 g) and roots (0.15 g) of rice were separately collected and ground with 3 mL of 0.1% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged at 12,000g for 20 min. The supernatant was mixed with 0.5% thiobarbituric acid (TBA) in 20% TCA (5 mL) and heated at 95 °C for 30 min, chilled, and centrifuged at 12,000g for 20 min. The TBARS content in supernatant was determined using a spectrophotometer.

Chlorophyll contents were measured according to the method described by Ma et al.[18] Fresh leaves (0.05 g) were leached in 5 mL of 80% acetone solution (pH 7.8) for 48 h. The mixture was centrifuged at 5000g for 5 min. The supernatant was collected, and chlorophyll content was determined by a spectrophotometer at OD_{649} and OD_{665}. The chlorophyll fluorescence parameters of rice leaves were determined by a Chlorophyll Fluorescence Imager (CFI, Technologica, UK).[21] After measuring minimum fluorescence (F_0) in the dark-adapted state (30 min), samples were illuminated with a short (1 s) saturating pulse (max. 6164 μmol m^{-2} s^{-1}) to elicit maximum dark-adapted fluorescence (Fm). The value of Fv/Fm was calculated by Fv/Fm = (Fm − F_0)/Fm. For examining the effective quantum yield of PSII photochemistry (ΦPSII) and non-photochemical quenching (NPQ), the light adaptation process was set up. The actinic light was turned on with light intensity of 500 μmol m^{-2} s^{-1}. The saturation pulse was turned on after light adaptation for 4−5 min, and the steady-state fluorescence (Fs) and instant maximum fluorescence (Fm′) were measured. ΦPSII and NPQ were calculated by the CFI system, where ΦPSII = (Fm′ − Fs)/Fm′ and NPQ = (Fm − Fm′) − 1.

To analyze the activity of rice roots, fresh roots (0.1 g) were soaked in 1 mL of 0.4% 2,3,5-triphenyltetrazolium chloride (TTC) and 66.7 mM phosphate buffer (pH 7.4) and then incubated at 37 °C under dark conditions for 2 h. The reaction was stopped by adding 2 mL of sulfuric acid (1 M). The roots were taken out, dried with filter paper, transferred to 5 mL of methanol, and incubated at 40 °C for 7 h. The supernatant was measured by a spectrophotometer at 485 nm.[22]

Analysis of Antioxidant and Detoxification Enzyme Activities in Rice. Fresh shoots and roots (0.3 g) were separately homogenized with ice-cold 3 mL extraction buffer containing 1 mM EDTA, 1.5% w/v polyvinylpyrrolidone, and 50 mM Tris−HCl (pH 7.8). The homogenate was centrifuged at 12,000g for 15 min. The supernatant was collected as a crude extract for analysis of enzyme activities.

The SOD activity was measured by monitoring inhibition of photochemical reaction of nitro blue tetrazolium (NBT) at 560 nm.[20] Briefly, the mixture solution with 50 mM phosphate buffer (pH 7.8, 2.4 mL), 195 mM methionine (0.2 mL), 1.125 mM NBT (0.2 mL), 3 μM EDTA (0.1 mL), 60 μM riboflavin (0.1 mL), and 40 μL of enzyme extract was placed under 4000 lx illumination for 15 min. For CAT, the enzyme extract (200 μL) was mixed with 50 mM phosphate buffer (pH 7.0, 2 mL) and 10 mM H_2O_2 (1 mL) and the CAT activity was determined by analyzing the rate of change in absorbance at 240 nm.[20] The POD enzyme extract (1 μL) was mixed with 50 mM phosphate buffer (pH 7.0, 2.4 mL), 0.5% guaiacol (0.5 mL), and 1% H_2O_2 (0.1 mL). The POD activity was determined by the change of absorbance at 470 nm due to guaiacol oxidation.[20] The activity of GST was determined following the change of absorbance at 340 nm.[23] The activity was assayed in a reaction solution composed of 100 mM phosphate buffer (pH 7.0, 3 mL), 40 mM 1-chlor-2,4-dinitrobenzene (CDNB, 75 μL), and 1 μL enzyme extract for 4 min. The mixture (250 μL) for assaying GT activity contained 100 μL of enzyme extracts, 2 mM UDP-glucose, and 0.04 mM p-nitrophenol. Reactions were initiated with an enzyme at 30 °C, terminated after 2 h by the addition of 250 μL methanol, and chilled to −20 °C for 0.5 h. The centrifuged supernatant was filtered through a 0.2 mm PTEF membrane for HPLC analysis.[24] The CYP450 enzyme activity was quantified using an Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Ruifan Biological Technology Co., Shanghai, ART NO:RFRF-Z321). The extraction and determination of P450 enzymes by ELISA were performed according to the method described by Lu et al.[25] Briefly, fresh plant tissues (50 mg) were collected and homogenized in liquid nitrogen. The extraction solution (0.1 mmol pH 7.4 PBS) was added (PBS volume was nine times the sample solution). The samples were extracted at 4 °C for 2 h and centrifuged at 3000g at 4 °C for 10 min. The supernatant (the rude enzyme) was collected for ELISA analysis. The 10 μL testing sample and 40 μL sample diluent were added to the well. Then, 100 μL of horseradish POD-conjugate reagent was added to each well. The mixture solution was covered with an adhesive strip and incubated at 37 °C for 60 min, aspirated each well, and washed for five times. Chromogen solution A of 50 μL and chromogen solution B of 50 μL were added to each well, gently mixed, and incubated at 37 °C under dark conditions for 15 min. The stop solution (50 μL) was added to each to stop the reaction. OD_{450} was read using a microtiter plate reader within 15 min.

Quantification of BNTZ in Rice. Fresh shoots (2 g) and roots (2 g) were collected and pestled with liquid nitrogen, respectively. Ultrapure water (5 mL) and acetonitrile (10 mL) were added to each sample. The sample was shaken for 30 min, added with 5.0 g of NaCl, and shaken for another 10 min. After that, the mixture was centrifuged at 3500g for 5 min. The supernatant (5 mL) was concentrated to dry in a vacuum rotary evaporator at 40 °C. The dried residue was dissolved with 2 mL of methanol, transferred to a 2 mL centrifuge tube with 50 mg of PSA and 25 mg of GCB, vortexed for 2 min, and centrifuged at 18,000g for 3 min. The supernatant was filtered by a 0.22 μm syringe membrane by high-performance liquid chromatography (HPLC). The HPLC instrument (Waters 515; Waters Technologies Co., Ltd.) equipped with a Hypersil reversed-phase C18 column (Thermo, 250 × 4.6 mm) and a UV detector were used for detection of BNTZ. The mobile phase contained a mixed solvent of acetonitrile and 0.1% phosphoric acid (35/65, v/v), and the flow rate was set at 0.8 mL/min; the wavelength was 218 nm. Bioconcentration factors were calculated

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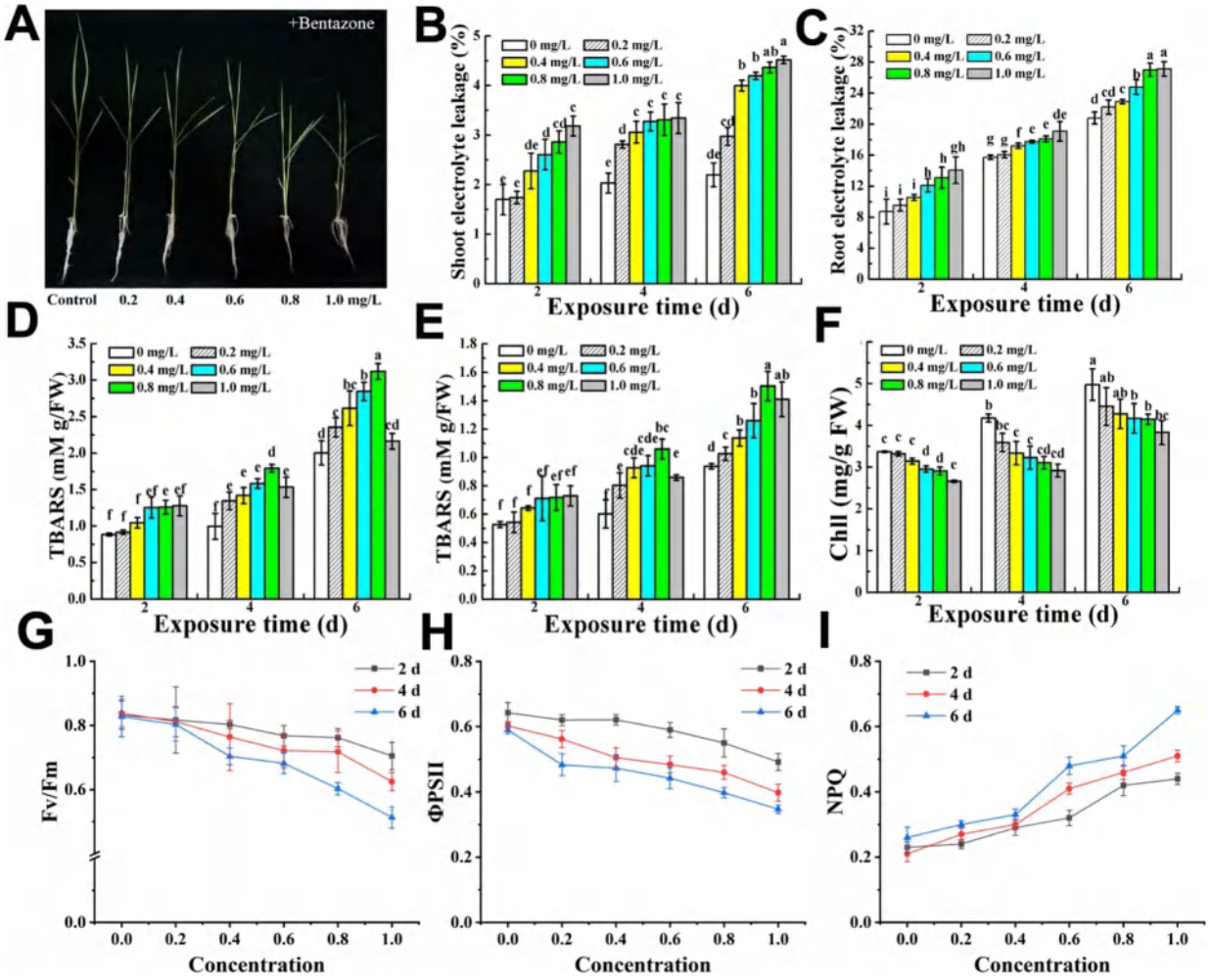

Figure 1. Analysis of toxic response of rice to bentazone (BNTZ). Rice seedlings at the three-leaf stage were exposed to BNTZ (0−1.0 mg/L) for 2, 4, and 6 days, respectively. Phenotypes of rice seedlings under the BNTZ stress (A). Membrane permeability of shoots (B) and roots (C). TBARS of shoots (D) and roots (E). Chlorophyll content (F). The maximum photochemical efficiency Fv/Fm of photosystem II (G). Effective quantum yield of PSII photochemistry (H). Non-photochemical quenching (I). Values are the means ± SD (n = 3). Means followed by different letters are significantly different within each biotype or treatment (p < 0.05, ANOVA).

by the ratio of the BNTZ concentration in rice to the concentration of BNTZ in the growth medium. The translocation factors were calculated by the ratio of the concentration of BNTZ in shoots of rice to the concentration of BNTZ in the roots of rice.[26]

Construction of RNA Libraries and Data Processing. Samples of shoots and roots were collected from 2, 4, and 6 days after treatment under 0 and 0.8 mg/L BNTZ exposure, respectively. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, California) and merged based on the treatment and untreatment.[18] Four libraries were constructed termed Shoot−BNTZ, Shoot+BNTZ, Root−BNTZ, and Root+BNTZ and sequenced using the Illumina HiSeq 2500, with three biological replicates.[27] Therefore, a total of 12 (4 × 3) were subjected to RNA sequencing. After deleting the low-quality bases, the clean reads were mapped to the rice genome (http://rice.plantbiology.msu.edu/index.shtml). We used p < 0.05 and fold change ≥2 as a threshold to determine the significance of gene expression differences and subjected it to Gene Ontology (GO) analysis (http://www.geneontology.org/). The expression level for each transcript was calculated by fragments per kilobase per million fragments mapped (FPKM).

Quantitative RT-PCR Analysis. Total RNA from shoots and roots of rice was isolated by TRIzol (Invitrogen). Each sample was incubated with RNase-free DNase I at 37 °C for 30 min. EasyScript First-Strand cDNA Synthesis SuperMix was used for reverse transcription reaction after one-step gDNA removal. The resultant cDNA was diluted for subsequent qRT-PCR. The reaction system contained 2 μL of cDNA, 10 μL of 2 × SYBR Premix Ex Taq, and 200 nM primers (Table S1). The reaction was performed as follows: 1 cycle of 95 °C for 30 s for denaturation, 40 cycles of 95 °C for 5 s and 60 °C for 30 s for annealing and extension.[28]

Characterization of BNTZ Metabolites and Conjugates in Rice. The rice tissues (shoots or roots) were extracted based on the method described above. The concentrated samples without purification were dissolved with 1 mL of methanol and filtered through a 0.22 μm syringe membrane for characterization of BNTZ metabolites and conjugates by HRLC-Q-TOF-MS/MS (5600, AB Science, Redwood, California, USA). The samples were isolated by the LC system (Shimadzu, Japan) equipped with a Poroshell 120 ECC18 (50 × 2.1 mm, 2.7 mm particle size, Agilent). The injection volume was 10 μL. The flow rate was 0.3 mL/min with the mobile phase of 0.1% formic acid (A) and acetonitrile (B). The gradient elution program was set as follows: 5% B (0−3 min), 5−30% B (3−18 min), 30−95% B (18−28 min), 95% B (28−30 min), 95−5% B (30−31 min), and 5% B (31−33 min). The column temperature was 35 °C. The TOF-HRMS/MS was operated in negative electrospray ionization mode (ESI−) with a 100−1000 m/z range and MS/MS (50−1000) mode.[27] The parameters were shown as follows: ion spray voltage floating, 5500 V; temperature, 500 °C; ion source gas pressures, 50 psi; declustering potential, 80 V; collision energy, 35 eV.

Statistical Analysis. Experiments with the data in the figures and tables were independently performed in triplicate. For each treatment,

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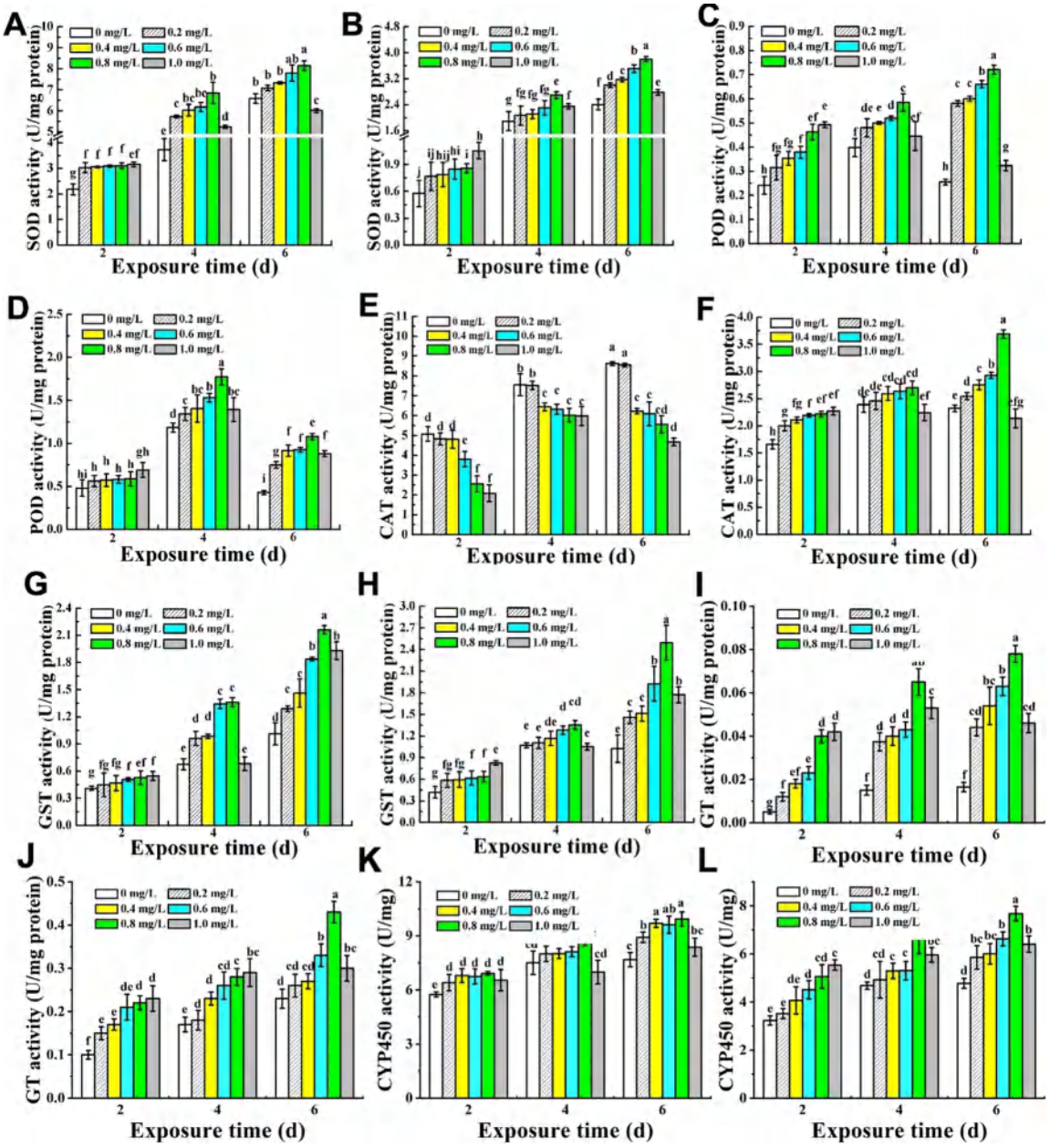

Figure 2. Effects of BNTZ on activities of antioxidant and detoxifying enzymes in rice tissues. Rice seedlings at the three-leaf stage were hydroponically cultured with BNTZ at 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/L for 2, 4, and 6 days, respectively. SOD activities of shoots (A) and roots (B). POD activities of shoots (C) and roots (D). CAT activities of shoots (E) and roots (F). GST activities of shoots (G) and roots (H). GT activities of shoots (I) and roots (J). CYP450 activities of shoots (K) and roots(L). Values are the means ± SD (n = 3). Means followed by different letters are significantly different between the genotypes of plants ($p < 0.05$, ANOVA).

more than 10 plants were used. SPSS (Version 22.0) was used to analyze all data. Significant differences between treatments were performed by using one-way analysis of variance (ANOVA). The least significant difference (LSD) was set as $p < 0.05$.

RESULTS

Effects of BNTZ on Rice Growth and Physiological Responses. Figure 1 depicts the growth, membrane permeability, malondialdehyde (MDA), and chlorophyll contents of rice exposed to 0−1.0 mg/L of BNTZ. BNTZ at 0.2−1.0 mg/L significantly inhibited the growth of rice seedlings (Figure 1A). A substantial increase in electrolyte leakage of biomembrane (membrane permeability) was observed with the increasing BNTZ concentration (Figure 1)B,C. Under 1.0 mg/L BNTZ exposure for 2, 4, and 6 days, the electrolyte leakage rates of rice shoots were 1.87, 1.65, and 2.06 folds over the control while the electrolyte leakage rates of rice roots were 1.61, 1.21, and 1.30 folds over the control, respectively. TBARS is equivalent to MDA used to evaluate the degree of lipid oxidation or oxidative stress. BNTZ exposure (0.2−0.8 mg/L) increased the MDA content in rice tissues. When the concentration of BNTZ was greater than 0.8 mg/L, the MDA content in rice was significantly decreased (Figure 1D,E). The elongation and biomass of rice shoots were reduced by 20.85−26.07% and 12.05−25.24% after 1.0 mg/L BNTZ treatment for 2, 4, and 6 days, respectively (Figure S1A,C). The elongation and biomass of roots were gradually shrunk with the increase of BNTZ concentrations and treatment time (Figure S1B,D). The activity of roots, which

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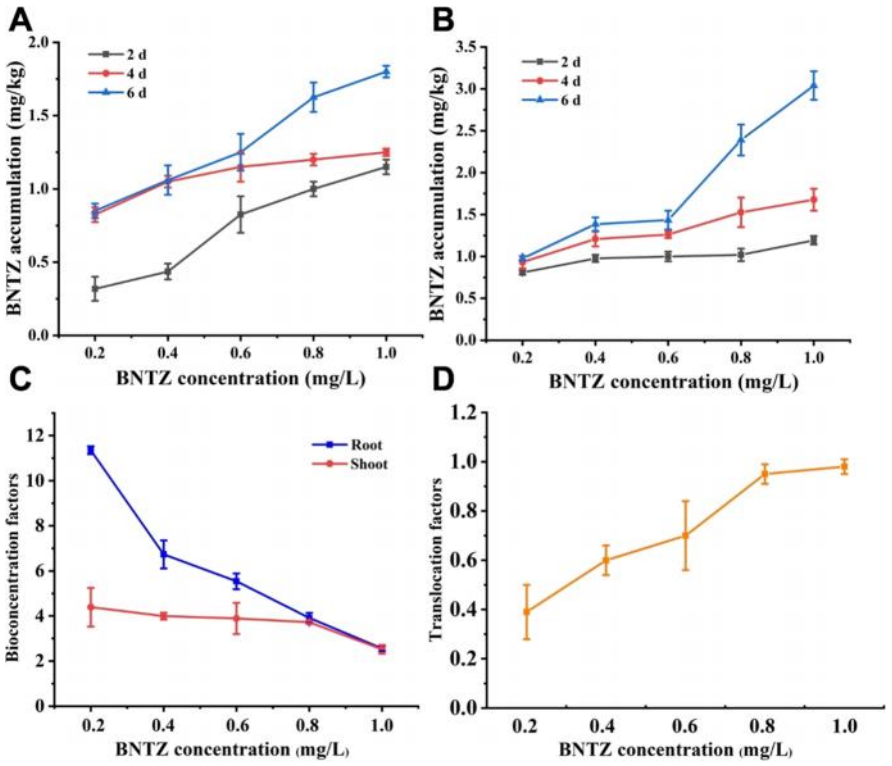

Figure 3. Accumulation, bioconcentration factors, and translocation factors of BNTZ in rice. Rice seedlings at the three-leaf stage were treated with 0−1.0 mg/L BNTZ for 2, 4, and 6 days, respectively. Accumulation of BNTZ in shoots (A) and roots (B). Bioconcentration factors (C) and translocation factors (D) of BNTZ. Bioconcentration factors were the ratio of BNTZ concentration in rice and growth medium. Translocation factors were the ratio of BNTZ in shoots and roots of rice. Values are the means ± SD (n = 3). Means followed by different letters are significantly different between the genotypes of plants ($p < 0.05$, ANOVA).

represented by the capability of TTC reduction, systematically increased with increasing BNTZ during the first 4 days. With treatment time, the root activity was inhibited at the higher concentration of BNTZ (1.0 mg/L) (Figure S1E). Compared with the control, the chlorophyll content significantly decreased with increasing BNTZ supply. Notably, the content of chlorophyll was dropped by 22.9% compared with the control group, with 1.0 mg/L BNTZ treatment for 6 days (Figure 1F). BNTZ stress affected not only chlorophyll biosynthesis but also photosynthetic electron transfer and thus impaired photosynthesis. The maximum photochemical efficiency Fv/Fm and effective quantum yield of ΦPSII in rice leaves after BNTZ treatment decreased with the treatment concentration. There were significant differences between different days of treatment; the lowest value for Fv/Fm and ΦPSII of rice leaves was detected 6 days after treatment (Figure 1G,H). In contrast, the NPQ value tended to increase with the BNTZ treatment time and concentrations (Figure 1I).

Compared with the controls, the activities of antioxidize enzymes SOD and POD in rice tissues initially increased with the increasing BNTZ concentrations after BNTZ exposure for 2 days; when the treatment was extended to 4−6 days, the SOD and POD activities showed a trend of initially increasing and then decreasing with the BNTZ concentrations (Figure 2)A−D. When plants were exposed to 0.8 mg/L BNTZ for 6 days, the SOD and POD enzyme activities were significantly induced. The SOD activities in shoots and roots of rice were 1.24 and 1.58 folds over the control. The reduced activities at mg/L of BNTZ may be due to the excessive accumulation of BNTZ in rice (Figure 2A,B). The POD activities in the shoots and roots of rice were 2.83 and 2.51 folds of the control (Figure 2)C,D. The CAT activity in shoots was not induced under BNTZ stress (Figure 2E), while in roots with 0.2−0.8 mL/L BNTZ for 2 and 6 days, the CAT activity was higher compared with control (Figure 2F). The detoxification enzyme activities of GST, GT, and CYP450 were also found to generally increase in the dose−response- and time-course-dependent manners (Figure 2G−L). With the extension of treatment time (4−6 days), the enzyme activities in rice seedlings were maximally induced at 0.8 mg/L BNTZ treatment. For example, when treated for 6 days, the GST, GT, and CYP450 activities in the shoots and roots of rice were 1.29−2.14 folds and 1.51−2.44 folds over the controls, respectively (Figure 2G−L).

Accumulation and Translocation of BNTZ in Rice. The spiked recoveries of three levels of BNTZ (0.05, 0.1, and 1.0 mg/kg) in the shoots, roots, and growth medium of rice were determined (Table S1). The average spiked recoveries of BNTZ in the shoots, roots, and growth medium ranged from 96.0 to 99.2%, 99.5 to 101.2%, and 95.5 to 98.3%, respectively. The relative standard deviations (RSDs) were lower than 10%, suggesting that the method was reliable for measuring the concentration and translocation of BNTZ in three matrices.

To determine the variation of BNTZ accumulation in rice, 10-day-old rice seedlings were treated with 0.2, 0.4, 0.6, 0.8, and 1.0 mg/L BNTZ for 6 days and the accumulation of BNTZ in shoots and roots was measured. The BNTZ accumulation gradually increased with the BNTZ exposure time and concentrations (Figure 3A,B). For example, the concentrations of BNTZ in shoots were 2.68-, 2.44-, 1.58-, 1.63-, and 1.60-fold higher at 6 days than those at 2 days. Similarly, the rice plants exposed to 1.0 mg/L accumulated more BNTZ than any other plants exposed to lower BNTZ concentrations. However,

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compared with shoots, the roots accumulated more BNTZ, possibly due to the direct contact of root tissues with BNTZ.

To study the uptake and translocation of BNTZ in rice, the values of bioconcentration factors and translocation factors were calculated. When the concentration of BNTZ ranged from 0.2 to 1.0 mg/L, the bioconcentration factors of shoots and roots declined from 11.35 to 2.56 (Figure 3C) whereas the translocation factor was proportionally increased from 0.39 to 0.98 with the treatments, indicating that BNTZ tended to retain in roots, rather than being transmitted to shoots (Figure 3D).

RNA-Seq Revealed Variation in Numerous-Gene Expression under BNTZ Stress. To get an insight into the genes in response to BNTZ, we undertook the RNA-Seq to profile four libraries, namely, Shoot−BNTZ, Shoot+BNTZ, Root−BNTZ, and Root+BNTZ. Rice seedlings at the three-leaf stage were employed, when the antioxidant enzyme activities were significantly induced. A total of 20.3−24.3 million clean reads were achieved after removing the low-quality reads. Of these, there were 33.8−47.1 million reads (83.3−97.1% of clean reads) aligned to the reference rice genome (japonica cv. Nipponbare, http://rapdb.dna.affrc.go.jp/download/irgsp1.html) (Table S2). Among the four libraries, 920, 1134, 632, and 864 transcripts were present only in Shoot−BNTZ, Root−BNTZ, Shoot+BNTZ, and Root+BNTZ libraries, respectively (Figure 4A,B), indicating that BNTZ exposure can reprogram the global gene expression and more transcripts expressed in roots than in shoots.

To better understand the effects of BNTZ exposure on gene expression in rice, the FPKM was used to present the gene expression abundance in the libraries ($p < 0.05$ and fold change ≥2 as the thresholds). Large amounts of genes were differentially expressed compared with the control (Figure 4C−F). Of these, 579 differentially expressed genes were upregulated and 193 differentially expressed genes were downregulated in the shoots under BNTZ exposure (Figure 4)C,D. Similarly, there were 1106 genes up-regulated and 1046 genes down-regulated in Root+BNTZ compared with Root−BNTZ. These results suggest that BNTZ treatment altered the transcription of many genes in rice.

Analysis of GO Functional Categories in BNTZ-Exposed Rice. To understand whether enrichment of BNTZ-responsive genes was associated with their biological functions, the Gene Ontology (GO) analysis of different expression genes (DEGs) was performed. Compared with the control, 595 DEGs in shoots and 1534 in roots were assigned to the GO terms. They were grouped into three categories, namely, biological process (BP), cellular component (CC), and molecular function (MF) (Figure S2). The "BP" classification includes organic substance metabolic and catabolic processes, regulation of response to stimulus, cellular biosynthetic process, and many other processes related to resistance to environmental stress. The gene expression in the plasma membrane, cytoplasm, and chloroplast belonging to "CC" was also affected by BNTZ. In addition, DEGs related to detoxification and stress response were assigned to items in "MF" categories such as oxidoreductase activity, monooxygenase activity, hydrolase activity, and glutathione transferase activity.

Several up-regulated DEGs involved in pesticide detoxification and metabolism were screened (Figure 5). There were 11 CYP450 genes and 2 laccase genes that were up-regulated in shoots and roots of rice potentially resistant to BNTZ. In addition, BNTZ significantly stimulated the expression of seven hydrolase and five oxidase genes involved in hydrolysis and

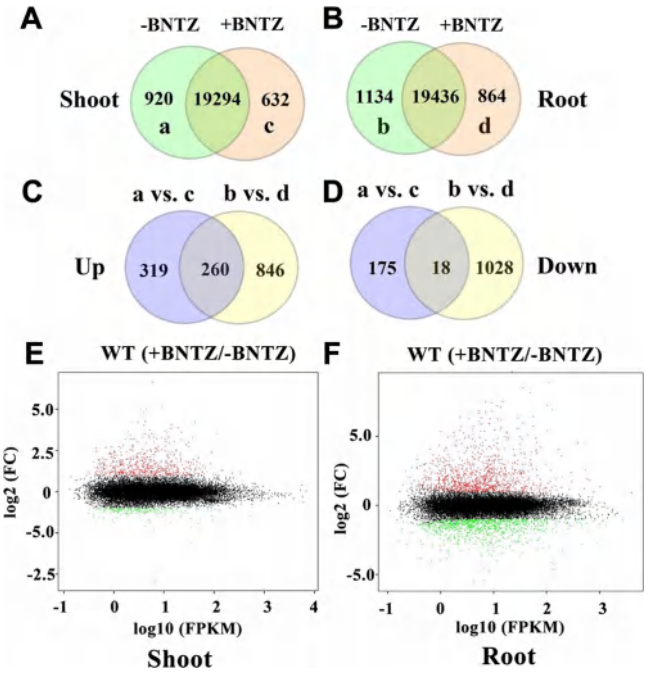

Figure 4. Differentially expressed genes in rice under BNTZ exposure. Rice seedlings at the three-leaf stage were exposed to 0 and 0.8 mg/L BNTZ for 6 days. Four libraries [a, Shoot−BNTZ (shoot control, BNTZ-free); b, Root−BNTZ (root control, BNTZ-free); c, Shoot +BNTZ (shoot treated with BNTZ); d, Root+BNTZ (root treated with BNTZ)] were constructed, and transcriptomes were determined by RNA-seq. Venn diagrams of genes specifically expressed in one library or both (A, B). The specific up-regulated and down-regulated genes that are unique and shared between the different pairwise comparisons (C, D). Threshold values fold change > 2, $p < 0.05$. Volcano diagrams showed up- and down-regulated genes in rice under the As the exposure of (−BNTZ) and BNTZ-exposed (+BNTZ) (E, F). The *y*-axis represents the log2 fold change under the mean normalized expression of all transcripts (*x*-axis). Green dots indicate the down-regulated genes, and red dots show the up-regulated genes.

oxidation. For phase II reaction, DEGs related to methyltransferase (MT), ACE, glucosyltransferase (GT), and GST were screened. MT and ACE can catalyze the formation of methylated or acetylated products of various substrates in plants to detoxify toxicity.[29] It was found that the up-regulated genes involved in detoxification of xenobiotics were more concentrated in GT and GST families in phase II reaction. In this study, 14 GT and 16 GST genes were up-regulated in shoots and roots of rice. Nine genes encoding transcription factors including NAC, AP2, and WRKY were highly expressed in rice shoots and roots after BNTZ exposure (Figure 5).

Validation of Genes from RNA-Sequencing by Quantitative RT-PCR. We identified many differentially expressed genes under BNTZ stress following RNA sequencing. To verify the accuracy of the transcriptome results, 12 genes from up-regulated expression genes related to the detoxification and metabolism of BNTZ were chosen for qRT-PCR analysis, based on the method described previously.[28,30] These genes included a laccase (LOC_Os11g42200), a hydrolase (LOC_Os01g70850), an oxidase (LOC_Os04g51150), an ACE (LOC_Os03g58010), a GT (LOC_Os09g34250), two cytochrome P450s (LOC_Os07g44140, LOC_Os01g52790), two GSTs (LOC_Os09g20220, LOC_Os11g03300), one SOD (LOC_Os08g02110), one POD (LOC_Os03g22020), and one CAT (LOC_Os03g03910) (Figure S3). The qRT-PCR

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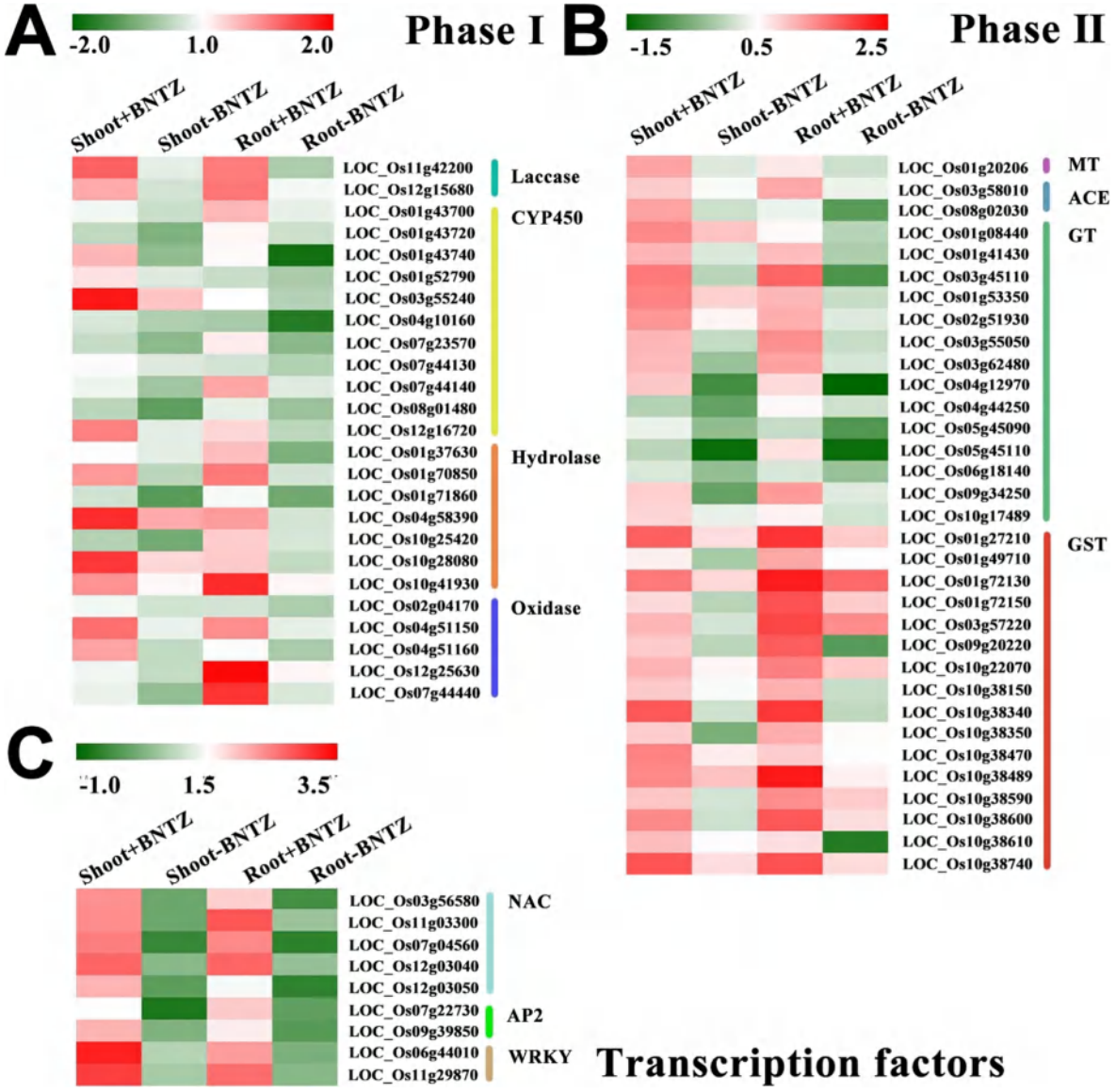

Figure 5. Heatmap summary of up-regulated DEGs possibly involved in BNTZ stress responses in rice. (A) Metabolic enzymes include laccase, cytochrome P450 (CYP450), hydrolase, and oxidase in phase I. (B) Metabolic enzymes include methyltransferase (MT), acetyltransferase (ACE), glucosyltransferase (GT), and glutathione S-transferase (GST) in phase II. (C) Transcription factors include NAC, AP2, and WRKY families.

results showed that the expression patterns of the 12 genes under BNTZ stress were consistent with those from RNA-seq, indicating that the data of RNA Seq were reliable.

A Set of BNTZ Metabolites and Conjugates in Rice Were Characterized. The $[M\text{-}H]^-$ ions of BNTZ and its metabolites and conjugates in rice tissues were characterized by HRLC-Q-TOF-MS/MS in a negative mode. The factors (theoretical m/z, actual m/z, δ, and retention time) were identified for BNTZ metabolites and conjugates (Table 1). The extracted ion chromatograms of the intermediate compounds were presented (Figure S4). Seven metabolites and 14 conjugates of BNTZ were eventually characterized based on the accurate MS data and the appropriate fragmentation patterns from MS^2 data (Table 1 and Figure S5 and S6).

We found a group of metabolites including four hydroxylated products (metabolite nos. 3, 5, 6, and 7) at the benzene ring (Figure S5D,F−H). Metabolites 5 and 7 were methylated and dehydrogenated while being hydroxylated. Two metabolites were formed into smaller compounds by removing the certain group from the BNTZ. Metabolite 1 (m/z 225) was formed by demethylation of BNTZ (Figure S5B). Metabolite 4 (m/z 136) was produced by removing sulfonyl and isopropyl groups from BNTZ (Figure S5E). In addition, there was a product (metabolite 2, m/z 257) derived through hydrolysis of the sulfonamide group in BNTZ (Figure S5C).

There were three BNTZ-acetylated conjugates (nos. 1, 10, and 12), in which conjugate 12 was formed by concomitant glycosylation and acetylation (Figure S6). The two amino acid conjugates (2 and 6) were formed by binding of BNTZ and BNTZ metabolites to hGSH and Cys/Asp, respectively. Furthermore, multiple glucose conjugates (3, 5, 7, 8, and 9), glucosamine conjugates (11 and 14), malonyl glucose conjugates (4), and methylated glucose conjugates (13) were detected in this study (Figure S6).

DISCUSSION

This study examined the toxic response of rice to BNTZ (0−1.0 mg/L) stress and the underlying mechanism for BNTZ

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Table 1. Summary of All Mass Spectrometry Data for BNTZ Metabolites and Conjugates in Rice

no.	acronym	chemical formula	t_R[a]	theor m/z, $[M-H]^-$	exptl m/z, $[M-H]^-$	delta (ppm)	distribution
1	BNTZ	$C_{10}H_{12}N_2O_3S$	17.78	239.0496	239.0497	0.6	S, R
2	FSA[b]	$C_{15}H_{10}ClF_3N_2O_6S$	23.51	436.9827	436.9837	2.3	
identification confidence	level 2	level 4	level 3	level 2	level 5	level 5	
no.	degradation products						
1	demethyl-BNTZ	$C_9H_{10}N_2O_3S$	13.28	225.0339	225.0330	−4.4	R
2	(2-(isopropylcarbamoyl)phenyl)sulfamic acid	$C_{10}H_{14}N_2O_4S$	16.57	257.0602	257.0597	−1.9	S, R
3	6-OH-BNTZ	$C_{10}H_{12}N_2O_4S$	14.48	255.0445	255.0447	0.6	S, R
4	2-aminobenzoic acid	$C_7H_7NO_2$	6.22	136.0404	136.0409	3.9	S, R
5	1-methyl-6,8-OH-BNTZ	$C_{11}H_{14}N_2O_5S$	15.47	285.0551	285.0552	0.3	S
6	6,8-OH-BNTZ	$C_{10}H_{12}N_2O_5S$	17.77	271.0394	271.0403	3.2	S, R
7	1-methyl-3-dehydrogenation-6-OH-BNTZ	$C_{11}H_{12}N_2O_4S$	19.00	267.0445	267.0446	0.3	R
no.	conjugates						
1	demethylation-*O*-acetylation-BNTZ	$C_{11}H_{12}N_2O_5S$	17.39	283.0394	283.0397	1.1	S, R
2	Cys/Asp-BNTZ	$C_{14}H_{18}N_4O_6S$	8.25	369.0874	369.0849	−6.8	S
3	2-amino-*N*-isopropylbenzamide-*O*-glucoside	$C_{16}H_{24}N_2O_7$	14.49	355.1511	355.1520	2.5	S, R
4	BNTZ-*O*-malonylglucoside	$C_{19}H_{24}N_2O_{12}S$	12.83	503.0977	503.0980	0.6	R
5	6-OH-BNTZ-*O*-glucoside	$C_{16}H_{22}N_2O_{10}S$	10.78	433.0922	433.0926	0.8	S, R
6	hGSH-BNTZ	$C_{21}H_{29}N_5O_9S_2$	20.32	558.1334	558.1332	−0.4	S, R
7	1-methyl-6-OH-BNTZ-*O*-glucoside	$C_{17}H_{24}N_2O_{10}S$	13.87	447.1079	447.1079	0	S, R
8	BNTZ-*O*-glucoside	$C_{16}H_{22}N_2O_9S$	12.31	417.0973	417.0977	0.8	S, R
9	BNTZ-*N*-glucoside	$C_{16}H_{22}N_2O_8S$	13.52	401.1024	401.1026	0.6	R
10	1-oxymethyl-BNTZ-*O*-acetylation	$C_{13}H_{16}N_2O_6S$	17.86	327.0656	327.0663	2.1	R
11	6-OH-BNTZ-*O*-glucosamine	$C_{16}H_{23}N_3O_9S$	12.17	432.1082	432.1043	−9.0	S, R
12	BNTZ-*O*-acetylglucoside	$C_{18}H_{24}N_2O_{10}S$	14.78	459.1079	459.1082	0.7	R
13	1-methyl-3-dehydrogenation-BNTZ-*O*-methyglucoside	$C_{18}H_{24}N_2O_9S$	15.85	443.1130	443.1130	0.1	S, R
14	2-amino-*N*-isopropylbenzamide-*O*-glucosamine	$C_{16}H_{25}N_3O_6$	14.61	354.1671	354.1647	−6.6	S, R

[a] t_R, retention time. [b] FSA, fomesafen, internal standard; S, R represent shoot and root, respectively.

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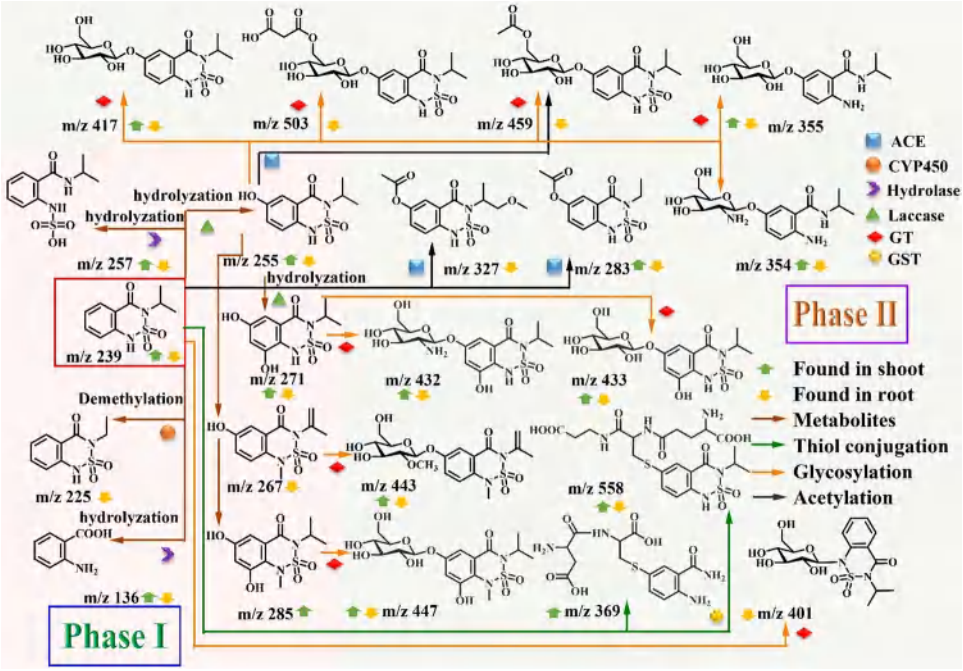

Figure 6. The proposed metabolic pathways of BNTZ in rice. All presented metabolites and conjugates of BNTZ were characterized using LC/Q-TOF-HRMS/MS.

catabolism. The biomass and chlorophyll contents were reduced, and the membrane permeability was increased under BNTZ stress in a time- or concentration-dependent manner (Figure 1, Figure S1). BNTZ stress interrupted photosynthetic electron transfer, promoted ROS accumulation, and caused lipid peroxidation. Because BNTZ treatment resulted in over-generation of oxygen radicals, this suggests that the damage of cellular integrity should be attributed to the increased oxidative stress and lipid peroxidation of rice tissues, which consequently impaired the rice growth and physiological responses. The reduction of TTC represents the increased dehydrogenase activity of plant roots,[22] and the increased root activity reflects the strong capability of BNTZ absorption and metabolism. Compared with shoots, roots accumulated more BNTZ (Figure 3), suggesting that the root activity was higher under the pesticide stress than under the control condition.

Chlorophyll is one of the most important photosynthetic pigments and closely related to photosynthesis. We found that BNTZ exposure reduced the chlorophyll content in rice leaves, which was associated with impaired photosynthesis in rice and reduced production of plant energy and materials. Our result is consistent with the previous report on potato under BNTZ treatment.[31] This result was also reinforced by the chlorophyll fluorescence parameters. The maximum photochemical efficiency (Fv/Fm) of PSII significantly decreased under BNTZ stress, indicating that the PSII reaction centers was impaired. ΦPSII reflects the actual photochemical efficiency of photosynthetic electron transport, which is the efficiency of PSII light energy capture when the PSII reaction center is closed.[32] ΦPSII also showed a decreasing tendency under BNTZ stress, confirming that the photosynthetic efficiency and accumulated energy for the dark carbon assimilation were negatively affected under the stress of BNTZ. NPQ signifies the situation that the light energy absorbed by the pigment of PSII antenna cannot be properly utilized for photosynthetic electron transport and dissipated in the form of heat.[21] In this study, the value of NPQ was moderately increased under BNTZ treatment, suggesting that accumulation of light energy in the photosynthetic center was affected by the inhibition of BNTZ. The self-regulatory mechanism of plants accelerates the dissipation of excess light energy and thus protects the overall growth and physiological function.

The anti-stress behavior was also validated through the enhanced activities of antioxidize enzymes (SOD, POD, and CAT) and detoxification enzyme GST (Figure 2). Because pesticide molecules can penetrate the cuticular wax layer of plant tissues, they can activate ROS, SOD, POD, and CAT, all of which are able to clean up the oxygen free radicals and protect plants from oxidative injury.[29,33] In addition to SOD, POD, and CAT, there are many other enzymes that can directly target the herbicide molecules and make them detoxified.[11] For example, activities of GST were obviously increased under BNTZ exposure (up to 0.8 mg/L). GST can actively participate the redox reactions and bind many toxic compounds for detoxification as well.[17] GT is another specific enzyme that transfers glucose molecules to receptors to form glycosidic bonds and regulates metabolism of exogenous chemical herbicides.[14] CYP450 also plays an important role in catalytic conversion of toxic substances in plants.[34,35] These results suggest that activities of the detoxic enzymes can be strongly induced and in turn involved in detoxification of BNTZ.

A global identification of transcriptomes helps understand gene expression and regulatory mechanisms for rice adaptation to BNTZ stress. It was found that there were more genes differentially expressed (up-regulated and down-regulated) in roots than in shoots, which confirmed that root is the major tissue for BNTZ uptake and detoxification. However, whether the regulated genes in roots or shoots are more effective for the pesticide degradation not only depends on the type of chemicals but also on the plant species. The rice seedlings treated with fomesafen showed more up-regulated DEGs in shoots for detoxification and metabolism of the pesticide.[29] The expression

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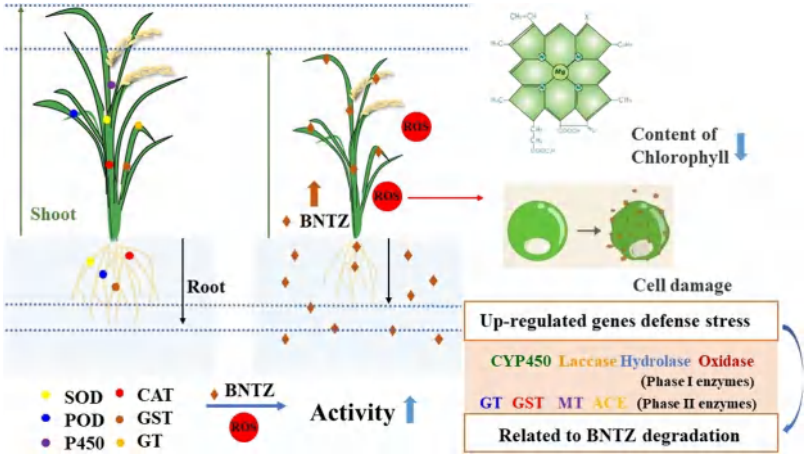

Figure 7. The proposed mechanism of BNTZ metabolism or degradation in rice.

levels of the same family genes in different plants also varied under different pesticides; for example, more genes were differentially expressed in the shoots of alfalfa under atrazine stress.[12] With regard to BNTZ, the upregulated DEGs such as laccases, CYP450s, GT, and GSTs are preferentially involved in the metabolism and degradation of BNTZ. Regarding phase I reaction components, laccases catalyze the oxidation of a variety of substrates[36] while CYP450 monooxygenases transform organic pollutants by dealkylation, epoxidation, and hydroxylation or biochemically convert them into highly polar, chemically active, and water-soluble compounds.[12] In phase II metabolism, GTs catalyze glycosylation of small molecules using the donor UDP glucose, while GSTs play an important role in decomposition of exogenous substances by catalyzing the nucleophilic attack of reduced glutathione-SH on various electrophilic molecules such as pesticides and carcinogens.[13] Furthermore, some genes encoding ABC transporters responsible for the subcellular compartmentalization and tissue distribution of exogenous toxic compounds across membrane are also highly induced.[17,37] ABC transporters belong to the phase III metabolism of pesticides in plants. We recently reported that some genes of ABC transporters were upregulated in response to four pesticides including fomesafen, mesotrione, bentazone, and ametryn,[38] suggesting that ABC transporters can be involved in the phase III metabolism of herbicide in plants.[17,37]

Seven BNTZ metabolites (in phase I) and 14 BNTZ conjugates (in phase II) were characterized by HRLC-Q-TOF-MS/MS to correlate with the phase I/II DEGs and their catalyzed metabolic products in rice (Figure 6). BNTZ was metabolized through hydroxylation, hydrolysis, acetylation, glycosylation, and thiolation. Among these, 6-OH-BNTZ and 6,8-OH-BNTZ were characterized in shoots and roots. The hydroxyl derivatives of BNTZ were previously reported in microbes of soil.[39] Laccase is a distinct kind of multi-copper-containing oxidase and detected in many bacterial and fungi species.[36] Laccases catalyze various toxic organics such as polycyclic aromatic hydrocarbons, herbicides, and polyphenol compounds.[40] Two genes encoding laccases (LOC_Os11g42200, LOC_Os12g15680) were significantly up-regulated under BNTZ stress (Figure 5). As OH-BNTZ metabolites were characterized, this allowed to speculate that the laccases were most likely involved in the metabolism of BNTZ, which preferentially converted BNTZ to the more active 6-OH and 8-OH derivatives. In addition, a hydrolysis metabolite of BNTZ (2-isopropylcarbamoyl-phenyl sulfamic acid, metabolite 2) was identified. The increased expression of hydrolases may promote the chemical reaction. Hydrolases degrade exogenous toxic pollutants by catalyzing the transfer of hydrogen atoms of water molecules to substrates.[41] Transformation of the hydrolase gene *ophB* into *Escherichia coli* facilitated degradation of chlorpyrifos.[42] An hydrolase purified from strain JZ-2 hydrolyzed the −CHCN-COO− group of fenpropathrin to generate cyano-3-phe-noxybenzyl alcohol and 2,2,3,3-tetramethylcyclopropane-carboxylic acid.[43] In this study, metabolite 2 was produced by hydrolysis of the sulfonyl group of parent BNTZ into the sulfonic acid group, and metabolite 4 (2-aminobenzoic acid) was formed by hydrolysis of −CO-NH−. The adealkylated metabolite (demethyl-BNTZ, metabolite 1) was identified. Its formation was likely relevant to the upregulated expression of CYP450s (Figure 5).

Metabolites associated with acetylation, glycosylation, and amino acids occur in phase II reaction and correlate with enzymes ACE, GT, and GST. Acetylation is an important process of pesticide metabolism in plants, which is catalyzed by acetyl-CoA or ACE.[29] Two loci (ACE), LOC_03g258010 and LOC_08g02030, were up-regulated significantly in roots (Figure 5). Activation of ACE-encoding genes contributes to rice tolerance to environmental stress and plant productivity.[44] ACEs were reported to target atrazine, isoproturon, and glyphosate.[45−47] Three acetylated conjugates (demethylation-*O*-acetylation-BNTZ, 1-oxymethyl-BNTZ-*O*-acetylation, and BNTZ-*O*-acetylglucoside) were identified in this study. BNTZ-*O*-acetylglucoside was formed by joint catalysis of ACE and GTs. The function of glycosylation is believed to increase water solubility of metabolites, which makes them easier to be metabolized in plants.[48] GTs catalyze different sugars attached to the target molecules.[49] In rice, several reports indicate the roles of GTs in regulating glycosylated conjugates of atrazine, isoproturon, acetochlor, and other pesticides.[14,28,29] Overexpression of *ARGT1* and *IRGT1* generated more glycosylated products and thus attenuated cellular injury of rice.[13,14] This study revealed that multiple GT genes were up-regulated under BNTZ stress and possibly catalyzed BNTZ to form glycosylation conjugates (Figures 5 and 6). These conjugates include glycosyl transfers, which usually occur at the nucleophilic oxygen of hydroxyl acceptors such as OH-BNTZ and *N*-linked glycosylated BNTZ. Linking BNTZ metabolites to amino acids may be another important metabolic pathway, by which two conjugates hGSH-BNTZ and Cys/Asp-BNTZ were detected. GSTs

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detoxified various herbicides by interacting with glutathione (GSH).[50] The formation of hGSH-BNTZ may relate the up-regulated expression of GSTs (Figure 5). The degradative products may be either compartmentalized in the vacuole or excluded out of the cells by phase III metabolism.[12,17]

In summary, this study provides several lines of evidence to understand the toxic and detoxic effects of BNTZ and underlying regulatory mechanisms (Figures 6 and 7). When rice was exposed to BNTZ, a series of toxic responses occurred in a time-course- or concentration-dependent manner. Rice can actively transport the BNTZ residue from the environment and accumulate in roots. An extensive survey of global BNTZ-responsive genes revealed that multi-family DEGs were involved in the process of BNTZ metabolism. Global BNTZ-responsive genes were identified by RNA-Seq, and the multi-family DEGs were found to involve the BNTZ metabolic process. Metabolites and conjugates of BNTZ were characterized by HRLC-Q-TOF-MS/MS, and the association between specific BNTZ-degraded products and detoxification enzymes was established. Our data may help understand the multi-pathways for BNTZ metabolism and degradation in rice crops and environments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c02535.

> Growth response analysis of rice under bentazone (BNTZ) exposure; GO analysis; quantitative RT-PCR validation of RNA-Seq changes; the extracted ion chromatograms of metabolites and conjugates of BNTZ in rice; seven metabolites of BNTZ identified based on the accurate MS data and the appropriate fragmentation patterns from MS^2 data; fourteen conjugates of BNTZ identified based on the accurate MS data and the appropriate fragmentation patterns from MS^2 data; the spiked recoveries of BNTZ in rice tissues and growth medium; data description of RNA-Seq reads in four libraries with three biological replicates by RNA sequencing; significantly enriched gene ontology (GO) terms in "Shoot+BNTZ vs. Shoot−BNTZ" and "Root +BNTZ vs. Root−BNTZ" libraries of rice; primers for genes used for validating gene expression in rice (PDF)

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Notes

The authors declare no competing financial interest.

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