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Combined Physio-Biochemical and Transcriptome Analyses Illuminate the Resistance Response of Rice Priming with Decoyinine against *Nilaparvata lugens*

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Abstract: The brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), is a notorious pest in rice production. The microbial secondary metabolite, decoyinine (DCY), is produced by *Streptomyces hygroscopicus*. Recent studies found that seed priming with DCY could enhance rice resistance to BPH and *Laodelphax striatellus*; however, the mechanism of enhancing insect resistance in rice remains unclear. Here, an integrated physio-biochemical and transcriptome analysis was performed on rice priming with DCY after BPH infestation. Defense-related enzymes activities such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), glutathione S transferase (GST), and polyphenol oxidase (PPO), concentrations of hydrogen peroxide (H_2O_2), callose, and free amino acids in rice priming with DCY were significantly increased than in control plants after BPH infestation. Concentrations of soluble sugars, sucrose, and glucose were higher in rice treated with DCY than in the control after BPH infestation; however, the concentration of malondialdehyde (MDA) was significantly decreased in rice treated with DCY after BPH infestation. In the transcriptome analysis, GO functional annotation and KEGG pathway analysis were enriched in defense response, transcription factors, secondary metabolites, reactive oxygen species, and cell wall organization and these data also support physio-biochemical results. The qRT-PCR results further verified the differential expressed genes related to DCY-treated rice responding to BPH. Meanwhile, it indicated that DCY might enhance the resistance of rice to BPH by regulating the rice WRKY transcription factor genes. Our results provide a basis for further exploring the molecular mechanism of the defense response of rice priming with DCY against BPH infestation and could provide valuable resources to control insect pests.

Keywords: *Nilaparvata lugens*; decoyinine; physio-biochemical index; transcriptome

1. Introduction

Rice (*Oryza sativa* L.) is the main food for more than half of the global population and is grown in more than 100 countries, with 90% of the total world's production from Asia [1,2]. However, rice production is damaged by various biotic and abiotic stresses throughout its lifetime. About ten percent of the rice yield is lost annually due to insect pests [3,4]. Among these pests, planthoppers, such as the brown planthopper (BPH) *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), is a notorious pest of rice in Asia and has caused significant losses annually and threatened countries' food security [5,6]. BPH directly injures rice plants by sucking phloem sap, resulting in a significant reduction in rice production [7]. BPH can also cause indirect damage by the spreading of various viruses (rice ragged stunt virus, rice grassy stunt virus, etc.) [8,9]. However, the long-term abuse of chemical pesticides has led to a series of problems such as pesticide resistance, pesticide residues,

and environmental contamination [10]. Therefore, it is urgent to develop new, safe, and environment-friendly pest control technology.

Biopesticides, the potential alternative to synthetic pesticides, can be classified into different categories, such as plant-incorporated protectants, biochemicals, and microbial pesticides [11–13]. *Streptomyces* are well-known and have been widely reported for their production of secondary metabolites with unique structures and modes of action, with major options for biocontrol [14]. Decoyinine (DCY) was discovered by Yuntsen H in 1954 and was originally isolated from *S. hygroscopicus* [15,16]. Our previous studies have shown that rice seed priming with DCY can confer rice higher resistance to the BPH and small brown planthopper (SBPH, *L. striatellus*) [17,18]. However, the potential mechanism of rice priming with DCY to improve pest resistance has been rarely reported.

The ultimate impact on insect performance notably depends on the interplay between a positive effect derived from enhanced plant growth and a negative effect derived from an induced resistance in the plant [19]. MDA content has been widely used as a biomarker of the degree of cell membrane damage [20]. Various biotic and abiotic stresses induce the rapid production of reactive oxygen species (ROS) in plants, particularly H_2O_2, which activates a battery of plant defense mechanisms [21]. Under steady-state conditions, the ROS are scavenged by various antioxidant enzymes, in which SOD, POD, GSTs, and CAT are important in maintaining a balance of ROS [22,23]. PPO and PAL catalyze the synthesis of insect-resistant secondary metabolites (phenols and lignin) in response to insect attacks [24]. Callose and flavonoid accumulation is inextricably linked to the plant's insect resistance [25,26]. The development and fitness of herbivore insects are tightly linked to the nutritional quality of their host plants. Soluble sugars and free amino acids of plants are important sources of nutrients for herbivores [27].

Our previous study showed that DCY induced some physiological changes to enhance rice resistance to SBPH [18]; however, the DCY-mediated physiological and molecular mechanisms against BPH remain unclear. Transcriptome analysis is an effective tool to determine plant-pest interactions [28], which may help us to understand the defense mechanisms of rice priming with DCY against BPH. In this study, we measured a series of physiological indicators including CAT, SOD, POD, GST, PPO, PAL, H_2O_2, MDA, callose, flavonoid, soluble sugars, sucrose, glucose, and free amino acids, which can elucidate the physiological mechanisms of insect resistance in DCY-treated rice plants. To further reveal the molecular mechanisms of rice priming with DCY responding to BPH, transcriptome sequencing was used to analyze the gene expression profiles in BPH-fed rice plants. Our results facilitate the understanding of plant resistance to herbivores as enhanced by microbial metabolites and develop the application of DCY in pest control.

2. Materials and Methods

2.1. Insect

BPH strains were obtained from the China National Rice Research Institute, Hangzhou, China, and raised on the seedlings of Taichung Native 1 (TN1) in a growth chamber (27 ± 1 °C, humidity 70 ± 5%, and light:dark 16:8 h).

2.2. Plant and Insect Treatments

The rice variety TN1 is susceptible to BPH, which was provided by China National Rice Research Institute, Hangzhou, China [17]. DCY was provided by the Shanghai Macklin Biochemical Technology Co., Ltd. Rice seeds were soaked in 50 mg/L DCY water solution or water for 24 h at 28 °C, 16 h:8 h photoperiod, and then sprouted in a dark environment for 24 h [17]. The rice seeds were grown in plastic buckets (30 cm in diameter and 28 cm in height) and placed in greenhouse. The plastic bucket was covered with 100 mesh nylon net (length × width × height = 100 cm × 100 cm × 100 cm) to prevent attacks by herbivores.

The rice plants were in four treatments: (1) without DCY and BPH (control), (2) with 50 mg/L DCY solution and without BPH (DCY), (3) without DCY and with BPH infestation (control + BPH), and (4) with 50 mg/L DCY solution and BPH infestation (DCY + BPH).

Before being used to infest rice, the 3rd-instar BPH nymphs were starved for 1 h. At elongation stage (60 days), 20 BPH nymphs were placed on parafilm bags (length $\times$ width = 5 cm $\times$ 5 cm) and attached to a rice plant sheath. For each treatment, sampling was repeated 5 times. The rice leaf sheaths, including herbivore-exposed local (damaged) parts, were sampled at 0, 24, 48, 72, and 96 h post-infestation, immediately maintained in liquid nitrogen, and then stored at -80 °C.

2.3. Assay of Defense-Related Enzyme Activity

Six defense-related enzymes including CAT, SOD, POD, GST, PPO, and PAL were measured. GST activity was assayed by the methods of 1-chloro-2, 4-dinitrobenzene [23]. Detailed methods for determining the activities of CAT, SOD, and POD refer to the methods of Han et al. [24]. Activities of PPO and PAL were measured by the methods of Cai et al. [29].

2.4. Measurements of MDA, H_2O_2, Sugars, Free Amino Acids, Flavonoid, and Callose Content

The content of MDA was measured according to the thiobarbituric acid method [30]. H_2O_2 content was determined by the method of ammonium molybdate spectrophotometry [31]. Soluble sugar content was assayed by the method of anthrone colorimetry [18]. The contents of glucose and sucrose were measured using the methods of Liu et al. [32]. The content of free amino acids was assayed using ninhydrin method [33]. Callose content was measured by aniline blue method [34]. Callose deposition in rice sheaths was measured by the methods of Liu et al. [35].

2.5. Rice RNA Library Construction, Sequencing, and Mapping

The rice leaf sheathes samples including control 48 h, DCY 48 h, control + BPH 48 h, and DCY + BPH 48 h, were used for total RNA extraction. The total RNA of three individual rice leaf sheathes from the same treatment was pooled as one replicate. Each treatment had three biological replicates. cDNA library preparation, sequencing, and mapping were performed by the Novogene Bioinformatics Technology Co. Ltd., Beijing, China. The genomes of *Oryza sativa* cultivar: TN1 (https://www.ncbi.nlm.nih.gov/bioproject/663050, accessed on 11 June 2021) were used for alignment of the clean reads. The RNA sequences of this experiment have been stored in the NCBI sequence read archive and the access number is PRJNA898387.

2.6. Analysis of Differential Expressed Genes (DEGs)

DEGs were performed for any two groups using the DESeq2 v1.20.0 [36]. DESeq2 mainly uses the model of negative binomial distribution to analyze the differential expression. DEGs with adjusted *p*-value (padj) < 0.05 and absolute log2 (fold change) $\geq$ 1 were defined as statistically significant [37].

2.7. Gene Ontology (GO) and KEGG Enrichment Analysis

GO and KEGG are databases of gene-related functions based on different classification ideas. GO enrichment describes DEGs from three levels: molecular function, cellular component, and biological process. KEGG mapped DEGs from the complex interrelationships between gene sets, genes, and metabolites.

2.8. Quantitative Real-Time PCR Analysis

Eighteen DEGs were selected for qRT-PCR validation and the *Ubiquitin* gene (AK059694.1) was used as the reference gene [38]. Primers are listed in Supplementary Table S1. The qRT-PCR was performed using the CFX96® Real-Time PCR Detection System (Bio-Rad3). Three independent biological replicates were used for each sample. The relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method [39].

2.9. Statistical Analysis

Differences in rice physiological indexes and gene expression levels were analyzed by Tukey's honestly significant difference (HSD) test ($p < 0.05$). The data were subjected to three-way analysis of variance (ANOVA) for the effects of DCY treatment, BPH infestation, BPH infestation time, and the interactions between the three treatments. Results were presented as the mean $\pm$ SE and all data analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Responses of Defense-Related Enzyme to BPH Infestation

The three-way ANOVA results showed that DCY treatment significantly influenced defense-related enzymes including CAT, SOD, POD, and PPO in rice plants' response to BPH infestation, except GST and PAL (Table S2). All defense-related enzymes showed no significant differences between the control and DCY plants at all-time points without BPH infestation. CAT activity was remarkably higher in DCY + BPH plants than in control + BPH plants at 24 h (37.08%), 48 h (45.17%), and 72 h (34.30%) post-BPH infestation (Figure 1A). Likewise, SOD activity was significantly higher in DCY + BPH plants than in control + BPH plants at 24 h (16.05%), 48 h (15.41%), and 72 h (18.65%) post-BPH infestation (Figure 1B). POD activity was remarkably higher in DCY + BPH plants than in control + BPH plants only at 24 h (26.00%) and 48 h (12.16%) post-BPH infestation (Figure 1C). Although GST activity was significantly higher in DCY + BPH plants than in the control + BPH plants at 48 h (Figure 1D), DCY treatment, BPH infestation, and BPH infestation time all showed no influence on GST activity in plants (Table S3). Similar results were shown in PAL activity (Figure 1F, Table S3). PPO activity showed significant increases in DCY plants compared to control plants at 24 (82.53%) and 48 h (58.64%) post-BPH infestation (Figure 1E).
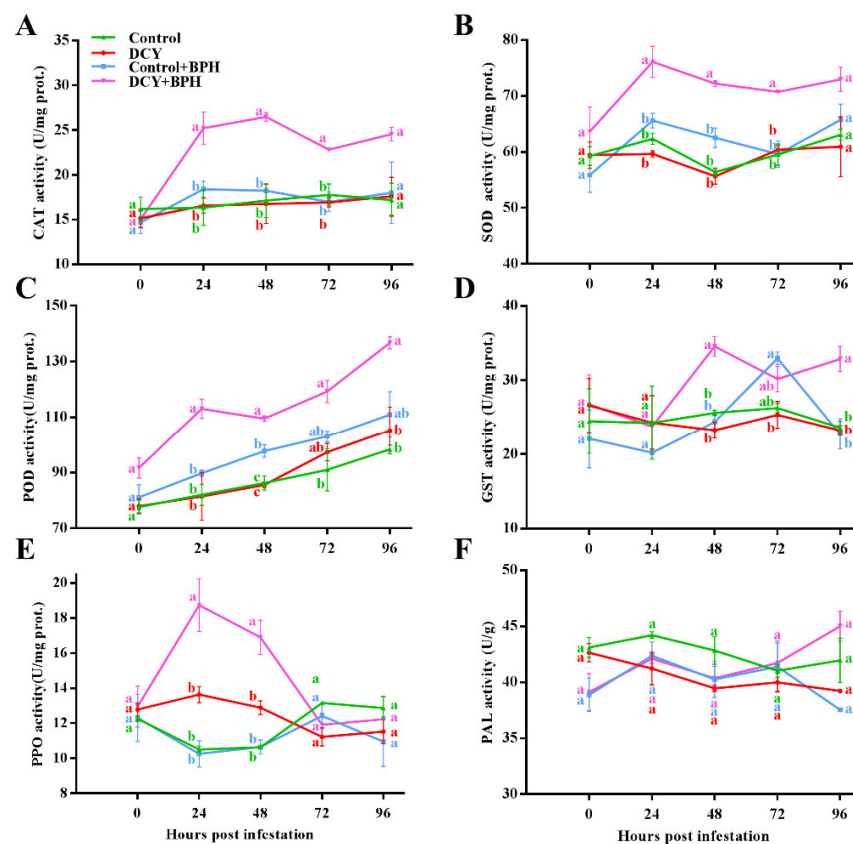

Figure 1. Changes in defense-related enzyme activity in rice sheaths' responses to DCY treatment and BPH infestation: (**A**) CAT, (**B**) SOD, (**C**) POD, (**D**) GST, (**E**) PPO, and (**F**) PAL. Data represent means $\pm$ standard error of three replicates. Different letters at a certain time point post-BPH infestation indicate significant differences ($p < 0.05$, Tukey's HSD test).

3.2. Responses of Defense-Related Compounds to BPH Infestation

The three-way ANOVA results showed that DCY treatment significantly influenced defense-related compounds including MDA, H_2O_2, callose, and flavonoid in rice plants' responses to BPH infestation (Table S2). The contents of MDA, H_2O_2, and flavonoid were not significantly different between the control and DCY plants at all-time points without BPH infestation. However, callose concentrations were significantly higher in DCY plants than in control plants at 0 h (15.29%), 24 h (27.54%), 48 h (14.44%), and 96 h (12.63%) without BPH infestation. The contents of callose were also significantly higher in DCY + BPH plants than in control + BPH plants at all-time points (0 h, 15.37%, 24 h, 13.28%, 48 h, 14.62%, 72 h, 13.78%, and 96 h, 5.98%, Figure 2C). Similar results are shown in Supplementary Figure S1. The MDA concentration in control + BPH plants showed a significant increase from 48 h to 96 h (48 h, 43.65%, 72 h, 46.45%, and 96 h, 68.08%) post-infestation than in DCY + BPH plants (Figure 2A). H_2O_2 concentration in the DCY + BPH plants showed a significant increase only at 24 h (39.07%) and 48 h (26.21%) post-BPH infestation over the control + BPH plants (Figure 2B). The flavonoid concentration in DCY + BPH plants showed a significant increase only at 48 h (37.35%) and 72 h (35.87%) post-BPH infestation over the control + BPH plants (Figure 2D).
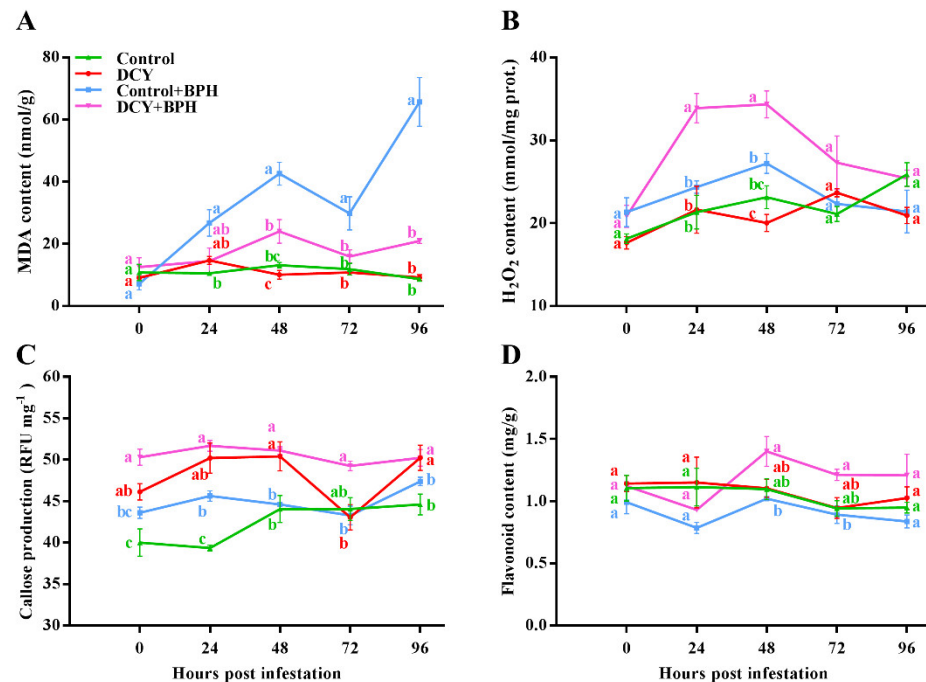

Figure 2. Changes in defense-related compound content in rice sheaths' responses to DCY treatment and BPH infestation: (**A**) MDA, (**B**) H_2O_2, (**C**) callose, and (**D**) flavonoid. Data represent means ± standard error of three replicates. Different letters at a certain time point post-BPH infestation indicate significant differences ($p < 0.05$, Tukey's HSD test).

3.3. Responses of Nutrient Contents to BPH Infestation

The three-way ANOVA results showed that DCY treatment significantly influenced the nutrient content including soluble sugar and sucrose in rice plants' response to BPH infestation (Table S2). The contents of soluble sugar, sucrose, glucose, and free amino acids were not significantly different between the control and DCY plants at all-time points without BPH infestation. Soluble sugar concentration significantly decreased in control + BPH plants than in DCY + BPH plants at 24 h (72.19%), 48 h (40.31%), and 72 h (74.16%) post-BPH infestation (Figure 3A). Similarly, sucrose concentration in control + BPH plants showed a significant decrease at 24 h (42.29%), 48 h (36.80%), and 96 h (83.10%) post-BPH infestation than in DCY + BPH plants (Figure 3B). Glucose concentration significantly decreased in control + BPH plants than in DCY + BPH plants only at 48 h (40.31%) and

96 h (74.16%) post-BPH infestation (Figure 3C). The contents of the free amino acid were remarkably higher in DCY + BPH plants than in the control + BPH plants only at 24 h (Figure 3D).
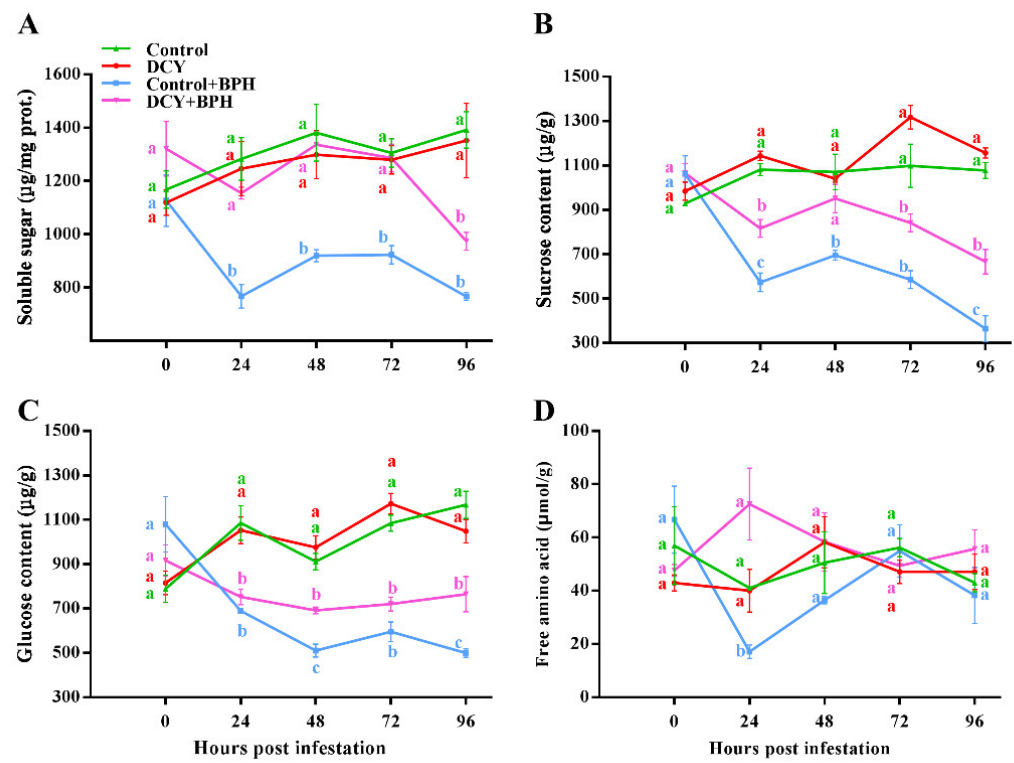

Figure 3. Changes in nutrient content in rice sheaths' responses to DCY treatment and BPH infestation: (**A**) soluble sugar, (**B**) sucrose, (**C**) glucose, and (**D**) free amino acid. Data represent means ± standard error of three replicates. Different letters at a certain time point post-BPH infestation indicate significant differences ($p < 0.05$, Tukey's HSD test).

3.4. Illumina Sequencing and Mapping of Reads

The transcriptome sequencing data of the 12 samples are shown in Supplementary Table S3. The number of raw reads obtained from 12 libraries ranged from 42,563,208 to 55,325,630 and the number of clean reads ranged from 40,782,836 to 54,164,088. The clean reads were successfully mapped to the reference genome of the *O. sativa* cultivar TN1. For each sample, the comparison efficiency ranged from 91.41% to 93.60% and at least 36,374,586 unique genes were referenced to the genome for functional annotation. The Q30 values in the 12 rice samples were all higher than 93.46%.

3.5. Transcriptome Analysis of Rice Treated with DCY in Response to BPH Infestation

Statistical analysis of DEGs in four comparative groups (DCY vs. control, DCYB vs. controlB, controlB vs. control, DCYB vs. DCY) was carried out (Figure 4A). BPH infestation altered the gene expression of the control rice plant significantly, including the upregulation of 1373 DEGs and the downregulation of 1972 DEGs (controlB vs. control). After the rice was treated with DCY, there were 396 DEGs, including 336 upregulated genes and 60 downregulated genes compared with the control. Following rice treated with DCY, BPH infestation showed more DEGs than in other comparisons (DCYB vs. DCY). There were 5402 DEGs, including 1870 upregulated genes and 3532 downregulated genes. Comparison of DEGs in groups (DCYB vs. controlB) revealed significant changes, including 287 upregulated genes and 225 downregulated genes (Figure 4A). Moreover, the number of DEGs between DCY vs. control and DCYB vs. controlB, DCYB vs. controlB and controlB vs. control, controlB vs. control and DCYB vs. DCY, DCYB vs. DCY, and DCY vs. control

was 39 (3 + 3 + 14 + 19), 172 (3 + 54 + 101 + 14), 2199 (2021 + 101 + 14 + 63), and 277 (14 + 63 + 19 + 181), respectively (Figure 4B).
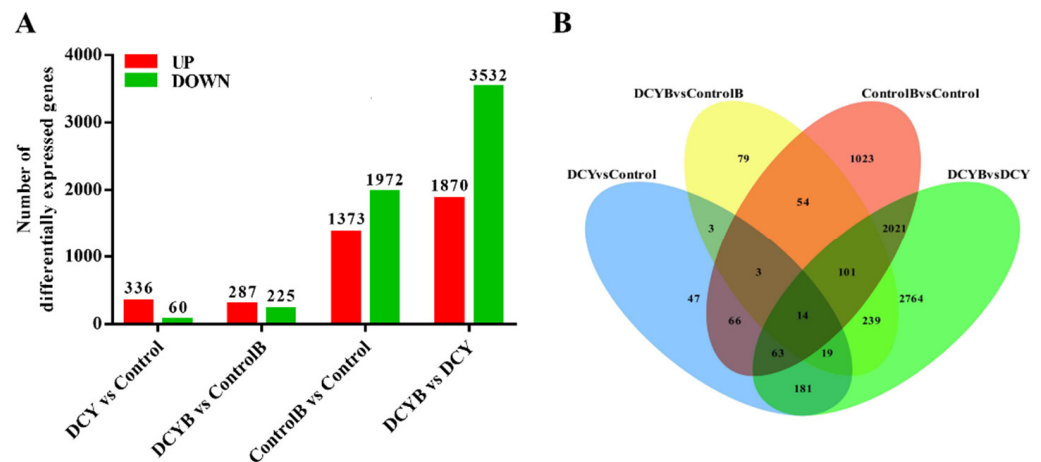

Figure 4. DEGs induced in rice plants by DCY and BPH infestation: (**A**) Comparison of upregulated and downregulated DEGs, and (**B**) Venn diagram of DEGs.

3.6. GO Function Annotation Analysis of DEGs

Without BPH infestation, the DEGs between DCY and control were annotated in cell wall modification (GO: 0042545), cell wall organization (GO: 0071555), and cell periphery (GO: 0071944) (Figure 5C). After BPH infestation, the DEGs in groups of controlB vs. control and DCYB vs. DCY were enriched in the cellular carbohydrate metabolic process (GO: 0044262), polysaccharide metabolic process (GO: 0005976), cellular glucan metabolic process (GO: 0006073), and cell wall (GO: 0005618) (Figure 5A,B). The GO analysis of the DEGs between DCYB and controlB showed that the main functional groups were annotated in the cellulose metabolic process (GO: 0030243), cellulose biosynthetic process (GO: 0030244), glucan biosynthetic process (GO: 0009250), and glucan metabolic process (GO: 0044042) and cell wall (GO: 0005618) and sequence-specific DNA binding (GO: 0043565) for molecular function (Figure 5D).

3.7. KEGG Pathway Enrichment Analysis of DEGs

Without BPH infestation, the DEGs in the group of DCY vs. control were annotated into the KEGG pathway, which was mainly enriched in six pathways. They were pentose and glucuronate interconversions, phenylpropanoid biosynthesis, carotenoid biosynthesis, vitamin B6 metabolism, diterpenoid biosynthesis, and fatty acid biosynthesis (Figure 6C). After BPH infestation, the DEGs in groups of controlB vs. control and DCYB vs. DCY were all enriched in eight pathways. They were alpha-Linolenic acid metabolism, ascorbate and aldarate metabolism, carotenoid biosynthesis, flavonoid biosynthesis, phenylalanine metabolism, phenylpropanoid biosynthesis, starch and sucrose metabolism, and tyrosine metabolism (Figure 6A,B). The KEGG analysis of the DEGs in the group of DCYB vs. controlB showed that the main pathways were annotated in alpha-Linolenic acid metabolism, phenylpropanoid biosynthesis, ABC transporters, MAPK signaling pathway-plant, butanoate metabolism, flavone and flavonol biosynthesis, tryptophan metabolism, flavonoid biosynthesis, and phagosome (Figure 6D).
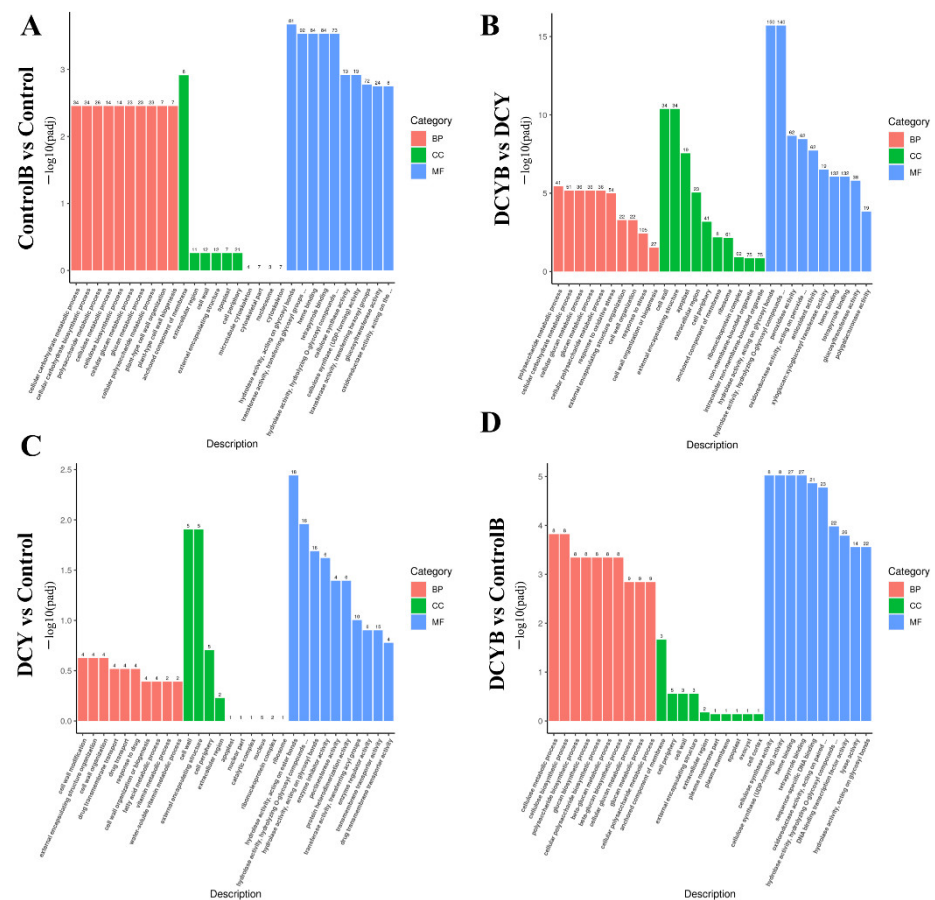
Figure 5. GO annotation classification chart of DEGs in different comparative groups. (**A**) ControlB vs. control: the group of DEGs between control + BPH and control rice plants; (**B**) DCYB vs. DCY: the group of DEGs between DCY + BPH and DCY rice plants. (**C**) DCY vs. control: the group of DEGs between DCY and control rice plants; (**D**) DCYB vs. controlB: The group of DEGs between DCY + BPH and control + BPH rice plants. The *x*-axis was functional classification, the *y*-axis was −log10 (padj). BP: biological process, CC: cellular component, MF: molecular function.

3.8. Representative Pathway DEGs Response to DCY and BPH Infestation in Rice

Thirty-six defense-related genes including 15 WRKY transcription factor genes (LOC4344296, LOC107276056, LOC4332755, LOC4351328, LOC4341678, LOC107277481, LOC107279783, LOC107277470, LOC4347070, LOC107276131, LOC4327651, LOC4349612, LOC4347069, LOC4327611, and LOC4339665), six cytochrome P450 genes (LOC9271617, LOC4326660, LOC4349113, LOC4324604, LOC4336711, and LOC9266682), a catalase gene (LOC4328073), four superoxide dismutase genes (LOC4346329, LOC4332082, LOC4344210, and LOC4339922), two peroxidase genes (LOC4324556 and LOC4337482), a glutathione S-transferase gene (LOC4349207), a phenylalanine ammonia-lyase gene (LOC4336415), two callose synthase genes (LOC4340315 and LOC4325900), two hexokinase genes (LOC4338010 and LOC4339361) and two glucose-6-phosphate 1-dehydrogenase genes (LOC4329889 and LOC4342988) were up-regulated in response to DCY treatment of BPH-infested plants compared with BPH-infested plants without DCY treatment (Figure 7). However, another bidirectional sugar transporter gene (LOC4338107) was down-regulated. Even with the DCY or control, all these genes were up-regulated without an infestation of BPH.
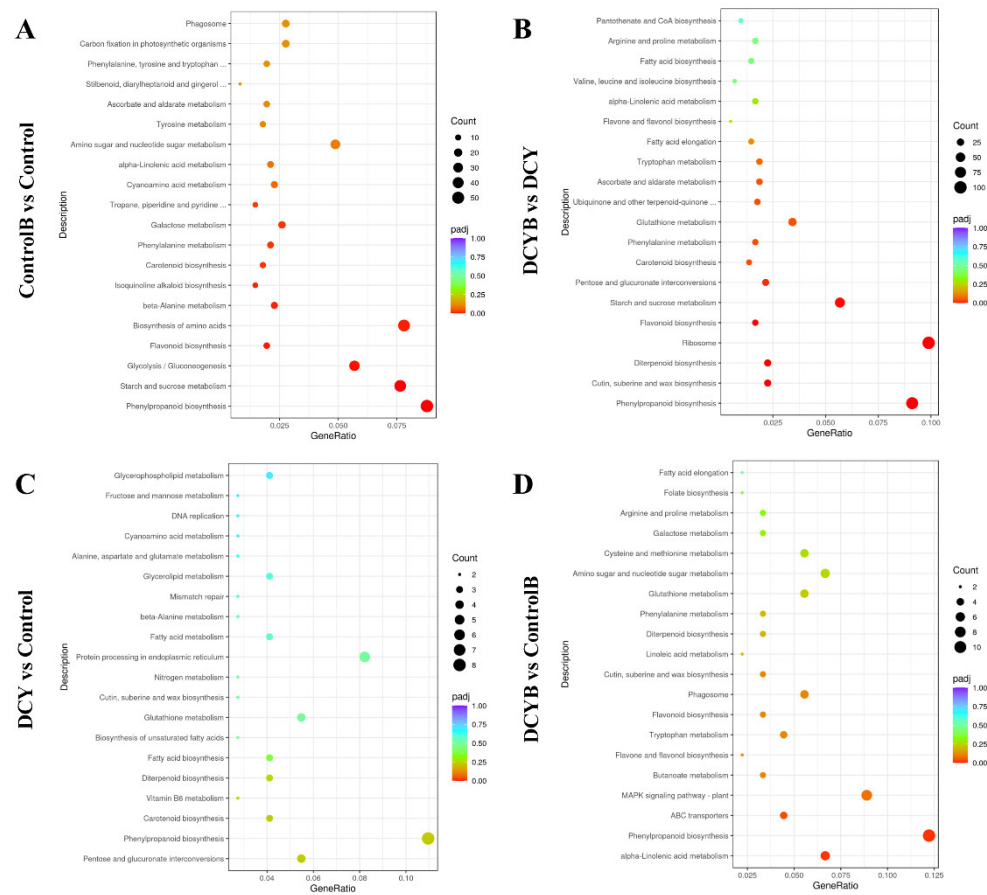
Figure 6. KEGG annotation classification chart of DEGs in different comparative groups. (**A**) ControlB vs. control: the group of DEGs between control + BPH and control rice plants; (**B**) DCYB vs. DCY: the group of DEGs between DCY + BPH and DCY rice plants. (**C**) DCY vs. control: the group of DEGs between DCY and control rice plants; (**D**) DCYB vs. controlB: The group of DEGs between DCY + BPH and control + BPH rice plants. The *x*-axis indicated enrichment factor, the *y*-axis was padj.
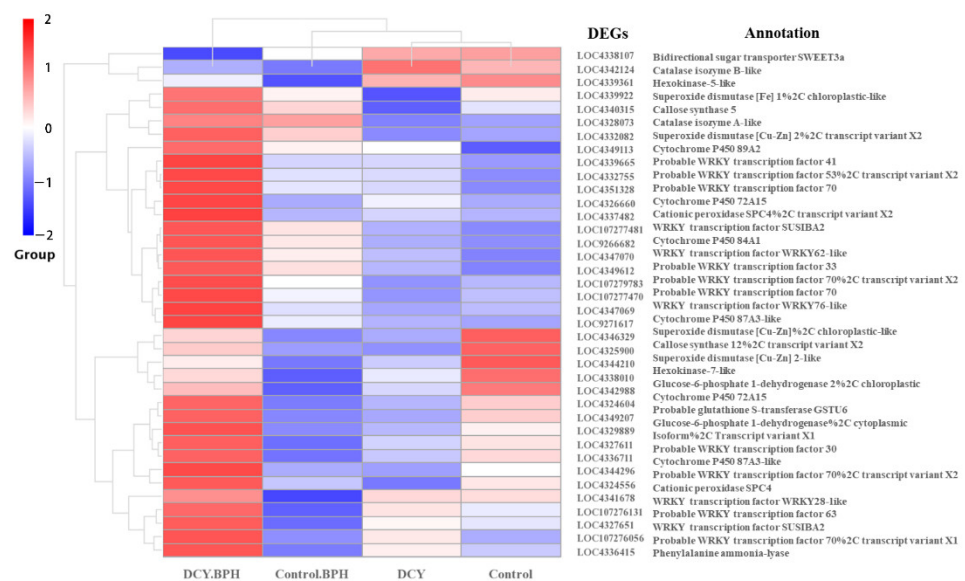

Figure 7. Fold change patterns of representative pathway genes. Each column shows a comparison and each row represents a gene. Colors represent fold-change values. Red represents upregulation, and blue represents downregulation.

3.9. Validation of DEGs by qRT-PCR

A total of 16 DEGs were selected to validate the data from the RNA-Seq analyses. These 16 genes included four WRKY transcription factor genes (LOC4332755, LOC107277481, LOC4347070, and LOC4347069), four cytochrome P450 genes (LOC9271617, LOC4326660, LOC4349113, and LOC4324604), two catalase genes (LOC4328073 and LOC4342124), a superoxide dismutase gene (LOC4346329), a peroxidase gene (LOC4324556), a glutathione s-transferase gene (LOC4349207), a callose synthase gene (LOC4340315), a phenylalanine ammonia-lyase gene (LOC4336415), and a glucose-6-phosphate 1-dehydrogenase gene (LOC4329889). The qRT-PCR analysis validated the expression trends of 16 DEGs shown by the RNA-seq analysis (Figure 8).
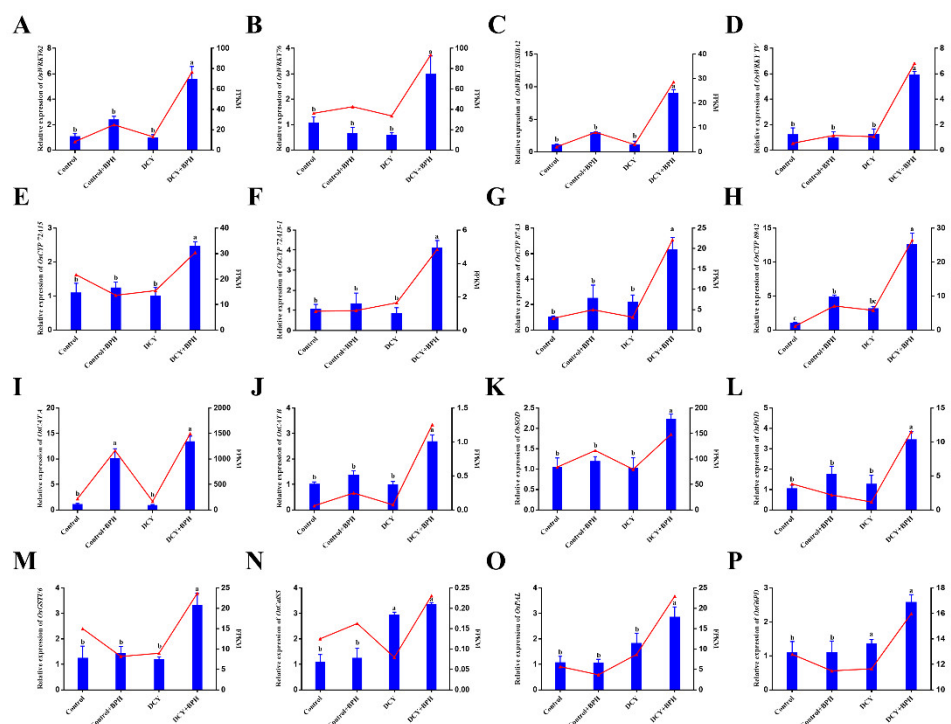
Figure 8. qRT-PCR verification of DEGs. The relative expression level of sixteen genes was determined by qRT-PCR and compared with the transcriptome results. (**A**). OsWRKY62, WRKY transcription factor WRKY62 (LOC4347070). (**B**). OsWRKY76, WRKY transcription factor WRKY76 (LOC4347069). (**C**). OsWRKY SUSIBA2, WRKY transcription factor SUSIBA2 (LOC107277481). (**D**). OsWRKY TV, WRKY transcription factor 53%2C transcript variant X2 (LOC4332755). (**E**). OsCYP 72A15, cytochrome P450 72A15 (LOC4326660). (**F**). OsCYP 72A15-1, cytochrome P450 72A15 (LOC4324604). (**G**). OsCYP 87A3, cytochrome P450 87A3 (LOC9271617). (**H**). OsCYP 89A2, cytochrome P450 89A2 (LOC4349113). (**I**). OsCAT A, catalase isozyme A (LOC4328073). (**J**). OsCAT B, catalase isozyme B (LOC4342124). (**K**). OsSOD, superoxide dismutase (LOC4346329). (**L**). OsPOD, cationic peroxidase (LOC4324556). (**M**). OsGSTU6, glutathione S-transferase GSTU6 (LOC4349207). (**N**). OsCalS5, callose synthase 5 (LOC4340315). (**O**). OsPAL, phenylalanine ammonia-lyase (LOC4336415). (**P**). OsG6PD, glucose-6-phosphate 1-dehydrogenase (LOC4329889).

4. Discussion

Biological control is a promising alternative to the use of synthetic insecticides. Particularly, microbial pesticides have peculiar advantages because they have a unique mode of action. In this study, we investigated the physiological and molecular mechanism of rice priming with DCY responding to BPH.

Rice plants have developed sophisticated defense systems against BPH in the long-term evolutionary arms race between the two. Rice can defend against BPH attacks through the production of various defense proteins or chemicals, which reduce the digestion and absorption of nutrients by insects [28,40,41]. Results showed that DCY treatment

significantly influenced a series of physiological indexes including MDA, H_2O_2, CAT, SOD, POD, PPO, soluble sugar, sucrose, callose, and flavonoid in rice plants in response to BPH infestation. Rice plants activate intricate networks of signaling cascades associated with the generation of ROS, such as superoxide anion, hydroxyl radical, and H_2O_2 in response to herbivores [42]. The second messenger, H_2O_2, can stimulate a cascade of reactions that lead to the expression of defense genes in plants, protecting the plants from herbivores via triggering chloroplast, peroxisome autophagy, and programmed cell death [43]. Our results have proved that H_2O_2 contents were significantly increased in DCY + BPH rice plants. High levels of lipid peroxidation caused by ROS can result in increased MDA; however, excessive MDA can damage the cell membrane and cause harm to plant growth and development. [20] Our results showed that DCY inhibited MDA accumulation in rice plants attacked by BPH and thus provided protection for the stressed plants. Excessive ROS also can cause serious damage to cells and tissue in plants. The antioxidant enzymes (CAT, SOD, and POD) play an important role in scavenging excessive ROS. SOD can transform superoxide anion to H_2O_2, while CAT and POD rapidly convert H_2O_2 into water, but they allow lower levels of H_2O_2 to remain so as to maintain signaling pathways [44,45]. The activities of CAT, SOD, and POD in the leaf blade and sheath of wild rice IRGC99577 significantly enhanced the inhibition of BPH [45]. Similarly, the activities of CAT, SOD, and POD in DCY + BPH rice plants significantly increased than in control + BPH plants. These results indicated that DCY played a role in protecting rice plants by priming activities of antioxidant enzymes. PPO catalyzes the oxidation of phenols or polyphenols to form corresponding quinones that can impair nutrient uptake by herbivorous insects [46]. We found that PPO activity was triggered after BPH infestation, which increased remarkably in DCY + BPH rice plants than in control + BPH plants. Therefore, we hypothesized that the priming of secondary metabolic enzymes by DCY might partially explain the increased resistance of rice plants to BPH and the impairment of BPH's fecundity [17]. PAL is involved in the metabolism of phenylpropanes in plants, and L-Phenylalanine decarboxylation results in the formation of a series of secondary metabolites, including phenolic acids, phenolic amines, lignin, and flavonoids [47]. Flavonoids can not only directly participate in the defense against pathogens and insects but also act as signaling molecules to induce the expression of plant defense genes [26]. Our results showed a higher concentration of flavonoid in DCY-treated plants than in control plants after BPH infestation. Callose, a β-1, 3-linked glucan, accumulates at the edge of the sieve hole of rice, and its content is one of the indexes to evaluate the insect-resistant ability of rice [48]. Our results also showed that DCY-treated rice had significantly higher callose content than untreated rice, no matter whether the rice was fed by BPH or not. Higher concentrations of soluble sugar can stimulate herbivores to feed on plants. Especially sucrose, glucose, and dextrose are known as feeding stimulants for BPH [49,50]. However, our results suggested that DCY-treated rice with high sugar content including soluble sugar, sucrose, and glucose after BPH infestation, which confirms the results of previous feeding choice experiments reporting that BPH don't like feed on DCY-treated rice [17]. It also suggests that DCY may act as a potential repellent and thus provide protection to rice plants.

GO functional annotation analysis showed that the most representative grouping was in the glycometabolism, cell wall components, and antioxidant activity. KEGG pathway enrichment analysis showed that phenylpropanoid biosynthesis, starch and sucrose metabolism, glycolysis and glycometabolism, flavonoid biosynthesis, amino acid biosynthesis, flavonoid and flavonol biosynthesis, and tryptophan metabolism are the most representative. These results coincide with the results of the above physiological indexes.

When rice plants were fed by BPH, the DCYB vs. control B transcriptome data showed significant differences in genes related to oxidative stress, the results of previous experiments also confirmed that the activities of defense enzymes (CAT, SOD, POD, and GST) in DCY-treated rice increased after BPH infestation. Meanwhile, these results were further confirmed by the significant upregulation of a series of rice oxidative stress-related genes including cytochrome p450 genes (OsCYP 72A15, OsCYP 72A15-1, OsCYP 87A3,

and OsCYP 89A2), catalase genes (OsCAT A and OsCAT B), superoxide dismutase genes (OsSOD), peroxidase genes (OsPOD), and glutathione s-transferase genes (OsGSTU6). The increase in defense enzyme-related genes and defense enzyme activities in rice is a key link in the defense of rice against biotic or abiotic stresses [51]. The results showed that the accumulation of ROS scavengers protected rice plants from detrimental effects, and the DCY treatment with BPH infestation changed the redox state of the resistance mechanism in rice. The GO functional annotation was representative of the anchored component of the membrane, cell periphery, and cell wall in comparative treatments of DCYB vs. control B. Meanwhile, the higher contents of callose and upregulation of the callose synthase gene (OsCalS5) also support this. Increased callose synthase activity leads to increased callose deposition, resulting in stronger plant defense against insects [25]. In addition, transcriptome data showed that 15 WRKY transcription factor family genes were significantly upregulated in DCY-treated plants with BPH infestation compared with infested control plants (Figure 7). Some genes involved in MAPK cascade-induced plant resistance, such as OsWRKY70, OsWRKY76, OsWRKY62, and OsWRKY30 were significantly up-regulated [52–55]. OsWRKY62 and OsWRKY76 have been reported as negative regulators of defense-related metabolites, providing evidence for an important role of the phenylpropanoid pathway in SA production in rice [52]. Many studies have shown that WRKY transcription factors are involved in the regulation of insect resistance in plants. In the model plant Arabidopsis thaliana, the transcription factors AtWRKY18 and AtWRKY40 protect against *Spodoptera littoralis* by upregulating the dependent COI1 gene and promoting the synthesis of glucosinolates [56,57]. Overexpression of OsWRKY89 in rice induces a waxy deposition on the surface of leaves to protect against the white-backed planthopper, *Sogatella furcifera* [58]. OsWRKY53 gene inhibits herbivorous-induced defense mechanisms through negative feedback regulation of MPK3/MPK6 expression [59]. It also has been reported that the OsWRKY70 gene in rice contributes to the indole-mediated activation of JA-dependent defense systems in plants against insects [60]. Therefore, we hypothesized that DCY may induce and regulate the rice WRKY transcription factor family genes in response to the BPH infestation.

5. Conclusions

In summary, rice priming with DCY could enhance the resistance to BPH by regulating a series of physiological indicators including oxidative stress-related enzymes (CAT, SOD, POD, and GST), secondary metabolites enzymes (PPO and PAL), nutrients (soluble sugars, sucrose, glucose, and free amino acids) and defense-related compounds (H_2O_2, callose, and flavonoid). Meanwhile, transcriptome data also showed that the genes of the related pathway were up-regulated. In addition, the function of WRKY transcription factor family genes may improve DCY-treated rice resistance to biotic stress. Our data will increase the understanding of the potential mechanism in defense responses of rice priming with DCY against insects provide important basic knowledge for insights into the molecular mechanisms, and can also promote the development of DCY as a new biological pesticide to control pests.

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