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Transcriptome-Based Identification and Characterization of Genes Associated with Resistance to Beta-Cypermethrin in *Rhopalosiphum padi* (Hemiptera: Aphididae)

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Abstract: Beta-cypermethrin is one of the most widely used pyrethroid insecticides. However, its continuous and extensive use developed pests' resistance to beta-cypermethrin. To identify candidate genes for potential resistance phenotypes and facilitate more targeted studies, we screened out a wide range of resistance-related genes by comparing multiple transcripts before and after the induction of multiple agents. In this study, transcriptomes were compared to elucidate the mechanisms and genetic basis of potential resistance between *Rhopalosiphum padi* (*R. padi*) sensitive (SS) and resistant (Beta-R) strains to beta-cypermethrin (resistance ratio: 4588.48). These two strains of aphids were treated with a spray solution of lethal beta-cypermethrin concentration (i.e., LC_{50}). To obtain diverse transcripts, we obtained 17,985,440–25,478,353 clean data from different transcript groups, of which 17,183 genes were annotated. Subsequently, these transcripts were divided into multiple groups for comparison purposes to obtain more comprehensive genes related to resistance. There were 178 to 2856 differentially expressed genes (DEGs) in these transcript groups. The DEGs, including the enriched ones, were classified according to the GO and KEGG Pathway databases. Besides, some drug-resistant DEGs were related to cuticle proteins and detoxification metabolic processes. Among them, 17 genes related to cuticle protein were upregulated and 20 were downregulated, 11 genes related to P450 were upregulated and 25 were downregulated, 7 genes related to UGT were upregulated and 15 were downregulated, 2 genes related to ABC transporter were upregulated and 4 were downregulated, 2 genes related to trypsin were upregulated and 1 were downregulated. Finally, qRT-PCR by DEGs confirmed the observed trend in the RNA sequencing expression profile, and most of the results were consistent between qRT-PCR and RNA sequencing (RNA-seq). The results of this study are highly significant in understanding the resistance phenomenon in *R. padi* and other similar wheat aphids, establishing the valuable basis for further research in the complex mechanism of *R. padi* resistance to beta-cypermethrin.

Keywords: beta-cypermethrin; *Rhopalosiphum padi*; transcriptome comparison; resistance mechanism

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1. Introduction

Due to their relatively low toxicity to humans and high insecticidal efficiency, pyrethroids are among the most widely used insecticides [1]. Beta-cypermethrin is one of the most com-

monly used synthetic pyrethroids to protect crops by reducing pest infestations, thereby minimizing yield losses. However, with the extensive use of pyrethroids, many pests have developed varying degrees of resistance to these insecticides [2,3]: for example, *Spodoptera litura* [4], *Musca domestica* [5], the maize fall armyworm [6] and the picture-winged flies [7]. Pyrethroids have also been used for a long time to control aphids such as *Aphis glycines* [8], *Aphis gossypii* [9] and wheat aphids [10]. However, Gong et al. monitored the resistance of wheat aphids to pyrethroids in 15 provinces of China in the period from 2018 to 2019, finding numerous wheat aphids' moderate or higher resistance to pyrethroids; the most notable among these aphids include *Sitobion avenae* and *Rhopalosiphum padi* [10]. In light of such previous studies, novel research on the resistance mechanism of beta-cypermethrin to pest is highly significant when such a study ensures the rational use of pyrethroids as well as suggesting ways of delaying pests' early resistance to pyrethroids [11].

Resistance to insecticides is a common adaptive strategy. The increased expression of metabolic resistance-related protein family genes and the decreased penetration rate of insect epidermis are two very important resistance mechanisms [12].

Penetration resistance refers to modifications in the cuticle that eventually slows down the penetration of insecticide molecules into insects' body. So far, two mechanisms of penetration resistance have been described: cuticle thickening and altering cuticle composition. Cuticular modifications occur due to the overexpression of diversified genes or exoskeleton proteins such as cuticular proteins, enzymes-catalyzed enzymatic reactions (*CYP4G16* and laccase 2) or ABC transporters that promote cuticular translocation [13]. Many experiments have demonstrated that epidermal penetration is integral to insects' resistance to pyrethroids. For example, *Blattella Germanica*, a beta-cypermethrin-resistant insect, has lower epidermal penetration than that of sensitive strains [14]. According to additional research, cuticular penetration is a significant factor in the development of fenvalerate resistance in common house mosquitoes such as *Culex pipiens pallens* [15].

Insects exhibit pyrethroid-resistance as a result of an increase in the expression levels of multiple metabolic detoxification enzymes. Pyrethroid insecticides are mainly metabolized by the oxidation of methyl, methylene, alkyl and aryl substituents, and the resulting metabolites are hydrolyzed by the corresponding alcohols, phenols, acids and glycine, sulfate, glucuronic acid or glucoside and ester bonds, which can be excreted by insects [11]. Oxidation requires phase I detoxifying enzymes of Cytochromes P450 (P450s) [16], phase II detoxifying enzymes of UDP–glucose–transferase (UGTs) [17] and glutathione–s–transferase (GSTs) [18], and ATP binding cassette transporters (ABCs) [19], while ester bond breaking requires esterases such as carboxyl/cholinesterase (CCEs) [20]. Among them, P450s are the superfamily of detoxifying enzymes, and they play a crucial role in the detoxification of many exogenous substances found in insects [21]. Many related studies have linked high expression levels of the cytochrome P450 gene with pyrethroid resistance in the aphid. For example, P450 plays an important role in pyrethroid resistance in *Aphis gossypii*, *Myzus persicae* and *Aphis glycines* [22–24]. Further studies on the resistance of UGT-related genes to insects are also in progress. For instance, the major phase II detoxification enzymes—Uridine diphosphate (UDP) and –glycosyltransferases (UGTs)—have been implicated in the glycosylation of lipophilic endobiotics and xenobiotics and thus, potentially lead to the evolution of insecticide resistance [25]. Moreover, some UGTs have also been found to be associated with insecticide resistance [26–28].

Rhopalosiphum padi (Hemiptera: Aphididae) (*R. padi)* is among the dominant wheat aphid populations found in wheat [29]. At present, the use of pyrethroids insecticides is the major technique to control the growth and spread of *R. padi* [30]. Thus, it is very necessary to study the resistance of aphids to pyrethroids. We assumed that insect resistance to insecticides might be the result of the co-regulation of multiple genes, and a significant number of genes can be searched more comprehensively through the transcriptome. Therefore, we used multiple transcriptome comparisons to compare the differentially expressed genes in multiple dimensions to expose key insecticide-related resistance genes more easily. For

example, Feng et al. used short-term induction of agents to select resistance-related genes in their insecticide resistance study on *Tetranychus cinnabarinus* [31], and Wang et al. also used short-term induction of agents to obtain one of the transcripts in their insecticide resistance study on *R. padi* [32].

In this study, a highly beta-cypermethrin-resistant *R. padi* population was found in the field while high-throughput RNA-seq was used to determine the transcriptome profile of beta-cypermethrin-resistant (Beta-R) and beta-cypermethrin-sensitive (SS) *R. padi*. We treated the two *R. padi* populations (SS and Beta-R) with a spray solution of lethal beta-cypermethrin concentration (i.e., LC_{50}) to produce different transcripts; then, we compared the transcripts of beta-cypermethrin-sensitive and beta-cypermethrin-resistant strains of *R. padi*, and we also compared the transcripts of beta-cypermethrin-susceptible and -resistant strains of *R. padi* before and after their exposure to beta-cypermethrin. Through this process, effective resistant genes were screened out. In consequence, the results of the study are extremely useful for understanding the beta-cypermethrin resistance mechanism of *R. padi* and developing more effective management strategies.

2. Materials and Methods

2.1. Aphid Strain

For the current study, the *R. padi* population used in the experiments was collected from Shizuishan in Ningxia (NXS) in 2018 (N39°05′57″, E106°44′51″), and the sensitive strain (SS) and beta-cypermethrin resistant strain (Beta-R) were obtained from different single-female populations. After rearing the population for a specific period of time, the lethal beta-cypermethrin concentration (i.e LC_{50}) of the single female population to beta-cypermethrin (95% purity, Beijing Green Agricultural Science and Technology Group Co., Ltd., Beijing, China) was evaluated; this measurement showed the lowest LC_{50} as the sensitive strain (SS), and the sensitive strain was not exposed to any chemical agent during the rearing period. The population with the highest LC_{50} was selected as the primary resistant strain, and selection for the beta-cypermethrin resistance strain was performed via spraying the aqueous solution of beta-cypermethrin once a week with concentrations their current lethal concertation (i.e., LC_{50}) for the pyrethroid insecticide. Meanwhile, the lethal concertation (i.e., LC_{50}) of *R. padi* resistance strain to beta-cypermethrin was evaluated every third generation. The high resistance strain (Beta-R) was established after continuous culture for multiple generations.

2.2. Bioassay

The LC_{50} of the pyrethroids (beta-cypermethrin) was tested with leaf-dip bioassays [33]. Beta-cypermethrin was dissolved in acetone and used to prepare five serial concentrations in water containing 0.1% Tween 80, respectively. Wheat leaves with about 60 apterous adult *R. padi* were dipped into insecticide solutions for 3–5 s; the residual droplets of the solution on the leaves were absorbed by dry filter paper and subsequently placed in a 90 mm diameter plastic Petri dish. The control was treated with water containing 0.1% Tween 80 and 0.1% acetone alone. Three replicates of approximately 60 individuals were used for each insecticide concentration, and 5 serial concentrations were used for each insecticide and a control. The mortality was checked after 24 h. During and after treatment, the aphids were maintained at 22–24 °C with a photoperiod of 16:8 (L:D) h and a relative humidity of 60 ± 10%.

2.3. RNA Isolation

We diluted beta-cypermethrin to the LC_{50} of the sensitive and resistant strains. The sensitive strain (SS) was divided into two groups; one group was treated with SS without any external treatment, while the other group was treated for short-term spray treatment with SS strain LC_{50} (3.22 mg/L) and it was named SS-T. The resistant strain Beta-R was also divided into two groups. One group was treated with Beta-R alone, while the other received a short-term spray treatment with Beta-R strain LC_{50} (14,774.89 mg/L) and was

designated Beta-R-T. Later, the samples (10 wingless adults from each treatment along with four replicates per treatment) were collected after 24 h. Altogether, 16 samples were collected for RNA-seq.

Total RNA was isolated by using RNA Easy Fast Tissue/Cell Kit (Tiangen, Beijing, China). The RNA concentration and purity were measured using a Nano Photometer N50 Touch (IMPLEN, München, Germany). The integrity of RNA was confirmed using Agarose gel electrophoresis.

2.4. Library Preparation and Sequencing

Illumina-based RNA-seq was performed on 4 treatments of *R. padi* and its 4 replicas for each treatment. Based on synthesis and sequencing technology, cDNA libraries were sequenced, and numerous reads were produced. These 'raw reads' are usually provided in FASTQ (FQ) format. To obtain clean reads, the adapter, ploy-N, and low-quality sequences were removed from raw reads. Q score, GC content, and sequence duplication level were calculated to obtain clean, high-quality reads, which were then utilized for all subsequent analyses. The clean reads were mapped to the *R. padi* genome (GenBank no. GCA_020882245.1), and the expression levels of the genes were calculated by using fragments per kilobase per million reads (FPKM) values [34].

2.5. Functional Annotation

KOBAS 2.0 software, and topGO R packages were used to compare selected transcript sequences against two databases: Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.6. Differentially Expressed Genes (DEGs) Analysis and Annotation

We evaluated four transcription profiles: "Beta-R-T compared with Beta-R", "SS-T compared with SS", "Beta-R compared with SS-T", and "Beta-R compared with SS" in order to identify the mechanism of *R. padi* resistance to beta-cypermethrin. Then, these four transcription profiles were divided into two groups for comparative analysis of DEGs. The first group ("Beta-R-T compared with Beta-R", "SS-T compared with SS") was the resistant group and sensitive group; when the aphids were briefly induced by beta-cypermethrin, the changes were observed in the various functions of resistant and sensitive strains. The second group ("Beta-R-T compared with SS-T" and "Beta-R compared with SS") was the beta-cypermethrin-induced group and the non-beta-cypermethrin-induced control group, to locate and screen a large number of drug-resistance genes.

Differential expression analysis was performed, using edgeR [35], to calculate the *p*-value of differential expression. The greater the significance of the gene expression difference, the smaller the *p*-value; hence, genes were identified as differentially expressed genes (DEGs). In order to control the false detection rate (FDR), it is necessary to filter genetic variations using a combination of *p*-value and FoldChange, with the screening condition being *p*-value 0.05 and $\log2 |\text{FoldChange}| > 1$. The functional annotation and classification of the genes were performed through the GO database, while biological pathway annotations were obtained by using the KEGG database.

2.7. Quantitative Real-Time PCR Analysis

Quantitative real-time PCR (qRT-PCR) analysis was conducted to validate the expression profiles of 10 randomly selected DEGs and one reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes [36]. DNAMAN was used to design specific primers to confirm the assembled sequences, followed by the use of a simple technique of reverse transcription PCR (the primers used in qRT-PCR are summarized in Table 1). Quantitative reverse transcription PCR (qRT-PCR) reactions were performed by using the reagent Super-Real PreMix Plus (SYBP Green) (Tiangen, Beijing, China) on a CFX Connect™ Fluorescent quantitative PCR detection system (Bio-Rad, Hercules, CA, USA). All qRT-PCR experiments were performed in triplicate using independent samples. The expression levels were

determined by the $2^{-\triangle\triangle Ct}$ method [37], using the geometric mean of the reference gene for normalization [36].

Table 1. Primers used for qRT-PCR validation of differentially expressed genes.

Gene Name	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
g9940	ACACATCGTTGGGAATCG	GCTCTGTAACCACGCTTTC
g24565	GCAGTTTGCTTTGCTTGC	TTCGGCTGTCTTGTAATCG
g488	CCGCACTTGCCCAATAC	AACCGCTGTGGTCATCG
g19171	TGACGCCACATACGACATC	CGATTCTCTTGGTGCCATC
g11992	GGTGGCCCAAGAGGTTGTATAGG	TGGACACTGAAGTTTCGCAAGAATG
g7371	TAAGGATTTCCCAGCGGA	CGTTTCTCGCCTGTATCAAG
g9824	GTTACAAGTTCGCTGGTTTC	CATTGCTGATGCCTCAAG
g7368	ATCACTACTCGCATCGCAC	AGATTCGGGTTCAACACC
g4263	ACAACAAGCCGTTCCCAAG	ATGGACCTTCGGAGACGAGT
g4506	CCTGTTCTTGGATTCCCTC	TGACATCGGTCGGTCTTT
g16174	TCCAGAGGTTCGTCAATG	GTTCAGAAAGATAACTGCCG
g12284	TGATTCGTCGCACCCAAAC	ACACGCCTTCCGTTGTCTTG
g7359	GTCATCAGGTTCTATCTATGGC	TCAGTGTGCCTTCCAAAC
g8765	TCCAGAGACATAGCGTTGC	AGACCACACACTGACTGACG
GAPDH(reference gene)	GCTCCATTAGCCAAGGTTATTC	CAGCACCTCTACCATCTCTCC

3. Results

3.1. Bioassay

As can be seen from Table 2, the LC_{50} of *R. padi* to SS was 3.22 mg/L of indoor. At the same time, the LC_{50} of *R. padi* to Beta-R increased continuously after continuous agent selection. At the 24th generation, the resistant strain reached 14,774.89 mg/L, and the resistance ratio was 4588.48.

Table 2. Sensitivity of resistant and sensitive strains of *R. padi* to beta-cypermethrin.

Strain	Generation [1]	LC_{50} (95%Cl; mg/L) [2]	RR [3]
SS	-	3.22 (1.97–5.26)	-
Beta-R	G24	14,774.89 (5808.17–37,603.98)	4588.48

[1] Generation number of spraying tide for *R. padi* resistant strains. [2] Median lethal concentration (LC_{50}), 95% confidence interval (95%CI). [3] Resistance ratio (RR).

3.2. Transcriptome Data Analysis

Illumina sequencing data are shown in Table 3. Following filtration, 22,191,059 to 25,478,353 clean readings were obtained from the SS strain, 19,307,241 to 22,881 clean readings were obtained from the Beta-R strain, 20,228,267 to 26,040 clean readings were obtained from the SS-T strain, and 17,985,440 to 22,214,579 clean readings were obtained from the Beta-R-T strain. In the Digital Gene Expression (DGE) library, clean readings' Q30 percentage and GC content ranged from 92.75% to 98.92% and 35.73% to 39.52%, respectively. On average, 77.51% of the clean reading was drawn to the *R. padi* genome database (https://bipaa.genouest.org/sp/rhopalosiphum_padi/analysis/16, accessed on 8 January 2022). Statistics were made on the distribution of reads compared to genomes in the samples (Figure 1). The distribution in the Exon region and the Intron region of the comparison sequences ranged from 84.4 to 91.9% and 3.6 to 7.1, respectively. Distribution in the Intergenic region was between 4.5% and 10.7%.

Table 3. Summary of Illumina RNA-sequencing data.

Samples [1]	Raw Reads [2]	Clean Reads [3]	Q30 (%) [4]	GC Content (%) [5]	Total Mapped [6]	Mapped Ratio (%) [7]
SS-1	25,645,404	25,388,540	92.75	38.53	25,388,540	96.99
SS-2	25,744,355	25,439,416	94.81	39.17	25,439,416	95.81
SS-3	25,802,229	25,478,353	94.75	38.64	25,478,353	95.79
SS-4	22,427,557	22,191,059	95.58	39.19	22,191,059	97.43
Beta-R-1	19,606,609	19,307,241	98.15	38.47	42,874,386	73.73
Beta-R-2	23,305,059	22,881,481	98.23	36.95	22,881,481	48.97
Beta-R-3	19,873,732	19,350,373	98.91	37.11	19,350,373	45.28
Beta-R-4	19,816,890	19,333,166	98.18	38.67	19,333,166	64.92
SS-T-1	20,228,267	19,995,591	95.81	39.52	19,995,591	97.38
SS-T-2	26,040,187	25,614,225	95.97	38.83	25,614,225	97.47
SS-T-3	25,027,865	24,448,408	95.72	39.14	24,448,408	97.24
SS-T-4	20,847,432	20,497,212	95.87	39.11	20,497,212	97.40
Beta-R-T-1	17,985,440	17,587,297	98.81	35.73	38,741,343	48.04
Beta-R-T-2	21,079,387	20,600,348	98.92	37.03	20,600,348	28.55
Beta-R-T-3	24,195,778	23,528,216	98.21	38.02	23,528,216	74.14
Beta-R-T-4	22,214,579	21,623,496	98.82	38.98	21,623,496	81.06

[1] Sample name: there were four groups of samples, SS, Beta-R, SS-T, and Beta-R-T, with four replicates in each group. SS was a *R. padi*-sensitive strain not induced by beta-cypermethrin, Beta-R was a *R. padi*-resistant strain not induced by beta-cypermethrin, and SS-T was a *R. padi*-sensitive strain induced by beta-cypermethrin. Beta-R-T was a *R. padi*-resistant strain induced by beta-cypermethrin. [2] The number of sequenced Raw reads. [3] The number of Clean reads obtained after filtering. [4] The percentage of bases with Phred values greater than 30 in the total base population. [5] G/C bases as a percentage of the total number of bases. [6] Total number of filtered Clean reads. [7] The proportion of the genome that is compared.

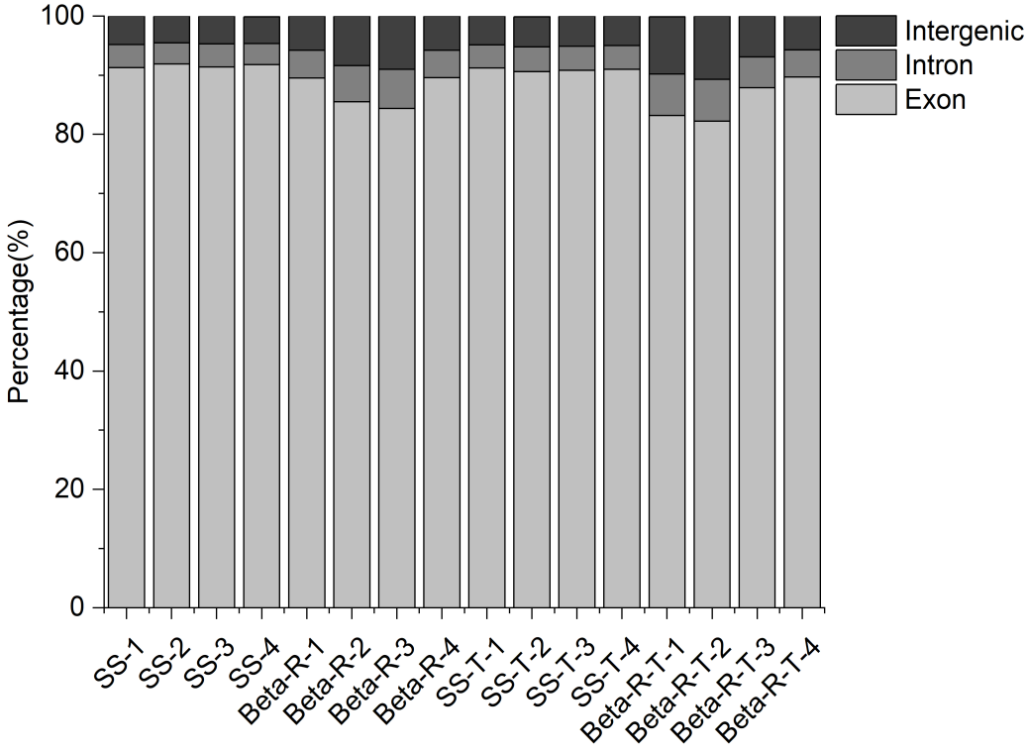

Figure 1. Distribution of unique mapping reads in exon, intron, and intergenic regions. SS was the *R. padi*-sensitive strain transcriptome without beta-cypermethrin induction, Beta-R was the *R. padi*-resistant strain transcriptome without beta-cypermethrin induction, and SS-T was the *R. padi*-sensitive strain transcriptome with beta-cypermethrin induction. Beta-R-T was the *R. padi*-sensitive strain transcriptome with beta-cypermethrin induction, with four replicates in each group.

3.3. Functional Annotation and Classification

Annotation results show that 17,183 (64.76%) of the 26,535 total unigenes were annotated against different databases. For different databases, 10,775 (40.61%) and 6408 (24.15%) unigenes were annotated in the GO and KEGG databases, respectively. Gene ontology (GO)

showed that 10,775 single genes were divided into 3706 subclasses, of which 2157 (58.20%) were biological process (BP) items, 276 (7.45%) were cellular component (CC), and (MF) 1273 (34.35%) were molecular function (Supplementary File S1). Most of the unigenes in the BP category were presumed to be the oxidation-reduction process (644), regulation of transcription, DNA templated (471), and proteolysis (423). The unigenes of the CC category were presumed to be an integral component of the membrane (3090), nucleus (904), and membrane (541). Those in the MF category were presumed to be nucleic acid binding (1479), ATP binding (1051), and Zinc Ion binding (749) (Figure 2A, Supplementary File S1). In the KEGG mapped results, the pathways were divided into six main categories; among these, genetic information processing (2598), metabolism (1597), and signaling and cellular processes (1113) accounted for the top three (Figure 2B, Supplementary File S2).

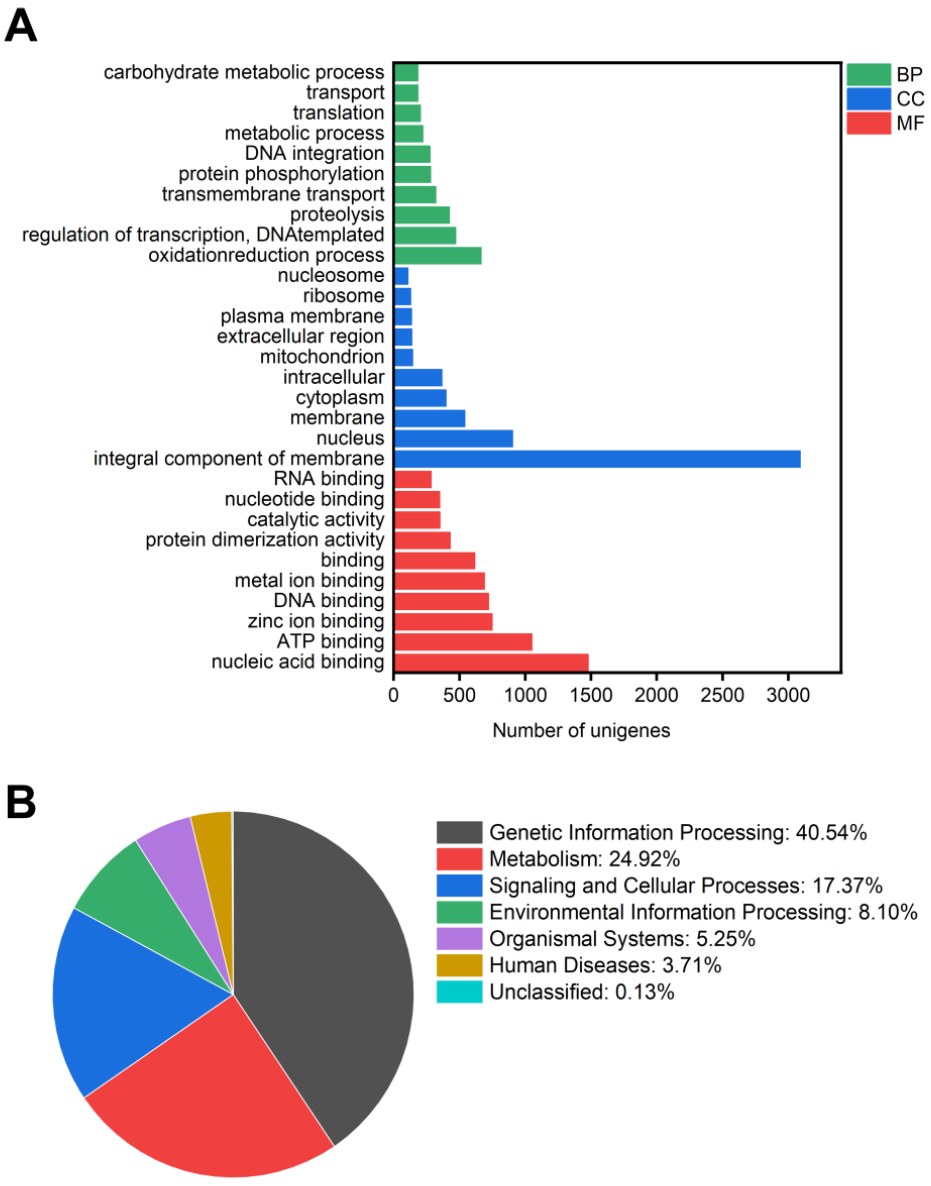

Figure 2. Results of unigenes against gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database. (**A**) Gene ontology (GO) annotation and classification of the *Rhopalosiphum padi* transcriptome. BP: biological process, CC: Cellular component, MF: Molecular Function. (**B**) Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation and pathways of the *R. padi* transcriptome.

3.4. Differentially Expressed Genes Analysis

Based on the *p*-value and log2 |Foldchange| value, DEGs of insecticide treatment samples and contrast samples identification comparison results are presented in Table 3. The number of DEGs obtained was different in different treatments. The least number of DEGs was obtained when resistant strains were induced and resistant strains were not induced (Beta-R-T compared with Beta-R) (178). The greatest number of DEGs was obtained when resistant strains were not induced. Meanwhile, sensitive strains were not induced (Beta-R compared with SS) (2856) (Table 4, Figure S1, Supplementary File S3).

Table 4. The number of upregulated and downregulated DEGs in each sample was compared.

Compare	Up	Down
Beta-R-T vs. Beta-R [1]	147	31
SS-T vs. SS [2]	584	297
Beta-R-T vs. SS-T [3]	1518	930
Beta-R vs. SS [4]	1758	1098

[1] DEGs obtained by comparing the transcriptional profiles of resistant *R. padi* strains were induced by beta-cypermethrin with those of resistant *R. padi* strains not induced by beta-cypermethrin. [2] DEGs obtained by comparing the transcriptional profiles of sensitive *R. padi* strains were induced by beta-cypermethrin with those of sensitive *R. padi* strains not induced by beta-cypermethrin. [3] DEGs obtained by comparing the transcriptional profiles of resistant *R. padi* strains were induced by beta-cypermethrin with those of sensitive *R. padi* strains induced by beta-cypermethrin. [4] DEGs obtained by comparing the transcriptional profiles of resistant *R. padi* strains were not induced by beta-cypermethrin with those of sensitive *R. padi* strains not induced by beta-cypermethrin.

3.5. GO Classification and KEGG Pathway Identification of DEGs

We analyzed the 178 annotated DEGs in Beta-R-T compared with the Beta-R group and 881 annotated DEGs in the SS-T compared with SS group; and 64 DEGs were found in the two beta-cypermethrin-treated groups; 114 DEGs were unique to the Beta-R strain, and 817 DEGs were unique to the SS strain (Figure 3). Based on results obtained using GO annotation and KEGG pathway analysis for the specific genes in the different groups, we found that the functional classification of DEGs was very different between them. The number of DEGs in the SS experimental group was greater than that in the Beta-R experimental group for GO terms.

Under the induction of beta-cypermethrin, in the Beta-R-T compared with the Beta-R group, a total of 73 DEGs were found to be annotated in 541 GO terms. The 762 DEGs, found in the SS strain experimental group (SS-T compared with SS), were mapped with 1881 GO terms (Supplementary File S4). Later, the 64 GO terms were shared with the DEGs of the two transcriptome comparison groups. Comparing the log2 Fold Change of 64 common genes between the two groups, we found that the expression levels of most genes in the resistant group were higher than those in the sensitive group (Supplementary File S5). Additionally, one unique GO term (structural constituent of cuticle) was found among the DEGs of the Beta-R experimental group. The change of cuticle structure may be the reason for the resistance of *R. padi* to beta-cypermethrin (Figure S2, GO terms were sorted by *p*-values from small to large, Supplementary File S4).

When the Beta-R-T group was compared with the Beta-R group, a total of 21 DEGs were annotated into 45 KEGG pathways. The 160 DEGs, found in the SS strain experimental group (SS-T compared with SS), were mapped with 194 KEGG pathways; and the number of DEGs in the sensitive group was the largest. As the bubble chart indicates, most of the top pathways belong to the categories of metabolism and organismal systems among which typical pathways include "Ascorbate and aldarate metabolism", "Starch and sucrose metabolism", and "Pentose and glucuronate interconversions". This indicates that the SS strain has a great impact when stimulated by chemical agents, and more genes are activated to synthesize energy and metabolize energy to help worms survive the crisis. In addition, the pathway annotations of the resistance and sensitive groups indicate that the pathways are marked with drug metabolism. There is also an ABC transporters pathway

in the resistance group that seems responsible for *R. padi*'s resistance. The mechanism is worthy of further study in this context (Figure 4, Supplementary File S6).

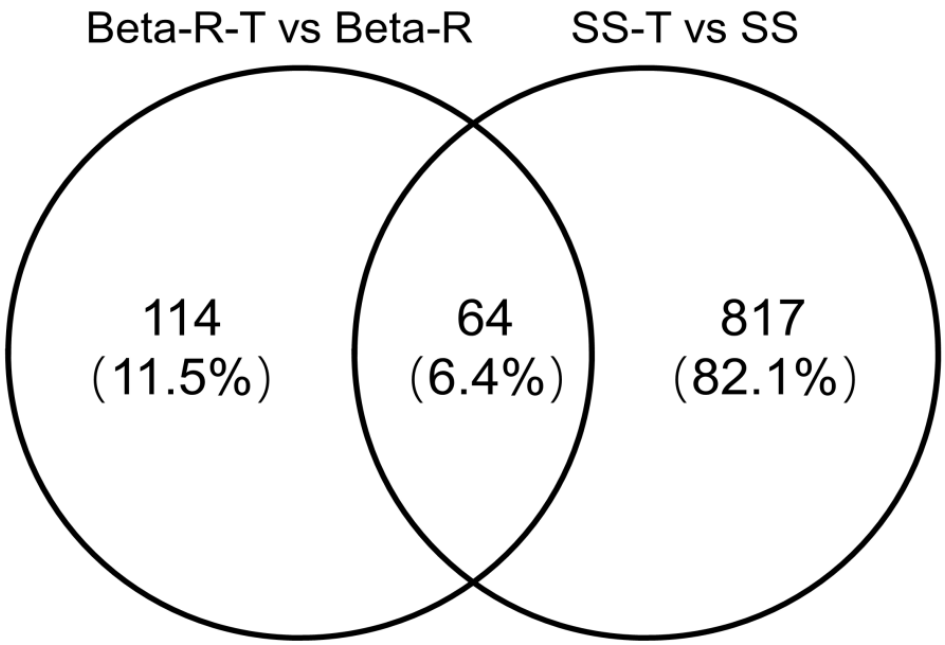

Figure 3. Venn map of differentially expressed genes (DEGs) in Beta-R-T vs. Beta-R group and SS-T vs. SS group. Beta-R-T vs. Beta-R: DEGs obtained by comparing the transcriptional profiles of resistant *R. padi* strains were induced by beta-cypermethrin with those of resistant *R. padi* strains not induced by beta-cypermethrin. SS-T vs. SS: DEGs obtained by comparing the transcriptional profiles of sensitive *R. padi* strains were induced by beta-cypermethrin with those of sensitive *R. padi* strains not induced by beta-cypermethrin.

3.6. Analysis of DEGs Associated with Drug Resistance and qRT-PCR Validation of DEGs

The insecticide-treated DEGs and control samples, which were considered to be insecticide tolerance-related genes, are listed in Table 5 and Supplementary File S7. Using Beta-R-T vs. SS-t, Beta-R vs. SS transcripts as the standard, Beta-R-T vs. Beta-R vs. SS transcripts were compared for all the DEGs associated with drug resistance in the auxiliary. We found that 17 out of 37 genes associated with cuticle protein were upregulated and 20 were downregulated; 11 cytochrome P450-related genes were upregulated while 25 were downregulated; 7 UDP-glycosyl transferase (UGT)-related genes were upregulated and 15 were downregulated (Supplementary File S7). We further found that there were 6 ABC transporter-related genes, of which 2 were upregulated and 4 were downregulated, and 3 trypsin-related genes, of which 2 were upregulated and 1 was downregulated (Supplementary File S7). Overall, we found that the total number of downregulated genes was more than the number of upregulated genes, and the number of DEGs, associated with insecticide tolerance in the "Beta-R vs. SS" group, was the highest, followed by cuticle protein, cytochrome P450, and UGTs. Lastly, four cuticle proteins, four cytochrome P450s, four UGTs, one ABC transporter, and one trypsin-related DEG were selected for qRT-PCR verification (Table 6).

Finally, we performed a qRT-PCR analysis of 14 DEGs associated with drug resistance and confirmed the expression trends observed in the RNA-Seq results (Figure 5), suggesting that the DEGs profiles were reliable.

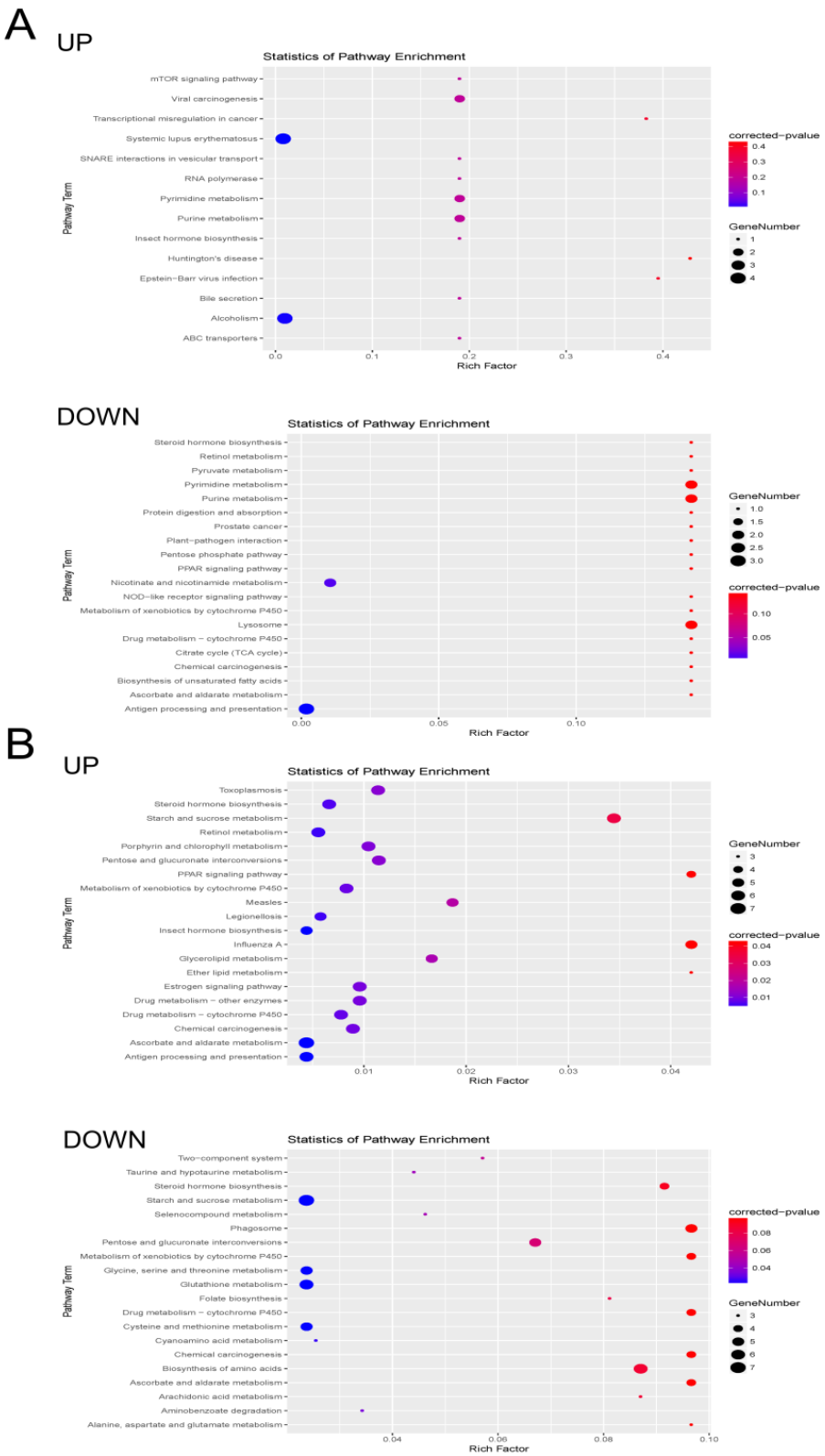

Figure 4. KEGG pathways annotation classification map of DEGs in SS and beta-R groups before and after beta-cypermethrin induction. (**A**) Beta-R-T vs. Beta-R: DEGs obtained by comparing the transcriptional profiles of resistant *R. padi* strains were induced by beta-cypermethrin with those of resistant *R. padi* strains not induced by beta-cypermethrin. (**B**) SS-T vs. SS: DEGs obtained by comparing the transcriptional profiles of sensitive *R. padi* strains were induced by beta-cypermethrin with those of sensitive *R. padi* strains not induced by beta-cypermethrin.

Table 5. Insecticide tolerance-related genes.

	Beta-R-T vs. Beta-R [1]		SS-T vs. SS [2]		Beta-R-T vs. SS-T [3]		Beta-R vs. SS [4]	
	Up	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**
Cuticle protein	4	0	7	2	7	12	11	14
Cytochrome P450	0	0	6	5	4	16	6	11
UDP-glucosyltransferases UGT	0	1	6	7	3	6	5	10
ATP-binding cassette (ABC) transporter	0	0	0	0	1	4	1	1
Trypsin	0	0	0	0	2	0	2	1

[1] DEGs obtained by comparing the transcriptional profiles of resistant *R. padi* strains were induced by beta-cypermethrin with those of resistant *R. padi* strains not induced by beta-cypermethrin. [2] DEGs obtained by comparing the transcriptional profiles of sensitive *R. padi* strains were induced by beta-cypermethrin with those of sensitive *R. padi* strains not induced by beta-cypermethrin. [3] DEGs obtained by comparing the transcriptional profiles of resistant *R. padi* strains were induced by beta-cypermethrin with those of sensitive *R. padi* strains induced by beta-cypermethrin. [4] DEGs obtained by comparing the transcriptional profiles of resistant *R. padi* strains were not induced by beta-cypermethrin with those of sensitive *R. padi* strains not induced by beta-cypermethrin.

Table 6. DEGs associated with insecticide resistance.

Gene ID	Accession Number	Description
g9940	XM_026951046.1	cuticle protein 19-like
g24565	XM_026952080.1	cuticle protein 7-like
g488	XM_027990008.1	cuticle protein 7-like
g19171	XM_026961146.1	cuticle protein-like
g11992	XM_027984718.1	P450 4C1-like
g7371	MW287284.1	cytochrome P450 6CY8 (CYP6CY8)
g9824	MN764890.1	cytochrome P450 CYP6DC1 (CYP6DC1)
g7368	MK534505.1	cytochrome P450 CYP6CZ1 (CYP6CZ1)
g4263	XM_027994410.1	UDP-glucuronosyltransferase 2C1-like
g4506	XM_026960509.1	UDP-glucuronosyltransferase 2B2-like
g16174	XM_026962565.2	UDP-glucuronosyltransferase 2C1-like
g12284	XM_026954189.1	UDP-glucuronosyltransferase-like
g7359	XM_026956515.1	ABC transporter G family member 23-like
g8765	XM_026949522.1	trypsin-1-like

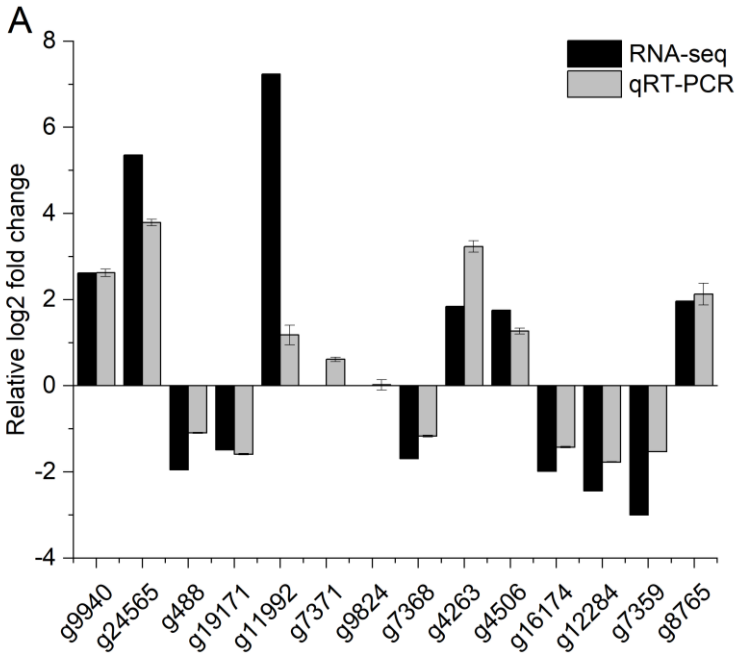

Figure 5. *Cont.*

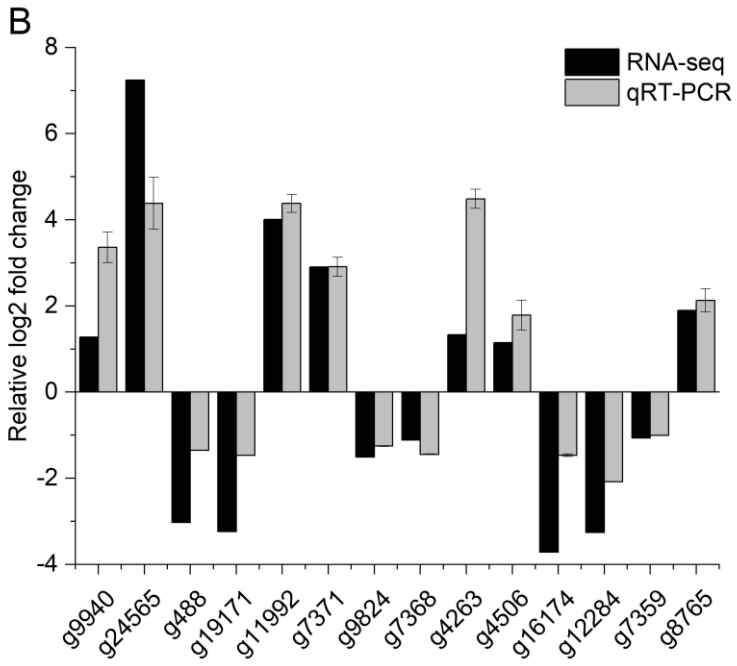

Figure 5. Quantitative real-time PCR (qRT-PCR) validation of the expression of differentially expressed genes identified using RNA sequencing. The expression levels were normalized to GAPDH genes. (**A**) Beta-R-T vs. SS-T: DEGs obtained by comparing the transcriptional profiles of resistant *R. padi* strains were induced by beta-cypermethrin with those of sensitive *R. padi* strains induced by beta-cypermethrin. (**B**) Beta-R vs. SS: DEGs obtained by comparing the transcriptional profiles of resistant *R. padi* strains were not induced by beta-cypermethrin with those of sensitive *R. padi* strains not induced by beta-cypermethrin.

4. Discussion

A common approach in studying the physiological adaptations that lead to insect resistance to insecticides is to compare the transcriptome of resistant strains to that of sensitive strains [38]. In our transcriptome experiment, to screen more comprehensive resistance-related genes, we treated the existing *R. padi*'s beta-cypermethrin resistant and sensitive strains with short-term agent treatment, respectively, to obtain drug-induced strains of resistant and sensitive strains.

Firstly, we conducted expression analysis and functional enrichment of GO and KEGG by comparing the two transcriptomes of DEGs before and after induction of antagonistic strains and sensitive strains. In this way, it is easier to expose the rapid changes in expression and function of resistance genes after encountering agents to identify the functions that lead to aphid resistance to insecticides. We found that the DEGs of the inducible resistant strain and non-inducible resistant strain treatment comparison groups were significantly less in number than those in the inducible sensitive strain treatment and non-inducible sensitive strain treatment comparison group. Furthermore, almost no DEGs, related to resistance, appeared in the inducible resistance strain treatment and non-inducible resistance strain comparison groups. This result contradicts Wang's obtained results, where the number of differentially expressed genes in resistant strains was higher than that in sensitive strains [32]. However, our research found that the expression level of common genes in the resistant groups was significantly higher than sensitive groups. This phenomenon requires further study of the specific reasons and real mechanisms. The functional annotation showed that these genes had different functions, most of which were related to the enhanced activity of detoxification enzymes and structural changes of cuticle proteins.

We then conducted two transcriptome comparisons between uninduced resistant and sensitive strains, and between induced resistant and sensitive strains. We found a large number of resistance-related genes. We found that most DEGs appeared in the comparison

of two transcriptomes between non-induced resistant strains and sensitive strains and induced resistant strains and sensitive strains, and the expression levels of some genes changed in the multi-group comparison, and the expression levels changed in the same trend. For example, regarding the cuticle protein gene g9944 and UGT gene g16174, this kind of gene should be studied as the key gene of insecticide resistance. We also found a large number of downregulated genes, which may be a balance to compensate for the fitness cost. This phenomenon is very interesting and requires further research.

The findings could indicate that the mechanism of drug resistance in *R. padi* is complex, resulting from the joint action of multiple resistance mechanisms. From the contact of the drug with the epidermal protein of the insect body to the digestion of trypsin after entering the insect body, to the final function of the detoxification enzyme gene oxidation hydrolysis upon exiting the body, the process is rigorous and scientific.

The cuticle protein exhibits unique properties and noticeable changes during all kinds of comparisons. The insect cuticle is the first line of defense against external chemicals, specifically the proteinaceous and chitinous procuticle [39]. Cuticular protein (CP) genes play an important role in insect development and defense [39]. As such, cuticular proteins have been reported to play crucial roles in the insecticide resistance and tolerance of a variety of insect species, including *Myzus persicae* [40], *Aphis gossypii* [41], and *Plutella xylostella* [42]. Our results demonstrated that epidermal protein-related genes were the most numerous and varied among the insecticide-related DEGs, as shown in Table 4. The downregulation of epidermal protein genes, as in this study, conforms with previous studies that reported the downregulation of several cuticle protein genes in the imidacloprid-resistant *Myzus persicae* [43]. The cuticular changes underlying insecticide resistance involve two main parameters: thickness and composition of the cuticle [13]. More significantly, cuticular thickening has been more commonly associated with resistance [44]; but there are recent reports that link cuticular composition to decreased xenobiotic penetration [45]. Therefore, on the basis of the results, it is suggested that cuticular reorganization could play a role in insecticide resistance for *D. citri*, though the specific roles of the CPs involved in alterations of cuticle structure or composition as specifically related to insecticide penetration require further research.

Cytochrome P450 is an enzyme with a variety of metabolic functions, and it is involved in detoxifying foreign chemicals such as pesticides [46]. The overexpression of the P450 monooxygenase enzyme is the most common mechanism of resistance to beta-cypermethrin in many insects [47–49]. In aphids, the P450 monooxygenase enzyme also plays an important role in insecticide detoxification and resistance [46,50,51]. In the present study, different treatments generated different DEGs; among these, the most P450s-related downregulated single genes were generated in the inducible resistant strain treatment and inducible sensitive strain treatment comparison group (17). Most of the P450-related upregulated single genes were generated in non-inducible resistant strain treatment and non-inducible sensitive strain treatment comparison group (6) (most of which were labelled as CYP4 and CYP6 family genes, as presented in Supplementary File S8). Both genes (CYP4 and CYP6) were more associated with pesticides than any other P450 family, suggesting that these genes play an important role in pesticide metabolism and detoxification [52,53]. Therefore, overexpression of these two genes may be related to the resistance and detoxification process of *R. padi* to beta-cypermethrin. For example, the CYP4 family gene of *Aedes albopictus* plays an important role in pyrethroid detoxification [54]. In houseflies, the CYP6 family gene was overexpressed in pyrethroid-resistant populations, which proved to be related to the pyrethroid resistance of houseflies [55]. Similarly, transcriptional overexpression of CYP6 family genes, associated with pyrethroid resistance, was also found in *Helicoverpa zea* as collected from the field [56]. Although our results demonstrated that overexpression of CYP4 and CYP6 family genes is associated with the detoxification of beta-cypermethrin in *R. padi*, further research is required to determine whether these P450s can metabolize imidacloprid and chlorpyrifos.

UDP-glucosyltransferases (UGTs) are detoxification enzymes that are involved in insecticide metabolism [57]. The phase II enzymes, UGTs act on the toxic by-products of the phase I metabolism; UGT-catalyzed conjugation of small lipophilic compounds with sugars is an important detoxification and homeostatic function in all living organisms [58]. For example, in thiamethoxam-resistant strains of *A. gossypii*, UGT was significantly upregulated relative to the susceptible strains [59], and UGTs were also implicated in the resistance of *Meteorus pulchricornis* to commonly used insecticides [60]. In our experiment, we used qRT-PCR to verify the expression levels of UGTs genes (g4263 g15391, g13098, g16174) that were differentially expressed in transcriptome data; it suggests the involvement of UGTs in beta-cypermethrin metabolism and transport. In the qRT-PCR assay of the annotated UGT detoxification enzymes, we found that g4263 expression was highly upregulated by the qRT-PCR assay of the UGT detoxification enzyme. UGTs play an important role in a variety of physiological and biochemical processes in insects, including the detoxification of substrates (such as plant allelochemicals and insecticides) [61,62]; our results indicated that UGTs may play a role in the tolerance and detoxification of insecticides.

ATP-binding cassette (ABC) transporters are a family of transmembrane proteins that mainly rely on ATP to generate energy for substrates. Here, ABC transporters, as the phase III detoxification enzymes, play a vital role in the detoxification and defense of various tissues and organs of the insect [63,64]. ABC transporters have been associated with pyrethroids resistance in insects [65]. In this study, insecticide-resistant DEGs were associated with ABC transporters, and most were downregulated single genes. Thus, the ABC transporter may play an important role in the detoxification process and insecticide tolerance of *R. padi.* In addition, trypsin-related genes, which accounted for a large group of DEGs in *S. avenae* [66], were upregulated in *Caligus rogercresseyi*, following exposure to deltamethrin [67]. In our study, trypsin DEGs were also found to be related to pyrethroid resistance, indicating that the trypsin enzyme is vital in the detoxification process and insecticide tolerance of *R. padi*.

Through this experiment, we found a large number of genes related to insecticide resistance, and we should screen out the key genes of resistance for further study in the follow-up experiments. We found that metabolites are the direct defense line of insects against the harm of drugs and can directly respond to the attack of drugs, to enhance insect resistance. Thus, metabolome analysis can assist transcriptome analysis to more accurately identify key genes associated with resistance [68]. Therefore, metabolism-related experiments should be included in subsequent experiments to assist in verifying the results of transcriptome analysis.

5. Conclusions

This study used RNA-seq to describe genes associated with beta-cypermethrin resistance in *R. padi*. The results indicate that aphid response patterns, during the development of beta-cypermethrin resistance, are complex as evidenced by changes observed in epidermal structure, binding, metabolic processes, and expression of genes associated with oxidative phosphorylation. Additional research is required to evaluate the specific role of these genes in the response of *R. padi* to pesticide exposure stress. As carried out in the current study, transcriptome analysis reveals resistance and analyses insecticide-related genes by transcriptome map; and it provides updated ideas regarding the resistance of *R. padi*. Moreover, understanding how insects develop resistance to insecticides and evaluating the related mechanisms of insecticide tolerance are useful steps for preventing and delaying the development of insecticide resistance in insects.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13020235/s1, File S1: Functional items annotated to genes in the GO database and the number of genes enriched for each item, File S2: Genes annotated to the KEGG database for functional classification and the number of genes enriched to that function, File S3: DEGs up- or downregulated in response to different treatment, File S4: Comparison of resistance group and sensitive group, File S5: DEGS GO enrichment in resistant and sensitive groups, File S6: DEGS KEGG enrichment in resistant and sensitive groups, File S7: Screening of different resistance-related genes, File S8: P450 DEGs family classification. Figure S1. Differential expression unigene (DEGs) analysis of beta-cypermethrin-high-resistant and beta-cypermethrin-susceptible *R. padi*. (A) Beta-R-T vs. Beta-R: The *R. padi*-resistant strains induced by beta-cypermethrin were compared with the *R. padi*-resistant strains not induced by beta-cypermethrin, (B) SS-T vs. SS: The *R. padi*-sensitive strains induced by beta-cypermethrin were compared with the *R. padi*-sensitive strains not induced by beta-cypermethrin, (C) Beta-R-T vs. SS-T: The *R. padi*-resistant strains induced by beta-cypermethrin were compared with the *R. padi*-sensitive strains induced by beta-cypermethrin, (D) Beta-R vs. SS: The *R. padi*-resistant strains not induced by beta-cypermethrin were compared with the *R. padi*-sensitive strains not induced by beta-cypermethrin. (a) Volcanic map of DEGs. Dots represent individual genes. Red dots represent upregulated genes and green dots represent downregulated genes. The blue dots indicate genes that are not differentially expressed. (b) Heat map analysis of DEGs hierarchical clustering. Red and blue indicate high and low expression in beta-cypermethrin resistant strains, respectively. The colors range from dark red to dark blue, indicating the size of the log2FPKM value. Figure S2. GO annotation classification map of DEGs in SS and beta-R groups before and after beta-cypermethrin induction. (A) Beta-R-T vs. Beta-R: The *R. padi*-resistant strains induced by beta-cypermethrin were compared with the *R. padi*-resistant strains not induced by beta-cypermethrin, (B) SS-T vs. SS: The *R. padi*-sensitive strains induced by beta-cypermethrin were compared with the *R. padi*-sensitive strains not induced by beta-cypermethrin. GO terms are sorted by *p*-values from small to large.

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