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Article

Transcriptome Profiling Analysis of Wolf Spider *Pardosa pseudoannulata* (Araneae: Lycosidae) after Cadmium Exposure

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Abstract: *Pardosa pseudoannulata* is one of the most common wandering spiders in agricultural fields and a potentially good bioindicator for heavy metal contamination. However, little is known about the mechanism by which spiders respond to heavy metals at the molecular level. In this study, high-throughput transcriptome sequencing was employed to characterize the *de novo* transcriptome of the spiders and to identify differentially expressed genes (DEGs) after cadmium exposure. We obtained 60,489 assembled unigenes, 18,773 of which were annotated in the public databases. Ultimately, 3450 cDNA simple sequence repeats were identified and validated as potential molecular markers in the unigenes. A total of 2939, 2491 and 3759 DEGs were detected among the three libraries of two Cd-treated groups and the control. Functional enrichment analysis revealed that metabolism processes and digestive system function were predominately enriched in response to Cd stress. At the cellular and molecular levels, significantly enriched pathways in lysosomes and phagosomes as well as replication, recombination and repair demonstrated that oxidative damage resulted from Cd exposure. Based on the selected DEGs, certain critical genes involved in defence and detoxification were analysed. These results may elucidate the molecular mechanism underlying spiders' responses to heavy metal stress.

Keywords: *P. pseudoannulata*; Cadmium; Transcriptome; RT-qPCR

1. Introduction

Cadmium (Cd) is one of the most abundant, ubiquitous, toxic heavy metal elements in the environment [1]. In particular, many agricultural soils are significantly influenced by Cd derived from anthropogenic activities in many developing country, such as China [2,3]. Cd can be absorbed by plant, and via food web accumulated in phytophagous insects and their predators [4,5], thus presenting a serious threat to ecosystem and human health. But the diseases resulted from the long-term exposure to the sub-lethal concentration of heavy metals are difficult to be diagnosed timely, such as Itai-itai and minamata diseases in Japan [6]. Therefore, how to monitor Cd pollution and assess its toxicological effects to organisms and environment has been an important environmental issue.

Many studies have indicated that spiders have the ability to accumulate and withstand high concentrations of Cd [6,7]. Accumulated Cd can affect spiders' biological traits, such as development and reproduction, biochemical and physiological processes [8,9,10]. Additionally, Cd can cause

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oxidative damage by stimulating the formation of free radicals and reactive oxygen species (ROS), resulting in oxidative stress [11], even can display strong genotoxic effects and may cause DNA damage to spiders at low concentrations [12]. There is growing interest to use spiders as heavy metal indicators and ecotoxicological studies [6,13,14]. However, little is known about the mechanism of spider responses to Cd at the molecular level [9,11,15].

In the recent years, the increasing use of high-throughput sequencing has provided us with an efficient and powerful platform to understand the biological mechanisms of arachnids [16,17,18]. Though the genes related to Cd response were widely studied in many vertebrate and invertebrate [19,20,21], only a few genes of spider responding to heavy metals have been reported previously. The wolf spider *Pardosa pseudoannulata* is one of the most common species of wandering spiders in agricultural fields in China, and act as one of the most important natural enemies to reduce pest populations, such as rice plant hoppers and leafhoppers [22]. Our previous study indicated Cd can significantly affect the fitness-related traits and activity of antioxidative enzymes of *P. pseudoannulata* [10]. To further understand the biological basis of *P. pseudoannulata* responding to Cd, there is a need to explore the transcriptomic biology of this spider species following Cd exposure.

The present study aimed to elucidate the molecular mechanisms and the critical genes involved in regulating spider responses to Cd stress. Accordingly, female adult spiders were exposed to 0, 0.2 and 2 mM $CdCl_2$ solutions for 7 days and their respective transcriptomes were compared for the first time，an abundance of differentially expressed Cd responsive genes were analysed. The molecular basis of the response to Cd stress was first comprehensively characterized in spider, and the resulting information would help in furthering our understanding of the toxicological mechanism of Cd, and using spiders as potential bioindicators of heavy metal contamination.

2. Results and Discussion

2.1. Sequence Analysis and De Novo Assembly

To study the mRNA expression dynamics of *P. pseudoannulata* exposed to different concentrations of $CdCl_2$ solution, we constructed and sequenced mRNA-seq libraries from adult female spiders treated with lower (0.2 mM, TL), or higher (2 mM, TH) concentration of Cd, and distilled water as control (TC), respectively. We obtained more than 6.44G high-quality bases with 89.64% Q30 and 42.05% GC content for each sample after removing the dirty reads from the raw reads, an unprecedented sequencing depth. These high-quality reads were *de novo* assembled using the Trinity method, and 60,489 unigenes were obtained with average length of 951bp. The N50 values of these transcripts and unigenes were 1,637 and 1,433 bp，respectively. All unigenes were longer than 300 bp with a length of 300 to 500 bp sequences representing the highest proportion, followed by 500–1000 bp sequences. A total of 16,257 (26.87%) unigenes were longer than 1000 bp, and 6,390 (10.56%) were longer than 2000 bp (Table 1). This large dataset will contribute to the biochemistry analysis of this species as well as identification of important functional genes.

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Table 1. The length distribution of contigs, transcripts and unigenes.

Length range	Contig	Transcript	Unigene
300-300	11,618,302 (99.43%)	11,907 (10.02%)	(0%)
300-500	31,712 (0.27%)	40,345 (33.95%)	27,245 (45.04%)
500-1000	18,712 (0.16%)	31,410 (26.43%)	16,985 (28.08%)
1000-2000	9,958 (0.09%)	20,515 (17.26%)	9,867 (16.31%)
2000 +	6,767 (0.06%)	14,674 (12.35%)	6,390 (10.56%)
Total number	11,685,452	118,853	60,489
Total length	555,856,808	119,999,996	57,521,612
N50 length	48	1,637	1,433
Mean length	47.57	1009.65	950.94

2.2. Annotation of Unigenes

A total of 18773 of all (31.04%) unigenes were annotated by a BLAST search and align against the sequences in the nonredundant database, SwissProt database, Pfam database, COG database, GO database, KOG database, KEGG database, with an E-value threshold of le-5. 5131(8.48%) unigenes were annotated against COG, 6876 (11.37%) against GO, 8297 (13.72%) against KEGG, 11804 (19.51%) against KOG, 13550 (22.40%) against Pfam, 10216 (16.89%) against Swissprot, 18477 (30.55%) against nr. Additionally, 41716 (68.96%) were not annotated and may represent new genes (Table 2). The number of annotated unigenes is lower than the results reported by other research groups [23,24], indicating that our knowledge of *P. pseudoannulata* genes is limited. Further research is needed to characterize these genes and explore their functions.

Table 2. Function annotation of the *P. pseudoannulata* transcriptome.

Annotated databases	Annotated_number	300≤length<1000	Length≥1000
COG	5131 (8.48%)	1312	3819
GO	6876 (11.37%)	2310	4566
KEGG	8297 (13.72%)	2484	5813
KOG	11804 (19.51%)	3694	8110
Pfam	13550 (22.40%)	4204	9346
Swissprot	10216 (16.89%)	3164	7052
nr	18477 (30.55%)	7477	11000
All	18773 (31.04%)	7709	11064

The E-value distribution of the top hits in the Nr database showed that 40.67% of the sequenced have strong homology (smaller than 1E-60), and 44.18% of the homology sequences ranged from 1E-60 to 1E-5 (Figure 1A). 20.99% of the sequences had hits with similarity higher than 80% against the Nr database, and 52.02% of the sequences with similarity higher than 60% (Figure 1B). The species distribution showed that 76.63% of unigenes matched to four species, with 68.64% of the unigenes had the highest homology to genes from *Stegodyphus mimosarum*, followed by *Ixodes scapularis* (4.36%), *Zootermopsis nevadensis* (2.15%), *Metaseiulus occidentalis* (1.49%) (Figure 1C), which indicated that the sequences of transcripts obtained from *P. pseudoannulata* in the present study were assembled and annotated correctly.

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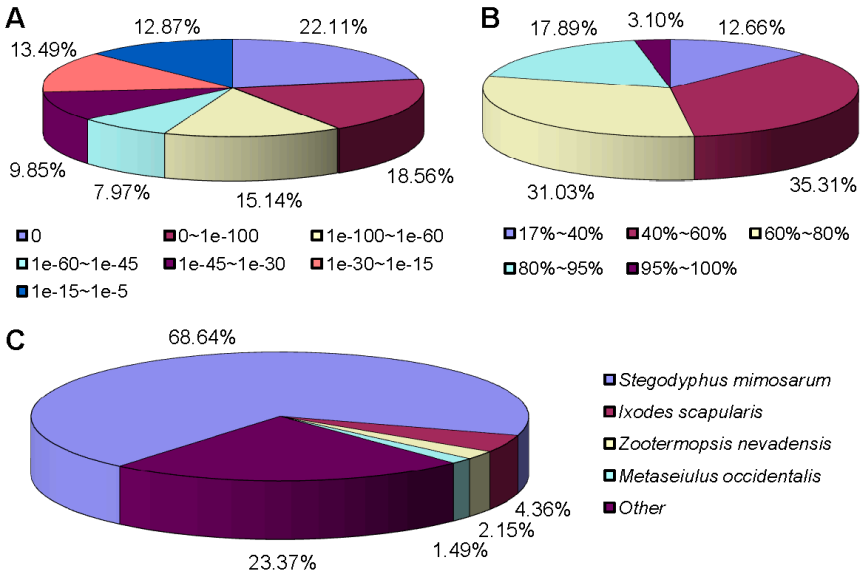

Figure 1. Characteristics of similarity search of unigenes against Nr databases. (A) E-value distribution; (B) Similarity distribution; (C) Species distribution.

The unigenes of *P. pseudoannulata* were mapped to 26,522 GO terms and categorized into 54 subcategories, among which 5,921 (22.32%) terms assigned to 17 subcategories in cellular components, 8,268 (31.17%) terms assigned to 18 subcategories in molecular function, and 12,333 (46.5%) terms assigned to 19 subcategories in biological processes, respectively (Figure 2). The 3 major sub-categories shown in Fig. 2 were "cell part" (GO: 0044464), "organelle" (GO: 0043226), and "macromolecular complex" (GO: 0032991) were in the cluster of cellular component. The 3 sub-categories of "catalytic activity" (GO: 0003824), "binding" (GO: 0005488), and "transporter activity" (GO: 0005215) were in the cluster of molecular function, and the 4 sub-categories of "cellular process" (GO: 0009987), "metabolic process" (GO: 0008152), "single-organism process" (GO: 0044699) and "biological regulation" (GO: 0065007) were in the cluster of biological process.

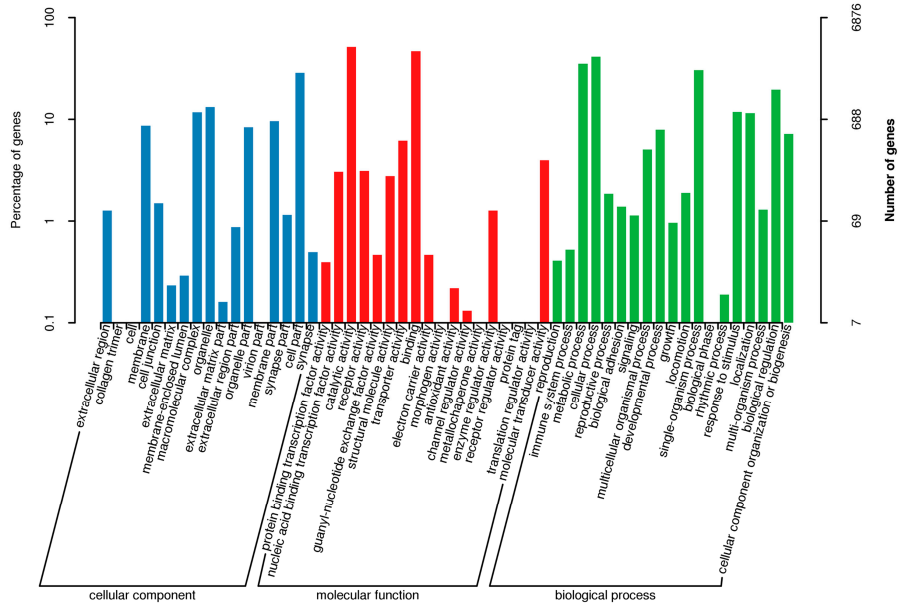

Figure 2. Gene Ontology (GO) functional classification. Unigenes were annotated for one of three categories: biological processes, cellular components and molecular functions.

In addition, 6878 unigenes had significant matches in COG database. Among the 25 COG categories，the cluster for general function prediction only (27.74%) was the largest category, follow by replication, recombination and repair (11.02%), transcription (7.93%), signal transduction mechanisms (7.78%), posttranslational modification, protein turnover, chaperones (5.64%), amino acid transport and metabolism (5.41%) and translation, ribosomal structure and biogenesis (5.18%) (Figure 3).

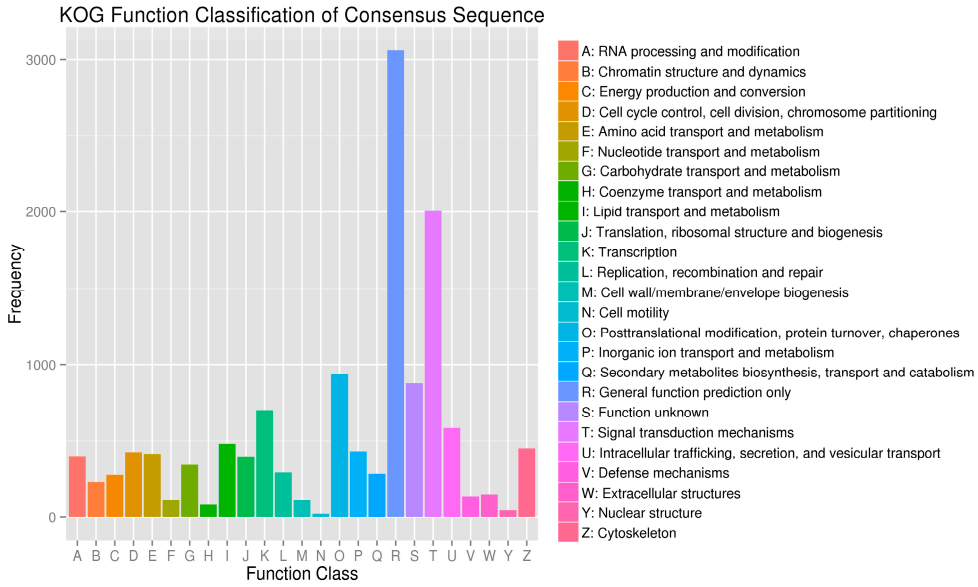

Figure 3. Clusters of orthologous (COG) classification. A total of 6685 unigenes were grouped into 25 COG classifications. The y-axis indicates the number of genes in a specific function cluster. The legend shows the 25 function categories.

A total of 11737 unigenes participated in 254 KEGG pathways (Table S1), which were assigned to five categories, including metabolism, genetic information processing, environmental information processing, cellular processes, and organismal systems. Among them, the largest number of unigenes are assigned to signal transduction (2134 unigenes), endocrine system (1012 unigenes), nervous system (732 unigenes), immune system (616 unigenes), carbohydrate metabolism (494 unigenes) and transport and catabolism (492 unigenes). These results provided a valuable clue for investigating functional genes and specific biological processes in spider research.

2.3. SSR Identification and Validation

16,257 unigene sequences longer than 1 kb were searched for SSRs. As a result, 5,168 sequences containing a total of 3450 SSRs were identified, with 516 of the sequences containing more than one SSR. About 96.32% repeat were perfect repeats among the identified SSRs (Table 3). Among all the SSRs, the repeat units mostly represented were mono-nucleotide and di-nucleotide, which accounting for 41.83% and 42.32%, respectively (Table 4).

225 types of perfect repeat sequences were identified. The most abundant mono-nucleotide repeat type was A/T (1427, 41.36%). The 3 types of di-nucleotide repeat motif with the highest frequencies were AT/AT (997, 28.90%), AC/GT (301, 8.72%), AG/CT (161, 4.67%). The dominant repeat motifs in the tri-nucleotide were AAT/ATT (158, 4.58%) and ATC/ATG (85, 2.46%) (Figure 4). Among the unigenes containing SSR, 2187 were suitable for SSR primer design. A total of 6561 pairs of SSR primer sequences were obtained using Primer 5.0.

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Table 3. Statistics of SSR searing in the *P. pseudoannulata* transcriptome.

Searching Item	Number
Total number of sequences examined	16,257
Total size of examined sequences (bp)	35,468,445
Total number of identified SSRs	3,450
Number of SSR containing sequences	2,805
Number of sequences containing more than 1 SSR	516
Number of SSRs present in compound formation	127

Table 4. Frequency of SSRs in *P. pseudoannulata* transcriptome.

Number of	Number of motif repeat											
nucleotides	5	6	7	8	9	10	11	12	13	>13	Total	%
Mono								489	301	653	1443	41.83%
Di		732	338	149	104	78	53	6			1460	42.32%
Tri	315	117	51	4	1					1	489	14.17%
Tetra	49	7									56	1.62%
Penta		2									2	0.06%
Total	364	858	389	153	105	78	53	495	301	654	3450	100%
%	10.55%	24.87%	11.28%	4.43%	3.04%	2.26%	1.54%	14.35%	8.72%	18.96%	100%	

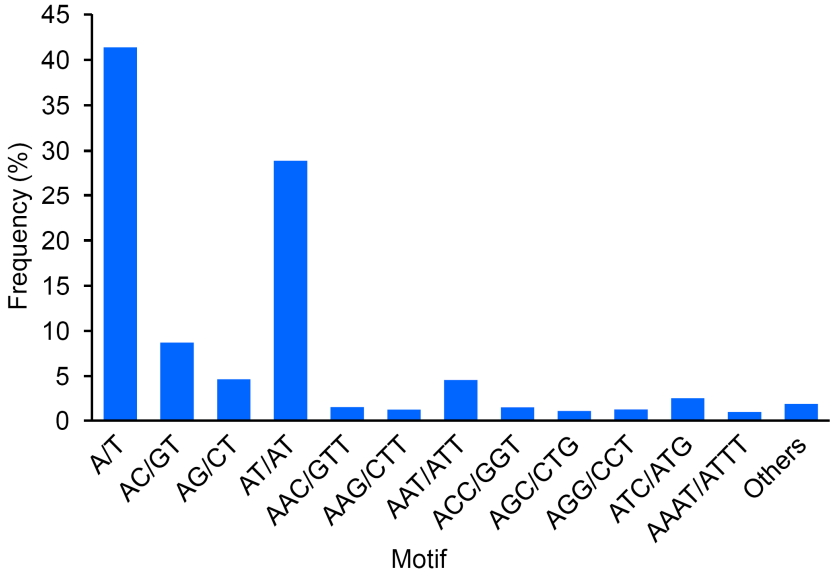

Figure 4. Frequency distribution of SSRs based on motif types.

The SSR frequency in the transcriptome of *P. pseudoannulata* was 21.22%, and the distribution density was 10.28 per kb (Table 1). The SSR frequency in this study is relatively higher than many of other insects and arachnid species [23,25]. These transcriptome-based SSRs mainly occur in the protein-coding regions of annotated unigenes, which can be used as a tool for identifying associations with functional genes and thus with phenotypes [26]. In general, tri-nucleotide repeats motifs have been reported to be the highest frequency in many insect species [27,28,29], but the dominant motifs in *P. pseudoannulata* are di-nucleotide repeats motifs (1460, 42.32%), which are much more than the

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tri-nucleotide repeats motifs (489, 14.17%) (Table 2). To some extent, this indicates that there is much difference of the genome structure between this spider and other insects.

To test the reliability of Solexa sequencing and the developed SSRs, a subset of 26 SSR primer pairs was randomly selected for RT-PCR analysis (Table S2). Twenty-five primer pairs resulted in successful PCR amplification. These results suggest that the quality of assembled unigenes were high, and SSRs identified in our study could be used for future analysis.

2.4. Differential Expression Analysis

To screen responsive genes, we calculated and compared expression levels among the three groups (TC, TL and TH groups), respectively, and thereby identified DEGs with each other, with an FDR < 0.01 and $\log_2$FC ≥ 1 taken as the selection criteria. As shown in Fig. 5A, there are a total of 2939 DEGs between the TC and TL groups, in which 2176 and 763 unigenes are up-regulated and down-regulated respectively. Between the TC and TH groups, there exist a total of 2491 DEGs, in which 1887 and 604 unigenes are up-regulated and down-regulated respectively. Comparing the TL group with the TH group, there are a total of 3759 DEGs, in which 1674 and 2085 unigenes are up-regulated and down-regulated respectively. There were much more up-regulated genes than down-regulated ones by comparing the two treatment groups with the control, but more down-regulated genes than up-regulated ones were detected in TL and TH groups. This implied that 0.2 mM Cd was more effective than 2mM Cd in enhancing gene expression. Through functional annotation, we separately obtained 1160, 883 and 1173 annotated DEGs in TC versus TL, TC versus TH and TL versus TH respectively. Notably, 202 genes were commonly regulated by different concentrations of Cd stress (Fig. 5B). Significantly, these DEGs could be used to discover genes responsive to Cd stress in the spider, and thereby to identify some biomarkers for monitoring heavy metal pollution [30].

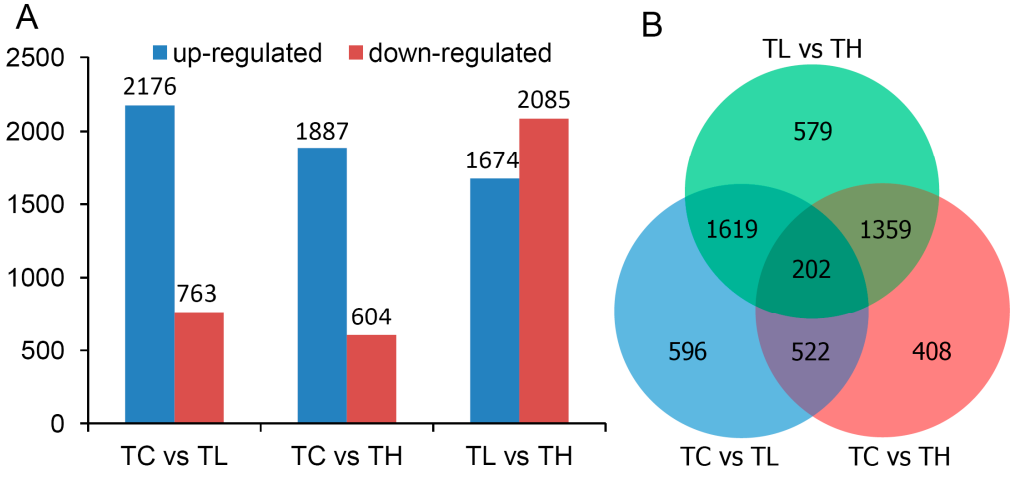

Figure 5. The differentially expressed genes numbers associated with different concentrations of Cd exposure.

2.5. Functional Enrichment Analysis of DEGs

The functional classification of DEGs were further analysed to explore the pattern of transcriptome regulation of *P. pseudoannulata* following Cd exposure. We performed GO enrichment analysis of DEGs that were identified between the groups (TC versus TL, TC versus TH and TL versus TH) among the three main clusters respectively, and thirty-eight, thirty-six and thirty-four subcategories under three main GO categories were obtained respectively (Table S3). The predominant enriched subcategories in all of the three groups were shown in Table 5.

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TopGO was further used for the enriched GO terms in the three primary clusters (enrichment significance KS <0.05). The topGo terms significantly enriched in the three groups related to biological processes included the "cell surface receptor signalling pathway" (GO: 0007166), "regulation of transmembrane transport" (GO: 0034762) and "regulation of localization" (GO: 0032879). Those related to molecular function included "catalytic activity" (GO: 0003824), "molecular transducer activity" (GO: 0060089), and "signal transducer activity" (GO: 0004871). Those related to cellular components included "membrane" (GO: 0016020) and "membrane-bounded organelle" (GO: 0043227) (Table S4).

Table 5. The predominant enriched subcategories in all the three groups.

biological process	molecular function	cellular component
"metabolic process" (GO: 0008152)	"catalytic activity" (GO: 0003824)	"cell part" (GO: 0044464)
"cellular process" (GO: 0009987)	"binding" (GO: 0005488)	"organelle" (GO: 0044422)
"single-organism process" (GO: 0044699)	"structural molecule activity" (GO: 0005198)	"macromolecular complex" (GO: 0032991)
"biological regulation" (GO: 0065007)	"transporter activity" (GO: 0005215)	"organelle part" (GO: 0044422)
"developmental process" (GO: 0032502)	"nucleic acid binding transcription factor activity" (GO: 0001071)	"membrane" (GO: 0016020)

The DEG annotation results in 26 COG classifications of TC versus TL, TC versus TH, and TL versus TH are shown in Table S5. In TC versus TL, the DEGs were mainly localized into the following four classifications: E (45, 18.44%), L (19, 7.79%), Q (19, 7.79%), and G (17, 6.97%). In TC versus TH, E (27, 15.61%), L (22, 12.72%), Q (18, 10.4%), and G (11, 6.36%) were the four primary classifications. In TL versus TH, L (37, 16.09%), E (29, 12.61%), P (17, 7.39%), and Q (16, 6.96%) were the four primary classifications. The DEGs annotation results from KEGG pathways type classification showed that 194, 120 and 153 pathways were differentially expressed in TC versus TL, TC versus TH and TL versus TH respectively. We then analysed the significant of the pathways using an enrichment factor and the Q-value; the results of the first 20 minimum Q-value pathways are displayed in Table S6.

When spiders are subjected to Cd stress, more energy is expended for defence (i.e., detoxifying the poisonous substance) [31,32,33]. Compared to the control groups, Amino acid metabolism (COG, E), carbohydrate metabolism (COG, G), lipid metabolism (COG, I) , energy metabolism (COG, C) and digestive system in COG and/or KEGG database were all shown to be significantly enriched, and most of the DEGs were significantly up-regulated, indicating the digestion and metabolism activities were actively induced to maintain the stability of their bodies when subjected to Cd stress.

The effects of heavy metal appear first at the molecular level and involve changes in polypeptide synthesis, the oxidation and denaturation of protein structures, DNA damage, intracellular respiration disorders and energy generation processes [11,12]. In extreme cases, high level of such damage suppresses metabolic processes and/or disintegrates cell organelles, leading to apoptosis [34,35,36]. When *P. pseudoannulata* were subjected to Cd stress, GO terms related to "metabolic process" (GO: 0008152), "catalytic activity" (GO: 0003824), "cell part" (GO: 0044464) and "organelle" (GO: 0044422), as well as DEGs involved in replication, recombination and repair (COG

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L), and KEGG pathways in lysosomes and phagosomes were significantly enriched. These findings may indicate that oxidative damage in spiders at the cellular and molecular levels results from Cd exposure.

2.6. Analysis of Genes Related to Cd Detoxification

Transmembrane metal transporters are assumed to play key roles in heavy metal transport and detoxification [37,38,39]. Numerous studies focused on metal transporters in both plants and animals have been reported [19,23,39,40]. ATP-binding cassette (ABC) family transporters are important heavy metal transporters and interacted with a wide range of chemicals including metals by pumping them across the cell membrane to maintain of cellular metal homeostasis [41,42]. In our study, the expression levels of ABC transporters were inhibited or induced. The expression of four of these genes was induced by Cd, one gene was suppressed in TC vs TL groups, one gene in TC vs TH groups was induced, and four genes in TL vs TH groups were down regulated. A relationship between ABC transporter expression and cadmium exposure has also been observed in other species [39,43,44].

Signal transduction is the main way for cells responding to heavy metal stress, when encounter an extra-cellular stimuli, the cell could active a variety of specific stress-responsive signalling proteins to protect the cell [39,40]**.** Comparing the enriched DEGs of *P. pseudoannulata* in association with varying concentrations of Cd exposure, the "cell surface receptor signalling pathway" (part of BP, GO:0007166) and "signal transducer activity" (part of MF, GO:0004871) were significantly enriched. Various signalling pathways have been demonstrated to be associated with Cd exposure [19,45]. MAPK pathway is usually known to be activated by Cd via ROS generation, which is associated with signal transduction in response to oxidative stress, thus, also plays an essential role in eliminating oxidative damaged cells [46,47]. KEGG annotation indicated involvement of the following signalling pathways in all three libraries: MAPK, AMPK, Notch, Hedgehog, Ras and TNF (Table S6). Most of the DEGs involved in the pathways were significantly upregulated with Cd exposure, confirming that the signal transduction of the spiders is activated by Cd contamination.

Heat shock protein families (Hsps) are critical factors during the process of environmental stress [19,48]. Genes encoding for Hsps, which play a vital role in the transport, folding, and assembly of proteins, are induced by various casual agents such as heavy metals [49,50,51]. In the present study, several forms of Hsps, including Hsp90, Hsp70, Hsp67, Hsp60, Hsp40 and Hsp20 genes were annotated in the unigene database. However, only the expression of Hsp70 and Hsp20 were significantly regulated, (Table S7). Consistent with the finding that hsp20 and hsp70 genes was substantially modulated in *Tigriopus japonicus* by heavy metals [50], and Hsp70 was significantly induced in *Cyprinus carpio* by Cd, indicating that both of the genes may be a good potential molecular biomarker for monitoring of heavy metal pollution [52].

Many studies have documented that Cd is often involved in oxidative stress resulting from the production of ROS [53,54]. Genes encoding detoxification enzymes played important role in preventing oxidative stresses and protecting organisms by the scavenging of ROS [55,56,57]. Glutathione metabolism played a pivotal role in protecting the organisms from heavy metal stress by quenching induced ROS, and GST is the most important enzyme of phase II detoxification and has a central role in defence against various environmental pollutants [58,59,60]. In the present study, several glutathione metabolism related to enzymes were found based on the assembled transcriptome background, two transcripts encoding GST were detected over-expressed compared

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with the control in pathway of glutathione metabolism, indicating their important roles in the defence against Cd stress. Same results were recorded in digestive gland of *Mizuhopecten yessoensis* following Cd exposure [19]. Three other genes in glutathione metabolism pathways, including 5-oxoprolinase, gamma-glutamyltransferase were detected and all up-regulated in TC vs TL and TC vs TH. However, the same tendency was not observed in TL and TH groups, it could be due to the sensitivity to dose of chemicals of antioxidative enzymes in some invertebrates. CYP450 is another critical detoxification enzyme considered to be a biomarker in many animals [19,30]. In our pathway enrichment analysis, totally 25 DEGs of CYP450 families were found both in TC vs TL and TC vs TH groups and most of them presented up-regulate, and these molecules may be used as biomarkers to assess the toxic effects on terrestrial invertebrates by heavy metals.

2.7. Validation of mRNA-seq Data by RT-qPCR

To further evaluate the DEG library, nine transcripts with clear annotation were randomly selected for analysis by RT-qPCR. The RT-qPCR results displayed the same expression tendency as the DEG libraries (Table 4). The expression profiles of these nine genes are shown in Table 6, including cytochrome P450 4C1, heat shock protein 70kDa, myb-related transcription factor, glutathione S-transferase, cytochrome P450 4c3, gamma-Glutamyltransferase ywrD, ABC transporter G family member 22 and Dimethylaniline monooxygenase.

Table 6. Validation of the RNA-Seq expression profiles of selected DEGs by RT-qPCR.

Transcript ID	Description	DEG library	Fold by RNA-Seq	Fold by qPCR
c59601.graph_c0	Cytochrome P450 4C1	TC vs TL	7.78	21.55
c82407.graph_c1	Heat shock protein 70kDa	TC vs TL	–2.53	0.26
c91015.graph_c0	Myb-related transcription factor	TC vs TH	7.39	4.65
		TL vs TH	5.26	2.25
c92582.graph_c0	Glutathione S-transferase	TC vs TL	2.80	4.41
c98395.graph_c0	Cytochrome P450 4c3	TC vs TL	9.06	42.27
		TC vs TH	8.47	15.51
c98430.graph_c0	gamma-Glutamyltransferase ywrD	TC vs TL	2.56	2.93
		TC vs TH	2.27	2.24
c101656.graph_c1	ABC transporter G family member 22	TC vs TL	2.24	4.96
		TL vs TH	–2.55	0.52
c91520.graph_c0	Glutathione S-transferase	TC vs TL	2.62	10.44
		TC vs TH	3.24	21.13
c87886.graph_c0	Dimethylaniline monooxygenase	TC vs TL	7.60	315.25
		TL vs TH	–3.81	0.29

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4. Materials and Methods

3.1. Animal Materials and RNA Extraction

Subadult *P. pseudoannulata* specimens were collected from farm fields in Ma'anshan Forest Park, Wuhan (30°52′N, 114°31′E), Hubei Province, China, in April 2014. Spiders were kept individually in cylindrical glass tubes (diameter 2 cm, height 12 cm) with a layer of sponge (1.5 cm thick) moistened with distilled water on the bottom and fed in a chamber at 26°C, relative humidity of 60–80% under a light: dark cycle of 14:10 h (lights turned on at 08:00). Two days post-maturation, female adult spiders were exposed to 0.2 and 2 mM $CdCl_2$ solution as their drinking water according to our previous study, and water for the control group [10]. We fed the spiders with *Drosophila melanogaster* and *Tendipes sp*., and replaced the moistened sponges every other day. Three biological replications were performed with each treatment containing at least six spiders. Seven days later, the treated spiders were immediately frozen in liquid nitrogen and stored at –70°C refrigerator for RNA extraction.

The entire body (containing the carapace and abdomen) of each spider was used for RNA extraction, and then equal quality RNA of the three replicates of each group were mixed for mRNA-sequencing. Total RNAs were extracted using TRIzol Reagent (Huayueyang Biotech Co., Ltd., Beijing, China) following the manufacturer's protocol. The extracted RNA was treated with RNase-free DNaseI (TaKaRa Biotech Co., Ltd., Dalian, China) to remove residual DNA. RNA integrity was checked through agarose gel electrophoresis (1.2%), and RNA concentration was estimated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA).

3.2. cDNA library Construction, Sequencing and De Novo Assembly

Magnetic beads with Oligo (dT) were used to isolate poly (A) from the high-quality total RNA samples. RNA sequencing libraries were generated using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA) with multiplexing primers, according to the manufacturer's protocol. The cDNA library was constructed with average inserts of 250 bp (150~250 bp), with non-stranded library preparation. The cDNA was purified using a QiaQuick PCR extraction kit (Qiagen, Inc., Hilden, Germany). The desired size of adaptor-ligated fragment was selected by agarose gel electrophoresis. Polymerase chain reaction (PCR) was performed to selectively enrich and amplify the selected cDNA fragments to construct cDNA libraries for paired end sequencing using the Illumina HiSeq 2500 (Biomarker Technologies Co., Ltd., Beijing, China). Low-quality reads were removed from the raw reads to generate clean reads. De novo transcriptome analysis of the clean reads were assembled using the short-read assembly program Trinity.

3.3. Unigene Functional Annotation

The BLASTX search (with E-value ≤ 1e-5) of each unigene larger than 200 nt was conducted against Nr, Swiss-Prot, KEGG, COG and GO databases, and the BLASTN search (E-value ≤ 1e-5) against the Nt to predict the function and metabolic pathways of unigenes.

3.4. Searching, Analysis and Detection of SSR Markers

Unigenes of *P. pseudoannulata* unigenes longer than 1 kb obtained in this study were subjected to SSR detection using MISA software (MIcroSAtellite identification tool, http://pgrc.ipk-gatersleben.de/misa/). The parameters were set as follows: a minimum of 12 repeats for mononucleotides, six for

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dinucleotides, and five for tri-, penta-, and hexanucleotide motifs. Total RNA of the spiders was extracted by using Trizol method (Taraka, Japan). One microgram of total RNA was used in reverse transcription in total volume of 20 μL in the presence of 6-mer random primer and oligo primer according to the protocol of Taraka. PCR was performed with 1 μL template for the PCR reaction at 94℃ for 5 mins, then 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s with 35 cycles in a 20 μL system. The PCR products were analysed by electrophoresis.

3.5. Differential Dene Expression and Functional Annotation Analyses

All clean reads were aligned to the unigene library using Bowtie software [61]. These results were employed to estimate expression level using RSEM software [62]. Fragments Per Kilobase of transcript per Million (FPKM) mapped reads were used to indicate the expression abundance of respective unigene [63]. FPKM could eliminate the influences that gene length and sequencing quantity difference for calculation gene expression, the gene expression results could be directly used to compare the gene expression differences between different samples.

EBSeq software [64] was used for differentially expressed gene (DEG) analysis. In the analysis procedure, we used recognized effective approaches such as the Benjamini–Hochberg method to correct the significance of the P-value [65]. To reduce the false positives among the expression values that occur when a large number of genes are analysed by successive independent statistical analyses, we used corrected P-values that called false discovery rate (FDR) as the key indicator for screening DEGs. In the process of screening, FDR <0.01 and $\log_2$fold change (FC) ≥1 were taken as the selection criteria.

Based on the gene expression levels of different samples, functional annotations for the DEGs were analysed. Then, GO annotation statistical analysis of DEGs in "biological process", "cellular component", "molecular function" three main categories between groups was implemented. GO enrichment analysis was used the topGO software [66] by the "elim" method with a minimum node size of 6. On the basis of COG and KEGG annotation, we statistically analysed the DEGs in COG function classifications and KEGG pathway classifications respectively. We further analysed DEGs in the KEGG pathway enrichment degree by the enrichment factor and corrected P-value (Q-value). Computation formula of enrichment factor is as follows, enrichment factor = (the number of DEGs in pathway / the number of DEGs)/(the number of all unigenes in pathway / the number of all unigenes in KEGG). The enrichment factor is bigger, the more significant of the enrichment level for DEGs, and the log value of Q-value is smaller, the more reliable of the enrichment significant for DEGs.

3.6 Validation of mRNA-seq Data

Total RNA was isolated from the previous samples and conducted as described above. Quantitative real-time PCR was performed on a Viia™ 7 Real-Time PCR System (ABI, USA) platform using the SYBR Premix Ex Taq™ II (Tli RNaseH Plus, ROX plus), (TaKaRa, Japan) following the manufacturer's instructions. The primer sets for each unigene were designed using Primer Premier 5.0 software (Tables S8). The amplification was achieved by the following PCR program of first denaturation 95℃ for 30s, followed by 40 cycles of 5 s at 95℃, 30s at 55℃, and 30s at 72℃, then a melt ing curve analysis was conducted from 60 to 95℃. All samples were tested in triplicate, and the experiments were performed on three biological replicates. The relative expression levels of the selected transcripts normalized to the internal control gene (*β-actin*) were calculated using the $2^{-\Delta\Delta Ct}$ method.

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4. Conclusions

In summary, the present study firstly represents a comprehensive transcriptome characterization of *P. pseudoannulata* following Cd exposure through high depth sequencing. A total of 60,489 assembled unigenes were obtained, 18773 of which were annotated in the public databases. 3450 cDNA simple sequence repeats were identified and validated as potential molecular markers. A total of 2939, 2491 and 3759 DEGs were detected among the two Cd-treated and control libraries in the spider. Metabolism processes and digestive system were predominately enriched with Cd exposure. Significantly enriched pathways in lysosome, phagosome, and replication, recombination and repair demonstrated the oxidative damage resulted from Cd at cellular and molecular levels. Based on the DEGs analysis, multiple candidate genes involved in defence and detoxification were successfully identified in response to Cd stress. These data would provide potential molecular targets in *P. pseudoannulata* for functional studies of genes responding to Cd stress and would serve as a valuable reference for identifying biomarkers in Cd pollution monitoring.

Supplementary Materials:

Supplementary materials can be found at www.mdpi.com/

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Author Contributions:

Conception and design of experiments: Yu Peng and Changchun Li. Performing the experiments: Changchun Li, Yong Wang and Jian Chen. Analysis of data: Changchun Li, Guoyuan Li, Yujun Dai. Preparing the manuscript: Changchun Li and Yu Peng.

Conflicts of Interest:

The authors declare no conflict of interest.

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