Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.03521

The toxico-transcriptomic analysis of nano-copper oxide on *gazami* crab: especially focus on hepatopancreas and gill

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Abstract

To explore the gene expression profiling in gazami crab's hepatopancreas and gill tissues which were challenged with CuO-NPs. Specimens of *gazami* crab *P. trituberculatus* were collected and challenged with CuO-NPs, then their hepatopancreas and gill tissues were dissected for RNA extraction. The cDNA libraries were synthesized and sequenced. De novo assembly of crab transcriptome was conducted for gene expression quantification and differential expression analysis. Finally the results were validated by RT-PCR. 56 unigenes displayed differential expression pattern in CuO-NPs treated hepatopancreas tissue. and 273 unigenes displayed differential expression pattern in CuO-NPs treated gill tissue. The gene expression pattern between control and CuO-NPs treated hepatopancreas was very closed to each other, while the gene expression pattern between control and CuO-NPs treated gill was more distinct. 13 genes were mutually exclusive to participate, and he data generated from RNAseq was well consistent with those obtained from RT-PCR. CuO-NPs could induce toxic effects in crab gills as well as in genetic level and the 13 genes might be the potential marker genes for CuO-NPs toxicology.

Keywords: CuO-NPs; hepatopancreas; gill; *gazami* crab.

Practical Application: The current study reveals toxico-transcriptomic analysis of Nano-copper oxide on hepatopancreas and gill of gazami crab.

1 Introduction

Metallic nanoparticles (MNs) have attracted scientists' interest due to their advantageous properties on biomedical and engineering fields. Metallic nanoparticles can be synthesized and modified with appropriate functional groups to make it possible for drug delivery. Meanwhile metallic nanoparticles have been considered as one of the main pollutants in the aquatic environment. These heavy metal contaminations in aquatic environments, particularly in freshwater systems, have posed severe risks because their ability to produce toxicity in aquatic organisms. This process happens mainly for the effects of particulates rather than the release of dissolved ions. Some major metallic nanoparticles in the aquatic environment have been demonstrated acutely toxic to across a wide spectrum of aquatic species including freshwater Hydra (Tortiglione et al., 2007), nematode (Ahn et al., 2014; Ma et al., 2009), Daphnia (Allen et al., 2010; Li et al., 2010), zebrafish (Asharani et al., 2008, 2011; Bar-Ilan et al., 2009) and mice (Gajdosíková et al., 2006; Kim et al., 2006; Ziady et al., 2003). Nano-copper oxide (CuO-NPs), which has advantages of good sterilization, catalytic properties, thermal stability, has been widely used in coatings, waste water treatment, sterilization, biomedical ceramic materials, and other fields. Hence, it can inevitably enter into the environment and ecological system, and the corresponding environmental toxicology effect will be induced. CuO-NPs can be accumulated in different tissues after being absorbed by animals. It's reported that the common mussel (*Mytilus galloprovincialis*) mainly accumulate in the digestive gland after suction of CuO-NPs (Gomes et al., 2012), while mussels (*Mytilus edulis*) mainly accumulate in gill (Hu et al., 2014). CuO-NPs usually has a bad effect on animal cells and tissues after being absorbed. *In vitro* studies have found that CuO-NPs are toxic to different cell lines, such as human's liver cells, renal cells as well as epithelial cells of the African clawed frog (*Xenopus laevis*), which will cause the stagnation of the cell cycle, affect cell proliferation and lead to apoptosis and so on (Wang et al., 2011; Xu et al., 2013; Thit et al., 2013).

Crustaceans are very sensitive to heavy metal pollution, and crabs have been considered as suitable bioindicator. Crabs can easily expose to heavy metals because they live in the sediments of aquatic environments. These crabs have been confirmed to accumulate some metallic nanoparticles in their main organs, such as hepatopancreas, gill, gonad, and hemocytes. Lately, hepatopancreas in Crustaceans is generally thought to be a key target organ for heavy metal toxicity and other environmental stresses. Studies have found that hepatopancreas plays an important role in responses to environmental stresses besides its function in digestion and metabolism, such as detoxification. The toxico-transctiptomic analysis for another freshwater crab (*Sinopotamon henanense*) was done in the previous research. The research was designed to obtain the crab transcriptomic analysis in *S. henanense*, and analyze differential gene expression profiles of hepatopancreas samples treated with Cadmium.

Received 26 Jan., 2021
Accepted 23 Feb., 2021
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But nowadays, only few studies used hepatopancreas as the target organ (Sun et al., 2016).

The swimming crab (*Portunus trituberculatus*) was used as biological subjects due to its larger output in China's offshore fishing areas. *P. trituberculatus* is one of the swimming crabs that inhabits the seafloor habitats with sand or pebbles and is being widely distributed in the coastal waters of China, Korea, and Japan. *P. trituberculatus* is one of the most common edible crabs in Japanese waters. It has been artificially propagated and stocked. Given its high commercial interest, extensive studies focus on the diseases causing large mortality and emulsification disease with metallic nanoparticles confirmed as its main causative agents (Yamauchi et al., 2003). In this study, we explored the transcriptomic response of hepatopancreas and gill tissues toward CuO-NP pollution in *P. trituberculatus*.

2 Materials and methods

2.1 RNA extraction

Specimens of *gazami* crab *P. trituberculatus* were collected from the city of Zhoushan in Zhejiang province, China. Female crab was approximately 9 months old with an average weight of 234 g (range from 203 to 252 g) and an average body length of 8.8 cm (range from 7.8 to 9.6 cm). Before CuO-NPs challenge, carbs were acclimated indoor in plastic tank (60 cm × 40 cm × 50 cm) at room temperature for 7 days. Crabs were then transferred into new tanks with 12L sea water and challenged with CuO-NPs at 40 ppm. The control crabs were cultured in the seawater without CuO-NPs. After 20 days, crabs were sacrificed and their hepatopancreas and gill tissues were dissected and flash-frozen in liquid nitrogen and stored at -80 °C prior to RNA extraction. Total RNAs were extracted by using the TRIZOL Kit (Invitorgen, Carlsbad, CA, USA) following manufacturer's instructions. Total RNA samples were then digested by DNase I to remove potential genomic DNA contamination. Integrity and size distribution were checked with Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA). Equal amounts of the high-quality RNA samples from each tissue were then proceed to perform cDNA synthesis and next generation sequencing.

2.2 Library construction and Illumina sequencing

Initially, about 2 μg of starting total RNAs were used to synthesize the cDNA libraries by following the standard protocols of the Illumina TruSeq RNA Sample Preparation Kit (Illumina). The final library had an average fragment size of 250 bp. After KAPA quantitation and dilution, the library was sequenced on an Illumina HiSeq X Ten to generate approximately 10M single-end clean reads with 50 bp reading length. The raw transcriptome sequences in the present study were deposited in the NCBI SRA database.

2.3 De novo assembly of crab transcriptome

We used previous published data downloaded from NCBI and in-house generated data to perform de novo assembly by using CLCBio software with default parameters settings. The transcriptome was assembled, combining 609,768,300 clean reads into 147,314 unigenes, ranging from 250 to 34,158 bp in length. The average length was 825 bp, the N50 length was 1073 bp. The assembled transcriptome unigenes were subjected to similarity search using BLAST2GO with an e-value cut off of 1e-6. About 31% assembled unigenes could be annotated.

2.4 Gene expression quantification and differential expression analysis

For gene expression comparison, three gill and hepatopancrease cDNA libraries were established for either control or CuO-NPs treated groups and subjected to generate about 10M single-end reads with 50 bp with three replicates, respectively. The cleaned reads of each RNA-seq library were mapped to the previous assembled unigenes with Bowtie program (Langmead, 2010). The counting of alignments was done using RSEM (Li & Dewey, 2011). The differential expression statistical analysis was done using the statistical method described in the R package (Cordero et al., 2012). Differentially expressed gene (fold changes > 2 and adjusted p-value < 0.00001) between two samples were identified with the software.

2.5 Principal Component Analysis (PCA)

The gene expression level (RPKM) for each contig from different treated groups were summarized into a single excel table and later imported into a SIMCA-P software) to perform PCA analysis, which was an excellent tool to reduce the complexity of multiple variants from high to low dimension.

2.6 qRT-PCR and RT-PCR

The concentration of total RNA was determined by spectrophotometry and checked RNA quality by running electrophoresis in RNA-denatured gels. For qRT-PCR, 1 μg of total RNA was reverse-transcribed with TAKARA PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) and then performed PCR with SYBR green dye (ABI SYBR® Select Master Mix, 4472908) in ABI Vii7 Real Time PCR machine according to the manufacturer's instructions. The primer sequences used to perform qRT-PCR were listed in Table 1.

2.7 Whole genome sequencing

In order to better annotate gene in swimming crab, we perform whole genome decoding by using shot gun approach. Genomic DNA was extracted from muscle tissue and then the high quality genomic DNA was subjected to construct library with average insert size of 350 bp. DNA degradation and contamination was detection by 1% agarose gel, DNA purity was checked by using the Nano Photometer Spectrophotometer and DNA concentration was checked by using Qubit 2.0 Flurometer. Later, genomic DNA was fragmented by using Ultrasonic Processor to produce sheared DNA fragments with approximately 350 bp. DNA fragments were then terminal repaired, add base A and add sequence adapter. Subsequently, preliminary quantitative by Qubit 2.0, library concentration was diluted to 1 ng/uL. Then library's insert size was verified by Agilent 2100, finally Q-PCR were implemented to ensure the effective quantitative concentrations of library. Finally,

the genomic DNA library was sequencing by using Illumina HiSeq X Ten with PE150 strategy to produce 45 Gb raw data. The raw genome reads were assembled, combining 309,008,720 clean reads into 427,510 contigs, ranging from 250 to 34,158 bp in length. The average length was 1,812 bp, the N50 length was 3,118 bp.

3 Results

3.1 Gene expression quantification and differential expression analysis

The gill and hepatopancrease transcriptome was sequenced at single end and their expression level was mapped to contigs which were assembled from the previous transcriptomic datasets (48Gb in size) that deposited in NCBI (Table 2). Mapping results show 56 unigenes (20 up and 36 down) displayed differential expression pattern in CuO-NPs treated hepatopancrease tissue. For gill tissue, there are 273 unigenes (150 up and 123 down) displayed differential expression pattern in CuO-NPs treated gill tissue.

Table 1. The primer sequences used to perform qRT-PCR.

Primer name	sequence (5' to 3')
18s rRNA-F	ATTGGTGTCCAGTTCGCAGC
18s rRNA-R	TGCGGCCCAGAACATCTAAG
contig_104069-F	TTCCCCACTAGCTTGACGGC
contig_104069-R	AATTCGACTCCGATGGCAGC
contig_104571-F	TCCCATTGAACAACTTTGGC
contig_104571-R	GGTGGAATCAAAGATGGCAA
contig_108326-F	AAAGTTTGGTTCCCAGTTTG
contig_108326-R	ATTGCCGATCTTTTTGACTC
contig_108562-F	CTGCCCTTCATTGGTCAGAG
contig_108562-R	AAGCAGAAGCTGGAACCCAT
contig_10951-F	CCGTGCGACACCTTCAACTT
contig_10951-R	TGGTGGCGTGACTCCATTCT
contig_146949-F	TTGTCCTCCAGTCTTTGTGA
contig_146949-R	TCACTGAGTTCCAAGGATCA
contig_18055-F	TCTCCATCCGGTCCTCCTTC
contig_18055-R	TGTCGCTCGGTCACCAACTT
contig_2188-F	CGTTGTGCCACAGTCCAGTG
contig_2188-R	CACTGGTGGTGATGTGCCTG
contig_5457-F	CACCCACCGACAAGCTCATG
contig_5457-R	TGACAGGCAGGAGTTGCAGG
contig_69949-F	CTGCGGACAATATTCCTTCT
contig_69949-R	TGCAGATGGTATGAAAGACG

3.2 Principal Component Analysis (PCA)

The gene expression pattern between control (K) and CuO-NPs treated hepatopancrease (W) was very closed to each other, while the gene expression pattern between control (KS) and CuO-NPs treated gill (WS) was more distinct (Figure 1). The same time, both control gill and CuO-NPs treated gill showed gene inductions. Therefore, we picked up the data generated from gill tissues to maximize the potential marker genes for CuO-NPs toxicology.

3.3 Differentially expressed genes

The gene expression difference in crab according to their fold change was studied. The fold change data showed more than 450 mutually exclusive genes in both K&W and KS&WS gene lists. Further the number of genes was narrowed down for analyzing closely via false discovery rate (FDR) to do biomarker validation. And just 43 genes (13.6%) in K&W and 260 genes (82.3%) in KS&WS were in the lists. It was confirmed that only 13 genes were mutually exclusive to participate in this study (Figure 2).

3.4 Validation of differentially expressed genes

To validate the feasibility of data collected from RNAseq analysis, we performed RT-PCR for potential marker genes. The results showed the up regulation of WS and down regulation of **KS** (Figure 3). For upregulated group, four out of five selected genes showed significantly upregulated in WS. For downregulated group, four out of five selected genes show significantly downregulated in WS. The CuO-NPs treated gill crab had up

Table 2. The previous transcriptomic datasets which deposited in NCBI.

SRA ID	Description	Sequencer	PE	data size (Gbp)
SRR1920180	Portunus trituberculatus testis transcriptome	Illumina HiSeq 2000	2X101	7.4
SRR1920182	Portunus trituberculatus ovary transcriptome	Illumina HiSeq 2000	2X101	6.1
SRR1630818	molting related transcriptome	Illumina HiSeq 2000	2X91	4.9
SRR1185310	RNA-seq of Portunus trituberculatus when exposed to ammonia	Illumina HiSeq 2000	1X100	3
SRR1013695	Transcriptome analysis of Portunus trituberculatus-non-challenged	Illumina HiSeq 2000	2X102	5.9
SRR1105793	RNA-seq of Portunus trituberculatus when exposed to ammonia	Illumina HiSeq 2000	1X100	2.9
SRR1013696	Transcriptome analysis of Portunus trituberculatus-high salinity stress	Illumina HiSeq 2000	2X102	7
SRR1013694	Transcriptome analysis of Portunus trituberculatus-low salinity stress	Illumina HiSeq 2000	2X102	5.8
SRR768319	Transcriptome Portunus trituberculatus	Illumina HiSeq 2000	2X91	4.9

PE: paired-end.

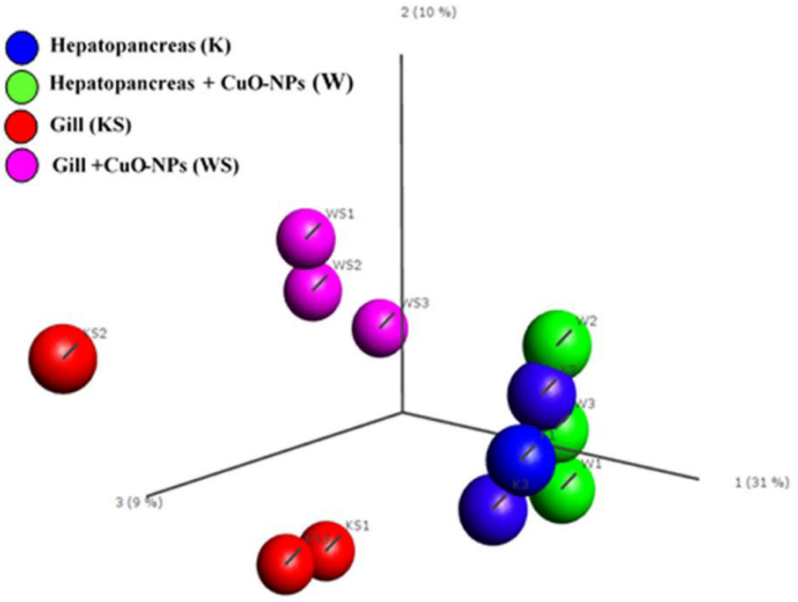

Figure 1. PCA analysis of different dispose groups.

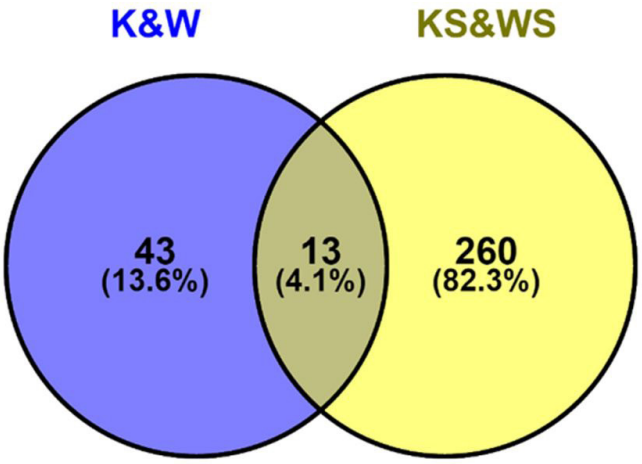

Figure 2. The fold change of gene expression difference in crab.

regulated gene compared with control gill. The same time, the control gill showed gradual decrease in gene regulation in the crab. Further, we identified the upregulating gene pathways and made tree structure on it (Figures 4 and 5).

4 Discussion

Gills are the primary sites for the absorption of many aquatic pollutants in fish and other invertebrates because gills are directly in contact with the surrounding water and pollutants. The metal content of the gills will be increased if they are exposed to both dissolved and nanoparticulate metals. The toxicity of these particles is largely manifest at the gills and it does not seem to be explained simply by particle dissolution. In this study, we detected more gene showing differential expressed pattern in hepatopancreas and gill of gazami crab after challenged with CuO-NPs. We sequenced gill and hepatopancreas transcriptome at single end and mapping their expression level to contigs. Both control gill and CuO-NP treated gill showed gene inductions. Further we studied the gene expression difference in crab according to their fold change and we confirmed that only 13 genes are mutually exclusive to participate in this study, which were the potential marker genes for CuO-NPs toxicology. Besides, we conducted validation for the results, andthe data generated from RNAseq was well consistent with those obtained from real time RT-PCR. We confirmed that the CuO-NPs could induce toxic effects in crab gills as well as in genetic level. Branchial uptake of ionic silver and copper has been well documented in freshwater fish. This uptake appears to occur primarily through apical membrane sodium channels and the copper transporter protein. Studies also have found that Nanocopper, one of the nanoparticulates, can induce a significant increase in gill filament width. There are various mechanisms for nanoparticulates to increase the gill metal level. These particles may be trapped in the mucus layer of the gill as demonstrated for larger particles. Even though these particles may not actually enter the cells, but mucus entrained particles can also increase the intracellular metal content by enhanced dissolution due to changes in water chemistry in the gill microenvironment including mucus complexation. The other possibility is nanoparticles are actually taken up by gill epithelial cell (Griffitt et al., 2009). Vulnerability of gills exposed to aquatic pollutants has been established in previous studies involving different fish species such as the European bullhead Cottus goblo (Dorts et al., 2011). Gill tissues of rainbow trout (Oncorhynchus mykiss) which were exposed to a sub lethal concentration of waterborne zinc also used to investigate the response in the gill tissues by differential screening of a heterologous cDNA array and protein profiling (Hogstrand et al., 2002). The low amount of Cadmium and Mercury accumulation in crab's gills and hepatopancreas lead to major biological problems and death too (O'Hara, 1973).

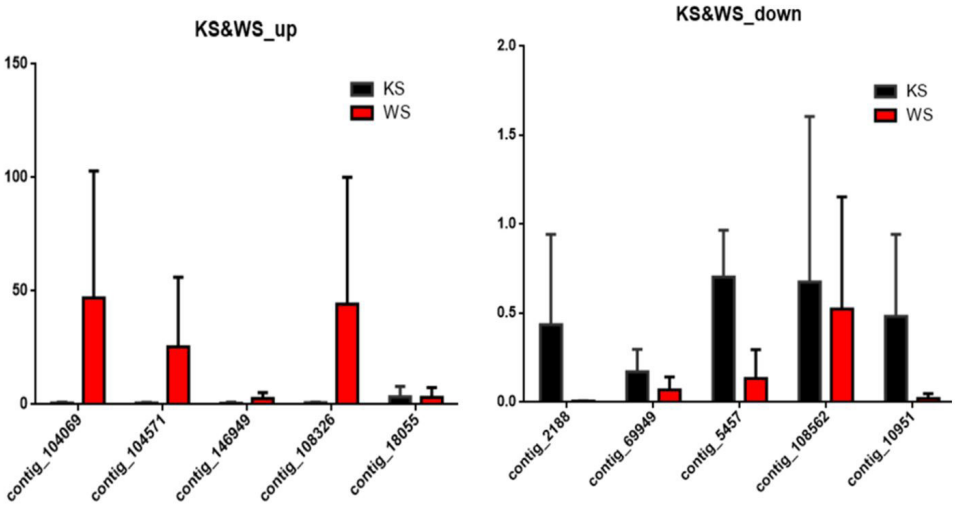

Figure 3. The results of real time RT-PCR verification of marker gene.

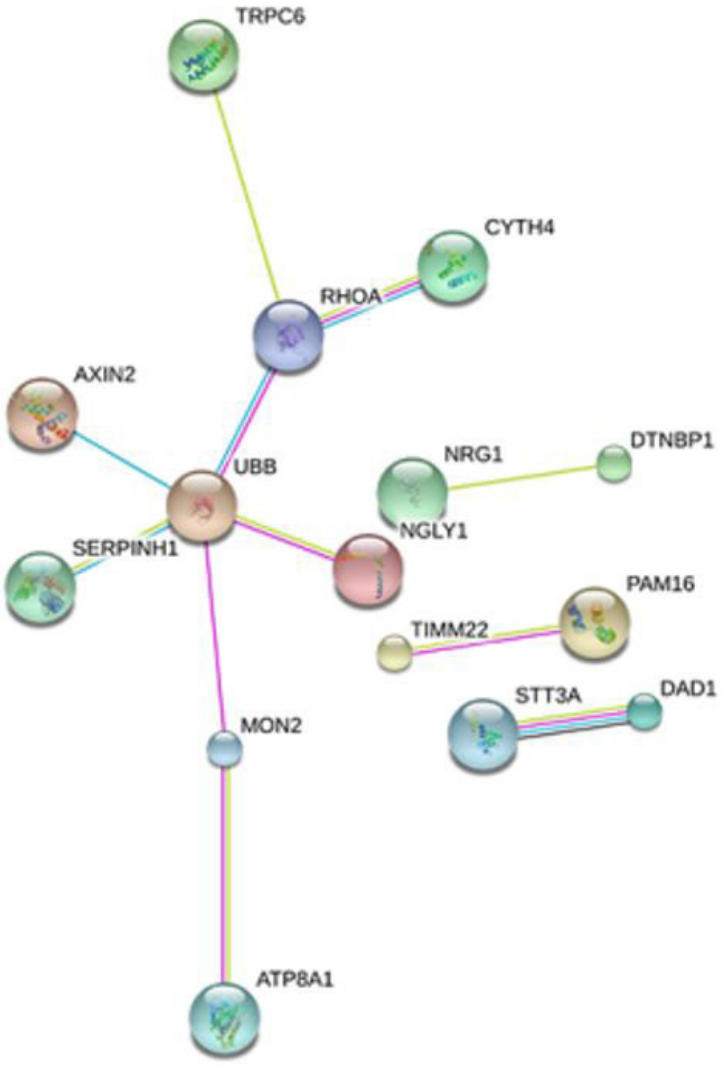

Figure 4. The pathways of upregulating gene.

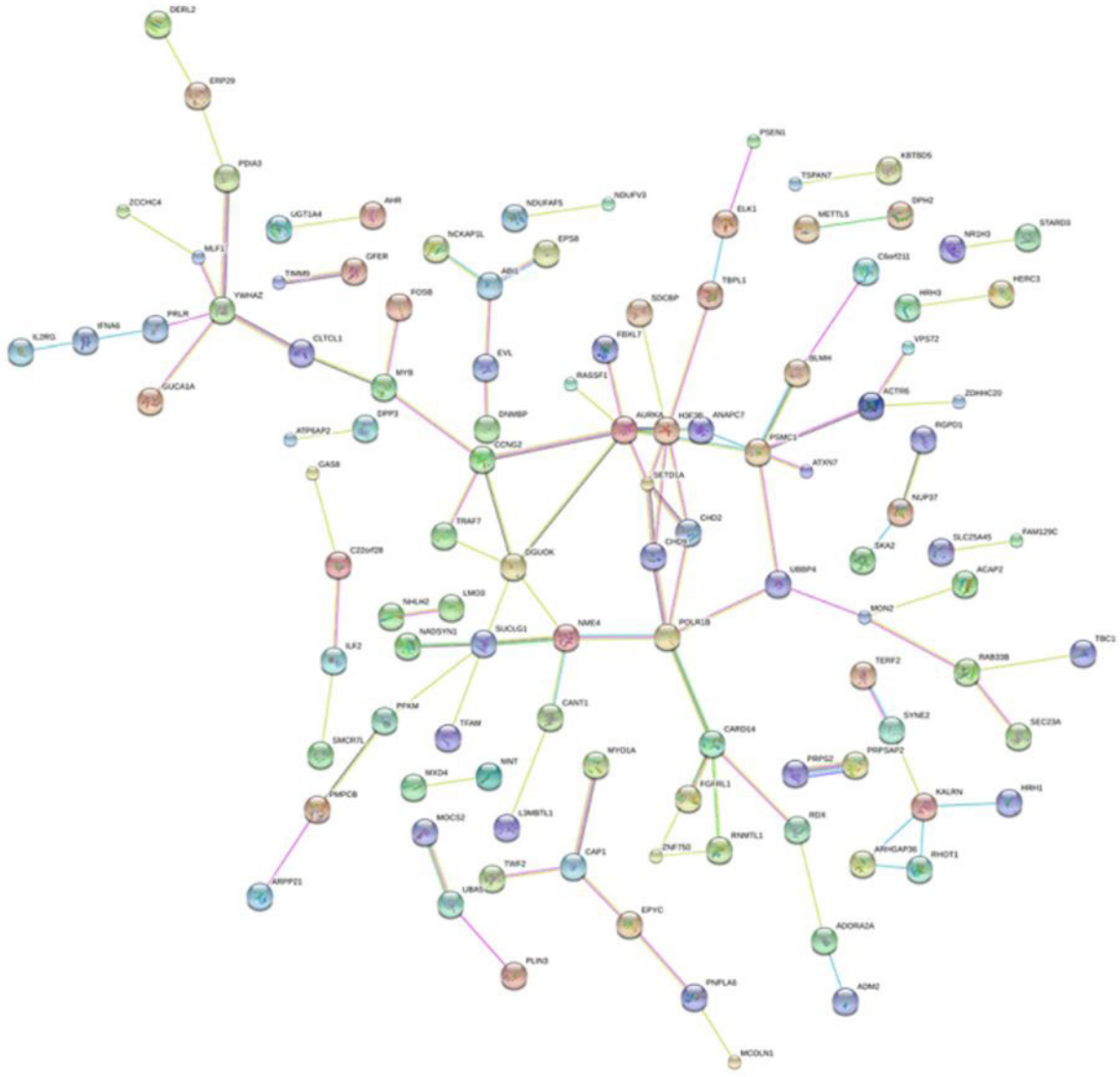

Figure 5. The tree structure of upregulating gene pathways.

Hepatopancreas has been reported to play an important role in responses to environmental stresses besides as detoxification. In this study, 56 unigenes (20 up and 36 down) displayed differential expression pattern in CuO-NPs treated hepatopancreas tissue, but the gene expression pattern between control and CuO-NPs treated hepatopancreas was very closed to each other. We need further study for the mechanism.

In summary, the current study helps to reveal the toxico-transcriptomic analysis of Nano-copper oxide on hepatopancreas and gill of gazami crab.

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