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Transcriptome profiling analysis of the seagrass, *Zostera muelleri* under copper stress

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Abstract

Copper (Cu) in an essential trace metal but it can also contaminate coastal waters at high concentrations mainly from agricultural run-off and mining activities which are detrimental to marine organisms including seagrasses. The molecular mechanisms driving Cu toxicity in

seagrasses are not clearly understood yet. Here, we investigate the molecular responses of the Australian seagrass, *Z. muelleri* at the whole transcriptomic level after 7 days of exposure to 250 µg Cu L^{-1} and 500 µg Cu L^{-1}. The leaf-specific whole transcriptome results showed a concentration-dependent disturbance in chloroplast function, regulatory stress responses and defence mechanisms.

This study provided new insights into the responses of seagrasses to trace metal stress and reports possible candidate genes which can be considered as biomarkers to improve conservation and management of seagrass meadows.

1. Introduction

Globally, coastal habitats are increasingly affected by a wide range of anthropogenic discharges which often contain high levels of trace metals (Cambridge & McComb 1984; Waycott et al. 2009; Leng et al. 2015). Once introduced to the marine environment, they are taken up by seagrasses via roots and shoots (Howley et al. 2006). Consequently, as a food source for many marine organisms, seagrasses represent a trophic pathway for the distribution of trace metals into the marine food chain (Ward 1987; Kalay, Ay & Canli 1999; Campanella et al. 2001; McGeer et al. 2004; Navratil & Minarik 2005). Trace metals are not only detrimental to the health of fishes, dugongs, turtles and marine invertebrates that feed on contaminated seagrass tissues, but also to human health as the trace metals subsequently find their way into our foods through contaminated seafood (Basha & Rani 2003; Canli & Atli 2003; Wang et al. 2005).

In particular, agricultural run-off and acid mine drainage often contain high concentrations of Cu (Tokar et al. 2013). Under normal conditions, Cu is vital for the function of many key enzymes and proteins including superoxide dismutase and plastocyanin (Katoh 1977; Barón, Arellano & Gorgé 1995; World Health Organization 1996; Kaufman Katz et al. 2003;

Nagajyoti, Lee & Sreekanth 2010; World Health Organization 2011). However, high concentrations of Cu in the environment can cause deleterious effects to living organisms including seagrasses at both physiological and molecular levels (Barón, Arellano & Gorgé 1995; Gupta & Gupta 1998; Theophanides & Anastassopoulou 2002; Yruela 2005; Stern 2010). More specifically, the alteration of the function of transporters and ion channels as a result of excess level of Cu can cause intra-cellular redox imbalances (Cambrollé et al. 2013; Tiecher et al. 2017) as well as cellular damage via the over-production of reactive oxygen species (Girotto et al. 2013; Tiecher et al. 2017).

Zostera muelleri is a fast growing species of seagrasses in the family of *Zosteraceae* found predominantly in coastal regions of Eastern and Southern Australia (den Hartog 1970; Kenworthy et al. 2006; Wissler et al. 2011; Davey 2017). The destructive effects of hyper-accumulation of Cu in the family of *Zosteraceae*, have been reported previously as irreversible suppression in photosynthesis efficiency (Prange & Dennison 2000; Macinnis-Ng & Ralph 2002, 2004b; Buapet et al. 2019) and over production of reactive oxygen species (ROS) (Greco et al. 2019; Buapet et al. 2019). In higher plants, regulatory scavenging mechanisms for the detoxification of Cu include chelation (Sancenón et al. 2003; Yruela 2009), alteration to less toxic ionic form (Gill & Tuteja 2010; Thounaojam et al. 2012) and sequestration into vacuoles (Himelblau & Amasino 2000). However, less in known about the toxicity responses of seagrasses to Cu stress.

Transcriptomic profiling can be useful to better understand the toxicology response of seagrasses to a range of environmental stress factors. Recent reports of complete genomes of *Z. muelleri* and *Z. marina* (Lee et al. 2016; Olsen et al. 2016) have opened new avenues to deepen our understanding about the molecular basis of stress responses in the family of *Zosteraceae* (Franssen et al. 2011 and 2014; Kong et al. 2014; Pernice et al. 2015; Schliep et al. 2015Kumar, Padula, et al. 2016; Pernice et al. 2016; Martin-Guirao et al. 2017; Ruocco et

al. 2017; Lin et al. 2018). However, with few exceptions (Davey et al. 2017; Procaccini et al. 2017; Ruocco et al. 2017; Lin et al. 2018), most of the differential gene expression studies so far are limited to few targeted genes and do not investigate the effect of certain stress factors at the whole transcriptome level. In addition, none of these studies have investigated the molecular responses of *Z. muelleri* to Cu stress.

According to the Australian trace metal field measurement study in South coast of New South Wales (2018), Cu is measured between 0.01 − 0.08 µg/L in water (McVay et al. 2018). However, the Cu toxicity level in the family of *Zosteraceae*, that negatively affects the physiology state of the plant was previously reported to be higher and within the range of 0.1 − 10 mg Cu L^{-1} (Clijsters & Assche 1985; Macinnis et al. 2002; Prange & Dennison 2000; Ralph & Burchett 1998b). Additionally, the physiological response of *Z. muelleri* under 250 and 500 µg Cu L^{-1} (corresponding to 3.9 and 7.8 µM, respectively) has been recently studied after 1, 3 and 7 days showing a concentration and time-dependent decline in effective quantum yield (ϕPSII) and maximum quantum yield (F_v/F_m) parameters (Buapet et al. 2019). In the same study, a RT-PCR investigation illustrated an elevation in ROS production, as well as up-regulation of the transcript expression of antioxidant enzymes including glutathione peroxidase (*gpx)*, catalase (*cat* – only for 250 µg Cu L^{-1}), superoxide dismutase (*Cu/Zn sod*) and ascorbate peroxidase (*apx*) after day 7 (Buapet et al. 2019).

Therefore, in this study we continued our investigation using whole transcriptomic analysis to further investigate how transcriptome of *Z. muelleri* altered under 250 µg Cu L^{-1} and 500 µg Cu L^{-1} after 7 days of Cu exposure and identify which genes were specifically expressed in response to this Cu stress.

2. Materials and Methods

2.1. Sample collection and aquaria setup

Samples were collected at Pittwater, New South Wales, Australia (33°38′45.6′′S. 151°17′12.8′′E) in July 2016 at the approximate depth of one meter. Whole vertical plants of *Z. muelleri* were collected and transferred to the aquarium facility at the University of Technology Sydney (UTS) within 2 – 3 hours of collection and in dark containers to avoid additional stress as previously described by Davey et al. (2017).

Aquaria were prepared according to Buapet et al. (2019). Briefly, six aquaria (two tank replicates for control and two for each treatments) were established with conditions mimicking the natural environment at the sampling time, i.e. salinity of 30 ppt and temperature of 21°C and diel cycle of 12 hour light : 12 hour dark with maximum light intensity of 200 μmol photons m^{-2} s^{-1} at midday. One LED aquarium light (Cidley 250W), one submerged pump (Elite mini, Hagen, Canada) and one air stone were also equipped for each tank. The sediment for planting seagrasses was as a mixture of 50% washed sand and 50% natural sediment (4 – 5 cm for each tank). Individual plants (30 – 40) were rinsed with artificial seawater (30 ppt) to remove epiphytes and transplanted randomly into each aquaria.

2.2. Experimental design

Plants were kept in 6 allocated aquaria to acclimatise to the closed-system conditions for 18 days with daily monitoring of photosynthetic efficiency using Diving-PAM (Walz GmbH, Effeltrich, Germany) until stable effective quantum yield (ϕPSII) was measured (data not shown) as previously mentioned in Buapet et al. (2019). Afterwards, a stock solution of $CuCl_2$ was prepared to make the final concentration of 250 μg Cu L^{-1} and 500 μg Cu L^{-1} and added to treatment tanks as a single dose at midday on day 0. Leaves of three biological replicates were harvested randomly from allocated tanks for each treatments on day 7 for RNA extraction. Collected leaves were rinsed with saline water, tap dried and were frozen in liquid N_2 prior to storage at – 80°C for further analysis.

2.3. RNA extraction

Frozen leaf tissue (80 – 100 mg) from each treatments (control, 250 and 500 μg Cu L^{-1}) were ground into a fine powder using a pre-chilled pestle and mortar. RNA extraction was performed using an Ambion PureLink RNA Mini kit (Fisher Scientific) according to the manufacturer`s instructions. On-column DNA digestion was performed during RNA extraction using Ambion PureLink DNase set (Thermo Fisher Scientific).

2.4. Library preparation and RNA sequencing

Nine RNA samples (three for control and three for each treatments) were sent to the Ramacciotti Center for Genomics (University of New South Wales, Australia) for quality control and sequencing. Quality and quantity measurement were conducted using a 2100 Bioanalyzer (Agilent Technology) with quality cutoff for RNA Integrity (RIN) numbers of > 6. Library preparation was performed using TruSeq mRNA standard total library preparation kit (Illumina) for sequencing of 380 million base-pair reads (42 million pair-end reads per sample) using a HiSeq2500 system from Illumina.

2.5. Genome-guided transcriptome assembly and annotation

The quality of raw reads were checked using FastQC software (version 0.11.05) (Andrews 2017; Davey et al. 2017). Adaptors and low quality reads were trimmed using Trimmomatic (version 0.2.35) with the following settings: ILLUMINACLIP: TruSeq3-PE2.fa: 2:30:10; LEADING:14; TRAILING:14; SLIDING-WINDOW: 4:10; MINLEN: 90 (Bolger, Lohse & Usadel 2014; Davey et al. 2017). Trimmed reads were aligned to the reference genome using the STAR RNA aligner (version 2.5.2b) (Dobin et al. 2013). Functional annotations of transcripts were taken from the *Z. muelleri* genome annotation file (Lee et al. 2016). Read

counts were created using HTSeq (version 0.6.1) (Love, Anders & Huber 2014) and the counts were imported into the R package DESeq2 (version 3.7) for differential gene expression analysis (Anders, Pyl & Huber 2015; Love et al. 2015). Conditions of 250 and 500 μg Cu L^{-1} were compared to the control and genes with corrected cut-off p-value of < 0.05 were reported. Heatmaps were generated from z-scaled variance stabilized counts from DESeq2 using R package ComplexHeatMap.

The genome of *Z. muelleri* is not completely annotated yet. Therefore, the FASTA file of significantly expressed genes in both 500 μg Cu L^{-1} and 250 μg Cu L^{-1} were submitted in Blast2Go (version 5.0.5) for gene identification based on the best hit in NCBI database (cutoff e-value of 1×10^{-3}) as well as for sister species, *Z. marina*. In case on multiple gene description, the common name between two searches were selected. GO distribution of expressed genes for both Cu treatments were exported for most induced biological process, molecular function and cellular process using Blast2Go data analysis.

Lastly, the functional classification of correspondent proteins were reported using BlastKOALA (version 2.1) https://www.kegg.jp/blastkoala/.

3. Results

3.1. Transcriptome assembly and functional annotation

Extracted RNA from control and treated samples generated an average of 22 million Illumina pair reads per sample. The quality check of raw reads (using FastQC package) gave an average quality score of 30 (1:1000 probability of incorrect base), which was sufficient for the further quantitative analysis of the transcriptome. After trimming low quality reads and adaptors using Trimmomatic, an average of 84.6% of the reads were mapped back to the reference genome unambiguously. The converted text files from HTSeq were used for differential expression analysis with DESeq2. DESeq2 results revealed significant changes in

the transcriptome expression of both 250 μg Cu L^{-1} and 500 μg Cu L^{-1} samples in comparison to the control (Figure 1). Among 39 and 96 differentially expressed genes in 250 μg Cu L^{-1} and 500 μg Cu L^{-1}, respectively (in comparison to control samples), 30 genes were in common to both treatments. Additionally, 6 genes were not identified in either BLAST search or in *Z. muelleri* (InterProScan database) for both treatments. For the remaining genes, a total of 76 genes were up-regulated and 29 genes were down-regulated in our Cu treatments (Table 1). The list of GO identification, their correspondent log 2 fold change as well as their DESeq2 results can be found in Appendix A-D.

The list of genes in both Cu treatments were searched for GO distribution using BLAST2GO (version 5.2.5) (Figure 2). The most highly induced biological processes in response to Cu was metabolic processes which comprise a range of metabolite categories including organic substances, nitrogen compounds, macromolecules and proteins. At the molecular function level, 70% of the genes were identified in binding category including genes involved in binding of ions, organic compounds, cofactors, ribonucleotides and ATP. The most affected cellular component were membrane (27%) mostly as integral and intrinsic components of membrane.

Lastly, the functional classification of correspondent proteins were investigated as a combined search for both treatments using BlastKOALA (Figure 3). Genetic information processing and biosynthesis of other secondary metabolites were the most affected pathways.

3.2. Changes in the expression of genes in *Z. muelleri* in response to Cu stress

Based on the results of functional classification of related proteins to our expressed genes, it is shown that genetic information processing and secondary metabolites biosynthesis were the most induced pathways in response to 250 μg Cu L^{-1} and 500 μg Cu L^{-1} and selected for further investigation. Additionally, according to Buapet et al. (2019), photosynthesis and

defense mechanisms (enzymatic and chemical) were induced in response to Cu stress and therefore, genes linked to chloroplast function and defense mechanisms were also targeted for further investigation in this study. As a result, a total of 16 significantly expressed genes were selected for the study of toxicity response of *Z. muelleri* under elevated levels of Cu (Table 2 and Figure 4).

Surprisingly, no genes related to photosystem subunits were expressed significantly in chloroplast compared to control after 7 days of Cu stress. However, two genes related to chloroplast function were up-regulated; the first gene was chloroplast caseinolytic protease (Clp) subunit 4 which was upregulated (-3.82 log 2 fold change) in 500 µg Cu L^{-1} only. The second gene was Dna J which was also upregulated in both Cu treatments (-1.7 and -1.8 log2 fold change in 250 and 500 µg Cu L^{-1}, respectively). The second gene was the enzyme, ferredoxin nitrite reductase. The expression of ferredoxin nitrite reductase was significantly down-regulated in both Cu treatments with 4.26 and 5.95 log2 fold change in 250 and 500 µg Cu L^{-1}, respectively.

Enzymatic defense mechanism (oxidative response) was shown to be affected after 7 days of Cu exposure in 500 µg Cu L^{-1} only. Peroxidases (P7-like, 12 and 5) were up-regulated with -2.62, -3.92 and -3.86 log 2 fold change, respectively. Glutathione s transferase (T1) was also up-regulated with -3.42 log 2 fold change.

A mixed pattern was recorded in two genes encoding enzymes involved in chemical defense mechanisms in both Cu concentrations; Cytochrome P450 (89A2-like) was up-regulated with an average log 2 fold change of -2.26 in both Cu concentrations whereas Cytochrome P450 (84 A1-like) was down-regulated with log 2 fold change of 1.93 at 500 µg Cu L^{-1} only. Proline dehydrogenase 2 was the second enzyme which also down-regulated with log 2 fold change of 2.49 at 250 µg Cu L^{-1} only.

Lastly, we identified up-regulation of heavy metal-associated isoprenylated plant protein 3-like (HIPP3) with an average of -1.6 log 2 fold change at 500 µg Cu L^{-1}.

4. Discussion

Investigation of differential gene expression of *Z. muelleri* in response to increased levels of Cu illustrated sensitivity of this seagrass species to Cu as previously reported in the family of *Zosteraceae* (Lee et al. 2004; Lin et al. 2018, Buapet et al. 2019). As a result, two main Cu-specific responses were observed in this study:

4.1. Elevated Cu concentrations impacted the chloroplast function and regulatory stress responses with no significant effect on photosystem subunits

There are previous reports showing that photosystem subunits especially PSII are sensitive to Cu stress in higher plants and seagrasses at the physiological level (Buapet et al. 2019; Cedeno-Maldonado, Swader & Heath 1972; Mohanty, Vass & Demeter 1989; Arellano et al. 1995; Jegerschoeld et al. 1995; Ralph & Burchett 1998; Prange & Dennison 2000; Macinnis-Ng & Ralph 2002a, 2004a; Dattolo et al. 2014). However, we did not find any statistical differences in the expression levels of any photosystem subunits in our results at the molecular level. Cu toxicity is reported to change the conformation and function of the photosystem over time (Yruela 2005). However, it seems like the significant damage will be mostly recorded at much higher Cu concentrations in short term studies. For example, Leng et al (2018) reported down-regulation of PSII subunits in grape vine leaves after 24 hours of Cu exposure at much higher concentrations of Cu (100 µM) than our experiment (3.9 and 7.8 µM). Additionally, Lin et al. (2018) reported Cu-induced transcriptomes associated with photosynthesis pathways in *Z. japonica* after 7 days exposure to 50 µM of Cu. Therefore, these results could be indicated that the destructive effects of Cu on photosystem subunits in

Z. muelleri might need longer exposure time to manifest significantly at the molecular level when Cu concentrations are less than 10 µM.

Although there were no significant damage recorded in photosystem subunits in our results, we investigated whether the repairing system of photosystem was activated in our Cu treatments. Previous studies have shown that phytohormones can have a direct or indirect role in regulating and repairing PSII under abiotic stress factors (Gururani, Venkatesh & Tran 2015). For example, in higher plants, auxin is shown to increase the ability of energy trapping by PSII reaction centres under Cu stress (Ouzounidou & Ilias 2005). Jasmonic acid is another suggested phytohormone with repairing activity for PSII under Cu stress in higher plants (Maksymiec, Wojcik & Krupa 2007).We had one gene in our results identified as GH3 auxin-responsive promoter/ jasmonic acid at 500 µg Cu L^{-1} and shown to be up-regulated. This result could indicate that the PSII was partially damaged and the repairing mechanism was activated at 500 µg Cu L^{-1}.

We also found two regulatory stress response genes in our results with concentration-dependent responses; the first one was DnaJ which has previously reported to have role in tolerance to oxidative stress in plants (Chen et al 2010). We identified up-regulated chaperone protein dnaJ 8, chloroplastic-like at 500 µg Cu L^{-1} as well as dnaJ homolog subfamily B member 1 at 250 µg Cu L^{-1}. The second gene was chloroplast caseinolytic protease (Clp) system (subunit 4) which plays a role in chloroplast homeostasis by regulating Cu transporter PAA2 in thylakoid (Shen et al 2007). This gene was also up-regulated in our results at 500 µg Cu L^{-1} only. Tapken et al (2015) suggested that Clp chaperones are susceptible to increase level of Cu and degrade PAA2 activity in *Arabidopsis* which might to be the similar case in *Z. muelleri* as well. However, we did not find any significance result in PAA2 Cu transporter activity to prove it.

Conversely, there was a strong down-regulation in Ferredoxin nitrite reductase observed in both Cu treatments. Ferredoxin nitrite reductase is shown to assist with chlorophyll biosynthesis by providing NH_4^+ for glutamate biosynthesis in chloroplast (Alipanah et al 2015). The fact that this enzyme is down-regulated in both Cu treatments might indicate that a possible role of glutamate biosynthesis pathway in repairing chloroplast in response to Cu stress was not active or damaged in *Z. muelleri* after 7 days of exposure.

4.2. Induced enzymatic and chemical defense mechanism in response to Cu stress

ROS production via Haber-Weiss and Fenton reaction is the first stress response towards most of the common stress factors in plants (Hall 2002; Halliwell 2006). Activation of two enzymatic defense mechanism to quench over-expression of ROS seemed to be active after 7 days of Cu exposure; Peroxidase (POX) and glutathione s transferase (GST). Peroxidase is a key enzyme in terrestrial plants for scavenging over-produced ROS (Hiraga et al. 2001). Glutathione s transferase (GST) also has a protective function and is a carrier for photochemicals (Edwards, Dixon & Walbot 2000). Both enzymes were up-regulated in our results at 500 µg Cu L^{-1} only. Cu exposure was previously hypothesized to inhibit some antioxidant enzymes activity by binding to their sulfhydryl groups of proteins and alter their structure (Yruela 2009; Pena et al. 2012, Buapet et al. 2019). However, peroxidase and glutathione s transferase seemed to be unaffected by Cu in *Z. muelleri*.

Secondary stress responses also play a significant role against the stress factors at later stages of toxicity (Jonak, Nakagami & Hirt 2004; Opdenakker et al. 2012; Jalmi et al. 2018). CytP450 is shown to have a role in detoxification of lead and cadmium in plant and fungi as suggested by Zhang et al. (2015). Our results indicated that CytP450 82 A2-like (and not 84 A1-like) may play a role in Cu detoxification in *Z. muelleri* in response to 500 µg Cu L^{-1} only.

297 Proline, is also shown to be involved in secondary defense mechanisms and protecting
298 cellular components during stress (Rhodes & Hanson 1993; Ashraf & Foolad 2007). Proline
299 dehydrogenase, which is involved in the transportation of proline to the mitochondria was
300 down-regulated at 250 μg Cu L^{-1} only in our results. Proline accumulation was previously
301 reported in detoxification of salt and drought stress by stabilizing sub-cellular structures and
302 scavenging free radicals (Öztürk & Demir 2002; Hsu, Hsu & Kao 2003; Kishor et al. 2005).
303 The mechanism is thought to be via breaking-down of proline inside mitochondria to induce
304 oxidative phosphorylation and ATP production to recover the damage. In our results, proline
305 dehydrogenase was down-regulated at 250 μg Cu L^{-1} only. Therefore, proline seemed not to
306 be involved in recovering any mitochondrial damage under Cu treatments used in this study.
307 Lastly, heavy metal-associated isoprenylated plant protein 3-like (HIPP3) which is previously
308 reported to have a role in regulation of biotic and abiotic stress response (Zschiesche et al
309 2015) was only shown to be significantly up-regulated at 500 μg Cu L^{-1}. Therefore, it seems
310 like HIPP3 could have a role in regulation of Cu stress in *Z. muelleri* in a concentration-
311 dependent manner.

312

313 It is important to note that because we used individual plants for this experiment, it is
314 possible that the plants' condition might have been affected compared to natural conditions
315 but the relative differences found between the plants exposed to the different treatments
316 remain valid. Some of the gene regulations observed at the whole transcriptome level in this
317 study were also observed using RT-qPCR in a study recently published (Buapet et al, 2019),
318 further supporting our RNA-seq results. To summarise, our results showed that the
319 concentration of Cu exposure seemed to play a critical role in Cu toxicity responses of *Z.*
320 *muelleri*. Enzymatic defense mechanism (peroxidase and glutathione s transferase), chemical
321 defense mechanisms (CytP450) and regulatory stress mechanisms were activated in Cu stress.

Our study provided a knowledge base for the development of specific biomarkers for Cu toxicity in the seagrass, *Z. muelleri*. However, future studies can enrich this data by investigating the limitations of this study. For example in a recent study, Buapet et al, 2019 found that Cu bioaccumulation in leaves of *Z. muelleri* plants saturated after day 3. Therefore it is possible that our results could reflect a progressive reduction in seawater Cu availability due to removal by sediments and/ or experimental system itself. Additionally, investigation of Cu-contaminated seagrass tissue from contaminated sites as biologically relevant samples can enrich our close-system experiment dataset. Furthermore, investigation of multiple Cu exposure in order to retain the specific Cu concentration throughout the experiment could also be informative when compared to single dose of Cu addition in this study.

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Table 1 (edited)

Table 1. Total number of expressed genes and ORFs under 500 µg Cu L^{-1} and 250 µg Cu L^{-1}.

	Up-regulated	**Down-regulated**
250 µg Cu L^{-1}	23	12
Uncharacterised (in both databases)	4	0
500 µg Cu L^{-1}	68	25
Uncharacterised (in both databases)	3	0
Total	**98**	**37**
Common	**22**	**8**
Total (unique)	**76**	**29**

Table 2 (edited)

Table 2. List of 14 differentially expressed genes selected for further investigation in this study.

	Gene ID	Description	*Z. marina* accession ID	Log2 fold change	
				250 µg/L	500 µg/L
	Chloroplast function				
1	0:maker-500_52247_3964_29592--0.7	ATP-dependent Clp protease proteolytic subunit 4, chloroplastic	KMZ63497.1		-3.850
2	1:maker-1730_58158_16459_58158--0.13	chaperone protein dnaJ 8, chloroplastic-like	N/A		-1.808
3					
4	1:maker-3083_30171--0.6	Ferredoxin--nitrite reductase, chloroplastic	KMZ61228.1	4.264	5.959
	Enzymatic defence mechanism				
5	0:augustus_masked-6109_21278--0.0- mRNA	Peroxidase P7-like	KMZ56929.1		-2.628
6	0:maker-625_101397_6977_101397--0.33- mRNA	Peroxidase 12	KMZ75156.1		-3.922
7	1:maker-1744_70048_1_49765--0.9-mRNA	Peroxidase 5	KMZ75156.1		-3.864
8	0:maker-1173_166881_1_148987--0.46-mRNA	Glutathione S-transferase T1	KMZ72094.1		-3.422
	Chemical Defence mechanism				
9	0:maker-1210_109775--0.31- mRNA	Proline dehydrogenase 2, mitochondrial-like	KMZ67632.1	2.491	

10	0:maker-14927_11295--0.5-mRNA-1	Cytochrome P450 89A2-like	N/A	-2.215	-2.362
11	0:maker-8118_116443--0.34-mRNA-1			-1.788	-2.174
12	1:maker-522_51695--0.11	cytochrome P450 84A1-like	N/A		1.935
	Regulatory stress response				
13	0:maker-1520_80468_39862_77238--0.6	heavy metal-associated isoprenylated plant protein 3-like (HIPP3)	KMZ67897.1		-1.448663975
14	0:snap_masked-2153_115725_1_52866--0.3				-1.901078429
15	0:maker-2866_46954--0.6	GH3 auxin-responsive promoter/ Jasmonic acid-amido synthetase JAR1	KMZ65778.1		-5.079917865
16	1:maker-3661_43599_1_15864--0.10	dnaJ homolog subfamily B member 1	KMZ75474.1	-1.707	

Figure 1 (edited)

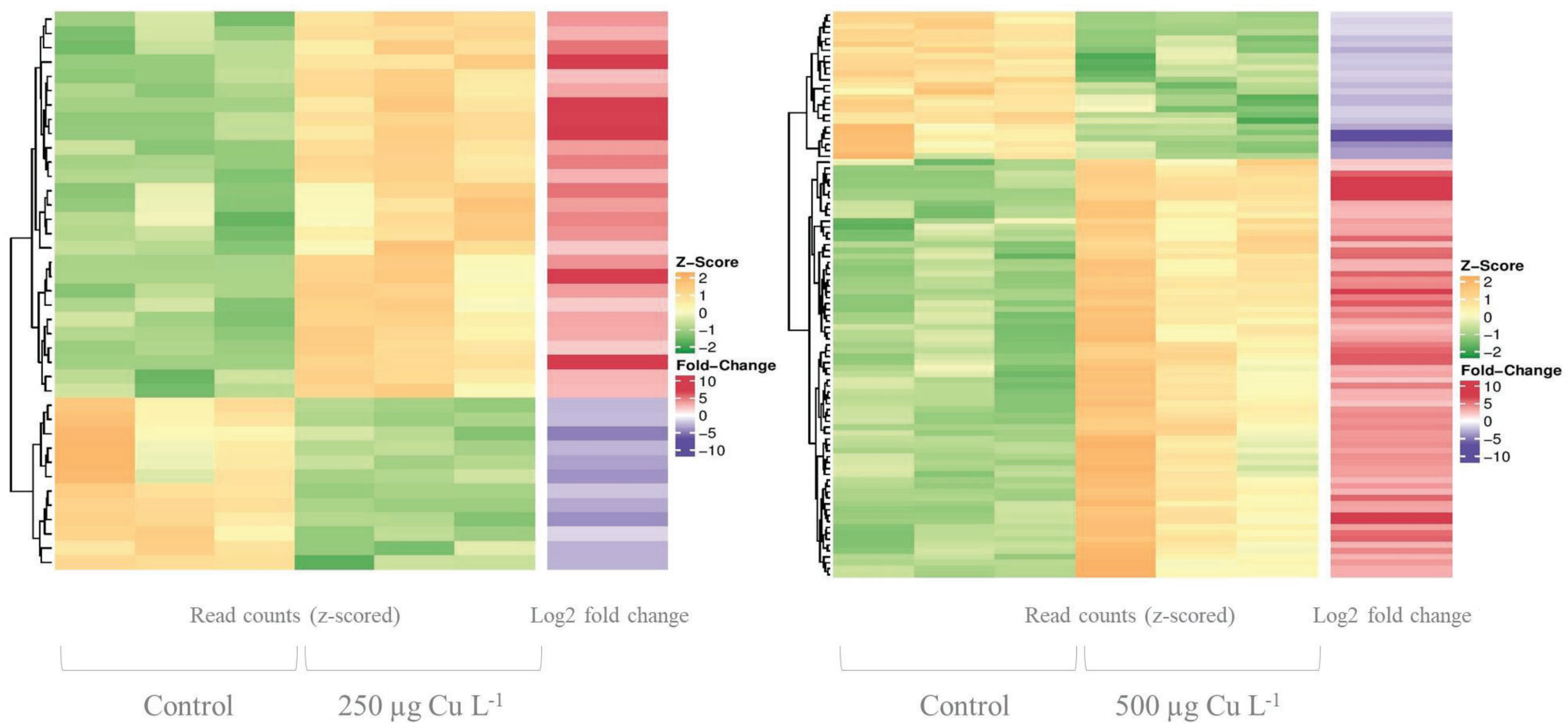
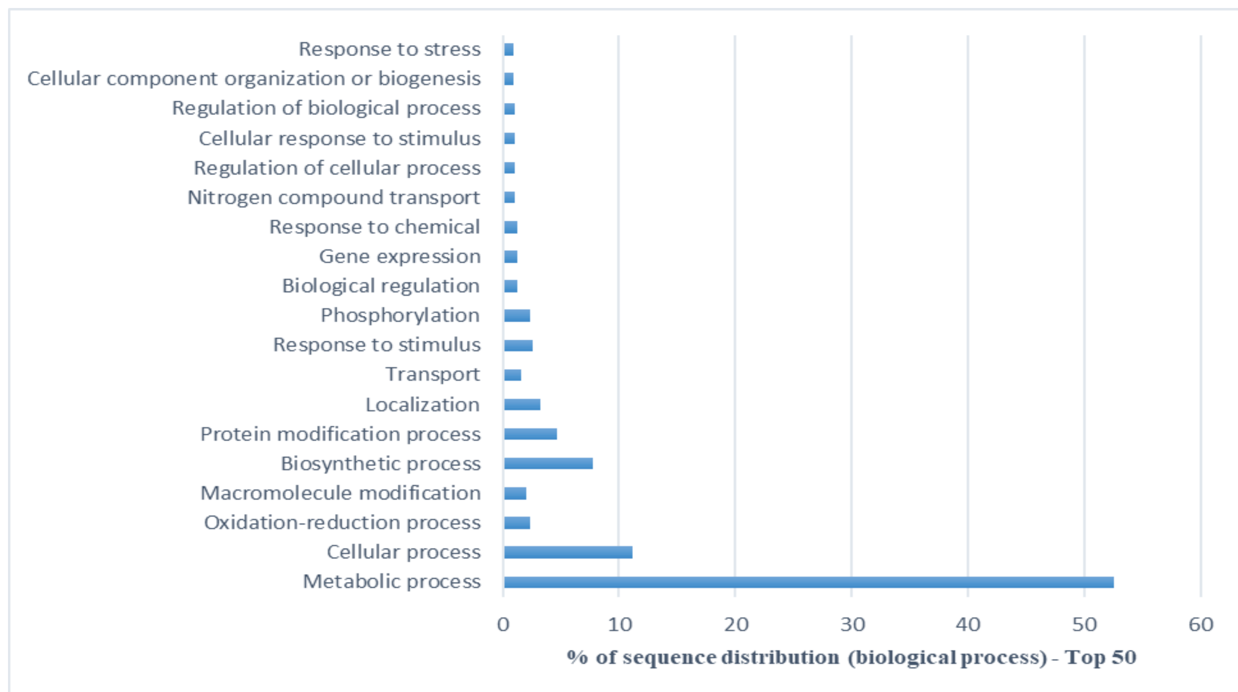


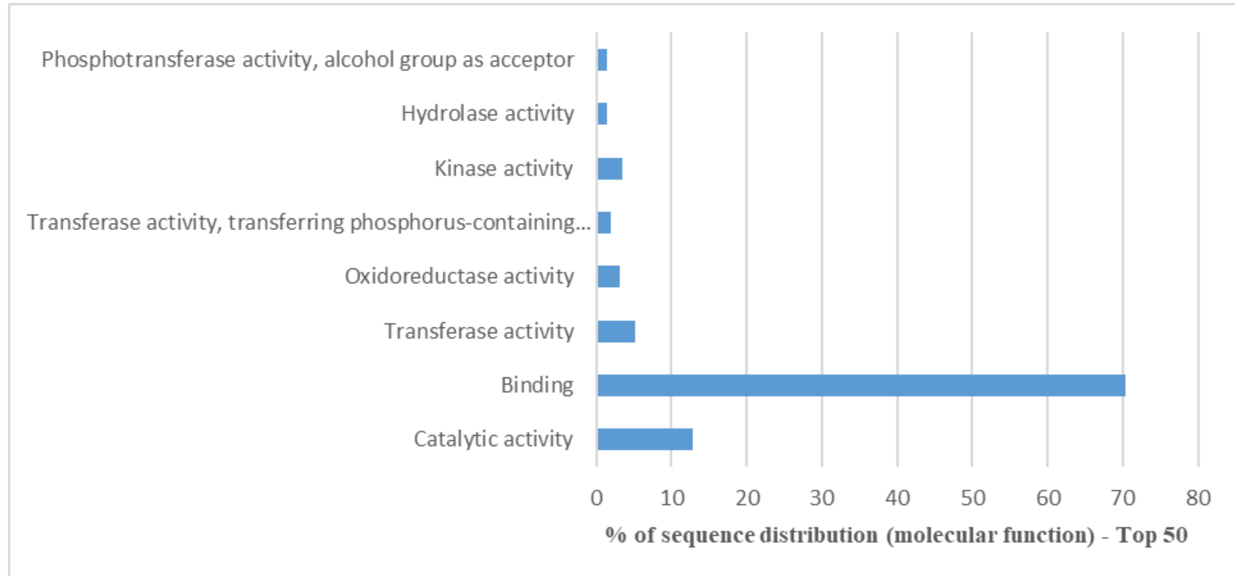
Figure 1. Overall representation of significantly expressed genes under Cu stress. Heatmaps of the log 2 fold change and z-score of normalized read counts for significantly differentially expressed genes in response to 250 µg Cu L^{-1} (left) and 500 µg Cu L^{-1} (right) after 7 days.

Figure 2 (edited)

A



B



C

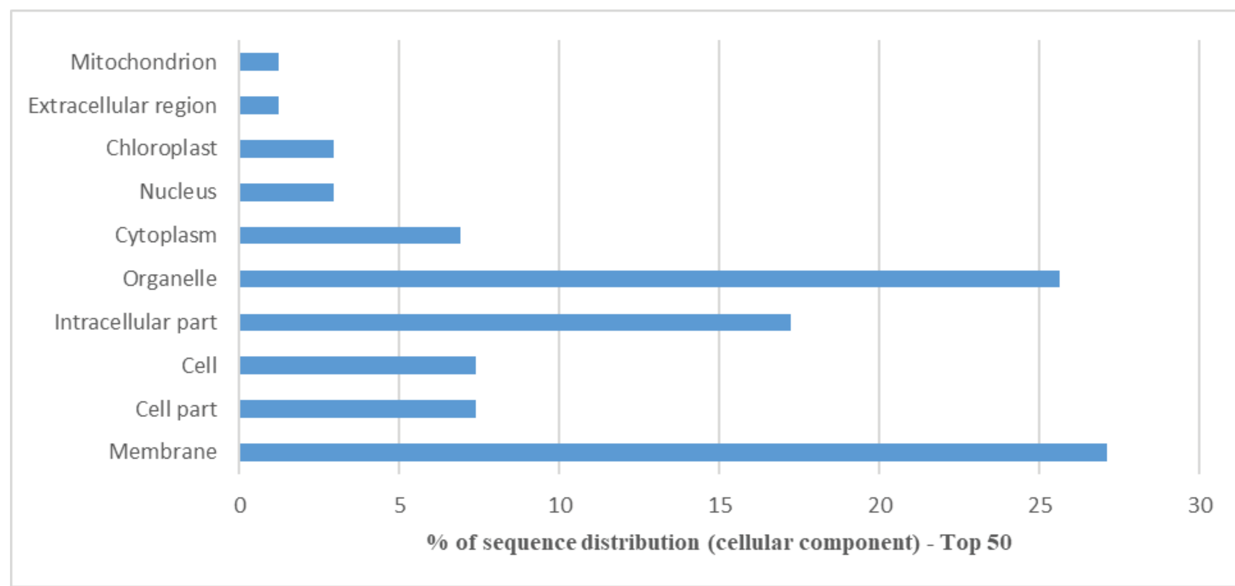


Figure 2. Gene ontology analysis. Sequence distribution of biological process (A), molecular function (B) and cellular component (C) of top 50 genes expressed in 250 µg Cu L^{-1} and 500 µg Cu L^{-1} after 7 days.

Figure 3 (edited)

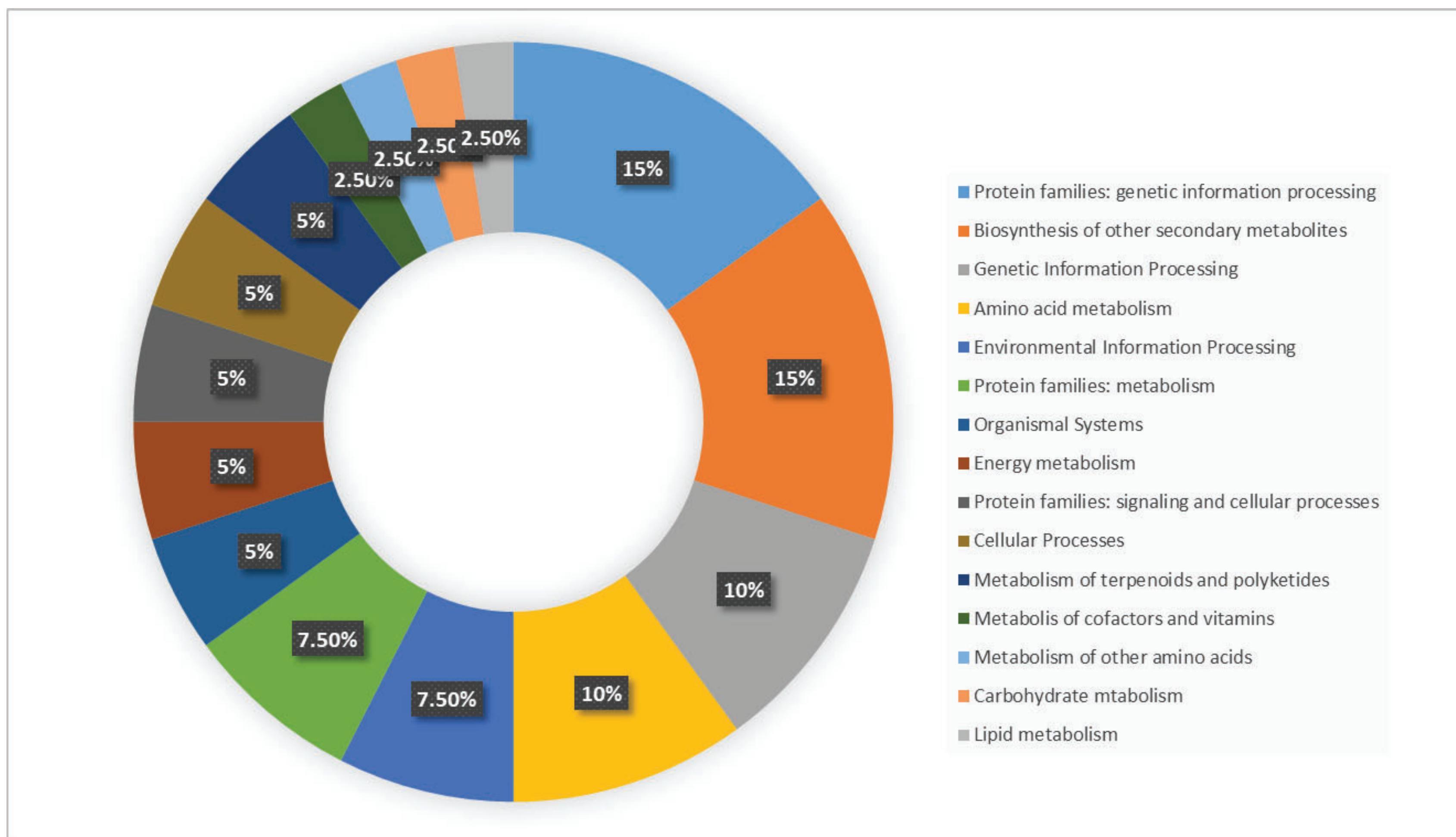


Figure 3. Most induced pathways in response to Cu stress. Functional classification of proteins in 250 µg Cu L^{-1} and 500 µg Cu L^{-1} after 7 days.

Figure 4 (edited)

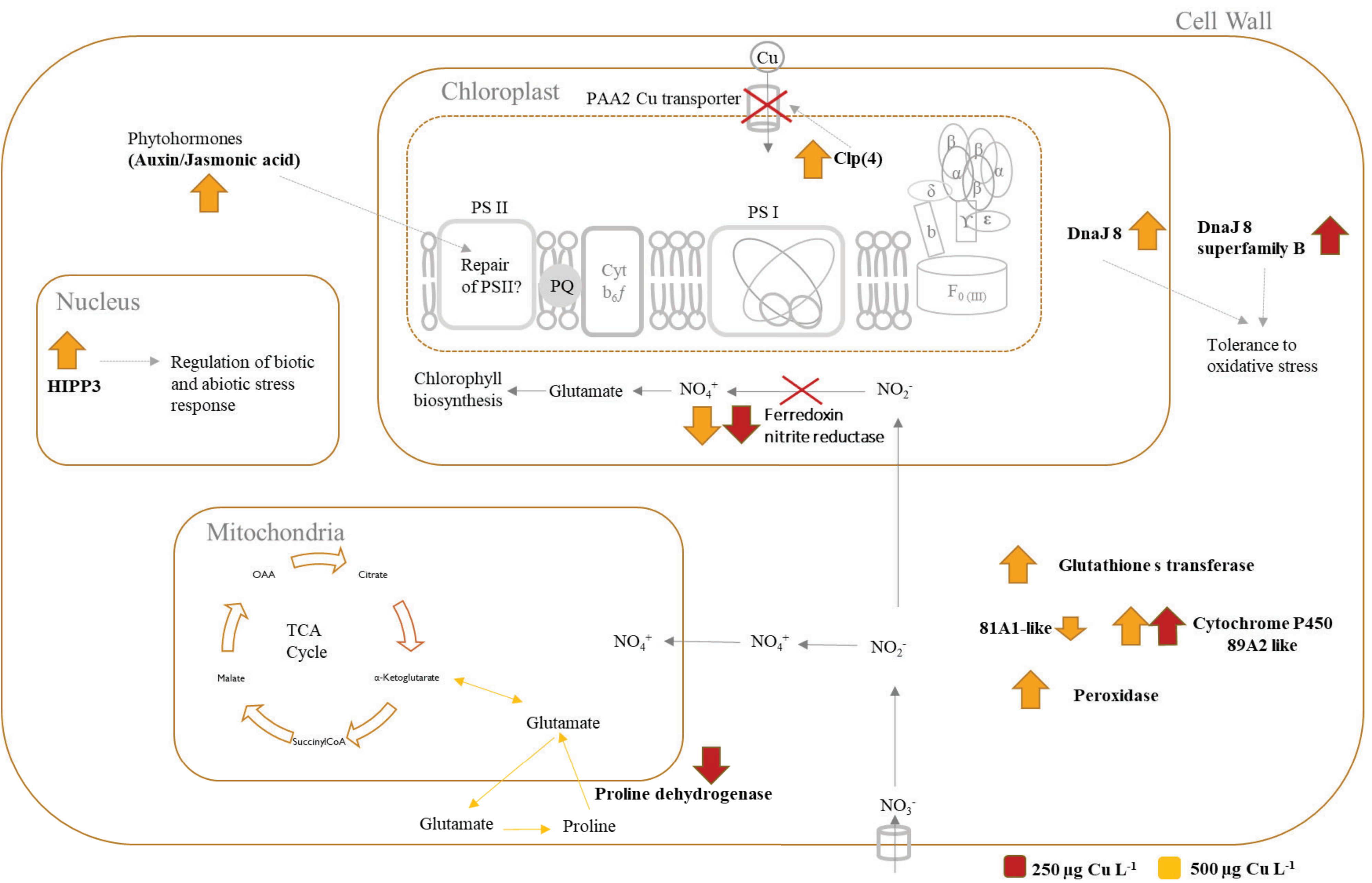


Figure 4. Possible Cu-induced pathways. Differentially expressed genes related to photosynthesis, carbon fixation, energy metabolism, enzymatic and chemical defense mechanism under 250 μg Cu L^{-1} and 500 μg Cu L^{-1}. **Chloroplast:** PAA2/HMA8: Cu transporter; CLP(4): chloroplast caseinolytic protease (Clp) subunit 4; DnaJ : Chaperone DnaJ. **Mitochondria:** TCA cycle: citric acid cycle; OAA: oxaloacetate; OAA : oxaloacetic acid. **Nucleus:** HIPP3: heavy metal-associated isoprenylated plant protein 3.