



# RNA-seq profiling of *Fugacium kawagutii* reveals strong responses in metabolic processes and symbiosis potential to deficiencies of iron and other trace metals

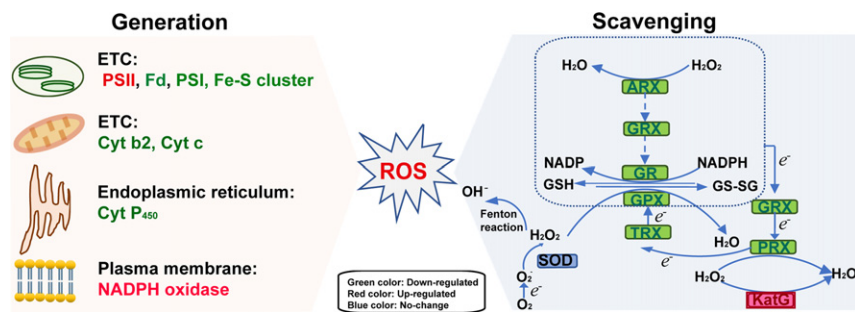
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## HIGHLIGHTS

- The influence of *Fugacium kawagutii* for trace metal in transcriptomic level exhibits the following order: Fe > Mn > Zn > Cu > Ni.
- Genes associated with extracellular matrix (ECM), cell surface structure and cell adhesion were up-regulated by trace metal deficiencies.
- Deficiency of trace metal (especially Fe deficiency) seems to repress growth and ability of ROS scavenging.
- While deficiency of trace metal seems to elevate energy metabolism, innate immunity, and cell adhesion.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

Article history:  
 Received 13 August 2019  
 Received in revised form 9 October 2019  
 Accepted 24 November 2019  
 Available online 26 November 2019

Editor: Lotfi Aleya

Keywords:  
*Fugacium kawagutii*  
 RNA-seq  
 Trace metals  
 Deficiency  
 ROS scavenging  
 Metabolism

## ABSTRACT

A healthy symbiotic relationship between corals and Symbiodiniaceae relies on suitable temperature and adequate nutrients including trace metals. Besides global warming, trace metal deficiency has been shown to cause coral bleaching, a phenomenon responsible for extensive coral reef degradation around the world. How trace metal deficiency impacts Symbiodiniaceae and coral symbiosis is poorly understood, however. In this study, we applied RNA-seq to investigate how *Fugacium kawagutii* responds to the deficiency of five trace metals (Fe<sup>2+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>). We identified 685 to 2805 differentially expressed genes (DEGs) from these trace metal deficiency conditions, among which 372 were commonly regulated by all the five trace metals and were significantly enriched in energy metabolism (e.g. fatty acid synthesis). Furthermore, genes associated with extracellular matrix (ECM), cell surface structure and cell adhesion were impacted, suggesting that the ability of recognition and adhesion of *F. kawagutii* may be altered by trace metal deficiencies. In addition, among the five metals, Fe<sup>2+</sup> deficiency exhibited the strongest influence, with Fe-rich redox elements and many antioxidant synthesis genes being markedly down-regulated, indicative of adaptive reduction of Fe demand but a compromised ability to combat oxidative stress. Overall, deficiency of trace metals (especially Fe) seems to repress

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## 1. Introduction

Mutualistic nutritional symbioses between coral animals and photosynthetic dinoflagellates of Symbiodiniaceae are the foundation of the highly biodiverse and productive coral reef ecosystem (Peixoto et al., 2017). A symbiotic Symbiodiniaceae provides its coral host with vital photosynthesis products that can meet up to 95% of the coral's energy requirements; in exchange, the coral host provides inorganic nutrients and serves as shelter (Muscatine and Porter, 1977) for the symbiont. This mutualistic relationship, however, is delicate and susceptible to the environment factors including thermal stress, high light and extreme salinity (Davy et al., 2012; Kuanui et al., 2015; Nielsen et al., 2018; Skirving et al., 2019). These stress conditions often lead to the expulsion, death, or pigment loss of the Symbiodiniaceae from the host, known as coral bleaching. Global warming and anthropogenic activities have been responsible for massive bleaching and coral degradation, and posed major threats to the precious coral reef ecosystem around the world (Hughes et al., 2017).

Reef corals live in tropical nutrient-poor waters, and they can experience limitation of nutrients, including trace metals (Measures and Vink, 2000; Obata et al., 2008). It has been shown that trace metal deficiency can also induce coral bleaching (Ferrier-Pages et al., 2018). Trace metals are essential components of electron transport chains or important cofactors for enzymes involved in various biological processes, such as chlorophyll synthesis, nitrate reduction, and photoprotection or photorepair (Twining and Baines, 2013; Andresen et al., 2018). Marine microalgae acquire trace metals from their ambient environment through the action of metal transporters and in some species by releasing chelating compounds like siderophores or similar machineries to facilitate the uptake (Morel and Price, 2003). The influence of trace metal availability on phytoplankton is well documented (e.g. Sunda, 2012; Hong et al., 2017), but its effects on Symbiodiniaceae is rarely investigated, especially at the molecular level. We recently reported that the growth of *Fugacium kawagutii* was limited by deficiency of antioxidant-associated trace metals in the following order of significance: Fe > Zn > Mn > Cu > Ni (Rodriguez and Ho, 2018), but the underlying cell metabolism remain undetermined.

In this study, we conducted transcriptome profiling for *F. kawagutii* grown under metal-replete and Fe-, Zn-, Cu-, Mn-, and Ni- deficient conditions to gain understanding on what metabolic pathways are significantly regulated by these metals and which of these metals is most influential in this species. Our results provide insights into the relative importance of these trace metals and the impact of Fe limitation on *F. kawagutii* in the context of symbiosis.

## 2. Materials and methods

### 2.1. Algal culture and trace metal treatments

*Fugacium kawagutii* (strain CCMP2468) was obtained from the National Center for Marine Algae and Microbiota, and was cultured in 500 mL polycarbonate (PC) bottles at 26 °C under a square-wave 12 h:12 h light: dark regime with 680  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  photon flux. The trace metal-defined medium was modified from the original L1 medium recipe (Guillard and Hargraves, 1993). Surface seawater collected from Taiwanese South East Asia Time Series Station (SEATS, 18°N 116°E) was passed through a column packed with Chelex® 100 resin to remove background trace metal contents and filter-sterilized using

0.22  $\mu\text{m}$  pore size filters to remove bacterial contamination prior to use. All culture vessels and other materials used for culturing were carefully washed with 2% Micro-90® solution, rinsed, soaked with 10% hydrochloric acid solution, and rinsed thoroughly with ultrapure water prepared using a Milli-Q system.

In this experiment, we designed five treatment groups and a control group to investigate the influence of availability of Cu, Zn, Mn, Ni, and Fe on the genes expression of *F. kawagutii* (Table S1). The control cultures were supplied with total dissolved metal concentrations of 250 nM for Fe and 10 nM for Cu, Mn, Ni, and Zn. These resulted in expected inorganic concentrations of 1.25 nM Fe', 0.5 pM Cu', 4.2 nM Mn', 6.7 pM Ni', and 12.5 pM Zn' upon addition of 20  $\mu\text{M}$  ethylenediaminetetraacetic acid (EDTA). The five treatment groups consisted of three treatments in which no Cu, Mn or Ni was provided (denoted as —Cu, —Mn, and —Ni, respectively) and two treatments in which either Zn or Fe was provided at 1/5 of its normal concentration (denoted as +1/5 Zn and +1/5 Fe, respectively). The differential treatments were designed because our prior physiological study indicated that total omission of Zn and Fe in the medium completely inhibited growth, but for convenience, all these conditions are described as deficiency conditions. All batch cultures were carried out in triplicates.

### 2.2. RNA extraction

When the cultures entered the mid-exponential growth phase, cells were harvested and total RNA was isolated as previously reported (Zhang et al., 2007). Briefly, cells were homogenized by using the Fastprep®-24 Sample Preparation System (MP Biomedicals, USA) with bead-beating (~ 3:1 mixture of 0.5 mm and 0.1 mm diameter ceramic beads) until sample was thoroughly homogenized (speed of 6 M/S for homogenized, 1 min/cycle for 3 cycles, chilled on ice for 1 min in between cycles) as confirmed microscopically. Then total RNA was extracted using Trizol reagent (Molecular Research Center, Inc., USA) coupled with further purification using Direct-Zol RNA Miniprep (Zymo Research, Orange, CA, USA). The concentration and the quality of extracted RNA were determined on NanoDrop (ND-2000 spectrophotometer; Thermo Scientific, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

### 2.3. Differential gene expression (DGE) profiling

The extracted RNA, with Integrity Number > 7.0, was then subjected to the RNA-seq sequencing (BGI Genomics Co., Ltd). Briefly, 1  $\mu\text{g}$  total RNA from each sample was used for poly (A)<sup>+</sup> - based selection using OligodT magnetic beads to enrich mRNA. After fragmenting the mRNA molecules using fragmentation buffer, random N6 primer was used in cDNA synthesis. Then double-stranded cDNA (dscDNA) was subjected to end-repair and 3'adenylated. The ligation products were purified and PCR amplified. Lastly the PCR products were denatured by heat and the single strand DNA is cyclized before sequencing on BGISEQ-500 platform. From the raw data, clean reads were obtained by removing low quality reads, reads containing only adapter, and reads containing poly-N in all the experimental samples. Rarefaction curve analysis was conducted to examine if depth of sequencing data was sufficient for genome-wide gene expression profile analysis.

We used *F. kawagutii* genome (Lin et al., 2015; Liu et al., 2018) and our updated reference gene database ([http://web.malab.cn/symka\\_new/index.jsp](http://web.malab.cn/symka_new/index.jsp)) and NCBI GenBank resource (accession number:

SRA148697) as the reference database. To optimize the analysis outcome, we amended the existing genome data to yield *F. kawagutii* reference gene database V3 (to be published elsewhere). Data mapping and functional annotation of the transcriptomic data were based on this new reference gene set.

#### 2.4. DEG analysis

The clean transcriptomic reads were mapped to reference gene database V3 using Bowtie (Langmead and Salzberg, 2012). The gene expression levels were calculated for each sample using RSEM (Li and Dewey, 2011) and gene with folds changes >2 and *P* value <.05 were accepted as DEGs between different samples. Significant DEGs were used to perform KEGG functional enrichment analyses with ClusterProfiler package (Yu et al., 2012). GO annotation results were visualized by WEGO 2.0 (<http://wego.genomics.org.cn>).

#### 2.5. RT-qPCR

Totally, 15 genes were selected for expression quantification using reverse-transcription quantitative PCR (RT-qPCR) to verify RNA-seq results. For each sample, 400 ng total RNA was used in cDNA synthesis using PrimeScript™ RT reagent Kit (Takara, Clontech, Japan) that contained the genomic erase buffer. Specific primers were designed (Table S2) based on the gene sequences and synthesized by BGI Genomics Co., Ltd. RT-qPCR was performed using iTaq™ Universal SYBR® Green Supermix on a CFX96 Real-time PCR System (Bio-Rad Laboratories, Hercules, USA). The reaction was carried out in a total volume of 12 µL containing 6 µL Supermix, cDNA equivalent to 5 ng of total RNA and 2.5 µM of each primer (Li et al., 2018).

#### 2.6. Statistic analysis

In order to evaluate the statistical significance of the differences observed between control and trace metal deficient groups, analysis of variance was carried out using SPSS statistics software package. All data presented are means with standard deviation calculated from the triplicated cultures in each trace metal deficient group or the control group.

#### 2.7. Accession number

All raw sequencing data from this study have been deposited in the GenBank's Sequence Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/>) under the accession number PRJNA550184.

### 3. Results

#### 3.1. Differential gene expression profiles under metal deficiencies

RNA-seq yielded 22 M clean reads for each of the six triplicated (totally 18) cultures. Approximately 61% of the RNA-seq reads found matches in reference gene database V3 (Table S3). From our RNA-seq data, peridinin chlorophyll-*a* binding protein (PCP), high-affinity nitrate transporter, nitrate transporter, ribulose biphosphate carboxylase/oxygenase (Rubisco), and fucoxanthin-chlorophyll *a-c* binding protein B

were highly and commonly expressed genes across all the trace metal culture conditions. And 685 to 2805 differentially expressed genes (DEGs) were identified for the trace metal deficient conditions, which accounted for 1.2% to 4.7% of total detected genes (Table 1). Of these, 372 DEGs (Table S4) were shared in five trace metal deficient conditions and significantly enriched (*P* < .05) in seven KEGG pathways including fatty acid biosynthesis, fatty acid metabolism, pyruvate metabolism, propanoate metabolism, nitrogen metabolism, glutamate metabolism and biosynthesis of amino acids (Fig. 1A, B).

When all the KEGG pathways that were enriched by DEGs found in any of the five trace metal deficient conditions were considered together, 27 pathways were identified, 18 of which were specific for the Fe-deficient condition (Fig. 1C). For instance, MAPK signaling pathway, autophagy, long-term depression, circadian entrainment and nucleotide excision repair were specifically regulated by the Fe deficiency, while RNA degradation pathway was only enriched in the Cu-deficient condition (Fig. 1C). Interestingly, no significantly enriched KEGG pathway was found specifically from Zn-, Mn- or Ni-deficient conditions. The exception was the up-regulation of mRNA surveillance pathway, which was significantly enriched by DEGs under deficiencies of Zn, Mn, and Fe. GO analysis, on the other hand, showed that the Fe-deficient group uniquely exhibited enrichment of rhythmic process, development process and virion, and the Mn-deficient group was uniquely enriched in molecular function regulator (Fig. 1D). This is despite the fact that the generally low GO annotation rate (14% - 17%) caused the GO enrichment profiles to be not significant (*P* > .05) for any of the metal deficient conditions.

#### 3.2. Transporter of trace metals and other nutrients

Totally, 42 transport related genes were identified from our transcriptomic data. Among these genes, 31 showed differential expression under Fe deficiency, 11 under Mn deficiency, 9 under Zn deficiency, 6 under Cu deficiency and 6 under Ni deficiency (Fig. S1, Table S5). For example, iron permease, ABC transporter A/F/G families, ammonium transporter 1/3, ammonia channel, amino acid permease and urea-proton symporter were up-regulated under Fe deficiency. In contrast, zinc transporter 5, magnesium transporter, nitrate transporter 2.5, ABC transporter B family and sulfate permease were down-regulated under Fe deficiency. Under deficiency of the other four metals, most of these transporter genes were not regulated except for ABC transporter A family that was regulated under all five trace metal deficiencies.

#### 3.3. Molecular components dependent on trace metal

Trace metals are essential cofactors of many enzymes. Here we found 43 genes specifying Fe-dependent enzymes that were regulated under Fe deficiency, 17 Mn-requiring enzyme genes that were regulated under Mn deficiency and 48 Zn-requiring genes that were regulated under Zn deficiency (Fig. 2, Table S6). However, no Cu- or Ni-dependent enzymes, even Cu-SOD and urease, showed regulation under Cu or Ni deficiencies respectively, suggesting possible replacement by functionally equivalent enzymes not requiring these trace metals. Interestingly, we found that most of the genes requiring iron cation or 4Fe—4S cluster as cofactors were down-regulated but genes requiring 2Fe—2S cluster or 3Fe—3S cluster were up-regulated under Fe-deficient condition, indicating an adaptive strategy to reduce Fe demand (Fig. 2A). All Mn- or Zn-dependent genes were up-regulated under Zn or Mn deficiencies (Fig. 2B), respectively, suggesting that these two metals were probably functionally interchangeable as cofactors.

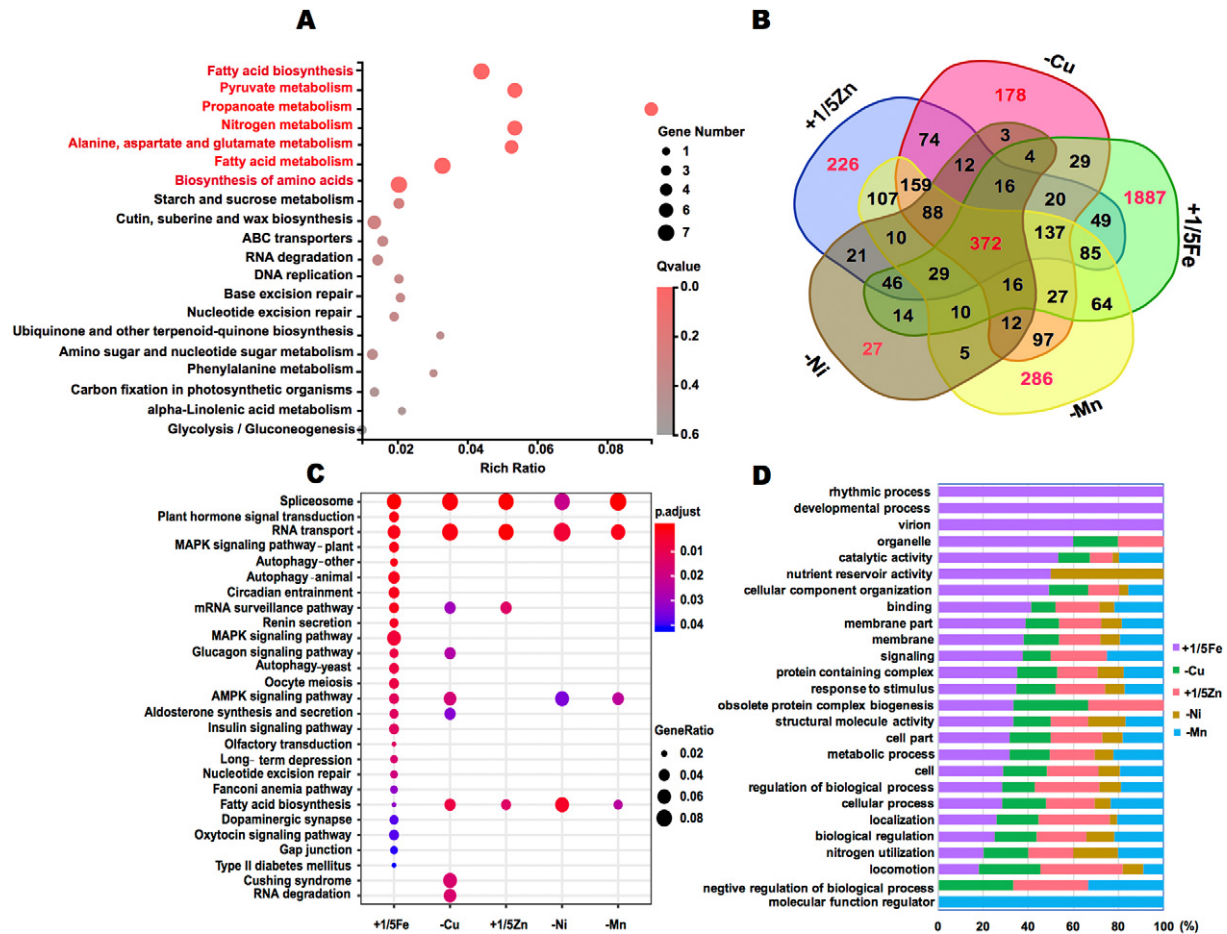
Nitrate is the only N source in our culture, so nitrite reductase requiring 4Fe—4S cluster as cofactor plays important roles in nitrate metabolism. Down-regulated nitrite reductase and nitrate transporter mentioned above, indicating nitrate assimilation was depressed under Fe deficiency (Fig. 2). Furthermore, ammonia channel, ammonium

**Table 1**  
Number of DEGs under trace metal deficiencies.

Samples for comparison	Up regulated	Down regulated	Total regulated (%) <sup>a</sup>
Control vs. +1/5Fe	1,435	1370	2,805 (4.7%)
Control vs. -Mn	1,468	36	1,504 (2.5%)
Control vs. +1/5Zn	1,402	49	1,451 (2.5%)
Control vs. -Cu	1205	39	1,244 (2.1%)
Control vs. -Ni	653	32	685 (1.2%)

<sup>a</sup> DEG account for total detected genes.





**Fig. 1.** Overall differential gene expression profile. (A) KEGG enrichment of 372 DEGs common regulated by five trace metal deficiencies. Red fonts depict significant enriched pathways ( $P < .05$ ) and the color strength represents the Q-value. (B) Venn diagram of DEGs in five low trace metal deficiencies. (C) Significant KEGG enrichment of DEGs from each trace metal deficiency ( $P < .05$ ), the color strength represents the adjusted p-value. (D) Relative abundance of GO terms (%) found in deficiency of each trace metal.

transporter 1/3, urea-proton symporter and glutamate synthase [NADH] were up-regulated, clearly a metabolic response by shifting to use ammonium or urea that do not require the Fe-dependent nitrite reductase (Fig. S1, Fig. 2).

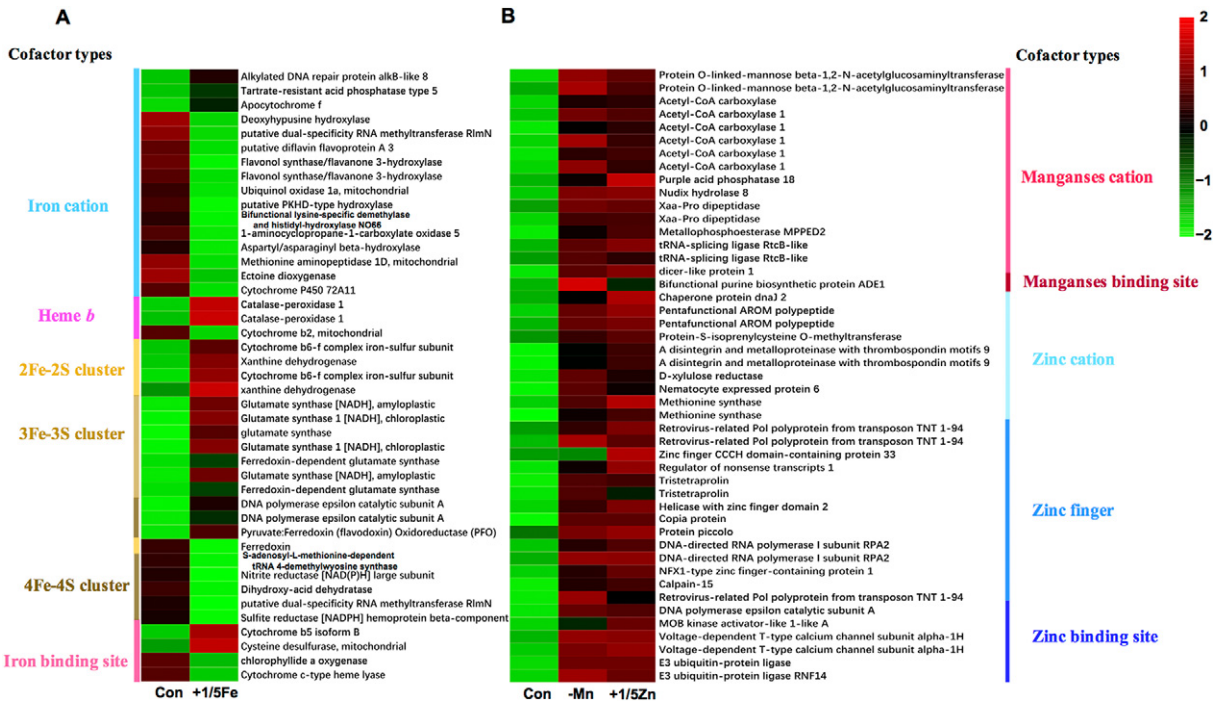
### 3.4. Electron transport chain (ETC) and energy metabolism

The ferredoxin and cytochrome *b6-f* complex iron-sulfur subunit (Fig. 2A) are main components of the ETC in the chloroplast, where  $Mn^{2+}$  is a cofactor of water-oxidizing complex (WOC) of photosystem II and  $Cu^{2+}$  is a cofactor of plastocyanin. In search for DEGs involved in photosynthetic electron transfer chain, we found photosystem I reaction center (PSI), ATP synthase (chloroplastic), and ferredoxin were down-regulated under Fe-deficient condition, while cytochrome *b6f* complex, fucoxanthin-chlorophyll *a-c* binding protein A/F, photosystem II reaction center (PSII) and Rubisco were up-regulated under Fe-deficient condition (Fig. 3). For the cell respiratory system, cytochrome *b2* and ATP synthase mitochondrial F1 complex assembly factor 2 were significantly down-regulated (fold change  $>2$ ,  $P < .05$ , as defined earlier in the paper), and cytochrome *c*-type heme lyase and ATP synthase (mitochondria) showed less dramatic down-regulation (fold change between 1.5 and 2,  $P < .05$ ) under Fe-deficient condition (Fig. 3). Most of these chloroplastic and mitochondrial ETC genes showed no changes in expression under deficiency of one of the other four trace metals, suggesting that ETC was influenced only by iron among the five trace metals examined.

The regulation of genes involved in ETCs and significant enrichment of fatty acid biosynthesis in KEGG pathways was indicative of Fe dependency of energy metabolism (Fig. 1). Further analysis revealed up-regulation of most genes involved in glycolysis and fatty acid biosynthesis under Fe deficiency, e.g. pyruvate kinase, pyruvate dehydrogenase, acetyl-CoA carboxylase, fatty acid desaturase, erythronolide synthase and NADP-dependent malic enzyme (Fig. 3).

### 3.5. ROS scavenging ability, immunity, stress responses

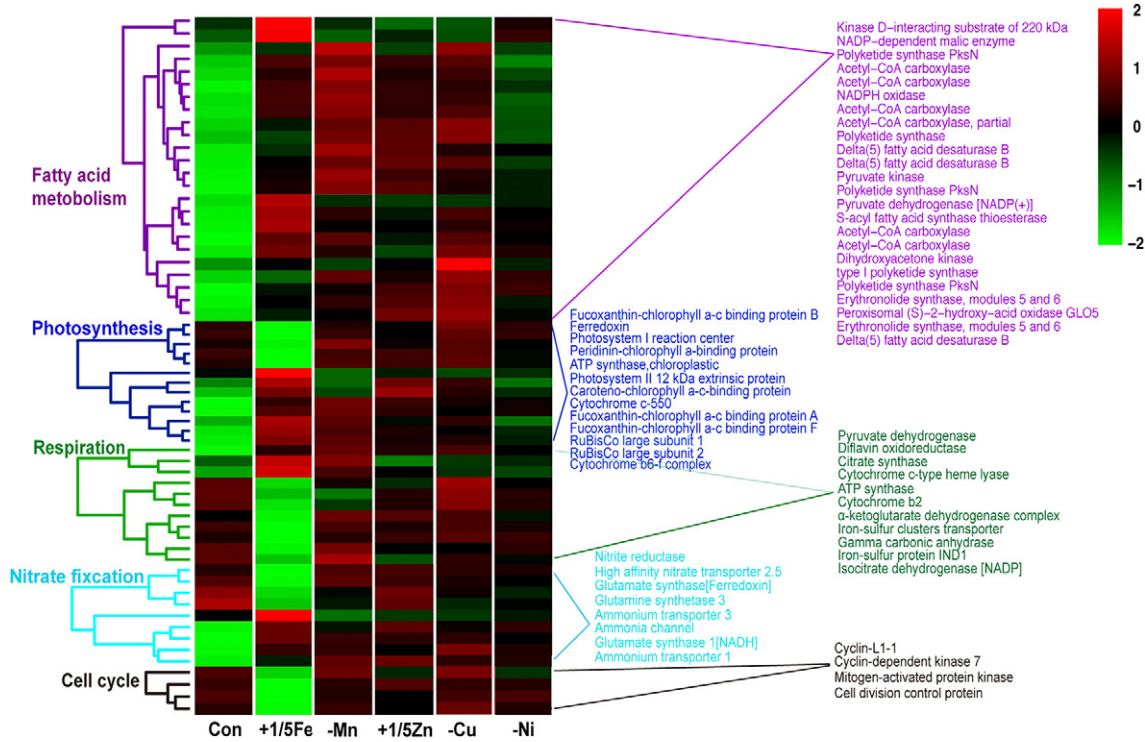
PSII and ferredoxin are reactive oxygen species (ROS) source site in photosynthetic organisms (Karuppanapandian et al., 2011; Pospíšil, 2017). A complex interplay between the host and the symbiont in a symbiotic entity is evident in response to oxidative stress (Lesser, 1997; Baird et al., 2009). We searched for ROS scavenging genes and found 30 genes that showed strong regulation (8; fold change  $>2$ ,  $P < .05$ , as defined earlier in the paper) or moderate regulation (22, fold change between 1.5 and 2,  $P < .05$ ) under trace metal deficiencies, especially under Fe deficiency (Table S7). Among the up-regulated genes, catalase-peroxidase (*katG*) was strongly up-regulated under Fe deficiency and moderately up-regulated under other four trace metal-deficient conditions, while zeta-carotene-forming phytoene desaturase (*zcfpd*) was strongly up-regulated under Mn deficiency and moderately up-regulated under other four trace metal-deficient conditions. Among the down-regulated genes, glutathione S-transferase L1 (*gstl*), thioredoxin (*trx*), peroxiredoxin (*prx*) and cytochrome P450 were strongly down-regulated under Fe deficiency, while glutathione



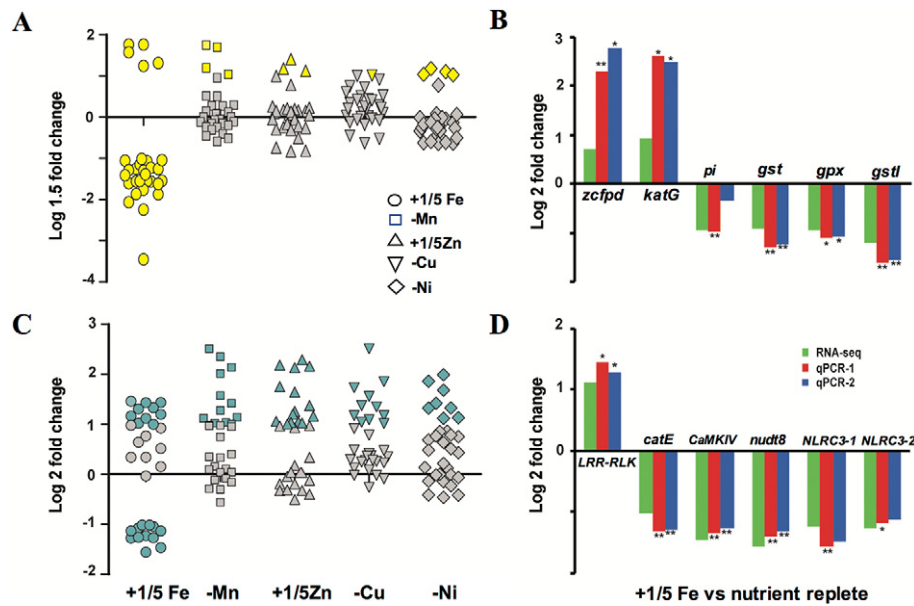
**Fig. 2.** Heatmaps showing changes in the expression levels of molecular components in response to deficiencies of trace metals. (A) Molecular components responding to iron deficiency. (B) Molecular components responding to deficiencies in manganese or zinc. The heatmap color strength represents homogenized gene expression (FPKM), from green (lowest), dark, red (highest). Con, control.

peroxidase (*gpx*), glutathione reductase, glutathione S-transferase (*gst*), ascorbate peroxidase (*apx*), peroxidase (*px*), and prolycopene isomerase (*pi*) were moderately down-regulated under Fe deficiency and no

regulation was observed under deficiencies of other four trace metals (Fig. 4A). Of these, *zcfpd*, *katG*, *pi*, *gpx*, *gst* and *gstl* were selected for further validation using RT-qPCR (Fig. 4B). *F. kawagutii* contains a diverse



**Fig. 3.** Heatmap showing changes in expression levels of genes involved in five functional categories. The heatmap color strength represents homogenized gene expression (FPKM), increasing from green (lowest), dark, to red (highest). Every cluster (left) represents one functional category. Con, control.



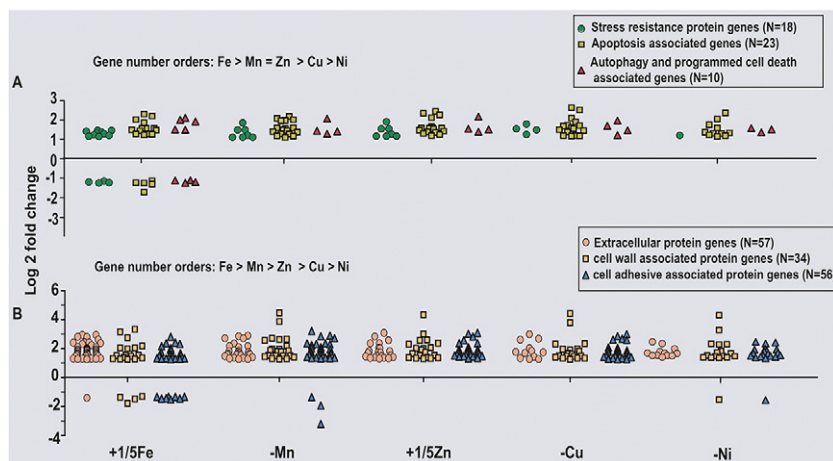
**Fig. 4.** Expression fold changes of selected genes under deficiencies of trace metals. Horizontal lines depict no change in expression, above line are genes up-regulated and below the line are genes down-regulated under trace metal deficiencies. (A) Genes involved in ROS scavenging under five trace metal deficiencies. Yellow color indicates fold change >1.5 and gray color indicated fold change <1.5. (B) RT-qPCR validation of six genes involved in ROS scavenging. *Zcfpd*: zeta-carotene-forming phytoene desaturase; *katG*: catalase-peroxidase; *pi*: polycopene isomerase; *gst*: glutathione S-transferase; *gpx*: glutathione peroxidase; *gstl*: glutathione S-transferase L1. (C) Genes involved in immune response under five trace metal deficiencies. Light blue means fold change >2. (D) RT-qPCR validation of six genes involved in innate immunity. *LRR-RLK*: leucine-rich repeat receptor-like protein kinases; *catE*: cathepsin E; *CaMKIV*: calcium/calmodulin-dependent protein kinase type IV; *nudt8*: nudix hydrolase 8; *NLRC3-1* and *NLRC3-2*: protein NLRC3. Note: qPCR-1 means *gapdh* as reference gene and qPCR-2 means *tubulin* as reference gene; \* $P < .05$ , \*\* $P < .01$ .

set of superoxide dismutase (SOD; Cu/Zn-, Mn/Fe-, and Ni-dependent) encoding genes (Lin et al., 2015). Here we identified 9 SODs, including 1 Fe/Mn- dependent, 7 Mn- dependent and 1 Cu/Zn- dependent SODs, but they displayed no differential expression under any of the five trace metal deficiencies relative to the control.

The innate immunity in scleractinian corals is important in regulating coral-Symbiodiniaceae symbiosis (Kvennefors et al., 2010; Zhou et al., 2017). It follows that molecules in the symbiont that have influence on the innate immunity may have impacts on the maintenance of the symbiosis. In this study, we found 31 immune associated genes, such as protein NLRC3 (*NLRC3-1* and *NLRC3-2*), nudix hydrolase 8 (*nudt8*), Cathepsin E (*catE*), calcium/calmodulin-dependent protein kinase type IV (*CaMKIV*), leucine-rich repeat receptor-like protein kinases (*LRR-RLK*) and ankyrin repeat domain-containing protein 17, that were regulated under trace metal deficiencies (Fig. 4C). Among them, 8 genes annotated as protein NLRC3, which is a negative regulator of the innate immune response, were down-regulated under Fe deficiency

(Table S8). No down-regulated (fold change <2) immunity associated gene was found in other four metal deficient conditions. Of these, *NLRC3-1*, *NLRC3-2*, *nudt8*, *catE*, *CaMKIV* and *LRR-RLK* were selected for further validation by RT-qPCR (Fig. 4D).

In addition, we found that 18 stress resistance genes, 22 apoptosis genes and 10 autophagy or programmed cell death associated genes were regulated under five trace metal deficient conditions (Fig. 5A, Tables S9, S10, S11). Of these, multidrug resistance-associated protein 1, protein FAM3B, mitogen-activated protein kinase kinase A, mitogen-activated protein kinase kinase 3, poly [ADP-ribose] polymerase 11 and autophagy protein 2 were up-regulated under deficiencies of five trace metals and no gene was down-regulated. In response to Fe deficiency, heat shock protein sti1-like, beta-glucosidase 42, chaperone protein DnaJ 2, zeaxanthin epoxidase, DnaJ-like subfamily C member 7, protein N-lysine methyltransferase METTL21A were up-regulated and heat shock protein-like pss1, Hsp90 co-chaperone Cdc37, WW domain-containing oxidoreductase, general stress protein



**Fig. 5.** Expression fold changes of selected genes regulated by trace metals. (A) Summary of genes involved in stress response under trace metal deficiencies. (B) Summary of genes involved in extracellular protein, cell wall synthesis or reconstruction and cell adhesive. N indicates gene number. Detailed information is available in tables S9 to S11.



13, WD40 repeat-containing protein and bifunctional apoptosis regulator were down-regulated.

3.6. Extracellular matrix (ECM), cell wall, and cell adhesion

The extracellular matrix (ECM) probably contains both proteins and polysaccharides, which play important roles in cell adhesion, cell-cell interaction and cell communication between symbiodiniaceae and host. In this study, 57 genes encoding extracellular proteins (e.g. extracellular matrix protein FRAS1, heparanase, outer membrane protein PmpB, fibroin heavy chain-like and SCO-spondin) and 56 cell adhesion or cell-cell recognition associated genes (e.g. adhesive plaque matrix protein, P-selectin, tenascin-X, classical arabinogalactan protein 9-like and fibronectin type-III domain-containing protein 3A) were found to significantly change expression under trace metal deficient conditions, especially under Fe deficiency (Fig. 5B, Table S12). Most of these were up-regulated except for outer membrane protein pmp6, integral membrane protein, reticulocyte-binding protein 2-like a, phosphatidylinositol 4-phosphate 5-kinase 4 and endoglucanase EG-1, which were down-regulated, under Fe deficiency. Among these cell adhesive or cell-cell recognition associated genes, 35 genes were responsive to Fe deficiency (27 up-regulated), 37 genes to Mn deficiency (34 up-regulated), 34 genes to Zn deficiency (32 up-regulated), 32 genes to Cu deficiency (32 up-regulated), and 20 genes to Ni deficiency (19 up-regulated). Six genes annotated as Svp1 (Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1), a novel cell adhesion molecule (Shefer and Benayahu, 2010), were also identified and found to be up-regulated by trace metal deficiencies (Fig. 5B, Table S13).

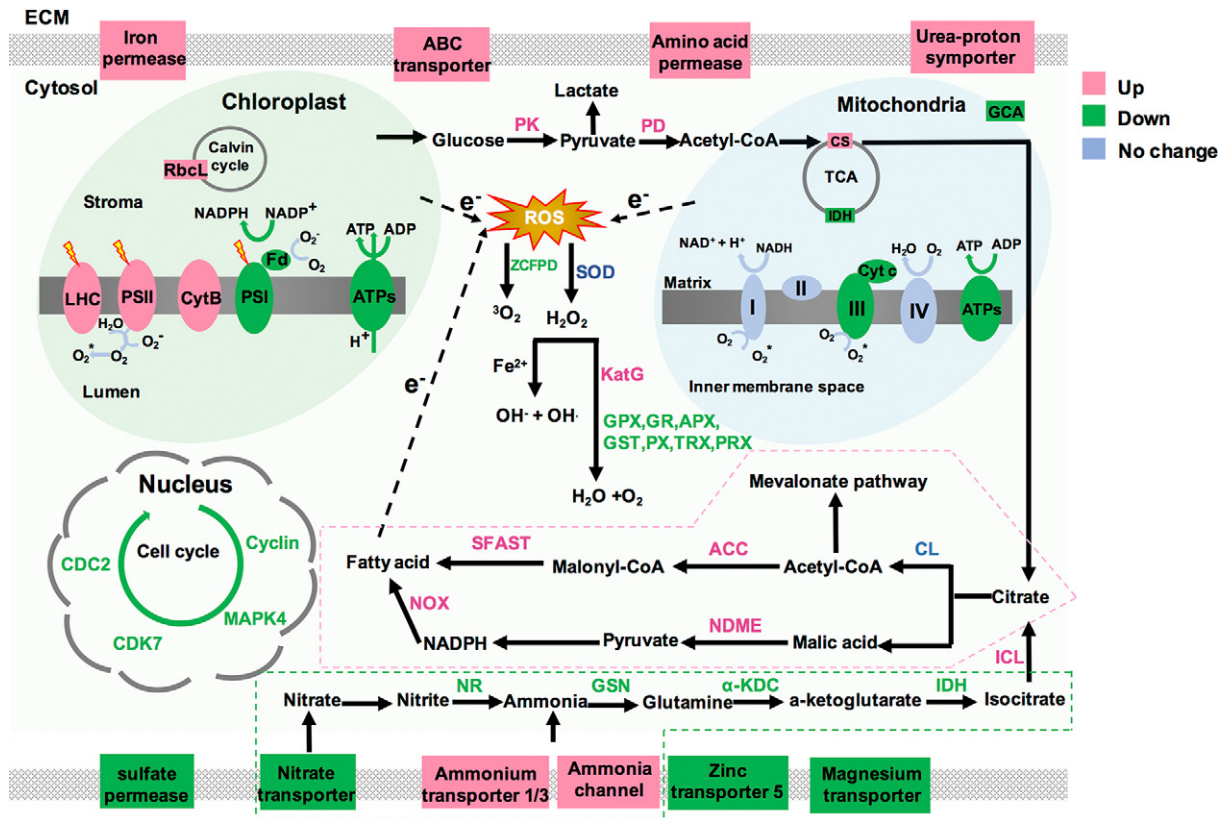
In addition, we found 34 genes encoding cell wall synthase or cell wall structure protein genes that were responsive to trace metal deficient conditions (Fig. 5B). Of these, genes encoding cell wall alpha-1,3-glucan synthase mok12, D-xylulose reductase, Callose synthase 7, D-alanine:D-alanine ligase, glucuronoxylan glucuronosyltransferase IRX7, glycine-rich cell wall structural protein-like and vegetative cell wall protein gp1-like were up-regulated under five trace metal deficiencies (Table S14). Together these results suggest that ECM and cell wall are sensitive to ambient stress rendering cell adhesion ability changeable as a result. Comparative analysis of total DEGs number revealed the influence by trace metal availability in the following order of significance, Fe > Mn > Zn > Cu > Ni.

3.7. Cell cycle genes

Under Fe deficiency condition, cyclin-dependent kinase 7 (CDK7), cyclin-L1-1, mitogen-activated protein kinase 4 (MAPK4) and cell division control protein 2 (CDC2) were down-regulated (Figs. 3, 6), consistent with the decrease in cell growth rate under Fe deficiency (Rodriguez and Ho, 2018). CDK7 is a regulator of the G1 to S phase transition of the cell cycle (Devos et al., 2015), and its down-regulation suggests that the cell cycle may be arrested in G1 phase under Fe deficiency. Interestingly, all these cell cycle genes showed no changes in expression level under deficiencies of other four trace metals.

3.8. Validation of RNA-seq results using RT-qPCR

We first developed reference genes by testing the expression stability of the candidate genes *gapdh*, *tubulin* and *actin*. These genes were



**Fig. 6.** Schematic representation of biological pathways in *F. kawagutii* under Fe- deficient condition. Photosystem II 12 kDa extrinsic protein (PSII), Rubisco (RbcL), cytochrome b6-f complex (CytB), photosystem I reaction center (PSI), ferredoxin (Fd); ATP synthase (ATPs), light harvest protein (LHC), pyruvate kinase (PK), pyruvate dehydrogenase (PD), citrate synthase (CS), isocitrate dehydrogenase (IDH), cytochrome b2 (III), cytochrome c-type heme lyase (CytC), gamma carbonic anhydrase(GCA), zeta-carotene-forming phytoene desaturase (ZCFPD), superoxide dismutase (SOD), catalase-peroxidase (KatG), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), ascorbate peroxidase (APX), peroxidase (PX), thioredoxin (TRX), peroxiredoxin (PRX), cyclin-dependent kinase 7 (CDK7), cyclin-L1-1 (Cyclin), cell division control protein 2 (CDC2), mitogen-activated protein kinase 4 (MAPK4), isocitrate lyase (ICL), citrate lyase (CL), acetyl-CoA carboxylase (ACC), S-acyl fatty acid synthase thioesterase (SFAST), NADPH oxidase (NOX), NADP-dependent malic enzyme (NDME), nitrite reductase (NR), glutamine synthase (GSN), α-ketoglutarate dehydrogenase complex(α-KDC), isocitrate dehydrogenase (IDH).

selected as candidates because they showed no significant changes in expression between replete and depleted conditions of the five trace metals in RNA-seq. RT-qPCR and geNorm (Vandesompele et al., 2002) were performed for these genes, and results showed that the *gapdh* and *tubulin* were stable in expression and we used them as reference genes for normalizing target gene expression (Fig. S2). Then we selected 6 genes associated with ROS scavenging (*zcfpd*, *katG*, *pi*, *gpx*, *gst* and *gstI*) and 6 gene associated with innate immunity (*NLRC3-1*, *NLRC3-2*, *nudt8*, *catE*, *CaMKIV* and *LRR-RLK*) for RT-qPCR. Results showed that these 12 genes all showed consistent expression patterns between the RT-qPCR and the RNA-seq results (Fig. 4B, D).

#### 4. Discussion

The symbiosis between corals and Symbiodiniaceae relies on compatible host-symbiont recognition and nutrient exchange, which are vulnerable to external environmental stressors including seawater warming, excess light, and eutrophication or nutrient deficiency (Ferrier-Pages et al., 2018; Skirving et al., 2019). Corals in tropical seas characteristically experience low levels of major inorganic nutrients such as nitrogen and phosphorus (Muscatine and Porter, 1977) and low concentration of essential trace metals (Measures and Vink, 2000; Obata et al., 2008). Recent studies indicated that trace metal deficiency could lead to coral bleaching (Ferrier-Pages et al., 2018) and bleaching increased under Fe deficiency (Shick et al., 2011), demonstrating that Fe and perhaps other trace metals as well are essential to maintaining the mutualistic relationship between corals and symbiodiniacean symbionts. In response to the dearth of information on molecular responses of Symbiodiniaceae to trace metal deficiency, we used *F. kawagutii* as a model and examined its responses to deficiency of five trace metals.

##### 4.1. Overall response under trace metal deficiencies

The slower growth rates of *F. kawagutii* under trace metal deficient conditions (Rodriguez and Ho, 2018) and the elevated expression of many stress genes under trace metal deficient conditions found in the present study together clearly indicated that the cultures were under trace metal stress. Under the deficiencies of the five trace metals examined in this study, 372 shared DEGs were regulated and significantly enriched in seven KEGG pathways (Fig. 1A), indicating that these are likely common response to trace metal stress, some of which might even be general stress response. The present study further indicated that the number of DEGs identified by comparing trace metal deficient cultures and control cultures was the greatest in Fe deficiency, then decreasing in the following order, Fe > Mn > Zn > Cu > Ni (Table 1). This result is broadly in line with previous report that the demand of *F. kawagutii* for the trace metals is also the highest for Fe, and decreases in the following order: Fe > Zn > Mn > Cu > Ni (Rodriguez and Ho, 2018). Thus, the DEG numbers generally reflect the relative importance of individual trace metal to *F. kawagutii* growth. However, it is of interest to note that Mn was ranked higher than Zn with respect to DEG numbers but lower than Zn with respect to growth demand. This discrepancy suggests that Zn is needed in greater amount but probably can be replaced by other metals whereas Mn is needed in smaller amount but its deficiency induced broader metabolic modification.

In dinoflagellates, <30% of genes have been shown to exhibit transcriptional changes, and the changes are relatively small, in response to environmental fluctuations or stresses (Lin, 2011; Barshis et al., 2014). Despite the general low expected transcriptional regulation, the finding that only 1.2% to 4.5% of detected genes were regulated under deficiencies of the 5 trace metals examined was surprising. There are two possibilities. First, the majority of genes in *F. kawagutii* may be regulated translationally or post-translationally. Second, the influence of deficiencies of the 5 trace metals is restricted to a small number of genes and pathways. Based on general dinoflagellate peculiar characteristics, the first possibility is more likely, underscoring the

importance of proteomic approach to address complete molecular regulation of stress response in this dinoflagellate in the future.

A small number of genes are highly represented in the EST datasets in dinoflagellate, e.g. *K. brevis* (Lidie et al., 2005) and *A. catenella* (Uribe et al., 2008). In *F. kawagutii*, PCP, high affinity nitrate transporter, nitrate transporter, Rubisco, fucoxanthin-chlorophyll *a-c* binding protein were highly and commonly expressed genes in nutrient replete and trace metal deficient conditions. These genes are important in light harvesting and nitrate utilization. And among these genes, PCP has been reported to be highly expressed in at least three dinoflagellate cultures, e.g. *Symbiodinium* Clade C3, *Lingulodinium polyedrum* strain 70 and *Alexandrium tamarense*-EST1 (Lin, 2011), indicating that dinoflagellates commonly have a high ability of light harvesting and photoprotection. The higher expression of nitrate transporters than ammonium transporter is particularly interesting, because in the genome of this species, NH<sub>4</sub> transporter gene is more abundantly duplicated than NO<sub>3</sub><sup>-</sup> transporter gene (Lin et al., 2015). The inconsistency suggests that while the species has a high potential of utilizing NH<sub>4</sub> (perhaps when living in symbiotic association with a host), when grown free of host and with NO<sub>3</sub><sup>-</sup> medium, gene expression strongly favors NO<sub>3</sub><sup>-</sup> transporter over NH<sub>4</sub> transporter.

##### 4.2. Lipid metabolism and cell surface features

Fatty acid content has previously been documented in Symbiodiniaceae in attempts to link thermal tolerance to thylakoid membrane lipid composition (Tchernov et al., 2004) and to develop lipid biomarker for stress (Kneeland et al., 2013). A recent transcriptome analysis of Symbiodiniaceae also revealed differences in expression of fatty acid metabolism and biosynthesis pathway genes potentially related to membrane composition and energy storage (Parkinson et al., 2016). In this study, we found fatty acid synthesis and metabolism was significantly enriched under trace metal deficiencies. And it is worth noting that 3 genes encoding delta fatty acid desaturase enzyme previously reported under thermal stress in *Fugacium* (Gierz et al., 2017) were found to be up-regulated under trace metal deficiencies (Fig. 3). Fatty acid desaturases are involved in synthesis of polyunsaturated fatty acid (PUFA), which are important in the acclimation of microorganisms to various ambient temperatures (Los and Murata, 1998). This implies the trace metal deficiencies may have similar influence as thermal stress on PUFA synthesis and changing membrane lipid composition may be a common response to ambient stress of Symbiodiniaceae. Most genes associated with membrane composition were regulated by deficiency of trace metals, further confirming the membrane composition was sensitive to change under metal deficiencies. Transmembrane protein 65 and outer membrane protein PmpB, for instance, were found to be up-regulated, and integral membrane protein transmembrane and coiled-coil domain-containing protein 4 were down-regulated. Thus, fatty acid biosynthesis and metabolism was significantly regulated by trace metal deficiencies, thermal stress (Gierz et al., 2017; Lin et al., 2019) and light intensity changes (Xiang et al., 2015), suggesting in concert that the membrane lipid composition of Symbiodiniaceae (even the thermal-resistant strains such as *F. kawagutii*) is sensitive to environmental stress.

For Symbiodiniaceae as endosymbionts, the ECM, which probably contains both proteins and polysaccharides, plays important roles in cell adhesion, cell-cell interaction and cell communication between symbionts and their hosts. It has been known that a novel cell adhesion molecule Svp1 (Shefer and Benayahu, 2010) is significantly reduced by increasing or decreasing light density in Symbiodiniaceae SSB01 cells, and this was implicated in influencing the potential of symbiosis (Xiang et al., 2015). While in the present study, we found that six genes encoding Svp1 were up-regulated under trace metal deficiencies in *F. kawagutii*. We also found 23 genes encoding the ECM protein, 3 genes encoding cell adhesive plaque matrix protein, 10 genes encoding trophinin and 1 gene encoding P-selectin were up-regulated under



trace metal deficiencies. One gene annotated as classical arabinogalactan protein 9-like, which is implicated in diverse developmental roles such as differentiation, cell-cell recognition, cell signaling and programmed cell death (Schultz et al., 2000), was also up-regulated under trace metal deficiencies. In addition, cell wall structure of Symbiodiniaceae was sensitive to light density (Xiang et al., 2015), exogenous glucose (Xiang et al., 2017), heat stress (Lin et al., 2019). In present study, glycine-rich cell wall structural protein-like, cell wall alpha-1,3-glucan synthase mok11/12, vegetative cell wall protein, pollen-specific leucine-rich repeat extensin-like protein 1 and D-alanine:D-alanine ligase (DDI) involved in cell-wall biosynthesis were up-regulated under deficiency of trace metals, suggesting that the cell wall structure can also be influenced by trace metals. Taken together, our data along with the previous data of others suggest that the ability of recognition and cell adhesion in *F. kawagutii* may be changed by trace metal deficiencies, especially in Fe deficiency. These have implications in the symbiotic relationships of *F. kawagutii* with corals or other organisms.

#### 4.3. Molecular components dependent on trace metals

Trace metals are required for many processes, including nitrate and nitrite reduction, chlorophyll synthesis, and the ETC of respiration and photosynthesis (Twining and Baines, 2013). Iron is involved in photosynthetic components of PSII, PSI, Cytochrome *b<sub>6</sub>f* complex and ferredoxin (Shi et al., 2007) and hence is a limiting nutrient for primary production on coral reefs (Sakka et al., 1999; Obata et al., 2008). Molecular studies have revealed that iron limitation in phytoplankton up-regulates PSII protein abundance (Hong et al., 2017) and impairs the electron transport capacity of PSII (Petrou et al., 2014). In our study, most DEGs identified in the photosystem were down-regulated under Fe deficiency, indicative of depressed photosynthetic capacity. It has been known that the ferredoxins and other carriers on the reducing side of PSI have sufficiently negative electrochemical potentials to donate electrons to oxygen resulting in the formation of ROS (Arora et al., 2002). The PSII, Cytochrome *b<sub>6</sub>f* complex and Cytochrome *c-550* were up-regulated, indicating elevation of light harvesting and electron transport, which can potentially promote photorespiration and ROS production, especially given that PSI, ferredoxin and ATP synthase genes were down-regulated. The Mn and Cu are also involved in ETC, but the DEGs associated with ETC were only identified from the Fe-deficient group. And most Mn- or Zn- dependent components were up-regulated on Mn- and Zn- deficient group, suggesting that these components probably can use other elements as cofactors when the primary cofactors are deficient (Fig. 2), making them less essential than Fe.

The iron-containing ferredoxin can be replaced by the non-iron protein flavodoxin in the photosynthesis electron transfer chain of algae (Pankowski and Mcminn, 2008). We found that ferredoxin gene was down-regulated under iron deficiency as expected, but observed no change in flavodoxin transcription. One possibility is that flavodoxin expression is regulated at translational level rather than transcriptional level, as are many genes in dinoflagellates (Lin, 2011). Another possibility is that the decrease in ferredoxin expression is compensated by another functional analog, which is unknown. In addition, *F. kawagutii* has been shown to maintain growth at low iron availability when other trace metals such as manganese, copper or zinc is available (Rodriguez and Ho, 2018). From the transcriptomic we found the zinc and manganese transporter were down-regulated, while iron permease and ABC transporter A/G/F families were up-regulated under Fe deficiency. This suggests that *F. kawagutii* is more likely to reduce Fe requirement and enhance Fe uptake to cope with Fe deficiency and maintain growth than replacing Fe with Zn and Mn, unless transporters of these two trace metals are regulated at the post-transcriptional level.

In algae, the assimilation of nitrate relies on iron-containing nitrite reductase (NiR) (Esen and Ürek, 2015). It is no surprise, therefore, that Fe deficiency caused down-regulation of *nir* and high affinity

nitrate transporter, as accumulated  $\text{NO}_3^-$  inside the cells as a result of *nir* down-regulation would trigger feedback response of the transport system. However, it is interesting to note that ammonia channel, ammonium transporter and urea-proton symporter were up-regulated in the Fe-deficient group. This is evidence that *F. kawagutii* might be sensing N stress (even though nitrate was supplied at an excess level) as a result of disabled nitrite reduction and that the dinoflagellate species possesses a strategy to shift toward ammonium or urea utilization to reduce Fe requirement and energy cost in N-nutrient assimilation under Fe-deficient condition.

#### 4.4. Defense response against the low trace metal concentration in *F. kawagutii*

Indirect and direct measurements have shown that oxidative damage and oxidative load within corals and their symbiotic dinoflagellates play a significant role in thermal and solar radiation bleaching (Lesser, 1997; Baird et al., 2009). In plants, ROS are formed by the inevitable leakage of electrons onto  $\text{O}_2$  from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a byproduct of various metabolic pathways localized in different cellular compartments (Sharma et al., 2012). Potentially toxic ROS are removed by antioxidant systems including most enzymatic antioxidants such as SOD and metal ions such as ferrous ion. As long as scavenging mechanisms are functional, ROS will not accumulate. Our transcriptomic data indicated that *gpx*, *gst*, *apx*, *prx*, *trx* and *pi* were all down-regulated in Fe-deficient group (Fig. 4). To make the matter worse, under the iron deficiency, NADP oxidase, cytochrome P450 and citrate synthase, enzymes that can potentially promote ROS production, were up-regulated (Fig. 3). Thus, Fe deficiency can potentially be a serious oxidative threat to *F. kawagutii*, in which case oxidative damage will occur, leading to metabolic dysfunction, cell destruction or mutation. None of these ROS scavenging genes was down-regulated under other four trace metal deficiencies, indicating the cell can hold a stable ability of ROS scavenging. In addition, the Fe/Mn-, Mn- and Cu/Zn- dependent SODs showed no differential expression in five metal deficiencies relative to the control. This apparently stable SOD antioxidant enzyme, if verified to be so at protein and activity levels, may serve as rescue mechanism in *F. kawagutii* cope with oxidative stress caused by Fe limitation and other types of stress. Together, these results suggest that *F. kawagutii* invests heavily in SOD production, and would have to reallocate Fe toward SOD by reducing synthesis of other Fe-dependent proteins.

In vertebrates, important components of immunity include tolerance of and resistance to microbes. Innate immunity in scleractinian corals plays an important role in the maintenance of coral-Symbiodiniaceae symbiosis (Kvennefors et al., 2010; Zhou et al., 2017). It is generally assumed that Symbiodiniaceae actively suppress the coral immune response to allow infection by the symbiont cells and symbiosis establishment. Little literature has described the counter-immunity mechanism of Symbiodiniaceae to sustain healthy symbiosis with corals. In our *F. kawagutii* transcriptomic data, we found genes putatively involved in immunity and found that most of them was up-regulated under deficiencies of the five trace metals examined here. No gene was found down-regulated under trace metal deficiencies except for Fe deficiency. Among these, 8 genes encoding protein NLR3, an intracellular sensor that negatively affected innate immune responses (Allen et al., 2012; Zhang et al., 2014), were down-regulated under Fe deficiency. The uptick change of their innate immune under trace metal deficiencies, especially under Fe deficiency, suggests that the deficiency may lead to strengthening of host immunity, and imaginably to the disruption of symbiosis and bleaching. However, this requires further experimental study using coral-Symbiodiniaceae holobionts as the subject.

## 5. Conclusions

By transcriptomic profiling, most trace metal dependent genes were affected under trace metal deficiencies, especially under Fe deficiency. DEGs encoding ECM, cell surface structure and cell adhesion protein were up-regulated in five trace metal deficiencies, suggesting that the ability of recognition and adhesion in *F. kawagutii* may be changed when the trace metal was not available in situ. Comparison of the number of DEGs detected in every treatment group indicated that iron was the most influential metal in *F. kawagutii*. Under iron deficiency, antioxidant machinery seemed to be weakened and ROS production enhanced, suggesting that oxidative damage will likely occur, leading to metabolic dysfunction, cell destruction or mutation. In addition, our data revealed up-regulation of innate immunity in *F. kawagutii*, implying a heightened potential of symbiont expulsion and symbiosis breakdown under trace metal deficient conditions, a perspective warranting further experimental inquiry. In summary, trace metal deficiency seems to promote energy metabolism, innate immunity, and cell adhesion and depress growth and the capacity of ROS scavenging, which when occurring to coral symbionts may induce coral bleaching.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2019.135767>.

## Author contribution

Tangcheng Li, Irene B. Rodriguez and Tung-Yuan Ho performed experiments. Tangcheng Li, Liying Yu, Xin Lin and Senjie Lin analyzed the transcriptomic data. Tangcheng Li and Senjie Lin wrote the manuscript.

## Declaration of competing interest

All the authors declare that there are no conflicts of interests regarding this article.

## Acknowledgement

We wish to thank Ling Li, Chentao Guo and Xiaohong Yang of Marine EcoGenomics Laboratory of Xiamen University, China for technical assistance in selecting reference genes. We also thank Jianwei Chen, Guilin Liu, Bin Geng of BGI Genomics Co., Ltd. for assistance with bioinformatics analysis of the RNA-seq data. This work was supported by Natural Science Foundation of China [grant numbers 31661143029, 41776116 41706116] and Fundamental Research Funds for the Central Universities of China [grant numbers 20720180101 (XL)].

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