PeerJ Manuscript to be reviewed

Physiological changes and gene responses during *Ganoderma lucidum* growth with selenium supplementation

Bo Zhang, Wei Tan*, Jie Zhou, Lei Ye, Dinghong Jia, Xiaolin Li*

Sichuan Institute of Edible Fungi, Chengdu 610066, China

* correspondence: Wei Tan tanweichengdu@126.com, Xiaolin Li kerrylee_tw@sina.com

Abstract: ~~As a *Basidiomycota*-phylum mushroom~~, *Ganoderma lucidum* is highly appreciated for its health and nutrition value. The present study cultivated *Ganoderma lucidum* as selenium transformation carrier, and revealed the physiological changes and gene responses by selenium supplementation through high-throughput RNA-Seq technology. As a result, selenium supplementation increased the stipe length and the cap size, but decreased the cap thickness of *G. lucidum*. And the mineral salt supplementation could greatly promote the formation of triterpene acids and selenium in *G. lucidum*. ~~Moreover~~, the highest yield was gained in the treatment with selenium content of 200 μg/g. Subsequently, the tissues of *G. lucidum* at budding and mature stages in this treatment group were sampled for transcriptome analysis and compared to those of a control group without selenium supplementation. ~~Finally~~, a total of 16113 expressed genes were obtained from the transcriptome of *G. lucidum*, and GO-annotated unigenes were mainly involved in molecular functions and KEGG-annotated ones were highly expressed in ribosomal pathway. Furthermore, genes involved in carbon metabolism pathway were most promoted by selenium at budding stage of *G. lucidum*, while gene expression was the highest in the pathway of amino acid biosynthesis at mature stage of *G. lucidum*. Specially, selenium-related genes in *G. lucidum*, such as GL23172-G, GL29881-G and GL28298-G, played a regulatory role in oxidoreductase, antioxidant activity and tryptophan synthesis. The results provide a theoretical basis for further study of selenium-enriched mushrooms and aid to development of Se-enriched foodstuff and health products made from fungi.

Key words: *Ganoderma lucidum*; Selenium; High throughput technology; Transcription

Introduction

G. lucidum is an edible ~~fungus~~ of *Basidiomycota* phylum, and its fruit bodies and spores are abundant in health and nutritious substances including polysaccharides, triterpenoids and nucleoside (*Yu & Zhai, 1979*; *Kinahan, Kowal & Grindey, 1981*). These substances are proved to strengthen immune system and inhibit tumor formation (*Wang, 2002*; *Sakamoto, 2016*), hence usually utilized in clinical medicine. With great medicinal value, *G. lucidum* becomes appreciated and has been cultivated in large areas in China. Moreover, a long history of *G. lucidum* cultivation contributes to sophisticated planting skills, as well as its high and stable yield (*Boh et al., 2007*). To satisfy the expanding market demand of *G. lucidum* products, exploration in improving its quality and growth efficiency is now pursued.

Selenium is a multifunctional bionutrient element, and is also recognized as the necessary trace element for important metabolic enzymes. Moreover, as a key component of glutathione peroxidases and selenoproteins, selenium shows great significance to human health with anti-oxidation, anti-tumor, enhancing immunity, etc (*Rayman, 2012*; *Rayman, Infante & Sargent, 2008*; *Rotruck et al., 1973*). The main existence state of selenium in nature is inorganic and active-in-plant in low concentrations. It's evidently demonstrated that inorganic selenium is more toxic and difficult to absorb compared to the organic state. So it's increasingly demanding to seek a way of transformation. A large number of edible mushrooms (e.g. *Flammulina velutipes*, *Pleurotus ostreatus* and

Ganoderma lucidum) were reported to be capable of selenium accumulation and transformation, becoming an ideal Se-enriched foodstuff (*De Souza et al., 1999*; *Hanson et al., 2003*; *Haddad et al., 2013*; *White, 2015*; *Zayed, Lytle & Terry, 1998*). Thus, researches have focused on mineral enrichment in edible fungi, with expectation of transforming the supplemented elements from inorganic to organic states. Zhao and Hartman planted *G. lucidum* and etc. with essential element addition (e.g. selenium and calcium) in the substrates, and finally harvested nutritional value-improved fruit bodies (*Hartman et al., 2000*; *Zhao et al., 2004*). Our previous study demonstrated the cultivated *Auricularia cornea* with a 100 μg/g supply of selenium in the substrate outperformed with high yield, rich crude polysaccharides and selenium content (*Li et al., 2019*).

~~At present~~, Illumina high-throughput technology is widely applied to transcriptome sequencing and exploration of gene composition and functions for mushrooms based on its unprecedented handling capacity, scalability and speed (*Patel & Jain, 2015*). Also, this technology is regarded as a necessary way to clarify the biosynthetic pathways of bioactive compounds that mushrooms produce (*Tomohiro 2021*). *Dong et al. (2021)* identified 17 candidate genes that were involved in triterpenoid biosynthesis with using high throughput method, getting a molecular understanding of *Phellinus igniarius*. *Duan, Bao & Bau (2021)* performed high-throughput transcriptome sequencing of a wild mushroom species *Leucocalocybe mongolica*, and discovered expression changes of some key CAZyme-related genes between mycelia and fruiting body organs. Additionally, real-time quantitative PCR becomes increasingly important in the quantitative detection of genes for its obvious advantages (*Pfaffl et al., 2002*). qPCR method has been used in detection of gene stability and verification of gene functions (*Zarivi et al., 2015*; *Li et al., 2019*).

Despite some studies on *G. lucidum* as a transformation carrier of mineral elements like selenium, the suitable concentration and mechanisms of selenium accumulation in *G. lucidum* should be further explored. So, physiological changes including mycelial growth rate, stipe length, cap size and thickness, fresh yield, contents of the crude polysaccharide, triterpenoids and total selenium in the mature fruit bodies and gene responses during *G. lucidum* growth by selenium supplementation were investigated with using high-throughput sequencing technology. ~~Meanwhile,~~ six differentially expressed genes, which were potentially selenium-dependent were selected for real-time reverse transcription PCR (RT-PCR) to validate the gene expression profiles in *G. lucidum* transcriptome.

Materials and methods

Ganoderma lucidum cultivation

The studied *G. lucidum* cultivar was Chuan Yuanzhi No. 1. The substrate was composed of 90% cottonseed hull, 5% wheat bran, 4% corn flour and 1% gypsum, and a sodium selenite (Na_2SeO_3) solution was supplemented to the substrate. The mixed substrate was put into polypropylene cultivation bags with the size of 17 cm×33 cm×0.005 cm. The final concentrations of sodium selenite in the substrate were determined to be 0 μg/g, 50 μg/g, 100 μg/g, 200 μg/g, 250 μg/g, and 300 μg/g, with the labels of GCK, G50, G100, G200, G250, G300, respectively. The substrate bags were then under sterilization at 98-100 ℃ for more than 18 hours. Afterwards, the bags were cooled to room temperature and then prepared for inoculation. The process of *G. lucidum* inoculation was done in a laminar flow cabinet with a sterile environment, ~~After inoculation, the cultivation bags were carried~~ into a culture room for ~~mycelia germination~~. With the mycelia full of bags, they were planted in the cultivation site at Zhaojia, Jintang, China (N 30°48′ 16.45″ , Ei 104°35′ 48.79″). The space of the cultivation site was ventilated, and previously cleaned and simply disinfected with lime.

Growth index investigation

The mycelial growth rate of *G. lucidum* was measured before the mycelia were filled with cultivation bags. Other

Use past tense in Material & Methods.

growth indexes including stipe length, cap size and thickness, fresh yield, crude polysaccharide, triterpenoids and total selenium concentration were ~~investigated~~ determined at the mature stage. The crude polysaccharide content was determined ~~by~~ using a phenol-sulfuric acid method (*Du et al., 2008*), while the triterpenoids was by UV-vis spectrophotometry (*Zhang 1987*). The statistical analysis was done ~~with~~ using Excel and SPSS13.0. Data with different lower-case letters showed significant differences (p-value <0.05) by the LSD method of a one-way ANOVA. This is out of place — belongs to the figure legend.

Sample collection for transcriptome analysis

The *G. lucidum* tissues of GCK and G200 were sampled at both budding and mature stages with prepared gloves, tweezers and knives, which were previously sterilized. There were ~~totally~~ four samples, labelled GCKb, GCKm, G200b and G200m, respectively. Each sample ~~has~~ had three replicates, and more than 500 mg of fresh tissues per replicate were collected and pooled. Afterwards, the samples were stored in liquid nitrogen with 2 mL Eppendorf tubes (Eppendorf, Germany), and then sent to Personalbio (Shanghai, China) for RNA extraction and transcriptome sequencing. The statistical power of this experimental design, calculated in R (version 4.1), ~~is~~ was 0.5696 in RNASeqPower.

RNA extraction, library preparation and sequencing

Total RNA of *G. lucidum* samples was extracted with a Qiagen RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. After assessing RNA quality ~~examination~~ and contaminated RNA elimination, the remaining RNA was cleaved into fragments of 200-300 bp in length. Then the RNA was reverse transcribed to cDNA with a Fast Quant RT Kit (TIANGEN, Beijing). PCR amplification was applied in the cDNA fragments enrichment, and an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) was for examination of 300-400 bp fragments during library generation. With libraries diluted to 2 nM uniformly and formed single stranded with alkaline denaturation, they were finally paired-end sequenced based on Illumina NextSeq 500 (Illumina, San Diego, CA, USA). All the raw sequences were deposited in the NCBI Sequence Read Archive (SRA) database with the accession NO. SRR5576791-SRR5576802.

Sentence does not make sense!

Assembly and annotation

Raw reads with FASTQ ~~type~~ format were checked and filtered ~~by~~ using the FastQC program(*Lu, Tzovaras & Gough 2021*). The adapters were removed, and then sequences shorter than 50 bp or lower than Q20 in quality score were removed. Afterward, de novo assembly was performed ~~by a short reads assembling~~ with the program Trinity (*Zhou et al., 2014*; *Grabherr et al., 2011*). A total of 3.72×108 raw reads were obtained in the ~~present~~ study, with approximately 0.99% of low-quality reads removed, and finally 3.69×108 high-quality reads were screened out. Besides, the statistical results of Q20, q30 and GC contents related to the obtained sequences were in Table S1. Furthermore, each sample contained 4.45 GB data, and 67.43% of high-quality sequences were aligned to the corresponding reference genome, so as to carry in-depth analysis of transcriptome data.

Sentence does not make sense.

High-quality sequences started to be spliced alternatively to obtain transcript ones by Inchworm, Chrysalis and Butterfly. The longest sequence in each cluster was treated as one unigene, and annotated against the databases of GO (Gene Ontology)(*Ashburner et al., 2000*), KEGG (Kyoto Encyclopedia of Genes and Genome)(*Kanehisa et al., 2000*), KOG (Cluster of eukaryotic Orthologous Groups), NR (Non-Redundant Protein Sequence Database) and SwissProt (Swiss-Prot protein)(*Boeckmann et al., 2003*). The clean sequences were aligned to analyze differential gene expression and enrichment. ~~The present study~~ We used KOBAS software to perform KEGG pathways enrichment analysis following the hypergeometric distribution principle using(*Gentleman et al., 2009*).

Gene expression analysis and validation

RSEM was used for expression quantification of RNA-Seq data with a reference of de novo assembled transcriptome, and the result of alignment was ~~finally~~ investigated (*Li & Dewey, 2011*). Based on RNA-Seq technology, Each unigene's FPKM value that represented the expected fragment numbe per kilobase of transcript sequence per million sequenced reads (*Trapnel et al., 2010*) was calculated as the expression level. ~~The~~ analysis of unigene expression difference was carried ~~on~~ with DESeq (Version 1.18.0) (*Li & Dewey, 2011*). The expressed genes with significant difference (DEGs) were screened and the threshold for screening was |log2(FoldChange)| > 1 and p-value < 0.05. Furthermore, a heatmap was drawn to display the expression pattern of each DEG across all the samples between the selenium-treated (G200) and control groups (GCK) with two-way hierarchical clustering based on the R package Pheatmap (*Tauno & Jaak, 2015*). Besides, four identified unigenes were selected for expression validation using the qPCR analysis. A Super RT Kit (TaKaRa, Osaka, Japan) was for RNA reverse transcribing, and Ribosomal Protein L4 was as reference to amplify and normalize gene expression for each qPCR using primers (*Xu et al., 2014*). Ultimately, each gene expression in one sample was confirmed with not less than three independent qPCR reactions.

Results

***G. lucidum* growth changes affected by selenium supplementation** — Do you not have images that you can include of how they grew?

Selenium supplementation significantly affected the physiological development of *G. lucidum* including agronomic traits and nutrient contents in the present study (Table 1). The mycelial growth was evidently promoted by selenium supplementation, and the treatment with the selenium concentration of 100 μg/g in the substrate ~~was the~~ fastest (6.38 mm/d), significantly faster than the control ($P<0.05$). Selenium supplementation showed a limited effect on *G. lucidum* shape characteristics, including the stipe length and cap size of fruit bodies, had no significant changes in each treatment. While, the cap thickness of Se-treated fruit bodies was smaller than that of the control, and those of G50, G100 and G250 were significantly thinner. ~~The yield investigation revealed the~~ highest fresh yield ~~of G200~~ (149.50 g/bag), which was 12.44% higher than the control, but the other treatments were with lower yields. In addition, different effects occurred on the active components of fruit bodies by selenium supplementation. Triterpenoid acids in the *G. lucidum* fruiting bodies were promoted, and the contents increased with selenium supplementation from 100 μg/g to 300 μg/g. The highest triterpenoid acids (0.91%) were found in the treatments with the selenium concentration of 50 μg/g and 300 μg/g in the substrate, significantly higher than the control. However, there was no significant difference between the polysaccharide contents in fruit bodies of each treatment, and G300 was the highest, reaching 0.61%.

Besides, the total selenium in ~~*G. lucidum*~~ fruit bodies increased first and then decreased with the selenium addition, and the Se-treated ~~ones~~ were significantly higher than the control in selenium accumulation. It peaked at 11.79 μg/g in the treatment with the selenium concentration of 250 μg/g in the substrate. ~~Moreover, the~~ Treatment with the lowest selenium concentration (G50) had the highest accumulation rate of sodium selenite (0.90%), and then it decreased by selenium supplementation. Thus, it demonstrated that *G. lucidum* ~~be~~ is capable of selenium absorption but with a cumulative threshold. Consequently, *G. lucidum* cultured in the substrate with 200 μg/g of selenium was selected for transcriptome analysis to explore gene responses by selenium supplementation at the budding and mature stages due to its highest yield and rich nutrients.

Functional annotation based on *G. lucidum* transcriptome

The gene expression profiles of *G. lucidum* between the selenium-treated samples at 200 μg/g concentration (G200)

Why "agronomic" traits? I realise you refer to crop production — but it really affects the growth and size.

and the control (GCK) at the budding and mature stages were analyzed using High-throughput RNA sequencing. A total of 16113 unigenes were finally obtained, ranging from 50 bp to 16,000 bp in length. To uncover more *G. lucidum*-related functional genes, five frequently used databases were employed for annotation including GO, KEGG, KOG, NR and SwissProt. As a result, the numbers of the annotated unigenes were 5554 (GO), 3139 (KEGG), 5760 (KOG),10874 (NR) and 6328 (SwissProt), accounting for 34.47%, 19.48%, 35.75%, 67.49%, 39.27%, respectively.

1) The unigenes in the *G. lucidum* transcriptome were totally annotated to 910 GO-terms during its growth period. And 58 of them were with more than 10% of the annotated genes, in which, the category of molecular function had the most terms(29), followed by the biological process (22) and cellular component (7) (Table S2). What's more, there were 22 GO-terms with over 1000 annotated genes, including binding, catalytic activity, metabolic process, cellular process and etc. Thus, the GO-annotation results indicated GO pathways were regulated by a large number of genes, playing important roles in primordia formation and fruit body maturation of *G. lucidum*.

2) To further understand the biological pathways during *G. lucidum* growth, the unigenes were mapped to the reference pathways in the KEGG database including cellular process, environmental information processing, genetic information processing, metabolism and organismal systems (Table 2). A total of 4285 and 4313 unigenes were annotated at budding and mature stages, respectively, and the unigenes related to metabolism were the most at both growth stages, accounting for 42.01% and 40.95%. Results showed that more unigenes in *G. lucidum* were up-regulated at budding stage with 200 μg/g sodium selenite addition in the substrate of all the five KEGG pathways. However, just the genetic information processing were with larger amount of up-regulated unigenes in Se-treated *G. lucidum* at the mature stage. Thus, selenium supplementation potentially activated physiological development during *G. lucidum* primordium formation more than at the mature stage.

3) Specially, three of the KEGG pathways were with more than 10 up-regulated genes in the control group at budding stage including biosynthesis of amino acids, carbon metabolism and glycine, serine and threonine metabolism (Table S3). Meanwhile, a total of 19 KEGG pathways had over 10 up-regulated genes with selenite supplemented in the substrate at budding stage, in which, "carbon metabolism" had the largest amount (32), followed by the pathways of "biosynthesis of amino acids" and "citrate cycle (TCA cycle)". However, a smaller amount of unigenes were up-regulated at the mature stage, and just 3 of the pathways were with more than 10 up-regulated genes in each treatment, representing "carbon metabolism", "peroxisome" and "amino sugar and nucleotide sugar metabolism" in the control group, and "biosynthesis of amino acids", "purine metabolism" and "pyrimidine metabolism" in the Se-treated samples. These database annotations provided basical biological information in *G. lucidium*, contributing to a better understanding of selenium accumulation in the studied fungus.

Differentially-expressed gene analysis

1) To generally uncover how selenium addition and growth stage affected the gene expression pattern of *G. lucidum*, a heatmap based on the RPKM method was drawn. As illustrated in Fig. 1, the growth stage had significant impacts on gene expression, separating the *G. lucidum* transcriptomes into two clusters. The interesting result was that the up-expressed genes at the budding stage of both treatments became down-expressed at the mature stage. More specifically, the control group had 1695 up-expressed genes at mature stage and 1402 up-expressed genes at budding stage (Fig. S1). However, only 975 and 1291 genes were up-expressed at mature and budding stage with selenium addition, respectively. Also, the gene expression pattern of *G. lucidum* was greatly changed by selenium addition. There were 3280 differentially expressed unigenes between the selenium-treated and control groups at the

budding stage, of which, 1612 upregulated and 1668 downregulated in response to selenium addition. And 912 upregulated and 1549 downregulated genes were detected with selenium supplementation at the mature stage.

2) The top 10 up-regulated genes in both treatments and genes with the highest expression at budding and mature stages were investigated in the present study (Table S4, Table S5) . The most up-regulated genes treated with selenium addition were GL23959-G and GL26604-G at budding and mature stages, respectively. The gene GL23959-G was related to the monomeric metabolic process, while the gene GL26604-G was involved in the activity of cation transmembrane transporter. Besides, the genes in the Se-treated *G. lucidum* with the highest expression at budding stage was GL21838-G, with relevance to the binding of organic ring compounds, peroxidase activity and stress response, and GL23307-G was the highest at mature stage. As was shown in the result, just two up-regulated genes were among the top 10 genes with the highest expression. And they were GL23263-G and GL24771-G, annotated in GO:0003824 etc.

3) To validate the gene expression profiles in *G. lucidum* transcriptome, six differentially expressed genes were selected for qPCR. Two of the selected genes GL23172-G and GL29881-G were up-regulated at budding and mature stages with selenium supplementation, and they played a role of regulating the oxido-reductase and antioxidant activities. Meanwhile, the gene GL23172-G, which was related to active oxygen metabolism, was found up-expressed at both growth stages of *G. lucidum*. What's more, the genes GL24625-G and GL28298-G were both involved in tryptophan synthesis, and one was down-regulated and another up-regulated during *G. lucidum* primordium formation. All of the six genes were successfully amplified, and the qPCR results were highly consistent with the DEG expression patterns of the transcriptome analysis, confirming the reliability of the RNA-Seq data (Table 3).

Discussion

Selenium supplementation affected *G. lucidum* agronomic traits

One key indicator of hyphal vitality is the growth rate, which also shows the hyphal adaptability to the surroundings. The present study revealed a faster growth rate of *G. lucidum* hypha with selenium supplemented in the substrate, demonstrating that selenium be capable of promoting *G. lucidum* growth at hyphal stage. However, the promotion wasn't infinitely increased with the increase of selenium content in the substrate, and it peaked at the concentration of 100 μg/g. As is reported by *Goyal, Kalia & Sodhi (2015)*, the growth of *G. lucidum* hypha is obviously affected by selenium supplementation, making the hypha thinner and their branches wider. And even the spore morphology is changed by the increase of selenium addition. It's thus clear that higher concentration of inorganic selenium may not be good for hyphal growth. In addition, selenium is found to be widely used in mushroom cultivation, and it can improve the agronomic characteristics of edible fungi (*Wu et al., 2015*). In the present study, selenium supplementation obviously made the cap of *G. lucidum* fruit bodies thinner, while the mineral had no significant effects on the stipe length and cap size. Besides, The yield of *G. lucidum* is the most important production indicator that farmers concern. The fresh weight of *G. lucidum* fruit bodies was the highest when the selenium concentration was 200 μg/g in the present study. Considering the better agronomic characteristics of the 200 μg/g Se-treated *G. lucidum*, this supplementation concentration can be optimal for *G. lucidum* cultivation. Thus, selenium has a comprehensive effect on *G. lucidum* growth and development, and it still needs further exploration into the unknown mechanism.

Nutrient contents in *G. lucidum* changed with selenium supplementation

The present study uncovered a continual increase of selenium in *G. lucidum* fruit bodies with selenium

supplementation. As is known, the majority of edible fungi (e.g. *G. lucidum*) have a strong ability to enrich mineral elements (*Drewnowska & Falandysz, 2015*; *Kala & Svoboda, 2000*), and selenium is a typical representative (*G secka, Siwulski & Mleczek, 2018*; *Reilly 1998*). Besides, selenium is proved to be an indispensable trace element for humans, playing an important role in our health. A dynamic change of selenium enrichment in *G. lucidum* fruit body was revealed, and the largest content was detected at the concentration of 250 μg/g in the present study. According to Goyal *et al.*, the selenium absorption by *G. lucidum* hypha shows an increasing trend with the increase of selenium supplementation(*Janssen 2006*), which will probably have a direct effect on the selenium accumulation in fruit bodies. And the effective adsorption of selenium by *G. lucidum* in our study is consistent with ~~Li's research~~ on selenium enrichment of algae (*Li, Guo & Li, 2003*). Additionally, ~~it's~~ found that sodium selenite (Na_2SeO_3) supplemented into the substrate can be transformed into organic state with bio-absorbing by edible fungi including *Agaricus bisporus*, *Lentinus edodes* and etc.(*Dernovics, Stefán & Kodor, 2002*; *Elteren, Woroniecka & Kroon, 1998*; *Ogra et al., 2004*; *Racz et al.,2000*; *Yoshida et al., 2005*).These states include organic selenoproteins, polysaccharides ~~and so on~~. Also, selenium has been proved to form conjugated complex with mushroom polysaccharides, significantly improving the biological activities of *G. lucidum* and other edible fungi, such as anti-tumor and free radical scavenging(*Shi et al., 2010*). It follows that *G. lucidum* enriches selenium, aiming to improve its medicinal value. Furthermore, the selenium state in the studied *G. lucidum* fruit bodies needs to further verify if they are ~~on~~ safe. As is reported, the suitable selenium intake of human body is 60 ug everyday. That is to say, only 7.91g dry fruit body per day can satisfy the selenium need if the studied *G. lucidum* of the highest yield is ~~eaten~~ with organic safety. ~~What's more,~~ the selenium supplementation also greatly promoted the triterpene acid contents in *G. lucidum* fruit bodies in the present study, contributing to its higher medicinal properties. In general, selenium can promote the formation of active substances in *G. lucidum* fruit bodies, and the results provide a theoretical basis for the selenium-enriched cultivation of *G. lucidum*.

Genes from *G. lucidum* transcription responded to selenium supplementation

~~The present study~~ obtained 16113 genes and annotated them into different databases including GO, KEGG and etc., from which the potential genes relating to selenium enrichment were mined. Consequently, catalytic activity is an important functional type during the growth of *G. lucidum*. ~~As is reported,~~ different enzymes (e.g. cellulose and lignin peroxidase) participate in the catalytic process during the growth of edible fungi, and biological processes like metabolism, nutrition and energy conversion are greatly determined by catalysis(*Buswell et al., 1996*; *Lechner & Papinutti, 2006*; *Lee et al., 2004*). The gene regulation of *G. lucidum* at the transcriptional level presented significant changes with selenium supplementation in ~~the~~ study. This supplemented element is reported to have antioxidant effect, protecting cells from free radical oxidation damages(*Serafin et al., 2006*). Besides, selenium is a cofactor of selenium-related enzymes (e.g. glutathione peroxidase)(*Malinowska et al.,2009*). ~~It's~~ demonstrated by Goyal *et al.* that there are selenium signals present in *G. lucidum* hypha, and they are existing mainly in selenoprotein state (*Janssen 2006*).

The changing genes and selenium-related genes played their roles.

~~Meanwhile,~~ some genes have different functions at different growth stages of organism (*Hsu et al., 2011*; *Muraguchi & Kamada, 2000*). *Yu et al. (2012)* studied the changes in gene expression and biological pathways from the mycelia to the initial primordial stages, ~~and proved the viewpoint~~. It was previously reported that a large number of genes involved in the pathway of bio-synthetic metabolism were up-regulated, while the genes relating to degradation activity presented up-expressed at the stage of fruit formation. ~~What's more~~, there was a higher expression of genes

coding for hydrophobins and lectins investigated at fruiting stage of *Agaricus bisporus* compared to that at undifferentiated hyphal stage by *Morin et al.(2012)*. And genes involved in stress signals (e.g. MAPK, cAMP) were found up-regulated at fruiting stage when the gene expressions at different growth stages of *Hypsizygus marmoreus* were compared (*Zhang et al., 2015*). Besides, among the six verified genes, GL28298-G participates in k00600 pathway, which has something to do with carbon accumulation of folic acid. As is seen in Fig. S2, k00600 pathway is located importantly in the carbon accumulation process of folic acid (2.1.2.1), greatly contributing to the synthesis of 5,10-methylenetetrahydrofuran and tetrahydrofolate. Moreover, two of the verified genes GL24625-G and GL28298-G were involved in tryptophan synthesis. To sum up, it revealed different gene expressions and biological pathways of *G. lucidum* in response to selenium supplementation in the present study, which aids to further study of the molecular mechanism in edible fungi.

Conclusion

Our study revealed a significant effect of selenium supplementation on the hyphal growth, morphological characteristics, yield and active substances in *G. lucidum*. And the most suitable selenium concentration for *G. lucidum* bag cultivation was selected at 200 μg/g. The transcription analysis uncovered the different expressions of some significant genes: the ribosome-related genes were most active during the primordium formation, and the genes related to amino acid biosynthesis were up-expressed during the fruit body maturation. More importantly, the expression of genes in different biological pathways was decided by the growth stage and selenium concentration. Some potential selenium dependent genes were unearthed, which played a regulatory role in oxidoreductase, antioxidant activity and tryptophan synthesis. These results provide a theoretical basis for selenium-enriched mushroom cultivation, helping develop foodstuff and health products of Se-enriched fungi.

Acknowledgments

This work was supported by China Agriculture Research System (CARS-20) and the Sichuan Mushroom Innovation Team (SCCXTD-2022-07).

References

Ashburner M, Ball CA, Blake JA, Botstein D, Cherry JM.2000. Gene ontology: tool for the unification of biology Consortium TGO.*Nature Genetics* **25:**25-29 DOI:10.1038/75556.

Boeckmann B.2003. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. *Nucleic Acids Research* **31:** 365-370 DOI:10.1093/nar/gkg095.

Boh B, Berovic M, Zhang J, Lin ZB. 2007. *Ganoderma lucidum* and its pharmaceutically active compounds. *Biotechnology Annual Review* **13:**265-301 DOI:10.1016/S1387-2656(07)13010-6.

Buswell J A, Cai YJ, Chang ST, Peberdy JF, Yu HS. 1996. Lignocellulolytic enzyme profiles of edible mushroom fungi. *World Journal of Microbiology and Biotechnology* **12(5):** 537-542 DOI:10.1007/BF00419469.

Dernovics M, Stefánka Z, Fodor P. 2002. Improving selenium extraction by sequential enzymatic processes for Se-speciation of selenium-enriched *Agaricus bisporus*.*Analytical and Bioanalytical Chemistry* **372(3):** 473-480 DOI:10.1007/s00216-001-1215-5.

De Souza MP, Huang CPA, Chee N, Terry N. 1999. Rhizosphere bacteria enhance the accumulation of selenium and mercury in wetland plants. *Planta* **209(2):** 259-263 DOI:10.1007/s004250050630.

Dong Y, Ma H, Sun L, Ye XF. 2021. De novo transcriptome analysis ofmedicinal mushroom *Phellinus igniarius* by RNA-seq. *International Journal of Medicinal Mushrooms* DOI:10.1615/IntJMedMushrooms.2021038960.

Drewnowska M, Falandysz J. 2015. Investigation on mineral composition and accumulation by popular edible mushroom common chanterelle (*Cantharellus cibarius*). *Ecotoxicology and Environmental Safety* **113:** 9-17 DOI:10.1016/j.ecoenv.2014.11.028.

Duan M, Bao H, Bau T. 2021. Analyses of transcriptomes and the first complete genome of *Leucocalocybe mongolica* provide new insights into phylogenetic relationships and conservation. *Scientific Reports* **11(1)** DOI:10.1038/s41598-021-81784-6.

Elteren JT, Woroniecka UD, Kroon KJ. 1998. Accumulation and distribution of selenium and cesium in the cultivated mushroom *Agaricus bisporus*-a radiotracer-aided study. *Chemospher* **36:**1787-1798 DOI:10.1016/S0045-6535(97)10064-9.

Fu LZ, Wu XQ, Li MY, Li HB, Wei HL, Wu QQ, Cheng JW, He L. 2008. Changes of crude polysaccharide and triterpene contents in *Ganoderma lucidum* at different growth and development stages. *Acta Edulis Fungi* **(03):**47-50 DOI:10.16488/j.cnki.1005-9873.2008.03.002.

G secka M, Siwulski M, Mleczek M. 2018. Evaluation of bioactive compounds content and antioxidant properties of soil-growing and wood-growing edible mushrooms. *Journal of Food Processing and Preservation* **42(1):** e13386 DOI:10.1111/jfpp.13386.

Gentleman R, Hornik K, Parmigiani G, Wickham H, Dordrecht S, London H, York N.2009. ggplot2 elegant graphics for data analysis 123. *Springer New York.*

Goyal A, Kalia A, Sodhi H S. 2015. Selenium stress in *Ganoderma lucidum*: A scanning electron microscopy appraisal. *African Journal of Microbiology Research* **9(12):** 855-862 DOI:10.5897/AJMR2014.7250.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng QD. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology* **29:** 644-652 DOI:10.1038/nbt.1883.

Haddad S, Hong J, Morrissy J, Zhang L. 2013. Use of selenium-contaminated plants from phytoremediation for production of selenium-enriched edible mushrooms.*Selenium in the environment and human health* 124-126 DOI:10.1201/b15960-56.

Hanson B, Garifullina GF, Lindblom SD, Wangeline A, Ackley A, Kramer K, Norton AP, Lawrence CB, Pilon-Smits EAH. 2003. Selenium accumulation protects *Brassica juncea* from invertebrate herbivory and fungal infection. *New Phytologist* **159(2):** 461-469 DOI:10.1046/j.1469-8137.2003.00786.x.

Hartman SC, Beelman RB, Simons S, van Griensven LJLD.2000. Calcium and selenium enrichment during cultivation improves the quality and shelf life of *Agaricus* mushrooms. *Science and Cultivation of Edible Fungi* **2:**499-505.

Hsu KH, Lee YR, Lin YL, Chu FH.2011. Cytochrome P450 genes in medicinal mushroom *Antrodia cinnamomea* TT Chang et WN Chou (higher Basidiomycetes) are strongly expressed during fruiting body formation. *International Journal of Medicinal Mushrooms* **13(6)** DOI:10.1615/IntJMedMushr.v13.i6.30.

Janssen PH. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes.*Applied & Environmental Microbiology* **72:**1719-1728 DOI:10.1128/AEM.72.3.1719-1728.2006.

Kala P, Svoboda L.2000. A review of trace element concentrations in edible mushrooms. *Food Chemistry* **69:**273-281 DOI:10.1016/S0308-8146(99)00264-2.

Kanehisa M, Goto S, Kawashima S, Okuno Y, Hattori M. 2000. The KEGG resource for deciphering the genome. *Nucleic Acids Research* **32:**D277-280 DOI:10.1093/nar/gkh063.

Kinahan JJ, Kowal EP, Grindey GB.1981. Biochemical and antitumor effects of the combination of thymidine and 1-beta-D-arabinofuranosylcytosine against leukemia L1210. *Cancer Research* **41(2):**445-51

DOI:10.1523/JNEUROSCI.2176-12.2012.

Lechner BE, Papinutti VL. 2006. Production of lignocellulosic enzymes during growth and fruiting of the edible fungus *Lentinus tigrinus* on wheat straw. *Process Biochemistry* **41(3):** 594-598 DOI:10.1016/j.procbio.2005.08.004.

Lee DH, Kim JH, Park JS, Choi YJ, Lee JS. 2004. Isolation and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom *Tricholoma giganteum*. *Peptides* **25:**621-627 DOI:10.1016/j.peptides.2004.01.015.

Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-seq data with or without a reference genome. *BMC Bioinformatics* **12:**323 DOI:10.1186/1471-2105-12-323.

Li XL, Yan LJ, Li Q, Tan H, Zhou J, Miao RY, Ye L, Peng WH , Zhang XP, Tan W, Zhang Bo. 2019. Transcriptional profiling of *Auricularia cornea* in selenium accumulation. *Scientific Reports* **9:**5641 DOI:10.1038/s41598-019-42157-2.

Li ZY, Guo SY, Li L. 2003. Bioeffects of selenite on the growth of *Spirulina platensis* and its biotransformation. *Bioresource Technology* **89:**171-176 DOI:10.1016/S0960-8524(03)00041-5.

Lu C, Tzovaras BG, Gough J. 2021. A survey of direct-to-consumer genotype data, and quality control tool (GenomePrep) for research. *Computational and Structural Biotechnology Journal* **(4)** DOI:10.1016/j.csbj.2021.06.040.

Malinowska E, Krzyczkowski W, Herold F, Łapienis G, Ślusarczyk J, Suchocki P, Kuraś M, Turło J. 2009. Biosynthesis of selenium-containing polysaccharides with antioxidant activity in liquid culture of *Hericium erinaceum*. *Enzyme and Microbial Technology* **44(5):** 334-343 DOI:10.1016/j.enzmictec.2008.12.003.

Morin E, Kohler A, Baker AR, Foulongne-Oriol M, Lombard V. 2012. Genome sequence of the button mushroom *Agaricus bisporus* reveals mechanisms governing adaptation to a humic-rich ecological niche. *Proceedings of the National Academy of Sciences* **109(43):** 17501-17506 DOI:10.1073/pnas.1206847109.

Muraguchi H, Kamada T. 2000. A mutation in the cln2 gene encoding a cytochrome P450 of *Coprinus cinereus* affects mushroom morphogenesis. *Fungal Genetics and Biology* **29(1):** 49-59 DOI:10.1006/fgbi.2000.1184.

Ogra Y, Ishiwata K, Encinar JR, obiński R, Suzuki KT. 2004. Speciation of selenium in selenium-enriched shiitake mushroom, *Lentinula edodes*. *Analytical and Bioanalytical Chemistry* **379(5-6):** 861-866 DOI:10.1007/s00216-004-2670-6.

Patel RK, Jain M.2015. NGS QC Toolkit : A Platform for Quality Control of Next-Generation Sequencing Data. *Encyclopedia of Metagenomics* 1-5 DOI:10.1007/978-1-4899-7478-5_348.

Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30:** e36 DOI:10.1093/nar/30.9.e36.

Racz L, Bumbalova A, Harangozo M, Tölgyessy J, Tome ek O. 2000. Determination of cesium and selenium in cultivated mushrooms using radionuclide X-ray fluorescence technique. *Journal of Radioanalytical and Nuclear Chemistry* **245(3):** 611-614 DOI:10.1023/A:1006738116887.

Rayman MP. 2012. Selenium and human health. *Journal of Shahid Sadoughi University of Medical Sciences* **379(9822):**0-1268 DOI:10.1016/s0140-6736(11)61452-9.

Rayman MP, Infante HG, Sargent M. 2008. Food-chain selenium and human health: spotlight on speciation. *British Journal of Nutrition* **100(2):** 238-253 DOI:10.1017/S0007114508922522.

Reilly C.1998. Selenium: a new entrant into the functional food arena. *Trends in Food Science & Technology* **9(3):** 114-118 DOI:10.1016/S0924-2244(98)00027-2.

Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hockstra DGHG. 1973. Selenium: biochemical role as a component of glutathione peroxidase. *Science* **179(4073):** 588-590 DOI:10.1126/science.179.4073.588.

401 **Sakamoto S, Kikkawa N, Kohno T, Shimizu K, Tanaka H, Morimoto S. 2016.** Immunochromatographic strip assay for
402 detection of bioactive *Ganoderma* triterpenoid, ganoderic acid A in *Ganoderma lingzhi*. *Fitoterapia* **114:**51-55
403 DOI:10.1016/j.fitote.2016.08.016.
404 **Serafin Muñoz AH, Kubachka K, Wrobel K, Gutierrez Corona JF, Yathavakilla SKV, Caruso JA. 2006.** Se-enriched
405 mycelia of *Pleurotus ostreatus*: distribution of selenium in cell walls and cell membranes/cytosol. *Journal of*
406 *Agricultural and Food Chemistry* **54(9):** 3440-3444 DOI:10.1021/jf052973u.
407 **Shi WL, Han H, Chen GZ, et al. Chen X, Hong YK, Chen LK,Chen D,Lu Z. 2010.** Extraction, characterization of the
408 polysaccharide extracts from Se-enriched *G. lucidum* (Se-GLP) and its inhibition against oxidative damage in ischemic
409 reperfusion mice. *Carbohydrate Polymers* **80(3): 774-778** DOI:10.1016/j.carbpol.2009.12.027.
410 **Tauno M, Jaak V. 2015.** ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component
411 Analysis and heatmap. *Nucleic Acids Research* **(W1):**W566-W570 DOI:10.1093/nar/gkv468.
412 **Tomohiro S. 2021.** Genetic sequence analysis and characterization of bioactive compounds in mushroom-forming fungi.
413 *Bioscience, Biotechnology, and Biochemistry* **(1):**1 DOI:10.1093/bbb/zbaa067.
414 **Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. 2010.**
415 Transcript assembly and abundance estimation from RNA-Seq reveals thousands of new transcripts and switching
416 among isoforms. *Nature Biotechnology* **28:**511 DOI:10.1038/nbt.1621.
417 **Wang YY, Khoo KH, Chen ST, Lin CC, Wong CH, Lin CH. 2002.** Studies on the immuno-modulating and antitumor
418 activities of *Ganoderma lucidum* (Reishi) polysaccharides: functional and proteomic analyses of a fucose-containing
419 glyco-protein fraction responsible for the activities. *Bioorganic & Medicinal Chemistry* **10:**1057-1062
420 DOI:10.1016/S0968-0896(01)00377-7.
421 **White PJ. 2015.** Selenium accumulation by plants. *Annals of Botany* **117(2):** 217-235 DOI:10.1093/aob/mcv180.
422 **Wu Z, Bañuelos GS, Lin ZQ, Liu Y, Yuan L, Yin X, Li M. 2015.** Biofortification and phytoremediation of selenium in
423 China. *Frontiers in Plant Science* **6:** 136 DOI:10.3389/fpls.2015.00136.
424 **Xu J, Xu ZC, Zhu YJ, Luo HM, Qian J. 2014.** Identification and evaluation of reference genes for qRT-PCR normalization
425 in *Ganoderma lucidum*. *Current Microbiology* **68:**120-126 DOI:10.1007/s00284-013-0442-2.
426 **Yoshida M, Sugihara S, Inoue Y, Chihara Y, Kond M, Miyamoto S, Sukcharoen B. 2005.** Composition of chemical
427 species of selenium contained in selenium-enriched shiitake mushroom and vegetables determined by high performance
428 liquid chromatography with inductively coupled plasma mass spectrometry.*Journal of Nutritional Science and*
429 *Vitaminology* **51(3):** 194-199 DOI:10.1016/j.jnutbio.2004.12.014.
430 **Yu GJ, Wang M, Jie H,Yin YL,Chen YJ, Shuai J, Jin YX, Lan XQ, Wong BHC, Liang Y. 2012.** Deep insight into the
431 *Ganoderma lucidum* by comprehensive analysis of its transcriptome. *PloS One* **7:**e44031-e44031
432 DOI:10.1371/journal.pone.0044031.
433 **Yu JG, Zhai YF. 1979.** Studies on constituents of *Ganoderma capense* IV. *Acta Pharmaceutica Sinica* **6:** 20.
434 **Zarivi O, Cesare P, Ragnelli AM, Aimola P, Pacioni G.2015.** Validation of reference genes for quantitative real-time PCR
435 in Périgord black truffle (*Tuber melanosporum*) developmental stages. *Phytochemistry* **116:**78-86
436 DOI:10.1016/j.phytochem.2015.02.024.
437 **Zayed A, Lytle CM, Terry N. 1998.** Accumulation and volatilization of different chemical species of selenium by plants.
438 *Planta* **206(2):** 284-292 DOI:10.1007/s004250050402.
439 **Zhang J, Ren A, Chen H, Zhao M, Shi L, Chen M, Wang H, Feng Z, Minou N. 2015.** Transcriptome analysis and its
440 application in identifying genes associated with fruiting body development in basidiomycete *Hypsizygus*
441 *marmoreus*. *PloS One* **10:** e0123025 DOI:10.1371/journal.pone.0123025.

442 **Zhang WJ. 1987.** Biochemical research technology of compound polysaccharide. *Shanghai Science and Technology Press*
443 7–10.
444 **Zhao L, Zhao G, Zhao Z, Chen P, Tong J, Hu X. 2004.** Selenium distribution in a Se-enriched mushroom species of the
445 genus *Ganoderma*.*Journal of Agricultural & Food Chemistry* **52:**3954-3959 DOI:10.1021/jf049965i.
446 **Zhou Y, Chen L, Fan X, Bian Y. 2014.** De novo assembly of *Auricularia polytricha* transcriptome using Illumina
447 sequencing for gene discovery and SSR marker identification. *PloS One* **9:**e91740 DOI:10.1371/journal.pone.0091740.
448

A graph would have been easier to follow.

Table 1 ***G. lucidum*** **agronomic traits and nutrient content of fruiting bodies in different treatments**

NO.	MGR (mm/d)	Length (cm)	Size (cm^2)	Thickness (cm)	Yield (g/bag)	CP (%)	TT (%)	TSC (μg/g)	AR (%)
GCK	5.26±0.37 c	3.13±0.70 a	35.53±10.20 bc	2.35±0.26 a	125.82±5.56 ab	0.49±0.08 a	0.72±0.06 b	0.18±0.03 e	-
G50	5.44±0.27 bc	3.73±0.59 a	28.94±8.17 c	1.75±0.14 c	116.77±3.32 b	0.60±0.09 a	0.91±0.01 a	5.19±0.41 d	0.90
G100	6.38±0.46 a	3.28±0.77 a	39.97±10.34 abc	2.07±0.13 b	83.01±7.32 c	0.41±0.05 a	0.67±0.02 b	7.48±0.07 c	0.46
G200	5.75±0.42 b	3.46±0.74 a	39.43±6.38 abc	2.13±0.30 ab	149.50±6.97 a	0.52±0.24 a	0.86±0.02 a	7.59±0.07 c	0.42
G250	5.54±0.65 bc	3.17±0.45 a	48.94±15.78 a	2.02±0.26 b	91.64±12.10 c	0.42±0.07 a	0.88±0.01 a	11.79±0.47 a	0.32
G300	5.37±0.10 bc	3.10±1.16 a	44.61±14.78 ab	2.17±0.28 ab	125.48±5.84 ab	0.61±0.07 a	0.91±0.01 a	9.73±1.06 b	0.30

Abbreviations: *NO.* cultivating formula; *MGR* mycelial growth rate; *Length* the length of a single random *G. lucidum* stipe; *Size* the size of a single random *G. lucidum* cap; *Thickness* the thickness of a single random *G. lucidum* cap; *Yield* the fresh yield per bag; *CP* the content of the crude polysaccharide in the mature fruit bodies; *TT* the content of the triterpenoids in the mature fruit bodies; *TSC* the total selenium concentration in the mature fruit bodies; *AR* accumulation rate of sodium selenite, *AR*=(TSC×Dry yield)/(Sodium selenite concentration×Dry substrate weight), Dry yield=Yield×0.38, Dry substrate weight=0.51kg.*GCK* the control group without the addition of selenium; *G50* the treatment group with 50 μg/g sodium selenite addition; *G100* the treatment group with 100 μg/g sodium selenite addition; *G200* the treatment group with 200 μg/g sodium selenite addition; *G250* the treatment group with 250 μg/g sodium selenite addition; *G300* the treatment group with 300 μg/g sodium selenite addition. Data with different lower-case letters display significant differences (p-value <0.05) by the LSD method using a one-way ANOVA. *MGR*, *Length*, *Size*, *Thickness* and *Yield* were replicated 8-10 times, while *CP*, *TT* and *TSC* were replicated 3 times.

Not all are abbreviations

Not abbreviated

1 **Table 2 Gene number investigation in KEGG database at budding and mature stages**

KEGG	GCKb Up Number	G200b Up Number	Gb Total number	GCKm Up Number	G200m Up Number	Gm Total Number
Cellular Processes	25	62	576	42	40	573
Environmental Information Processing	19	102	437	54	27	461
Genetic Information Processing	15	76	815	30	58	826
Metabolism	246	419	1800	230	191	1766
Organismal Systems	23	138	657	98	32	687

2 Abbreviations: *GCKb* the control group without the addition of selenium at budding stage; *G200b* the treatment group with 200 μg/g sodium selenite addition at
3 budding stage; *GCKm* the control group without the addition of selenium at mature stage; *G200m* the treatment group with 200 μg/g sodium selenite addition at
4 mature stage.

5

Table 3 Gene expression profiles in *G. lucidum* transcriptome based on qPCR

Group	Gene ID	DESeq analysis based on RNA-seq		Validation of the DEGs by qPCR analysis		
		Log2Fold Change	p-value	GCK(2-⊿⊿Ct)	G200(2-⊿⊿Ct)	p-value
G200b vsGCKb	GL29881-G	4.33	1.30E-16	1.00±0.00	1.18E+04±0.00	3.09E-12
	GL23172-G	3.02	5.27E-07	1.03±0.09	9.11±0.00	3.38E-05
	GL24625-G	-1.82	1.61E-4	1.00±0.00	4.31E-04±0.00	2.67E-04
	GL28298-G	2.80	6.68E-09	1.00±0.01	10.09±0.00	2.30E-06
G200m vsGCKm	GL23172-G	-1.47	2.55E-02	1.01±0.02	0.40±0.00	2.79E-02
	GL29881-G	-3.36	3.37E-06	1.00±0.00	0.32±0.01	6.70E-03

2-⊿⊿Ct displays relative gene expression level using RPL4 as a reference genein qPCR analysis. Data are presented as means±standard deviation of three replicates. Abbreviations: *GCK* control group without selenium addition in the substrate; *G200* treatment group with 200 μg/g of seleniumaddition in the substrate; *m* at the mature stage; *b* at the budding stage.

The title does not make sense. You present both expression profile and qPCR data. So it is not based on qPCR.

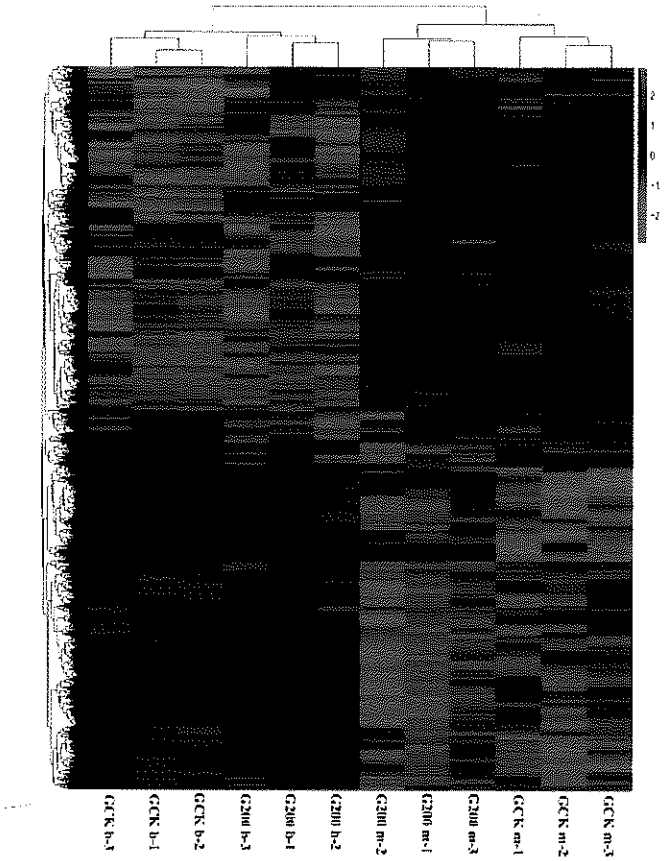

Fig. 1 A heatmap showing the log2(FoldChange) values of the selenium-responsive DEGs (n=3) in *G. ludium* samples.

The DEGs and samples were subject to bidirectional clustering analysis using the R package Pheatmap based on the Euclidean distance and complete linkage clustering. The up-expressed DEGs are coloured in red and the down-regulated DEGs in green, respectively.