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Comparative Transcriptome of Isonuclear Alloplasmic Strain Revealed the Important Role of Mitochondrial Genome in Regulating *Flammulina filiformis*

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Abstract: The golden–needle mushroom *Flammulina filiformis* is one of the most precious cultivated edible fungi in the world. Despite recent progress in the study of *F. filiformis*, there is still a gap in the regulation of the mitochondrial genome during browning, which poses a serious threat to the golden–needle mushroom industry. Comparative transcriptome analysis of two isonuclear alloplasmic strains showed that changes in the mitochondrial genome lead to different gene expression and key biological pathways at different stages in the two isonuclear alloplasmic strains. Furthermore, transcriptome analysis revealed that the mitochondrial genome has a significant role in the regulation of a multitude of critical metabolic pathways relating to the browning of *F. filiformis* fruiting bodies. Functional enrichment analysis showed that the differentially expressed genes were mainly involved in many vital processes of mitochondria, mitochondrial membrane, and multiple amino acid metabolisms of *F. filiformis*. Taken together, the current study highlights the crucial role of the mitochondrial genome in the growth of *F. filiformis* and could be beneficial to genetic breeding of elite varieties of edible fungi.

Keywords: transcriptome; isonuclear alloplasmic; Enokitake; mitochondrial genome; *Flammulina filiformis*

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1. Introduction

Flammulina filiformis, formerly known as *F. velutipes*, is commonly referred to as golden–needle mushroom, winter mushroom, and Enokitake [1]. This fungus is typically found in the wild growing on the dead trunks and stumps of broadleaf trees such as willows, elms, and poplars. Most wild *F. filiformis* have a reddish–brown color and a relatively thick stipe. In contrast, the cultivated *F. filiformis* are usually characterized by having a smaller cap and a thinner, stickier stipe surface when touched. Furthermore, the stipe of cultivated *F. filiformis* is more likely to be more tender and fragile compared to the wild variety [2]. It is clear that the shape of *F. filiformis* is subject to change depending on the concentrations of oxygen and carbon dioxide in its environment. When the oxygen concentration is higher, the cap of *F. filiformis* tends to be larger in size and the color of the cap is darker. On the other hand, if the carbon dioxide concentration is higher, the cap of *F. filiformis* will be smaller and its color will be lighter [3]. In recent years, *F. filiformis* has become increasingly popular in the market due to its unique flavor, nutritional benefits, and medicinal properties. The polysaccharides and secondary metabolites present in *F. filiformis* have been found to be effective at providing antibacterial, immunomodulatory, and antitumor effects [4]. Through

extensive research on mushroom breeding technology, it is feasible to cultivate edible fungi varieties that possess a variety of features, such as high yield, improved disease resistance, increased adaptability, and better quality. These developments make it possible to generate edible fungi that have greater economic value and superior quality, significantly elevating the economic profitability of the edible fungi industry.

Mushroom breeding is a long and laborious process that involves selecting the most desirable traits from different mushroom parents and crossbreeding them in order to produce the desired variety. Mycologists make use of the strain isolated from the cultivated *F. filiformis* varieties and utilize breeding strategies such as domestication and hybridization to obtain varieties with economic value [5]. Scientists can modify environmental factors such as temperature, light, air, and humidity to achieve the desired outcome, and thus optimize the yield of their mushroom [2,6–10]. Nevertheless, few studies have been conducted on the regulation of *F. filiformis* development and the formation of its fruiting body formation at the genetic level. Consequently, further studies are necessary to gain a better understanding of the genetic basis of mushroom breeding and to enhance the quality of the mushroom products. Mushroom breeding technology has enabled development of edible fungi varieties with high yields, strong disease resistance, and good quality, thus increasing their economic value and improving the economic benefits of the edible fungi industry.

In comparison with traditional breeding methods, mining functional gene strategies may offer a novel approach to modern molecular breeding. This strategy is based on the use of modern genetic tools to identify and characterize genes controlling important agronomic traits. By understanding the genetic basis of these traits, breeders can better select and combine genes from different varieties to create new varieties with desired characteristics. In our previous study, we discovered a method that can generate two distinct strains of *F. filiformis* with the same nucleus but different cytoplasm. These homonuclear and heterogeneous strains, cultured through protoplast mononucleosis, demonstrate remarkable variations in terms of their morphological characteristics, such as the color of the cap and the degree of browning of the fruiting stalk [11]. Notably, the characteristics of isonuclear alloplasmic strains of *F. filiformis* displayed remarkable variations during cultivation, particularly the color of the cap and the degree of browning of the fruiting stalk. This indicates that the mitochondrial genome has significant influence on the growth and development of *F. filiformis*. Whereas most of the genes in fungi are located on the chromosomal nucleus and are passed down to successive generations through sexual and asexual reproduction, the cytoplasm also contains a smaller mitochondrial genome. Interestingly, cytoplasmic genes have been demonstrated to have different genetic patterns from nuclear genes. Previous studies have highlighted the heterogeneity caused by cytoplasmic genomic mutations, and the possibility of transmitting these heterogeneities to successive generations [12]. Consequently, it is essential to investigate the role of the mitochondrial genome in the growth and reproduction of *F. filiformis* in order to make substantial progress in modern molecular breeding.

Mitochondria are essential organelles involved in a variety of cellular activities, including energy production, metabolism, biosynthesis, cell growth, differentiation, cell signaling, and senescence [13,14]. Mitochondria are the primary source of energy for most eukaryotic cells, such as animals, plants, and fungi [15–17]. Although most of the genetic information of eukaryotic cells is kept in the nucleus, mitochondria also possess all their genes in circular DNA molecules, which have their own replication and transcription mechanisms [18,19]. Most mitochondrial proteins are coded by nuclear genes, which are synthesized in the cytoplasm and then transported into mitochondria [20,21]. These nuclear gene–encoded proteins are responsible for transcription and translation of most subunits of the respiratory chain complexes found in the mitochondrial inner membrane. These respiratory chain complexes are essential for the generation of a proton gradient and ATP production on the membrane [22,23]. Consequently, the introduction of nuclear–encoded proteins into mitochondria is essential for mitochondrial function [24]. Furthermore, the mitochondrial genome can activate transcription factors, which are then transported to the

nucleus as molecular signals to regulate certain nuclear genes and modify the transcription of mitochondrial proteins [20,25,26].

At present, yeast has been proved to be the predominant organism for mitochondrial (mt) comparison and population genomics. This is due to the fact that the complete genome sequence of yeast mt has enabled researchers to investigate mt diversity both within and across closely related species [27,28]. With continuous advancements in research into fungi and the ongoing development of sequencing technology, scientists have increasingly appreciated the essential role of mtDNA in genetic breeding [29]. To date, the complete mitochondrial genome of various edible mushrooms have been analyzed and annotated, including *Agaricus bisporus*, *Agrocybe aegerita*, *Grifola frondose*, *Sparassis crispa*, and *Ganoderma lucidum*, constituting a strong foundation for further investigations into mitochondrial functions [11,30–33]. This study uses RNA–Seq to investigate the transcriptome of the isonuclear alloplasmic strains of *F. filiformis* across four main developmental stages. The specific aims of this study were to explore the effect of the mitochondrial genome on the growth and development of the isonuclear alloplasmic strains, analyze the difference mechanism, and, consequently, provide a reference for better understanding the mitochondrial genome function of high–yielding varieties.

2. Materials and Methods

2.1. Strain Samples

The isonuclear alloplasmic strains of *F. filiformis* used in this study are deposited in the Edible Fungi Center of Shanxi Agricultural University. The monocytic strain Y1, with its yellow nucleus and yellow cytoplasm, was isolated from the white *F. filiformis* strain f0012, which originated from Japan, while the monocytic strain Y33, which has a yellow nucleus and white cytoplasm, was isolated from the yellow *F. filiformis* strain f0027 (Sanming 1). To obtain the dikaryon strain, as illustrated in Figure 1, the activated Y1 and Y33 strains were inoculated onto the surface of a 90 mm diameter dish containing PDA medium and incubated in a dark incubator (24 °C) for seven days. Subsequently, the dikaryon strain with clamp connections on one side of Y33 was identified and then inoculated onto the surface of a PDA medium. This was cultured in the dark at 24 °C for an additional seven days, resulting in the binuclear strain Y33–J1, which has the same cytoplasm as the yellow *F. filiformis* strain Y33 being present on the side of strain Y33. The same method was employed to obtain the binuclear strain J1–Y33, which has the same cytoplasm as the yellow *F. filiformis* strain Y1. Thus, J1–Y33 and Y33–J1 have the same nucleus, but different cytoplasms.

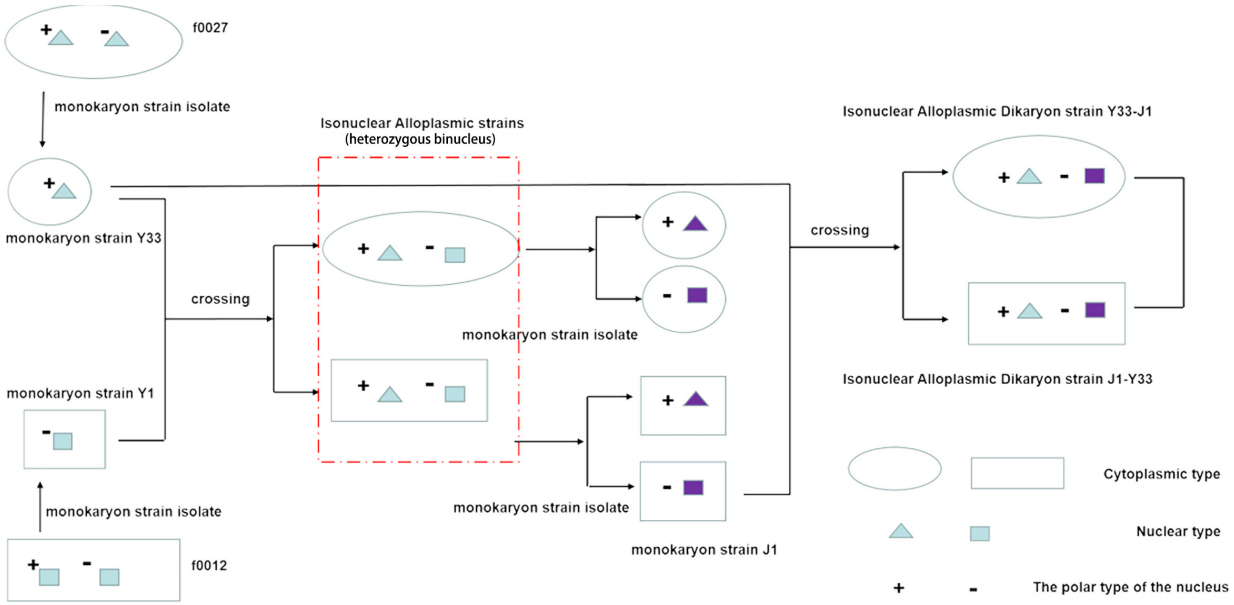

Figure 1. The process for constructing isonuclear alloplasmic strains of *Flammulina filiformis*.

2.2. Reference Genome

The genome of *F. filiformis* Fv01–10 was downloaded from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genome, accessed on 10 January 2023) and used as a reference genome for mapping the Illumina reads.

2.3. Culture and Collection of Samples

The *F. filiformis* strains were inoculated at the center of PDA medium and incubated in the dark at 24 °C. To guarantee the uniformity of the samples, routine management was employed during the cultivation process. Subsequently, sample collections were conducted at four different growth stages, including mycelium growth (M1, M2), primordial stage (P1, P2), young mushroom (YF1, YF2), and fruiting body (FB1, FB2) (Table 1). For each sample, three biological replicates were used to ensure the accuracy of the results.

Table 1. The developmental stage of isonuclear alloplasmic strains of *F. filiformis*.

Strain	Developmental Stage	Label
J1–Y33	The mycelium growth stage of *F. filiformis*	M1
Y33–J1	The mycelium growth stage of *F. filiformis*	M2
J1–Y33	The primordia formation stage of *F. filiformis*	P1
Y33–J1	The primordia formation stage of *F. filiformis*	P2
J1–Y33	The young mushroom stage of *F. filiformis*	YF1
Y33–J1	The young mushroom stage of *F. filiformis*	YF2
J1–Y33	The fruiting body stage of *F. filiformis*	FB1
Y33–J1	The fruiting body stage of *F. filiformis*	FB2

2.4. Total RNA Extraction and Transcriptome Sequencing

The total RNA of *F. filiformis* was extracted using Trizol reagent and then used to assess the integrity of the sample by 1% agarose gel electrophoresis. Subsequently, an Agilent 2100 Bioanalyzer was used for detecting the concentration of total RNA and its corresponding RNA integrity number (Rin). The NanoDrop Ultra Micro ultraviolet spectrophotometer was utilized to investigate the purity of the total RNA. The process of transcriptome sequencing involves extracting RNA from the organism of interest, converting it into cDNA, and fragmenting the cDNA. The RNASeq library (2 × 150 bp) was sequenced on the Illumina NovoSeq 6000 platform (Illumina, San Diego, CA, USA).

2.5. Transcriptome Analysis

Raw reads were processed using Trimmomatic v0.36 to remove adaptors and low–quality bases [34], and then these cleaned reads were aligned to the reference genome using Hisat2 v2.1.0. Subsequently, htseq–count was utilized to convert the mapped reads into a count matrix with gene and sample information [35]. To select differentially expressed genes (DEGs), the R package DESeq2 was employed with a false discovery rate (FDR) of $\leq$0.05 and an absolute value of log2(Fold Change) of $\geq$1 [36]. Finally, the R clusterProfiler package was used to conduct KEGG pathway and gene ontology (GO) enrichment analyses of the differentially expressed genes [37].

2.6. Quantitative RT–PCR (qRT–PCR) Validation

To validate the results of the transcriptome data, eight genes were randomly selected for RT–qPCR analysis. The primer was designed using Primer Premier 5 (Supplementary Table S2). The Universal SYBR® Green Supermix kit was used for qRT–PCR. A 20 µL reaction system was prepared with 2 × QPCR mixture (10 µL), primer (10 nmol), template cDNA (1 µL), and sterile water without nuclease (8 µL). The qRT–PCR procedure was as follows: pre–denaturation at 95 °C for 25 s; denaturation at 95 °C for 5 s, annealing at 60 °C for 5 s, 40 cycles. The fusion curve of the amplification product (65~95 °C, rising at 0.5 °C/5 s) was drawn to determine whether the reaction product was a single product. All

experiments were repeated three times for each sample. The relative expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method.

3. Results

3.1. Morphological Characteristics of the Isonuclear Alloplasmic Strains in F. filiformis Mycelia and Fruit Body Morphology Observation

Monocytic strain Y1 was isolated from the white *F. filiformis* strain f0012, and monocytic strain Y33 was isolated from the yellow *F. filiformis* strain f0027 (Sanming 1). When cultivated in the same period (15 days from the current base), the maximum length of Y33–J1 was approximately 10 cm, while that of J1–Y33 was approximately 13 cm. J1–Y33 (yellow) had a rapid growth rate, low browning at the base, and was easily able to open the cap; on the other hand, Y33–J1 (white) had a slow growth rate, serious browning at the base, and opened the cap with difficulty (Figure 2).

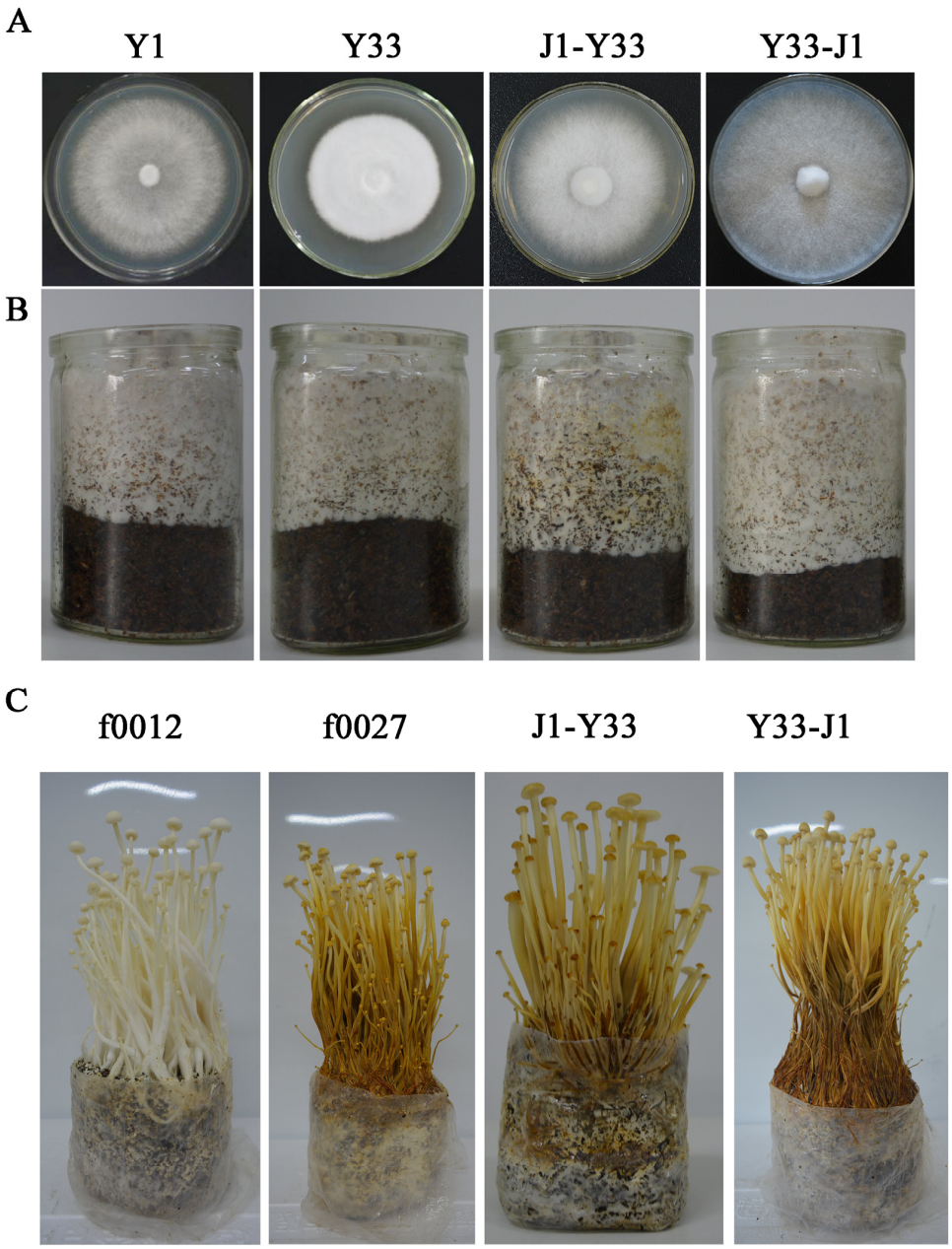

Figure 2. Biological characteristics of the isonuclear alloplasmic strains of *Flammulina filiformis*: (**A**) colony morphology; (**B**) mycelial growth rate in cultivation bottles; (**C**) fruit body morphology.

3.2. Analysis of Differentially Expressed Genes (DEGs)

To investigate the transcriptomic responses of each strain during different growth stages, RNA–Seq libraries were constructed from mRNA of the isonuclear alloplasmic strains J1–Y33 and Y33–J1 of *F. filiformis* during the mycelium growth stage (M1, M2), the primordia formation stage (P1, P2), the young mushroom stage (YF1, YF2), and the fruiting body stage (FB1, FB2). The total raw reads, total clean reads, clean bases, and GC content (%) of the transcriptome sequencing for the isonuclear alloplasmic strains J1–Y33 and Y33–J1 of *F. filiformis* are listed in Table S1. To explore the effects of mitochondria in *F. filiformis* during different growth stages, we conducted a comparative analysis of the transcript differences in isonuclear alloplasmic strains during the four growth periods (Table 2). According to the results, totals of 4388, 4239, and 3959 differentially expressed genes (DEGs) were identified in the stage of primordia formation (P1 vs. M1), the young mushroom stage (YF1 vs. M1), and fruiting body stage (FB1 vs. M1) of *F. filiformis* J1–Y33. In contrast, totals of 4502, 4442, and 3843 DEGs were observed in the stage of primordia formation (P2 vs. M2), the young mushroom stage (YF2 vs. M2), and fruiting body stage (FB2 vs. M2) of *F. filiformis* Y33–J1. In addition, we found that 1032 upregulated genes and 686 downregulated genes were identified in M2 compared to M1, 290 upregulated genes and 256 downregulated genes in P2 compared to P1, 314 upregulated genes and 482 downregulated genes in YF. These results suggest that the mitochondrial genomes of *F. filiformis* may have important roles in regulating the growth and development of different stages (Figure 3).

Table 2. Differentially expressed genes (DEG) of isonuclear alloplasmic strains of *F. filiformis*.

Label	Upregulated Genes	Downregulated Genes	DEGs
P1_vs._M1	2072	2316	4388
P2_vs._M2	2152	2350	4502
YF1_vs._M1	1999	2240	4239
YF2_vs._M2	2090	2352	4442
FB1_vs._M1	1777	2182	3959
FB2_vs._M2	1811	2032	3843
M2_vs._M1	1032	686	1718
P2_vs._P1	290	256	546
YF2_vs._YF1	314	482	796
FB2_vs._FB1	479	486	965

3.3. Functional Annotation of Differentially Expressed Genes

To further analyze the biological functions of the differentially expressed genes (DEGs), GO and KEGG enrichment analysis was conducted (Figure 4, Figures S1–S20). GO enrichment analysis revealed that the DEGs between the isonuclear alloplasmic strains Y33–J1 and J1–Y33 of *F. filiformis* during the mycelium growth stage (M2 vs. M1) were significantly enriched in three categories: biological process (BP), cellular component (CC), and molecular function (MF). Under the BP category, the DEGs were found to be enriched in anion transport (28 DEGs), transmembrane transport (49), anion transmembrane transport (18), organic anion transport (24), ascospore formation (18), cell development (22), cofactor transport (9), positive regulation of transcription by RNA polymerase I (7), reactive oxygen species metabolic process (7), and cell–substrate adhesion (5). As for the CC category, the DEGs were enriched in mitochondrial envelope (53), envelope (67), organelle envelope (67), mitochondrion (122), mitochondrial membrane (47), organelle inner membrane (38), mitochondrial inner membrane (36), intrinsic component of plasma membrane (15), intrinsic component of membrane (86), and organelle envelope lumen (12). Lastly, the MF category revealed that the DEGs were mainly enriched in transporter activity (46), transmembrane transporter activity (37), amide transmembrane transporter activity (6), antioxidant activity (7), organic anion transmembrane transporter activity (12), RNA polymerase II repressing transcription factor binding (5), anion transmembrane transporter activity (13), inorganic molecular entity transmembrane transporter activity (23), ion transmembrane transporter

activity (25), and repressing transcription factor binding (5) (Figure 4). Through GO enrichment analysis, it was found that the DEGs were enriched in various biological processes, cellular components, and molecular functions, such as anion transport, transmembrane transport, anion transmembrane transport, organic anion transport, oxidoreductase activity, and peptidase activity. These findings reveal the molecular mechanisms underlying the morphological differences between Y33–J1 and J1–Y33 and can be further used to improve the quality and yield of *F. filiformis*.

The DEGs between the isonuclear alloplasmic strains Y33–J1 and J1–Y33 of *F. filiformis* during the primordia formation of *F. filiformis* (P2 vs. P1) were found to be significantly enriched in the BP, CC, and MF categories. These DEGs are likely to be involved in the regulation of secondary metabolism, cellular protein metabolism, and proteolysis during primordia formation. This suggests that the regulation of these processes plays an important role in the formation of primordia in *F. filiformis*. It is worth discussing further how the DEGs are involved in the regulation of secondary metabolism, cellular protein metabolism, and proteolysis during primordia formation. Further research is needed to elucidate the precise roles of these DEGs in the regulation of these processes and how they interact with each other. In addition, further research could be performed to investigate the roles of other DEGs in the primordia formation of *F. filiformis*.

The results of the GO enrichment analysis demonstrate that the DEGs between the isonuclear alloplasmic strains Y33–J1 and J1–Y33 of *F. filiformis* during the young mushroom stage (YF2 vs. YF1) of *F. filiformis* significantly affect the expression of a variety of genes related to biological processes, cellular components, and molecular functions. These results suggest that the DEGs play an important role in regulating the development and growth of the young mushroom. Furthermore, these results may provide better understanding of the molecular mechanisms underlying the growth and development of *F. filiformis*, which could be a valuable source of information for further research. The GO enrichment analysis provides valuable information about the molecular mechanisms underlying the growth and development of *F. filiformis*. This is an important step in understanding the genetic basis for the growth and development of this species, which could lead to potential biotechnological applications. Additionally, further research is needed to determine the exact mechanisms through which the DEGs affect the growth and development of *F. filiformis*, and to explore the potential applications of these results.

These results suggest that the DEGs between the isonuclear alloplasmic strains Y33–J1 and J1–Y33 of *F. filiformis* during fruiting body stage (FB2 and FB1) significantly affect the expression of genes related to meiotic cell cycle, which plays a key role in the development of *F. filiformis*. In addition, analysis of the cellular components showed that the DEGs also affect the expression of genes related to the cell surface, protein–DNA complex, and extracellular region, indicating that the DEGs have an important role in the regulation of cell processes. The DEGs also affect the expression of genes related to oxidoreductase activity, transition metal ion binding, DNA binding, and endopeptidase activity. These findings suggest that the DEGs between fruiting body stage (FB2 and FB1) of isonuclear alloplasmic strains of *F. filiformis* play a critical role in the regulation of various metabolic activities. It is worth discussing how these DEGs regulate the meiotic cell cycle and affect the metabolic activities of *F. filiformis*. Further research is needed to identify the specific mechanisms by which the DEGs affect these processes. However, more research is needed to understand the exact mechanisms involved in these processes. In particular, further research should focus on elucidating the molecular pathways that are involved in the regulation of mitochondria and redox process. Additionally, further investigation into the effects of different environmental factors on the development of fruiting body is also needed.

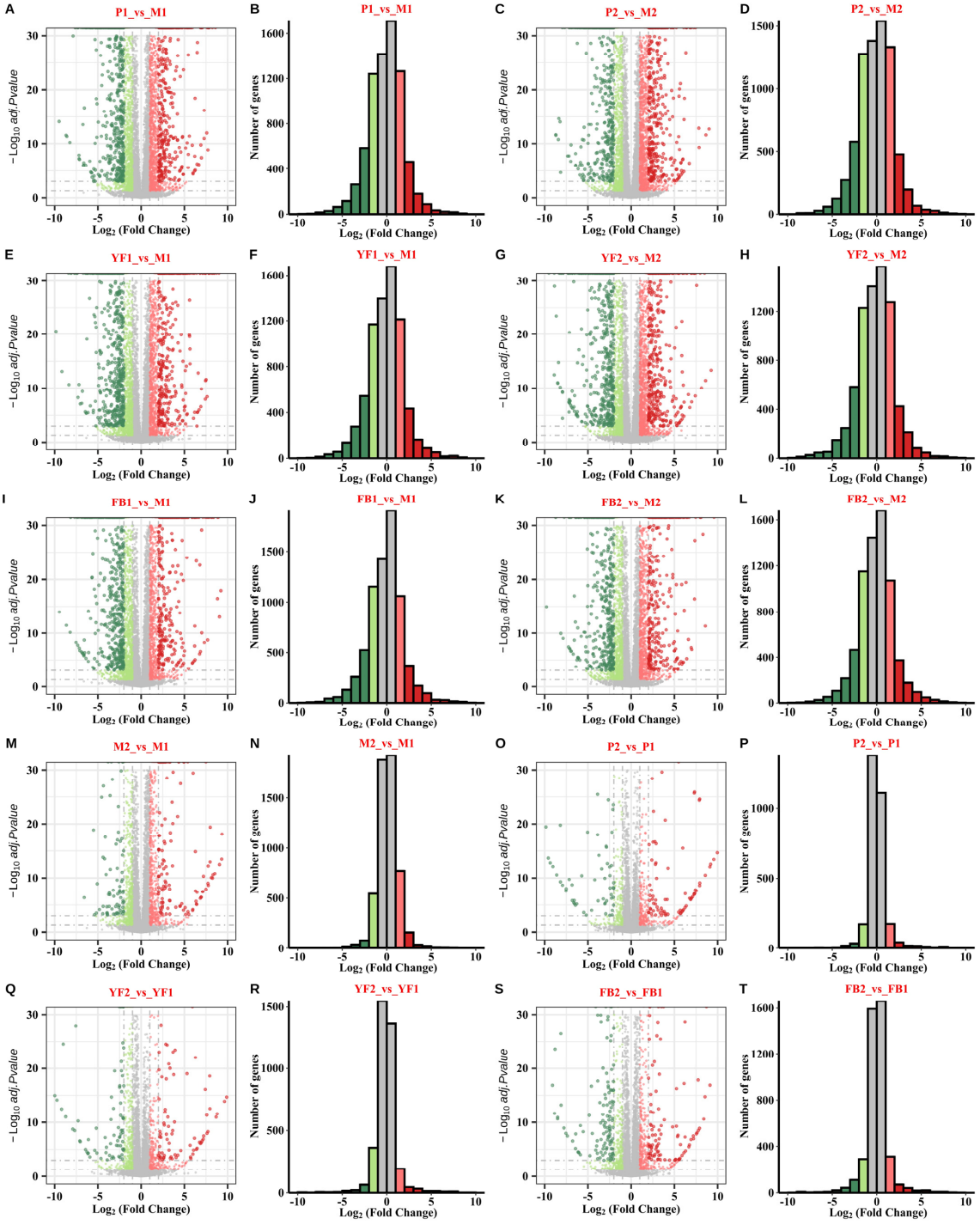

Figure 3. The global gene expression pattern of the isonuclear alloplasmic strains in *Flammulina filiformis* mycelia and fruit body is depicted in the volcano plot, with the y-axis indicating the mean expression value of log10 (adjust *p*–value, also referred to as false discovery rate (FDR)), and the x-axis displaying the log2(fold change value). Red dots represent the significantly differentially expressed transcripts (FDR < 0.05) that were upregulated under the given treatments, while green dots signify significantly differentially expressed transcripts (FDR < 0.05) that were downregulated. Gray dots, meanwhile, denote transcripts whose expression levels did not reach statistical significance (FDR > 0.05) or the absolute value of log2(fold change value) was smaller than 1. Volcano plots show the gene expression pattern for the isonuclear alloplasmic strains in *F. filiformis* (**A**,**C**,**E**,**G**,**I**,**K**,**M**,**O**,**Q**,**S**). Histogram plots showing the gene expression pattern for the isonuclear alloplasmic strains in *F. filiformis* mycelia and fruit body are also presented (**B**,**D**,**F**,**H**,**J**,**L**,**N**,**P**,**R**,**T**).

Figure 4. Comparative gene ontology (GO) analysis of the differentially expressed genes (DEGs) between the transcriptome of the isonuclear alloplasmic strains in *Flammulina filiformis* mycelia and fruit body was carried out, with the results being summarized in three main GO categories (biological process, cellular component, molecular function). The count denotes the number of differentially expressed genes annotated in the GO category, while the x-axis indicates various mutants and the y-axis indicates the GO term. The color signifies the −log10(q value) for each GO category.

3.4. KEGG Enrichment Analysis of Differentially Expressed Genes

KEGG enrichment analysis revealed that the DEGs of the isonuclear alloplasmic strains Y33–J1 and J1–Y33 of *F. filiformis* during mycelium growth stage (M2 vs. M1) mainly enriched in glutathione metabolism (10); sulfur metabolism (6); tryptophan metabolism (11); cyanoamino acid metabolism (6); methane metabolism (7); glycerophospholipid metabolism (11); nitrogen metabolism (6); arginine and proline metabolism (10); phenylalanine metabolism (8); ether lipid metabolism (4); MAPK signaling pathway–yeast (18); peroxisome (13); mitophagy–yeast (8); ubiquinone and other terpenoid–quinone biosynthesis (4); cysteine and methionine metabolism (8); glycine, serine, and threonine metabolism (9); glycosylphosphatidylinositol (GPI)–anchor biosynthesis (4); fructose and mannose metabolism (5); glycerolipid metabolism (8); biosynthesis of unsaturated fatty acids (4); protein export (4); pentose and glucuronate interconversions (6); amino sugar and nucleotide sugar metabolism (10); starch and sucrose metabolism (9); inositol phosphate metabolism (4); tyrosine metabolism (4); and various types of N–glycan biosynthesis (3). It further highlights that the mycelium growth stage (M2 vs. M1) of isonuclear alloplasmic strains of *F. filiformis* indicated a significant influence on a wide range of metabolic processes (Figure 5).

KEGG enrichment analysis showed that the isonuclear alloplasmic strains Y33–J1 and J1–Y33 of *F. filiformis* during the primordia formation stage (P2 vs. P1) significantly affected the expression of many genes involved in the indole alkaloid biosynthesis; various types of N–glycan biosynthesis; n–Glycan biosynthesis; valine, leucine, and isoleucine degradation; glutathione metabolism; pentose and glucuronate interconversions; arginine and proline metabolism; glycine, serine, and threonine metabolism; and oxidative phosphorylation. Similarly, young mushroom stage (YF2 vs. YF1) of isonuclear alloplasmic strains of *F. filiformis* significantly affected the expression of many genes associated with cysteine and methionine metabolism; the two–component system; n–Glycan biosynthesis; protein processing in endoplasmic reticulum; sulfur metabolism; proteasome; ABC transporters; pantothenate and CoA biosynthesis; inositol phosphate metabolism; pentose and glucuronate interconversions; starch and sucrose metabolism; glycerophospholipid metabolism; oxidative phosphorylation; and glycine, serine, and threonine metabolism.

KEGG enrichment analysis revealed that the isonuclear alloplasmic strains Y33–J1 and J1–Y33 of *F. filiformis* during the young mushroom stage (YF2 vs. YF1) had a significant impact on the expression of many genes associated with various metabolic pathways. These pathways included the cysteine and methionine metabolism (6), the two–component systems (5); n–Glycan biosynthesis (4); protein processing in endoplasmic reticulum (8); sulfur metabolism (3); various types of N–glycan biosynthesis (3); proteasome (4); ABC transporters (3); pantothenate and CoA biosynthesis (3); inositol phosphate metabolism (3); pentose and glucuronate interconversions (3); starch and sucrose metabolism (4); glycerophospholipid metabolism (3); oxidative phosphorylation (4); and glycine, serine, and threonine metabolism (3). These results are highly significant, as they suggest that the expression of genes involved in these metabolic pathways is highly regulated and may play a critical role in the development and growth of *F. filiformis* during the young mushroom stage. Further research is needed to determine the precise mechanisms by which these metabolic pathways are regulated and how they affect the growth and development of this species.

KEGG enrichment analysis demonstrated that the isonuclear alloplasmic strains Y33–J1 and J1–Y33 of *F. filiformis* during the fruiting body stage (FB2 vs. FB1) had a marked influence on the expression of numerous genes related to the various types of N–glycan biosynthesis (5); base excision repair (5); amino sugar and nucleotide sugar metabolism (9); ubiquinone and other terpenoid–quinone biosynthesis (3); protein export (3); nucleotide excision repair (4); DNA replication (4); galactose metabolism (3); pentose and glucuronate interconversions (4); methane metabolism (3); peroxisome (6); tyrosine metabolism (3); valine, leucine, and isoleucine degradation (3); meiosis–yeast (6); the two–component systems (3); cell cycle–yeast (7); pyruvate metabolism (3); purine metabolism (4); starch

and sucrose metabolism (4); and glycerolipid metabolism (3). This indicates that the two strains of *F. filiformis* underwent considerable critical metabolism during the fruiting body stages of mushroom formation.

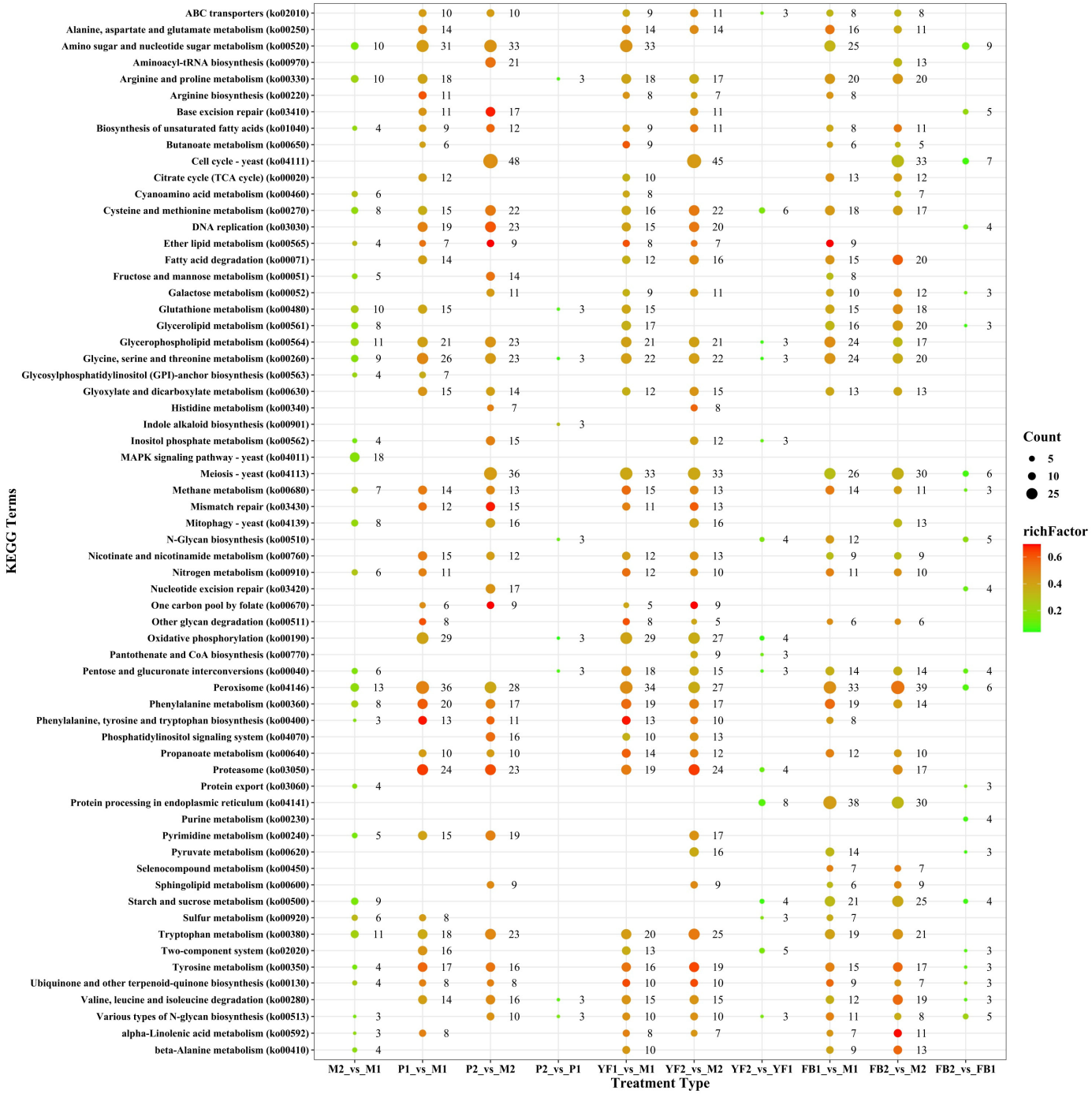

Figure 5. Comparative KEGG pathways analysis was conducted on the DEGs between the transcriptome of the isonuclear alloplasmic strains in *Flammulina filiformis* mycelia and fruit body. The x-axis shows the various mutants, while the y-axis reflects the KEGG pathway. The count indicates the number of DEGs enriched in the pathway, and the color indicates the enrichment factors of each pathway.

3.5. Validation of DEGs by Quantitative RT–PCR

It was discovered that in the YF1 and YF2, P1 and P2, FB1 and FB2 stages, genes related to the mitochondrial membrane in a cellular component were significantly enriched. Upon further analysis of the expression patterns of genes related to mitochondria, it was observed that many crucial genes associated with mitochondria displayed varying levels of expression in the isonuclear alloplasmic strains for both the young mushroom and fruiting body stages (Figure 6). The result showed that GB009999 (FMP32, DUF1640–domain–containing protein); GB000362 (ABC1 family); GB001398 (belongs to the mitochondrial carrier); GB006100 (OAC1, belongs to the mitochondrial carrier); GB003573 (COQ1, belongs to the FPP GGPP synthase family); GB004098 (HER2, allows the formation of correctly charged Gln–tRNA(Gln) through the transamidation of misacylated Glu–tRNA(Gln) in the mitochondria); GB006890 (CRC1, belongs to the mitochondrial carrier); GB003472 (COQ6, FAD–dependent monooxygenase required for the C5–ring hydroxylation during ubiquinone biosynthesis); GB000811 (ICP55, peptidase M24); GB006766 (COX6A, cytochrome c oxidase); GB009201 (mitochondrial carrier required for the biosynthesis of heme, possibly by facilitating 5–aminolevulinate (ALA) production); GB006357 (SAC1, SacI homology domain); GB008448 (COX12, the terminal oxidase in mitochondrial electron transport); GB010069 (HFD1, belongs to the aldehyde dehydrogenase family); GB004610 (SYM1, belongs to the peroxisomal membrane protein PXMP2 4 family); GB008257 (SFC1, belongs to the mitochondrial carrier); GB002887 (QCR2, ubiquinol–cytochrome C reductase complex core protein 2); GB006846 (belongs to the mitochondrial carrier), GB007656 (GUT2, DAO–domain–containing protein); GB003065 (POR1, eukaryotic porin); GB004152 (CDC48, belongs to the AAA ATPase family) were significantly upregulated. The expression profiles of some genes observed in transcriptome analysis were validated by RT–qPCR, with nine genes associated with the mitochondrial membrane (GB001767, GB005431, GB006100, GB006766, GB007198, GB008257, GB009214, GB009768) exhibiting consistent upregulation compared to RNA–Seq data (Figure 7). This result indicates that many genes related to the mitochondrial membrane are broadly upregulated in the isonuclear alloplasmic strains in *F. filiformi* during the growth stage, suggesting that these genes related to mitochondrial membrane are required for the growth of the strains. Further study would be needed to explore the underlying molecular mechanisms of this process and the exact role of the mitochondrial membrane in the growth of the strains.

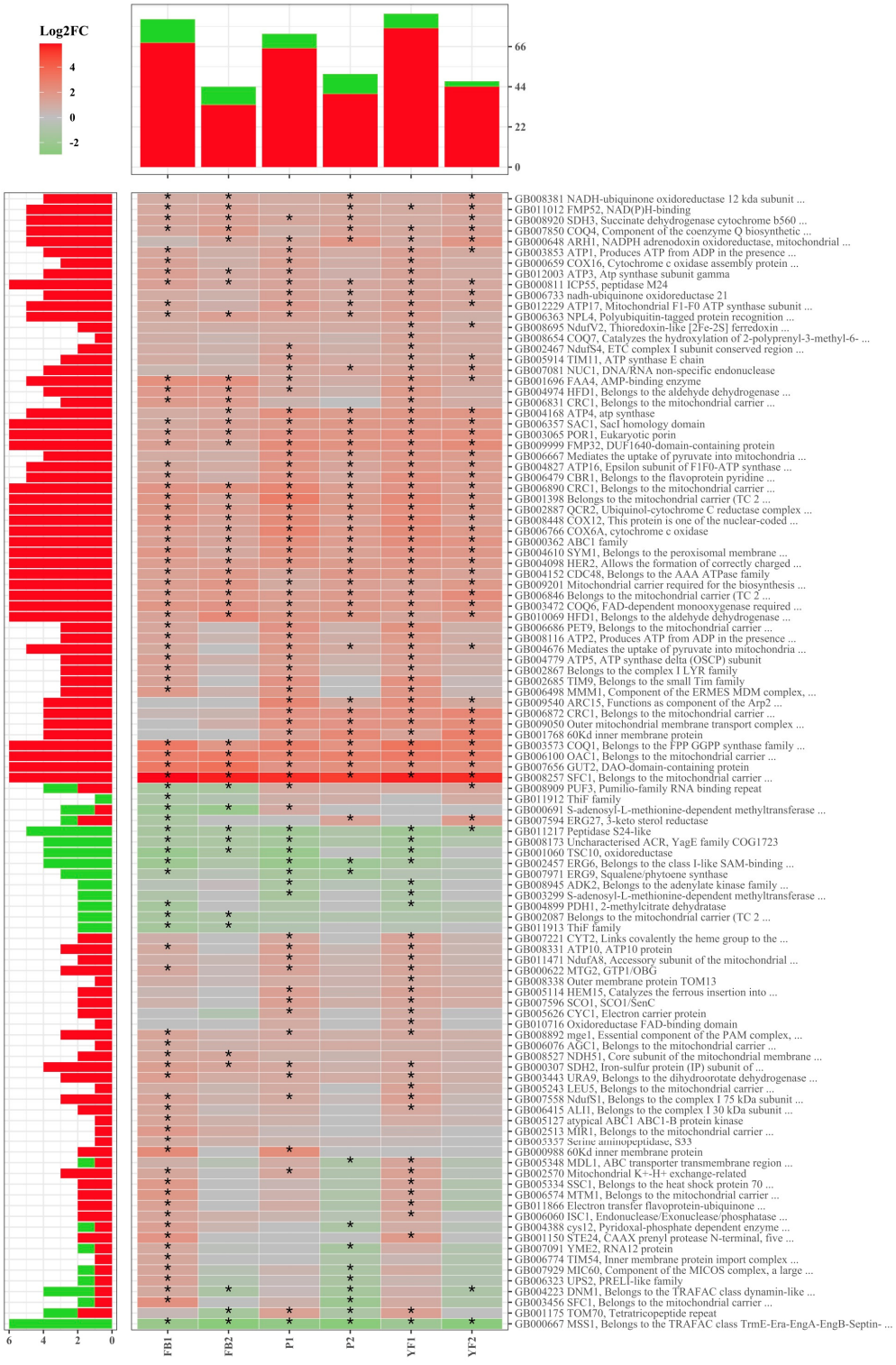

Figure 6. Multiple genes related to the mitochondrial membrane were significantly altered between the transcriptomes of the isonuclear alloplasmic strains in *Flammulina filiformis* mycelia and fruit bodies (FB1 vs. M1, FB2 vs. M2, P1 vs. M1, YF2 vs. M2, P2 vs. M2, YF1 vs. M1). The expression pattern of genes related to mitochondrial membrane in *F. filiformis* was investigated across different growth stages. The bar plot above the heatmap displays the differentially expressed genes (DEGs) for each treatment. The bar plot on the right illustrates the number of DEGs. Red indicates upregulation, gray indicates no differential expression, and green indicates downregulation. The asterisk in the heatmap corresponds to an absolute value of log2(FC) >= 1.0, with a false discovery rate (FDR) of less than 0.05.

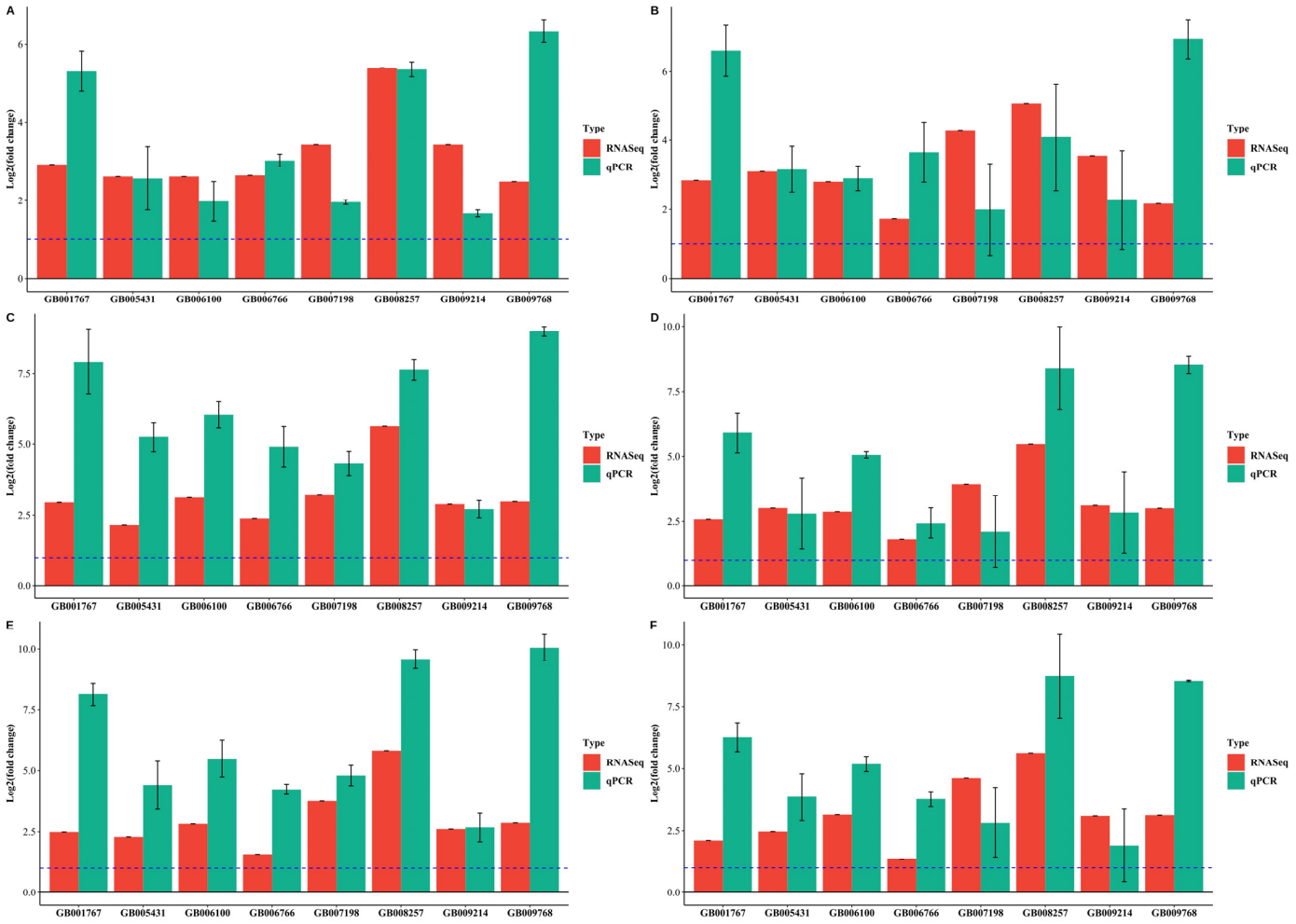

Figure 7. The expression of the gene related to the mitochondrial membrane in isonuclear alloplasmic strains in *Flammulina filiformis* mycelia and fruit bodies (P1 vs. M1, P2 vs. M2, YF1 vs. M1, YF2 vs. M2, FB1 vs. M1, FB2 vs. M2). The blue dotted line in the figure represents log2(FC) = 1. (P1 vs. M1 (**A**), P2 vs. M2 (**B**), YF1 vs. M1 (**C**), YF2 vs. M2 (**D**), FB1 vs. M1 (**E**), FB2 vs. M2 (**F**)).

4. Discussion

This work performed transcriptome analysis of the isonuclear alloplasmic strain during the mycelium growth stage, the primordia formation, the young mushroom stage, and the fruiting body stage of *F. filiformis*. Through comparative analysis of the transcription group of the mycelium stage, the primordial stage, young mushroom stage, and fruiting body stage, this study seeks to explore the molecular mechanism and regulation network of the globe mitochondrial genome of the mushroom, and provides a theoretical basis for the selection of new varieties of *F. filiformis*. The results of this study showed that different mitochondria lead to distinct phenotypes of mycelia, cap color, and degree of browning of the fruiting body stipe. It was found that the gene expression on the multichannel pathway of different cytoplasm has changed substantially. The expression of mitochondrial–related genes of different cytoplasm cells was also altered. The activity of the endoninase and the hydrolyzed enzyme activity of the lingosic acid plays an important role in the difference in mitochondrial function, which directly affects the energy metabolism of cells, and thus causes significant differences in the shape of the iconic strain. This variation in cytoplasm directly impacts the closely related metabolism and synthesis of proteins and other macromolecular components, such as enzymes and amino acids, which further causes the change in morphological features [11].

Mitochondria acts as energy factories in cells, and isonuclear alloplasmic strains of *F. filiformis* may have an impact on the differential expression of genes associated with the mitochondria. Through transcriptome analysis of M1 vs. M2, P1 vs. P2, YF1 vs. YF2, and FB1 vs. FB2, we identified 4025 differentially expressed genes (DEGs). Upon further investigation, it was found that in M1 vs. M2, there was a significantly enriched expression of processes such as ubiquinone metabolism, ubiquinone biosynthesis, ubiquitin hydrolase activity, RNA binding, serine peptidase activity, serine endopeptidase inhibitor activity, serine endopeptidase activity, and serine hydrolase activity. Notably, ubiquinone (coenzyme Q) is involved not only in the transport of electrons and protons in the mitochondria and electron transport related to ATP synthesis, but also plays a key role as a reducing form of antioxidant (panthenol), which helps to prevent lipid peroxidation on biological membranes. Panthenol, a fat–soluble antioxidant, is synthesized de novo by animal cells and has an enzyme mechanism that enables regeneration of antioxidants from their oxidation form, thus inhibiting lipid peroxidation [38–40]. This suggests that ubiquinone and its reducing form panthenol play a crucial role in the cellular defense against oxidative damage during the development of *F. filiformis*.

The proliferation of cells is closely related to the process of substantial synthesis of a serine and the rapid operation of glycolysis. This metabolic process can be observed in the formation of filamentous fungi from young mushrooms to the fruiting body stage, which involves the rapid division and proliferation of cells. This process requires the consumption of a large amount of carbon source substances. Comparisons between FB1 and FB2 and YF1 and YF2 revealed multiple DEGs involved in the metabolism of serine family amino acids [41,42]. Serine is a crucial part of the cellular metabolic network, connecting the single carbon cycle with glycolysis to support the balance between oxidants and antioxidants and to facilitate cell proliferation. Additionally, serine has antioxidant properties that are activated through multiple molecular pathways. It is the precursor of glycine and cysteine, both of which are the main substrates for the synthesis of the cellular antioxidant glutathione (GSH), and may play an important role in the adaptive response to oxidative stress. Moreover, serine is a key component in the synthesis of NADPH, a major cell reductant [43]. The de novo synthesis of serine is of critical importance in the maintenance of metabolic processes, preserving the redox homeostasis at the cellular and mitochondrial levels. Inhibition of serine synthesis can lead to mitochondrial dysfunction and oxidative stress damage, which further highlights the importance of serine–related metabolism for sustaining the antioxidant capacity of filamentous F cells [44].

The wild–type strain of *F. filiformis* is a yellow strain that is distinct from the white varieties that are currently in circulation. To date, however, the exact mechanism behind the color change between different strains has yet to be elucidated. In order to determine the underlying cause, a comparative analysis was conducted between FB1 and FB2, P1 and P2, and YF1 and YF2, leading to the identification of multiple DEGs (differentially expressed genes) related to the metabolism of aspartic acid family amino acids. The results of this study suggest that the intermediate products of central metabolism, particularly the citric acid cycle, vary between different strains. In eukaryotic cells, the citric acid cycle takes place in the matrix of mitochondria and it plays a critical role in regulating the expression of nucleic acid–binding proteins, transcription factors, signal molecules, and enzyme regulators. For example, superoxide dismutase (SOD2), a key enzyme involved in the reactive oxygen species (ROS) in mitochondria, can reduce cellular oxidative damage by eliminating ROS. This may be an adaptive strategy employed by *F. filiformis* to regulate the browning process. It is therefore hypothesized that the variations in the citric acid cycle, as well as the consequent regulation of the aforementioned proteins, could be key factors in the color change between the yellow and white strains of *F. filiformis*. Further studies are needed to explore the exact role of the citric acid cycle and its related proteins in the color change in *F. filiformis* [45]. The fundamental dissimilarity between the phenotypes of the isonuclear alloplasmic strains of *F. filiformis* is that their cytoplasmic mitochondrial genomes are distinct. Consequently, we established that there is no variation in the genes associated

with mitochondrial membrane in the isonuclear alloplasmic strains at the primordial stage through the GO pathway analysis of transcriptome data. Nevertheless, it is intriguing that in the young mushroom and fruiting body stage, compared with YF2 and FB2, the genes related to mitochondrial membrane are significantly upregulated. In particular, the mitochondrial inner membrane gene, which is mainly involved in mitochondrial energy conversion, may have an influence on *F. filiformis*.

5. Conclusions

Through the structuring and comparison of isonuclear alloplasmic strains, this study has illustrated that mitochondria may play a role in affecting mycelial growth rate, the color of the cap, and the degree of browning of the fruiting body stipe in *F. filiformis*. Moreover, the analysis of the transcriptome has revealed that the divergence in mitochondrial genomes can lead to a change in the gene expression profile of homonuclear and heterogeneous strains. Based on this, it can be inferred that tyrosine metabolism, lysine degradation, glutathione metabolism, proteasome, tryptophan metabolism, steroid biosynthesis, oxidative phosphorylation, phenylalanine metabolism, cysteine and methionine metabolism, and peroxisome may be the key biological pathways for controlling the degree of browning via the interaction between the nuclear gene and the mitochondrial gene of *F. filiformis*. Such research could provide more knowledge on the role of mitochondria in *F. filiformis*, thereby establishing the way for the development of superior new varieties.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13040998/s1, Table S1. Summary of the transcriptome sequencing of the isonuclear alloplasmic strain of F. filiformis. Table S2. The primers used in this study. Figure S1. The Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) between the fruiting body stage of *F. filiformis* J1–Y33 (FB1) and the mycelium growth stage of *F. filiformis* J1–Y33 (M1) was conducted. Figure S2. KEGG enrichment analysis of the differentially expressed genes (DEGs) between the fruiting body stage of *F. filiformis* J1–Y33 (FB1) and the mycelium growth stage of *F. filiformis* J1–Y33 (M1) is presented. Figure S3. The Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) between the fruiting body stage of *F. filiformis* Y33–J1 (FB2) and the fruiting body stage of *F. filiformis* J1–Y33 (FB1) was conducted. Figure S4. KEGG enrichment analysis of the differentially expressed genes (DEGs) between the fruiting body stage of *F. filiformis* Y33–J1 (FB2) and the fruiting body stage of *F. filiformis* J1–Y33 (FB1) is presented. Figure S5. The Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) between the fruiting body stage of *F. filiformis* Y33–J1 (FB2) and the mycelium growth stage of *F. filiformis* J1–Y33 (FB1) was conducted. Figure S6. KEGG enrichment analysis of the differentially expressed genes (DEGs) between the fruiting body stage of *F. filiformis* Y33–J1 (FB2) and the mycelium growth stage of *F. filiformis* J1–Y33 (FB1) is presented. Figure S7. The Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) between the mycelium growth stage of *F. filiformis* Y33–J1 (M2) and the mycelium growth stage of *F. filiformis* J1–Y33 (M1) was conducted. Figure S8. KEGG enrichment analysis of the differentially expressed genes (DEGs) between the mycelium growth stage of *F. filiformis* Y33–J1 (M2) and the mycelium growth stage of *F. filiformis* J1–Y33 (M1) is presented. Figure S9. The Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) between the primordia formation stage of *F. filiformis* J1–Y33 (P1) and the mycelium growth stage of *F. filiformis* J1–Y33 (M1) was conducted. Figure S10. KEGG enrichment analysis of the differentially expressed genes (DEGs) between the primordia formation stage of *F. filiformis* J1–Y33 (P1) and the mycelium growth stage of *F. filiformis* J1–Y33 (M1) is presented. Figure S11. The Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) between the primordia formation stage of *F. filiformis* Y33–J1 (P2) and the mycelium growth stage of *F. filiformis* Y33–J1 (M2) was conducted. Figure S12. KEGG enrichment analysis of the differentially expressed genes (DEGs) between the primordia formation stage of *F. filiformis* Y33–J1 (P2) and the mycelium growth stage of *F. filiformis* Y33–J1 (M2) is presented. Figure S13. The Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) between the primordia formation stage of *F. filiformis* Y33–J1 (P2) and the primordia formation stage of *F. filiformis* J1–Y33 (P1) was conducted. Figure S14. KEGG enrichment analysis of the differentially expressed genes (DEGs) between the primordia formation stage of *F. filiformis* Y33–J1 (P2) and the primordia formation stage of *F. filiformis* J1–Y33 (P1)

is presented. Figure S15. The Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) between the young mushroom stage of *F. filiformis* J1–Y33 (YF1) and the mycelium growth stage of *F. filiformis* J1–Y33 (M1) was conducted. Figure S16. KEGG enrichment analysis of the differentially expressed genes (DEGs) between the young mushroom stage of *F. filiformis* J1–Y33 (YF1) and the mycelium growth stage of *F. filiformis* J1–Y33 (M1) is presented. Figure S17. The Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) between the young mushroom stage of *F. filiformis* Y33–J1 (YF2) and the mycelium growth stage of *F. filiformis* Y33–J1 (M2) was conducted. Figure S18. KEGG enrichment analysis of the differentially expressed genes (DEGs) between the young mushroom stage of *F. filiformis* Y33–J1 (YF2) and the mycelium growth stage of *F. filiformis* Y33–J1 (M2) is presented. Figure S19. The Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) between the young mushroom stage of *F. filiformis* Y33–J1 (YF2) and the young mushroom stage of *F. filiformis* J1–Y33 (YF1) was conducted. Figure S20. KEGG enrichment analysis of the differentially expressed genes (DEGs) between the young mushroom stage of *F. filiformis* Y33–J1 (YF2) and the young mushroom stage of *F. filiformis* J1–Y33 (YF1) is presented.

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