Biotechnology and Bioprocess Engineering 28: 406-418 (2023)
DOI 10.1007/s12257-022-0207-5

pISSN 1226-8372
eISSN 1976-3816

RESEARCH PAPER

Dissecting Metabolic Regulation in Mycelial Growth and Fruiting Body Developmental Stages of *Cordyceps militaris* through Integrative Transcriptome Analysis

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Received: 5 July 2022 / Revised: 4 September 2022 / Accepted: 20 September 2022

Abstract Dissecting the cellular metabolism of *Cordyceps militaris* is important for the efficient production of bioactive compounds of a target with medicinal and industrial applications. However, the metabolic functions during developmental stages in this fungus at a system level are still unexplored. In this study, we aimed to reveal the metabolic functions and regulation of *C. militaris* TBRC6039 relevant to its developmental stages, including mycelial growth (MY) and fruiting body (FB) stages through integrative transcriptome analysis. The transcriptome analysis showed that 9,256 genes of *C. militaris* were expressed in both stages. Of them, 1,877 genes, residing primarily in the cell division cycle and amino acid, carbohydrate, and lipid metabolisms, exhibited significant differences in transcript levels between the MY and FB stages. Through integration with genome-scale networks analysis, the unique reporter metabolites (*e.g.*, α-D-glucose, β-D-glucose, D-galactose, triacylglycerol, and diacylglycerol) and key regulators (*e.g.*, AtfA, Atf2, and Yap1 transcription factors) were identified in *C. militaris* when grown at FB stage, linking to up-regulation of the metabolic genes involved in galactose and polysaccharide metabolisms, as well as glycerolipid and glycerophospholipid biosynthesis. Moreover, the high cordycepin content is related to the upregulated genes in lipid metabolism during the FB stage. Our findings suggest that the transcriptional regulation of these metabolic pathways played a crucial role in specific developmental stages of *C. militaris*. This study serves for cultivation process improvement for overproduction of valuable metabolites in *C. militaris* through an emerging systems and synthetic biology approach.

Keywords: *Cordyceps militaris*, fruiting body, transcriptome analysis, reporter metabolite, transcription factor

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1. Introduction

Cordyceps militaris is an entomopathogenic fungus belonging to Ascomycetes. It has been widely used in traditional Chinese medicine because it contains various bioactive compounds, *i.e.*, cordycepin, adenosine, mannitol, polysaccharides, sterols, and various amino acids [1-3]. Among these, cordycepin (3'-deoxyadenosine), a nucleoside analog, has attracted the most attention due to its therapeutic potential, such as anti-tumor, anti-cancer, anti-diabetic, anti-inflammatory, anti-fungal, anti-microbial, anti-viral,

immunomodulatory and anti-hyperlipidemic activities [4,5]. In addition to medicinal applications, *C. militaris* extract has been used as an ingredient in several functional foods, nutraceutical, and cosmetic products. The increasing demand for *C. militaris* leads to the development of a fermentation process for enhancing the production of the targeted metabolites.

Nowadays, solid-state fermentation (SSF) is the main process for producing fruiting bodies of *C. militaris* for commercial purposes. Much attention has also been given to the developing of submerged fermentation and surface liquid cultivation of *C. militaris*. However, *C. militaris* is often cultured in surface liquid cultivation, which provides high cordycepin productivity [6]. Various parameters of the cultivation conditions of *C. militaris* were investigated to optimize the efficient cultivation process, such as medium composition, light, temperature, and pH. It has been reported that apart from the fungal strains, the contents of targeted metabolites (*e.g.*, cordycepin and adenosine) were depended on the cultivation conditions. The effects of various carbon sources and carbon/nitrogen ratios on the cordycepin production by *C. militaris* were studied [7-10]. It has been documented that light is a crucial environmental factor for developing fruiting bodies and the pigment formation in fruiting bodies [11]. So far, the regulatory mechanism underlying the developmental stages of this fungus is still unclear. The metabolites associated with developmental stages of cultivated *C. militaris* were investigated by metabolomics analysis [12], showing that the aging stage of the fruiting body (FB) enriched in cordycepin, mannitol, and γ-aminobutyric acid.

With the advancement of high-throughput sequencing technology, the genomes and transcriptomes of *C. militaris* strains have been analyzed [9,13-15]. A difference between mycelial cells and fruiting bodies of *C. militaris* was identified through transcriptome and proteome analysis, revealing that the cordycepin pathway was more active in mycelial cells [16]. Using transcriptome analysis, most studies focused on the *Cordyceps* species grown at particular developmental stages (*e.g.*, mycelium, developing FB, and mature FB) by SSF using different types of media [16-18]. Prospectively, better understanding of the metabolic control across developmental stages of this fungus on a productive medium by SSF would improve the content of cordycepin and other valuable metabolites in terms of fungal mass production and product quality. The metabolic responses in the carotenoid and cordycepin biosynthetic pathways of *C. militaris* growths by surface liquid cultivation under light condition were investigated. Consequently, the light-responsive gene regulatory network (GRN) for cell growth and development, and secondary metabolite production was constructed [19,20]. Therefore, this study aimed to reveal the metabolic functions and regulation contributing to different developmental stages, including mycelial growth (MY) and FB, of *C. militaris*. The transcriptomic analysis and targeted metabolite profiling of *C. militaris* grown at the developmental stages were performed. In addition, the genome-scale integrated networks analysis was implemented for exploring the potential metabolic routes and key regulators attributing to the developmental stages. This study permits further development of SSF for overproduction of valuable metabolites in *C. militaris* through an emerging synthetic biology approach.

2. Materials and Methods

2.1. Fungal strain and cultivation

C. militaris strain TBRC6039 was used in this study. It was grown in a rice medium consisting of an equal amount of rice grains and defined glucose medium (1:1, w/v) under the static condition at 22 ± 2°C. For inducing the FB formation, the fungal cultivations were conducted under dark condition for 14 days and then subjected to interval light exposure by switching the light (~1,000 lux) and dark conditions at an interval of 12 h. All cultivations were performed in three biological replicates.

The cultured samples were harvested at different cultivation times for growth profiling and metabolite measurement. For genome-wide transcriptome analysis, the harvested samples of the MY stage (14-day culture) and FB stage (56-day culture) were immediately frozen in liquid nitrogen and then stored at −80°C before RNA extraction.

2.2. Growth and metabolite measurement

The harvested fungal samples were dried overnight using a freeze dryer at −90°C, and then subjected to fungal mass and metabolites measurement.

The fungal mass of *C. militaris* was measured by using an indirect method, as previously described [21-23], in which glucosamine content was analyzed to estimate the fungal growth. For measurement of adenosine and cordycepin contents, the dried sample of *C. militaris* culture was mixed with water, sonicated for 30 min, and then subjected to high-performance liquid chromatography analysis using a HiQSil C18HS column (300 mm × 4.6 mm, 5 μm) at 40°C, and a UV detector at wavelength of 260 nm. The elution conditions were set using 15% (v/v) methanol solution as a mobile phase at a flow rate of 0.6 mL/min for 25 min.

The acid-heating method was employed to extract the carotenoid from the dried sample. The carotenoid content was measured by the colorimetric method using absorbance at 445 nm [24].

2.3. RNA extraction and transcriptome sequencing

The harvested mycelial cells and fruiting bodies were immediately immersed in liquid nitrogen for total RNA extraction. Sample (~500 mg) was ground using a cold mortar and pestle, and subjected to total RNA extraction and purification using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the instruction manual. Total RNA amount was assessed by measuring absorbance at 260 and 280nm. The quality of total RNA was determined by gel electrophoresis (Bio-Rad, Hercules, CA, USA), NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA, USA). Subsequently, the qualified total RNA was treated with DNase I to remove genomic DNA, and then magnetic beads coated with oligo-deoxythymidylate (oligo(dT)) primers were used for mRNA isolation. For the construction of the sequencing library, mRNA was mixed with the buffer, and then the fragmented mRNAs were used as templates for cDNA library construction. The library was subsequently connected with paired-end adaptors and sequenced using the Illumina NovaSeq 6000 system (Illumina, San Diego, CA, USA). Raw RNA-Seq data were kept in FASTQ format and were checked by FastQC [25]. The paired-end reads containing poly-N were filtered, and the adapters sequences were trimmed using the BBDuk program [26] to obtain clean RNA-Seq data. For RNA-Seq mapping, the clean reads were mapped to the *C. militaris* CM01 genome [15] using the Burrows-Wheeler Aligner package [27]. SAMtools was used for estimating mapped reads to all reference genes [28]. As a result, the FASTQ files, including detailed read sequences, were deposited in NCBI Sequence Read Archive under the BioProject accession number PRJNA832515 (BioSample accession: SAMN27864466, SAMN27864467, SAMN27864468, and SAMN27864469).

2.4. Differentially expressed genes analysis and functional annotation

All genes with mapped reads were subjected to calculate fragment per kilobase of transcript per million mapped reads (FPKM), which is a normalized estimation value of gene expression based on RNA-Seq data [29]. The expressed genes were indicated by FPKM value ≥ 1. For functional annotation, Joint Genome Institute via MycoCosm portal [30], EuKaryotic Orthologous Groups, Kyoto Encyclopedia of Genes and Genomes (KEGG), InterPro, and Gene Ontology (GO) databases were used for assigning the functional expressed genes.

The differentially expressed genes (DEGs) analysis between the MY and FB stages of *C. militaris* was performed using the DESeq2 R package with Q-value < 0.05 [31]. Genes with a value of log2fold change $\geq |1|$ and false discovery rate (FDR) < 0.001 were identified as significant DEGs. The analysis of GO and KEGG pathway enrichment analysis was also performed based on hypergeometric test with a threshold value of p value < 0.05. The clustering algorithm was further performed to identify similar expression patterns across two different stages of MY and FB. Euclidean distance under the complete-linkage hierarchical clustering method for the DEGs expression patterns was visualized by the pheatmap R package [32].

2.5. Identification of reporter metabolites and reporter regulators on different mycelial growth and fruiting body developmental stages by genome-scale integrated networks

The genome-scale integrated networks were employed for identifying reporter metabolites and reporter regulators relevant to the fungal development at different growth stages of *C. militaris*. Initially, the enhanced genome-scale metabolic network (GSMN) of *C. militaris* (*i*RT1467) generated by Thananusak *et al*. [20] was integrated with the identified DEGs of the MY and FB stages using the consensus gene-set enrichment analysis towards reporter metabolites analysis under the piano R package [33,34]. The potential metabolites with a distinct up-directional p value < 0.05 were designated as reporter metabolites.

Considering the identification of reporter regulators, the genome-scale GRN was used, which consisted of 71 transcription factors (TFs) as gene regulators, and 31,703 pairwise interactions of TFs and relevant target genes [19]. Further, the DEGs of the MY and FB stages were integrated with the genome-scale GRN using the consensus gene-set enrichment analysis towards reporter regulator analysis under the piano R package [33,34]. The potential TFs with a distinct up- and down-directional p value < 0.01 were identified as reporter regulators.

3. Results and Discussion

3.1. Signature characteristics of *C. militaris* during growth and development

The growth characteristics of *C. militaris* grown at the MY and FB stages were systematically analyzed by integrating their phenotypes with the genome-wide transcriptome data. A phenomenological difference in both stages of *C. militaris* cultures is shown in Fig. S1. It was found that the mycelia of *C. militaris* were formed when grown under dark conditions for 14 days, which was defined as the MY stage (Fig. S1A). After light exposure for 56 days, the fruiting bodies were generated, wherein was designated as the FB stage (Fig. S1B). The cell mass of *C. militaris* increased along with the cultivation times, and the total cell mass

content was maximal at the FB stage (Fig. S1C). The contents of the cordycepin and carotenoid concomitantly increased along the cell growth of *C. militaris*, which were the highest at the FB stage (Fig. S1D and S1E). The maximal cordycepin content was 44.54 ± 0.62 mg/g cell mass, which was 3.4 times higher than the MY stage (Fig. S1D). In addition, the carotenoid content at the FB stage (2.734 ± 0.25 mg/g cell mass) was significantly higher than that of the MY stage grown under dark conditions (Fig. S1E). The result indicated that the light exposure not only triggered the FB formation but also influenced the carotenoid biosynthesis. Likely, the overproduction of cordycepin and carotenoid metabolites were tightly related to the FB formation of *C. militaris*, which was induced by light stress at a particular stage of fungal growth. It is noted that the contents of adenosine (Fig. S1F), a precursor of cordycepin biosynthesis [35], were not significantly different between the MY and FB stages. This observation is consistent with the previous reports [12,36].

3.2. Genome-wide transcriptome of *C. militaris*

To investigate the transcriptional changes related to the FB formation of *C. militaris*. The *C. militaris* mRNA pools isolated from the MY and FB cultures were sequenced using an Illumina NovaSeq 6000 system. As shown in Table 1, total clean reads were retrieved with an average sequencing depth of 46.12 ± 4.25 million paired-end reads with a %GC average of 56.32 ± 0.19. The percentage of mapped reads of 78.82 ± 1.03 was obtained based on the *C. militaris* CM01 genome [15]. For all biological replicates, Pearson's correlation coefficient (> 0.97) was achieved indicating the good quality of sequencing results (Fig. S2). The analysis of transcriptome data showed the distribution of FPKM value across all genes (Fig. 1A and Table S1). Mostly, the genes with moderate expression levels (10 ≤ FPKM < 100) were found in both MY and FB stages. Notably, the MY stage had a greater number of highly expressed genes (FPKM ≥ 100), which could be simply explained that the MY stage was a more active growth stage, while the FB stage had the greater number of lowly expressed genes (1 ≤ FPKM < 10). Considering the genes with FPKM ≥ 1, the 9,256 expressed genes were identified. Of them, there were 9,119 and 9,000 genes expressed in the MY and FB stages, respectively. With overlapping analysis, a total of 8,863 genes were expressed in both stages (Fig. 1B). In addition, the functional assignment of the genes in *C. militaris* are provided in Table S2.

Table 1. Mapping results of *Cordyceps militaris* transcriptome

Sample	Total clean reads (M)	GC (%)	Number of mapped reads (M)	Percentage of mapped reads
MY-1	49.71	56.41	39.87	80.21
MY-2	39.97	56.53	31.57	78.98
FB-1	47.68	56.20	37.14	77.89
FB-2	47.11	56.12	36.84	78.20
Average	46.12 ± 4.25	56.32 ± 0.19	36.36 ± 3.47	78.82 ± 1.03

Values are presented as number only or mean ± standard deviation.
MY: mycelial growth, FB: fruiting body.

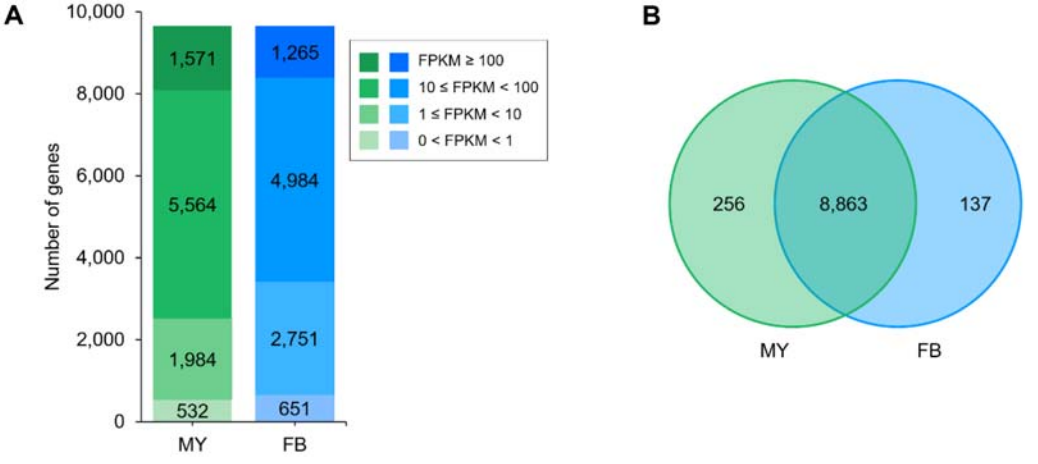

Fig. 1. Transcriptome data of *Cordyceps militaris* grown at mycelial and fruiting body stages. (A) Bar chart shows the distribution of genes having different FPKM values at the different stages. (B) Venn diagram shows the expressed genes of each stage. MY: mycelial stage, FB: fruiting body stage, FPKM: fragment per kilobase of transcript per million mapped reads.

3.3. Significant differentially expressed genes of *C. militaris* across mycelial growth and fruiting body developmental stages

To identify the significant genes of *C. militaris* cultures grown at MY and FB stages, DEGs analysis by DESeq2 using |log2fold change| ≥ 1 and FDR < 0.001 as thresholds was performed. As illustrated in Fig. 2A and Table S3, the number of significant DEGs across MY and FB stages was 1,877 genes, including 942 and 935 genes up-regulated in FB and MY, respectively. All significant genes were further classified into three main functional categories, namely biological process (BP), cellular component (CC), and molecular function (MF) based on GO classification as shown in Fig. 2B and Table S4. For BP, the significant genes lied in seven categories, including cellular process (354 genes), metabolic process (311 genes), localization (40 genes), biological regulation (5 genes), developmental process (3 genes), reproduction (3 genes), and response to stimulus (5 genes). For CC, the significant genes were found in the largest categories related to membrane (399 genes), and intracellular anatomical structure (348 genes). Other gene categories were related to cell, macromolecule

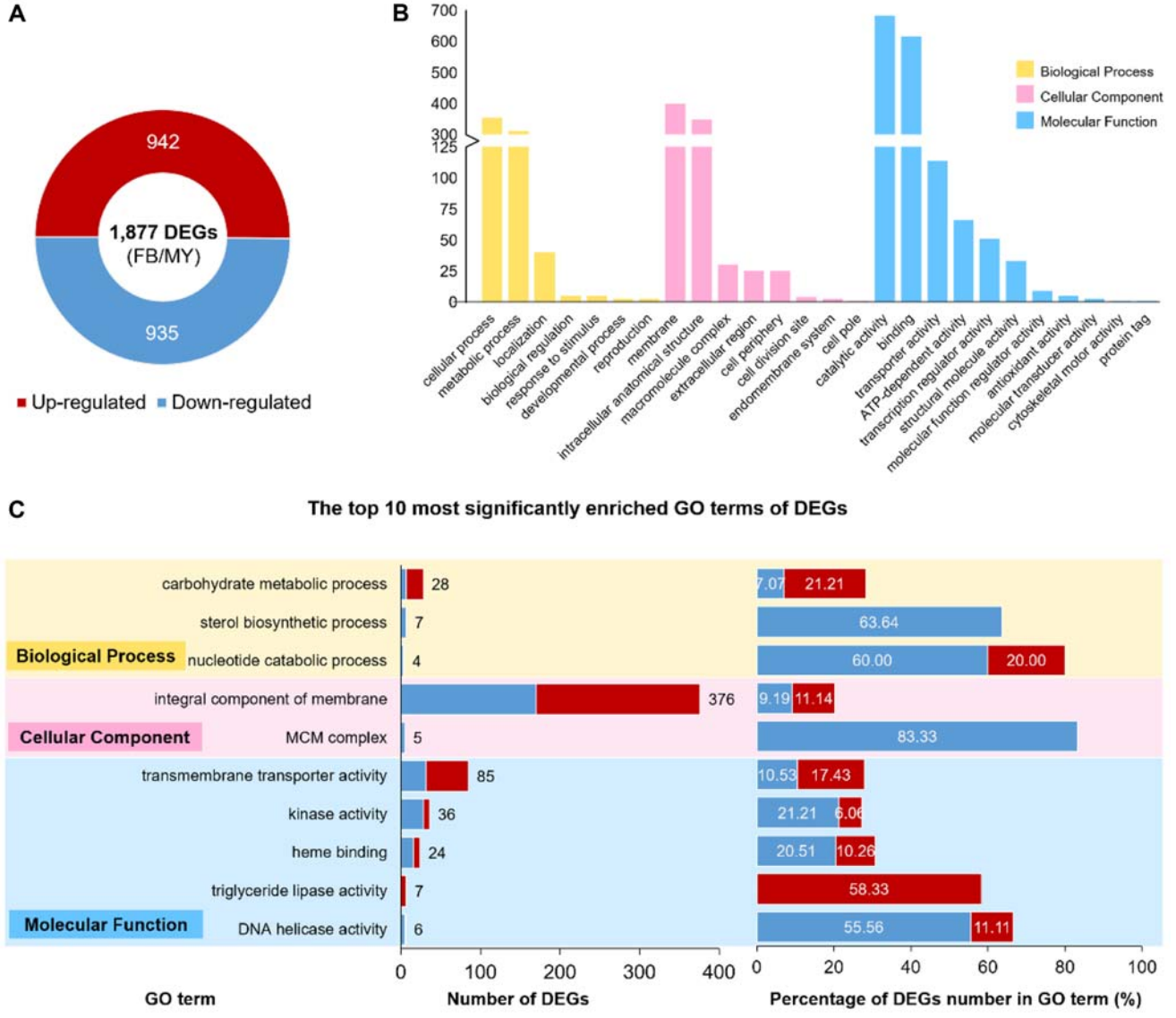

Fig. 2. Number of significant DEGs between two developmental stages of *Cordyceps militaris*, and their gene ontology (GO) functional classification. (A) Pie chart represents the number of DEGs between the MY and FB cultures. (B) GO term assignments for the identified DEGs. (C) Top 10 most significantly enriched GO terms of DEGs. The blue bars represent the downregulated genes in the FB culture, whereas the red bars represent the upregulated genes in the FB culture. The percentage of DEGs number in each GO term was calculated by dividing the number of DEGs to the number of total genes assigned to the specific GO term. DEGs: differentially expressed genes, MY: mycelial stage, FB: fruiting body stage.

complex, and extracellular region, including 30, 30, and 25 genes, respectively. For MF, the significant genes with functional roles in the catalytic activity (684 genes) and binding activity (616 genes) were grouped into the largest category.

GO term enrichment analysis was performed to identify which GO terms are over-represented in significant genes. The top 10 significantly enriched terms are illustrated in Fig. 2C and Table S4. The top enriched BP categories were carbohydrate metabolic process, sterol biosynthetic process, and nucleotide catabolic process. Of these, the highest number of significantly expressed genes involved the genes encoding for carbohydrate metabolic processes (28 genes), among which 21 were upregulated, and the remaining seven genes were downregulated in FB. Observably, CCM_02883 encoding for β-galactosidase (EC: 3.2.1.23), CCM_04624 encoding for glucosamine-6-phosphate deaminase (EC: 3.5.99.6), and CCM_01904 encoding for α,α-trehalase (EC: 3.2.1.28) were significantly upregulated at the FB stage, which the fold changes were 23.67, 10.54, and 8.72, respectively. These findings suggest that energy demand was acquired for FB development and cellular metabolism compared to the MY stage. Interestingly, it was found that 63.64% of all genes in the *C. militaris* genome annotated with the GO term sterol biosynthetic process were downregulated at the FB stage (7 genes), which included CCM_08656 and CCM_04684 encoding for sterol 24-C-methyltransferase (EC: 2.1.1.41), CCM_08633 encoding for 7-dehydrocholesterol reductase (EC: 1.3.1.21), CCM_03387 and CCM_02751 encoding for Δ(14)-sterol reductase (EC: 1.3.1.70), CCM_07765 encoding for C-8 sterol isomerase (EC: 5.-.-.-), and CCM_00310 encoding for hydroxymethylglutaryl-CoA synthase (EC: 2.3.3.10). The transcript results presumably referred to the decline in the biosynthesis of sterols at the FB stage of *C. militaris*, which was consistent with the previous study, mentioning that eight genes involved in steroid biosynthesis were downregulated in *C. militaris* cultivated under light condition. It has also been postulated that the blue-light receptor white collar-1 (CmWC-1) might suppress the expression of those related genes to switch the vegetative growth state to primordia differentiation [11]. For the nucleotide catabolic process, two genes (CCM_02831 and CCM_00622) encoding for 5'-nucleotidase (EC: 3.1.3.5) involved in the cordycepin biosynthesis were differentially expressed. Notably, CCM_02831 was upregulated, while CCM_00622 was downregulated in the FB culture compared to the MY culture that might be a result of the regulation in gene transcription process [37].

The significant genes encoded for membrane-bound proteins are the top genes enriched in the CC category. Among them, 376 genes were enriched in the integral component of membrane, accounting for about 20% of the total number of genes assigned to this term, whereas five genes were mainly enriched in the minichromosome maintenance (MCM) complex involved in the cell cycle. These genes, including CCM_06744, CCM_02923, CCM_06218, CCM_03199, and CCM_05573 encoding for DNA replication licensing factors (EC: 5.6.2.3), were downregulated at the FB culture. Moreover, the top enriched genes in the MF category encoded for the proteins with transmembrane transporter activity, DNA helicase activity, triglyceride lipase activity, heme binding, and kinase activity. As observed, over 50% of all genes annotated with the GO terms, which displayed DNA helicase activity (66.67%) and triglyceride lipase activity (58.33%) were differentially expressed when alteration between MY and FB. Noticeably, most of the significant genes enriched in MCM complex and DNA helicase activity were downregulated at the FB stage, indicating that cell cycle and division-related genes were more active in the MY culture. Interestingly, seven significant genes, including CCM_00256, CCM_09597, CCM_03046, CCM_03562, and CCM_04970 encoding for the secretory lipase (EC: 3.1.1.3), and CCM_02791 and CCM_08202 encoding for the patatin-like phospholipase (EC: 3.1.1.4) were upregulated at the FB stage. Generally, lipids are structural constituents of cell membranes (*e.g.*, phospholipids and glycolipids) and their storage compartments. There have been several reports on the fungal lipases as potential virulence factors of plant or insect pathogens [38,39]. The pathogenicity of the entomopathogenic fungi also depends on the existed enzymatic system, consisting of lipases, proteases and chitinases, which are responsible for degrading the insect's integument [40]. As such, it is unsurprising that *C. militaris* possess a broad range of hydrolytic enzymes specific for neutral lipid and phospholipid substrates. It is reasonable that *C. militaris* might secrete these lipases to the culture broth for hydrolyzing the lipids that existed in the medium, which was further utilized as a nutrient and energy source for growth and development at the FB stage.

3.4. Differential expression patterns of differentially expressed genes associated with the growth and developmental stages of *C. militaris*

Hierarchical clustering analysis was applied for 1,877 significant genes based on the transcript levels of DEGs across the MY and FB stages. In overall, they were divided into two gene clusters with different expression patterns according to the highly expressed genes found in the developmental stages (Fig. 3A and Table S5), in which 935 and 942 genes were highly expressed at the MY and FB stages belonging to the Cluster 1 and 2, respectively. In addition, the KEGG pathway enrichment analysis could

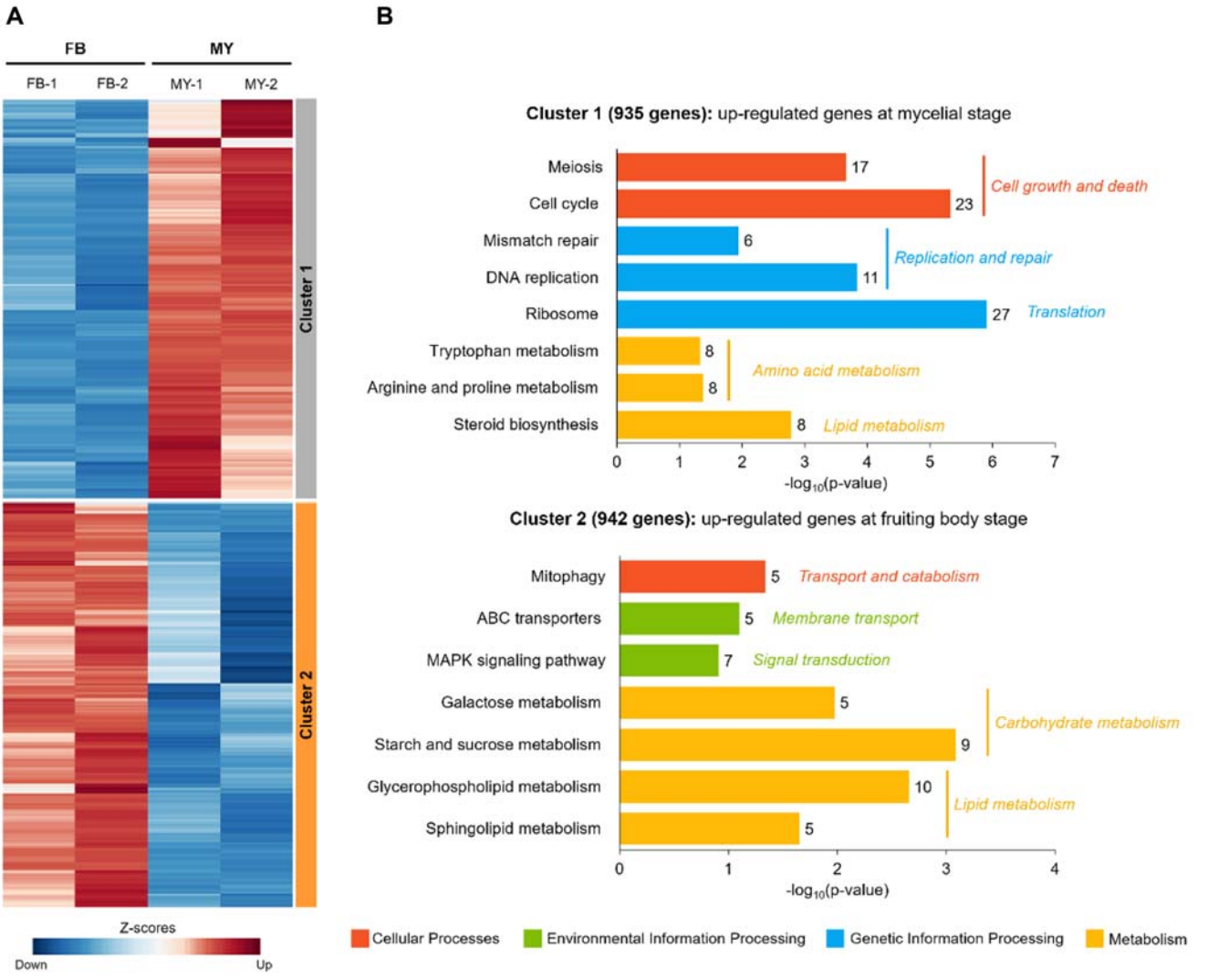

Fig. 3. Cluster analysis of the significant DEGs between different developmental stages of *Cordyceps militaris*. (A) The heat map diagram shows different gene expression patterns. (B) The most significantly enriched KEGG pathways of each gene clusters are represented as -log10(p value). The number on the bars shows the number of genes enriched in the corresponding pathways. DEGs: differentially expressed genes, KEGG: Kyoto Encyclopedia of Genes and Genomes, MY: mycelial stage, FB: fruiting body stage.

identify the pathways, which were over-represented in each gene cluster. The most significant enriched KEGG pathways of each gene expression cluster are shown in Fig. 3B and Fig. S3. The significant genes were categorized into four main functional classes, including cellular processes, environmental information processing, genetic information processing, and metabolism. It was found that the genes involved in the functional groups of cell growth and death, replication and repair, translation, amino acid metabolism, and lipid metabolism pathways were significantly enriched in Cluster 1, particularly found in the MY stage. Meanwhile, the genes in the groups of transport and catabolism, membrane transport, signal transduction, carbohydrate metabolism, and lipid metabolism pathways were significantly enriched in Cluster 2 found in the FB stage. It is noted that the genes involved in the lipid metabolism pathway were significantly enriched in both gene clusters but participated in different metabolic pathways, *i.e.*, the genes involved in the steroid biosynthesis were enriched in Cluster 1. In contrast, the genes responsible for the glycerophospholipid and sphingolipid metabolisms were enriched in Cluster 2.

In Cluster 1, the genes involved in the cell division cycle, as well as the pathways of amino acid metabolism and steroid biosynthesis were enriched (Fig. 3B and Table S6). Recently, the cell cycle of yeast (*Saccharomyces cerevisiae*) was studied by quantitative multi-omics analysis, showing that the biosynthetic precursors were synthesized on demand during cell division, and the amino acid metabolism was also synchronized with cell division [41]. In addition, it was found that the abundance of ergosterol

enzymes was detected in the mitotic phase of yeast, leading to up-regulation of the sterol biosynthesis during the mitosis of cell cycle [42]. Accordingly, we suggest that the amino acid metabolism and steroid biosynthesis might be attributed to the cell division processes in the MY stage of *C. militaris*.

For Cluster 2, the genes involved in the signal transduction pathway, which encoded proteins localized at the cell membrane, and act as a transporter, were enriched (Fig. 3B). For example, the genes coding for pheromone receptors, PreA and PreB (CCM_01499 and CCM_06752, respectively), were upregulated in the FB stage. Our result is consistent with the previous study by Yin *et al*. [16], indicating their importance for FB formation in *C. militaris*. It has also been reported that the pheromone receptors induced sexual reproduction by stimulating a G-protein-initiated signaling pathway in *S. cerevisiae* [43]. In addition, the genes involved in carbohydrate metabolism (galactose, starch and sucrose metabolisms) and lipid metabolism (glycerophospholipid, and sphingolipid metabolisms) were also enriched in the FB stage as shown in Fig. 3B. Among them, several significant genes encoding polysaccharide-degrading enzymes were upregulated in the FB stage, such as CCM_08811 encoded glucoamylase (EC: 3.2.1.3), CCM_06740 encoded α-Glucosidase (EC: 3.2.1.20), CCM_02883 encoded β-galactosidase (EC: 3.2.1.23), and CCM_04122 and CCM_09237 encoded glycosylphosphatidylinositol-anchored cell wall β-1,3-endoglucanase (EC: 3.2.1.39). It has been reported that β-1,3-glucanase plays a major role in fungal cell wall softening during hyphal branching in filamentous fungi [44]. These results presumably indicated that the hydrolases were highly active in the FB stage of *C. militaris*, which might be required for the cell wall biogenesis for FB growth or the hydrolysis of nutrients from the surrounding material. However, this hypothesis should be further systematically verified. Considering the lipid metabolism, the upregulated genes in the FB stage mainly participate in the pathways of glycerophospholipid, and sphingolipid metabolism. Most of them encoded the lipid synthetic enzymes. For example, CCM_07138 encoded phosphatic acid phosphatase (EC: 3.1.3.81, EC: 3.1.3.4), CCM_08202 encoded patatin-like phospholipase, and CCM_03231 encoded sphingomyelin phosphodiesterase (EC: 3.1.4.12). This could indicate that *C. militaris* might require a high energy for the FB development.

To gain a more comprehensive understanding of the growth and developmental process in *C. militaris*, we also overviewed the significant DEGs in each gene cluster based on the homology analysis of our previous study [19]. As listed in Table 2, the upregulated genes encoding for protein functions involved in the growth and developmental process were found in both gene clusters. Two genes were detected in Cluster 1, including CCM_01128 (ortholog of *flbB* gene encoding for Basic-Zipper-Type (bZIP-type) TF (FlbB), and CCM_04514 (encoding for GATA TF LreA). The TF, FlbB, regulates the asexual development in *Aspergillus nidulans*, and its proper temporal and quantitative expression is required for *brlA* transcription [45]. For Cluster 2, three genes were upregulated at the FB stage, including CCM_04531 (*VeA*), CCM_05395 (*LaeA*), and CCM_08391 (*WetA*). The CCM_04531 (*VeA*) and CCM_05395 (*LaeA*) encode proteins that are known to form the VeA/LaeA complex in *A. nidulans*, which regulates the balance of asexual and sexual development as well as secondary metabolism by linking the light response of the cell [46]. This indicates that *C. militaris* was exposed to light and then sensed the signal via light receptors to these growth and developmental process-related genes. The CCM_08391 (*WetA*) encodes the developmental regulatory protein WetA, which has been reported to play a global regulatory role in the conidial development [47].

3.5. Metabolic regulation at fruiting body stage of *C. militaris*

To dissect how carbohydrate and lipid metabolisms contribute to the metabolic regulation at the FB stage of *C. militaris*,

Table 2. List of significant genes encoding for the functional protein involved in the growth and developmental process and their orthologs

Cluster	Gene ID	Functional protein	Ortholog[a]
1	CCM_01128	Hypothetical protein	bZIP transcription factor (*Cordyceps fumosorosea* ARSEF 2679, *Beauveria bassiana* ARSEF 2860)
1	CCM_04514	GATA transcription factor LreA	Vivid PAS protein VVD (*Cordyceps javanica*), *lreA* (*C. fumosorosea* ARSEF 2679)
2	CCM_04531	Sexual development activator VeA	*veA* (*Beauveria brongniartii* RCEF 3172, *C. javanica*)
2	CCM_05395	Methyltransferase LaeA	Methyltransferase *laeA* (*C. fumosorosea* ARSEF 267, *C. javanica*)
2	CCM_08391	Developmental regulatory protein WetA	Developmental regulatory protein WetA (*Fusarium phyllophilum*)

[a]Gene functions were assigned based on the previous study by In-on *et al.* [19].

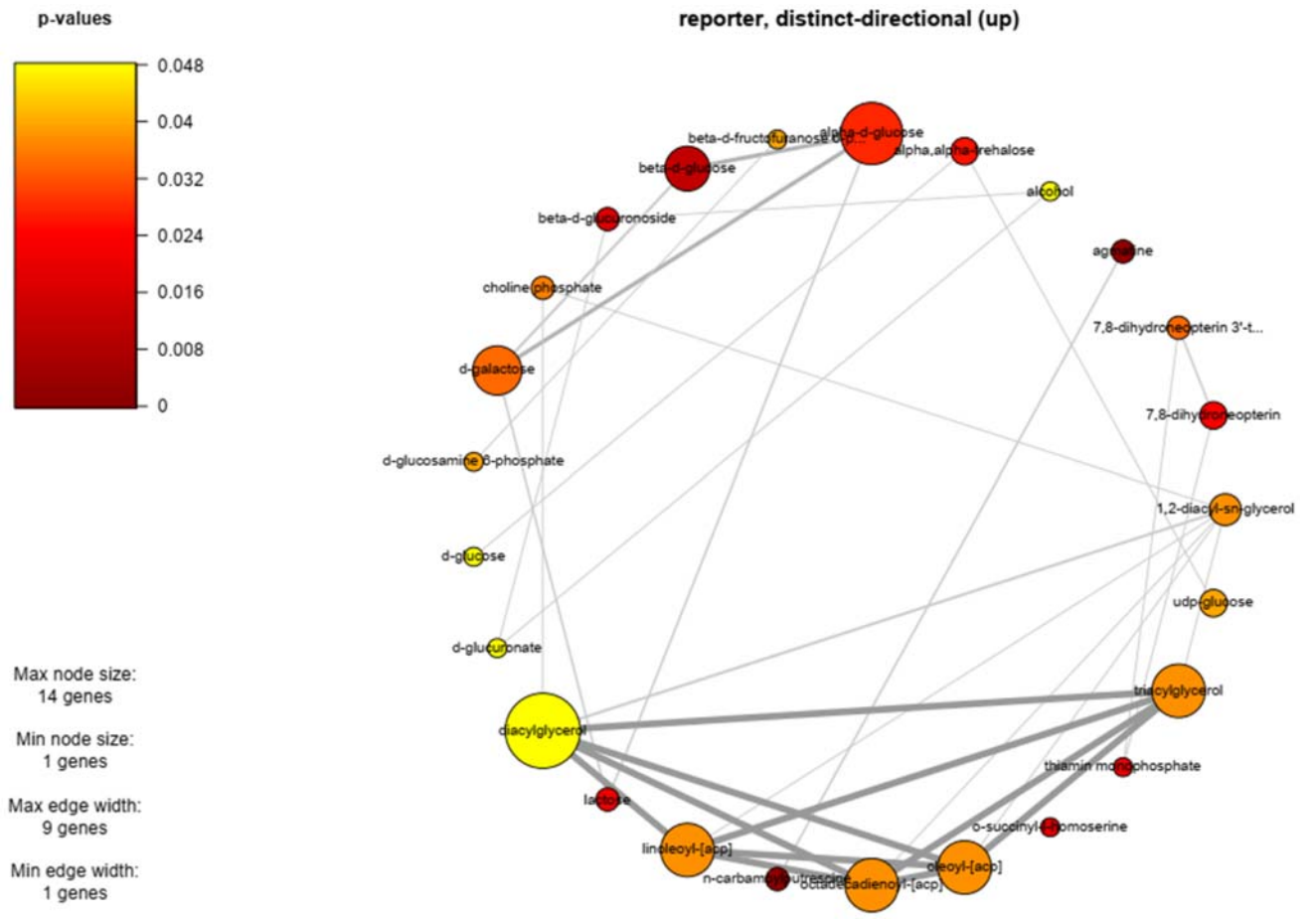

Fig. 4. Subnetwork of reporter metabolites of *Cordyceps militaris* at fruiting body stage. The size of the nodes (circles) indicates the number of genes in the gene set. The thickness of edge represents the number of shared genes. The p values were taken from the distinct up-directional p value from the reporter metabolites analysis.

the integrative analysis was performed using the transcriptome data obtained in this study and the GSMN of *C. militaris* (*i*RT1467) as a scaffold (Tables S7-S10). As expected, it is seen that most of the significant reporter metabolites at FB stage involved in carbohydrate and lipid metabolic pathways, such as α-D-glucose, β-D-glucose, D-galactose, α,α-trehalose, uridine diphosphate glucose (UDP-glucose), linoleoyl-[acyl-carrier protein] (linoleoyl-[acp]), octadecadienoyl-[acyl-carrier protein] (octadecadienoyl-[acp]), oleoyl-[acyl-carrier protein] (oleoyl-[acp]), triacylglycerol (TAG), choline phosphate, and diacylglycerol (DAG) (Fig. 4 and Table S11).

Focusing on the reporter metabolites participating in the carbohydrate metabolism (*i.e.*, α-D-glucose, β-D-glucose, D-galactose, α,α-trehalose, and UDP-glucose), we found that the genes coding for glucohydrolases were mainly up-regulated at the FB stage. Most of them involved in the metabolism of galactose and polysaccharide, for example CCM_08811 encoding for glucoamylase (EC: 3.2.1.3), CCM_06740 encoding for α-glucosidase (EC: 3.2.1.20), CCM_01167 or CCM_09241 encoding for trehalose-phosphatase (EC: 3.1.3.12), and CCM_02756 or CCM_04632 encoding for 1,3-β-glucan glucohydrolase (EC: 3.2.1.58). These enzymes play a role in the polysaccharide degradation. In addition, the genes encoding for sugar transporters were also upregulated at the FB stage, such as CCM_01166, CCM_03583, CCM_05726, and CCM_08696. The results indicated that *C. militaris* might secrete the glucohydrolases out of the cells to hydrolyze polysaccharides (*e.g.*, starch) in the culture medium, and then simple sugar was taken up into the cells via sugar transporters for energy supply in the growth and developmental process, particularly in the FB formation.

It was also found that the significant reporter metabolites at the FB stage involved in the lipid metabolic pathway (*e.g.*, glycerolipid, and glycerophospholipid biosynthesis subnetworks), for examples, linoleoyl-[acp], octadecadienoyl-[acp], oleoyl-[acp], TAG, choline phosphate, and DAG. Most significant genes relevant to these metabolites participated in the lipid synthesis pathway. For example, CCM_08323 encoding for the phospholipase C (EC: 3.1.4.3) was up-

regulated at the FB stage with a log2fold change of 2.32. This enzyme is responsible for hydrolyzing 1,2-diacylglycerol-3-phosphate yielded a DAG, the substrate for *de novo* lipid synthesis via the Kennedy pathway and the TAG biosynthesis [48]. Moreover, the genes encoding for secretory lipases (*e.g.*, CCM_03046, CCM_03562, CCM_04970, and CCM_09597) were upregulated at the FB stage. These enzymes hydrolyze neutral lipid (TAG) into free fatty acid (FFA). These FFAs diffuse via membrane and transport with an acyl carrier protein to newly synthesize lipid in the fungal cell. It has been reported that the neutral lipids in lipid droplets were required during cordycepin accumulation in *C. militaris* [5]. Moreover, Xia *et al*. [35] also stated that both Cns1 and Cns2 are co-localized on lipid droplets in *C. militaris* cells. This might explain the phenomenon of the high cordycepin content in relevance to the upregulated genes in lipid metabolism during the FB stage of *C. militaris* that the cordycepin might be highly accumulated in lipid droplets in fruiting bodies (Fig. S1D). However, the expression of cordycepin-producing genes, *cns1* (CCM_04436) and *cns2* (CCM_04437), were lower at FB with a fold change of 0.67 and 0.99, respectively, indicating that the simultaneous accumulation of lipid and cordycepin contents in the FB stage was co-regulated by other mechanisms in addition to the transcriptional level. Furthermore, it might be a protective mechanism of the fungal cell due to cordycepin is a self-toxic compound for the cordycepin-producing fungi. It has been reported that lipid droplets enclosed cordycepin to overcome its interaction with targets inside the cell, and permit the secretion of cordycepin into the extracellular matrix via specific transporter [5].

According to the targeted metabolites (*e.g.*, adenosine, cordycepin, and carotenoid) measured in this study (Fig. S1D-S1F), none of them were identified as a reporter metabolite at the FB stage. Observably, the expression of genes involved in adenosine production, such as genes encoding for 5'-nucleotidase (EC: 3.1.3.5) and adenosine deaminase (EC: 3.5.4.4), were upregulated and downregulated in FB, respectively (Table S12). This could explain why the adenosine content in both stages was not significantly different. Contrary to *cns1* and *cns2*, the expression of CCM_04438 (*cns3*), a cordycepin-producing gene, was higher at FB with a fold change of 1.55. For carotenoid-producing genes, CCM_06728 encoding for torulene dioxygenase (EC: 1.13.11.59) and CCM_09155 encoding for β-apo-4'-carotenal oxygenase (EC: 1.2.1.82) were highly expressed in FB stage with a fold change of 1.20 and 2.48, respectively. These expression results are coincided with the carotenoid content as shown in Fig. S1E.

3.6. Identified reporter regulators involved in growth and developmental process in *C. militaris*

In this study, the genome-scale GRN of *C. militaris* was used as a scaffold (Table S13) for integrative analysis with our transcriptome data across the MY and FB stages to identify further the reporter regulators involved in the growth and developmental process. As listed in Table 3, seven reporter regulators or TFs were identified, namely bZIP TF (AtfA), cyclic AMP-dependent TF (Atf2), yeast AP-1-like TF (Yap1), chromatin-associated proteins containing the high-mobility-group (HMG) box domain, OefC protein, GATA TF NreB-*Penicillium chrysogenum* (nreB), and C6 zinc finger domain-containing protein. Here, the most of key regulators, such as AtfA, Atf2, Yap1, OefC, NreB, and C6 zinc finger domain-containing protein were upregulated under FB stage (Table S14). Elaborately, AtfA (CCM_09124) has been reported as a regulator involved in oxidative stress tolerance [49] and other sexual development-related genes in fungi [50,51]. The Atf2 (CCM_09413) is a transcriptional activator that regulates the transcription of genes involved in DNA damage response [52]. Promisingly, we observed that Yap1 (CCM_09377) was significantly identified, which involves in stress response pathway [49]. This is consistent with an earlier report on Yap1 relevant to stress and development in filamentous fungi [53]. For the others, OefC (CCM_05966), NreB (CCM_04712) and C6 zinc finger domain-containing protein (CCM_00925) were responsible for the asexual

Table 3. The reporter regulators involved in the growth and developmental process of *Cordyceps militaris*

Gene ID	Function[a]	Gene symbol
CCM_09124	bZIP transcription factor (AtfA)	*CmAtfA*
CCM_09413	Cyclic AMP-dependent transcription factor (Atf2)	*CmAtf2*
CCM_02275	Chromatin-associated proteins containing the HMG box domain	*CmHmg*
CCM_09377	Yeast AP-1-like transcription factor (Yap1)	*CmYap1*
CCM_05966	OefC protein	*CmOefC*
CCM_04712	GATA transcription factor NreB-*Penicillium chrysogenum*	*nreB*
CCM_00925	C6 zinc finger domain-containing protein	-

[a]Gene functions were assigned based on Clusters of Orthologous Groups of proteins, Kyoto Encyclopedia of Genes and Genomes and Gene Ontology annotations and the previous study [19].

development [54], the nitrate assimilation [55], growth and development [56], respectively. For the remaining regulator, chromatin-associated protein containing the HMG box domain was downregulated under FB stage (Table S14). The HMG box domain protein, FvHmg1, was reported as a negative regulator in FB formation and development of *Flammulina velutipes* [57]. Inhibition of FvHmg1 function led to normal fully FB formation and development. Consistently, this study showed that chromatin-associated protein containing the HMG box domain (CCM_02275) was also downregulation.

4. Conclusion

In this study, the metabolic regulation of different developmental stages of *C. militaris* was revealed by integrative transcriptome analysis. The differential gene expression patterns were detected in the MY and FB stages. The cell cycle and division genes were transcriptionally regulated during MY. The involvement of the metabolic genes in carbohydrate and lipid supply as well as in the signal transduction pathway in the FB development was explored. Besides, the identified key regulators and reporter metabolites were elaborated to be attributes in the growth and developmental stages of *C. militaris*. Our findings would expand the fundamental knowledge on the metabolic changes in cell physiology of this industrially important fungus that provides a perspective on the product optimization of the high-value desirable metabolites.

Acknowledgements

The authors would like to thank Department of Zoology, SciKU Biodata Server, Faculty of Science, Kasetsart University for supports, resources and computing facilities. This research and innovation activity is funded by National Research Council of Thailand (NRCT). R.T. gratefully acknowledges supports from the Science Achievement Scholarship of Thailand (SAST) and Interdisciplinary Graduate Program in Bioscience, Faculty of Science, Kasetsart University. W.V. would like to thank Kasetsart University Research and Development Institute (KURDI), Omics Center for Agriculture, Bioresources, Food, and Health, Kasetsart University (OmiKU) and International SciKU Branding (ISB), Faculty of Science, Kasetsart University for supports.

This research was funded by The Thailand Research Fund, grant number RSA6180001 and National Research Council of Thailand (NRCT).

Author's Contributions

R.T. performed experiments, analyzed the data, prepared figures and tables, and wrote the manuscript. K.L. assisted in experimental design, data interpretation and revised the manuscript. N.R. analyzed and interpreted the data, and revised the manuscript. M.K. assisted in data interpretation and revised the manuscript. W.V. designed all experiments, interpreted all results, supervised throughout the study and revised the manuscript. All authors reviewed and approved the final manuscript.

Ethical Statements

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Neither ethical approval nor informed consent was required for this study.

Electronic Supplementary Material (ESM)

The online version of this article (doi: 10.1007/s12257-022-0207-5) contains supplementary material, which is available to authorized users.

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