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Research article

Transcriptome analysis revealing the effect of *Bupleurum scorzonerifolium* Willd association with endophytic fungi CHS3 on the production of saikosaponin D

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ABSTRACT

Saikosaponin D (SSd) is a naturally active product with strong pharmacological activity found in *Bupleurum scorzonerifolium* Willd. Studies have shown that endophytic fungi have great potential as sources of natural medicines. *Fusarium acuminatum* (CHS3), an SSd-producing endophytic fungus, was isolated from *B. scorzonerifolium*. To elucidate the effect of host plants on the production of SSd in CHS3, CHS3 was co-cultured with suspension cells of *B. scorzonerifolium* and SSd was detected using high-performance liquid chromatography (HPLC). Transcriptome sequencing (RNA-Seq) of CHS3 before and after co-culture was performed using an Illumina HiSeq 2500 platform. The results indicated that the content of SSd synthesised by CHS3 increased after co-culture with suspension cells of *B. scorzonerifolium*. Transcriptome analysis of CHS3 with differentially expressed genes (DEGs) showed that 1202 and 1049 genes were upregulated and downregulated, respectively, after co-culture. Thirty genes associated with SSd synthesis and 11 genes related to terpene backbone biosynthesis were annotated to the Kyoto Encyclopaedia of Genes and Genomes (KEGG). Combined with transcriptome data, it was speculated that the mevalonate (MVA) pathway is a possible pathway for SSd synthesis in CHS3, and the expression of key enzyme genes (*HMGR, HMGCS, GGPS1, MVK, FDFT1, FNTB*) was validated by qRT-PCR. In conclusion, the endophytic fungus CHS3 can form an interactive relationship with its host, thereby promoting SSd biosynthesis and accumulation by upregulating the expression of key enzyme genes in the biosynthesis pathway.

1. Introduction

Saikosaponin D (SSd) is a triterpenoid which has been found to have physiological and pharmacological effects, such as anti-cancer, anti-inflammatory, anti-depression, and treatment of neurodegenerative diseases [1–4]. Currently, SSd is primarily extracted from plants, and its production is limited by long growth times and low yields. Owing to the shortage of resources and increasing demand, it

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is still challenging to meet the needs of the medicinal field. This requires new alternative resources, such as endophytic fungi, to produce SSd.

Endophytic fungi are highly biodiverse and versatile microbial communities that are ubiquitous in nature [5]. Studies have shown that endophytic fungi are harmless microorganisms that colonise and grow in plants. These fungi form symbiotic relationships with host plants for mutual benefit [6]. Endophytic fungi and their beneficial properties have been recognised and explored since 1898, but reports on endophytic fungi from 1898 to 1980 are limited [7]. In 1993, paclitaxel-producing endophytic fungi were discovered [8]. Subsequently, the synthesis of bioactive compounds in plants was found to rely on endophytic fungi. At the same time, the same active components as that of host plants can be obtained from endophytic fungi, such as the vitexin-producing *Cajanus cajan,* rhein-producing *Rheum palmatum* L, and huperzine A-producing *Huperzia serrata* [9–11]. Therefore, secondary metabolites of endophytic fungi have potential nutritional and medicinal value and may play an important role in pharmaceutical engineering and clinical practice [12]. However, there is little information on the metabolic communication between endophytic fungi and host plants, and their regulatory mechanisms remain unclear.

RNA-Seq is an efficient and rapid research method that can solve various problems such as the in-depth discovery of new genes, identification of metabolic pathways, identification of gene families, and evolutionary analysis. The transcriptome is the link between genetic information and biological functions and is the main target of research on alterations in gene expression [13]. Recently, RNA-Seq has become a faster method of gene mining and has been used to study host plant–pathogen interactions owing to the rapid development of genomics research and the reduction of sequencing costs [14].

In a previous study, an SSd-producing endophytic fungus, *F. acuminatum* (CHS3), was isolated from *B. scorzonerifolium* [15]. To reveal the regulatory mechanism of host plants on SSd synthesis in CHS3, a co-culture model of endophytic fungi and plant suspension cells was established, and the regulatory mechanism of the plant on the gene expression involved in SSd synthesis in CHS3 was analysed by RNA-Seq. Our research team analysed the relationship between CHS3 and its host plant during SSd synthesis. This study provides a theoretical foundation and new method for increasing SSd production in the future.

2. Materials and methods

2.1. Biological materials and culture conditions

F. acuminatum (CHS3), an SSd-producing endophytic fungus, was isolated from *B. scorzonerifolium* and was preserved at the Biotechnology Laboratory of Heilongjiang University of Traditional Chinese Medicine (Fig. 1a). The seeds of B. *scorzonerifolium* were

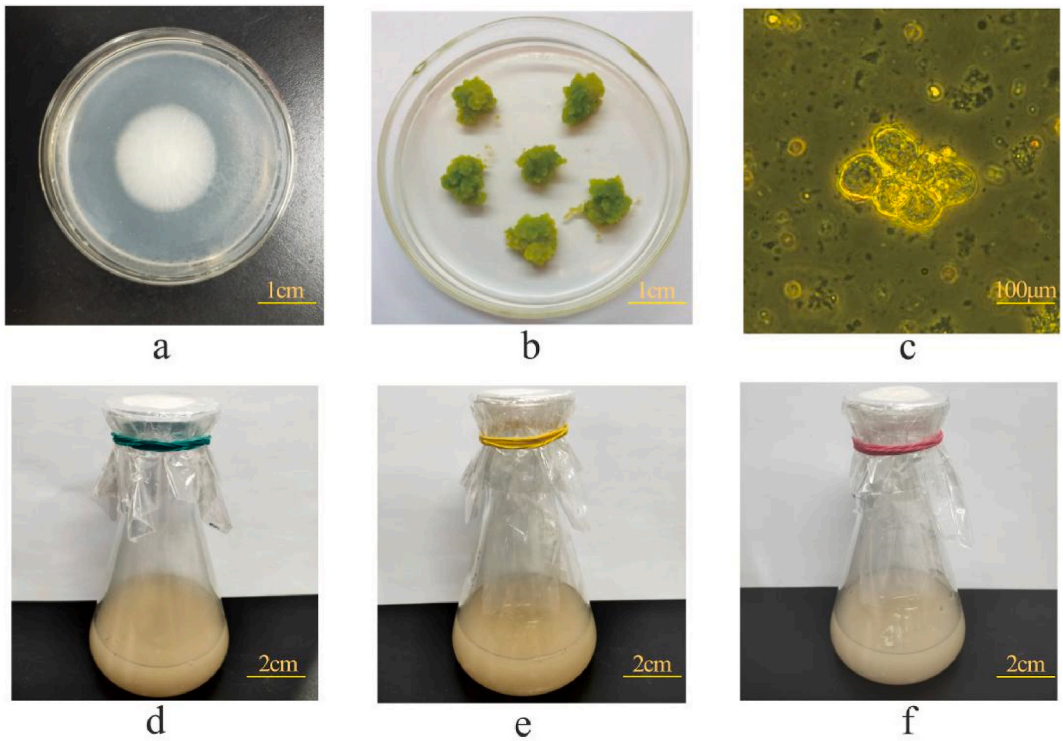

Fig. 1. Endophytic fungus and host plant materials. (a) Positive side of CHS3 strain; (b) The callus of *B. scorzonerifolium*; (c) The suspension cells of *B. scorzonerifolium*; (d) Endophytic fungus CHS3 control group (EF-K); (e) The supernatants induction group (EF-Y); (f) *B. scorzonerifolium* suspension cell co-culture group (EF-G).

collected by our team and authenticated by Prof. Zhenyue Wang (College of Pharmacy, Heilongjiang University of Chinese Medicine) [16].

To investigate the effect of *B. scorzonerifolium* on SSd production of CHS3, three groups were tested—the co-culture group: CHS3 culture with *B. scorzonerifolium* suspended cells (group named EF-G after the Chinese word Gongjun), the induction group: CHS3 culture with supernatants of cultured *B. scorzonerifolium* suspended cells (EF-Y after the Chinese word Youjun), and the control group: CHS3 pure culture (EF-K after the Chinese word Kongjun) (Fig. 1d–f). In the EF-G group, the co-cultured suspension cells of plants were separated from the cells of the endophytic fungi using dialysis membranes which allowed the exchange of larger molecules or information. The suspense cells (2 g) which were induced from the hypocotyl of germinated seeds of *B. scorzonerifolium* (Fig. 1b and c), were added to 100 mL MS medium (KT 0.9 mg/L, 2,4-D 0.1 mg/L, 6-BA 2.5 mg/L), and were cultured at 25 °C, 120 rpm for 10 days. The single colony of CHS3 was transferred into potato dextrose broth (PDB) and cultured at 28 °C for 4 days under dark conditions at 160 rpm then used as a seed. CHS3 seeds (5 mL) were mixed with 100 mL of liquid MS medium and cultured at 28 °C at 160 rpm for three days. The dialysis membrane was boiled in solution A ($NaHCO_3$ 2 %, EDTA 1 mmol/L, PH 8.0) for 10 min, then rinsed twice with distilled water, and finally boiled with solution B (EDTA 1 mM, PH 8.0) for 10 min. After cooling, the dialysis membrane was loaded with 5 mL of *B. scorzonerifolium* suspension cells or 5 mL of supernatants from cultured suspension cells, and was completely immersed in precultured endophytic fungal medium continuous co-culture at 28 °C at 160 rpm for three days (Fig. 1). All interaction experiments were performed in triplicate.

2.2. Extraction and determination of SSd from CHS3

Cultured CHS3 were collected by vacuum filtration, and the sample solution of each group was prepared by methanol extraction using an ultrasonic method [17]. Standard substances for SSd were obtained from Shanghai Yuanye Biotechnology Co., Ltd. The sample was then prepared for HPLC analysis according to the chromatography method described in the Chinese Pharmacopoeia (2020). The contents of SSd were determined using a Thermo U3000-DAD HPLC series (Thermo Fisher Scientific, Waltham, MA, USA) with an ODS-C18 analytical column (250 mm × 4.6 mm, 5 μm; GL Sciences, Shanghai, China). Chromatography-grade acetonitrile: water (25:75; v/v) was filtered through membrane was used as the mobile phase. Then, 20 μL of sample was injected at a flow rate of 1.0 mL/min; the column temperature was 30 ± 0.2 °C, and the detection wavelength was 210 nm.

2.3. RNA isolation, library construction, and sequencing

Total CHS3 RNA was extracted using a Total RNA Extractor Extraction Kit (Shanghai Promeg Biological Products Co., Ltd., Shanghai, China). After Qubit and electrophoresis detection, the mRNA library was constructed and entrusted to Shanghai Shenggong Bioengineering Service Co. Ltd. using the bridge PCR single-molecule amplification method on the Illumina HiSeq 2500 platform to complete transcriptome sequencing.

2.4. Sequence processing, annotation, and analysis

After sequencing, the quality was checked using FastQC software 0.11.2, and linker sequences and low-quality original data were removed. Then, the sequence with a low-quality value was filtered using Trinity software 2.4.0, paired-ends were spliced, and repeated sections were removed to obtain high-quality sequence data longer than 200 bp. The sequences were compared with the NR, NT, and KEGG databases to obtain annotation information of unigenes, and the metabolic pathway was analysed according to DEGs expression pattern cluster analysis, GO enrichment analysis, and KEGG enrichment analysis (screening criteria: qValue <0.05; $|\text{FoldChange}| > 2$).

2.5. Quantitative real-time PCR validation

Primers were designed according to the sequences of the different genes (Table 1). The cDNA was synthesised using a Reverse Transcription Kit (dsDNA) (Biosharp, Beijing, China). The amplification reactions were performed with the GoTaq® qPCR Master Mix (Promega, Madison, USA) in the LineGene 9600 Series (Bioer, Hangzhou, China) following the manufacturers' instructions. The relative gene expression level was calculated using the formula of $2^{-\triangle\triangle CT}$ with the GAPDH gene of *F. acuminatum* as a reference.

Table 1
Primers of key enzyme genes of CHS3 SSd synthesis.

Gene	Forward	Reverse
FDFT1	GTGGCATAACGCAGTACACG	CTTCTCCAGGGGTATCGTCA
GGPS1	GTTACGCACATCCTCCTC	GCTGTTCCGCTTCAAGAT
HMGR	TCCAGAGCAAGCACATTAG	GCCATTCCACGAGCAATA
MVK	CACTGGCTGTATCCTTGAA	CCGCTTGATACCAATGTTG
HMGCS	GCAAGCGAATTGGTCTCTTC	TTGGGGGTGTAGTCCTTCTG
FNTB	GTCCTCGGGTCTCGCTATTG	CAGGATAGCCTCTTCAGCCG
GAPDH	CGGCAAGACCATCAAGTTCTACT	CTTGTCAATGGTGGTGAAGACAC

3. Results

3.1. Detection of SSd produced by CHS3

The SSd standard was used as a control. As shown in Fig. 2, the characteristic absorption peak of SSd extracted from the CHS3 in different groups at 210 nm was determined by HPLC. Samples were collected at 1, 2, and 3 d to analyse the SSd content in CHS3 after co-culture. The peak area of each sample are listed in Supplementary Table 1. The results showed a significant increase in SSd content following co-culture with suspension cells. After three days of co-culture, the SSd contents of endophytic fungi in the control group (EF-K), induction group (EF-Y), and co-culture group (EF-G) were 3.093 ± 0.023 μg/g, 5.854 ± 0.174 μg/g, and 13.410 ± 0.120 μg/g, respectively (Fig. 3). These results indicate that suspended cells can significantly induce SSd biosynthesis and accumulation in CHS3 cells.

3.2. RNA-seq analysis of transcriptome samples of CHS3

After sequencing the transcriptional groups, the original sequencing data for CHS3 were obtained. To ensure the quality of the analysis, it was necessary to carry out data quality control, filter out the low-quality sequences with connectors inside, and get the clean data, in which the Q30 base percentage of each sample was not less than 96.04 % and the GC base content was not less than 51.84 % (Table 2). The pairwise Pearson's correlation coefficients of the three replicates × three groups are shown in Fig. 4a, which indicates a highly similar gene expression pattern between the sequencing data of each sample. The assembled transcripts were de-redundant, and 79,353 spliced transcripts and 27,180 redundant sequences were obtained. The total lengths were 341,729,131 bp and 37,210,036 bp, N50 measured 8524 bp and 4220 bp, and the average length of the transcript was 4306.44. The unigene measured 1369.02 bp (Table 3). As shown in Fig. 4c, the majority of the unigenes (46.144 %) were 200 to < 300 bp in length, and only 18.959 % of the reads were >2000 bp in length.

3.3. Transcriptome annotation of CHS3

After the de-redundant sequences were obtained, 27,180 unigenes were submitted to seven public databases for comparison to obtain functional annotation information. Analyses of the CHS3 transcriptome showed that 14,930 unigenes (54.93 %) had significant matches in the NR database; 2274 (8.37 %) in the KEGG database; 7549 (27.77 %) in the PFAM database; 12,517 (46.05 %) in the Swiss-Prot database; 7630 (28.07 %) in the KOG database; 16,987 (62.50 %) in the NT database and 14,463 (53.21 %) in the GO database (Fig. 4b). According to statistics, 22,102 genes (81.32 %) were successfully annotated in at least one database, and 1334 genes (4.91 %) were successfully annotated in all databases. Comparison with the NR database showed that some unigenes in CHS3 were highly similar to those in other species (Fig. 4d). The largest number of homologous genes were identified in *Fusarium langsethiae*.

Annotated CHS3 genes were functionally classified using the GO assignment program. Functional annotation of genes was divided

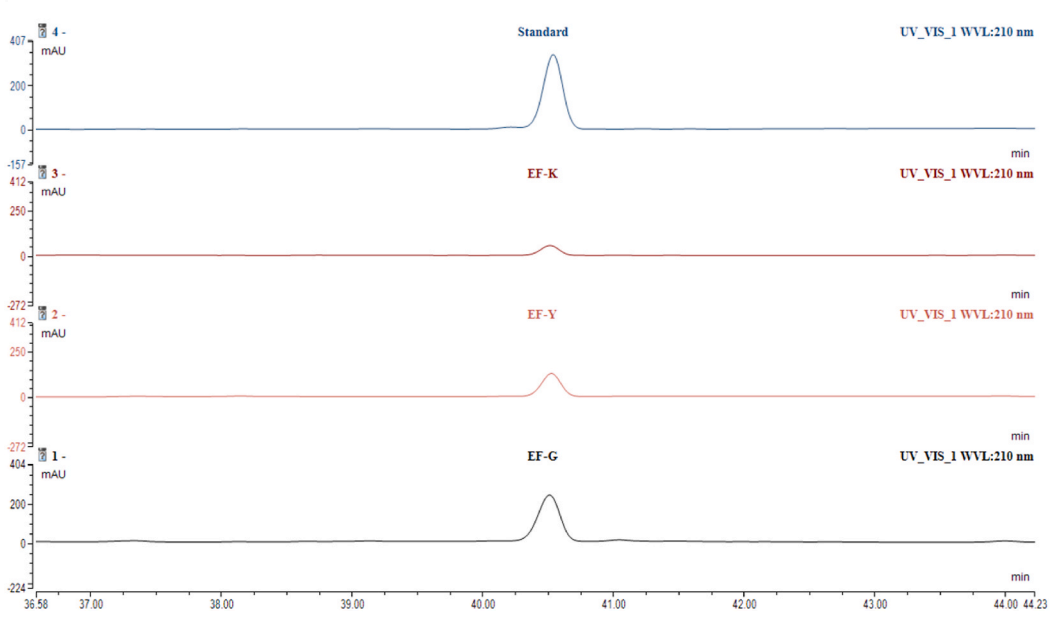

Fig. 2. Analytical results of different components by HPLC. The abscissa is retention time and the ordinate is absorbance. 1: EF-G, 2: EF-Y, 3: EF-K, 4: Standard.

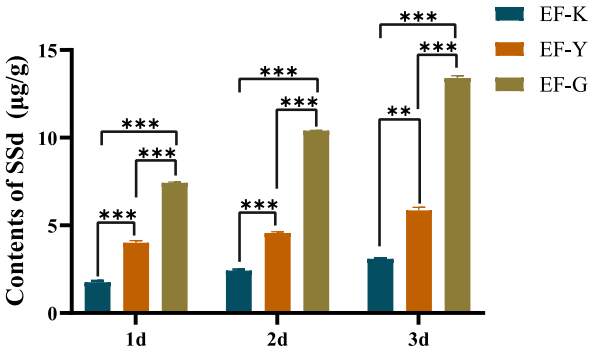

Fig. 3. Effects of different groups on the contents of SSd in CHS3. ***represents a significant difference between different groups (between-group independent-sample *t*-test, $P < 0.001$). **represents a significant difference between different groups (between-group independent-sample *t*-test, $P < 0.01$). Error bars represent the means ± SD (n = 3).

Table 2
The assessment table of CHS3 sample sequencing data.

Group	Sample	Total Reads Count	Total Bases Count (bp)	Average Read Length (bp)	Q30 Bases Ratio (%)	GC Bases Ratio (%)
Control	Kongjun 1	48,375,486	6,987,669,988	144.45	96.32 %	51.84 %
	Kongjun 2	61,966,070	8,952,807,125	144.48	96.04 %	52.11 %
	Kongjun 3	53,994,308	7,840,546,978	145.21	96.62 %	52.05 %
Induction	Youjun 1	51,817,226	7,497,288,588	144.69	96.59 %	52.52 %
	Youjun 2	61,754,156	8,796,290,062	142.44	96.79 %	52.60 %
	Youjun 3	53,465,368	7,703,490,617	144.08	96.62 %	52.64 %
Co-culture	Gongjun 1	52,856,104	7,621,102,456	144.19	96.60 %	52.52 %
	Gongjun 2	57,190,956	8,272,857,936	144.65	96.60 %	52.68 %
	Gongjun 3	52,132,270	7,535,312,208	144.54	96.71 %	52.68 %

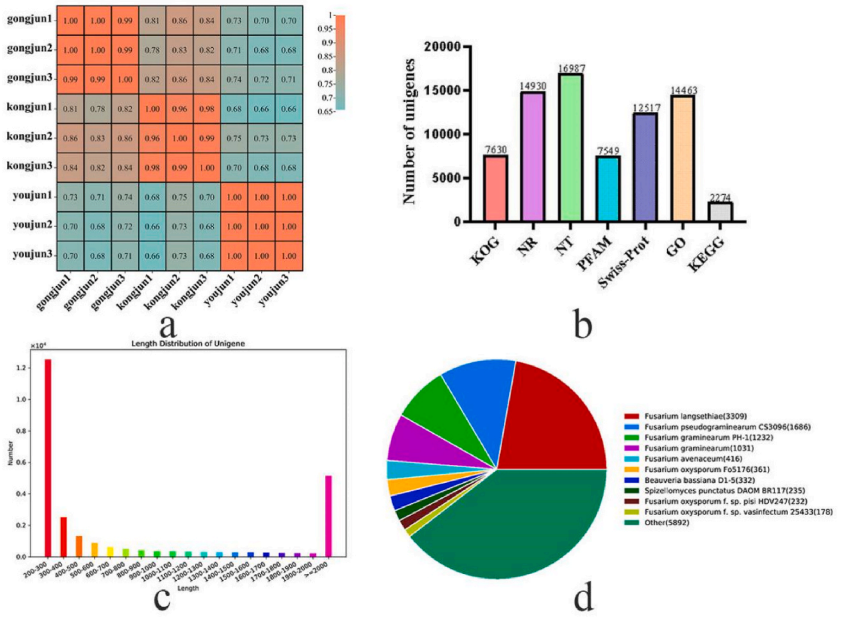

Fig. 4. Illumina sequencing and transcriptomes of CHS3 with various treatments. (a) Pairwise Pearson's correlation coefficients of the sequencing data from three groups x three replicates. (b) The size distributions of CHS3 unigenes. (c) The annotation of unigenes based on various databases. (d) The species distribution of the annotated unigenes.

into three main categories: biological processes, molecular functions, and cellular components. Generally, multiple terms are assigned to identical transcripts. These sequences were enriched for 66 main GO terms (Fig. 5). For the biological processes, there was a high percentage of genes associated with metabolic and cellular processes. Within the molecular function category, the assignments were

Table 3
Splicing result statistics.

	No.	≥500bp	≥1000bp	N50	N90	Max Len	Min Len	Total Len	Average Len
Transcript	79,353	58,782	53,083	8524	2684	52,656	201	341,729,131	4306.44
Unigene	27,180	10,785	7982	4220	381	52,656	201	37,210,036	1369.02

mostly distributed in terms of catalytic activity and binding. Among the cellular components, the dominant GO terms were grouped into either cell or cell parts. The large number of sequences involved in cell parts and metabolic processes indicate that CHS3 can actively produce important bioactive compounds, such as SSd.

The assignments of clusters of orthologous groups (KOGs) were used to evaluate the effectiveness of the annotation process and the transcriptomic library. A total of 7630 proteins were classified into different processes in CHS3 (Fig. 6). Within the 25 KOG categories, a high proportion of the clusters were "General function prediction only" (1,022, 13.40 %), "Posttranslational modification, protein turnover, chaperones" (862, 11.30 %), "Translation, ribosomal structure, and biogenesis" (688, 9.02 %), "Energy production and conversion" (608, 7.97 %), "Signal transduction mechanisms" (577, 7.56 %), and "Carbohydrate transport and metabolism" (440, 5.77 %). In addition, genes belong to secondary metabolite biosynthesis, transport, and catabolism (396, 5.19 %).

Unigene metabolic pathway analysis of CHS3 was performed using Kyoto Encyclopaedia of Genes and Genomes. A total of 2274 unique genes were annotated, and the pathways were classified into metabolism, cellular processes, environmental information processes, and genetic information processes (Fig. 7). The dominant unique transcripts were "translation" (349), followed by "signal transduction" (338). In addition, there are 30 genes involved in the "metabolism of terpenoids and polyketides" that may be related to the synthesis of SSd in CHS3.

3.4. Functional categorization of the DEGs in CHS3

DEGS analysis indicated that 1447 and 1202 genes were upregulated when comparing EF-Y vs. EF-K and EF-G vs. EF-K, respectively. The number of downregulated genes was 1109 and 1,049, respectively (Fig. 8a and b).

According to the comparison of EF-G vs. EF-K and EF-Y vs. EF-K, all DEGs were enriched in 56 functional groups related to various biological processes that were mainly involved in cellular composition and metabolism, especially the metabolism of saikosaponins. These groups included "cellular process" (GO: 0009987), "cell" (GO: 0005623), "cell part" (GO: 0044464), "metabolic process" (GO: 0008152), "catalytic activity" (GO: 0003824), "binding" (GO: 0005488) and "organelle" (GO: 0043226). These results are consistent with those of the SSd content determination, which revealed the effects of the co-culture on different biological processes at different stages.

In total, 161 pathways were identified using KEGG and DEGs analyses. There were 11 genes annotated in terpenoid backbone biosynthesis (ko 00900), and four genes were significantly upregulated. Three genes were annotated for sesquiterpenoid and triterpenoid biosynthesis (ko 00909) and one gene was significantly upregulated (Table 4). However, only two genes were upregulated between EF-Y and EF-K, and there were no differentially expressed genes involved in sesquiterpenoid and triterpenoid biosynthesis (ko

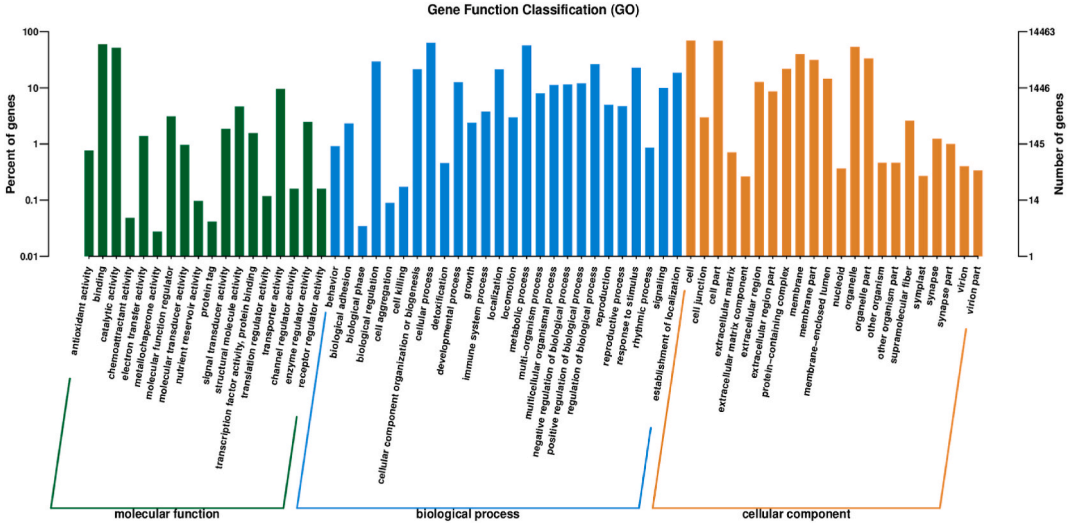

Fig. 5. GO categorization of non-redundant unigenes of CHS3. The horizontal axis is the secondary classification of GO, and the vertical axis is the number of genes in this category (right) and its percentage in the total number of genes annotated (left). Different colours represent different orthologues. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

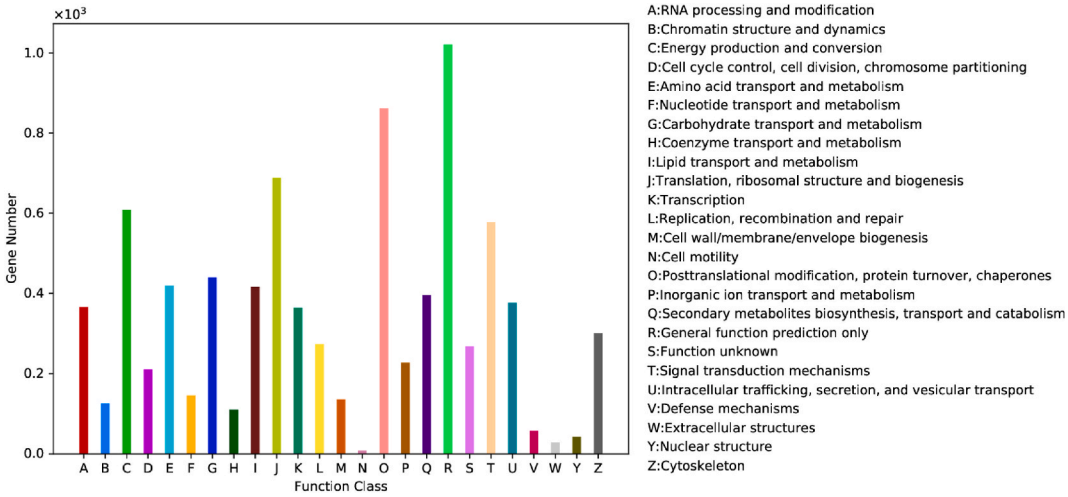

Fig. 6. KOG annotation of putative proteins of CHS3. Each colour on the horizontal axis represents a functional classification of KOG, as detailed in the note on the right. The vertical axis is the number of genes annotated to the classification. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

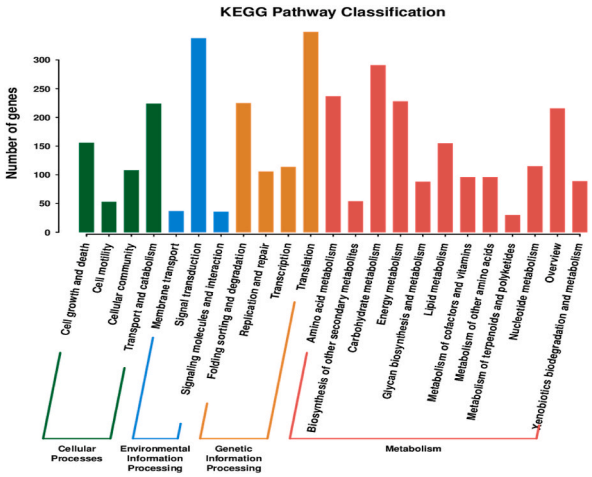

Fig. 7. KEGG pathway classification histogram of CHS3. The horizontal axis is the name of the metabolic pathway involved, and the vertical axis is the number of genes annotated to that pathway.

00909). These results suggest that the co-cultured suspension cells can significantly affect SSd biosynthesis of SSd in CHS3.

After screening, six key enzyme genes of CHS3 SSd synthesis were selected for validation by qRT-PCR, namely hydroxymethylglutaryl-CoA reductase *(HMGR)*, hydroxymethyl glutaryl coenzyme A synthase (*HMGCS*), farnesyl diphosphate farnesyltransferase (*FDFT1*), geranyl diphosphate synthase, type III (*GGPS1*), smevalonate kinase (MVK), and protein farnesyltransferase subunit beta (*FNTB*). Compared to the control group, the gene expression of key enzymes in the co-culture group was significantly upregulated (Fig. 9a–f). The comparison of RNA-seq and qRT-PCR data showed high consistency, which proved the validity of RNA-seq data for genes with distinct transcript abundance.

3.5. Prediction of the metabolic pathway of saikosaponin synthesis in CHS3

To date, there have been few reports on the biosynthetic pathways of secondary metabolites, particularly triterpenoid saponins, in fungi. There are two main terpenoid biosynthetic pathways in plants: the MVA pathway, which occurs in the cytoplasm, and the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, which occurs in plastids. These two pathways are crucial for formation of the triterpenoid backbone of the five-carbon intermediates isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). A total of 14 genes were annotated in terpenoid backbone biosynthesis (Ko 00900) and sesquiterpenoid and triterpenoid biosynthesis (ko 00909) (Supplementary Table 2). Based on the assignment of genes to the terpenoid trunk in the biosynthesis pathways, we speculated that the MVA pathway was the most important pathway for CHS3 synthesis of saikosaponins (Fig. 9g). At the same time, two key enzymes, (E)-

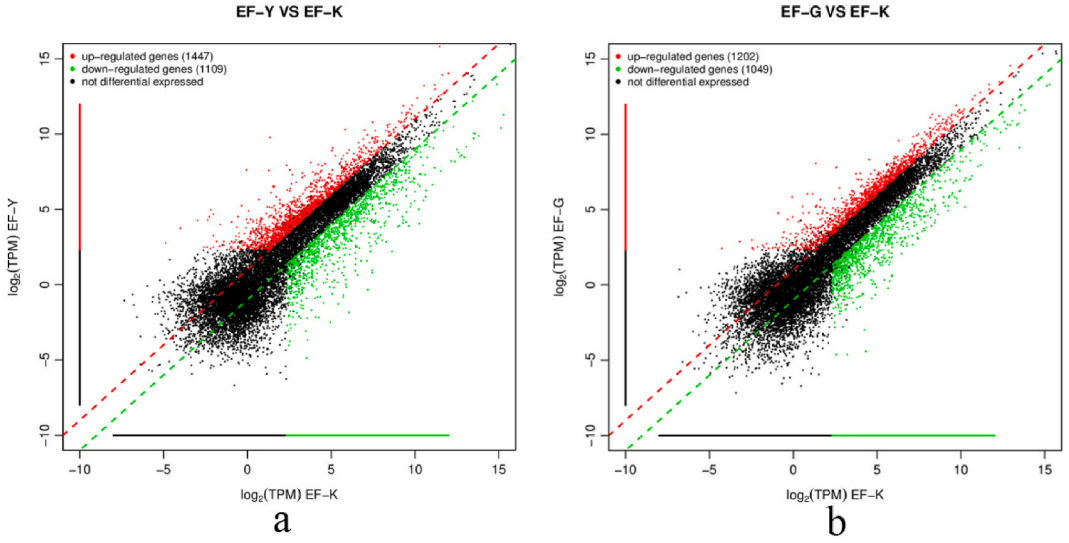

Fig. 8. Scatter diagram of the difference of gene expression of CHS3. (a) Comparison between induction group and control group: EF-Y vs EF-K; (b) Comparison between co-culture group and control group: EF-G vs EF-K. The horizontal and vertical axes are log2 (TPM) values of two groups of samples, respectively. Each dot in the picture represents a gene, and the closer to the origin, the lower the expression. Among them, red indicates an upregulated gene, green indicates a downregulated gene, black indicates a non-differential gene, and upregulation/downregulation is relative to horizontal axis sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Different genes in the metabolic pathway of saikosaponin biosynthesis in CHS3.

EC number	Function	Pathway
1.1.1.34	HMGR; hydroxymethylglutaryl-CoA reductase (NADPH)	ko00900
2.3.3.10	HMGCS; hydroxymethylglutaryl-CoA synthase	ko00900
2.5.1.1/2.5.1.10 /2.5.1.29	GGPS1; geranylgeranyl diphosphate synthase, type III	ko00900
2.7.1.36	MVK; mvaK1; mevalonate kinase	ko00900
2.5.1.21	FDFT1; farnesyl-diphosphate farnesyltransferase	ko00909
2.5.1.58	FNTB; protein farnesyltransferase subunit beta	ko00900

4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (IspG) and 4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase (IspH), were detected in the MEP pathway.

3.6. Cytochrome P450 (P450) and glycosyl transferase (GT) in SSd biosynthesis

Cytochrome P450 (P450) and glycosyltransferase (GT) are also involved in the SSd biosynthesis pathway and are the most important modifying enzymes. Almost all of the available 2,3-oxidosqualene undergo several modifications by GT and P450 before forming SSd. After co-culture, there were 110 P450 genes and 25 GT genes detected, most of which were highly expressed, and those with the highest enrichment were listed in Table 5.

4. Discussion

CHS3 cells were co-cultured with suspended cells to simulate the in vivo environment of endophytic fungi living in medicinal host plants. The results showed that the production of secondary metabolites by endophytic fungi is regulated by the host plant, and that there is communication signal transmission between them, increasing the synthesis of SSd in CHS3.

Transcriptome analysis is the overall study of the structure, function, expression, and transcriptional regulation of genes at the RNA level. It refers to the sum of all mRNA transcribed into cells at a specific function at the gene level [18]. RNA-seq is rapidly developing and has a strong practicability. Thus far, there has been a consensus on the application of RNA-Seq to the study of gene expression in many endophytic fungi, including *Aspergillus montevidensis* ZYD4, *Colletotrichum gloeosporioides* ES026, and *Aspergillus aculeatinus* Tax-6 [19–21]. To date, the analysis of saikosaponin synthesis genes in fungi using RNA-Seq has not been reported.

Based on the transcriptome data, we speculated that the biosynthesis of saikosaponins in CHS3 occurred mainly through the MVA pathway, which is consistent with the general synthesis of triterpenes. The last two enzymes, IspG and IspH, are also involved in the MEP pathway. It has been suggested that the two pathways mutually crosstalk through the regulatory role of IDI, which maintains

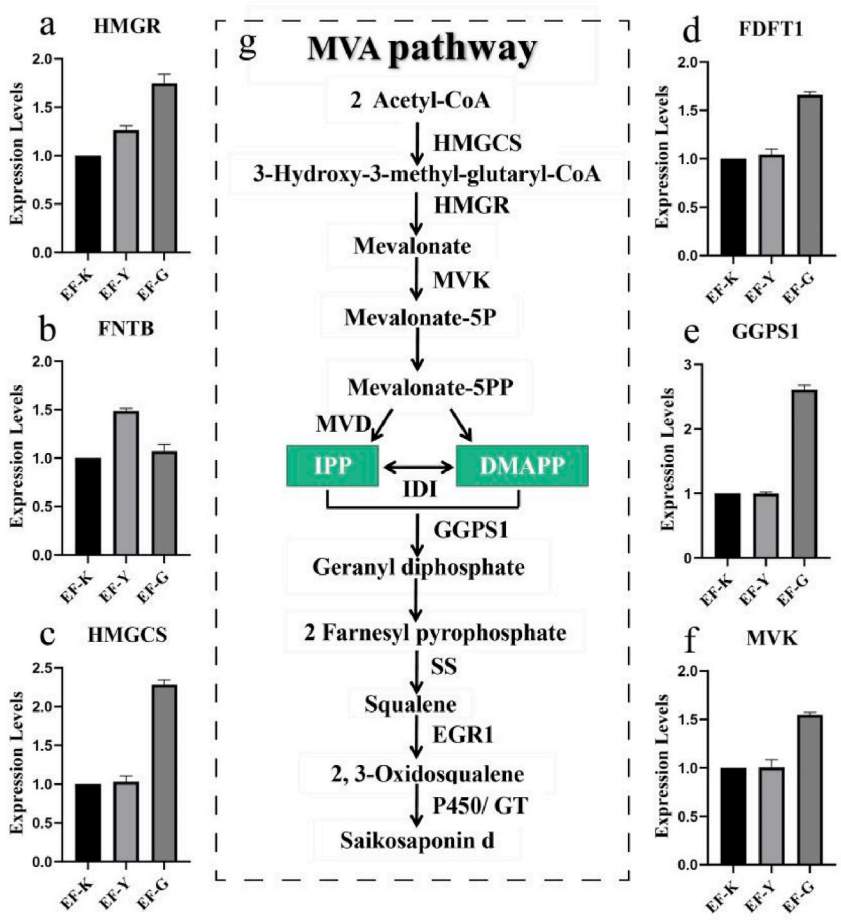

Fig. 9. Metabolic pathway of saikosaponin synthesis by CHS3 and the comparison of RT-PCR $2^{-\triangle\triangle Ct}$ values of key enzymes in the saikosaponin metabolism pathway. *FDFT1*, farnesyl diphosphate farnesyltransferase; *HMGR*, hydroxymethylglutaryl-CoA reductase, *FNTB,* protein farnesyltransferase subunit beta; *GGPS1,* geranyl diphosphate synthase, type III; *HMGCS*, hydroxymethyl glutaryl coenzyme A synthase; MVK, mevalonate kinase; *MVD,* diphosphcarate deboxylase; IDI, isopentenyl-diphosphate Delta-isomerase; SS, squalene synthase; P450, Cytochrome P450; GT, glycosyltransferase.

Table 5
Detection of upregulated cytochrome P450 and glycosyltransferase in degs.

Gene id	log2Fold	qValue	Function
TRINITY_DN11315_c0_g5	2.512	1.03E-78	Cytochrome P450 CYP2 subfamily
TRINITY_DN10947_c0_g1	2.005	4.02E-67	Cytochrome P450
TRINITY_DN11127_c1_g6	4.142	3.46E-53	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies
TRINITY_DN10233_c0_g1	2.928	2.39E-122	Cytochrome P450 CYP3/CYP5/CYP6/CYP9 subfamilies
TRINITY_DN10945_c1_g1	1.347	0.002198	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies
TRINITY_DN10263_c0_g1	1.992	1.49E-49	Cytochrome P450 CYP3/CYP5/CYP6/CYP9 subfamilies
TRINITY_DN7854_c0_g1	6.062	5.77E-18	Cytochrome P450 CYP2 subfamily
TRINITY_DN11031_c2_g2	2.231	2.91E-69	Cytochrome P450
TRINITY_DN9434_c0_g1	1.805	4.83E-60	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies
TRINITY_DN9673_c0_g2	1.121	0.000370	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies
TRINITY_DN10890_c1_g2	1.231	3.65E-23	UDP-glucuronosyl and UDP-glucosyl transferase
TRINITY_DN10942_c0_g1	1.432	4.20E-19	Glucosyltransferase- Alg6p

appropriate levels of IPP and DMAPP in the cytoplasm and plastids [22,23]. Therefore, it is speculated that the upstream pathway of MEP may play a pivotal role in the synthesis of saikosaponin precursors under co-culture conditions, and its specific regulatory mechanism needs to be further explored.

Triterpenoids were derived from C5 isoprene units of IPP and DMAPP through a "head-to-tail'' connection [24]. Phosphomevalonate kinase (PMK) is a crucial ATP-dependent enzyme in the MVA pathway, which can directly affect the biosynthesis of IPP and

DMAPP [25]. In the KEGG pathway database, triterpene biosynthesis requires PMK. However, no genes encoding this enzyme have been identified in the transcriptome data. Orthologous BLAST results showed that the single gene, TRINITY_DN11130_c1_g5, encodes PMK, which may be involved in the transformation of 5-phosphomevalonate to 5-pyrophosphomevalonate.

In the present study, we sequenced the transcriptomes of *B. scorzonerifolium* suspension cells grown on endophytic fungi. Compared to the EF-Y group, the EF-G group had a more significant effect on genes related to SSd biosynthesis. In the EF-G group, the expression levels of five key enzymes (*HMGR, HMGCS, GGPS1, MVK, FDFT1*) involved in the SSd biosynthesis pathway were upregulated. The EF-Y group did not show any significant effects on these genes; only *FNTB* was upregulated. This was consistent with the changes in the active component contents of the two groups after treatment. *HMGCS* and *HMGR* are crucial enzymes in the MVA pathway [26,27]. Sesquiterpene production is positively correlated with the expression of these two genes [28,29]. Therefore, the key enzymes upregulated in the saikosaponin biosynthesis pathway may play a similar role in increasing the SSd content. These results provide new insights into the molecular processes of saikosaponin biosynthesis and important resources for the construction of cell-synthesis plants with high SSd production by genetic engineering.

P450 and GT are required for triterpenoid biosynthesis [30]. UDP-glucuronosyltransferase promotes triterpenoid saponin synthesis [31]. Among these, UGT74M1 has been identified to be involved in the biosynthesis of triterpenes in *Saponaria vaccaria* [32]. HaCYPi3, CYP716, and TwCYP712K1 of the P450 family may be involved in triterpene metabolism and thus participate in the triterpene synthesis pathway, in which TwCYP712K1 has been shown to act on celastrol [33–35]. Transcriptome data showed that ten P450 genes and two GT genes were upregulated after co-culture. The suspension cells may regulate SSd biosynthesis by upregulating the expression of genes encoding these post-modification enzymes. Because P450 and GT are encoded by a large number of multi-gene families, it is difficult to predict the specific post-modifications involved in the biosynthesis of saikosaponins, which require further verification.

5. Conclusions

In this study, through the analysis of the results of HPLC detection, it was found that the co-culture of endophytic fungi and suspension cells of *B. scorzonerifolium* could increase the SSd content in endophytic fungi. Five key genes that increased SSd content in endophytic fungi were identified through transcriptome sequencing and verified by qRT-PCR. After co-culture, genes (*HMGR, HMGCS, GGPS1, MVK, FDFT1, FNTB*) were significantly upregulated, indicating that host plants promoted saikosaponin biosynthesis and accumulation by mediating the expression of key genes involved in the CHS3 biosynthesis pathway. Subsequent analysis of the related key enzyme genes further elaborated on the mechanism of saikosaponin production by endophytic fungi. The co-culture module imitated the in vivo metabolic communications between the host plant and endophytic fungi and revealed the mechanism of symbiotic interactions in SSd-producing CHS3. The large amount of data obtained in this study provides a powerful platform for future studies on the metabolic communication mechanisms between host plants and their endophytic fungi. However, further research is required to obtain more comprehensive and detailed information on communication mechanisms.

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Ethics statement

Review and/or approval by an ethics committee was not needed for this study because [This work is about the effect of B. scorzonerifolium on the production of SSd by endophytic fungi and does not address the ethical considerations of animal, cell, and human experimentation.].

Data availability statement

All data generated or analysed in this study are included in the published article. In addition, the transcriptome data of endophytic fungi (CHS3) have been uploaded to the Mendeley database (https://doi.org/10.17632/x25zzdptrh.1) and can be found online at https://data. mendeley. com/datasets/x25zzdptrh.

CRediT authorship contribution statement

Guangjie Liu: Writing – original draft, Project administration. **Yuanzhen Liu:** Writing – review & editing, Formal analysis. **Zhongmeng Li:** Visualization, Validation. **Yubin Ren:** Investigation. **Bo Liu:** Methodology. **Ning Gao:** Validation, Supervision. **Yupeng Cheng:** Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33453.

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