Genomic-wide analysis and prediction of genes involved in biosynthesis of polysaccharide and bioactive secondary metabolites in high-temperature-tolerance of wild *Flammulina filiformis*

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Abstract

Background: *Flammulina filiformis* (=Asian "*F. velutipes*") is a popular commercial edible mushroom. Many bioactive compounds such as polysaccharides and sesquiterpenoids with medicinal effects have been isolated and identified, but their biosynthesis and regulation in molecular level is unclear. In this study, we sequenced the genome of the wild strain *F. filiformis* Liu 355, predicated the biosynthetic gene clusters (BGCs) and profiled these genes expression between wild and cultivar strains and among different development stages of the wild strain of *F. filiformis* by a comparative transcriptomic analysis. Results: The results revealed that the genome of the *F. filiformis* was 35.01 M bp in length and annotated with 10396 gene models. 12 putative terpenoid gene clusters were predicted, 12 sesquiterpenes synthase genes belonged to four different groups and two type I PKS (polyketide synthase) gene clusters were identified from *F. filiformis* genome. The gene number related terpenoid biosynthesis is higher in wild strain (119 genes) than cultivar strain (81 genes) and most of them are up regulated in primodium and fruiting body of the wild strain, while PKS genes are usually up-regulated in the mycelium of wild strain. Moreover, genes encoding UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase involved in polysaccharide biosynthesis have relative high transcripts both in mycelium and fruiting bodies of *F. filiformis*. Conclusions: We identified candidate genes involved in the biosynthesis of polysaccharide and terpenoid bioactive compounds and profiled these genes expression during the development of *F. filiformis*. This study expands our knowledge for understanding the biology of *F. filiformis* and provides valuable data for elucidating the secondary metabolism regulation of the special strain of *F. filiformis*.

Background

Flammulina filiformis (= Asian *F. velutipes*), a species endemic to Asian region, as known as Enokitake, winter mushroom or golden needling mushroom, belongs to the family Physalacriaceae, Agaricales [1]. Recent new phylogenetic result based on multi-gene markers and morphological comparison demonstrated that "*F. velutipes*" in eastern Asia is not identical to the European winter mushroom *F. velutipes*, and should be treated as a separate species, namely *F. filiformis*, including all cultivated strains of "*F. velutipes*" in East Asia and those from South Korea and Japan with genome sequences [2]. Thus, we used the name "*F. filiformis*" instead of the Asian *F. velutipes* in our study.

F. filiformis is one of the most important and popular edible mushrooms available commercially in China. It has been widely cultivated and consumed in oriental countries owing to its high nutritional value and attractive taste. It was reported that China is currently the largest producer of *F. filiformis*, with an annual production of 2.4 million tons [3]. *F. filiformis* also possesses tremendous pharmaceutical value and many bioactive constituents have been identified such as polysaccharides [4, 5, 6], flavonoid [7], sesquiterpenes, glycosides, proteins, phenols and so on [8, 9, 10]. These compounds have been demonstrated have antitumor, anticancer and anti-atherosclerotic, thrombosis inhibition, anti-aging and antioxidant effects [11, 12]. In addition, as a typical white-rot fungus, *F. filiformis* can effective degrade lignin and produce alcohol dehydrogenase, and thus make them potential for bioethanol production [13].

In past decades, researches on the genus of *Flammulina* have been carried out on phylogenetic taxonomy [1, 14, 15], genetic diversity [16,17], nutritional and chemical constituents [18,19], pharmacological bioactivity [20, 21] and artificial cultivation [22, 23, 24]. Most of studies showed that *F. velutipes* possesses relative high content of carbohydrates, protein, amino acids and low fats or lipids, thus it generally was recognized as low energy delicacy [25]. In addition, bioactive polysaccharides (e.g. glucans and heteropolysaccharides), immunomodulatory protein (e.g. FIP-fve) and multiple bioactive sesquiterpenes were also isolated and identified from the extract, mycelium and fruiting bodies of *F. velutipes* [26]. Tang et al. [12] reviewed the compounds derived from the *F. velutipes* and their divers biological activities. More and more studies on chemical compounds and biological activity of the mushroom supported that *F. velutipes* should be exploited as a great source for development of functional foods, nutraceuticals and even pharmaceutical drugs [27].

Development of "omic" technology provided the powerful tools to understand the biology of edible mushroom including effective utilization for cultivation substrate (lignocellulose) [28, 29], the mechanism of fruiting body's formation and development and adaption for environment such as high temperature or cold stress [30, 31, 32]. For example, genome sequencing of the cultivars of *F. velutipes* from Korea and Japan revealed the high capacity for lignocellulose degradation [28, 33]. Transcriptomic and proteomic analysis of *F. velutipes* identified key genes associated with cold and light stress-fruiting body formation and fruiting body morphogenesis [34]. These researches provided important information for its breeding and commercial cultivation of *F. velutipes*.

Recent advances in genome sequencing have revealed millions of putative biosynthetic gene clusters (BGCs) are hidden in fungal genomes [35]. Genome mining efforts have also allowed us understanding the silenced or activated biosynthetic pathway in microbe combined with the development of bioinformatics software such as antiSMASH, SMURF and PRISM [36]. For instances, genome-wide investigation of 66 cosmopolitan strains of *Aspergillus fumigatus* revealed 5 general types of variation in secondary genes cluster [37]. Identification of the tricyclic diterpene antibiotic pleuromylin gene clusters in genome-scale increased antibiotic production in *Clitopilus passeckerianus* [38]; Prediction of gene clusters involved in biosynthesis of terpenoid/PKS in medicinal *Hericium erinaceus* by genome and transcriptome sequencing discovered a new family of diterpene cyclases in fungi [39, 40] and identification of the candidate cytochromes P450s genes cluster possible related to triterpenoid biosynthesis in medicinal mushroom *Ganoderma lucidum* by genome sequencing improved the production of the medicinal effective compounds [41, 42].

However, as a popular edible mushroom that have wide spectrum of interesting biological activities, little is known for synthesis and regulation of bioactive secondary metabolites of *F. filiformis*. In previous experiments, we collected a wild strain of *F. filiformis* Liu355 from Longling, Yunnan and demonstrated that it could tolerate relatively high temperature during fruiting body formation (at 18 °C–22 °C) in the laboratory and it is prior to the commercial strains of *F. filiformis* (Asian commercial *F. velutipes* usually produce fruiting body under low temperature ≤ 15 °C) [16]. Thus, the wild strain is a potential and important genetic material for future breeding engineer because it can save large amounts of energy. Most interestingly, the chemical composition of the wild strain was different from other commercially cultivated strains of *F. filiformis* with more unique chemical compounds. Total 13 new sesquiterpenes with nor-eudesmane, spiroaxane, cadinane, and cuparane skeletons were isolated and identified from the wild strain Liu355 [9].

Thus, the aims of our study are to explore the genetic feature of this interesting wild strain of *F. filiformis* in genomic scale, to identify gene clusters associated with biosynthesis of bioactive secondary metabolites and to profile these candidate genes expression difference during the development of *F. filiformis*. This

research will facilitate our understanding for its biology of the wild strain and provide a useful datasets for molecular breeding, as well for improving compounds production and producing novel compounds by next heterologous pathway and metabolic engineering in future.

Results And Discussion

General features of the F. filiformis genome

Prior to our study, three “genomes” assigned to *F. velutipes* were publically available including the relative complete genome of the KACC42780 from Korea, L11 from China and a draft genome of the TR 19 from Japan. In this study, we sequenced the genome of wild strain of *F. filiformis* by small fragments library construction and did a comparative genome analysis on secondary metabolism gene clusters. The assembled genomes of the wild *F. filiformis* were 35.01Mbp with approximately 118-fold genome coverage. Total 10396 gene models were predicted, with an average sequence length of 1445 bp. The genome size and the number of predicted protein encoding genes are very similar to the public reference genome of *F. filiformis* (= Asian *F. velutipes*) (Table 1). Functional annotation of the predicted genes showed that more than half of predicted genes were annotated in NR database (6383 genes) and 1972, 2582, 837, 5794 genes were annotated in database SwissProt, KEGG, COG and GO, respectively. We identified 107 cytochrome P450 family genes, 674 genes encoding secretory proteins and 287 genes in CAZy database.

There have 17293 pan-genes among four strains of *F. filiformis* and pan-genome core comprised 4074 genes (on average 23.5% of each genome) (Fig. 1A). The proportion (23.5%) of core genes in pan-genome analysis is similar to pan-genome analysis of 23 *Corallorhiza* spp. [43]. But possibly, the number was lower than the actual number because that these genomes were not sequenced to completion. 3104 out of orthologous genes was annotated in KEGG database, 2722 genes have annotations in the GO database and 1055 genes are specific to the wild strain Liu355.

Functional characteristics of F. filiformis predicted genes

A KEGG enrichment analysis was performed to determine the functions of predicted genes of *F. filiformis*. The result showed that the highest number of genes of *F. filiformis* is involved in genetic information processing and translation (253 genes), followed by metabolism (carbohydrate metabolism with 243 genes). 21 genes were found participate into terpenoid and polyketides biosynthesis (Additional file 1: Figure S1).

Transcriptomic analysis and gene expression

We studied the gene expression differences across different development stages of *F. filiformis* including the monokaryotic (MK), dikaryotic mycelium (DK), primordium (PD) and fruiting body (FB) in transcriptomic level. Meanwhile, the DK of the cultivar strain of *F. filiformis* (CGMCC 5.642) also was transcriptomic sequenced. Three biological replicates were designed for each sample. An average clean data for each sample is 8.07–9.32 G. We mapped the clean reads to genome of *F. filiformis* Liu 355 using the HISAT software and get relative high total mapping rate (92.63%). In addition, the expression variation between samples was the smallest between MK and DK and was the greatest between the MK and FB of *F. filiformis* (Additional file 1: Figure S2).

Among 10396 gene models of *F. filiformis*, 9931 genes models were expressed (FPKM>5) across the four different tissues (MK, DK, PD and FB) of the wild strain and the mycelium of a cultivar strain of *F. filiformis*. 6577 genes were commonly expressed in all tissues and 151 genes were specific expressed in the cultivar strain, and 152,116,46, 199 genes were specific expressed in MK, DK, PD and FB of the wild strain of *F. filiformis*, respectively (Fig. 1B). Tissue-specific and high expression transcripts in *F. filiformis* Liu 355 were listed in Additional file 2: Table S1. Two new genes encoding ornithine decarboxylase (involved in poyamine synthesis) are highly expressed in mycelium of cultivar strains (Novel01369, Novel01744) and genes encoding oxidoreductase also has the highest expressed level (gene 830, FPKM>1000). Genes encoding agroclavine dehydrogenase, acetylxylin exterase, beta-glucan synthesis-associated protein and arabinogalactan endo-1,4-beta-galactosidase protein are significantly high expressed in fruiting bodies (FB) of the wild strain *F. filiformis* with more than 20–100 fold change compared to mycelium. It was known that agroclavine dehydrogenase was involved in the biosynthesis of fungal ergot alkaloid ergovaline [44]; and beta-glucan synthesis-associated protein is probably linked the polysaccharide biosynthesis of fungal cell wall. Highly expression of these genes indicated that it probably play an important roles in fruiting bodies development and compounds enrichment.

Total 5131 genes (51.67%) were up- or down regulated at least in one stage transitions such as from mycelium to primordium (PD vs DK, 3889 genes) and from primordium to fruiting body (FB vs PD, 3308 genes) (Fig. 1C). During primordial formation stage, 1780 genes are up-regulated and most of the genes belong to oxidoreductase activity (Go:0016491), hydrolase activity (Go: 0004553) and carbohydrate metabolism (Go: 0005975), down-regulated genes mainly was enriched with transmembrane transport (Go:0055085). During fruiting body development stage, genes related to fungal-type cell wall (Go:0009277) and structural constituent of cell wall (Go:0005199) are up regulated, reflecting the dramatic changes of cell wall structure during the development process. In addition, Go term enrichment of differentially expressed genes (DEGs) between wild strain Liu355 and cultivar strain CGMCC 5.642 showed that most genes displayed a similar expression profile, but peptide biosynthetic and metabolic process (Go: 0006518; Go:0043043), amide biosynthetic process (Go: 0043604) and ribonucleoprotein complex (Go: 1901566) are up-regulated in cultivar strain of CGMCC 5.642.

KEGG enrichment analysis showed that DEGs involved in glutathione metabolism is significantly enriched in dikaryotic mycelium of wild strain Liu 355 compared to cultivar strain (Fig. 2). Thirty-three DEGs, including genes encoding glutathione S-transferase, ribonucleoside-diphosphate reductase, 6-phosphogluconate dehydrogenase, cytosolic non-specific dipeptidase, gamma-glutamyltranspeptidase, glutathione peroxidase etc. participated in this pathway. Study on the glutathione metabolism of the filamentous fungus *Aspergillus nidulans* indicated that glutathione itself and glutathione metabolic enzymes play a crucially important role in the germination of conidiospores, as well as remarkable contribute to the general stress tolerance of fungi [45]. High expression of genes related to glutathione metabolism in wild strain of *F. filiformis* implied the strain probably has strong environment adaptation and is potential better breeding resource. In addition, during the primordial and fruiting body development stage, MAPK signaling pathway (45 DEGs) and starch and

sucrose metabolism (26 DEGs) is significantly enriched. Tyrosine metabolism, biosynthesis of secondary metabolites and glycosphingolipid biosynthesis are also significantly enriched in fruiting bodies formation stage.

Genes involved polysaccharide biosynthesis of F. filiformis

Polysaccharides (PSs) are an important and bioactive component of *F. filiformis* and other edible and medicinal mushrooms [46]. Glucan is well studied PSs and can be produced by different fungi, such as lentinan, ganoderan, grifolan, and schizophyllan [47]. Previous studies showed that *F. velutipes* polysaccharide (FVP) have good immunomodulation activities, anti-proliferation and improved the learning and memory impairment in rats [4, 5]. Recently, an interesting result showed that FVP even could be used as a material of edible film for packaging of food and vegetables because of their excellent features of film-forming and edibility safety [48].

The biosynthetic pathways of polysaccharide (PSs) involved synthesis of nucleotide sugar precursors, assembly of repeating monosaccharide units, and the polymerization process [42]. Although the exactly biosynthetic pathways of mushroom PSs remain unclear, polysaccharide synthesis is not a template-driven process, but instead the structures are determined primarily by the complement of polysaccharide-modifying enzymes present in any organism [49]. Phosphoglucumutase (PGM) and UDP-glucose pyrophosphorylase (UGP) are known as important enzymes in the biosynthetic pathway of nucleotide sugar precursors [46]. In addition, based on identification of intermediate compounds and activities of synthesis-related enzymes, researchers constructed a simplified biosynthetic pathway of mushroom PSs and inferred several enzymes such as glucokinase (GK), phosphoglucose isomerase (PGI), fructose-1,6-bisphosphatase (FBPase) and UDP-Glc pyrophosphorylase (UGP) probably involved in the biosynthetic pathways of PSs of edible mushroom [42, 46]. These enzymes play key roles in glycolysis and gluconeogenesis pathway and starch and carbohydrate metabolism.

We identified total 80 genes related to PSs biosynthesis involved glycolysis and gluconeogenesis in KEGG pathway (KEGG map 00010) [50] at genome level, including Glucose-6-phosphate isomerase (GPI), Fructose-1,6-bisphosphatase (FBP), Mannose-6-phosphate isomerase (PMI). The expression profile of these genes showed that genes encoding Zinc-type alcohol dehydrogenase are both up-regulated in mycelium of wild strains compared to cultivar strain and in fruiting body stage compared to mycelium of wild strain of *F. filiformis* (Additional file 1: Figure S3 and Additional file 2: Table S2). Genes encoding glycerol 2-dehydrogenase (gene9557, gene2028), 7-bisphosphatase (gene 2929), alcohol dehydrogenase (gene7891-D2, gene 9773-D2) and aryl-alcohol dehydrogenase (gene 4871, gene 612) are up-regulated in mycelium of the wild strain. Gene encoding mannose-1-phosphate guanylyltransferase (gene 11132-D3) have highest expression level in mycelium of wild strain with more than 200-fold change compared to cultivar strain. Genes encoding glycerol 2-dehydrogenase (gene 894) and sugar phosphatase are up-regulated expression in fruiting bodies stage of wild strain.

Moreover, based on predicted metabolic enzymes related to PSs biosynthesis of *Ganoderma lucidum* [46], we identified 21 putative essential enzyme involved in PSs biosynthesis in *F. filiformis* using protein sequencing homology search method (Table 2), including glucose-6-phosphate isomerase, hexokinase, mannose-6-phosphate isomerase, UDP-glucose dehydrogenase, galactokinase and transketolase etc. Among them, genes encoding UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase and fructose-bisphosphate aldolase have relative high transcripts in all samples analyzed (FPKM>100). These candidate genes will be functional verification by experiment in future, combined the quantification of PSs in different tissue.

Carbohydrate active enzymes (CAZymes)

Secreted carbohydrate-degrading enzymes are crucial enzymes for fungal biology, both for fungal cell wall turnover and for degradation of external carbohydrate sources. They are also responsible for the biosynthesis, degradation and modification of oligo- and polysaccharides as well as of glycoconjugates [51]. We performed sequencing similarity search against the CAZy database using Blastx ($E < 10^{-5}$) and totally identified 287 carbohydrate-active related genes in *F. filiformis* genome, including 149 Glycoside-Hydrolases (GH), 48 Auxiliary Activities (AA) containing redox enzymes, 36 glycosyltransferases (GT), 29 Carbohydrate-binding module (CBM), 13 Carbohydrate Esterases (CE) and 12 Polysaccharide Lyases (PL). The number of secreted carbohydrate-degrading enzymes (mostly GHs) is nearly four times than polysaccharide biosynthetic enzyme (GT), indicating their dependence on external carbohydrate sources for growth and development as wood-rot fungi. Genes encoding beta-1,3-glucan-binding protein (GH16) are most abundant with 23 members in *F. filiformis* genome. Previous studies showed that GH16 enzymes are involved in the formation of the chitin-b-1,3-glucan complex, suggesting a role in the processing of fungal cell-wall polysaccharides [51]. The expression profile of these genes related to CAZys is diverse (Fig. 3). Seven genes of 23 GH16 family are up-regulated expression in DK mycelium stage compared to MK (three genes annotated as candidate glycosidase and four genes as beta-glucan synthesis-associated protein), four genes of GH16 family are up-regulated in fruiting bodies compared to primordium.

Besides GH16, seven out of the 14 GH5 members (four genes annotated as Glucan 1,3-beta-glucosidase, two genes annotated as endoglucanase and another annotated as mannan endo-1,4-beta mannosidase) are differentially up-regulated in DK mycelium of the wild strain compared to MK mycelium and five genes belong to GH5 family are up-regulated in primordium stage compared to mycelium. Eight members out of 11 GH43 are up-regulated in primordium compared to mycelium.

Predicted bioactive secondary metabolism genes clusters of F. filiformis

Besides the macronutrients and micronutrients present in the *F. filiformis*, a large number of structurally diverse bioactive secondary metabolites, especially various novel sesquiterpenes and norsequiterpenes were identified from the mycelium and fruiting bodies of *F. filiformis* [9]. The genes involved in sesquiterpenes biosynthesis were described previously [9]. In this study, the gene cluster encoding terpenoid and PKS were re-examined using the genome and transcriptome resource. In total, 12 gene clusters related to terpenoid biosynthesis and two gene clusters for polyketide biosynthesis were predicted (Fig. 4, Additional file 2: Table S3). Compared with other three cultivar strains (KACC42780, TR 19 and L11 with genome sequencing), the numbers of gene clusters involved in terpene, PKS, NRPS and Siderophore biosynthesis are different and the gene number related to terpene synthesis is higher in wild strain Liu355 (119 genes) than cultivar strain L11 (81 genes) in our study (Table 3).

119 genes of 12 terpenoid clusters divided into 10 clades according to their expression level (Additional file 1: Figure S4). Most genes in clade II are up-regulated in primodium stage of wild strain Liu 355 compared to mycelium stage, including encoding 4,5-DOPA dioxygenase extradiol-like protein (gene 3103) and squalene synthase (gene 3428) involved in biosynthesis of squalene, a precursor of terpenoid compounds. Genes in VIII-X clades are significantly differently expressed in mycelium of wild strain Liu 355 compared to cultivar strain and in the fruiting body stage compared with mycelium of wild strain Liu 355, including key enzymes involved in terpenoid biosynthesis such as protoilludene synthase, candidate peroxisomal acyl-coenzyme A oxidase, L-amino acid amidase and cytochrome P450. It probably explained more diverse bioactive compound in wild strains than cultivar strain in previous study.

Putative genes for sesquiterpenoid biosynthesis in F. filiformis

Sesquiterpene compounds are mainly bioactive secondary metabolites in *Flammulina*. Chemistry investigation of six strains of *F. filiformis* in previous reports revealed that the wild strain Liu355 contained many new sesquiterpene skeletons, including cuparene-type and sterpurane type sesquiterpenes [9]. However, little is known about sesquiterpene synthases (STSs) in Basidiomycota until the mostly recent years, although these fungi can produce diverse bioactive sesquiterpenes. Thanks to the identification of sesquiterpene synthases from *Coprinus cinereus* (Cop1–6) and *Omphalotus olearius*, it provided a useful guidance for subsequent development of *in silico* approaches for the directed discovery of new sesquiterpene synthases and their associated biosynthetic genes [52]. In our study, we performed a genome-scale homologous search with sesquiterpene synthases of *O. olearius*, *C. cinereus* and *Hericium erinaceus*, 12 homologue sequences with considerable similarity (e-value < 10^{-5}) to the known biochemically characterized sesquiterpene synthases were identified in the genome of *F. filiformis*. The phylogenetic analysis showed that these genes consist of four clades (Additional file 1: Figure S5, Additional file 2: Table S4). Eight genes out of 12 genes related to sesquiterpene biosynthesis have closely phylogenetic relationship with genes identified from cultivar *Flammulina* in the previous study [9]. 12 STSs genes of *F. filiformis* include five genes encoding delta (6)-protoilludene synthase (gene1663-D2, gene9115, gene2784 and gene9115-D2, gene 6325-D2), two genes encoding trichodiene synthase (gene1140, gene 2254), two genes encoding alpha-muurolene synthase (gene1358-D2, gene1358), and one gene encoding glucose-6-phosphate isomerase. Among them, genes encoding delta (6)-protoilludene synthase are up-regulated in fruiting body stage compared to mycelium and also up regulated in DK mycelium compared to MK of the wild strain Liu355 (Table 4).

Putative genes for polyketides biosynthesis in F. filiformis

Polyketides are major group of secondary metabolites isolated from bacteria and fungi and fungi in particular have been important sources of natural product polyketide pharmaceuticals. Structural complexity of these polyketides makes them interesting and useful bioactive compounds and these same features also make them difficult and expensive to prepare and scale-up using synthetic methods [53]. In past, understanding of the biosynthesis and regulation of polyketide in fungi are extremely difficult because of lacking of established genetic tools. Recent researches illustrated that *Saccharomyces cerevisiae* have potential as a tool for mining, studying and engineering fungal polyketide synthases during to their many advantages benefits such as unicellular organism well suited for large scale fermentation, limited native secondary metabolism and a number of developed genetic tools for protein expression and pathway construction [53].

The diverse structures of polyketides are biosynthesized from short-chain carboxylic acid units by polyketide synthases (PKSs), PKSs have been classified into type I, type II and type III based on their product profiles and catalytic domain architecture [54]. By gene clusters prediction using the antiSMASH, we found 30 genes of two gene clusters responsible for Type I PKS. They mainly located in a single scaffold 24 and 78 in *F. filiformis* genome, respectively (Fig. 4, Additional file 2: Table S3). The two gene clusters both included core genes encoding polyketide synthase (gene 8217 and gene 1373). Genes located scaffold 78 are most up-regulated in wild strain mycelium compared with cultivar strain, including erythronolide synthase (gene 1374), polyketide synthase (gene1373), benzoate 4-monooxygenase (gene 1372), indicating that polypeptide compounds are probably abundant in mycelium of this mushroom and especially in wild strain.

Cytochrome P450s in F. filiformis genome

It is reported that a more divergent cytochrome P450 oxidase could be involved in secondary biosynthesis [55]. So, we searched the genome of the *F. filiformis* for proteins with a P450 conserved domain using NCBI's CDD tool and BLASTp [39]. We yielded 107 genes in cytochrome P450 family, including nine putative trichodiene oxygenase, 31 O-methylsterigmatocystin oxidoreductase, five benzoate 4-monooxygenase, two linoleate 10R-lipoxygenase, two ent-kaurene oxidase, lanosterol 14-alpha and flavonoid hydroxylase and other candidate cytochrome P450. 102 genes out of them have diverse expression profile across different tissue of *F. filiformis* (Additional file 1: Figure S4). 26 CYP450 genes are up-regulated in mycelium of wild strain compared to cultivar strain and the gene 54280-D3 have most high expression level with more than 500 fold change. 21 CYP450 genes are up-regulated in fruiting bodies stage compared to mycelium and gene encoding benzoate 4-monooxygenase have highest transcript level with 15 fold change. In primordium formation stage, gene encoding docosahexaenoic acid omega-hydroxylase is highest differentially expressed.

Heat shock protein correspond to temperature change in F. filiformis

Besides with unique compounds, the wild strain of *F. filiformis* Liu 355 can produce the fruiting body at relative high temperature than current commercial strains, implying it is a potential excellent breeding resource. A temperature downshift (cold stimulation) is considered to be one of the most important and essential environmental factors for the fruiting initiation and fruiting body formation of *F. velutipes* [34]. In generally, mycelia of *F. velutipes* can grow vegetative at 20–24 °C and fruiting at an optimum temperature for 12–15 °C [32]. In our study, the wild strain of Liu 355 can grow fruiting bodies at 18 °C at the laboratory. Therefore, it is potential excellent genetic material for *F. velutipes* breeding. Proteomic sequencing revealed that the expression of proteins related to energy metabolism (e. g. catalase, glucose-6-phosphate isomerase, trehalase and beta-glucosidase), amino acids biosynthesis (e. g. argininosuccinate synthase) and signal pathway (e.g. BAR adaptor protein) are dramatically increased after long-term cold stress [32, 34]. In addition, histidine kinases, response regulators, and sometimes histidine-containing phosphotransfer proteins were also reported play crucial roles in response to cold stress in cyanobacterium *Synechocystis* sp. and *Arabidopsis* [56, 57]. Transcriptomic sequencing revealed that histidine kinase and proteins involved in MAPK pathway

(e.g. STE protein kinase, MAPK kinase) and Ca^{2+} signal transduction pathway (calcium-dependent protein kinase) were differentially expressed in *F. velutipes* [31].

The heat shock protein (HSP) family was known that it positively correlated with the organism thermotolerance [58]. In this study, 28 genes annotated as HSP were identified in *F. filiformis* genome (Fig. 5). Among them, six genes are significantly up-regulated in wild strain Liu 355 compared to cultivar strain and encoding HSP12, HSPC4, HSP104, LHS1 and GRP78, respectively. HSP12 is part of a group of small heat shock proteins (HSP) that function as chaperone proteins and are ubiquitously involved in nascent protein folding by protecting proteins from misfolding and partially characterized as a stress response and expression of HSP12 protein was observed in response to cold stress [59]. In *S. cerevisiae* and *C. albicans*, HSP104 in association with HSP40 and HSP70 helps in reactivation and aggregation of denatured protein, by providing disaggregated protein to HSP40 and HSP70 as a substrate [60]. Expression of HSP104 and HSP70 is regulated by Hsf (heat-shock factor) interaction, which can be stimulated by heat stress in yeast [58]. However, the exactly molecular function of HSP in the high-temperature-tolerance of wild *F. filiformis* and adaptive mechanisms for relative high temperature need further study.

Conclusion

In our study, the genome and transcriptome sequencing, assembly and annotation of a high-temperature-tolerance of wild *F. filiformis* was carried out and the gene clusters associated with polysaccharides, terpenoid and polyketide biosynthesis were predicted. Comparative genomic analysis with other three Asian cultivars strains of *F. velutipes* (= *F. filiformis*) revealed that the wild strain has a similar genome size and relative much more putative gene numbers related to secondary metabolites biosynthesis. Most of genes related terpenoid biosynthesis are up regulated in primodium and fruiting body of the wild strain, while PKS genes are usually up-regulated in the mycelium of wild strain but the exactly regulation pathway is unresolved in this study.

Six genes belongs to the heat shock protein (HSP) family were significantly up-regulated in wild strain Liu 355 compared to cultivar strain, including HSP12, HSPC4, HSP104, LHS1 and GRP78, which is possible responsible for high-temperature-tolerance of wild *F. filiformis* developing fruiting body in relative high temperature but these genes expression in other different strain of *F. filiformis*, especially in strain with low-temperature development will be verified in next step.

Our study provides an important genetic dataset for the potential breeding materials of *F. filiformis* and put a foundation for better understanding the biology of *F. filiformis*.

Methods

Culture of strains

The wild strain Liu355 was isolated from the fruiting body of *F. filiformis* collected from Longling, Yunnan province, southwestern China and its ITS sequence was listed under GenBank accession number KP867925 [9]. The haploid monokaryotic strain *F. filiformis* Liu355 (deposited in mycological lab of Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences) was prepared by protoplast mononuclear method and was grown on potato dextrose agar (PDA) at room temperature for 2–3 weeks in the dark (Additional file 1: Figure S6). The fruiting bodies were obtained in sterile plastic bottles containing on growth substrate (cotton seed hulls 78%, wheat bran 20%, KH_2PO_4 0.1%, MgSO_4 0.1%, sucrose 1% and ground limestone 1%, with a moisture content of 70%) at 25°C for 30 d, followed by cold-stimulation at 18°C and 90% humidity until primordial occurred. Cultures were maintained at low temperature (18°C and 75% humidity) to allow full fruiting body development [61]. In addition, the genomic data of two cultivars strains from Korea (KACC42780, Bioproject PRJNA191921) and Japan (TR19, Bioproject PRJDB4587) were available from NCBI public database and genomic sequences of strain L11 (Bioproject PRJNA191865) was friendly provided by the Mycological Research Center, College of Life Sciences, Fujian Agriculture and Forestry University [62].

Genome, transcriptome sequencing and analysis

Total genomic DNA of *F. filiformis* was extracted from the mononuclear mycelium in the PDA medium using the Omega E. Z. N. A. fungal DNA midi kit (Omega, USA) according to manufacturer's instructions. Total DNA obtained was detected by agarose gel electrophoresis and quantified by Qubit. The strain of *F. filiformis* was sequenced using 350 bp paired-end reads on an Illumina HiSeq 4000 by PE150 strategy. Library construction and sequencing was performed at the Beijing Novogene Bioinformatics Technology Co. Ltd (Beijing, China). After removing adapter and low quality sequence using FastQC, the high quality reads were mapped into the reference genome sequence of *F. filiformis* L11 (Bioproject PRJNA191865) using BWA software. Functional annotation of the predicted genes was performed using BLAST against Gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), SwissProt and NCBI non-redundant (NR) protein database [39]. Pan-genome analysis was carried out using the standalone CD-HIT tool to cluster orthologous proteins [63].

For transcriptomic sequencing, total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocols. Four samples were prepared including monokaryotic mycelium (MK), dikaryotic mycelium (DK), primodium (PD) and fruiting bodies (FB) of the strains Liu355 and each sample had three biological replicates. All samples were subjected to RNA-Seq on the Illumina HiSeq2000 platform (Illumina, San Diego, CA, USA). Bioinformatics analysis was done based on clean data with high quality and the RNA-seq reads were mapped to the *F. filiformis* genome (Liu355) using TopHat v2.0.1253 [64]. HTSeq v0.6.1 software was used to count the read numbers mapped to each gene [65]. The FPKM value was used to calculate gene expression, and the upper-quartile algorithm was used to correct the gene expression. Gene differential expression analysis was performed using the DESeq R package (1.18.0) using a corrected p-value [66]. Genes with an adjusted P-value < 0.05 were assigned as differentially expressed. Hierarchical clustering of gene expression was conducted using Genesis 1.7.7 [67].

Prediction of gene clusters

The biosynthetic gene clusters were predicted using antiSMASH 3.0 software [68]. The antiSMASH currently offers a broad collection of tools and databases for automated genome mining and comparative genomics for wide variety of different classes of secondary metabolites [69]. In addition, homology sequence search method (Blast) was also used for identification of the genes related to terpenoid biosynthesis. The sesquiterpene synthases were identified based on multiple sequence alignments and phylogenetic analyses developed by Schmidt-Dannert group [52].

Abbreviations

BGCs: Biosynthetic gene clusters; *PKS*: Polyketide synthase; *KEGG*: Kyoto Encyclopedia of Genes and Genomes; *COG*: Clusters Orthologous Groups; *GO*: Gene Ontology; *CAZY*: Carbohydrate-Active Enzymes; *MK*: monokaryotic; *DK*: dikaryotic; *PD*: primordium; *FB*: fruiting body; *FPKM*: Fragments Per Kilobase of transcript per Million fragments mapped; *MAPK*: mitogen-activated protein kinase; *PSs*: Polysaccharides; *FVP*: *F. velutipes* polysaccharide; *PGM*: Phosphoglucumutase; *UGP*: UDP-glucose pyrophosphorylase; *GK*: glucokinase; *PGL*: phosphoglucose isomerase; *GPI*: Glucose–6-phosphate isomerase; *FBP*: Fructose–1,6-bisphosphatase; *PMI*: Mannose–6-phosphate isomerase; *GH*: Glycoside-Hydrolases; *AA*: Auxiliary Activities; *GT*: glycosyltransferases; *CBM*: Carbohydrate-binding module; *CE*: Carbohydrate Esterases; *PL*: Polysaccharide Lyases; *STSs*: sesquiterpene synthases; *CDD*: Conserved Domain Database; *CYP*: Cytochrome P450 proteins; *HSP*: The heat shock protein; *Hsf*: heat-shock factor; *ITS*: internal transcribed spacer; *PDA*: potato dextrose agar; *DEGs*: differentially expressed genes (DEGs).

Declarations

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Author's contributions

JC and SG designed the experiments. JL, YT, BL, XL, YL, WX performed the experiment. ZY and HL collected the sample and provided the strain. BX sequenced and provided the reference genome of *F. velutipes* L11. JC, KM and XZ analyzed the data, JC contributed to text writing and literature search and SG reviewed and revised the manuscript.

Availability of data and materials

The datasets supporting the results of this article are included with in the article and additional files. The genomic and transcriptomic data have been deposited to GenBank database with the dataset identifier PRJNA531555 for genome and PRJNA530834 for transcriptome.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Genomic features of four strains of *Flammulina filiformis* (=Asian *F. velutipes*).

Strain voucher	Liu355	L11	TR19	KACC42780
Accession number	PRJNA531555	PRJNA191865	PRJNA191921	PRJDB4587
strain original	Wild, Yunnan, China	Clutivated, Fujian, China	Cultivated, Japan	Cultivated, Korea
Genome size (Mb)	35.01	34.33	34.79	35.64
Genome Coverage	118×		132×	37.2×
No. of Scaffolds	2040	1858	5130	11
No. of Contigs	2060	28 590	6 405	500
Genes number	10 396	11 526	10 096	11 038
Gene total length (bp)	15 027 318(42.92%)	17 020 883 (49.58%)	14 905 273 (42.84%)	15 924 075 (44.68%)
Gene average length	1 445	1 477	1 476	1 443
G+C content(%)	52.31	52.46	52.35	52.31
P450	107	144	-	-
CAZy	270	315	-	392
Secretory Protein	674	-	-	-
Transposon pre number	204	215	245	285

Due to technical limitations, table 2 is only available as a download in the supplemental files section

Table 3. Putative genes and gene clusters related to secondary metabolism biosynthesis for the *F. filiformis*

Strains	Liu 355 (wild strain)		L11(cultivar)		KACC42780(Korea)		TR19(Japan)	
Clusters	Clusters_number	gene_number	Clusters_number	gene_number	Clusters_number	gene_number	Clusters_number	gene_number
Siderophore	2	5	1	5	1	15	1	4
NRPS	1	17	1	15	1	44	1	13
Terpene	12	119	10	81	17	139	15	115
typelPKS	2	32	1	18	2	34	2	30
cf_putative	6	71	3	53	52	1286	39	696

Table 4. Expression level of 10 genes encoding enzymes involved into sesquiterpenoid biosynthesis in *F. filiformis*

#FPKM value is the mean expression value of three biological replicates

#Abbreviation: MK: monokaryontic mycelium; DK: Dikaryontic mycelium; FB: fruiting body; PD: primordia

#black bont means genes significantly up-regulated;

#Note: among 12 genes, the expression of 2 genes (gene1358;gene2785) have't been detected in varous tissues.

Gene ID	chromosome	length	wild_FB_FPKM	wild_primordia_FPKM	wild_MK_FPKM	wild_DK_FPKM	clutivar_DK_FPKM	log2ratio wild_DK/cul DK)
gene1358-D2	Scaffold262	1005	45.38	48.94	124.54	82.53	38.31	0.86
gene3100	Scaffold10	2559	66.43	69.62	104.75	95.85	80.55	0.01
gene1663-D2	Scaffold157	1035	1.87	0.17	1.34	0.27	3.49	-3.90
gene9115	Scaffold2	1038	111.29	4.54	2.69	21.74	10.70	0.77
gene1140	Scaffold110	891	5.16	4.34	2.22	5.34	4.36	0.07
gene4450	Scaffold150	936	101.68	38.30	6.24	29.57	22.48	0.15
gene2254	Scaffold201	996	0.82	0.26	0.09	0.21	171.85	-9.93
gene10498-D2	Scaffold62	864	0.14	0.06	0.14	0.02	0.01	0.71
gene9115-D2	Scaffold49	1038	87.98	3.11	3.31	23.40	16.45	0.25
gene6325-D2	Scaffold61	1017	43.08	3.28	0.89	42.16	11.23	1.66

Figures

Figure 1

Fig. 1 Venn diagram showing the numbers of orthologue genes or differentially expressed genes. (A) The numbers of orthologue genes between four strains of *F. filiformis*. L11(China) in red, TR19 (Japan) in purple, KACC42780 (Korea) in yellow and Liu355 (China) in green. (B). The numbers of differentially expressed genes (DEGs) in various comparative groups of *F. filiformis*. Fruiting body (FB) in blue, Primordium in red, Monokaryotic mycelium (MK) in green, Dikaryotic mycelium (DK) in yellow and cultivar strain of *F. filiformis* in brown. (C). Venn diagram showing the numbers of DEGs at adjacent development stage of *F. filiformis*. Blue color represented the number of DEGs of fruiting body (FB) versus primordium (PD) and red color represented primordium (PD) versus dikaryotic mycelium (DK). Abbreviation: MK: monokaryotic mycelium; DK: dikaryotic mycelium; PD: primordium; FB: fruiting body.

Figure 2

Fig. 2 KEGG pathway enrichment analysis of differentially expressed genes (DEGs) during *F. filiformis* development. Left columns: pathway enrichment at mycelium stage of wild strain Liu355 compared to cultivar strain CGMCC 5.642; Middle columns: pathway enrichment at primordium stage compared to mycelium stage of wild strain Liu355; Right columns: pathway enrichment at fruiting body stage compared to primordium stage. Abbreviation: MK: monokaryontic mycelium; DK: dikaryontic mycelium; PD: primordium; FB: fruiting body.

Figure 3

Fig. 3 Hierarchical clustering analysis of 287 CAZy of *F. filiformis* between wild strain Liu355 and cultivar strain CGMCC 5.642 and among four development stages of wild strain Liu 355. Expression ratios were plotted in a heatmap on a log2 scale. The red and green colors indicate up- and down-regulation, black represents no significant expression change and grey represents missing data The abbreviation: MK, monokaryotic mycelium; DK, Dikaryotic mycelium; PD, primordium; FB, Fruiting body. GH, Glycoside-Hydrolases; AA, Auxiliary Activities containing redox enzymes; GT, glycosyltransferases; CBM Carbohydrate-binding module; CE, Carbohydrate Esterases (CE); PL, Polysaccharide Lyases.

Figure 4

Fig. 4 Identification of the putative gene clusters for terpene and polyketides (PKS) in *F. filiformis* genome by antiSMASH software. Genes with SwissProt functional annotation were marked in red color.

Figure 5

Fig. 5 Hierarchical clustering analysis of 28 putative heat-shock protein encoding genes in *F. filiformis* genome between wild strain Liu355 and cultivar strain CGMCC5.642 and among four development stages of wild strain Liu 355. Expression ratios were plotted in a heatmap on a log2 scale. The red and green colors indicate up- and down-regulation, black represents no significant expression change and grey represents missing. The abbreviation: MK, monokaryotic mycelium; DK, Dikaryotic mycelium; PD, primordium; FB, Fruiting body.

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