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Transcriptomic analysis of the white rot fungus *Polyporus brumalis* provides insight into sesquiterpene biosynthesis

Su-Yeon Lee[a], Myungkil Kim[a], Seon-Hong Kim[b], Chang-Young Hong[b], Sun-Hwa Ryu[a,*], In-Gyu Choi[b,*]

[a] Division of Wood Chemistry & Microbiology, Department of Forest Resources Utilization, Korea Forest Research Institute, Seoul 130-712, Republic of Korea
[b] Department of Forest Sciences, College of Agriculture and Life Sciences, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-921, South Korea

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ABSTRACT

Object of this study was to identify genes and enzymes that are involved in sesquiterpene biosynthesis in the wood rotting fungus, *Polyporus brumalis*. Sesquiterpenes, β-eudesmane and β-eudesmol, were produced by the mycelium of *P. brumalis* cultured in modified medium. However, theses final products were not observed when the fungus was grown in potato dextrose medium. We used next generation sequencing (NGS) to identify differentially expressed genes (DEGs) related to terpene metabolism. This approach generated 25,000 unigenes and 127 metabolic pathways that were assigned to Kyoto Encyclopedia Genes Groups (KEGG). Further analysis of samples from modified medium indicated significant upregulation of 8 unigenes involved in the mevalonate (MVA) and methylerythritol phosphate (MEP) biosynthetic pathways. These pathways generate isopentenyl pyrophosphate (IPP) and farnesyl pyrophosphate (FPP), which are precursors for the synthesis of sesquiterpenes. Furthermore, genes encoding germacrene A synthase, which facilitate the cyclization of FPP, were only differentially expressed in mycelium from fungi grown in modified medium. Our data provide a resource for studying the molecular mechanisms underpinning sesquiterpene biosynthesis and terpene metabolism.

1. Introduction

Terpenoids group has important protective roles in plants, and they can also be used as pharmaceutical agents. In particular, sesquiterpenes, which are composed of three isoprene units, exhibit diverse antioxidant, antimicrobial and antitumor bioactivities (Alan et al., 2006). Sesquiterpene compounds are generally produced from plant sources and are components of plant essential oils. Eudesmane type sesquiterpenes in essential oils derived from *Cryptomeria japonica* and *Chamecyparis obtusa*, two representative coniferous species from South Korea and Japan, have been shown to have antifungal activity (Moiteriro et al., 2013; Yang et al., 2015). Compounds within the eudesmane family of terpenes, including more than 100 members, have low molecular weights and are composed of a unique skeleton. Theses natural products are difficult to produce via artificial synthesis, particularly if large quantities are desired (Ke and Phill, 2009).

Microorganisms provide another route for biosynthesis of terpene compounds as natural products (Berger, 2009). A number of metabolites isolated from fungi have been utilized in various medical applications or as leading structures for the development of pharmaceutical products. Wood-rotting fungi, which are widely distributed in nature, have secondary metabolic pathways, and numerous metabolites have been discovered through studies of fungal chemistry (Sanjay et al., 2009; Janos, 2004). We previously found that *Polyporus brumalis*, which is a polypore fungus that grows on fallen braches from deciduous trees and is distinguishable as a white rot fungus, is unique in its ability to synthesize eudesmane-type sesquiterpenoids as secondary metabolites. In this study, extended work of our previous study was conducted by performing transcriptome analysis in order to identify genes and enzymes involved in sesquiterpene synthesis. With the development of next generation sequencing (NGS) technology, several macrofungi have been sequenced and analyzed. Genome of the white rot fungus *Phanerochaete chrysosporium* (Wymelenber et al., 2010) has been firstly sequenced, genomes and transcriptomes of several species of wood decaying fungi have been researched (Chen et al., 2012; Martinez et al., 2009; Yakovlev et al., 2013).

* Corresponding authors.
E-mail addresses: shryu@forest.go.kr (S.-H. Ryu), cingyu@snu.ac.kr (I.-G. Choi).

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Table 1
Two media are formulated to profiling differentially expressed genes between grouped samples.

Nutrients of medium	Medium			
	Modified medium	g/L	PDB medium	g/L
Carbon	Glucose ($C_6H_{12}O_6$)	10 g	Potato starch dextrose	4 g20 g
Nitrogen	Ammonium tartrate ($C_4H_{12}N_2O_6$)	0.2 g	–[a]	
Phosphate	Potassium phosphate (KH_2PO_4)	2 g	–	
Magnesium	Magnesium sulfate ($MgSO_4$)	0.5 g	–	
Calcium	Calcium chloride ($CaCl_2$)	0.1 g	–	

[a] None addition.

Table 2
Grouped samples for extraction of total RNA and differentially expressed genes analysis.

Group	Cultivation days		
	5 day	7 day	12 day
Modified group[a]	Sample 1	Sample 2	Sample 3
PDB group[b]	Sample 4	Sample 5	Sample 6

[a] *P. brumalis* mycelium were inoculated in the modified medium.
[b] *P. brumalis* mycelium were inoculated potato dextrose broth medium.

However, despite these efforts, the synthetic pathways responsible for terpene metabolites in these fungi remain unclear. Knowledge-based prediction of metabolic pathways has revealed that isopentenyl pyrophosphate (IDI) and farnesyl pyrophosphate (FPP) are intermediates in the synthesis of 300 structurally diverse sesquiterpene including those eudesmane-types. Furthermore, mevalonate (MVA) and methylerythritol phosphate pathways (MEP) are known to mediate pathways for the synthesis of IPP and FPP (Lange et al., 2000). The MVA pathway is a well-documented source of precursors in biosynthetic pathway in bacteria, fungi, plants and animals (Lichtenthaler, 1999). Although well studied in eubacteria and plants (Rohmer et al., 2003), the role of the MEP pathway in fungi, and its relationship to sesquiterpene synthesis, is much less clear. To address this, we designed a transcriptomic study to identify genes that were selectively induced according to the culture conditions in which *P. brumalis* was grown.

2. Materials and methods

2.1. Mycelium

P. brumalis was provided from Korea Forest Research Institute, Seoul, Korea. We identified *P. brumalis* isolated by KFRI, with the internal transcribed spacer (ITS) and a BLST search of the Gene Bank database. Pre-inoculation of mycelium was performed in Potato Dextrose Agar medium in a stationary incubator at 28 °C for seven days. Fully grown mycelium on nine agar plates was separated using platinum wire after seven days. And then they were transferred into container of ultra-homogenizer with distilled water 20 ml. After homogenizing for 30 s; mycelium was obtained as suspension form. Unit of fungal suspension was calculated by measuring as dry weight (mg/ml) at drying machine for 3 h at 121 °C. Inoculation was started adding the fungal suspension (dry weight of suspension: 0.04 g/ml) of *P. brumalis* in 100 ml two different media, the modified medium and the potato dextrose broth (PDB) medium, for 5 days in the dark at 28 °C in an shaking incubator at 80 rpm (Table 1).

As shown in Table 2, all samples of mycelium were grouped according to medium to verify the differentially expressed genes (DEGs) in terpene metabolism. Because of metabolites, the sesquiterpene of *P. brumalis* mycelium were only synthesized in the modified medium, which included inorganic components. On days 5, 7, and 12 of culture, the mycelium was separated from the media for extraction of RNA. Then, the aqueous medium was extracted by the addition of 100 ml of the ethyl acetate followed by 15 min of shaking on the extractor.

Table 3
Summary of RNA-seq and de novo assembly of mycelium o *P. brumalis* transcriptome.

	Number of unigenes	Length	N50	Largest length
Modified group	23,876	21,621,857	1,403	11,305
PDB group	25,119	22,911,371	1,427	11,308

2.2. Analysis of metabolites

Analysis of gas chromatography (GC) equipped with FID and MS detectors were performed for qualitative and quantitative analysis of fungal metabolites. DB-5 column (dimension: 30 m × 0.25 mm, coating thickness: 0.25 um) was used stationary phase of GC and He (1 ml/min) was used carrier gas. The working conditions were referenced our previous study (Lee et al., 2015). Condition was as follows: injection at 300 °C and, detector at 250 °C and variation of oven temperature from 40 to 280 °C at 5 °C/min with initial and final holding time of 10 min. The split ratio was 5:1 and the range was from 50 to 800 *m/z*. A mass spectrum of each peak was identified based on NIST 08 (National Institute of Standard and Technology) library. Also, the metabolites were compared with relative retention times of *n*-alkane (C_8–C_{30}) mixture in DB-5 column.

2.3. cDNA library construction and sequencing

Total RNA of mycelium was obtained using liquid nitrogen and extraction kit (Hybrid-RTM, GeneAll) from two groups; modified group and PDB group. The total RNA qualification of each sample was performed by RNA 6000 Pico chip (Agilent Bio analyzer). Passed RNA shown quality above 7 ~ 10 of RNA integrity number. Purification of poly A containing mRNA was performed using poly T-oligo-attached magnetic beads. And mRNA was fragmented using ion chelating factor (K^{2+}) at 94 °C for 5 min. The fragment was proceeded immediately to synthesize for first strand cDNA. 1st cDNA from RNA sequence was generated by SuperScript III Reverse Transcriptase (Invitrogen, 18080-044) with random primer. 2nd cDNA was synthesized with dUTP at 16 °C for 2 h. Purification of 1st and 2nd cDNA was conducted using Agencourt RNAClean XP magnetic beads. End repair and A-tail of cDNA were carried out with Adenylate 3′ Ends at 37 °C for 30 min. To eliminate the uracil, UDG digestion was conducted adding UDG mix. Selection of library size of 400–500 bp was identified using Gel electrophoresis. Finally, validation of library was checked using Agilent Bio analyzer.

2.4. Mapping assembly of the transcriptome and DEGs analysis

Raw fastq data of RNA-sequence was extracted above quality score 20 using Illumina Hiseq 2500 by preprocessing. A reference sequence demonstrating the gene sequence was created by de novo assembly using the Trinity software (http://trinityr-naseq.sourceforge.net). To eliminate redundant sequence, a sequence