


Identification and Characterization of Two New S-Adenosylmethionine-Dependent Methyltransferase Encoding Genes Suggested Their Involvement in Stipe Elongation of *Flammulina velutipes*

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ABSTRACT

Two new SAM-dependent methyltransferase encoding genes (*fsmt1* and *fsmt2*) were identified from the genome of *Flammulina velutipes*. In order to make a comprehensive characterization of both genes, we performed *in silico* analysis of both genes and used qRT-PCR to reveal their expression patterns during the development of *F. velutipes*. There are 4 and 6 exons with total length of 693 and 978 bp in *fsmt2* and *fsmt1*, respectively. The deduced proteins, i.e., FVSMT1 and FVSMT2 contained 325 and 230 amino acids with molecular weight 36297 and 24894 Da, respectively. Both proteins contained a SAM-dependent catalytic domain with signature motifs (I, p-I, II, and III) defining the SAM fold. SAM-dependent catalytic domain is located either in the middle or at the N-terminal of FVSMT2 and FVSMT1, respectively. Alignment and phylogenetic analysis showed that FVSMT1 is a homolog to a protein-arginine omega-N-methyltransferase, while FVSMT2 is of cinnamoyl CoA O-methyltransferase type and predicted subcellular locations of these proteins are mitochondria and cytoplasm, respectively. qRT-PCR showed that *fsmt1* and *fsmt2* expression was regulated in different developmental stages. The maximum expression levels of *fsmt1* and *fsmt2* were observed in stipe elongation, while no difference was found in mycelium and pileus. These results positively demonstrate that both the methyltransferase encoding genes are involved in the stipe elongation of *F. velutipes*.

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1. Introduction


S-adenosylmethionine (SAM)-dependent methyltransferases can transfer methyl group from SAM to many kinds of biological molecules. This reaction is universal in eukaryotes and prokaryotes and plays crucial roles in many important biological processes, such as small molecule biosynthesis and methylation of DNA and protein [1–3]. DNA methylation is carried out by cytosine methyltransferase in eukaryotic organisms with varied methylation rates depending on organisms. In animal and fungi, certain DNA methyltransferases are responsible for methylation maintenance; while, in *Arabidopsis*, DNA methyltransferases seem to have functional redundancy [1].

In animals, DNA methylation not only plays crucial roles in basic developmental processes, such as early development, embryonic stem cell development, and adulthood [4], but also can affect specific gene expression in some diseases [5,6]. Protein methylation also plays important roles in many important biological processes, such as neural crest

migration [7], bacterial virulence [8], and regulation of estrogen receptor [9].

The SAM-dependent methyltransferase is essential for biosynthesis of small molecules, especially some important secondary metabolites and medicinal molecules [10]. Research on SAM-dependent methyltransferases in fungi mainly focuses on secondary metabolite biosynthesis and genetic modification. The biosynthesis of some important fungal secondary metabolites, such as fungal toxins [11,12], 2,4,6-trichloroanisole causing cork taint in cork industry [13,14], and C-9-methylated glucosylceramides [15], is performed by SAM-dependent methyltransferases. Members of SAM-dependent methyltransferases are also reported to play important roles in lignin metabolism [16]. SAM-dependent methyltransferases play prominent roles in fungal epigenetics. For example, SAM-dependent methylation affects mating through modification in pheromone signal pathway in the corn smut disease-causing pathogen (*Ustilago maydis*) [17]. SAM-dependent methyltransferase mutations in

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fungi, could cause changes in phenotypes or DNA and RNA methylation [1]. More recently, a heterodimeric methyltransferase is reported to influence the balance between asexual and sexual development, and secondary metabolite production [18,19].

In this article, we report two novel SAM-dependent methyltransferases from the mushroom-forming basidiomycete; *Flammulina velutipes*. We have also provided the characteristic properties of the SAM-dependent methyltransferases based on bioinformatics analysis, substrate prediction, and their expression patterns during the development process of *F. velutipes*.

2. Materials and methods

2.1. Genes identification and sequence analysis

The SAM-dependent methyltransferase encoding genes were identified in the genome sequence (data not uploaded) of dikaryotic Nongjin 6 strain. The ZOOM software (Bioinformatics Solutions Inc., Waterloo, Canada) was locally used to analyze the gene structure by using a pair-end alignment mode. The deduced amino acid sequences were obtained by using clonemanager 8.0 (http://www.scied.com/pr_cmpro.htm). The theoretical isoelectric point and molecular weight of peptides were predicted by using ExPASy (<http://web.expasy.org/protparam/>). Subcellular location of the deduced protein was predicted by using the iPSORT WWW Service (<http://ipsort.hgc.jp/>) and WoLF PSORT Prediction service (<http://www.genscript.com/tools/wolf-psort>).

GENEDOC software (<https://www.softpedia.com/get/Science-CAD/GeneDoc.shtml>) was used for subsequent process and output, and DNAMAN (<https://www.lynnon.com/>) was employed for the calculation of protein sequence identity. The evolutionary relationship between different SAM-dependent methyltransferases was inferred by using the Neighbor-Joining method [20]. The involved SAM-dependent methyltransferase sequences derived from plants, bacteria, ascomycetes, and basidiomycetes in the NCBI database (<https://www.ncbi.nlm.nih.gov/>). The MEGA7 software was used to conduct the multiple protein sequence alignment by using the ClustalW algorithm and to generate an evolutionary tree [21].

2.2. Substrate preference prediction of FVSMT1 and FVSMT2

Substrate prediction for FVSMT1 and FVSMT2 was conducted based on previous studies [3,22]. A total of 147 biochemically characterized methyltransferases, including 61 from plants and 86 from yeast, were used to construct a database for local alignment.

Sequences of FVSMT1 and FVSMT2 were used as query sequences.

2.3. Cultivation of *F. velutipes* and sample collection

A dikaryotic strain Nongjin 6 of *F. velutipes* was obtained from crossbreeding between two compatible monokaryons (NJ6-3 and NJ6-21) in our lab. The substrate composition was as follows: cotton seed hull, sawdust (*Quercus* sp.), wheat bran, and gypsum, with a proportion of 30:49:20:1. The final water content was 60%. Sterilization of substrate was carried out at 121 °C for 2.5 h. The spawn was inoculated into substrate bags and incubated at the set parameters for fruiting bodies formation [23]. Samples, i.e., mycelium, primordium, stipe, and pileus, were collected at different developmental stages. The mycelial samples were collected before cold induction. The primordial samples were collected before stipe and pileus differentiation. The stipe samples were collected at the elongation stage [24], and the pileus tissues were collected 15 days after the primordium formation. Each collected sample was not less than 0.25 g. Samples from four biological replicates were also collected in similar manner as mentioned above.

To determine the expression pattern of putative SAM-dependent methyltransferase encoding genes, the stipe samples were also collected for total RNAs extraction from the fast elongation region (FER) and the slow elongation region (SER) in according to previous studies [24,25]. The FER is adjacent to the apical point where the stipe and the pileus connect to each other, about 1 cm in length, and the SER locates under the FER [25]. The FER and SER samples were cut from the FER and SER, respectively. Four biological duplicates were prepared for each sample.

2.4. RT-PCR analysis

Plant RNA Kit (OMEGA Biotech, Karnataka, India) was used for the total RNA extraction and TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR kit (Transgen Biotech, Beijing, China) was used for cDNA synthesis. The cDNA was diluted with ddH₂O as 1:4 and stored at -20 °C for quantitative real-time PCR (qRT-PCR). The TransStart Top Green qPCR SuperMix (Transgen Biotech) was used for qRT-PCR analysis and reaction mixture was prepared as mentioned by Tao et al. [26]. The qRT-PCR conditions are as follows: denaturation at 95 °C for 3 min, PCR program consisted of 39 cycles, including 95 °C, 10 s and 56 °C, 30 s.

The qRT-PCR primers for *fvsm1* are; SMT1F (AGGCTCCGATCCATTAAGTC) and SMT1R (CGCCAGTGAAGTATACCCTATG); and primers of *fvsm2* are SMT2F (ACCTCTAAAGTCACTGTGCTC) and SMT2R (GTCCGCGTCAATGAAGATAAAG). The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was used as reference and primers used for this gene are: *gpdF* (CCTCTGCTCACTTGAAGGGT) and *gpdR* (GCGTTGGAGATGACTTTGAA). All primers were designed using PrimerQuest (<http://sg.idtdna.com/PrimerQuest/Home/Index>). The $2^{-\Delta\Delta CT}$ method was employed to calculate the relative expression level [26].

The sequences for *fvsm1* and *fvsm2* were deposited to GenBank under accession numbers with MF169207 and MF169208, respectively.

3. Results

3.1. Identification and characterization of *fvsm1* and *fvsm2*

The structure of the predicted SAM-dependent methyltransferase encoding genes (*fvsm1* and *fvsm2*) was confirmed by the locally used ZOOM software (As shown in [Supplementary Material](#)). *fvsm1* has six exons, with a total length 978 bp, encoding a protein FVSMT1 with 325 amino acids. *Fvsm2* has four exons, with a total length 693 bp, encoding a protein FVSMT2 with 230 amino acids. FVSMT1, with molecular weight 36297.26 Da and pI 7.78, was predicted to locate in mitochondria. FVSMT2, with molecular weight 24894.65 and pI 4.81, was predicted to distribute in cytoplasm.

The phylogenetic analysis showed that FVSMT1 and FVSMT2 belonged to different methyltransferase types. The analysis also showed that methyltransferases in basidiomycetes clustered to two distinct clades, and FVSMT1 and FVSMT2 were located within these two separate basidiomycete clusters (Figure 1). The sequence search service in EMBL-EBI (<http://pfam.xfam.org/search/sequence>) showed that FVSMT1 and FVSMT2 belonged to Methyltransferase_11 family (PF08241) and Methyltransferase_3 family (PF01596), respectively. Interestingly, methyltransferases (KIY61808 and KIY71359) of *Cylindrobasidium torrendii* FP15055 ss-10 are also distributed into these separate clusters of basidiomycetes, which indicate that even methyltransferase from the same fungal species, but are evolved in different evolutionary process.

Protein sequence alignment showed that FVSMT1 and FVSMT2 contained a single SAM-dependent domain and shared high similarity with methyltransferases in their own alignment groups. Similarity between FVSMT1 and FVSMT2 to other methyltransferases (MTs) within their respective

groups ranged from 56.06 to 63.44%, and 56.07 to 80.43%, respectively. However, the similarity between FVSMT1 and FVSMT2 is only 10.77%.

The unitary SAM-dependent catalytic domain in FVSMT1 and FVSMT2 contains four highly conserved signature motifs (I, p-I, II, and III) defining the SAM fold. Distribution of SAM catalytic domain in FVSMT1 and FVSMT2 is similar in Class III and Class I, respectively, of PMTs (phosphoethanolamine *N*-methyltransferase) which includes enzymes from *Plasmodium* spp., (263–266 amino acid in length) and *Caenorhabditis elegans* (~500 amino acid) with a single SAM-dependent catalytic domain located either in the middle or at the *N*-terminal (Figure 2(A,B)). On the basis of conserved domain analysis and phylogenetic analysis, we conclude that *fvsm1* and *fvsm2* are two different SAM-dependent methyltransferases in *F. velutipes*.

The alignment results of FVSMT1 with six methyltransferases from yeast in the integrated methyltransferome showed *E* value ranging from $3e-148$ to 0.008 (Table 1). The similarity between FVSMT1 and the six yeast methyltransferase was from 27.33% ($E=7e-28$) to 63.44% ($E=3e-148$). However, no methyltransferase similar to FVSMT1 was found in the plant methyltransferases (see [Supplementary Material](#), *fvsm1blast*). Thus, we suggest that FVSMT1 is a homolog of protein-arginine Omega-*N*-methyltransferase (accession NO.: YBR034C) from yeast [3]. On the other hand, FVSMT2 showed high affinity with cinnamoyl CoA *O*-methyltransferase clade [22], with *E* value ranging from $7e-019$ to $1e-023$ (Table 1). FVSMT2 exhibited no significant similarity with yeast methyltransferases ($E > 0.84$, see [Supplementary Material](#), *fvsm2blast*). So, we propose that FVSMT2 belongs to methyltransferases using caffeoyl-CoA as preferred substrate.

3.2. Expression patterns of *fvsm1* and *fvsm2*

The results of qRT-PCR assay showed that the expression of *fvsm1* and *fvsm2* were regulated in different developmental stages in *F. velutipes*. We found no expression difference of *fvsm1* and *fvsm2* between mycelial and pileus. At primordial stage, the *fvsm1* and *fvsm2* were upregulated by 3.34 and 1.02 folds, respectively. However, the maximum expression levels of *fvsm1* and *fvsm2* were observed in stipe elongation stage ($p < 0.01$), and found 18.21 and 8.77 folds higher than that in mycelia, respectively. A similarity in expression pattern has also been observed between *fvsm1* and *fvsm2* at different developmental stages and tissues (Figure 3(A,B)).

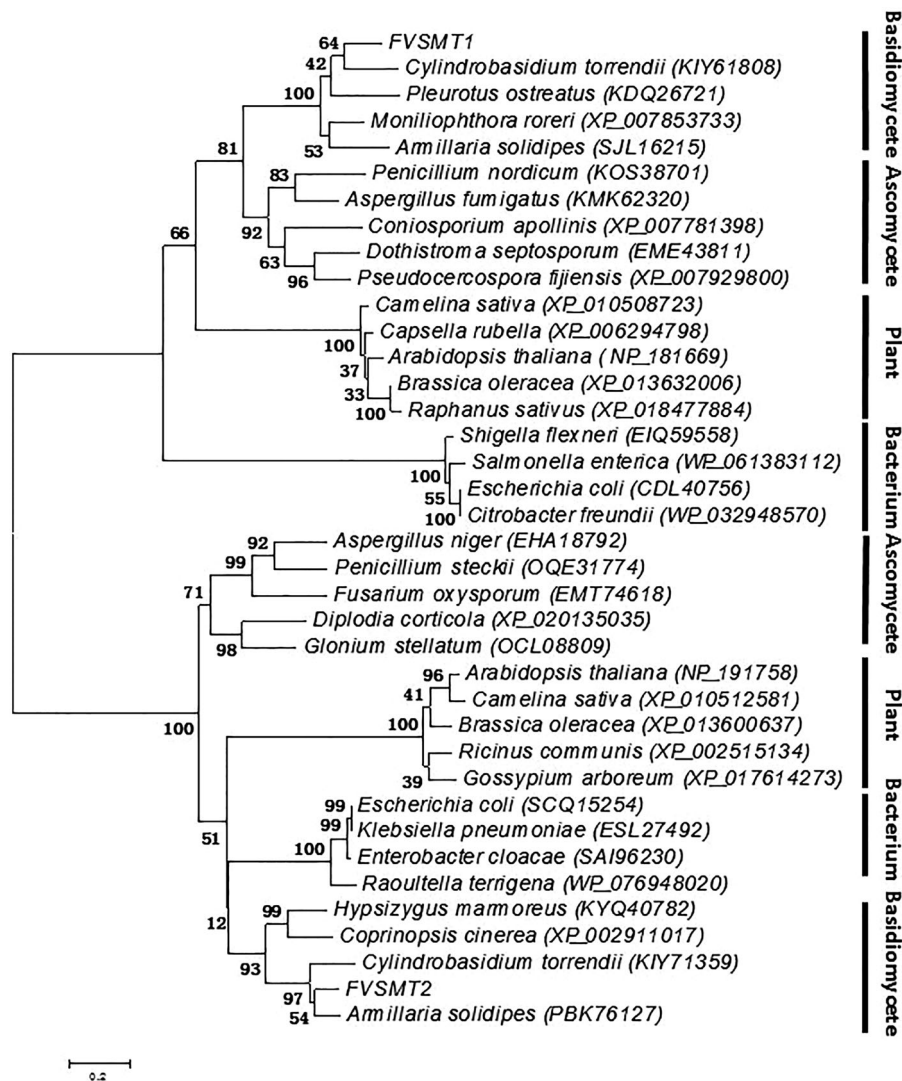


Figure 1. Phylogenetic analysis of two novel (FVSMT1 and FVSMT2) SAM-dependent methyltransferases of *F. velutipes* with other basidiomycetes, ascomycetes, plants, and bacteria. The tree is constructed by the neighbor-joining algorithm. The reliability of the tree branching is tested by conducting 1000 pseudo-replicate bootstrap re-samplings.

Since both methyltransferase encoding genes showed the highest expression level in stipe development, further investigation into expression in different parts of the elongating stipe was carried out. Expression levels of *fvsm1* and *fvsm2* in FER were about 2.46 and 9.11 times of that in SER, respectively (Figure 3(C,D)).

4. Discussion

Despite the global importance of SAM-dependent methyltransferase in various functions, their details have remained elusive in *F. velutipes*. In this study, two SAM-dependent methyltransferase encoding genes, *fvsm1* and *fvsm2*, were identified from *F. velutipes*. We have analyzed the genomic structure of both the genes and predicted some functions of their deduced proteins.

Alignment results showed that a set of consensus sequence motifs (I, post-I, II, and III) were found in

FVSMT1 and FVSMT2, which define the SAM binding site of various methyltransferases [27,28]. Unlike the bipartite plant PMTs, FVSMT1 and FVSMT2 of *F. velutipes* appear to contain single N-terminal or central methyltransferase domain, respectively. Alignment of the consensus motifs showed that the C-terminals of both FVSMT1 and FVSMT2 lack the sequence regions indicative of a methyltransferase domain.

The substrates of SAM-dependent methyltransferases are of wide range, such as proteins, nucleic acids, lipids, and many intermediate products in secondary metabolism. A number of studies show different specificities in the methyl transfer reaction [29–32], depending on enzyme structure and specific residues [33–37]. Based on protein sequence alignment against the methyltransferase, FVSMT1 is probably a protein-arginine Omega-N-methyltransferase, whereas FVSMT2 is predicted to be caffeoyl-CoA-3-O-methyltransferase. As implicated by plant FVSMT2

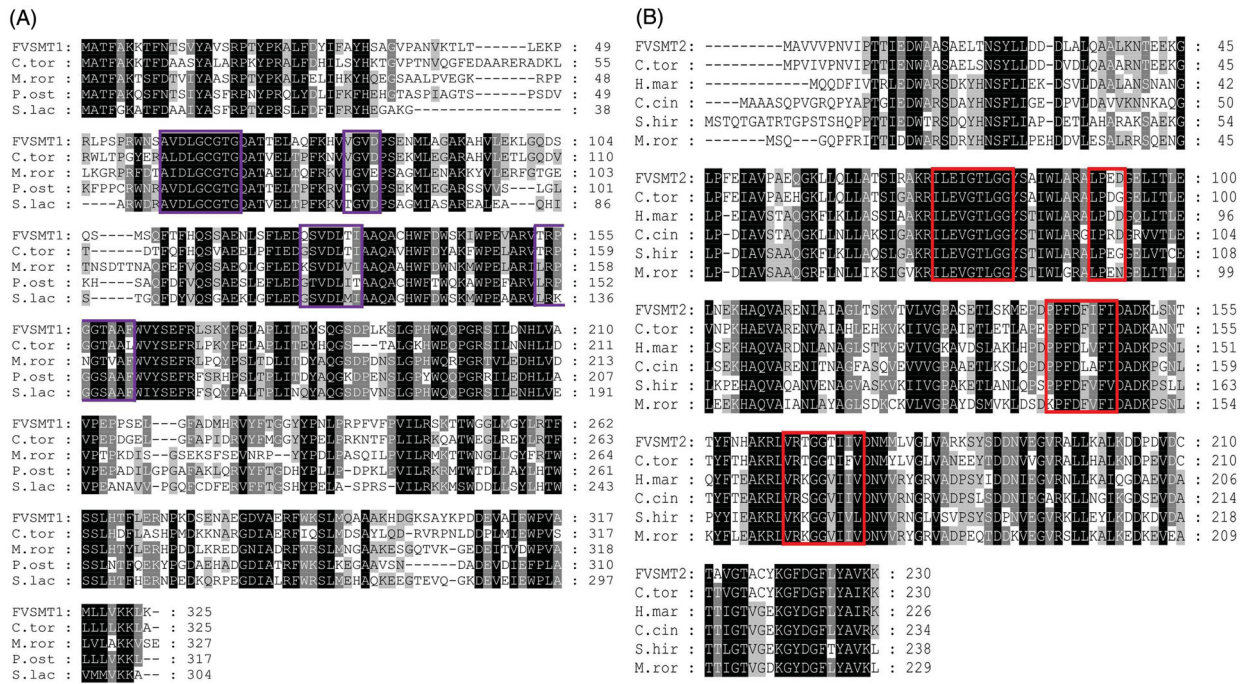


Figure 2. (A, B) Multiple protein sequence alignment and conserved motif analysis of FVSMT1 and FVSMT2. In both the groups of FVSMT1 and FVSMT2, the species names are abbreviated. Amino acids shaded in black represent high identity and those in gray lower identity. For FVSMT1 group, the accession numbers of *C. tor*, *M. ror*, *P. ost*, and *S. lac* are K1Y61808, KTB40671, KDQ26721, and XP_007323430, respectively. For FVSMT2 group, the accession numbers of *C. tor*, *H. mar*, *C. cin*, *S. hir*, and *M. ror* are K1Y71359, RDB17094, XP_002911017, XP_007301230, and ESK94813, respectively. Four specific conserved motifs (I, p-I, II, III) in aligned FVSMT1 and FVSMT2 are boxed in blue and green color, respectively.

Table 1. Alignment results of FVSMT1 and FVSMT2 with integrated methyltransferome.

Reference Methyltransferome for FVSMT1	E value (E < 0.01)	Reference Methyltransferome for FVSMT2	E value (E < 0.01)
YBR034C	3e – 148	U13151	1e – 023
YER175C	7e – 028	M69184	7e – 023
YHR209W	6e – 025	Z54233	1e – 022
YML008C	2e – 006	AJ224894	2e – 022
YOL096C	0.002	U38612	2e – 022
At5g55250	0.008	U20736	4e – 022
—	—	AF036095	2e – 021
—	—	L22203	1e – 019
—	—	AY145521	7e – 019

E values more than 0.01 are not presented in the table.

homologues in lignin biosynthesis [22], fungal FVSMT2 may also participate in phenylpropanoid biosynthesis, catalyzing caffeyoyl-CoA to produce feruloyl-CoA [38].

In addition to the versatile functions of SAM-dependent methyltransferases in development, less has been reported about the expression regulation of SAM-dependent methyltransferase encoding genes. The qRT-PCR assay showed that both *fsmt1* and *fsmt2* were developmentally regulated and had similarity in expression pattern at different developmental stages and tissues in *F. velutipes*. However, phylogenetic analysis showed that these genes have independent origins. Three SAM-dependent methyltransferase encoding genes (*AtHOL*, *AtHOL2*, and *AtHOL3*) had been identified with high protein sequence similarity and

tissue-specific expression patterns from *Arabidopsis thaliana* [39], indicating that they might play important roles in specific tissues. Honey bee was reported to express a SAM-dependent methyltransferase, AmJHAMT, differentially in queen larvae than worker larvae during the periods of caste development. These results indicated that AmJHAMT may play an important role in honey bee caste differentiation [40]. While in fungi, little have been discussed about expression patterns of SAM-dependent methyltransferase encoding genes except those show a metabolic cycle-dependent expression pattern [3]. Results showed that the expression levels started to escalate and became highest for both *fsmt1* and *fsmt2* in stipe, so we conclude that the expression of these genes is developmentally regulated.

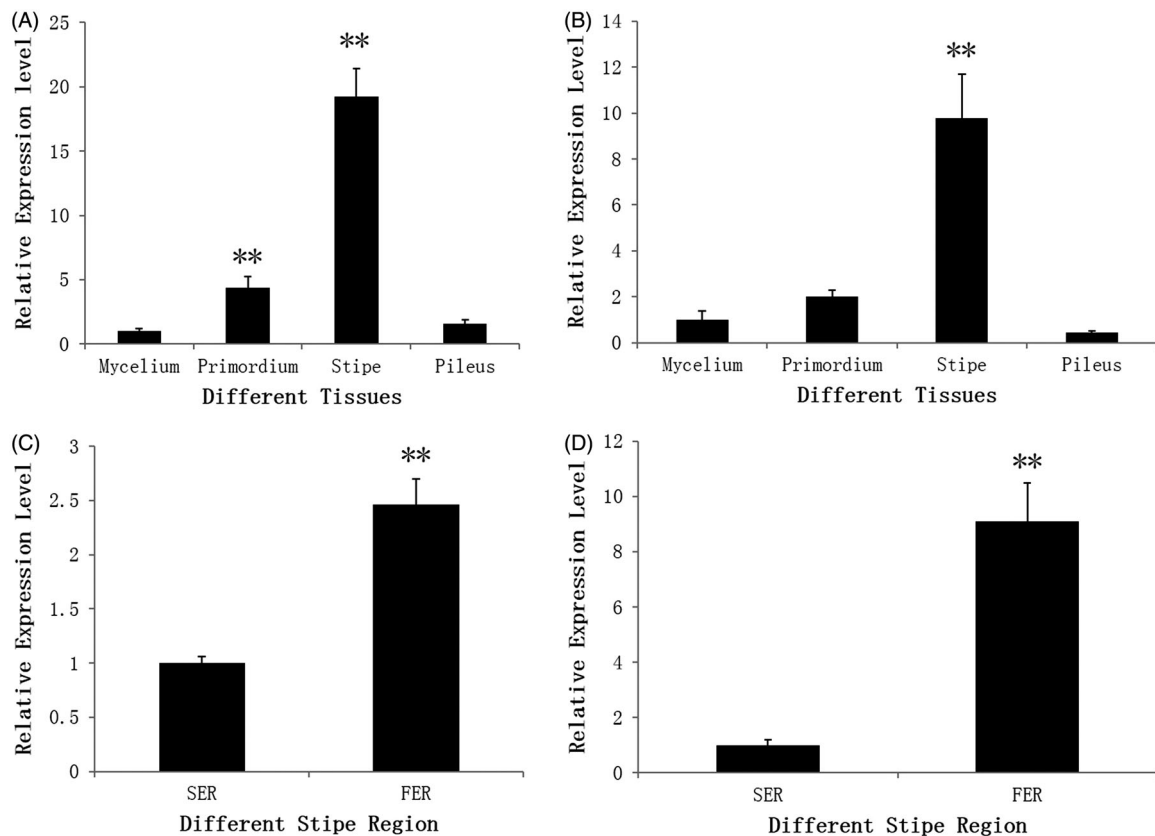


Figure 3. Expression patterns of *fvsmt1* and *fvsmt2*. (A) Relative expression levels of *fvsmt1* in different tissues; (B) Relative expression levels of *fvsmt2* in different tissues; (C) Relative expression levels of *fvsmt1* in the slow elongation region (SER) and fast elongation region (FER) of stipes; (D) Relative expression levels of *fvsmt2* in the SER and fast elongation region (FER) of stipes. Values are indicated as the mean \pm SE ($N=4$). One-way ANOVA was used to test the significant difference of the expression level in different tissues and different regions of stipe ($p < 0.01$ indicated by **).

Although the expression pattern of *fvsmt1* and *fvsmt2* is similar to each other, but they encode SAM-dependent methyltransferase with different predicted functions. FVSMT1 probably acts as arginine methyltransferase that regulates important cellular processes, such as RNA processing, signal transduction, and DNA repair [2]. FVSMT2 is predicted as caffeoyl-CoA methyltransferase, probably participating in phenylpropanoid biosynthesis.

In summary, we present the first comprehensive study of the SAM-dependent methyltransferase encoding genes (*fvsmt1* and *fvsmt2*) from *F. velutipes*. Results support that FVSMT1 and FVSMT2 are two different SAM-methyltransferase, belonging to separate classes that are involved in a variety of cellular processes and active in fungal morphogenesis and development.

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Disclosure statement

All authors have no conflict of interest to declare.

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