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Characterization and expression pattern of homeobox transcription factors in fruiting body development of straw mushroom *Volvariella volvacea*

Wei Wang [a], Li Wang [a], Bingzhi Chen [b], Irum Mukhtar [b], Baogui Xie [b, *], Zhuang Li [a, **], Li Meng [a, ***]

[a] Shandong Provincial Key Laboratory of Agricultural Microbiology, College of Plant Protection, Shandong Agricultural University, Tai'an 271018, China
[b] Mycological Research Center, Fujian Agriculture and Forestry University, Fuzhou 350002, China

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ABSTRACT

A number of homeobox transcription factors (TFs) play critical role in regulating developmental processes of fungi. However, studies on TFs in fruiting body development of mushroom forming species, *Volvariella volvacea*, are still at initial stage. Here, we report homeobox TFs in the whole-genomic sequence of *V. volvacea* and expression analysis of the homeobox TFs during a series of developmental stages. Homeobox TFs were identified using InterPro terms and Fungal Transcription Factor Database (FTFD) from the genome of *V. volvacea* and quantitative real-time PCR were used for gene expression analysis. Based on phylogenetic analysis, the homeobox TFs of *V. volvacea* were divided into two groups and showed close relationships with the TFs of other Basidiomycetes. Eight differentially expressed homeobox *TFs* were selected by digital gene expression analysis from 47 putative homeobox *TFs*, including five up-regulated genes in primordia and three down-regulated genes in fruiting elongation stage of *V. volvacea*. *VvHox1*, *VvHox2*, and *VvHox3* might be participating in fruiting body elongation. It can be assumed that *VvHox3* might be involved in volva development. Moreover, five *TFs* (*VvHox4-VvHox8*) might be contributing in primordia formation. Results indicated that differentially expressed homeobox TFs are significant candidates for fruiting body development study in *V. volvacea*.

1. Introduction

The straw mushroom (*Volvariella volvacea*) is an important edible mushroom presenting highly tasty and nutritional values with an estimated annual production of 330 000 tons in China, occupying over 80 % of global production (Bao et al., 2013; Cai et al., 1999), but erratic fruiting presents a serious challenge for its cultivation and breeding. According to previous studies, several factors containing mating type genes, transcription factors, mitogen-activated protein kinase, hydrophobin, and cytochrome oxidase can play important role in fruiting body formation of fungi (Raudaskoski and Kothe, 2010; van Wetter et al., 2000; Wu et al., 2016; Zhang et al., 2017). Two different and unlinked mating type loci, named MAT-A and MAT-B, can be found in the majority of mushroom forming fungi. The MAT-A encoding homeodomain proteins, can work as transcription factor to active downstream pathway, and MAT-B encoding pheromones and receptors. Although *V. volvacea* is always believed as homothallic fungus (Chang and Yau, 1971), Chen et al. (2016) recently indicated the possible coexistence of heterothallic and homothallic life cycles. Functional MAT-A loci encoding homeobox proteins were identified in *V. volvacea*. However, four pheromone receptors without polymorphism were not expected to participate in mating type determination (Chen et al., 2016). This also indicate the complex fruiting body formation mechanism of *V. volvacea*.

Homeobox TFs, containing homeodomain, can regulate the morphologic polymorphism in plant, animal and fungi (Acampora et al., 2000; Kim et al., 2002; Regulski et al., 1985). Previous studies have reported that homeobox genes are essential for conidiogenesis of the rice blast fungus *Magnaporthe oryzae* (Kim et al.,

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: mrcfafu@163.com (B. Xie), liz552@126.com (Z. Li), mengli0121@126.com (L. Meng).

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2009; Liu et al., 2010). Pelkmans et al. (2017) reported that, transcription factors Bri1 and Hom1 can stimulate vegetative growth in *Schizophyllum commune*, while biomass formation is repressed by Wc-2, Hom2, and Fst4 (Ohm et al., 2013). Moreover, mutant with a deletion of the homeodomain gene *hom2* cannot produce aggregates, primordia, and fruiting bodies. Mutant with deletion of the homeodomain gene *hom1* form smaller fruiting bodies but in higher numbers (Ohm et al., 2010, 2011). Additionally, homologs of the *S. commune* TFs have been identified in *Agaricus bisporus*, *Laccaria bicolor* and *Coprinus cinerea*. More studies showed that homeobox TFs could be involved in fruiting body development in several members of mushroom forming fungi (Vonk and Ohm, 2018).

The molecular regulation of fruiting body formation in mushroom is still limited. At present, availability of the genomic sequences of *V. volvacea* has provided a new platform to study molecular regulation on the whole genome wide (Chen et al., 2013). In this study, 47 homeobox TFs were identified in *V. volvacea*, and the gene expression levels of eight candidates were determined in heterokaryotic mycelia, primordia, egg and elongation developmental stages. These results provide preliminary evidence that homeobox TFs could be involved in fruiting body formation and development of *V. volvacea*.

2. Materials and methods

2.1. Strains and growth conditions

The heterokaryotic strain H1521 (obtained by a cross between homokaryotic PYd15 and PYd21 strains) used in this study was deposited in the Agricultural Culture Collection of China (ACCC52633). Fungal mycelia were grown in potato dextrose broth at 35 °C for five days and harvested by filtration. The mycelia (MY) of strain H1521 were inoculated into cotton waste and incubated at 32 °C. Samples were collected at five stages: primordia (PR; 8 d after inoculation), button stage (BU; 10 d after inoculation), egg stage (EG; 13 d after inoculation), fruiting body elongation stage (EL; 13 d after inoculation), and maturation stage (MA: 14 d after inoculation). Samples collected at different developmental stages were stored in liquid nitrogen.

2.2. Identification of homeobox TFs

The draft genomic sequence of *V. volvacea* PYd21 was available under accession no. PRJNA171553 at NCBI (Chen et al., 2013). Homeobox TFs were identified using InterPro term (IPR001356, IPR003120, and IPR009057) for homeodomains via the pipeline of the Fungal Transcription Factor Database (FTFD) (http://ftfd.snu.ac.kr/) (Park et al., 2008).

2.3. RNA extraction

Total RNAs of mycelia, primordia, egg, fruiting body elongation and maturation stages were extracted using pBIOZOL Plant Total RNA Extraction Reagent, according to the manufacturer's protocol (BioFlux, USA). Isolated RNAs from different samples were treated with the RNeasy plant mini kit to remove potential genomic DNA contamination (QIAGEN, Germany). Integrity and concentration of the isolated RNA were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Quality and concentration of the isolated RNAs were also evaluated by agarose gel electrophoresis and NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA).

2.4. Digital gene expression analysis

RNA library construction and sequencing were performed as previously described (Chen et al., 2013; Meng et al., 2013; Tao et al., 2014). The expressed sequence tags of the differentially expressed genes were obtained by mapping the tag library to genomic sequences using ZOOM Studio 1.5 software (Lin et al., 2008). Later, uniquely mapped tags were selected using the Basic Local Alignment Search Tool (BLAST) (blast-2.2.25+), while tags mapped to multiple genes were filtered out. The number of tags was calculated and normalized to the number of transcripts per million clean tags (TPM) (Subramanian et al., 2005; Morrissy et al., 2009; 't Hoen et al., 2008). Fold change of gene expression in combination with false discovery rate (FDR) control was used to distinguish differentially expressed genes between samples (FDR $\leq$ 0.001; $\log_2$-fold change $\geq$ 1). The heat map of gene expression was constructed using the software Multiple Experiment Viewer (MeV 4_8_1).

2.5. Quantitative real-time PCR (qRT-PCR)

cDNA was used for qRT-PCR analysis. Total RNAs obtained (mention in section 2.3) was used for cDNA synthesis with a RevertAid First Strand cDNA Synthesis Kit (ThermoFisher, USA) according to the manufacturer's protocol. Synthesized cDNA was stored at −70 °C. For qRT-PCR, each reaction mixture (25 μL volume) contained 0.5 μL of each primer (10 μM), 12.5 μL 2× TransStart™ Top Green qPCR SuperMix, 0.5 μL Passive Reference Dye/PCR Enhancer (50×), 1 μL cDNA (1000 ng/μL) template, and 10 μL ddH_2O. Thermal cycling conditions were as follows: 30 s at 94 °C, followed by 40 cycles of 5s at 94 °C and 30 s at 60 °C. Primers for homeobox TFs genes i.e., *Vvhox1* to *Vvhox8*, and the reference gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, GenBank accession number: DQ140384), were designed with Primer Premier 5.0 (Table 1). The $2^{-\triangle\triangle Ct}$ method was used for qRT-PCR data analysis (Livak and Schmittgen, 2001). All experiments were conducted in triplicate.

2.6. Phylogenetic tree construction of homeobox TFs

The gene sequences of homeobox TFs genes were obtained based on the coding sequences and corresponding genomic sequences. Multiple sequence alignment of homeobox TFs sequences was performed using Clustal X (Thompson et al., 1994). For phylogenetic relationship of the *V. volvacea* homeobox TFs with

Table 1
Primers used in this study.

Primer	Sequences (5′-3′)
Vvhox1-qPCR-F	GCACGGCATTCCCTTCAA
Vvhox1-qPCR-R	CTGGAGTATGGTTGGTGATGG
Vvhox2-qPCR-F	CAACTTCTTCGTCATCTTCGTCATCCT
Vvhox2-qPCR-R	GCCTATAACCTGTCTACTGCTGTTCTG
Vvhox3-qPCR-F	CCAAGAAGGTCGTCATCAAGGAT
Vvhox3-qPCR-R	GCACATCAGGAAGCGGCTAG
Vvhox4-qPCR-F	CTTGAGGTAAGCGAGGAAGGATATTGTT
Vvhox4-qPCR-R	GAGAATTGGCGGAAGGTGGAGATG
Vvhox5-qPCR-F	GTCTGGTGAGAGCGGTGTGGAT
Vvhox5-qPCR-R	GGAAGGCATAGGAGGATAAGGAGGAA
Vvhox6-qPCR-F	GCTATGGTCTCTTCTGTCACAC
Vvhox6-qPCR-R	TCGGTTGCTGGTTAGTGGTT
Vvhox7-qPCR-F	TCCGTTGCGTCCGTTCCGTTA
Vvhox7-qPCR-R	GTTCCACATTGACACAGCCACCT
Vvhox8-qPCR-F	AGAGAACTGCCGACTACTAGAGGAG
Vvhox8-qPCR-R	CCACACTTGTTGCCCGACCAT
GAPDH-qPCR-F	ATTGGCGTGGTGGTCGTAG
GAPDH-qPCR-R	ACGGAAACATCAAGGGTAGGG

other Basidiomycetes members sequences, a neighbor-joining (NJ) phylogenetic tree was constructed using MEGA 5.0 software (Saitou and Nei, 1987; Tamura et al., 2011) with 1000 bootstrap replicates.

3. Results

3.1. Homeobox TFs in V. volvacea

Altogether 214 putative *TFs* were identified among 11 534 predicted genes from draft genome of *V. volvacea* via the pipeline of the FTFD. Putative 214 TFs were classified into 30 TF families (Fig. 1; Supplementary Table 1). The largest family among all was Zn2Cys6 type with 48 genes, while almost half of the TFs families, were contained only one or two genes. A total of 47 homeobox TFs sharing conserved Homeobox (IPR001356; IPR003120) or Homeodomain-like (IPR009057) domain were selected from 214 predicted TFs.

3.2. Gene expression analysis of homeobox TFs

Six Digital Gene Expression Profiling (DGE) libraries were sequenced from independent samples of six distinct developmental stages: MY, PR, BU, EG, EL and MA of *V. volvacea*. The DGE data for all stages were submitted to the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), with accession numbers GSE43019 and GSE43297, respectively.

The clean reads from the six libraries were aligned to the draft genome of *V. volvacea* to establish gene expression profiles. Mapping results of clean reads against 11 534 predicted genes in draft genome of *V. volvacea*, showed that at least 8927 genes (77.4 % of the genome) were expressed in each developmental stage, while 5079 genes (44.0 % of the genome, 56.9 % of expressed genes) were expressed as common in all six stages (Table 2; Supplementary Table 2).

In the genome of *V. volvacea*, there were 47 homeobox TFs encoding genes. Transcription of 21 of these genes were detected in different stages using DGE (Fig. 2; Supplementary Table 2). These 21 homeobox TFs were classified into two groups based on expression levels calculated by K-mean analysis. There were 17 genes in group 1, and 4 genes in group 2 (Fig. 2).

Table 2
Major characteristics of DGE libraries.

Stages	Clean Tags	Expressed Genes	Expressed Genes/Genomic Genes
MY	5 701 781	7490	64.9 %
PR	5 659 262	7584	65.8 %
BU	5 612 361	6972	60.4 %
EG	5 978 522	7355	63.8 %
EL	5 972 497	6558	56.9 %
MA	5 967 968	6834	59.3 %

Note: MY: mycelia; PR: Primordia; BU: Button stage; EG: Egg stage; EL: Elongation stage; MA: Maturation stage.

Based on DGE analysis, eight differentially expressed (FDR $\leq$ 0.001; $\log_2$-fold change $\geq$ 1) homeobox TFs were selected. Sequences of these identified homeobox TFs (*Vvhox1-Vvhox8*) genes were submitted to GenBank under the accession numbers: JX843776, KY441407, KY441406, KY441409, KY441405, KY441404, KY441410 and KY441408. The expression levels of five genes i.e., *Vvhox4*, *Vvhox5*, *Vvhox6*, *Vvhox7* and *Vvhox8,* in group 1 (identified as *GME10392, GME1763*, *GME860, GME11623,* and *GME8409* in the database, respectively; Fig. 2), were significantly up-regulated from mycelia to primordia stage. Whereas the expression of other three genes i.e, *Vvhox1*, *Vvhox2* and *Vvhox3* (identified as *GME918*, *GME8013*, and *GME2319* in the database, respectively; Fig. 2), were significantly down-regulated in egg stage followed by elongation stage.

These results indicated that homeobox TFs genes may play stage-specific regulatory roles during the development of *V. volvacea.* The stage-specific expression patterns might facilitate the function of homeobox TFs in the transcription of genes regulating morphological variation in *V. volvacea* during fruiting body formation and development. Additionally, we used quantitative real-time PCR (qRT-PCR) to analyze these genes' expression in mycelia, primordia, egg and elongation stages (Figs. 3 and 4).

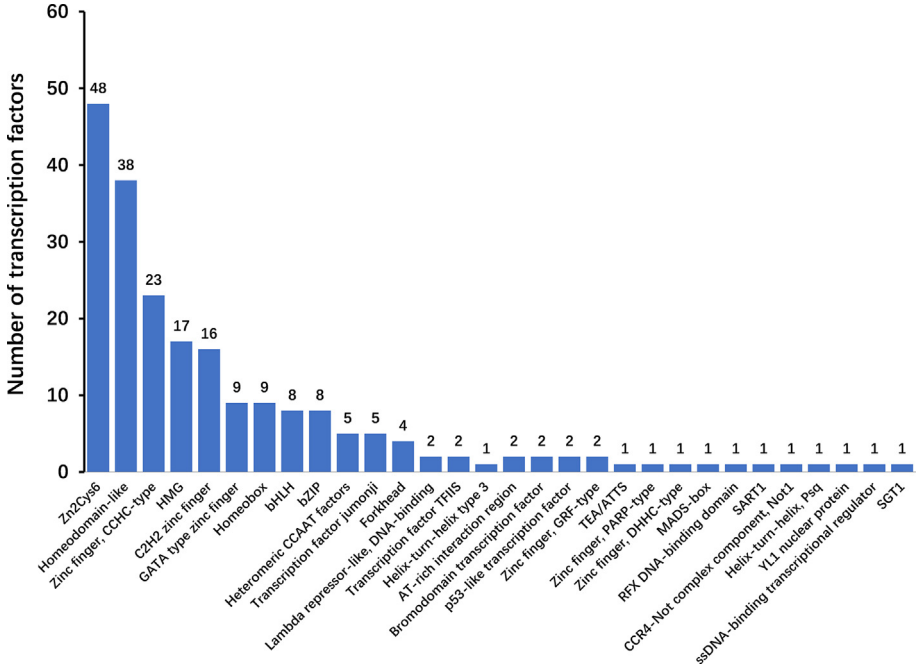

Fig. 1. Transcription factor families in *Volvariella volvacea*. 214 transcription factors were distributed in 30 families. The number of genes in each family were marked on the top of column.

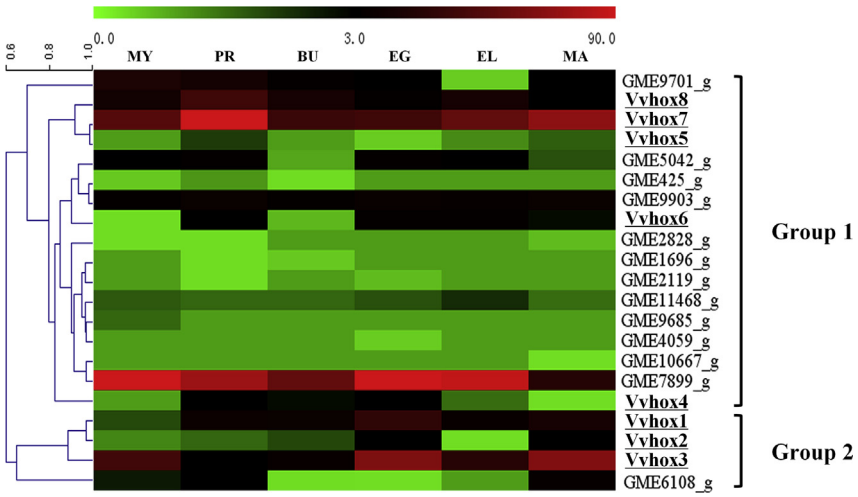

Fig. 2. Expression map of 21 homeobox transcription factors genes in different developmental stages in *Volvariella volvacea*. Units for the color scale bar were TPM values of analyzed genes. The variation in color shows the expression level based on TPM values of these genes. Genes were grouped together with similar expression patterns. The Pearson correlation coefficient was showed on top of the tree on the left. The raw expression profile information is put in Supplementary Table 2. Eight differentially expressed (FDR $\leq$ 0.001; $\log_2$-fold change $\geq$ 1) homeobox transcription factors (Vvhox1-Vvhox8) are marked in bold with underline. MY: mycelia; PR: Primordia; BU: Button stage; EG: Egg stage; EL: Elongation stage; MA: Maturation stage. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The results of qRT-PCR were consistent with the gene expression profile analyses.

We have also measured gene expression level of *Vvhox1, Vvhox2* and *Vvhox3* in different tissues (cap, stipe and volva) at egg and elongation stages using qRT-PCR (Fig. 5). The highest expression levels of *Vvhox1* and *Vvhox2* appeared in the stipe tissues, collected at elongation stage. While, expression of *Vvhox2* was up-regulated in the stipe, and lower in the cap and volva tissues at the elongation stage. These results suggest that *Vvhox1* and *Vvhox2* homeobox TFs may regulate stipe elongation. At least, they could involve in this process.

3.3. Phylogenetic analysis of homeobox TFs

The phylogenetic tree was constructed including 57 homeobox TFs from nine other members of Basidiomycetes along with

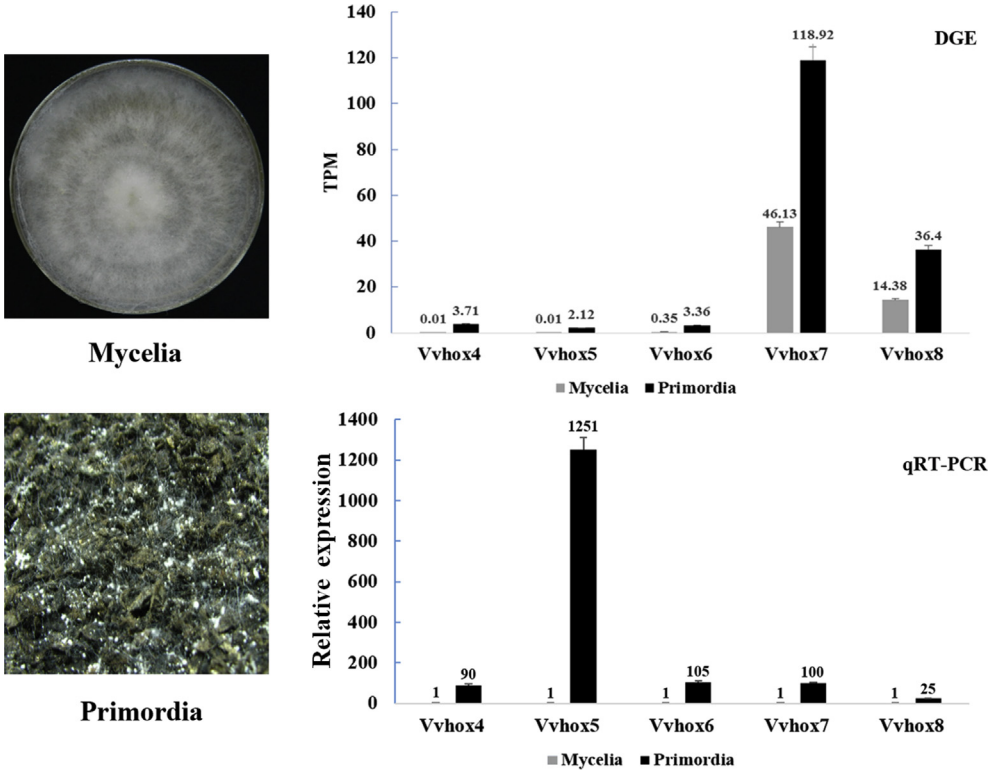

Fig. 3. Gene expression levels of homeobox transcription factors *Vvhox4-Vvhox8* in mycelia and primordia. Sample stages are shown on the left. DGE and qRT-PCR results are shown on the right. Values are marked on the top of column.

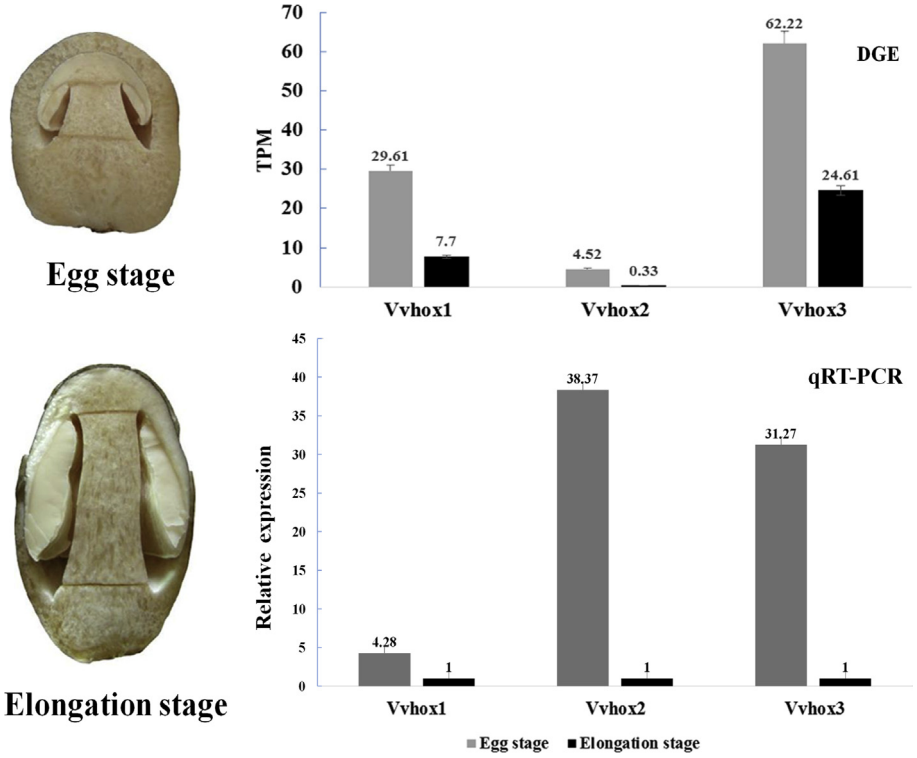

Fig. 4. Gene expression levels of homeobox transcription factors *Vvhox1-Vvhox3* in egg and elongation stages. Sample stages are shown on the left. DGE and qRT-PCR results are shown on the right. Value are marked on the top of column.

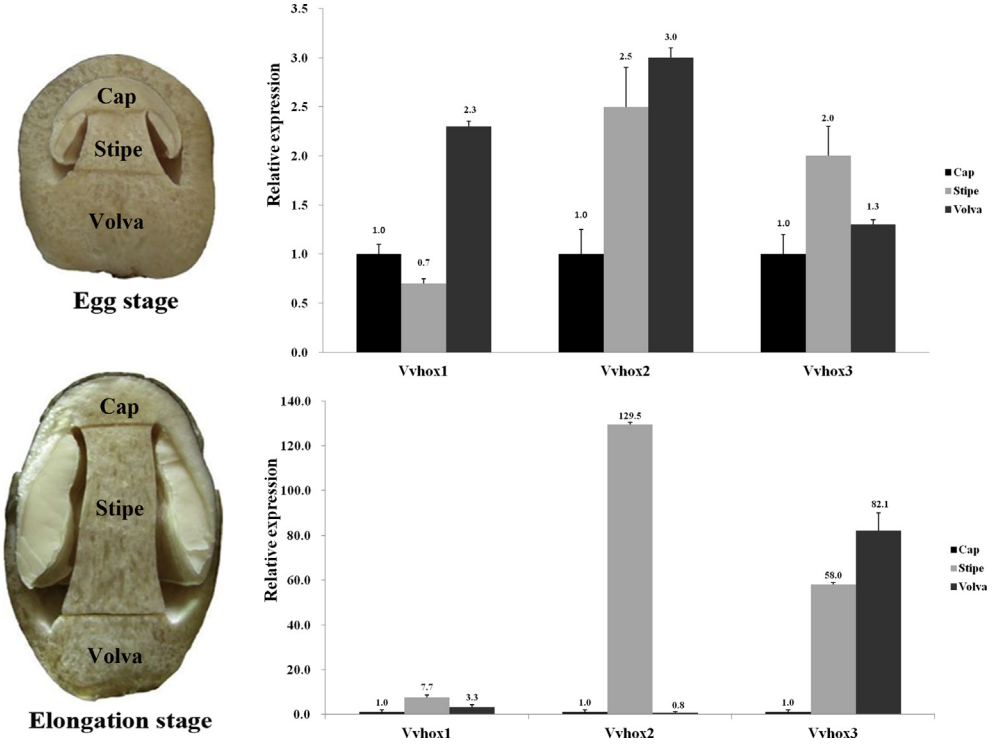

Fig. 5. The gene expression levels of homeobox transcription factors *Vvhox1-Vvhox3* in the various tissues of *Volvariella volvacea*. Tissues in two stages are shown on the left. The corresponding qRT-PCR results were showed on the right. Value was marked on the top of column.

V. volvacea. NJ tree showed that the homeobox TFs were classified into six separate groups, while eight TFs from *V. volvacea* were clustered into five groups (Fig. 6). In group 1, Vvhox6 was positioned in separate sub-group that contains non-mating type proteins, while other subgroup represents HD2 proteins belonging to MAT-A. Furthermore, Vvhox6 was shown up into a single-

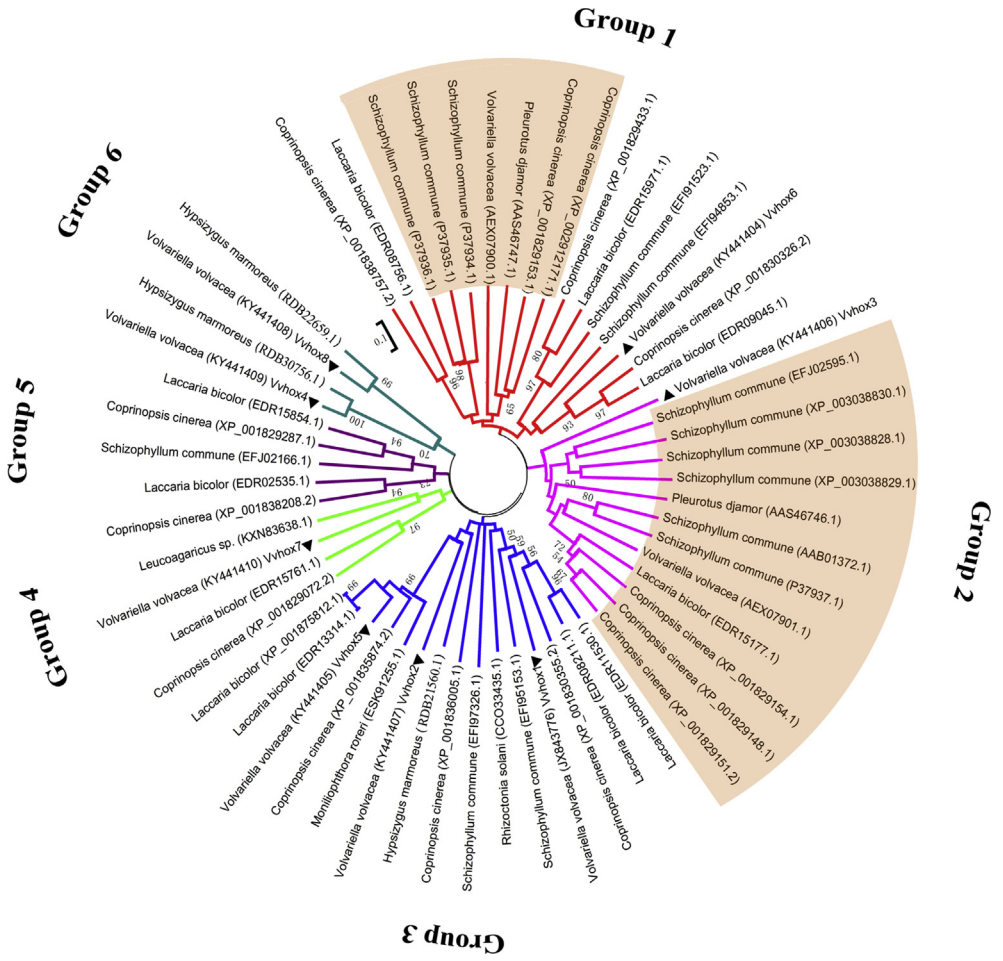

Fig. 6. Phylogenetic tree of 57 homeobox transcription factors from nine fungi of Basidiomycota. Homeobox transcription factors of *Volvariella volvacea* are marked with black triangles. GenBank accession number of each homeobox transcription factor is in brackets. HD transcription factor genes involved in mating are found in group 1 (HD2 genes; orange shadow) and group 2 (HD1 genes; orange shadow). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

branch, also indicating its independent origin. Interestingly, Vvhox3 was classified with HD1 proteins of MAT-A in group 2 (Fig. 6), however, Vvhox6 and Vvhox3 were not identified as mating type genes in previous studies (Chen et al., 2016). In group 4, Vvhox7 might be homologous of transcriptional activator Myb from *Leucoagaricus* sp. (Genbank accession number: KXN83638.1) (Fig. 6). As shown in group 6, Vvhox4 and Vvhox8 were positioned with ISWI chromatin-remodeling complex ATPase ISW2 (Genbank accession number: RDB30756.1) and SWR1-complex protein 4 (Genbank accession number: RDB22659.1) from *Hypsizygus marmoreus* respectively.

4. Discussion

V. volvacea is an edible mushroom with high economic and nutritional values. This mushroom has a long cultivation history in China and other Asian countries, where it is cultivated on commercial scales. *V. volvacea* has a complex life cycle and It can occur independently of its MAT-A-controlled (homeobox genes) bipolar mating system, enabling homothallic and heterothallic life cycles (Chen et al., 2016).

The basidiocarps of *V. volvacea* are roughly classified into six different developmental stages, such as pinhead, tiny button, button, egg, elongation, and mature stages (Chang, 1964). During the entire process, the primordia (tiny button) and elongation stages have the greatest research significance, as they represent two important developmental milestones. Primordia stage is between the mycelia and the fruiting body, during which vegetative growth transforms to reproductive growth. During the elongation stage, the fruiting body can exhibit significant morphological changes. In our previous study, it was suggested that cell elongation occurred predominantly in the elongation and mature stages (Luo and Xie, 2004). However, still limited information available about the genes involved in the growth and development of *V. volvacea*.

In the kingdom Fungi, homeobox transcription factors (TFs) play crucial role during multicellular development and in numerous signaling pathways. The homeobox TFs were the first identified in mating type loci, which regulate sexual development, while other homeobox genes were shown to be involved in fruiting body development in several members of Ascomycota and Basidiomycota (Vonk and Ohm, 2018). In the whole genomic sequence of *V. volvacea*, 47 homeobox TFs, including homeobox-like genes which have similar domain (Holland et al., 2007; Hedgethorne et al., 2017), were identified among 214 predicted homeobox TFs (Fig. 1), while 21 homeobox TFs were classified into two groups based on their expression models (Fig. 2).

To date, many homeobox TFs genes have been identified in different fungal species. *hom1*, the homologous gene of *VvHox1* in *S. commune*, is involved in fruiting body development. In previous study, it was found that dikaryon (heterokaryon) stage in

S. commune can develop more, although smaller reproductive structures than those of the wild type if *hom1* is deleted (Ohm et al., 2010). We inferred that *VvHox1* of *V. volvacea* may work similar as *hom1* of *S. commune* because this gene is highly conserved in Agaricomycetes. The egg stage is the initiation point for tissue differentiation in fruiting body of *V. volvacea*. Both DGE and qRT-PCR also revealed higher expression level of *VvHox1* in egg than elongation stage (Fig. 4).

Gene expression patterns can provide important clues for gene function. The homeobox TFs genes exhibit great difference in abundance among different organisms to exert different physiological functions. In present investigation, eight selected homeobox TFs genes showed different expression patterns. These may regulate specific development progress through the regulation of different target genes. We have found that the expression level of *VvHox1*, *VvHox2*, and *VvHox3* were down-regulated from egg to elongation stage, suggests that these genes may be negative regulators in elongation stage (Fig. 4). However, in the elongation stage, *VvHox1*, *VvHox2*, and *VvHox3* showed lower expression levels in cap, while higher expression levels in stipe tissue. These three genes may involve in stipe development at elongation stage. In addition, *VvHox3* may also be involved in volva development (Fig. 5). Primordia formation is the initial step of fruiting body. So, the regulators in this process must be worth to be studied. Homeobox TFs (*Vvhox4-Vvhox8*) were identified and detected significant up-regulated expression level from mycelia to primordia stage (Fig. 3). These genes might be involved in the primordia formation.

Phylogenetic analysis of 57 homeobox TFs from nine fungi of Basidiomycota including *V. volvacea*, showed that, HD transcription factor genes involved in mating were found in group 1 (HD2 genes; orange shadow) and group 2 (HD1 genes; orange shadow) (Fig. 6). These genes likely split during early fungal evolution in accord with Vonk and Ohm's results that obtained from 2113 homeodomain gens in 222 fungal genomes (Vonk and Ohm, 2018). Interestingly, *Vvhox3* was not identified as a mating type gene even it was clustered with HD1 genes together in group 2 (Fig. 6) (Chen et al., 2016).

In this study, the homeobox TFs genes that clustered in the same group did not show similar expression patterns. One possible reason is that close homeobox TFs in the phylogenetic tree are similar in TF domains, but may not be in full-length amino acids or rest domains which may have other functions. The phylogenetic and expression analyses of homeobox TFs genes provided an insight into the comprehensive functional characterization of the TFs gene family for *V. volvacea*. The functional exploration of homeobox TFs genes will also helpful the better understanding of gene regulation in mushrooms by homeobox TFs and marker-assisted breeding programs in the future. In the continuity of investigation on *TFs*, we would like to conduct a study on precise functions of home *TFs* in fruiting body development process by gene knock-out, RNAi and gene overexpression system in fungi.

5. Conclusion

Homeobox TFs are important regulators in developmental process of fungi. In the present study, we investigated the digital gene expression profiling of homeobox TFs genes and speculated their role(s) at different fruiting development stages in *V. volvacea*. Analyses of differential expression profiles between different fruiting body stages in *V. volvacea* allowed us to identify TF genes that showed significant changes in their expression levels at different stages. We identified 47 putative homeobox TF genes and of these, eight homeobox TF genes were further analyzed as those that were most likely to be related to fruiting body development in *V. volvacea*. We speculate about the role of homeobox TF genes in fruiting body development on the expression patterns of these genes at different development stages. Homeobox *TFs* can be suitable candidates for future investigations on gene functional research in mushrooms.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2018.10.008.

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