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Identification and expression analysis of *Pofst3* suggests a role during *Pleurotus ostreatus* primordia formation

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ABSTRACT

Only a few transcription factors involved in mushroom development have been reported till now, Therefore, identification of transcription factors in common edible mushroom has commercial and scientific importance. In this study, the *Pofst3* gene from *Pleurotus ostreatus* was cloned and characterized. Bioinformatics analysis showed that Pofst3 protein had 71% sequence similarity with fst3 of model mushroom *Schizophyllum commune*. Furthermore, the function of *Pofst3* gene was analyzed by overexpression and antisense silencing in *P. ostreatus* via *Agrobacterium*-mediated transformation. Expression verification of *Pofst3* in transformants through qRT-PCR showed that compared with the wild type strains, the transcription level was about 1.26 ~ 9.59 and 0.01 ~ 0.30 fold in *Pofst3* overexpressing and silencing strains, respectively. Petri dish and bag cultivation tests of transgenic strains showed that the number of primordia and the type of fruiting bodies of *Pofst3* overexpressing strains were consistent with the wild type strains, i.e. fewer primordia and larger fruiting bodies; the number of primordia formed by *Pofst3* silencing strains were more than those of wild type strains, but fruiting bodies were smaller. It was very likely that *Pofst3* was involved in the regulation of *P. ostreatus* development through inhibiting the formation of clusters of primordia.

1. Introduction

The importance of mushroom-forming fungi in agriculture, human health and ecology underscores their biotechnological potential for a wide range of applications. Although these are primarily of economic value because of their use as food (Kothe, 2001; Kües and Liu, 2000), mushrooms also produce antitumor and immunostimulatory molecules (Kothe, 2001; Kües and Liu, 2000) as well as enzymes used for bioconversions (Lomascolo et al., 1999). Moreover, they have been identified as promising cell factories for the production of pharmaceutical proteins (Berends et al., 2009).

Despite their economic importance, relatively little is known about how their fruiting bodies are formed. Because the basidiomycetes *C. cinerea* and *Schizophyllum commune* can be genetically modified, or cultured under laboratory conditions, studies on *C. cinerea* (Kües, 2000; Kamada, 2002) and *S. commune* (Ohm et al., 2010, 2011) have provided important insights into the development of mushrooms.

Mushroom formation starts with the aggregation of aerial dikaryotic hyphae. These aggregates develop into primordia, which further form mature fruiting bodies (Ohm et al., 2010, 2011). Functional analysis has shown that many transcription factors are differentially expressed during sexual development of *S. commune*. Yet, our understanding of developmental regulation of commercial mushrooms, such as *Pleurotus ostreatus*, at a molecular level is still limited. Efficient production of edible mushrooms relies on the precise control of fruiting body development, and hence identification of the molecular mechanism of fruiting body development has commercial and scientific importance.

The edible oyster mushroom, *P. ostreatus*, is one of the most widely cultivated mushrooms (Kües and Liu, 2000). The relationship between mycelial growth, mating type (Larraya et al., 2001) and their corresponding quantitative trait locus (QTL) (Larraya et al., 2002) has been studied. A developmentally expressed metalloprotease was isolated and its function was found to be related to fruiting body formation (Joh et al., 2004). The cDNA libraries of two developmental stages of *P. ostreatus* mycelia and fruiting bodies were constructed and 1256 unigenes were isolated (Lee et al.,

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2002). To extend the study of lee, Joh reported the isolation of genes expressed during eight distinct developmental stages of *Pleurotus osreatus*. In total, 11761 EST sequences (PoESTs) were generated, from which 4060 unigenes were obtained by redundancy analysis, representing 30–40 % of the entire genome of *P. ostreatus* (Joh et al., 2007).

Establishing the EST database of *P. ostreatus* and identification of developmentally expressed genes would be able to provide valuable genetic and biochemical information for the understanding developmental processes. However, up till now, there are few reports on the key regulatory genes involved in the development of *P. ostreatus* and other edible mushrooms. In this paper, the full length *Pofst3* cDNA was obtained through the RACE method in *P. ostreatus*. The *Pofst3* function was examined during development of *P. ostreatus* through overexpression and antisense silencing methods.

2. Materials and methods

2.1. Strains and plasmids

The *P. ostreatus* dikaryotic strain New 831 was provided by College of Life Science, Henan Agricultural University. The vegetative mycelium was maintained at 25 °C on potato dextrose agar (PDA; Difco, USA) covered with autoclaved cellophane membrane in a Petri dish. Vegetative mycelia and fruiting bodies of *P. ostreatus* New 831 were produced as previously described (Qi et al., 2017). For the selection and maintenance of transformants, CYM medium was supplemented with hygromycin (Roche, Mannheim, Germany) at 50 μg/mL. *Agaricus tumefaciens* EHA105 (Bomaide, Beijing, China), grown in YEB medium (Sigma–Aldrich, Shanghai, China) containing 100 μg/mL kanamycin and 50 μg/mL rifampicin (Sigma–Aldrich, Shanghai, China), was used to transform *P. ostreatus*. *Escherichia coli* DH5α (Takara, Dalian, China) was used as a host for plasmid construction and grown in Luria–Bertani broth (LB, Difco, Franklin Lakes, USA) or on LB plates which were supplemented with ampicillin (0.1 mg/mL), X-gal (40 μg/mL), and IPTG (10 μg/mL) when required. The plasmid pMD®19-T (Takara) was used as cloning vector. The vector pPo-GPD was constructed by modifying pCAMBIA 1300 which was purchased from the YRGene Company (Changsha, China), the original binary vector.

The medium used for *Agrobacterium*-mediated transformation was CYM, composed of: peptone 2.0 g, yeast extract 2.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KH_2PO_4 0.46 g, K_2HPO_4 1 g, glucose 20 g, and distilled water added to 1 L; MM: K-buffer 10 mL, M–N buffer 20 mL, 20 % Glucose (W/V) 10 mL, 0.01 % $FeSO_4$ (W/V) 10 mL, 20 % $(NH_4)_2SO_4$ (W/V) 2.5 mL, 1 % $CaCl_2 \cdot 2H_2O$ (W/V) 1 mL, and distilled water added to 1 L; IM: K-buffer 10 mL, M–N buffer 20 mL, 20 % Glucose (W/V) 5 mL, 0.01 % $FeSO_4$ (W/V) 10 mL, 20 % $(NH_4)_2SO_4$ (W/V) 2.5 mL, 1 % $CaCl_2 \cdot 2H_2O$ (W/V) 1 mL, 50 % Glycerol (W/V) 10 mL, 40 mM MES 8.528 g, and distilled water added to 1 L; CM: K-buffer 10 mL, M–N buffer 20 mL, 20 % Glucose (W/V) 2.5 mL, 0.01 % $FeSO_4$ (W/V) 10 mL, 20 % $(NH_4)_2SO_4$ (W/V) 2.5 mL, 1 % $CaCl_2 \cdot 2H_2O$(W/V) 1 mL, 50 % Glycerol (W/V) 10 mL, 40 mM MES 8.528 g , agar 20 g , and distilled water added to 1 L.

The SA medium used for transgenic *P. ostreatus* plate test referenced as described by Lee et al. (2011).

2.2. RNA isolation and full-length cDNA cloning

Total RNA was extracted using the RNAiso™ Plus (Takara) according to the manufacturer's protocol. The total RNA was reverse transcribed using the SMART™ RACE cDNA Amplification Kit (Clontech, USA). The *fst3* gene of *S. commune* (NCBI Protein ID: XP_003031320.1) was compared with *P. ostreatus* genome in the JGI (http://genome.jgi-psf.Org) and the primers, *fst3*-F and *fst3*-R (Table 1), were designed depending on the homologous sequence. The PCR product was cloned into the pMD19-T vector and transformed into *E. coli* DH5α for sequencing (BGI, Beijing, China).

Based on the obtained middle sequence, the specific primers (5′GSP-1, 5′GSP-2, 3′GSP-1, 3′GSP-2) for 5′-RACE and 3′-RACE were designed (Table 1). The 5′-RACE and 3′-RACE was carried out in accordance with the instructions of the SMART™ RACE cDNA Amplification Kit. Subsequently, products were purified with AxyPrep™ DNA gel extraction kit (Axygene, Hangzhou, China) and sequenced. The full-length *Pofst3* cDNA was amplified with the first strand cDNA as template and *fst3*-tF *and fst3*-tR (Table 1) as specific primers, and subsequently was cloned into pMD19-T, named pMD19-*Pofst3*.

2.3. Bioinformatics analysis

Sequence similarity was analyzed using BLAST from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The open reading frame (ORF) was found using NEW GENSCAN (http://genes.mit.edu/GENSCAN.html). Protein motifs were identified using MOTIF Search (http://www.genome.jp/tools/motif/) and the Conserved Domain Database from NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Secondary structure was predicted using ProtPamm (http://www.expasy.ch/tools/protpamm.htmL). Multiple sequence alignment was generated using ClustalX, and phylogenetic analysis was conducted using MEGA 6.1 (http://www.megasoftware.net/). The protein function was predicted through Interpro (http://www.ebi.ac.uk/interpro/scan.html).

2.4. Pofst3 *gene expression analysis*

Total RNA from different growth stages, mycelia, primordia and young fruiting bodies, was extracted, and the first strand cDNA was synthesized by PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China). To determine the expression level of the *Pofst3* gene in different samples, quantitative real-time PCR (qRT-PCR) was performed according to the protocol of SYBR Green Master Mix (Takara, Dalian, China) on the Roche Light Cycler 480 Real-Time PCR System (Roche, Germany) with *actin* as native control. The qRT-PCR conditions were: 94 °C for 30 s followed by 40 cycles at 94 °C for 30 s and 60 °C for 35 s. Each qRT-PCR reaction was carried out at independent triplicate trials. The expression ratios were calculated according to the $2^{-\Delta\Delta Ct}$ method (Qi et al., 2017), and data were presented as mean values of three replicates with the corresponding standard deviations. Statistical analysis was performed using one-tailed Student's t test. Values were considered significant when the P value was ≤ 0.05.

2.5. Vector modification

The original pCAMBIA1300 was a binary vector with hygromycin and kanamycin resistance under the control of the Cauliflower mosaic virus (CaMV) 35 S promoter. To replace the 35 S promoter with *P. ostreatus* glyceraldehyde-3-phosphate dehydrogenase gene (GPD) promoter, a pair of primers, *gpd*-up and *gpd*-down (Table 1), were designed based on the published *gpd* promoter sequence (Genebank accession No. GU062704). Using total DNA isolated with the DNeasy Plant Mini Kit (Qiangen China, Shanghai) as template, the GPD promoter was amplified. Then the PCR product was cloned into pMD19-T, and recombinant vector named T-gpd. Because the enzyme restriction sites were *Xho* I at both ends of the *hph* fragment, the *hph* fragment was re-cloned and the *Spe* I enzyme restriction site was introduced upstream (Table 1). Using T-gpd as template, one fragment of GPD promoter with *Bst* XI and *Spe* I restriction sites, and the other with *Sac* I and *Kpn* I restriction sites

Table 1
Primers for PCR amplification in this study.

Primers	Sequences (5′ → 3′)	Descriptions
fst3-F	GGAAGTCCGTCGAACTCTGC	For *Pofst3* homologous conserved sequence amplification.
fst3-R	GGGTTCTTCAGAGCTTGCATC	
3′*GSP*-1	CTCCGCAGGGTCAGGTCCTATCCA	For RACE.
3′*GSP*-2	GATGTGTGCTACGGAGATTGGTGTC	
5′*GSP*-1	ACAAGGAAGGGCAGGAGGAGGCATC	
5′*GSP*-2	CCTTGTTCAACCCAGTAGGCGAGTC	
fst3-tF	GAAACCGAGAACAGCTCCCAAT	For full-length *Pofst3* cDNA isolation.
fst3-tR	TCTAGTAAAGCGAGACAGGGATG	
gpd-up	CGTTCGTGACTCGCAATA	For *gpd* promoter amplification.
gpd-down	AGTCACAAGGATGGGTGGTTG	
gpd-Bst XI-up	CCACCATGTTGGAGTCACAAGGATGGGTGGTTG	For *gpd* promoter restriction sites design (The restriction sites were underlined).
gpd-Spe I-down	CTAGACTAGTCGTTCGTGACTCGCAATATCA	
gpd-Sac I-up	CGAGCTCCGTTCGTGACTCGCAATA	
gpd-Kpn I-down	CGGGGTACCAGTCACAAGGATGGGTGGTTG	
hph- Spe I-up	CTAGACTAGTCTTTCGCAGATCCCG	For *hph* restriction sites design (The restriction sites were underlined).
hph-Xho I-down	CGGCTCGAGCTTGTCGCTCGACAGATCC	
fst3-Xba I -up	GCTCTAGAATGAAATGTGATTTCCCGAAAGGT	Primers with restriction sites for the expression vector construction (The restriction sites were underlined).
fst3-Kpn I -down	GGGGTACCCTAGTAAAGCGAGACAGGGATGGCAG	
fst3-Kpn I -up	GGGGTACCATGAAATGTGATTTCCCGAAAGGT	
fst3-Xba I -down	GCTCTAGACTAGTAAAGCGAGACAGGGATGGCAG	
hph-up	TACTTCTACACAGCCATC	Primers for *hph* integration analysis.
hph-down	ATGTAGGAGGGCGTGGAT	
Pgpd-up	GAATCGTTATCTCGGTGGTGTCTG	Primers for *Pgpd-hph* integration confirmation.
Phph-down	CGCATATGAAATCACGCCATGTAGTG	
P_{gpd}-*fst3*$^{+}$-down	GGACGGGAAGTAGAATCATCAGC	Primer for *Pgpd- fst3*$^{+}$ integration confirmation.
P_{gpd}-*fst3*$^{-}$-down	GCGATTGACGACAGGCATACTC	Primer for *Pgpd- fst3*$^{-}$ integration confirmation.
β-actin-up	ATCCACGAGACAACATACAAC	Primers for qRT-PCR analysis the *Pofst3* expression in transformants.
β-actin-down	GATAGAACCACCAATCCAGA	
RT *fst3*-up	GTTGCTGTTGTTGGTATCCGAAGG	Primers for qRT-PCR analysis the *Pofst3* expression in transformants.
RT *fst3*-down	GCATAGTGGAAGGCAGGAAACC	

were amplified. The *hph* fragment with *Spe* I and *Xho* I restriction sites was amplified with pCAMBIA1300 vector as template.

The vector pCAMBIA1300 was digested with *Bst* XI and *Xho* I, the *gpd* promoter was digested with *Bst* XI and *Spe* I, and the *hph* fragment was digested with *Xho* I and *Spe* I. The digested pCAMBIA1300, GPD promoter and the *hph* fragment were recombined through the T4-DNA ligase. The preliminary modification recombinant vector was introduced into competent *E. coli* DH5α and screened. The preliminary modification pCAMBIA1300 and the other GPD promoter were digested with *Sac* I and *Kpn* I, and the digestion product was recombined. As above, the recombinant vector was introduced into competent *E. coli* DH5α and screened, the positive clones were further determined by colony PCR and sequencing. The modification pCAMBIA1300 vector with two GPD promoters (one control the *hph* gene, the other drive the pre-cloned target gene), pPo-GPD was constructed (Supplementary Fig. 2).

2.6. Expression vector construction

To clone the *Pofst3* gene into the plasmid pPo-GPD, *Xba* I and *Kpn* I restriction sites (Table 1) were designed in the gene specific primers for the *Pofst3* amplification. The template for amplification was PMD19-*Pofst3*. The fragment for expression vector construction is a 2388 bp ORF of the 2549 bp full length cDNA. Using *fst3-Xba* I-up/*fst3-Kpn* I-down primers and *fst3-Kpn* I-up/*fst3-Xba* I-down primers, the *Pofst3* fragments for the overexpression and antisense vectors construction were obtained, respectively. After digestion with *Xba* I and *Kpn* I, the fragments were forward and backward cloned into the plasmid pPo-GPD to generate the *Pofst3* gene expression cassette driven by the homologous *P. ostreatus gpd* promoter, and the recombinant overexpression and antisense vectors were named pPo-GPD-*Pofst3*$^{+}$and pPo-GPD-*Pofst3*$^{-}$, respectively (Supplementary Fig. 3). These vectors were finally introduced into *A. tumefaciens* EHA105 by electroporation (Bio-Rad, Hercules, USA).

2.7. Agrobacterium-mediated transformation

As transformation explants, *P. ostreatus* New 831 protoplasts were prepared as Qiu previously described (Qiu et al., 2010). The *A. tumefaciens* EHA105 harboring the pPo-GPD-*fst3*$^{+}$ or pPo-GPD-*fst3*$^{-}$ plasmid was cultivated at 28 °C in 1 mL LB for 1 d with the selective antibiotics (100 μg/mL kanamycin and 50 μg/mL rifampicin), and then transferred the cultures to the 100 mL MM medium containing 100 μg/mL kanamycin, at 28 °C for 2 d to an OD_{600} of 0.6–0.8. The collected bacterial cells were suspended in induction medium (IM, 200 μM acetosyringone plus, without antibiotics) to an OD600 of 0.2 and incubated for an additional 6 h (150 rpm, 28 °C) to pre-induce virulence of *A. tumefaciens*.

Prepared protoplasts and pre-induced *A. tumefaciens* were mixed as the ratio 1000:1 and plated onto cellophane disks on solid CM (containing 200 μmol/L acetosyringone) for co-cultivation. After 3 d incubation at 25 °C, the cellophane membranes with protoplast and bacterial colonies were transferred onto selection and regeneration medium (CYM with 0.6 M mannitol) containing 100 μg/mL hygromycin and 300 μg/mL cefotaxime. The plates were incubated at 25 °C until stellate aristae colonies grew out to select putative transformants. The untransformed bacteria control and

wild type protoplast for selection were used in all experiments. The transformants were selected three times under the hygromycin and cefotaxime pressure. Putative *Pofst3* overexpressing strains named *Pofst3*$^{+}$, antisense silencing named *Pofst3*$^{-}$.

2.8. Molecular analysis of transformants

After several rounds of hygromycin resistance screening, the putative transformants were transferred to CYM medium containing Petri dishes without hygromycin and cultured at 25 ± 2 °C for 7 d. After the mycelia were fully grow over the Petri dishes, mycelia were collected with a spatula, transferred to a Petri dish, and washed with sterile distilled water. The mycelia were then dried on autoclaved filter paper. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen China, Shanghai) according to the manufacturer's instructions. For the molecular evaluation of the transformants, PCR analysis for the integration of *hph* and *Pofst3* genes was conducted using primers *hph*-up/*hph*-down (Theoretical amplification length is 853 bp), *Pgpd*-up/*Phph*-down (891 bp), *Pgpd*-up/*Pgpd-fst3*$^{+}$-down (815 bp), and *Pgpd*-up/*Pgpd-fst3*$^{-}$-down (978 bp) (Table 1), respectively. The PCR condition followed was: 94 °C for 5 min followed by 38 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

2.9. QRT-PCR analysis the expression of Pofst3 in transformants

Total mycelium RNA from wild and transgenic *P. ostreatus* was isolated and reverse transcribed as above. qRT-PCR analysis for the expression of *Pofst3* gene was conducted using primers *β-actin*-up/down and RT *fst3*-up/down (Table 1), respectively. The *actin* gene was used as native control. The qRT-PCR conditions were: 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s and 60 °C for 31 s. Each qRT-PCR reaction was carried out at independent triplicate trials. The expression ratios were calculated according to the $2^{-\triangle\triangle Ct}$ method (Qi et al., 2017), and data were presented as mean values of three replicates with the corresponding standard deviations. Statistical analysis was performed using one-tailed Student's t test. Values were considered significant when the *P* value was $\leq$0.05.

2.10. Transgenic P. ostreatus petri dish cultivation

Cottonseed hull and water were used in a proportion of 1:1.2 to prepare culture material. Then, 33 g of wet material was evenly distributed to dishes with a diameter of 9 cm. 115 °C, high pressure sterilization was performed for 2 h. For SA medium, each dish contained 20 ml. From the obtained strains, three *Pofst3*$^{+}$ and three *Pofst3*$^{-}$ strains with significant increase and decrease in *Pofst3* expression were selected and activated through CYM solid culture medium on Petri dishes, respectively. After the strains were activated two times later, mycelium pellets with a diameter of 1 cm were taken with a perforator and transferred to the center of SA and cottonseed shell culture medium, while the wild type strain was used as control. Cultures were incubated under darkness and 25 °C, until the plates were fully colonized by the mycelia. After 5 d, dishes with cottonseed hull cultures were treated with low temperature and light to stimulate fruiting. The wild type strain and three *Pofst3*$^{+}$ and three *Pofst3*$^{-}$ strains were used for this test, each strain was cultured on 10 Petri dishes. Phenotyping was performed by visual comparison, without statistical tests.

2.11. Transgenic P. ostreatus bag cultivation

Cottonseed hull and water were used in a proportion of 1:1.2 to prepare culture material. Then, 500 g of wet material was placed into polypropylene bags (14 × 26 cm). 115 °C, high pressure sterilization was performed for 2 h. After the wild type, three *Pofst3*$^{+}$ and three *Pofst3*$^{-}$ strains were activated through CYM solid culture medium, the mycelium pellets with a diameter of 1 cm were placed in holes dug with a perforator. Then each bag was inoculated with 5 pellets. Each strain was cultured in 20 bags. Bags were incubated under darkness and 25 °C, until the bags were fully colonized by the mycelia. After 5 d, the bags were transferred to another room for subsequent routine fruiting management. Eight days later Pileus diameters of five fruiting bodies from each bag (totally, five bags from each strain) were measured and statistical analysis was performed using Student's t test, considering a $P \leq 0.05$ as significant.

3. Results

3.1. Isolation and characterization of the full length Pofst3 cDNA

Using the specific primers designed for the *P. ostreatus* homolog of the *S. commune fst3* gene, a cDNA fragment was obtained by PCR amplification with reverse transcribed RNA isolated from *P. ostreatus* mycelia. The cDNA 3′ and 5′ ends were cloned with the specific primers for RACE designed based on the obtained partial sequence. The full-length cDNA was amplified with the primers designed based on the connected 3′ and 5′ end sequence. Sequencing showed that the full-length cDNA was 2549 bp, named *Pofst3* (GenBank accession: KJ716894). Sequence analysis showed that the full-length cDNA contained a 2388 bp open reading frame, encoding 795 amino acids. The theoretical isoelectric point and molecular weight of the putative Pofst3 were 5.88 and 88 kDa, respectively. The secondary structure of Pofst3 mainly consists of irregular curling, accounting for 51.95 %; alpha helix and extended strand are the largest structural elements in the protein, alpha helix (Hh) 38.36 %, extended strand (Ee) 9.69 %. The secondary structure contained no beta fold. Conserved domain analysis showed that Pofst3 belonged to the MHR transcription factor superfamily, which had a typical zinc finger DNA binding domain. Through protein sequence alignment analysis, we found that Pofst3 had high sequence identity and similarity with a number of putative proteins in many basidiomycetes (Fig. 1). Among them, the Pofst3 and the transcription factor Fst3 (NCBI Protein ID: XP_003031320) of *S commune* had 62 % sequence identity and 71 % sequence similarity (Supplementary Fig. 1). Therefore, Pofst3 of *P. ostreatus* might have the similar function with *S. commune* Fst3, which was involved in the regulation of fruiting body development (Ohm et al., 2011).

3.2. The Pofst3 expression patterns during P. ostreatus development

Relative expression of *Pofst3* was analyzed in mycelia, primordia and young fruiting bodies stages of *P. ostreatus*. *Pofst3* was expressed in all three developmental stages, and the difference was significant ($P \leq 0.05$) (Fig. 2). The expression of *Pofst3* in primordia and young fruiting bodies was significantly higher than in mycelia, which indicated that Pofst3 might be involved in the development of fruiting bodies.

3.3. Expression of Pofst3 gene in transgenic P. ostreatus

The expression vectors pPo-GPD-*Pofst3*$^{+}$and pPo-GPD-*Pofst3*$^{-}$ were constructed (Supplementary Fig. 3) and transformed into the protoplasts of *P. ostreatus* mediated via *Agrobacterium*. A number of hygromycin resistant strains were selected based on hygromycin

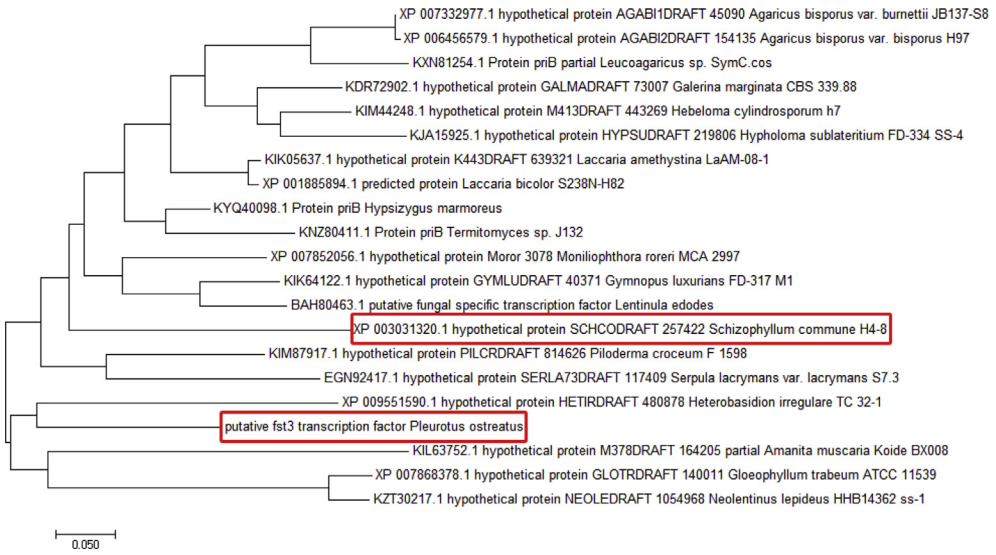

Fig. 1. Phylogenetic tree constructed based on the protein sequence of Pofst3 from *P. ostreatus*. The boxes noted are sequences of *S. commune* and *P. ostreatus*, respectively.

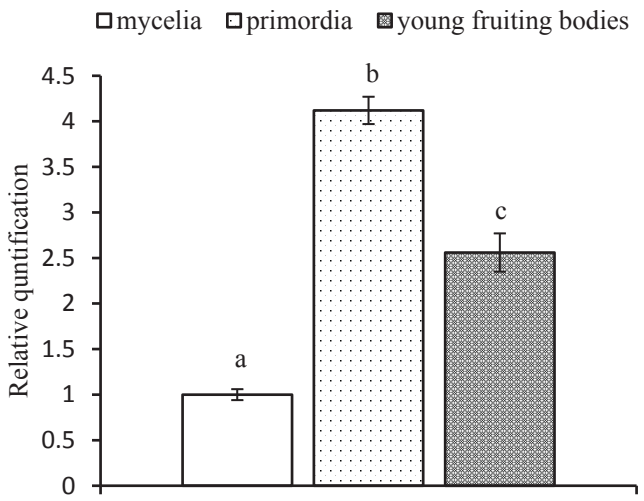

Fig. 2. qRT-PCR analysis of *Pofst3* expression patterns during *P. ostreatus* different developmental stages. Relative quantification is reported as fold expression against reference sample (mycelia stage), the RQ value equal to 1. Statistical analysis was performed using Student's t test. *P ≤ 0.05, significant differences among different developmental stages marked with different letters.

resistance selection strategy (Supplementary Fig. 4). To confirm the integration of the *Pofst3* expression cassette into the genome of *P. ostreatus*, eight transformants showing hygromycin resistance were selected randomly from forty-eight transformants for PCR analysis. As showed in Supplementary Fig. 5, fragments with the expected length were found in all analyzed transformants. The PCR and sequencing results indicated that the *Pofst3* overexpressed strains (*Pofst3*$^{+}$ strain) and antisense silenced strains (*Pofst3*$^{-}$ strain) were successfully obtained.

QRT-PCR was used to measure the expression of *Pofst3* in *Pofst3*$^{+}$ and *Pofst3*$^{-}$ strains. In the four *Pofst3*$^{+}$ strains, the expression of *Pofst3* gene was 1.25–9.59 fold higher than that of the wild type strain, and the level of expression in *Pofst3*$^{-}$ strains was only 0.01–0.30 fold compared to that of the wild type ($P \leq 0.05$) (Fig. 3). Thus, *Pofst3* expression in *Pofst3*$^{+}$ or *Pofst3*$^{-}$ strains increased or decreased significantly, which indicated that the *Pofst3* gene expressed effectively under the control of *gpd* promoter.

3.4. Phenotypic characteristics of transgenic P. ostreatus

Six transgenic strains of *Pofst3*$^{+}$ and *Pofst3*$^{-}$ were selected and cultured in the SA medium (Fig. 4A) and cottonseed shell Petri dishes (Fig. 4B). The effect of *Pofst3* expression on primordium formation and fruiting body development was analyzed. The result of plate cultivation test showed that the number of primordia of *Pofst3*$^{+}$ strains was almost the same to those of wild types, primordia could develop into larger adult fruiting bodies, and the number of primordia of *Pofst3*$^{-}$ strains was more than those of wild types and *Pofst3*$^{+}$ strains, the size of the primordia was uniform. The result of the bag cultivation showed that the number of the primordia of *Pofst3*$^{+}$ strains was similar to those of the wild types, and the size of the mature fruiting bodies was not uniform, while the number of primordia of *Pofst3*$^{-}$ strains was more than those of the other two type strains, and mature fruiting bodies were uniform, but the individuals were smaller (Fig. 4C). Pileus diameters of young fruiting bodies from each type *P. ostreatus* were measured and analyzed, and the average pileus diameters of *wild type* strain, *Pofst3*$^{+}$ strains, *Pofst3*$^{-}$ strains were 3.15 ± 0.14 cm, 3.27 ± 0.21 cm, and 2.34 ± 0.28 cm, respectively, which showed that there was no statistically significant difference in average pileus diameter between wild type and *Pofst3*$^{+}$ strains, while the diameter of the *Pofst3*$^{-}$ strain was significantly smaller than that of the wild type and *Pofst3* overexpressing type (Fig. 5).

Limited availability of nutrients can only provide for development of limited numbers of primordia. It is possible that transcription factor Pofst3 regulates the development of *P. ostreatus* by restraining mycelia from forming excessive numbers of primordia and guaranteeing that the primordia can successfully compete for the limited nutrients available. Therefore, when *Pofst3* was silenced in *Pofst3*$^{-}$ strains, the inhibition of primordium formation by transcription factor Pofst3 was eliminated, resulting in more primordia than in the wild type. These results on transgenic *P. ostreatus* suggest that Pofst3 perhaps inhibits the formation of clusters of mushrooms.

4. Discussion

Mushroom-forming fungi differentiate by sensing several environmental factors for fruiting body formation. For fruiting body induction, nutrient, temperature and light conditions are critical

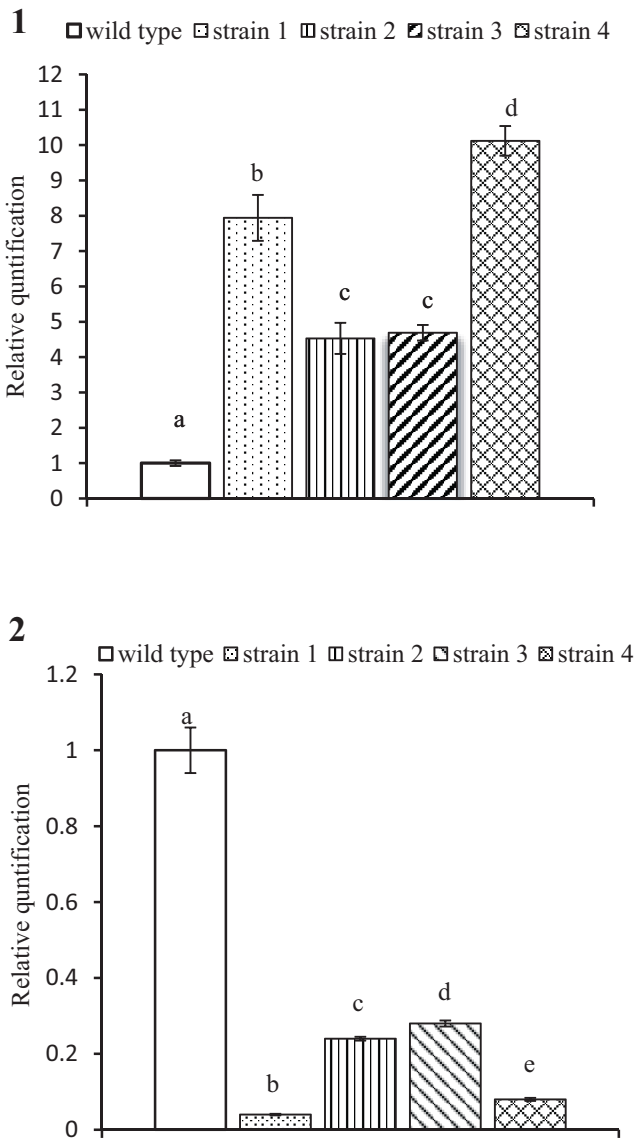

Fig. 3. qRT-PCR confirmation of *Pofst3* expression in transgenic *P. ostreatus*. Relative quantification is reported as fold expression against reference sample (wild type *P. ostreatus*), the RQ value equal to 1. Statistical analysis was performed using Student's t test. *P ≤ 0.05, significant differences among different strains marked with different letters. 1: *Pofst3*$^{+}$ strain; 2: *Pofst3*$^{-}$ strain.

environmental factors (Sakamoto, 2018). However, transcription factors (TFs) that are involved in mushroom development have been little known, so far. The well-studied homeodomain proteins encoded in the matA mating type locus play a critical role in the formation of a fertile dikaryon (Kües et al., 1992; Stankis et al., 1992). Two additional transcription factors involved in fruiting body formation have been identified in *C. cinerea*. The Exp1 transcription factor is involved in cap expansion and autolysis (Muraguchi et al., 2008), whereas Pcc1 functions in matA regulated development (Murata et al., 1998; Murata and Kamada, 2009). Mutations in pcc1 resulted in a completion of sexual differentiation independent of the mating-type genes. The role of transcription factor genes that are differentially expressed during mushroom development in *S. commune* has been more systematically studied (Ohm et al., 2010, 2011). The transcription factor genes, *hom1*, *hom2*, *gat1*, *c2h2*, *fst3*, *fst4*, and *bri1* of *S. commune*, all play an important role in the developmental process (Ohm et al., 2011). The homeodomain gene *hom2* and the DNA binding bright domain protein gene *bri1* deletion strains grow symmetrically in blue light (similar to dark-grown wild type dikaryons) and do not produce aggregates, primordia, and fruiting bodies (Ohm et al., 2011). The strain Δ*fst4*Δ*fst4* grows irregularly in the light, like the wild-type, but does not produce aggregates (Ohm et al., 2010). Strains in which the Cys2His2 zinc finger protein gene *c2h2* has been inactivated are arrested at the aggregate stage, while deletion strains of *fst3*, *gat1* and *hom1* form smaller fruiting bodies but in higher numbers (Ohm et al., 2010, 2011). The zinc finger protein Fst3 was proposed to play a role in the repression of the growth of fruiting bodies from primordia, while the GATA type zinc finger protein Gat1 and the homeodomain protein Hom1 have been proposed to play a role in the expansion of the fruiting bodies (Ohm et al., 2011). Homologs of *S. commune* transcription factors have been identified in *Agaricus bisporus* (Pelkmans et al., 2016), *Laccaria bicolor* (Martin et al., 2008) and *C. cinerea* (Kamada et al., 2010). Expression studies suggest the existence of a core regulatory program for fruiting body development in these and other basidiomycetes (Ohm et al., 2010; Morin, 2012; Plaza et al., 2014; Sipos et al., 2017; Nagy et al., 2018), which is supported by the finding that over-expression of the *c2h2* homolog of *A. bisporus* results in faster fruiting body development (Pelkmans et al., 2016). Transcription factors Bri1 and Hom1 of *S. commune* are involved in late stages of mushroom development, while Wc-2, Hom2, and Fst4 function early in development. Bri1 and Hom1 also stimulate vegetative growth, while biomass formation is repressed by WC-2, Hom2, and Fst4, which suggests that some TFs involved in mushroom formation also modulate vegetative growth (Pelkmans et al., 2017). Blue light in *Neurospora crassa* is detected by the white collar (WC) complex, which consists of the White collar 1 and White collar 2 (WC-1 and WC-2) proteins, and Both WC-1 and WC-2 encode GATA-type transcription factors (Talora et al., 1999). The blue light receptor complex of *S. commune* consists of WC-1 that has a blue light sensing domain and the transcription factor WC-2 (Wu et al., 2018). Inactivation of *wc-1* and/or *wc-2* results in a blind phenotype (Ohm et al., 2013). Two genes, *dst1* and *dst2*, homologs of *WC-1* and *WC-2*, are associated with the mushroom photomorphogenesis of *C. cinerea* encoding putative photoreceptors for blue light (Terashima et al., 2005; Kamada et al., 2010). TFs involved in the development were also reported in *Armillaria* species. For example, the expression of *ARMOST-01275* peaked in rhizomorphs and stipes, whereas a zf-Mynd TF was highly upregulated in rhizomorphs and all fruiting body stages (Sipos et al., 2017).

Although some progress has been made in the study of development related transcription factors in *S. commune* and *C. cinerea*, few studies have been reported in commercial edible fungi. The photoreceptor genes (*Le.phrA* and *Le.phrB*) from the basidiomycete *Lentinula edodes* were cloned and sequenced (Sano et al., 2007, 2009), and transcriptome analysis suggested that the mechanism of brown film formation in the *L. edodes* mycelium was dependent on photoreceptors (Tang et al., 2013). The gene *Cmwc-1*, homologous to *N. crassa wc-1*, from the genome of *Cordyceps militaris* was cloned, and its structure and expression in different strains were compared (Yang and Dong, 2014), and function analysis suggested that CmWC-1 might switch the vegetative growth state to primordia differentiation by suppressing the expression of related genes (Yang et al., 2016).

Here, the full length *Pofst3* cDNA from *P. ostreatus* was cloned. Protein sequence alignment analysis showed that Pofst3 and the transcription factor Fst3 of *S. commune* had 62 % sequence identity and 71 % sequence similarity (Supplementary Fig. 1). This is the first report on cloning and analysis of the homolog of *fst3* in *P. ostreatus*.

For *Pofst3* of *P. ostreatus*, functional analysis showed the same result as for *fst3* in *S. commune* (Ohm et al., 2010) and *L. bicolor*

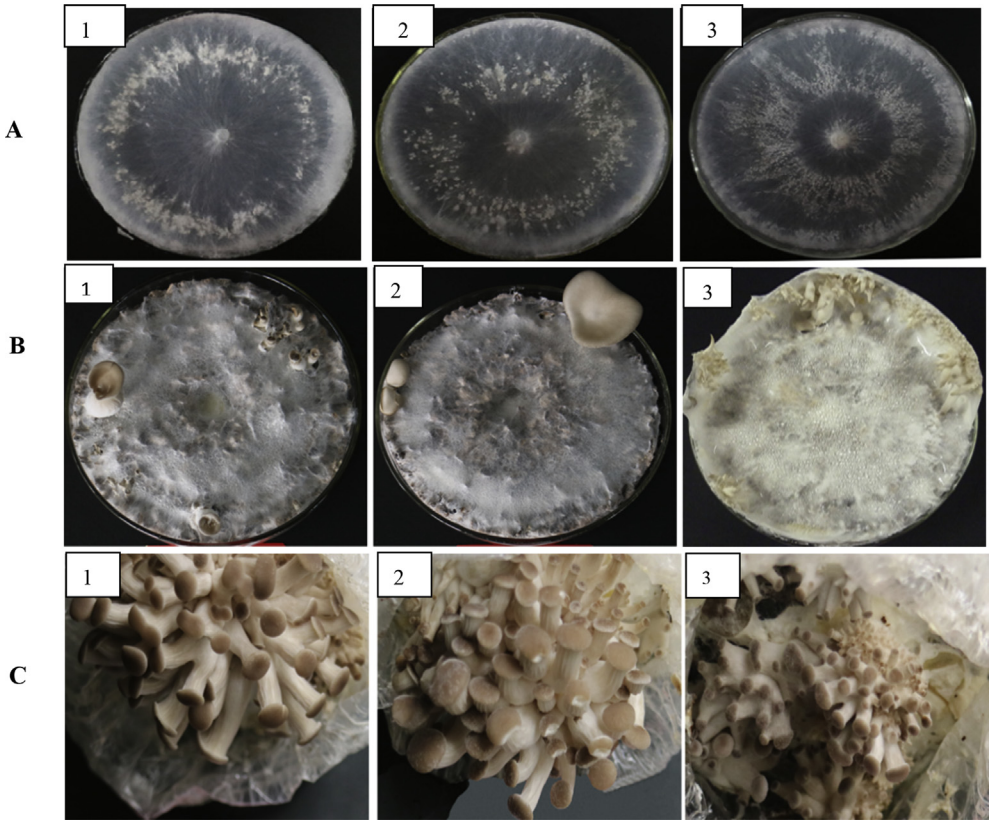

Fig. 4. Cultivation characteristics of transgenic *P. ostreatus*. (A) strains cultivated on SA medium plates; (B) strains cultivated on cottonseed hull plates; (C) strains cultivated on cottonseed hull bags. 1: Wild type strain; 2: *Pofst3*$^{+}$ strain; 3: *Pofst3*$^{-}$ strain.

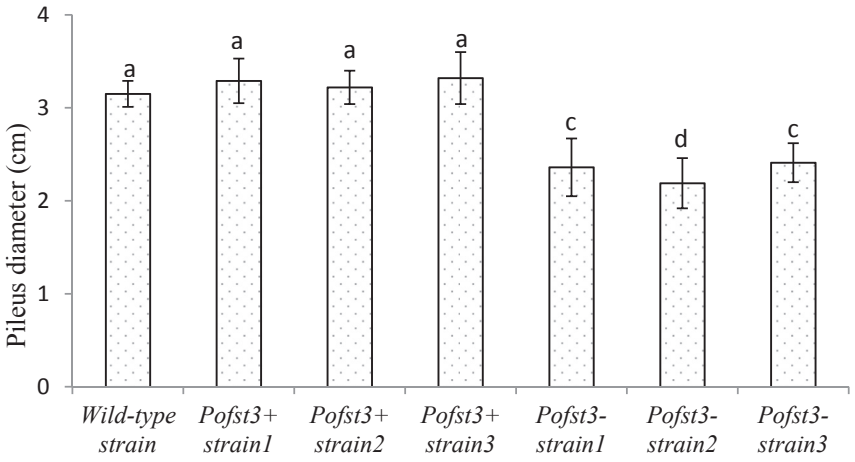

Fig. 5. Pileus diameters of bag cultivated transgenic *P. ostreatus*. Five individual pileus diameters of young fruiting bodies from each bag of each type *P. ostreatus* were measured. Five bags for each type *P. ostreatus*. Values in columns with the different letter in different type strains are significantly different at $P \leq 0.05$, by Student's t test.

(Martin et al., 2008). Transgenic *P. ostreatus* plate and bag cultivation showed that compared with wild type, the number of primordia of *Pofst3*$^{+}$ strains were similar to those of wild types, and the primordia could develop into larger adult fruiting bodies; while the number of primordia of *Pofst3*$^{-}$ strains was significantly more than those of wild types and *Pofst3*$^{+}$ strains, and the size of the primordia was uniform (Fig. 4). The *fst3* gene was upregulated in young fruiting bodies of *L. bicolor* compared to free-living mycelium (Martin et al., 2008). In mature fruiting bodies of *L. bicolor*, the expression level of *fst3* remained constant compared to immature fruiting bodies (Martin et al., 2008). In *S. commune*, the Δ*fst3*Δ*fst3* dikaryon formed more, albeit smaller, reproductive structures than those of the wild type. As spatial and temporal regulation of fruiting-body formation and sporulation were not altered in the Δ*fst3*Δ*fst3* strain, and it indicated that Fst3 perhaps inhibited the formation of clusters of mushrooms (Ohm et al., 2010). Unlike these limited reports on development related transcription factors of model mushrooms (Ohm et al., 2010, 2011; 2017; Muraguchi et al., 2008), in addition to observing the phenotypes of primordia and young fruiting bodies of transgenic and wild type *P. ostreatus*, we

quantified the size of young fruiting bodies before and after *Pofst3* overexpression and silencing (Fig. 5). Pileus diameter measurements clearly revealed that the size of young fruiting bodies formed after gene silencing was smaller than that of wild type and overexpression strain. It seems likely that *Pofst3* is involved in the regulation of *P. ostreatus* development through inhibiting the formation of clusters of primordia.

This is one of the few reports on the transcription factor in *P. ostreatus*, and the result will lay a foundation for further study of the developmental mechanisms of *P. ostreatus*, and provide some theoretical reference for the research of other commercial edible mushrooms. After all, over-expression and antisense silencing are only indirect ways to verify gene function. Recently, we are trying to analyze the differences in the development of *P. ostreatus* before and after gene deletion by means of gene knockout. Finding the target genes regulated by Pofst3 and further analyzing the molecular network regulated by this transcription factor is our ultimate goal.

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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.2018.12.008.

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