A WD40 Protein Encoding Gene Fvcpc2 Positively Regulates Mushroom Development and Yield in Flammulina velutipes

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Ascomycota and Basidiomycota are two closely related phyla and fungi in two phyla share some common morphological developmental process during fruiting body formation. In Neurospora crassa, the Gβ-like protein CPC-2 with a seven-WD40 repeat domain was previously reported. By transforming CPC-2 ortholog encoding genes, from 7 different fungal species across Ascomycota and Basidiomycota, into the cpc-2 deletion mutant of N. crassa, we demonstrate that all tested CPC-2 ortholog genes were able to complement the defects of the cpc-2 deletion mutant in sexual development, indicating that CPC-2 proteins from Ascomycota and Basidiomycota have the similar cellular function. Using Flammulina velutipes as a model system for mushroom species, the CPC-2 ortholog FvCPC2 was characterized. Fvcpc2 increased transcription during fruiting body development. Knockdown of Fvcpc2 by RNAi completely impaired fruiting body formation. In three Fvcpc2 knockdown mutants, transcriptional levels of genes encoding adenylate cyclase and protein kinase A catalytic subunit were significantly lower and colony growth became slower than wild type. The addition of cAMP or the PKA-activator 8-Bromo-cAMP into the medium restored the Fvcpc2 knockdown mutants to the wild-type colony growth phenotype, suggesting that the involvement of cAMP production in the regulatory mechanisms of FvCPC2. Knockdown of Fvcpc2 also weakened transcriptional responses to sexual development induction by some genes related to fruiting body development, including 4 jacalin-related lectin encoding genes, 4 hydrophobin encoding genes, and 3 functionally-unknown genes, suggesting the participation of these genes in the mechanisms by which FvCPC2 regulates fruiting body development. All three Fvcpc2 overexpression strains displayed increased mushroom yield and shortened cultivation time compared to wild type, suggesting that Fvcpc2 can be a promising reference gene for Winter Mushroom breeding. Since the orthologs of FvCPC2 were highly conserved and specifically expressed during fruiting body development in different edible mushrooms, genes encoding FvCPC2 orthologs in other mushroom species also have potential application in breeding.

Keywords: Flammulina velutipes, WD40 protein, sexual development, mushroom breeding, Neurospora crassa
INTRODUCTION

Among the six phyla of the fungal kingdom, Ascomycota members (produce sexual spores enclosed in a special sac called an ascus) and Basidiomycota members (produce external basidiospores on a basidium during sexual life) are closely related and together form the fungal subkingdom of Dikarya (Hibbett et al., 2007; Kirk et al., 2008). They evolved into two different branches only around 500 million years ago (Berbee and Taylor, 2010; Oberwinkler, 2012). The two phyla share commonness in the morphological process during fruiting body development (Pelkmans et al., 2016).

Fungal fruiting is an elaborate process which is corporately controlled by both genetics and environmental factors (Kües, 2000). As previously reported, common regulators of fruiting body development exist between these two phyla. For instance, the light perception mechanism plays a pivotal role in light-dependent fruiting. The blue light receptor WC-1 regulates the formation of perithecia (fruiting bodies) in the ascomycetes Neurospora crassa and Trichoderma reesei (Chen et al., 2012; Linden et al., 1997). Its orthologs in the basidiomycete mushroom Schizophyllum commune and Coprinopsis cinerea are required for the initiation and maturation of basidioma, respectively (Terashima et al., 2005; Ohm et al., 2013). Another common regulatory system of fruiting between these two phyla is pheromone signaling. Pheromone reception and its signal transduction are essential for mating and for fruiting initiation in many species of these two phyla (Debuchy et al., 2010; Raudaskoski and Kothe, 2010). The membrane-located heterotrimeric G-protein complex participates in pheromone signaling in many fungal species (Lengeler et al., 2000; Brown and Casselton, 2001; Li et al., 2007). The Gβ subunit of heterotrimeric G-proteins belong to WD40 repeat protein family, in which each of WD40 domains folds into a β-propeller structure, providing a platform for protein-protein interaction (Jain and Pandey, 2018; Neer et al., 1994). In addition to Gβ subunit, WD40 repeat proteins also have many other members.

In N. crassa, a Gβ-like protein CPC-2 with a seven-WD40 repeat domain controls amino acid synthesizing enzymes and proprote�타림 development (Krüger et al., 1990; Müller et al., 1995). Several CPC-2 orthologs in other fungal species of the Ascomycota and Basidiomycota phyla, including Cpc2 of Schizosaccharomyces pombe (Won et al., 2001), Ascl of Candida albicans (Liu et al., 2010), CpcB of Aspergillus nidulans (Kong et al., 2013), Gib2 of Cryptococcus neoformans (Palmer et al., 2006), and Rak1 of Ustilago maydis (Wang et al., 2011), were also reported. Cpc2 regulates the cell cycle and sexual differentiation in S. pombe (McLeod et al., 2000; Jeong et al., 2004; Paul et al., 2009), CpcB participates in the hyphal growth and cleistothecium development in A. nidulans (Hoffmann et al., 2000; Kong et al., 2013) and Rak1 controls mating in U. maydis (Wang et al., 2011). These studies indicate that CPC-2 and its orthologs are involved in fruiting in different fungal species in both Ascomycota and Basidiomycota. However, it is unknown whether they have the same cellular function. Although proprote�타림ic and cleistothecium of Ascomycota fungi are dramatically different from basidioma of Basidiomycota fungi in structure, they are all specialized fruiting body structures with similar biological functions. CPC-2 and CpcB regulate fruiting body development in N. crassa and A. nidulans, respectively (Müller et al., 1995; Kong et al., 2013). Therefore, it is possible that their orthologs in Basidiomycota fungi regulates mushroom development.

Although orthologs of CPC-2 have been predicted in the genomes of several mushroom species, including model mushroom species (C. cinerea and S. commune) and cultivated mushrooms (Flammulina velutipes, Volvariella volvaeca, Pleurotus ostreatus, Ganoderma lucidum, and Lentinus edodes) (Ohm et al., 2010; Stajich et al., 2010; Chen et al., 2012; Grigoriev et al., 2012; Bao et al., 2013; Park et al., 2014; Riley et al., 2014; Sakamoto et al., 2017), none of them has been functionally characterized. Similarly as that cpc-2 is up-regulated after crossing in N. crassa (Lehr et al., 2014), CPC-2 orthologs have an increased expression during fruiting body development in many mushroom species (Ohm et al., 2010; Yu et al., 2012; Cheng et al., 2013; Liu et al., 2015; Zhang et al., 2015; Sakamoto et al., 2017), suggesting that CPC-2 orthologs are likely involved in fruiting body development in these mushroom species. If it is true, CPC-2 ortholog encoding genes have potential to be a new group of reference genes for mushroom breeding. To date, the number of reported genes involved in mushroom development is very limited and most of them were only studied in model mushrooms (C. cinerea or S. commune). Identification of new genes critical for fruiting body development in commercially cultivated mushroom is necessary.

Due to the available genetic techniques (Kim et al., 2010; Okamoto et al., 2010; Hatooh et al., 2013; Shi et al., 2016), the world-wide cultivated Winter Mushroom (F. velutipes, which was recently renamed as Flammulina filiformis; Wang et al., 2018) has been used as a model to study mushroom development (Park et al., 2014; Lu et al., 2016). In this study, we intended to test whether CPC-2 orthologs are functionally conserved for fruiting body development in the two phyla Ascomycota and Basidiomycota. We also aimed to analyze the role of CPC-2 orthologs in fruiting body development in mushroom species using F. velutipes as a model system.

MATERIALS AND METHODS

Strains and Media

The dikaryotic strain F19 and the monokaryotic strain L11 of F. velutipes, the cDNA of V. volvacea, G. lucidum, and P. ostreatus and the binary vector pBHg-BCA1 were provided by Professor Baogui Xie (Fujian Edible Fungi Germplasm Resource Collection Center of China) (Liu et al., 2015; Wang et al., 2015). The cDNA of Saccharomyces cerevisiae, C. albicans, and C. neoformans were obtained from Professor Fengyan Bai, Guanghua Huang, and Linqi Wang (Institute of Microbiology, Chinese Academy of Sciences), respectively.

CYM medium (w v⁻¹, 0.2% yeast extract, 0.2% tryptone, 1% maltose, and 2% glucose) was used for routine growth of F. velutipes (Lin et al., 2013). For fruiting body development, F. velutipes strains were cultured in the composted sawdust substrate (52.5% cottonseed hulls, 25% wheat bran, 15% sawdust, 5% corn flour, 2% gypsum, and 0.5% ground limestone) with 61%
moisture content (Wang et al., 2015). *Escherichia coli* DH5α was used for propagation of plasmids, and *Agrobacterium tumefaciens* AGL-1 was used for transforming the plasmids into *F. velutipes*.

Strains FGSC#4200 (wild type, mating type α) and FGSC#13695 (the deletion mutant of cpc-2, mating type α, heterokaryon) of *N. crassa* were obtained from the Fungal Genetics Stock Center (FGSC; University of Kansas Medical Center). These strains were routinely grown on Vogel's minimal medium with 2% sucrose for conidial production or on synthetic crossing medium (SCM) with 0.2% sucrose for protoperitheciun and peritheciun formation (Davis and Serres, 1970). The 150 mm slants of solid agar medium (2% agar, 0.5% sucrose, 0.1 × SC and 1 mM iodoacetate) were used to separate the homokaryon (Ebbole and Sachs, 1990).

**Homokaryonization of the cpc-2 Deletion Mutant**

FGSC#13695 is a heterokaryon of the cpc-2 deletion mutant which could produce normal protoperitheciun as wild type. Therefore homokaryonization was performed to obtain the absolute homokaryons of microconidia (Ebbole and Sachs, 1990). Firstly, the mycelia plug (d = 5 mm) of FGSC#13695 was inoculated on slants and grown at 25°C with a 12 h light/dark cycle for 10 days to produce microconidia. Secondly, the microconidia were harvested from each slant into 2.5 ml of sterile water and filtered through a 5 μm PVDF filter units (Sterile Millex Filter Units; Merck KGaA, Darmstadt, Germany). Thirdly, the microconidia (2000 spores) were spotted on the Vogel's/sorbose agar plate and incubated at 28°C for 2–3 days. Lastly, individual colonies were transferred to Vogel's slants for confirming homokaryonization using PCR by three disparate primers (cpc2-ORF-F/R, cpc2-up-F/R, and cpc2-down-F/R) and fertility tests. The final sterile homokaryon strain was used as the Δcpc-2 strain in this study. The primers of homokaryonization were shown in Supplementary Table S1.

**Sequence Acquisition**

The sequence of Fv-cpc2 (GenBank Accession No. KY815023) was acquired from the genome of *F. velutipes* strain L11. The sequence of Vv-cpc2 (GenBank Accession No. MN075138) was obtained from the genome of *V. volvacea* strain PYd21. The sequence of CPC-2 orthologs encoding genes (with GenBank No. NM_001182616.1, XM_019475482.1, XP_012053792.1, GQ293361.1, and KDDQ27929.1, respectively) from *S. cerevisiae*, *C. albicans*, *C. neoformans*, *G. lucidum*, and *P. ostreatus* were obtained from National Center for Biotechnology Information (NCBI)1.

**Total DNA Extraction**

For DNA extraction of *N. crassa*, the mycelia were cultured in Vogel’s liquid medium (1 × Vogel’s salts with 2% glucose) at 28°C for 24 h and extracted by the ethanolic perchlorate method (Metzenberg and Baisch, 1981).

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**Complementation of the cpc-2 Deletion Mutant of *N. crassa***

The vector pCB1532, which contains a sulfonilurea resistance allele of the Magnaporthe grisea ILV1 as a selectable marker (Sweigard et al., 1997), was used in the construction. In order to achieve functional complementing plasmids, the coding sequences of Fv-cpc2, cpc2, asc1, Glcpc2, Vvcpc2, Pocpc2, gib2 of cpc-2 orthologs from *F. velutipes*, *S. cerevisiae*, *C. albicans*, *G. lucidum*, *V. volvacea*, *P. ostreatus*, and *C. neoformans*, were used. The native promoter and terminator regions of cpc-2 from *N. crassa* were used as the promoter region and terminator region, respectively. The plasmid with cpc-2 of *N. crassa* was used as the positive control.

A schematic representation of the complementing construction is shown in Supplementary Figure S1. A 1551 bp downstream regulatory region of cpc-2 was amplified by PCR with primer pair cpc2-DS-F/R from the genomic DNA of *N. crassa* strain FGSC#4200 and inserted into pCB1532 by homologous recombination (ClonExpress MultiS One Step Cloning Kit; Vazyme, Nanjing, China) to generate plasmid pre-pCB1532-cpc2. This plasmid was used as a precursor in the following construction.

To construct the complementing vector with *N. crassa* cpc-2, the full length of cpc-2 (3459 bp) was amplified by PCR with primer pair cpc2-CM-F/R from the genomic DNA of *N. crassa* strain FGSC#4200. The PCR product was introduced into pre-cpc2-pCB1532 by homologous recombination to generate the final vector Nccpc-2-pCB1532.

To construct the complementing vector with *F. velutipes*, the cpc-2 promoter and terminator were amplified by PCR using primer pairs NCCpc2-Promoter-F/R and NCCpc2-Terminator-F/R from the genomic DNA of *N. crassa* strain FGSC#4200, respectively. The open reading frame region of Fv-cpc2 (948 bp) was amplified by PCR with primer pair Fv-cpc2-F/R from the cDNA of *F. velutipes* strain L11. These three fragments were inserted into pre-cpc2-pCB1532 by homologous recombination to obtain the final plasmid, Fv-cpc2-pCB1532. The construction of plasmids Sccpc2-pCB1532, Caas1-pCB1532, Glcpc2-pCB1532, Cngib2-pCB1532, Vvcpc2-pCB1532, and Pocpc2-pCB1532 was performed following the same method as for Fv-cpc2-pCB1532. These plasmids were confirmed by PCR and sequencing. The primers employed for plasmid construction were shown in Supplementary Table S1.

The above accomplished plasmids were transferred into the Δcpc-2 strain via electroporation (Margolin et al., 1997). Then the transformants were selected by chlorimuron ethyl (15 μg ml⁻¹) and confirmed with PCR. Finally, the complemented strains with cpc-2 of *N. crassa* and CPC-2 ortholog encoding genes from *F. velutipes*, *S. cerevisiae*, *C. albicans*, *G. lucidum*, *V. volvacea*, *P. ostreatus*, and *C. neoformans* were achieved and were named as cpc-2; Δcpc-2, Fv-cpc2; Δcpc-2, cpc2; Δcpc-2, asc1; Δcpc-2.
Glpc2;Δcpc-2, Vvpc2;Δcpc-2, Pocpc2;Δcpc-2, and gib2;Δcpc-2, respectively. The primers for verifying complemented strains are shown in Supplementary Table S1.

Measurement of Hyphal Growth Rate and Conidial Production

The growth rate of N. crassa in race tubes filled with Vogel’s minimal medium was measured as previously described (Davis and Serres, 1970). The fungal growth of 4 days was recorded per 12 h by marking the visible colony edge on race tubes. Then the hyphal growth rate was calculated as average hyphal extension per day. Three replications were taken independently.

The conidial production of N. crassa was measured in test tubes each containing 2 ml Vogel’s solid medium. A mycelial plug (d = 5 mm) of wild type FGSC#4200 or cpc-2 mutants was inoculated into one test tube. After incubation at 28°C for 10 days, spores in each test tube were suspended into 2 ml sterile water. Then conidia were counted with hemocytometer under a BX51 microscope (Olympus, America).

Crosses and Fertility Tests of N. crassa

The crosses and fertility tests were performed in N. crassa using standard techniques (Davis and Serres, 1970). The female parents were inoculated on the plates first. Specifically, the conidial suspension (2 µl, 1 × 107 spores ml⁻¹) was spotted onto the center of SCM plate and plates were grown at 25°C in dark for 7 days. Then 2 µl conidial suspension of wild-type strain (FGSC#2225) with the opposite mating type A was spotted on the colony surface of the female parent strains, respectively. The crossed plates were incubated at 25°C in dark for another 7 days to induce the formation of perithecia.

Plasmid Construction and Transformation in F. velutipes

The binary vector pBHg-BCA1 containing the resistance gene of hygromycin was used in the construction of the Fvpc2 overexpression plasmid and RNAi plasmid as previously described (Wu et al., 2019). The promoter of glyceraldehyde-3-phosphate dehydrogenase Pgd and terminator of trpC (TrpC) were used to control the expression of Fvpc2 or the sense and antisense sequence of Fvpc2 in both plasmids (Kuo et al., 2004; Sekiya et al., 2013; Kim et al., 2015).

To construct the overexpression plasmid, Pgd and TrpC were first amplified by PCR with the primer pairs Pgd-F/R and TrpC-F/R from the genomic DNA of F. velutipes strain L11 and plasmid pCSN44 (Fungal Genetics Stock Center1; University of Kansas Medical Center), respectively (Nishikawa et al., 2016). The open reading frame region of Fvpc2 (1273 bp) was then amplified by PCR with the primer pair pd1-F/R from the genomic DNA of strain L11. The above three fragments were introduced into pBHg-BCA1. Finally, the overexpression plasmid, Fvpc2-OE, was achieved.

To construct the Fvpc2 RNAi plasmid, Pgd and TrpC were first amplified as described above. Then, a 340 bp antisense sequence of Fvpc2 was obtained from F. velutipes L11 by PCR with primer pair Fvpc2-antisense-F/R. These three fragments were inserted into pBHg-BCA1 to form the vector pre-Fvpc2-RNAi. After that, the sense sequence linked with the spacer (from the 113rd nucleotide to the 502nd nucleotide, 390 bp) was amplified from the genomic DNA of strain L11 by PCR with primer pair Fvpc2-sense-F/R, and ligated by BamHI (New England Biolabs, Beverly, MA, United States) into pre-Fvpc2-RNAi to produce the final Fvpc2-RNAi vector. The primers for plasmid construction were shown in Supplementary Table S2.

Plasmids Fvpc2-OE and Fvpc2-RNAi were transformed into F. velutipes dikaryotic strain F19 by the A. tumefaciens-mediated transformation method (Wu et al., 2019), respectively. The positive transformants were detected by PCR with primers Pgd-detect-F and TrpC-detect-R. The primers for transformant verification are shown in Supplementary Table S2.

Fruiting Body Production in F. velutipes

The fruiting body was produced on the composted sawdust substrate (Wu et al., 2019). In order to mimic the industrial production of Winter Mushroom, the parts of fruiting bodies out of the vessel were harvested and the fresh weight was used to record the yield of each vessel. Biological efficiency for each strain was calculated by the formula: BE (%) = (weight of fresh basidiomata/weight of dry substrate) × 100 (Harith et al., 2014; Xie et al., 2017). All the results were obtained by measuring basidioma harvested from six vessels for each strain and the average values were then calculated. These cultivation experiments were implemented for three times independently.

RNA Extraction and Gene Transcription Analysis

To obtain N. crassa samples at vegetative growth stage for RNA extraction, conidial suspension (2 µl, 1 × 107 spores ml⁻¹) of each strains (FGSC#4200, Δcpc-2, cpc-2;Δcpc-2, and Fvpc2;Δcpc-2) was spotted on the center of the cellophane-covered Vogel's plates and incubated at 28°C for 24 h. After 24 h, mycelia were collected for RNA extraction. To obtain N. crassa samples at sexual developmental stage, conidia were spotted on the center of the cellophane-covered SCM plates and incubated at 25°C for 5 days in dark. After 5 days, the mycelia were collected for RNA extraction. The mycelial samples were frozen in liquid nitrogen and ground into fine powder.

To detect the Fvpc2 transcript levels in F. velutipes, the samples were prepared following the method below. Mycelial plugs (d = 5 mm) of strains (F19, Fvpc2 overexpression strains and Fvpc2 RNAi strains) were respectively inoculated on the center of cellophane-covered CYM agar plates at 25°C. After 7 days, mycelia were harvested for RNA extraction.

To detect the genes responsive to Fvpc2 knockdown in F. velutipes, the mycelia of wild type strain F19 and Fvpc2 knockdown mutants were harvested from composted sawdust substrate (SM) after cold stimulation for fruiting body development.
The harvested mycelia were frozen in liquid nitrogen and ground into fine powder. Total RNA extraction was performed by the standard TRIzol protocol (Invitrogen, Carlsbad, CA, United States). Then 2 µg of total RNA were used to synthesize cDNAs by a cDNA synthesis kit (FastQuant RT Kit with gDNAse, TIANGEN, China) according to the manufacturer’s protocol. Quantitative real-time PCR (qPCR) was implemented on a CFX96 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, United States) with SYBR green detection (KAPA SYBR® FAST qPCR Kits; KAPA BIOSYSTEMS, Boston, MA, United States) following the manufacturer’s instructions. Three independent replicates were performed for each cDNA sample and the average threshold cycle was calculated. The expression of genes was normalized to that of β-tublin in N. crassa. The expression of genes was normalized to that of β-actin in F. velutipes (Tao et al., 2016). The 2−ΔΔCt calculation was used for the calculation of relative expression levels (Livak and Schmittgen, 2001). The significance of the differences between each of two samples were estimated by an unpaired two-tailed Student’s t-test using GraphPad Prism 7.0 and a p-value of less than 0.05 was considered as significant. The primers employed for qPCR are shown in Supplementary Table S3.

RESULTS

Identification of the CPC-2 Ortholog

FvCPC2 in F. velutipes

CPC-2 of N. crassa belongs to the WD40 protein family. A total of 51 WD40 proteins were predicted in the genome of F. velutipes strain L11, however, only one CPC-2 ortholog was predicted. A gene coding the CPC-2 ortholog was found in the genome of F. velutipes and named as Fvpc2 (GenBank Accession No. KY815023). The genome sequence of Fvpc2 is 1273 bp containing an open reading frame of 948 bp interrupted by four introns of 50, 50, 177, and 48 bp, respectively. Fvpc2 encodes a protein with 315 amino acid residues which displays 73% amino acid identity with CPC-2 of N. crassa (99% coverage). In addition, the amino acid sequence of Fvpc2 also shares highly identity with CpcB of A. nidulans, Cpc2 of S. pombe and Gib2 of C. neoformans (Figure 1B). Domain prediction with SMART showed that FvCPC2 was exclusively built up of seven WD40 repeats which formed seven β-propeller blades in three-dimensional structure (Figures 1A,C). qPCR analysis showed that the transcriptional level of Fvpc2 in primordia were 5.71-fold more than in mycelia at the

FIGURE 1 | Sequence and structure analysis of FvCPC2 protein. (A) The domain organization of FvCPC2 protein. Seven WD40 repeats were predicted in FvCPC2 protein by SMART (http://smart.embl-heidelberg.de/). (B) The Sequence alignment of FvCPC2, CPC-2, CpcB, Cpc2, and Gib2 in WD repeats domain. (C) The structural model of FvCPC2. The structural model of FvCPC2 was compared with Gib2 from Cryptococcus neoformans by SWISS-MODEL (https://www.swissmodel.expasy.org/).
vegetative growth stage (Figure 2; \( p = 0.0000026, n = 3 \)), consistent with the previously reported RNA-seq data (Liu et al., 2015), suggesting that FvCPC2 is likely to participate in fruiting body development.

**CPC-2 Orthologs From Seven Fungal Species Can Functionally Replace the Role of N. crassa CPC-2 in Vegetative Growth and Asexual Sporulation**

In order to test whether FvCPC2 are functionally similar to N. crassa CPC-2, a strain (Fvpcp2;Δcpc-2) was created, in which Fvpcp2 of F. velutipes was transformed into the cpc-2 deletion mutant (Δcpc-2) of N. crassa (Supplementary Figure S1). Meanwhile, another strain (cpc-2;Δcpc-2), in which N. crassa cpc-2 was transformed into the Δcpc-2 mutant and used as a control strain. Similar to the previous report (Garud et al., 2019), the Δcpc-2 strain grew slower and produced less conidia than N. crassa wild type. Both the Fvpcp2;Δcpc-2 strain and the cpc-2;Δcpc-2 strain displayed the wild-type colony growth rate and wild-type conidial production ability (Figure 3), indicating Fvpcp2 can functionally replace the role of N. crassa CPC-2 in vegetative growth and asexual sporulation.

We further respectively transformed the genes encoding cpc-2 orthologs from 6 other fungal species, including S. cerevisae, C. albicans, C. neoformans, V. volvacea, P. ostreatus, and G. lucidum, into the Δcpc-2 mutant (Supplementary Figure S2). The sequence conservation of these CPC-2 orthologs was shown in Supplementary Figure S3. All transformants displayed the wild-type phenotype in colony growth and conidial production (Supplementary Figure S2), demonstrating that the CPC-2 orthologs from a wide range of fungal species across Ascomycota and Basidiomycota can functionally replace the role of N. crassa CPC-2 in vegetative growth and asexual sporulation.

**Fruiting Body Development**

Fruiting body development in the Δcpc-2 mutant of N. crassa was blocked and it failed to produce protoperithecia (Müller et al., 1995). We observed the sexual development of all above mentioned strains. As shown in Figure 4, the wild-type strain produced normal protoperithecia after fruiting induction for 3–5 days, while the Δcpc-2 strain could form hyphal aggregation but failed to generate protoperithecia. After crossing with a wild-type strain with the opposite mating type, wild type produced abundant perithecia on the colony surface but no peritheciun.
appeared in the \( \Delta cpc-2 \) strain (Figure 4). The \( cpc-2;\Delta cpc-2 \) strain displayed wild-type phenotype and produced normal protoperithecia and perithecia. The \( Fvcpc2;\Delta cpc-2 \) strain could produce normal protoperithecia and perithecia but the formation of protoperithecia was 2 days later than wild type and the \( cpc-2;\Delta cpc-2 \) strain. The wild-type strain and the \( cpc-2;\Delta cpc-2 \) strain formed protoperithecia after three and half days of sexual development induction on the SCM media, while the \( Fvcpc2;\Delta cpc-2 \) strain needed five and half days.

Similar to the \( Fvcpc2;\Delta cpc-2 \) strain, all other complement transformants, each of which had one of CPC-2 ortholog genes from six other fungal species in the \( \Delta cpc-2 \) background, were able to form protoperithecia and perithecia (Supplementary Figure S4), but the formation of protoperithecia in these strains was delayed 1–4 days compared to wild type and the \( cpc-2;\Delta cpc-2 \) strain. Nevertheless, above results together indicate that the CPC-2 orthologs can functionally replace the role of \( N. crassa \) CPC-2 in fruiting body development.

**FvCPC2 Can Functionally Replace the Role of \( N. crassa \) CPC-2 in Transcriptional Regulation**

We detected the expression of more than 70 genes which were reported in fruiting body development in \( N. crassa \) by qRT-PCR (Kim and Nelson, 2005; Sun et al., 2012, 2019; Garud, 2013; Chinnici et al., 2014; Lehr et al., 2014; Wang et al., 2014), and found that \( poi-2 \), an essential gene for female fertility in \( N. crassa \), had a dramatic response to \( cpc-2 \) deletion (more than 100-fold change) after fruiting induction in \( N. crassa \) (Figure 5), indicating that CPC-2 regulates transcription of \( poi-2 \). To see whether \( FvCPC2 \) can functionally replace the role of CPC-2 in transcriptional regulation, transcript levels of \( poi-2 \) were analyzed by qPCR in wild type, the \( \Delta cpc-2 \) strain, the \( cpc-2;\Delta cpc-2 \) strain and the \( Fvcpc2;\Delta cpc-2 \) strain. As shown in Figure 5, \( poi-2 \) had a significant transcriptional increase in response to fruiting induction. However, its significant transcriptional increase did not appear in the \( \Delta cpc-2 \) strain. In contrast, both the \( cpc-2;\Delta cpc-2 \) strain and the \( Fvcpc2;\Delta cpc-2 \) strain had the significant transcriptional response in \( poi-2 \) to fruiting induction (Figure 5), indicating \( F. velutipes \) \( Fvcpc2 \) can functionally replace the role of \( cpc-2 \) in transcriptional regulation of \( poi-2 \) in \( N. crassa \).

**Generation of Mutants for \( Fvcpc2 \) Overexpression and Knockdown in \( F. velutipes \)**

Above results suggests that CPC-2 orthologs among the fungal species from the Ascomycota phylum and the Basidiomycota phylum are functionally conserved in fruiting body development. To understand the function of \( Fvcpc2 \) in \( F. velutipes \), the \( Fvcpc2 \) overexpression vector \( Fvcpc2-OE \) and the \( Fvcpc2 \) knockdown vector \( Fvcpc2-RNAi \) were constructed, respectively. The binary vector pBHg-BCA1, the promoter of glyceraldehyde-3-phosphate dehydrogenase (\( P_{gpd} \)) (Kuo et al., 2004) and the terminator of \( trpC \) (\( T_{trpC} \)) (Sekiya et al., 2013; Kim et al., 2015) were used to construct these plasmids as previously described (Wu et al., 2019).

The two vectors were introduced into a dikaryotic wild-type \( F. velutipes \) strain F19, respectively. Three \( Fvcpc2 \) overexpression

![FIGURE 4](image_url) Complementation of \( N. crassa \) \( \Delta cpc-2 \) with \( Fvcpc2 \) in fruiting body development. The wild-type strain FGSC#4200, the \( cpc-2 \) deletion mutant (\( \Delta cpc-2 \)) and complemented mutants (\( cpc-2;\Delta cpc-2 \) and \( Fvcpc2;\Delta cpc-2 \)) were inoculated on SCM plates and incubated at 25°C in the dark. After 7 days, the opposite mating type strain of wild type (FGSC#2225, mating type A) was spotted on the colony surface of each female strain. Then the plates were cultivated at 25°C under light for another 7 days. The images of protoperithecia and perithecia were captured on the 7th and 14th day by stereomicroscope, respectively. Perithecia and protoperithecia were marked by solid and hollow arrows, respectively. The scale bar is 100 μm.
strains Fvcpc2OE5, Fvcpc2OE33, and Fvcpc2OE124 were obtained, in which Fvcpc2 transcript levels were increased to 2.59-, 2.06-, and 2.09-fold, respectively, relative to the wild-type strain F19 (pFvcpc2OE5 = 0.0005, n = 3; pFvcpc2OE33 = 0.0023, n = 3; pFvcpc2OE124 = 0.0063, n = 3). Three Fvcpc2 knockout mutants Fvcpc2RNAi11, Fvcpc2RNAi41, and Fvcpc2RNAi43, were obtained, in which Fvcpc2 transcript levels were decreased by 69.4, 67.7, and 69.7%, respectively, relative to F19 (pFvcpc2RNAi11 = 0.0005, n = 3; pFvcpc2RNAi41 = 0.0009, n = 3; pFvcpc2RNAi43 = 2.4172E-05, n = 3) (Figure 6B). The six strains were used in the following study.

**FvCPC2 Positively Regulates Vegetative Growth in *F. velutipes***

When grown on CYM, all three Fvcpc2 overexpression strains did not display obvious difference from the wild-type strain F19 in colony growth, mycelial density and colony color (Figure 7A). However, the colony growth of Fvcpc2 knockout strains, especially Fvcpc2RNAi41 and Fvcpc2RNAi43, was slower than wild type. As shown in Figure 7B, the growth rates in Fvcpc2RNAi11, Fvcpc2RNAi41, and Fvcpc2RNAi43 were reduced by 42.1 ± 3.3%, 31.6 ± 2.1%, and 21.8 ± 5.8%, respectively, compared to F19 (pFvcpc2RNAi11 = 7.2068E-05, n = 3; pFvcpc2RNAi41 = 0.0025, n = 3; pFvcpc2RNAi43 = 0.0022, n = 3).

The difference in vegetative growth between Fvcpc2 mutants and the wild-type strain F19 was also observed in the composted sawdust substrate. All strains were cultured at 25°C. After 13 days, the mycelia of the wild-type strain and Fvcpc2 overexpression strains completely occupied the substrate, while the mycelia of three Fvcpc2 knockout mutants reached only two-thirds of the vessels (Figure 8). In fact, the mycelia of Fvcpc2 knockout mutants needed 8 more days to cover the entire vessel than the wild-type strain. Above results indicate that FvCPC2 plays a positive role in hyphal growth of *F. velutipes*.
FIGURE 7 | Effects of Fvcpc2 overexpression and knockdown in vegetative growth on agar plates. (A) Mycelial plugs (d = 5 mm) of wild type (F19), Fvcpc2 overexpression strains (Fvcpc2OE#5, Fvcpc2OE#33, and Fvcpc2OE#124) and Fvcpc2 knockdown strains (Fvcpc2RNAi#11, Fvcpc2RNAi#41, and Fvcpc2RNAi#43) were inoculated onto the center of CYM plates and cultured at 25°C for 7 days, respectively. (B) Growth rates of wild type (F19), Fvcpc2 overexpression and knockdown mutants. The colony edge was marked each 24 h and the growth rates were calculated based on 7 days of growth. Values shown are means of three biological replicates. Standard deviations are indicated with error bars. The significant levels of Fvcpc2 mutants in growth rate were calculated to wild type by t-test and marked as ** (0.001 < p < 0.01) or *** (p < 0.001). (pFvcpc2OE#5 = 0.6892, n = 3; pFvcpc2OE#33 = 0.4876, n = 3; pFvcpc2OE#124 = 0.8501, n = 3; pFvcpc2RNAi#11 = 7.2068E-05, n = 3; pFvcpc2RNAi#41 = 0.0029, n = 3; pFvcpc2RNAi#43 = 0.0022, n = 3).

FIGURE 8 | Effects of Fvcpc2 overexpression and knockdown in vegetative growth in composted sawdust substrate. Wild-type strain F19, Fvcpc2 overexpression strains (Fvcpc2OE#5, Fvcpc2OE#33, and Fvcpc2OE#124) and Fvcpc2 knockdown strains (Fvcpc2RNAi#11, Fvcpc2RNAi#41, and Fvcpc2RNAi#43) were inoculated into the culture vessels each containing 150 ± 5 g composted sawdust substrate. The photographs of each strain were captured after 13 days of growth at 25°C.
**FvCPC2 Positively Regulates Fruiting Body Development in *F. velutipes***

Fruiting body development of *F. velutipes* strains grown in the composted sawdust substrate was observed. After 21 days of cultivation, the mycelial mat on the sawdust surface was scrapped off to stimulate fruiting body development. The primordia in wild type appeared 7 days after stimulation (on the 28th day), while the *Fvcpc2* overexpression strains formed primordia one day earlier than wild type and they obviously produced more primordia than wild type. The optimal time for mushroom harvest in wild type appeared on the 41st day, while the mushrooms in *Fvcpc2* overexpression strains could be harvested 3 days earlier than wild type.
type (Figure 9). Thus, overexpression of Fvcpc2 could shorten the cultivation time by 7.3%.

All the Fvcpc2 knockdown strains failed to produce fruiting bodies (Figure 9). Even when the cultivation time was prolonged to 45 days, fruiting body was still not formed. The deficiency of mushroom formation in Fvcpc2 knockdown strains and the shortened cultivation time in Fvcpc2 overexpression strains were observed in another two independently repeated experiments (Supplementary Figure S4).

Overexpression of Fvcpc2 Increases Mushroom Yield in F. velutipes

The fruiting bodies of the wild-type strain and Fvcpc2 overexpression strains were harvested on the 41st day. As shown in Table 1, Fvcpc2 overexpression strains did not display obvious difference with the wild-type strain in fruiting body number. The average number of fruiting bodies for wild type and all Fvcpc2 overexpression strains was about 68 per vessel.

However, the stipe height of Fvcpc2 overexpression strains (Fvcpc2OE#5, Fvcpc2OE#33, and Fvcpc2OE#124) was significantly increased compared to wild type. The average height of stipes in the wild-type strain was 6.95 ± 0.12 cm, while the average height of stipes in the Fvcpc2OE#5, Fvcpc2OE#33, and Fvcpc2OE#124 strains was 7.98 ± 0.07 cm, 7.76 ± 0.13 cm, and 7.88 ± 0.13 cm, respectively (Table 1). Thus, the height of stipes could be increased by 14.8% in the Fvcpc2OE#5 strain which has the highest expression of Fvcpc2 among these Fvcpc2 overexpression strains.

The yield and biological efficiency were significantly also increased in Fvcpc2 overexpression strains relative to wild type. As shown in Table 1, the average yield per vessel in the wild-type strain, Fvcpc2OE#5, Fvcpc2OE#33, and Fvcpc2OE#124 was 23.37 ± 0.85 g, 26.57 ± 0.69 g, 24.90 ± 0.70 g, and 25.49 ± 0.87 g, respectively. The strain (Fvcpc2OE#5) with the highest expression level of Fvcpc2 had the highest yield and biological efficiency, both of which were increased by 13.69% relative to wild type. Among three independently repeated experiments, the yield was increased at least by 6.57% in the Fvcpc2OE#5 strain (Table 1).

Fvcpc2 Regulates Genes Involved in cAMP Signaling Pathway in F. velutipes

The CPC-2 ortholog Gib2 in C. neoformans functions as a typical Gβ subunit, which regulates the cellular cAMP (cyclic AMP) level (Palmer et al., 2006). The cAMP level mediates vegetative growth and fruiting body formation in C. cinerea, S. commune and V. volvacea (Kinoshita et al., 2002; Kües et al., 2004; Lu et al., 2015). In order to detect whether Fvcpc2 is related to cAMP production, transcript levels of gene10451 and gene8023, which respectively encode adenylate cyclase and protein kinase A catalytic subunit-2, were comparatively analyzed in all Fvcpc2 knockdown strains and the wild-type strain after fruiting induction in F. velutipes. In all Fvcpc2 knockdown strains, transcript levels of both gene10451 and gene8023 were significantly lower than wild type (Figure 10).

The reduced expression of adenylate cyclase and protein kinase A catalytic subunit-2 might result in an insufficient supply
FIGURE 10 | Transcriptional responses of gene10415 and gene8023 to Fvcpc2 knockdown. Transcript levels of gene10415 (coding adenylate cyclase) and gene8023 (coding kinase A catalytic subunit-2) were determined in wild type (F19) and the Fvcpc2 knockdown strain Fvcpc2RNAi#43 by qPCR after fruiting induction in F. velutipes. The results presented are means of three biological replicates with error bars and significant level of the indicated genes in Fvcpc2RNAi#43 compared to wild type were calculated by t-test and marked as ** (0.001 < p < 0.01) or *** (p < 0.001). In Fvcpc2RNAi#11, p\text{gene10451} = 0.0010, n = 3; p\text{gene8023} = 7.7599E-05, n = 3. In Fvcpc2RNAi#41, p\text{gene10451} = 0.0011, n = 3; p\text{gene8023} = 0.0004, n = 3. In Fvcpc2RNAi#43, p\text{gene10451} = 0.0014, n = 3; p\text{gene8023} = 0.0003, n = 3.

FIGURE 11 | Effects of cAMP or 8-Bromo-cAMP addition in colony growth of F. velutipes WT and Fvcpc2 knockdown strains. Mycelial plugs (d = 5 mm) of wild type (F19) and Fvcpc2 knockdown strains (Fvcpc2RNAi#11, Fvcpc2RNAi#41, and Fvcpc2RNAi#43) were inoculated onto the center of CYM plates with or without 0.8 µM cAMP or 6 µM 8-Bromo-cAMP (8-Br-cAMP), respectively. The plates were cultured at 25°C for 7 days for photography.

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of cAMP for normal hyphal growth. To test this possibility, effects of cAMP and the PKA-activator 8-Bromo-cAMP in colony growth of wild type and all Fvcpc2 knockdown strains were observed (Figure 11). When 0.8 µM cAMP or 6 µM 8-Bromo-cAMP was added into the CYM plate, the colony growth of wild type was not obviously affected. However, the colony of all Fvcpc2 knockdown strains obviously promoted by addition of cAMP or 8-Bromo-cAMP. Their colonies grew faster on plates amended with cAMP or 8-Bromo-cAMP than on control plates, further suggesting that the slower growth phenotype in these mutants might be due to insufficient production of cAMP.

FvCPC2 Positively Regulates Four Lectin Genes in F. velutipes

To understand how FcCPC2 regulates the fruiting body development, transcriptional levels of some genes related to basidioma development, were comparatively analyzed by qPCR in wild type and the Fvcpc2 knockdown strain (Fvcpc2RNAi#43) with the lowest Fvcpc2 expression after fruiting induction.

According to previous studies, lectins promote hyphal aggregation during fruiting body development of mushrooms (Wang et al., 1998; Kües and Liu, 2000). Nine lectin genes were found in F. velutipes and one of them, Fv-JRL1 (encodes jacalin-related lectin), was previously characterized. Overexpression of Fv-JRL1 promoted vegetative growth and basidioma development, while knockdown of Fv-JRL1 impaired
basidioma development in *F. velutipes* (Lu et al., 2016). Our analysis showed that 4 of 9 lectin genes had difference in transcriptional levels between wild type and *Fvcpc2RNAi* strains. The transcriptional level of *Fv-JRL1* in the *Fvcpc2* knockdown strain was significantly lower than that in wild type (Figure 12A). Transcriptional levels of three other lectin encoding genes (gene9094, gene10415 and gene10856) were also lower in *Fvcpc2RNAi* than in wild type. Transcriptional levels of these 4 lectin genes in *Fvcpc2RNAi* were reduced at least by 51.47% relative to wild type.

**FvCPC2 Positively Regulates Other Genes Related to Fruiting Body Development**

Two genes *FVFD16* and *FVFD30* are specifically expressed during fruiting body development in *F. velutipes* (Kim and Azuma, 1999; Kim et al., 1999). *FVFD16* has a homolog encoded by gene543 in *F. velutipes*. We analyzed their transcript levels and found that the transcript levels of *FVFD30*, *FVFD16*, and gene543 were significantly down-regulated in the *Fvcpc2* knockdown strain compared to wild type (Figures 12C,D).

**FvCPC2 Positively Regulates Four Hydrophobin Genes in *F. velutipes***

Ten hydrophobin encoding genes (Hyd-1 to Hyd-10) were specifically expressed in the primordium stage in *F. velutipes* (Kim et al., 2016). We analyzed transcripts of all these genes by qPCR and found that 4 of 10 hydrophobin encoding genes, including *Hyd-1* (also reported as *fv-hyd1*) (Yamada et al., 2005), *Hyd-3*, *Hyd-4*, and *Hyd-6*, had transcriptional responses to the *Fvcpc2* knockdown. As shown in Figure 12B, transcriptional levels of *Hyd-1*, *Hyd-3*, *Hyd-4*, and *Hyd-6* in *Fvcpc2RNAi* were reduced at least by 49.23% relative to wild type.
DISCUSSION

Although fruiting structures are distinct between Ascomycota and Basidiomycota, they are two closely related phyla separated only for about 500 million years ago (Berbee and Taylor, 2010; Oberwinkler, 2012). Two phyla share some common regulatory mechanisms in fruiting body development, including the blue light receptor WC-1 (Linden et al., 1997; Terashima et al., 2005; Chen et al., 2012; Ohm et al., 2013; Yang et al., 2016), the pheromone signaling (Debuchy et al., 2010; Raudaskoski and Kothe, 2010) and the heterotrimeric G-protein complex (Lengeler et al., 2000; Brown and Casselton, 2001; Li et al., 2007). Our study revealed that CPC-2 proteins also mediate fruiting body development in both phyla. Since overexpression of FvCPC2 could increase mushroom yield and shorten the cultivation time in Winter Mushroom, this study discovered a new gene for mushroom breeding.

The biological functions of CPC-2 and its orthologs were investigated individually in several different fungal species (Krüger et al., 1990; Müller et al., 1995; Gerbasi et al., 2004; Palmer et al., 2006; Liu et al., 2010; Wang et al., 2011, 2014; Kong et al., 2013; Cai et al., 2015), but it was unknown whether these proteins have the same cellular function. By transforming CPC-2 ortholog encoding genes, from 7 different fungal species across Ascomycota and Basidiomycota, into the cpc-2 deletion mutant of N. crassa, we demonstrate that all tested CPC-2 orthologs are able to complement the defects of the cpc-2 deletion mutant in sexual development, indicating these CPC-2 orthologs can functionally replace CPC-2 in N. crassa. Thus, at the protein level, CPC-2 and its orthologs might have the same cellular function. As WD40 domains provide the platform for protein-protein interaction, proteins interacting with CPC-2 orthologs should be structurally similar to those CPC-2-associated proteins in N. crassa. Since cpc-2 in N. crassa, cpcB in A. nidulans and FvCPC2 in F. velutipes are required for fruiting body development (Müller et al., 1995; Kong et al., 2013), CPC-2 and its orthologs are functionally conserved in regulation of sexual development in both Ascomycota and Basidiomycota and they are likely to be key regulatory components in morphological development of sexual reproduction. To fully understand their regulatory mechanisms in morphological development, the proteins interacting with CPC-2 orthologs and the signaling pathway(s) involved with CPC-2 orthologs are necessary to be identified in future.

By analyzing transcriptional levels of genes related to fruiting body development in F. velutipes, we demonstrate that FvCPC2 is required for transcriptional responses to sexual development induction. Although we did not investigate the genome-wide effects of FvCPC2 knockdown in gene transcription, our limited data indicate the importance of FvCPC2 in transcriptional regulation during morphological development of sexual reproduction in Winter Mushroom. The regulatory mechanism of FvCPC2 might be involved in the following aspects.

First, FvCPC2 might be required for the normal production of cellular cAMP. The cAMP level mediates vegetative growth and fruiting body formation in C. cinerea, S. commune, and V. volvacea (Kinoshita et al., 2002; Kües et al., 2004; Lu et al., 2015). The second messenger cAMP, produced by adenylate cyclase, is involved in many developmental processes in fungi (Lengeler et al., 2000). Both FvCPC2 and cpc-2 regulated the transcription of genes encoding adenylate cyclase and protein kinase A catalytic subunit, and Gib2 positively regulates the cellular cAMP (cyclic AMP) level in C. neoformans (Palmer et al., 2006), suggesting that the regulation of cAMP production is a conserved function of the CPC-2 proteins. Our data suggest that the production of cAMP is insufficient in these FvCPC2 knockdown mutants because the FvCPC2 knockdown made colony growth slower than wild type and the addition of cAMP or the PKA-activator 8-Bromo-cAMP into the medium restored the FvCPC2 knockdown mutants to the wild-type colony growth phenotype. In addition, as Gib2 stabilizes Gap1 in C. neoformans (Palmer et al., 2006), FvCPC2 might function in the stabilization of Gap protein in F. velutipes and thus guarantee the normal concentration of cAMP in cells. Therefore, the regulation of cAMP production is likely to be an important regulatory mechanism of FvCPC2 in morphological development. CPC-2 functions as the downstream of GNA-2 and the upstream of GNBI in hyphal growth and perithecial development in N. crassa, respectively (Garud et al., 2019). Since FvCPC2 has the similar role as CPC-2 of N. crassa in vegetative growth and fruiting body development, FvCPC2 might interact with the G protein signaling as CPC-2 does in F. velutipes.

Second, FvCPC2 regulates fruiting body development likely through mediating the expression of hydrophobins. Hydrophobins are fungal specific secretory proteins which could self-assemble into amphipathic layers at hydrophilic-hydrophobic interfaces (Wessels, 1996). The formation of amphipathic layers is beneficial for mycelia to form air channels, which probably promote gas exchange for fruiting bodies (Wosten and Wessels, 1997; Wessels, 2000). The important role of several hydrophobin encoding genes in the fruiting body formation have been demonstrated in mushroom species Agaricus bisporus, P. ostreatus, and L. edodes (De Groot et al., 1996; Ågnerdöttir et al., 1998; Ng et al., 2000). In F. velutipes, ten hydrophobin genes (Hyd-1 to Hyd-10) had increased expression during fruiting body development (Ando et al., 2001; Yamada et al., 2005; Kim et al., 2016). Relatively high transcriptional levels of these hydrophobin genes during fruiting body formation might be necessary for related morphological development. We found that four of them are positively regulated by FvCPC2 and FvCPC2 is critical for their normal expression during fruiting body development. Thus, maintenance of transcriptional levels of these four hydrophobin genes by FvCPC2 might play a role in fruiting body development. Loss of regulation by FvCPC2 in these hydrophobin genes might reduce hydrophobin production and further affect mushroom development.

Third, FvCPC2 regulates fruiting body development likely through promoting the expression of lectin genes. Lectin genes are up-regulated in fruiting body development in Pleurotus cornucopiae, C. cinerea, and Agrocybe aegerita (Kaneko et al., 1993; Oguri et al., 1993; Boulianne et al., 2000; Luan et al., 2010). In Sclerotium rolfsii, the interaction of lectin with the cell wall-associated putative endogenous lectin receptor promotes the aggregation of mycelia to form sclerotial bodies.
In *F. velutipes*, the lectin encoding gene *Fv-JRL1* and its three homolog encoding genes were dramatically upregulated at the primordium formation stage (Liu et al., 2015). *F. velutipes* *Fv-JRL1* positively regulates hyphal growth and fruiting body development (Lu et al., 2016). Thus, enhanced lectin production might be required for normal mushroom formation. Our results showed that reduction of *Fvpc2* expression could decrease transcription of these lectin genes. Therefore, *FvCPC2* plays a critical role in maintaining normal expression of these lectin genes during mushroom development. Transcriptional upregulation of these lectin genes by *FvCPC2* might promote lectin production and be important for fruiting body development.

In addition to these genes with relatively clear roles in morphological development, *FvCPC2* also regulate other genes with unknown functions. *FVFD16* and *FVFD30* are two genes specifically expressed during fruiting body development of *F. velutipes* (Kim and Azuma, 1999; Kim and Azuma, 2000). Our results showed that *FVFD16*, *FVFD30* and a gene encoding a *FVFD16* homolog were downregulated in response to *Fvpc2* knockdown. Although the functions of these *Fvpc2*-regulated genes in morphological development remain to be clarified, the regulation of their expression by *Fvpc2* might have an impact in fruiting body development.

In *N. crassa*, *poi-2* is an essential gene for sexual development. Disruption of *poi-2* caused vegetative growth deficiency and female sterility (Kim and Nelson, 2005). Our results showed that the expression of *poi-2* was increased by about 200-fold after sexual induction. CPC-2 regulates the expression of *poi-2* during sexual development because the increased expression in *poi-2* upon sexual induction disappeared in the *cpc-2* deletion mutant. *Fvpc2* can replace the role of *cpc-2* in the transcriptional regulation of *poi-2*, suggesting the signal transduction processes involved with CPC-2 proteins are likely conserved among different fungal species. However, the homologs of POI-2 were not hunted by alignment of amino acid sequence in *F. velutipes* and other mushroom species. Thus, the downstream genes regulated by CPC-2 proteins might vary from one fungal species to another.

The capacity of yield and production cost are the core concerns for mushroom industry. In addition to yield increase, overexpression of *Fvpc2* could also reduce the cultivation time by 3 days in Winter Mushroom. Since the formation of mushrooms for *F. velutipes* needs low temperature (15°C), the production of Winter Mushrooms has high electricity cost and equipment depreciation. The shorter cultivation cycle will dramatically reduce the energy consumption and promote the efficacy of equipment utilization. Thus, *Fvpc2* can be a promising reference gene for Winter Mushroom breeding. The orthologs of *FvCPC2* were highly conserved and widespread in different edible mushrooms. Most of the *FvCPC2* ortholog encoding genes specifically expressed during fruiting body development in mushroom species (Yu et al., 2012; Zhang et al., 2015; Sakamoto et al., 2017). Thus, genes encoding *FvCPC2* orthologs in other mushroom species may also have potential application in breeding.

**CONCLUSION**

In conclusion, the study demonstrates that CPC-2 and its orthologs are functionally conserved among different fungal species across Ascomycota and Basidiomycota and discovered a new gene with an essential regulatory role in mushroom development and a potential use in mushroom breeding.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article/Supplementary Material.

**AUTHOR CONTRIBUTIONS**

SL contributed conception and design of the study. TW performed experiments. SL and TW wrote and edited the manuscript. TW, ZZ, CH, SW, and LZ performed the statistical analysis. All authors contributed to manuscript revision, read and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00498/full#supplementary-material

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SL contributed conception and design of the study. TW performed experiments. SL and TW wrote and edited the manuscript. TW, ZZ, CH, SW, and LZ performed the statistical analysis. All authors contributed to manuscript revision, read and approved the submitted version.
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**Conflict of Interest:** LZ is employed by Shandong Jinniu Biotech Company Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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