**Pleurotus ostreatus** bHLH transcription factors regulate plant growth and development when expressed in Arabidopsis

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**ABSTRACT**

Fungi interact with plants in different ways in nature; however, it remains largely unclear if fungus gene may affect plant growth and development. Basic helix-loop-helix (bHLH) transcription factors are found in almost all organisms including plants and fungi. By using bioinformatics analysis, we found that there are 12 genes in *Pleurotus ostreatus* which encode bHLH transcription factors, namely *P. ostreatus* basic helix-loop-helix1 through 12 (PobHLH1-PobHLH12). By taking PobHLH1, 2, 5 and 8 as examples, we examined whether PobHLHs may regulate gene expression and plant growth and development when expressed in Arabidopsis. We found that all the four PobHLHs examined were localized in the nucleus; however, PobHLH2, 5 and 8 activated whereas PobHLH1 repressed reporter gene expression in transfected protoplasts. On the other hand, ectopic expression of PobHLH5 or 8 inhibited plant growth and development, whereas transgenic plants expressing PobHLH1 and PobHLH2 were largely morphologically similar to the wild-type plants.

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**1. Introduction**

Fungi, including yeasts, molds and mushrooms, are prehistoric eukaryotic organisms in the history of evolution, and are separated from other eukaryotes such as plants and animals. It is presumed that fungi colonized land long before terrestrial plants, and that they play fundamental roles in nutrient cycling and exchange in the ecosystem by decomposing organic matter (Brundrett 2002). Fungi interact with plants in different ways; some of them are pathogens to plants, whereas some others can establish a mycorrhizal relationship with plants. Infection of cultivated plants by pathogenic fungi may cause extensive damage to plants and thus result in great loss to agricultural productivity. As an example, rice blast caused by fungus *Magnaporthe oryzae* led to great agricultural loss (Talbot 2003). On the other hand, most of the plant species can form a mycorrhizal relationship with fungi for nutritional purposes (Bonfante 2003; van der Heijden et al. 2015). However, as ancient eukaryotic organisms to plants, it remains largely unknown whether fungi genes, when introduced into plants, by their interaction may directly affect plant growth and development.

Transcription factors are proteins that can bind directly or indirectly to the promoter regions of their target genes, thus regulating the expression of their target genes (Latchman 1997). Transcription factors may function alone or in association with other transcription factors or co-regulators to regulate gene expression (Lee and Young 2000). For example, auxin response factors (ARFs) can directly bind to the promoter regions of auxin-responsive genes, whereas Aux/IAA proteins can bind indirectly to the promoter regions of auxin-responsive genes via interacting with ARFs, and the interplay of ARFs and Aux/IAA proteins regulates auxin signaling in plants (Guilfoyle and Hagen 2007; Guilfoyle 2015). Transcription factor genes are only a small portion of the total genes in an organism; for example, there are only ~6% and ~10% genes in Arabidopsis and human respectively, encoding transcription factor (Riechmann et al. 2000; Babu et al. 2004). However, because transcription factors are essential for the regulation of gene expression, they are found in all living organisms (van Nimwegen 2003).

Basic helix-loop-helix (bHLH) transcription factors are one of the transcription factor families that have been found in all eukaryotes, including fungi, animals and plants (Heim et al. 2003; Skinner et al. 2010). The numbers of bHLH transcription factors in different organisms are different, with only 5 in yeast, 35 in *C. elegans*, 56 in *Drosophila*, ~150 in Arabidopsis and more than 170 in humans (Riechmann et al. 2000; Ledent and Vervoort 2001; Venter et al. 2001; Mewes et al. 2002; Heim et al. 2003; Toledo-Ortiz et al. 2003). Members of the bHLH transcription factor family are defined by the bHLH domain, which contains two functionally distinct regions: the N-terminal basic region that is involved in the DNA binding and the C-terminal HLH region that is involved in protein–protein dimerization (Heim et al. 2003; Toledo-Ortiz et al. 2003; Skinner et al. 2010). The bHLH transcription factors have diverse functions in different organisms. In mammals, for example, bHLH transcription factors control different cellular processes such as cell proliferation and cell lineage establishment (Grandori et al. 2000; Massari and Murre 2000; Skinner et al. 2010). In plants, bHLH transcription factors are involved in the regulation of plant growth and development, as well as plant response to environmental stimuli (Heim et al. 2003; Zhao et al. 2012). Structural, functional and phylogenetic analyses of the bHLH proteins have already been performed in mammals as well as in plants (Bailey et al. 2003; Heim et al. 2003; Toledo-Ortiz et al. 2003; Skinner et al. 2010).
Pleurotus ostreatus is one of the fungi whose whole genome has been sequenced (Riley et al. 2014; Alfaro et al. 2016; Castanera et al. 2016), but the function of the genes in *P. ostreatus* remained largely uncharacterized. We report here the identification and characterization of bHLH transcription factors in *P. ostreatus*. We found that there are 12 genes in *P. ostreatus* encoding bHLH transcription factors, that PobHLH1, 2, 5 and 8 were localized in the nucleus, and that they either functioned as a transcriptional activator or as a repressor when examined in transfected Arabidopsis protoplasts. We also found that PobHLH transcription factors differentially regulate plant growth and development when introduced into Arabidopsis by plant transformation.

2. Materials and methods

2.1. Identification of bHLH transcription factors in *P. ostreatus* and phylogenetic analysis

*P. ostreatus* bHLH transcription factors were identified by using the ‘GO’ catalog on MycoCosm (http://genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.home.html). The GO:0003674 molecular function’ term was first checked under the ‘GO’ catalog, followed by the ‘GO:0030528 transcription regulator activator’ term and finally the ‘GO:0003700 transcription factor activity’ term. The bHLH transcription factors were then identified by checking the annotation for each of the gene models under the ‘GO:0003700 transcription factor activity’ term. The entire amino acid sequences of the PobHLHs were obtained and subjected to phylogenetic analysis on Phylogeny (www.phylogeny.fr) by using the ‘One Click’ mode with default settings.

2.2. Plant materials and growth conditions

Fresh fruiting bodies of *P. ostreatus* were provided by the Mycological Institute of Jilin Agricultural University, and were used for RNA isolation. The Arabidopsis Columbia (Col) ecotype was used for protoplast isolation and plant transformation. For protoplast isolation and plant transformation, Arabidopsis seeds were sown directly into soil pots. For transgenic plant selection and phenotypic observation, T1 seeds were sterilized and sown on 1/2 MS plates containing antibiotics. All plants were grown in a growth room at 20°C with a 16/8 hr photoperiod at approximately 120 μmol m⁻² s⁻¹.

2.3. RNA isolation and RT-PCR

Total RNA from the fruiting bodies of *P. ostreatus* was isolated using Trizol (Ambion) by following the procedure provided by the manufacturer. Total RNA from leaves of 7-week-old Col wild-type and Arabidopsis transgenic plants was isolated using the EazyPure Plant RNA Kit (TransGen Biotech) by following the manufacturer’s protocol. One μg total RNA was subjected to cDNA synthesis by Oligo(dT)-primed reverse transcription, by using the Eazy Script First-Strand cDNA Synthesis Super Mix (TransGen Biotech) according to the manufacturer’s instruction. Synthesized cDNA was used for PCRs.

2.4. Plasmid construction

The reporter constructs LexA-Gal4-GUS and Gal4-GUS, and the effector constructs LD-VP, GD, GD-GL3 and GD-OPF1-N used for protoplast transient transfection assays have been described previously (Tiwari et al. 2004; Wang et al. 2007, 2008, 2014; Wang and Chen 2008; Wang X et al. 2015).

To generate the 35SS:HA-PobHLH, 35SS:GD-PobHLH and 35SS:GFP-PobHLH constructs, the full-length open-reading frame of the corresponding PobHLH gene was amplified by RT-PCR using RNA isolated from Fresh fruiting bodies of *P. ostreatus*, and the PCR products were cloned in frame with an N-terminal HA, GD or GFP tag, respectively, into the pUC19 vector under the control of the 35S promoter (Tiwari et al. 2004; Wang et al. 2005). The primers used are: PobHLH1, 5′-CAACATAGGTACACGATCATCAGCACAC-3′ and 5′-CAAGAGCTCTCAATAGCCTTTCTGAGGCTGACGG-3′; PobHLH2, 5′-CAACCATATGTCGACGAGCTCTCAATAGCCTTTCTGAGGCTGACGG-3′; PobHLH5, 5′-CAACATATGACATCTCCTCCACATCATGGCGAAGCCAG-3′ and 5′-CAAAGAGCTCTCCTCCTCCTCCAATTCC-3′ and 5′-CAAGAGCTCTCCTCCTCCTCCAATCATCATCATGTCCACATGACATCTCCTCCACATGGCGAAGCCAG-3′; PobHLH8, 5′-CAACATATGACATCTCCTCCACATCATCATGTCCACATGACATCTCCTCCACATGGCGAAGCCAG-3′ and 5′-CAAAATCGATTTAGAACGAGGCCGTGGGC-3′.

The corresponding 35SS:HA-PobHLH construct was double digested with the restriction enzyme PstI and SacI, and subcloned into the binary vector pFPZ211 for plant transformation. The 35SS:GD-PobHLH and 35SS:GFP-PobHLH constructs in pUC19 vector were used for protoplast transient transfection assays.

2.5. Plant transformation and transgenic plant selection

Wild-type Col Arabidopsis plants were used for transformation by the floral dip method (Clough and Bent 1998). About 5-week-old plants with several mature flowers on the main inflorescence were transformed with corresponding constructs via *Agrobacterium tumefaciens* GV3101.

Transgenic plants were selected on antibiotics plates containing 1/2 MS (Murashige & Skoog) with vitamins and 1% (w/v) sucrose, solidified with 0.6% (w/v) phytoagar. T1 seeds were sterilized and sown on 1/2 MS plates containing 50 mg/ml Kanamycin and 100 mg/ml Carbencillin. The plates were kept at 4°C in darkness for two days before being transferred into a growth room. Seven-day-old seedlings were transferred into soil pots and kept in a growth room with the growth conditions described above.

Phenotypes of the transgenic plants were examined in the T1 generation during the whole life cycle of the plants. Photographs were taken at different growth stages, RNA was isolated from leaves of 7-week-old plants and RT-PCR was used to examine the expression level of corresponding *PobHLH* genes in the transgenic plants. ACTIN2 (ACT2) (amplified by primers 5′-TATTTCCCACTGAGCATTTGTTTGGTACACGATCATCAGCACAC-3′ and 5′-GGTGGCAAGTGCTGTGTATTCTTCTTT-3′) was used as a control in PCRs.

2.6. Protoplast isolation, transfection and GUS activity assay

The procedures for Arabidopsis leaf mesophyll protoplast isolation, transfection and GUS activity assays have been
described previously (Tiwari et al. 2003; Wang et al. 2005; Tian et al. 2015; Wang X et al. 2015; Dai et al. 2016). Briefly, plasmid DNA of the reporters and effectors were isolated using the Gold Hi Endo Free Plasmid Maxi Kit (CWBiO) by following the manufacturer’s procedure, and co-transfected into protoplasts isolated from rosette leaves of ∼4-week-old Col wild-type Arabidopsis plants. The transfected protoplasts were incubated in dark at room temperature for 20–22 h, and then GUS activities were measured by using a Fluoroskan FInstruments Microplate Reader (MTX Lab Systems, Inc., Vienna, Virginia, USA).

Two different protoplast transfection assay systems were used in this study: the activator assay system and the repressor assay system. In the activator assay system, plasmids of the corresponding effector GD-PobHLH or GD alone and the reporter Gal4:GUS were cotransfected into the isolated protoplasts. The corresponding GD-PobHLH will be recruited to the Gal4 promoter region of the Gal4:GUS reporter gene via the fused GD domain, leading to the activation of the reporter gene in case the GD-PobHLH functions as an activator. In the repressor assay system, plasmids of the activator LD-VP, the corresponding effector GD-PobHLH or GD alone and the reporter LexA-Gal4:GUS were cotransfected into the isolated protoplasts. The activator LD-VP will be recruited to the LexA promoter region of the LexA-Gal4:GUS reporter gene via the fused LD domain, resulting in the activation of the reporter gene. The corresponding GD-PobHLH will be recruited to the Gal4 promoter region of the LexA-Gal4:GUS reporter gene via the fused GD domain, leading to the repression of the expression of reporter gene activated by the LD-VP activator, in case the GD-PobHLH functions as a repressor. The experiments were repeated at least three times with similar results.

3. Results
3.1. Identification of bHLH transcription factors in P. ostreatus
According to the ‘GO’ catalog on MycoCosm (http://genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.home.html), there are a total of 5850 genes in P. ostreatus that have been annotated, and 147 of them are encoding transcription factors. By examining each and all of the 147 transcription factor-encoding genes, we found 12 of them encoding bHLH transcription factors, which are collectively named as P. ostreatus basic helix-loop-helix1 through 12 (PobHLH1-PobHLH12) (Figure 1). The corresponding gene locus numbers for the PobHLHs identified are as follows: PobHLH1, estExt_Genewise1-Plus_C_031831; PobHLH2, estExt_Genewise1Plus_C_031929; PobHLH3, estExt_fgenesh1_pg_C_010203; PobHLH4, estExt_fgenesh1_kg.C_020077; PobHLH5, estExt_Genemark1.C_010384; PobHLH6, estExt_genemark.C_110334; PobHLH7, estExt_fgenesh2_pg.C_110277; PobHLH8, e_gw1.03.2699.1; PobHLH9, gw1.08.1595.1; PobHLH10, gw1.09.1179.1; PobHLH11, fgenesh1_pg.11. #_207; PobHLH12, estExt_fgenesh1_pm.C_040210.

As shown in Figure 1, structures of the PobHLH genes vary from one another. The exon number ranges from 1 to 8, with PobHLH12 having one and PobHLH12 having eight

Figure 1. Gene structure of the bHLH transcription factor genes in P. ostreatus. Data were obtained from MycoCosm (http://genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.home.html). Boxes indicate exons, and lines indicate introns. Red boxes indicate CDS, and blue boxes indicate 5′-UTR or 3′-UTR. Numbers above the exons or below the introns indicate the length of the exons or introns in bp. *, full-length CDS is not identified yet.
exons. However, it should be noted that the full-length coding sequences of PobHLH9 and PobHLH10 have not been annotated yet; it remains unknown how many exons they might have.

Similar to all the bHLH transcription factors identified so far, all the 12 PobHLHs contain a bHLH domain, though the location of the bHLH domain is different in the PobHLH proteins (Figure 2(a)). Phylogenetic analysis using full-length amino acid sequences of the PobHLHs showed that PobHLH1, 2, 4, 6, 7, 8 and 10 formed a subgroup, and PobHLH3, 5, 9, 11 and 12 formed another subgroup (Figure 2(b)).

### 3.2. Pobhlh2, 5 and 8 function as transcription activator, and PobHLH1 as repressor

The bHLH transcription factors are found in almost all organisms including fungi. It is known that bHLH transcription factors regulate multiple aspects of plant growth and development as well as plant metabolism (Zhao et al. 2012). Thus, we wanted to examine if PobHLHs may affect plant growth and development when expressed in Arabidopsis. PobHLH1, 2, 5 and 8 were chosen for this study. Based on the phylogenetic tree, PobHLH1, 2 and 8 are closely related and they form a clade, whereas PobHLH5 is from another subgroup. Thus, we were able to examine whether closely related PobHLHs may have similar functions.

![Figure 2](image)

**Figure 2.** The bHLH transcription factor genes in *P. ostreatus*. (a) Conserved bHLH domains in the PobHLH transcription factors. (b) Phylogenetic analysis of PobHLH transcription factors. The entire amino acid sequences of the PobHLH proteins were used for phylogenetic analysis, and the phylogenetic tree was generated by using ‘One Click’ mode with default settings on Phylogeny (www.phylogeny.fr). Branch support values are indicated above branches. The bar indicates branch length.

In plants, some bHLH transcription factors have been shown to be transcriptional activators (Wang and Chen 2008; Liu et al. 2015), and some are repressors (Nakata et al. 2013; Nakata and Ohme-Takagi 2013; Tian et al. 2015). To examine if PobHLHs may regulate plant growth and development, we examined if PobHLHs may function as transcriptional activators or repressors. As transcription factors have to be located in the nucleus for their proper function, we first examined PobHLHs’ subcellular localization by using protoplast transient transfection assay. Plasmids of GFP-PobHLHs were transfected into Arabidopsis protoplasts isolated from rosette leaves, and GFP florescence was observed after the transfected protoplasts were incubated overnight in darkness. We found that all the four PobHLHs examined are predominantly localized in the nucleus (Figure 3(a)).

We then examined transcriptional activities of PobHLHs in transfected protoplasts. The plasmids of effector GD-PobHLHs or GD control, and the reporter Gal4:GUS were co-transfected into protoplasts, and GUS activities were measured after incubation of the transfected protoplasts overnight in darkness. We found that co-transfection of GD control has little, if any, effect on the expression of the reporter gene, while co-transfection with the GD-PobHLH2, 5 or 8 resulted in the activation of the reporter gene (Figure 3(b)), suggesting that PobHLH2, 5 and 8 function as transcription...
activators. On the other hand, by co-transfecting plasmids of the activator LD-VP, effector GD-PobHLHs or control GD, and the reporter gene LexA-Gal4:GUS, we found that co-transfection of GD-PobHLH1 resulted in repression of the reporter gene activated by LD-VP (Figure 3(b)), indicating that PobHLH1 functions as a transcription repressor.

3.3. Transgenic Arabidopsis plants expressing PobHLH1 or 2 are morphologically similar to the wild-type plants

Having shown that the PobHLH2, 5 and 8 function as transcription activator, and PobHLH1 as repressor when tested in transfected protoplasts (Figure 3(b)), we further examined if they may differentially affect plant growth and development when introduced into plants. We thus generated transgenic plants expressing each of the four PobHLHs in the wild-type Col background. The expression of each PobHLH was driven by the 35S promoter.

Transgenic plants were selected on plates containing Kanamycin, and were transferred into soil pots for phenotypic observation. The transgenic plants were observed at all the different growth stages for any morphological defects. For more than 60 PobHLH1 transgenic plants obtained, we did not observe any obvious morphological phenotypes. Figure 4 shows four independent transgenic lines with different expression levels of PobHLH1. It is clear that these transgenic lines are morphologically indistinguishable from the wild-type plant at all the growth stages observed (Figure 4(a)). Similarly, we also obtained more than 60 PobHLH2 transgenic plants, none of which showed morphological difference to the wild type. Shown in Figure 5 are four independent transgenic lines with different expression levels of PobHLH2.

3.4. Ectopic expression of PobHLH5 or 8 affects multiple aspects of plant growth and development

Transgenic plants expressing PobHLH1 or PobHLH2 were morphologically similar to the wild-type plants. However, morphological changes were observed in transgenic plants expressing PobHLH5 or PobHLH8 when compared with the Col wild-type plants.

As shown in Figure 6, curled leaves were observed in the 35S:PobHLH5 transgenic plants, and the plants’ growth was faster at seedling stage when compared with that of the Col
wild-type plants. At a mature stage, the 35S:PobHLH5 transgenic plants showed a dwarf phenotype with smaller rosettes, shorter stems and smaller flowers (Figure 6). We also found that fertility in the transgenic plants was reduced, and the transgenic plants with high expression level of PobHLH5 could not produce any seeds (Figure 6).

Expression of PobHLH8 under the control of the 35S promoter in Arabidopsis also affected plants’ growth and development, but only in the late developmental stages. As shown in Figure 7, both 18-day-old and 30-day-old plants were largely indistinguishable from the Col wild-type plants. However, the PobHLH8 transgenic line with higher expression level produced dramatically reduced stems, with abnormal flowers which could not produce any seeds (Figure 7). We also noted that although the PobHLH8 transgenic line with relatively lower expression level was morphologically similar to the Col wild-type during the whole life cycle, it produced short siliques (Figure 7).

4. Discussion

P. ostreatus is a saprophytic fungus and an active lignin degrader in nature. Its whole genome sequence has been released recently (Riley et al. 2014; Alfaro et al. 2016; Castanera et al. 2016), which makes it possible to identify and characterize gene family in this fungus. The bHLH transcription factor family is one of the transcription factor families that have been found in all eukaryotes (Heim et al. 2003; Skinner et al. 2010). In this study, we report the genome-wide identification and characterization of bHLH transcription factors in P. ostreatus.

The numbers of bHLH transcription factors encoding genes vary from one organism to another, with only 5 in yeast and more than 170 in humans (Riechmann et al. 2000; Ledent and Vervoort 2001; Venter et al. 2001; Mewes et al. 2002; Heim et al. 2003; Toledo-Ortiz et al. 2003). We found that there are a total of 12 genes in P. ostreatus encoding bHLH transcription factors, and the gene structures vary in these genes, with only one exon in PobHLH2 and eight in PobHLH5 (Figure 1). On the other hand, all the 12 PobHLHs contain the bHLH domain (Figure 2), which defines the bHLH transcription factor family. These results suggest that the bHLH transcription factors may be conserved in the evolution of fungi, plants and animals.

It has been shown that some bHLH transcription factors, for example GLABRA3 (GL3), AtbHLH112 and AtMYC2, function as transcription activators (Wang and Chen 2008; Skinner et al. 2010; Liu et al. 2015), whereas some others such as bHLH129, JASMONATE-ASSOCIATED MYC2-LIKE1 (JAM1), JAM2 and JAM3 function as repressors (Nakata et al. 2013; Nakata and...
By using protoplast transient transfection assays, we found that all the four PobHLH transcription factors examined including PobHLH1, PobHLH2, PobHLH5 and PobHLH8 are nuclear proteins, and that PobHLH2, PobHLH5 and PobHLH8 function as transcription activators, and PobHLH1 as repressor (Figure 3). These results indicate that bHLH transcription factors in *P. ostreatus* may also act to activate or repress gene expression.

In both animals and plants, bHLH transcription factors have been shown to be involved in the regulation of growth and development. For example, in mammals, bHLH transcription factors regulate cell proliferation as well as cell lineage establishment (Lee and Young 2000; Massari and Murre 2000; Skinner et al. 2010). In plants, bHLH transcription factors regulate different aspects of plant growth and development (Heim et al. 2003; Zhao et al. 2012). By generating transgenic plants expressing PobHLH genes, we found that although PobHLH1 function as transcription repressor and PobHLH2 as activator in transfected protoplasts (Figure 3), none of them affected plant growth and development (Figures 4 and 5). On the other hand, both PobHLH5 and PobHLH8 activated reporter gene expression in transfected protoplasts (Figure 3), and both of them affected plant growth and development when ectopically expressed in Arabidopsis (Figures 6 and 7). However, we also found that PobHLH5 and PobHLH8 differentially affected plant growth and development, PobHLH5 affected plant growth and development at all the growth stages examined (Figure 6), whereas PobHLH8 affected plant growth and development only at late stages (Figure 7). Considering that the interaction between transcription factors is complicated (Karin 1990), the different effects between the PobHLHs on plant growth and development may be caused by activation of potential target genes, as well as different interactions between PobHLHs and Arabidopsis transcription factors.

It is worth mentioning here that we also observed the offspring (T2 generations) of the 35S:PobHLH5 and 35S:PobHLH8 transgenic Arabidopsis plants which showed defects but were still able to produce seeds. We found that none of the offspring showed any morphological difference to the Col wild-type plants (data not shown). It will be of great interest to examine why the effects of PobHLH5 and PobHLH8 on plant growth and development could not pass to subsequent generations.

Nevertheless, this study showed that there are 12 genes in *P. ostreatus* encoding bHLH transcription factors, that these transcription factors can regulate reporter gene expression in transfected protoplasts when recruited to the promoter region of the reporter gene and that some of the transcription factors can regulate plant growth and development when ectopically expressed in Arabidopsis. These results provide the first insight of the bHLH transcription factors in *P. ostreatus*, and will be helpful in future studies for characterization of their functions.

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**Disclosure statement**

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