INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18F–118/2018/20–11–2549–2555
DOI: 10.17957/IJAB/15.0813
http://www.fspublishers.org

Full Length Article

C2H2 Transcription Factor *brlA* Regulating Conidiation and Affecting Growth and Biosynthesis of Secondary Metabolites in *Aspergillus clavatus*

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Abstract

Aspergillus clavatus is a promising candidate suited for industrial production with its capacity of producing a variety of important metabolites. However, the research of gene functions in *A. clavatus* is still stagnant. Cloning a 1290 bp *brlA* gene, sequence analysis revealed that it encoded a C2H2 type transcription factor in *A. clavatus*. Deletion of the gene by building a *brlA*-deleted plasmid, numerous significant alterations occurred in growth, colonial morphology, microstructure, and conidiation. The HPLC analysis showed that secondary metabolism was also changed in the *brlA*-deleted mutant. The results indicated that *brlA* gene regulated conidiation and it was also involved in the regulation of growth and biosynthesis of secondary metabolites in *A. clavatus*. © 2018 Friends Science Publishers

Keywords: *Aspergillus clavatus*; *brlA* gene; Conidiation; Growth; Secondary metabolites

Introduction

Considerable efforts have been devoted to research on *Aspergillus clavatus* in the past decades owing to its high capacity for secreting various enzymes, such as extracellular acid protease, alkaline protease and endo-xylanase (Punt *et al*., 2002; Tremacoldi and Carmona, 2005; Squina *et al*., 2009; Silva *et al*., 2011). A novel thermostable antifungal peptide and two novel hepatocellular carcinoma cycle inhibitory cyclodepsipeptides were isolated from *A. clavatus* (Skouri-Gargouri and Gargouri, 2008; Jiang *et al*., 2013), which make it become one of the important sources of significant valuable polypeptides. *A. clavatus* has the capacities for the degradation of low-density polyethylene and biosynthesis of antimicrobial silver nanoparticles and anisotropic gold nanotriangles applied in nanomedicine (Saravanan and Nanda, 2010; Verma *et al*., 2010, 2011; Gajendiran *et al*., 2016). In our laboratory, *A. clavatus* was collected because it can produce lovastatin, a kind of cholesterol lowering drugs, but meanwhile, it can also produce patulin, a kind of mycotoxins.

A. clavatus has a complex life cycle, and the asexual life cycle is divided into two major stages, namely hyphae growth stage and conidiation stage (the main means of reproduction). It is found that conidiation possesses precisely timed and genetically programmed properties, and requires activating *brlA* gene in the *Aspergillus nidulans* (Timberlake, 1980; Adams *et al*., 1988, 1990; Timberlake, 1991). Studies of *A. nidulans* show that *brlA* is an extremely important gene in central regulatory pathway (CRP) of conidiation (Adams *et al*., 1988; Mirabito *et al*., 1989; Park and Yu, 2012). There is a "bristle-like" structure that produces an elongated stalk and fails to develop vesicles or any other subsequent structures in *brlA* null mutant (Clutterbuck, 1969; Boylan *et al*., 1987; Park and Yu, 2012). By contrast, growth cessation and formation of viable conidia directly from the hyphae tips will occur when there is over-expression of *brlA* gene in vegetative cells (Adams *et al*., 1988; Han *et al*., 1993). It is found that *brlA* gene encodes a C2H2 zinc-finger transcription factor (TF) that governs conidiation processes combined with other regulators in *A. nidulans* (Adams *et al*., 1990; Lee and Adams, 1994; Etxebeste *et al*., 2010). In *Aspergillus fumigatus*, a series of data suggest that *brlA* might provide a certain impact on vegetative growth, in addition to its role in spore development (Mah and Yu, 2006; Twumasi-Boateng *et al*., 2009). Moreover, it has been reported that biosynthesis of some secondary metabolites is correlated to *brlA* gene in *A. fumigatus* (Lim *et al*., 2014).

To explore the functions of the *brlA* gene in *A. clavatus*, the *brlA* gene was deleted. Variations in growth, colonial morphology, microstructure, conidiation and metabolites were analyzed. The results revealed that *brlA* regulated conidiation and it was also involved in the regulation of growth and biosynthesis of secondary metabolites in *A. clavatus*.

Materials and Methods

Plasmids and Strains

pMD-19T vector was purchased from Takara (Dalian, Japan). pKO1B (Fig. 1) (Lu *et al*., 2014) and pCB1003 were presented by Zhejiang University. *A. clavatus* Ac-32 serving

To cite this paper: Han, X., C. Xu, Q. Zhang, B. Jiang, J. Zheng and D. Jiang, 2018. C2H2 transcription factor *brlA* regulating conidiation and affecting growth and biosynthesis of secondary metabolites in *Aspergillus clavatus*. *Int. J. Agric. Biol*., 20: 2549–2555

as a recipient was obtained from the mouldy fruit and was stored in China Center for Type Culture Collection (CCTCC, No.M2015504) (Han and Jiang, 2017). *Agrobacterium tumefaciens* AGL-1 and *Escherichia coli* DH5α were preserved by our laboratory.

Cloning and Analysis of the *brlA* Gene

The *brlA* gene sequence and the whole genome shotgun sequence of *A. clavatus* were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). *A. clavatus* Ac-32 genomic DNA serving as the template for amplification was extracted with the method of cetyltrimethylammonium bromide (CTAB). A pair of primers, brlA-F and brlA-R (Table 1), was designed to amplify the *brlA* gene. Amino acid sequence encoded by *brlA* was also predicted on the NCBI website (http://www.ncbi.nlm.nih.gov/), and its tertiary structure was predicted with SWISS-MODEL (https://swissmodel.expasy.org/). Homology of the gene sequence and the deduced amino acid sequence was analyzed using software NCBI.

Construct the *brlA* Gene Deletion Vector

A gene deletion plasmid containing *brlA*-deleted structure was constructed for the target gene deletion. A 933 bp 5' flanking region, a 940 bp 3' flanking region and 1406 bp *hph* gene were fused by using double-joint PCR to generate the *brlA*-deleted cassette (*Bam*H I - 5' flanking region - *hph* gene - 3' flanking region - *Hin*d III) (Fig. 2a and Fig. 3a) (Yu *et al*., 2004). The *brlA*-deleted cassette was purified and cloned into vector pMD19-T, namely pMD19-TB. The pMD19-TB plasmid was transformed into competent *E. coli* DH5α and cloned accompanied by propagation of *E. coli*. After that, the reproductive pMD19-TB plasmid was extracted from *E. coli* and digested with *Bam*H I and *Hin*d III. The *brlA*-deleted cassette containing restriction enzyme cutting sites was isolated purified, and ligated into linearized pKO1B plasmid that has been digested with *Bam*H I and *Hin*d III by T4 DNA ligase to generate plasmid pKOBA. The pKOBA plasmid was transformed into competent *A. tumefaciens* AGL-1 using a freeze-thaw method (Chen *et al*., 1994).

Deletion of *brlA* Gene in *A. clavatus* Ac-32

Homologous replacement was applied for the deletion of *brlA* gene in *A. clavatus* Ac-32. The pKOBA vector was transformed into *A. clavatus* Ac-32 with the method of *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Han and Jiang, 2017), and the *brlA*-deleted cassette was integrated into *A. clavatus* Ac-32 genome for replacing the *brlA* with *hph* gene, which is shown detailedly in Fig. 2b.

PCR Characterization and Southern Blot Analysis

PCR analysis and Southern blot were performed to confirm the homologous replacement event. Primer sets hph-F/hph-R, brlA-F/brlA-R and 5UTR-F/3UTR-R (Table 1) were used for amplification from genomic DNA of one putative *brlA*-deleted mutant using, respectively. PCR products were examined by 1% (w/v) agarose gel. The genomic DNA of the putative *brlA*-deleted mutant was digested with *Eco*R I, subsequently, separated by electrophoresis in 0.7% agarose gel at 25 V and a lower temperature for 12 h. The *hph* gene probe (synthesized by Sangon, Shanghai) was labeled with digoxigenin (DIG) for signal detection. Probe labeling, hybridization, and immunological detection were performed according to the manufacturer's protocol (Roche, Germany).

***brlA* Gene Expression Analysis**

The *brlA* gene expression was ascertained using reverse transcription PCR with primers brlA-F and brlA-R (Table 1). The *GAPDH* gene was used as a reference gene. Total RNA was isolated from mycelia cultured on PDA plate using RNAiso Plus Kit (Takara, Dalian, Japan) according to the manufacturer's protocol. And reverse transcription of RNA into cDNA was performed with PrimeScript® Reverse Transcriptase (Takara, Dalian, Japan) according to the manufacturer's protocol. Subsequently, cDNA served as the template for amplification using primer sets GAPDH-F/GAPDH-R and brlA-F/brlA-R (Table 1). PCR products were examined by 1% (w/v) agarose gel.

Phenotypic Characterization, Microstructure and Fluorescence Analysis

The *brlA*-deleted mutant was cultivated on PDA plate without antibiotics for 7 d at 30°C. Subsequently, colony shape and color were detected. Hyphae prepared on glass slides were examined by an optical microscope. Expression of the *green fluorescent protein* (*GFP*) gene from pKOBA plasmid was evaluated with Leica TCS sp5 laser confocal fluorescence microscope at 488 nm excitation light. Hyphae of the *brlA*-deleted mutant were prepared on glass slides after 5 d growth on PDA plate without antibiotics at 30°C for fluorescence detection.

Growth and Secondary Metabolites Analysis

Weights of dry mycelia from 1 to 8 d liquid fermentation were obtained to depict the growth curves of the *brlA*-deleted mutant and wild type. Colony diameters from 1 to 8 d cultivation were recorded to describe the growth rates of the *brlA*-deleted mutant and wild type. HPLC was performed to detect yields of lovastatin and patulin in fermentation broth using a reverse-phase C18 column (Venusil XBP, 5 μm, 4.6 mm × 250 mm). Gradient elution was carried out to separate lovastatin using acetonitrile (solvent A) and water (pH 2.5, adjusted by phosphoric acid) (solvent B). After injection of 20 μL sample solution, solvent A was retained 75% (v/v) at the first five minutes, reduced to 65% (v/v) at the second five minutes, 55% (v/v) at the third five minutes, and 45% (v/v) at the fourth five minutes. On the contrary, solvent B was

Table 1: PCR primers used in this study

Name	Sequences(5' → 3')	Descriptions
brlA-F	ATGAGATCGCAGAGTAAC	For amplification of 1290 bp *brlA* gene
brlA-R	TCACTCATCCCAGCCATC	
hph-F	CGAGAGAAGATGATATTGAAGGAGC	For amplification of 1406 bp hygromycin B phosphotransferase (*hph*) gene
hph-R	TCTTGTTCGGTCGGCATCTACTCTA	
5UTR-F	CGGGATCCCG-ATCGTTCCCCTTGCTGGTAT	For amplification of 933 bp 5' flanking region of *brlA* gene, and adding *Bam*H I and 18 bp fragment of *hph* gene at 5'-end and 3'-end of 5' flanking region respectively
5UTR-R	CAATATCATCTTCTCTCG-ATCCTCGTTGTCTTCGAC	
3UTR-F	ATAGAGTAGATGCCGACA-ATCAGAACAGAGAAGAT	For amplification of 940 bp 3' flanking region of *brlA* gene, and adding *Hin*d III and 18 bp fragment of *hph* gene at 3'-end and 5'-end of 3' flanking region respectively
3UTR-R	CCCAAGCTTGGG-TAGGGCCAGTTTGGTTTACT	
GAPDH-F	ATGGGAGTCAACCACACCAG	For amplification of 551 bp *GAPDH* gene acting as reference gene
GAPDH-R	TTGTCGTACCAGGAAACGAG	

increased accompanied by solvent A reduction. The flow rate of mobile phase was 1.0 mL/min, and the temperature of the column was maintained at 28°C. Absorbance was monitored using UV-VIS detector at 238 nm (Zhao *et al*., 2014). Patulin was separated and detected with following protocol: 10% acetonitrile aqueous solution (v/v) was acted as mobile phase, the flow rate was 0.8 mL/min, the temperature of the column was maintained at 28°C, and the wavelength of detector was maintained at 276 nm (Zhou *et al*., 2012).

Results

Analysis of *brlA* Gene of *A. clavatus*

A 1290 bp *brlA* gene of *A. clavatus* Ac-32 was cloned (Fig. 3a). Sequence analysis revealed that the sequence of *brlA* gene of *A. clavatus* Ac-32 was consistent with that of *A. clavatus* that was stored in GenBank with accession number XM_001268617, and had 66.7% similarity with that of model organism *A. nidulans*. The sequence of deduced 429 amino acids encoded by *brlA* of *A. clavatus* Ac-32 had 69.9% similarity with that of *A. nidulans*. The *brlA* gene of *A. clavatus* Ac-32 encoded a C2H2 type transcription factor including two α-helices and two antiparallel β-folds (the tertiary structure predicted with SWISS-MODEL shown in Fig. 4).

Generation of *brlA*-deleted Mutant

52 transformants were obtained, but only one mutant was obviously different from wild type, as shown in Fig. 5. The putative *brlA*-deleted mutant was characterized by PCR, results shown in Fig. 3b. No *brlA* gene band but a bright *hph* gene band indicated that a successful homologous replacement event occurred in the putative *brlA*-deleted mutant. Genome DNA of the *brlA*-deleted mutant and wild type was digested with *Eco*R I, and hybridized using *hph* gene probe, results shown in Fig. 3c. Only one hybridized band in approximately 4.5 kb fragment of the *brlA*-deleted mutant indicated that a single copy of *hph* gene was carried by the *brlA*-deleted mutant.

GFP Gene and *brlA* Gene Expression Analysis

When the pKOBA was transformed into *A. clavatus* Ac-32 the *GFP* gene could be expressed. As shown in Fig. 6, the green fluorescence only detectable in the *brlA*-deleted mutant indicated that the pKOBA plasmid was integrated into genome DNA of *A. clavatus* Ac-32 and the *GFP* gene has been expressed. The expression level of *brlA* was tested with reverse transcription PCR. As shown in Fig. 7, the expression level was too low to detect in *brlA*-deleted mutant, which indicated that the *brlA* gene has been destroyed. It was demonstrated again that the homologous replacement event has occurred in the mutant.

Phenotypic Characterization, Microstructure Analysis

The *brlA*-deleted mutant was significantly different from wild type. In phenotype, compared with wild type that had a thick layer conidia on loose mycelia appearing green circinate and radial grooves, the *brlA*-deleted mutant had no conidia but relatively dense white mycelia with lots of mycelia that was exposed in air appearing a "bristle-like" structure (Fig. 5) (Park and Yu, 2012). It can be deduced that *brlA* gene was involved in conidiation and pigment biosynthesis (Twumasi-Boateng *et al*., 2009). In microstructure, wild type had intumescent vesicles with conidiation structures and conidia on it at the end of stalks, while the *brlA*-deleted mutant just had elongated stalks without vesicles or any other subsequent structures (Fig. 8) (Clutterbuck, 1969; Park and Yu, 2012). Based on these evidences, it can be proved that *brlA* gene appropriately regulated the developmental program of conidiation in *A. clavatus*.

Growth and Secondary Metabolites of Analysis

The growth rate and the kinds of secondary metabolites were changed after deletion of the *brlA* gene in *A. clavatus*. Compared with wild type, the growth rate of the *brlA*-deleted mutant cultivated in PDA plate was slower (Fig. 9a). In PDB liquid medium, the growth stages of the *brlA*-deleted mutant were observably

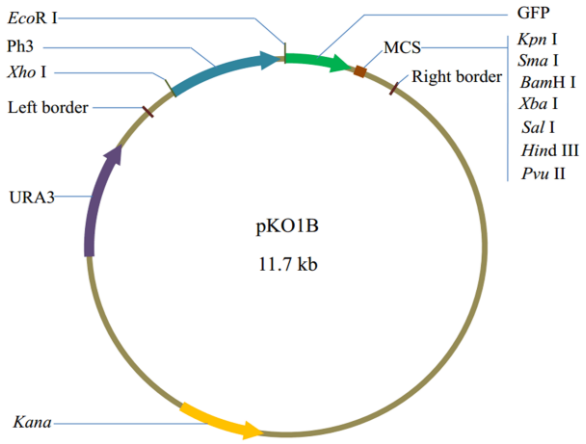

Fig. 1: The construction of pKO1B plasmid. It contains kanamycin resistance gene (*Kana*) and multiple cloning sites (MCS) that are contributed to remolding plasmid

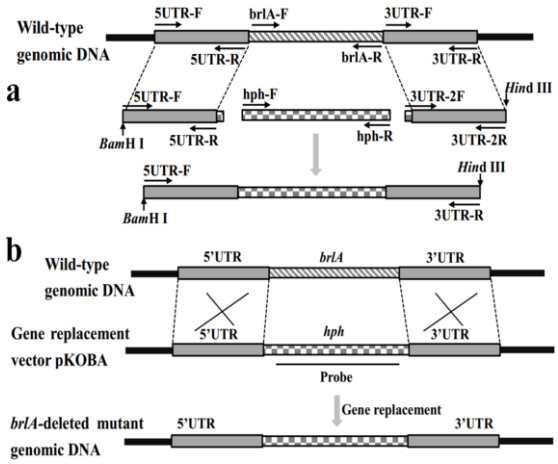

Fig. 2: Deletion of *brlA* gene in *A. clavatus* Ac-32. **a** Building of *brlA*-deleted cassette. The 5'-flanking region and 3'- flanking region were from *A. clavatus* Ac-32 genomic DNA, and *hph* gene was from pCB1003 plasmid. **b** Schematic representation of homologous recombination strategy yielding *brlA*-deletion mutant. Probe used for Southern blot analysis is shown as black lines

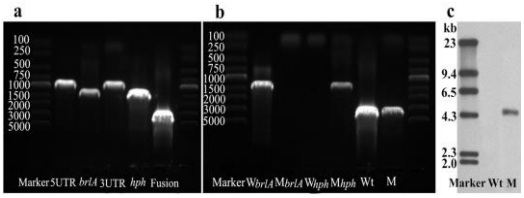

Fig. 3: PCR characterization and Southern blot analysis. **a** Fragments used for building *brlA*-deleted cassette. Lane 1, marker; lane 2, 5'-flanking region; lane 3, *brlA* gene; lane 4, 3'-flanking region; lane 5, *hph* gene; lane 6, fusion of three fragments. **b** Confirmation of homologous recombination event by PCR. Lane 1, marker; lane 2 and 3, brlA-F/brlA-R for amplification; lane 4 and 5, hph-F/hph-R for amplification; lane 6 and 7, 5UTR-F/5UTR-R for amplification; lane 2, 4 and 6, genomic DNA of wild type serving as template for amplification; lane 3, 5 and 7, genomic DNA of putative *brlA*-deleted mutant serving as template for amplification. **c** Southern blot analysis of the putative *brlA*-deleted mutant using *hph* probe. Lane 1, DNA marker; lane 2, wild type; lane 3, the *brlA*-deleted mutant

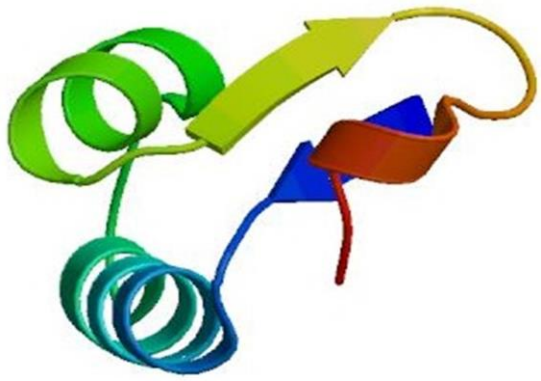

Fig. 4: Predicted tertiary structure of C2H2 transcription factor encoded by *brlA* gene of *A. clavatus* Ac-32

different from with wild type (Fig. 9b). Moreover, conidiation is closely related to the production of certain mycotoxins (Calvo *et al.*, 2002). Certain mycotoxins could not be produced by the *brlA*- deleted mutant that was failed to develop conidia, which was a potential reason that the maximum weight of dry mycelia of the *brlA*-deleted mutant (11.80 ± 0.45 g/L) was more than that of wild type (10.86 ± 0.98 g/L). The HPLC analysis revealed that heights and areas of some peaks were different from wild type, especially using 276 nm wavelength for detection (Fig. 10), which ascribed to the deficiency of *brlA* gene blocking the synthesis of certain compounds and leading to the accumulation of the upstream compounds. Lovastatin and patulin, two important secondary metabolites, were both increased after the deficiency of *brlA* gene in *A. clavatus* (Fig. 9c). (Yields of lovastatin and patulin were increased 18.36% and 20.96%, respectively). Therefore, it can be inferred that the *brlA* gene was related to the growth and biosynthesis of secondary metabolites.

Discussion

In the past decades, the conidiation process has been researched detailedly in the model organism *A. nidulans*. *brlA* gene, the best-characterized developmental regulatory gene, attracted more attention for its special location in the regulatory pathway of conidiation (Han *et al.*, 1993; Sewall, 1994; Han and Adams, 2001).

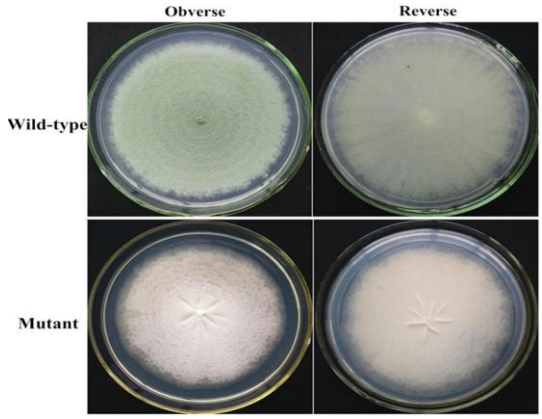

Fig. 5: Comparison of obverse and reverse of the wild type and the *brlA*-deleted mutant in colony phenotype

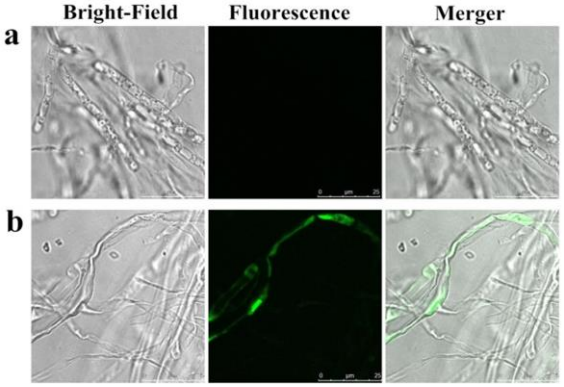

Fig. 6: Fluorescence characteristic analysis. **a** Wild type. **b** The *brlA*-deleted mutant

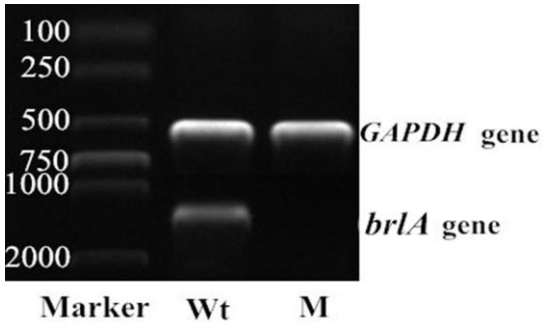

Fig. 7: The determination of *brlA* gene expression by reverse-transcription PCR. Lane 1, marker; lane 2, wild type; lane 3, the *brlA*-deleted mutant

Moreover, there were also some reports about the functions of *brlA* gene in pathogen *A. fumigatus* (Twumasi-Boateng *et al*., 2009; Etxebeste *et al*., 2010; Tao and Yu, 2010). In the regulation of conidiation, the *brlA* gene had similar regulatory mechanism in *A. nidulans* and

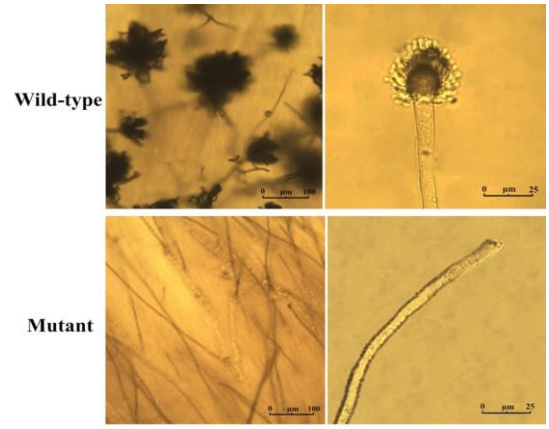

Fig. 8: Microstructure of wild type and *brlA*-deleted mutant cultivated in PDA plate for 7 d

A. fumigatus (Emri *et al*., 2005; Park and Yu, 2012), but it was strikingly different when it comes to regulation of secondary metabolism (Calvo *et al*., 2002; Lim *et al*., 2014). In addition, few reports suggested that *brlA* gene had an effect on growth rate in *Aspergillus* spp. In this paper, the conidia were failed to develop, and the growth and the secondary metabolites altered obviously when deleted the *brlA* gene in *A. clavatus*. It revealed that the function of *brlA* gene was similar in the regulation of conidiation, but different in growth and secondary metabolism in *Aspergillus* spp.

brlA, *abaA* and *wetA* genes composed a central regulatory pathway (CRP) of conidiation in *A. nidulans* (Timberlake, 1980, 1991; Aguirre, 1993). *brlA* gene located the upstream of the CRP, and directed the expression of *abaA* and *wetA* (Mirabito *et al*., 1989; Sewall, 1994). *brlA* gene encoded a C2H2 Zinc (II) transcription factor (TF), a nucleic acid binding protein, which regulated the expression of downstream genes by binding on nucleic acid in *A. nidulans* (Adams *et al*., 1990; Aguirre, 1993). Activation of *brlA* gene was an essential step of conidiation, which was required for hyphae transformation into conidiophore in *A. nidulans* (Chang and Timberlake, 1992; Lee and Adams, 1995; Emri *et al*., 2005). The conidiation pattern of *A. nidulans* has been summarized detailedly by Etxebeste and Park (Etxebeste *et al*., 2010; Park and Yu, 2012). *brlA* gene was not well conservative in *Aspergillus* spp. In our study, *BrlA* gene of *A. clavatu*s had 66.7% similarity with that of *A. nidulan*s, and also encoded a C2H2 TF. The type of TF encoded by *brlA* gene in *A. clavatus* was consistent with that in *A. nidulans*, which was a precondition of the functional consistency. When deleted the *brlA* gene, the alteration of conidiation in *A. clavatus* was similar to that in *A. nidulans*. It can be deduced that the *brlA* gene function of *A. clavatus* was similar to that of *A. nidulans* in the regulation of conidiation. Further, the conidiation pattern of *A. nidulans* could also be used in *A. clavatus*.

The results in this paper suggested that *brlA* gene

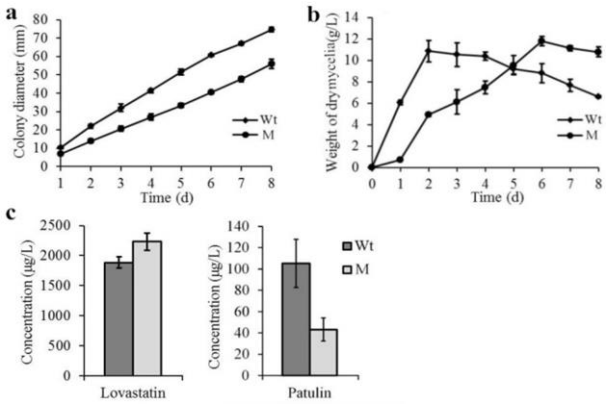

Fig. 9: The growth and secondary metabolites of wild type and the *brlA*-deleted mutant. **a** Colony diameters from 1 - 8 d cultivation in PDA plate. **b** Weights of dry mycelia from 1 - 8 d fermentation in 50 mL PDB liquid medium. **c** Yields of lovastatin and patulin after 7 d cultivation in PDB liquid medium. Each group of experiments was installed three repetitions

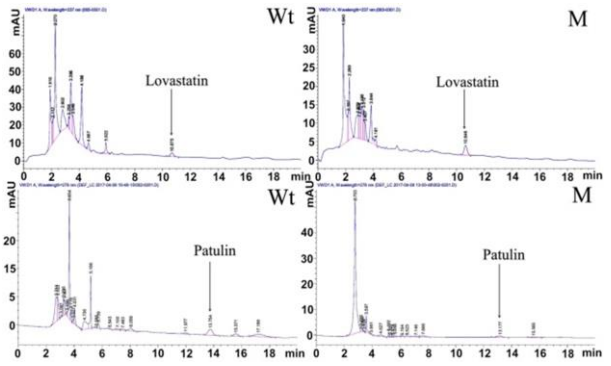

Fig. 10: HPLC analysis of lovastatin and patulin produced by wild type and *brlA*-deleted mutant after 7 d cultivation in PDB liquid medium. Samples of fermentation broth were diluted with quadruple volume methanol and filtrated with 0.45 μm filter membrane before used. Each group of experiments was installed three repetitions

provided a certain impact on growth and the secondary metabolism, in addition to its roles in conidiation. The synthesis of secondary metabolites altered when deleted the *brlA* gene in *A. clavatus*, and similar results also occurred in *A. fumigatus* (Twumasi-Boateng *et al*., 2009; Lim *et al*., 2014), which was due to the deficiency of *brlA* gene impacting the expression of related gene that regulated the synthesis of secondary metabolites. *A. clavatus* can produce several secondary metabolites at the same time. It is also possible that deletion of *brlA* resulted in elevated production of certain metabolites while it also inhibited the production of others.

Conclusion

In summary, *brlA* regulated conidiation, and it also affected growth and biosynthesis of secondary metabolites in *A. clavatus*. This study could provide a basis for controlling the synthesis of secondary metabolites by controlling conidiation in *A. clavatus*, and it would have significance in industrial production.

Acknowledgments

This work was supported by the grants from the National Natural Science Foundation of China (NO. 31570013 and NO. 31270061).

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(Received 04 June 2018; Accepted 09 July 2018)