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Research Paper

A B-type histone acetyltransferase Hat1 regulates secondary metabolism, conidiation, and cell wall integrity in the taxol-producing fungus *Pestalotiopsis microspora*

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In filamentous fungi, many gene clusters for the biosynthesis of secondary metabolites often stay silent under laboratory culture conditions because of the absence of communication with its natural environment. Epigenetic processes have been demonstrated to be critical in the expression of the genes or gene clusters. Here, we report the identification of a B-type histone acetyltransferase, Hat1, and demonstrate its significant roles in secondary metabolism, conidiation, and the cell wall integrity in the fungus *Pestalotiopsis microspora*. An *hat1* deletion strain shows a dramatic decrease of SMs in this fungus, suggesting *hat1* functions as a global regulator on secondary metabolism. Moreover, the mutant strain *hat1Δ* delays to produce conidia with significantly decreased number of conidia, while shows little effect on vegetative growth, suggesting that it plays a critical role in conidiation. The hypersensitivity of *hat1Δ* to Congo red demonstrates that disruption of *hat1* impairs the integrity of cell wall. Overexpression of the wild-type *hat1* allele enhances conidiation by boosting the number of conidia. This is the first report on the role of a B-type histone acetyltransferase in fungal secondary metabolism and cell wall integrity.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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Introduction

Filamentous fungi are well known for their capacity to produce a multitude of low molecular weight bioactive compounds known as secondary metabolites (SMs). These natural molecules have long been exploited for the purpose of hunting new antibiotics, anticancer and anti-viral agents, and others [1]. In the environment, they can provide a selective advantage for the fungal hosts. Reflecting this, the gene clusters coding for enzymes responsible for the biosynthesis of these SMs are frequently "silent" under laboratory conditions. Accumulating evidence suggests that covalent modifications of chromatin, for instance, phosphorylation, ubiquitination, ADP-ribosylation, and methylation, are important regulatoty mechanisms used by fungi to modulate the expression of genes involved in secondary metabolism [2]. Among them, acetylation is the best-characterized mechanism [3].

In eukaryote, the highly conserved histone acetyltransferase (HAT) complexes have a central role in diverse biological processes, such as chromatin assembly [4, 5], gene silencing [6], DNA repair [7], and cell-cycle progression [8]. HATs are also found as an integral subunit of the elongating RNA polymerase II holoenzyme [9]. Thus, function of HATs is required for cell growth and differentiation, and even the circadian rhythm [10]. The regulation by HATs on

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secondary metabolism in fungi are also observed in several fungal organisms. For example, an initial study examining the aflatoxin gene cluster in *Aspergillus parasiticus* found a temporal link between histone acetylation and the transcription of the aflatoxin gene cluster [11]. Nützmann et al. have demonstrated the role of acetyltransferases in the expression of orsellinic acid gene cluster in *Aspergillus nidulans* by deletions 36 of the 40 acetyltransferases in the genome [12]. The overexpression of *A. nidulans* histone acetyltransferase *Esa* A can increase the production of SMs in this fungus [13]. Moreover, histone deacetylase (HDAC) inhibitors (e.g., Trichostatin A) treatment alter transcriptional level of several SM genes and SMs production in several fungi [14–17].

Generally, two families of histone acetyltransferase have been recognized, that is, type A or type B [18]. A-type HATs are located in the nucleus and acetylate nucleosomal histones within chromatin, while B-type HATs are originally found to be a distinct class of cytoplasmic enzymes that acetylate free, but not chromatin-associated histones [19]. Among the B-type HATs, for example, Hat1, Rtt109, and HatB3.1, in yeast, Hat1 is the only B-type HAT that is conserved from yeast to human [5, 20]. It was identified as a HAT in a temperature-sensitive yeast mutant [21] and is a paradigm of the GCN5-related N-acetyltransferase (GNAT) superfamily [22]. Previous study demonstrated that the deletion of the Hat1 gene in budding yeast resulted in no observable phenotypes [21, 23], but defects in chromatin assembly and DNA repair [24, 25]. The human equivalent Hat1 was also identified [26]. Though there were reviews and papers related to regulation of secondary metabolism in fungi by HATs, little is known about the function of Hat1 on SMs by far [3, 20, 27, 28].

Pestalotiopsis microspora NK17 was isolated by our laboratory as a taxol-producer [29]. Pestalotiollide B, a derivative of dibenzodioxocinones (a new class cholesterol ester transfer protein) has lately been isolated from the culture of NK17 at high level [30]. To investigate the regulation of the secondary metabolism, we aim to examine the role of HAT family in the biosynthesis of SMs in our fungus NK17. In this work, we identified a putative B-type in NK17 genome which showed high homology to Hat1 subfamily, thus, designated as *hat1*. Through targeted deletion of *hat1* in this fungus, we showed that *hat1* played a critical role in the biosynthesis of secondary metabolites, conidiation, and cell wall structure, but showed relatively little effect on vegetative growth.

Materials and methods

Strains and culture conditions

The taxol-producing fungus *P. microspora* NK17 was originally isolated by our lab [18]. Its uracil auxotrophic strain, NK17-*ura3Δ*, was created by this lab [31] and used as the recipient strain in this study. The fungal strains were routinely cultured in Potato Lactose Broth (PLB) at 28 °C, 180 rpm, or on PLA (PLB supplemented with 2% (w/v) agar) at 28 °C.

The plasmid pOSCAR (Invitrogen, CA) harboring the *ccdB* gene was propagated in *Escherichia coli* strain DB3.1 [32]. The propagation of the vector, pA-Ura3-OSCAR, and other manipulations were generally conducted in *E. coli* DH5α. *Agrobacterium tumefaciens* LBA4404 was used for *A. tumefaciens*-mediated transformation (ATMT) of NK17. All bacterial strains were grown in Luria–Bertani (LB) medium added with appropriate antibiotics at 28 °C (for *A. tumefaciens*) or 37 °C (for *E. coli*), 180 rpm when needed. Inducing medium (IM) and Yeast Nitrogen Base (YNB) as a minimal were used for the transformation of NK17.

Construction of deletion vector

The deletion vector of *hat1* was constructed by the OSCAR method described by Paz et al. [32]. The plasmid, pA-Ura3-OSCAR, containing *pm-ura3* that could complement the uracil auxotrophic deficiency of NK17-*ura3Δ*, worked as maker vector in BP clonase reaction. A pair of primers, Hat1-up(F)/Hat1-up(R) (Table 1), containing attB2r and attB1r sequence, respectively, were designed to amplify the 663 bp 5′ flanking region of *hat1*. The primers, Hat1-down(F)/Hat1-down(R), containing attB4 and attB3 sequence, respectively, were designed to amplify the 789 bp 3′ flanking fragment of *hat1*. The two PCR fragments were then gel purified separately using the AxyPreP DNA Gel Extraction Kit (Axygen, USA).

To create the *hat1* deletion construct, a 5 μl BP clonase reaction mixture was set up to include 20 ng of each purified 5′ and 3′ *hat1* fragments, 60 ng DNA of pA-Ura3-OSCAR and pOSCAR (respectively) plus 1 μl BP clonase II enzyme (Invitrogen, CA, USA). After incubation at 25 °C overnight, the reaction was terminated by adding 0.5 μl proteinase K (20 $\mu g\,\mu l^{-1}$). The reaction mixture was then transformed into *E. coli* DH5α by heat shock. Bacterial colony was covered on LB plate added with 100 $\mu g\,ml^{-1}$ spectinomycin following overnight incubation at 37 °C. Two pairs of primers, Hat1-up(F)/Ura3-orf(R) and Ura3-orf(F)/Hat1-down(R), were used in PCR verification of deletion construct, pOSCAR-*hat1* (Supporting Information Fig. S1).

Table 1. Primers used in the study.

Primer	Primer sequence, 5′→3′
Hatl-up(F)	GGGGACAGCTTTCTTGTACAAAGTGGAACCGCTGAGCCGTTTACTTAG
Hatl-up(R)	GGGGACTGCTTTTTTGTACAAACTTGTTTTAGGTGATGACTTGTGCC
Hatl-down(F)	GGGGACAACTTTGTATAGAAAAGTTGTTACTGCCTCTTTCTGTGCGTC
Hatl-down(R)	GGGGACAACTTTGTATAATAAAGTTGTGGTGGGTGGTTCATTTAGTG
Hatl-down(CR)	GGGGACTGCTTTTTTGTACAAACTTGTGGTGGGTGGTTCATTTAGTG
Hatl-up(OF)	GGGGACAGCTTTCTTGTACAAAGTGGAACTCCAAACTCACAGCTAAA
Hatl-down(OR)	GGGGACAACTTTGTATAATAAAGTTGTCGTCAAAGGGGAGTAGATG
Hatl-orf(F)	CAACAGCACTCTGAAGGAAT
Hatl-orf(R)	TTGGGGGTGATAGACAGTTC
Hatl-uofu(F)	AACCGCCATAAGTCACGTCA
Hatl-dofd(R)	TGACATTCATCCTTTCGGGT
Ura3-orf(F)	GTCAAGACATCTGTTACCGTGG
Ura3-orf(R)	AGTCCTTGTGCTCCCGTG
Ura3-up(F)	GGGGACAACTTTGTATAGAAAAGTTGTTGAACGACAGAGCCCCATC
Ura3-down(R)	GGGGACAACTTTGTATAATAAAGTTGTTTCACTGGCAAGTAGGTA
Hvg(F)	GCCCTTCCTCCCTTTATT
Hyg(R)	TGTTGGCGACCTCGTATT
Actin-(F)	GTCGCTGCCCTCGTTATC
Action-(R)	CGAGAATGGAACCACCGAT

Disruption of *hat1* by ATMT

The *hat1* deletion construct pOSCAR-*hat1* was transformed into *A. tumefaciens* LBA4404 by following a protocol described previously with minor modification [31, 33]. *A. tumefaciens* LBA4404 transformants harboring pOSCAR-*hat1* were co-cultured with 10^6 conidia of NK17-*ura3Δ* at 28 °C on nitrocellulose filter, which was spread on IM plate supplemented with 50 mg L^{-1} uracil and 40 mg L^{-1} acetosyringone (AS, Sigma, St. Louis, MO). After induction for 2 days, the filter with transformants was transferred to YNB plate supplemented with cefotaxime (100 mg L^{-1}) following incubation for 2 days at 28 °C for sporulation. The individual fungal transformant was obtained through single-spore isolation.

Characterization and analysis of the transformants

Total DNA from each transformant was extracted from mycelium growing in 100 ml PLB for 3–4 days, as described by Hao et al. [34]. PCR amplification and Southern blot hybridization were used to characterize the *hat1Δ* candidates. For PCR analysis, the following pairs of primer were used: Hat1-orf(F)/Hat1-orf(R), Hat1-uofu(F)/Ura3-orf(R), Ura3-orf(F)/Hat1-dofd(R), and Hat1-uofu(F)/Hat1-dofd(R) (Table 1).

Southern blot analysis was conducted to confirm: (A) The absence of *hat1* in transformant; (B) Homologous recombination occurred only in *hat1* site, no other sites were disturbed; and (C) There is only one copy of *hat1* in NK17. Approximately 20 μg of total DNA from each strain was digested with *Kpn* I, resolved on a 0.8% agarose gel and then conducted alkaline transfer to the Magmaprobe Nylon Transfer Membrane-N^+ (Osmonics, Minnetonka, MN). A 789 bp probe generated by PCR amplification with primer pair Hat1-down(F)/Hat1-down(R) and a 348 bp probe with primer pair Ura3-orf(F)/Ura3-orf(R) (Fig. 2B) were used in Southern blot hybridization. All the steps involved in Southern blotting followed the protocol of the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche China, Shanghai, China).

Phenotype observation and secondary metabolism profiling for *hat1Δ*

To characterize the variation in phenotype and secondary metabolite biosynthesis between NK17 and *hat1Δ*, the conidia from each strain with identical number were cultivated in PLB and on PLA, respectively, for phenotypic characterization at the indicated date points. To profile the secondary metabolites, the conidia were cultured in 200 ml PLB liquid medium at 28 °C, 180 rpm for 196 h. Then the fermentation solutions, mycelia and spores were extracted by equal volume of dichloromethane. The suspension mixture in dichloromethane was condensed into 1 ml methanol by vacuum-rotary evaporation. The concentrated solution was analyzed on high performance liquid chromatography (HPLC) after being filtered using organic Millipore filter (0.45 μm). The protocol for HPLC profiling was described by Yu et al. with mobile phase methanol/H_2O (70:30, v/v) at a flow rate of 1 ml min^{-1} [33].

RNA extraction and determination of *hat1* expression

To characterize the temporal expression of *hat1*, total RNA was extracted from NK17 mycelia growing in PLB for 2, 3, 4, 6, and 8 days using TRIzol Regent (Invitrogen, CA). The first-strand of cDNA was synthesized following the manufacturer's instruction of M-MLV RTase cDNA Synthesis Kit (Takara, Dalian, China). The expression of *hat1* was confirmed by reverse-transcription PCR (RT-PCR) for 28 cycles as determined beforehand with primers, Hat1-orf(F)/Hat1-orf(R), and cDNA as template. The *act1* (encoding for actin) of NK17 was used as control. Primers used here are listed in Table 1.

Restoration and overexpression of *hat1*

The complement construct pOSCAR-*hat1*-C was constructed by BP Clonase reaction including 40–50 ng NK17 *hat1* allele, 30–40 ng of NK17 *ura3* allele, 60 ng of pA-Hyg-OSCAR (Invitrogen, CA), 60 ng of pOSCAR, and 1 μl BP clonase II enzyme mix. The NK17 *hat1* and *ura3* alleles were amplified with primer pairs Hat1-up(F)/Hat1-down(CR) and Ura3-up(F)/Ura3-down(R) (Table 1), respectively. NK17 genomic DNA was used as template. The schematic of the above disruption and complementation of *hat1* were shown in Supporting Information Fig. S2. The confirmation of correct complement strains *hat1Δ*-C was shown in Supporting Information Fig. S2C–E.

The overexpression construct pOSCAR-*hat1*-O was also constructed by BP Clonase protocol which included 40 ng NK17 *hat1* allele, 60 ng pOSCAR4 (unpublished, Supporting Information Fig. S3), and 1 μl BP clonase II enzyme mix, adding double distilled water to 5 μl. NK17 *hat1* allele was amplified with primers Hat1-up(OF)/Hat1-down(OR). Through ATMT, this *hat1* allele was inserted randomly in NK17 genome. Confirmation of pOSCAR-*hat1*-O and the overexpression strain were shown in Supporting Information Fig. S3.

Drug and stress sensitivity tests

For the susceptibility test of NK17, *hat1Δ*, and *hat1Δ-C* strains, sensitivity assay to fluconazole was performed by incubating strains on Complete Medium (CM) at 28 °C (the same below) supplemented with 30, 60, and 90 $\mu g\,ml^{-1}$ fluconazole, or bialaphos sodium at concentrations of 5, 15, and 30 $\mu g\,ml^{-1}$, hygromycin B at 30, 50, and 80 $\mu g\,ml^{-1}$. Growth inhibition for $CuSO_4$ was performed at final concentrations of 0.5, 1.5, and 2.5 mM. The susceptibility tests on SDS, Congo red and sorbitol were performed at 0.07% (w/v), 0.05% (w/v), and 2 M, in that order.

Results

Characterization of a B-type histone acetyltransferase encoding gene *hat1* in *P. microspora* NK17

The genome of *P. microspora* NK17 has been totally sequenced (unpublished). The availability of the sequence information assures the identification of the histone acetyltranferase-encoding gene *hat1* in NK17 (GenBank accession No. KU573774). The *hat1* gene, 1633 bp in length, consists of three exons and two introns and encodes a putative polypeptide of 504 amino acids (Fig. 1A). RT-PCR analysis demonstrated that *hat1* was constitutively expressed in NK17 (Fig. 1C). Structural analysis of Hat1 revealed this protein contained only one putative functional domain at the N-terminus of Hat1 superfamily (9–163 amino acids) (Fig. 1A). Amino acid sequence alignment indicated that Hat1 shared high identity with B-type homologs in other filamentous fungi, for example, 47.35% similarity with its *Fusarium oxysporum* homolog (EMT64021.1) (Fig. 1B). The high similarity among these sequences suggested the putative function of *hat1* in NK17. In addition, these homologs so far identified as B-type HATs in the fungi allow us to classify Hat1 in NK17 as a B-type HAT, which is generally responsible for acetylation of newly synthesized histones in cytoplasm. Moreover, Hat1 was also classified in GNAT (Gcn5-related N-acetyltransferase) superfamily of HATs according to the conserved acetylation-related motif [3].

Confirmation of *hat1* deletion, restoration, and overexpression strains

The *hat1* in NK17-*ura3Δ* was replaced by the selection maker *ura3* gene via homologous recombination to obtain deletion transformants. PCR screening was conducted to confirm correct deletion strains. The locations of four primer pairs used for PCR amplification are shown in Fig. 2B. Two, Nos. 4 and 7, of the five transformants were identified as the putative *hat1* deletion strains through PCR (Fig. 2A). Then, no. 4 was picked for Southern blot analysis. Southern blot hybridization confirmed that strain no. 4 was a correct transformant as anticipated (Fig. 2C). The restoration and overexpression of *hat1* were obtained by procedures described in Materials and Methods and identified by PCR amplification (Supporting Information Fig. S2) or RT-PCR (Supporting Information Fig. S3C).

Effects of *hat1* on conidiation

To characterize the phenotype of *hat1Δ*, we cultivated NK17, *hat1Δ*, and *hat1Δ*-C strains in PLB. As shown in Fig. 3A, the deletion of *hat1* delayed conidiation of *hat1Δ*.

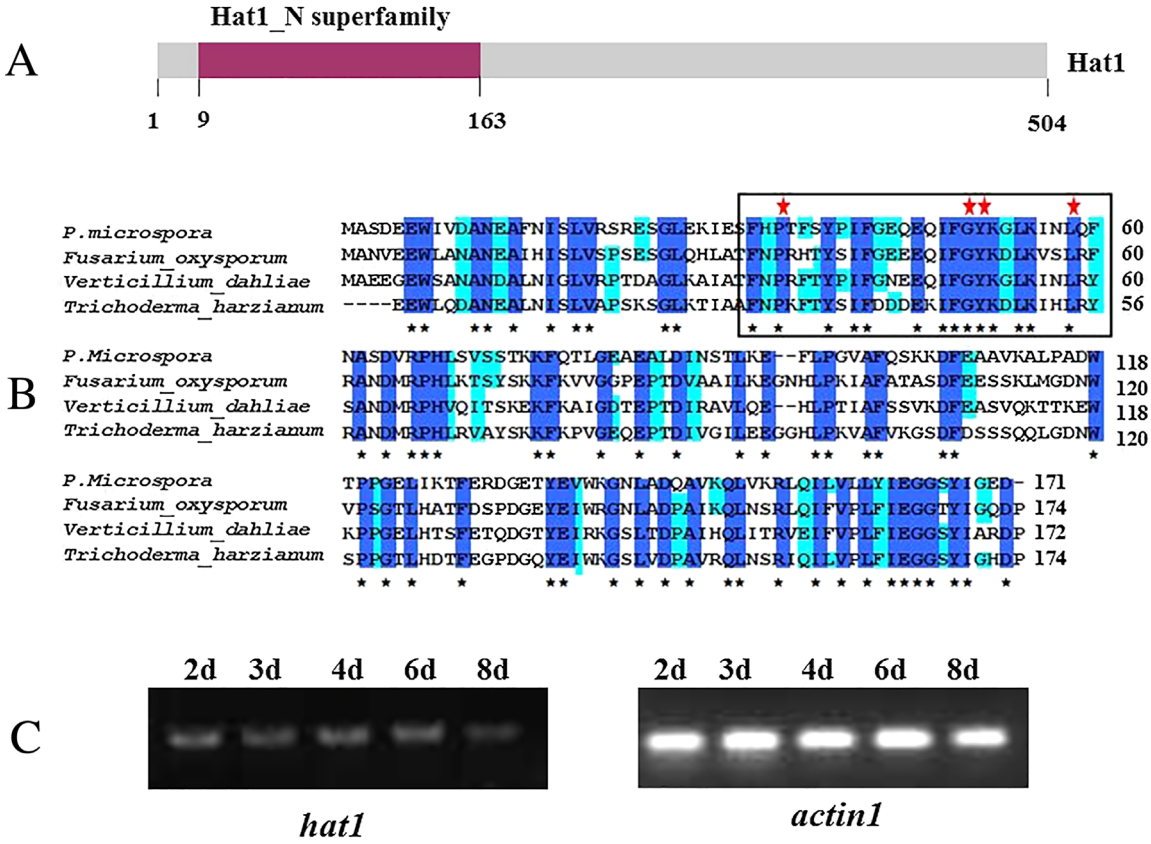

Figure 1. (A) Schematic diagram of the structure of Hat1 protein from *P. microspora*. (B) Amino-acid sequence (partial sequence) alignments of Hat1 homologs from different fungi. The identical amino acids are starred and highlighted in blue, and the less conserved amino acids are shown in turquoise. The framed sequence represents the putative Hat1 functional domain. (C) Reverse transcription PCR amplification of *hat1* expression at different time points of the culture. The expression of *act1* was used as an internal control.

The wild-type NK17 and *hat1Δ*-C started to produce conidia around 96 h, whereas few conidia were produced by *hat1Δ* up to 108 h. Melanin is a dark brown or black pigments produced by this fungus, in particular, in the reproduction structure such as conidia [35]. Therefore, the less production of pigment in *hat1Δ* than that of NK17 and *hat1Δ*-C implied a decrease in the number of conidia in *hat1Δ* as well (Fig. 3A). We conducted quantification for conidia production on plate. The mutant strain *hat1Δ* had a an average of $3.17 \pm 0.25 \times 10^6$ conidia per plate ($p < 0.01$), whereas NK17 had a number of $5.61 \pm 0.34 \times 10^6$ conidia per plate ($p < 0.01$) (Fig. 3B). The complement strain *hat1Δ-C* restored the level of melanin and the production of conidia with a number of $5.37 \pm 0.28 \times 10^6$ conidia per plate ($p < 0.01$) (Fig. 3B). Interestingly, overexpression of *hat1* considerably enhanced conidiation (Fig. 4) with a number of $7.14 \pm 0.45 \times 10^6$ conidia each plate ($p < 0.01$) (Fig. 3B). However, little difference in mycelial growth and morphology were observed among three strains (Fig. 3C). The results demonstrated that *hat1* is required for conidiation while dispensable for the vegetative growth.

Sensitivity to external stressful conditions

HAT proteins in fungi have a role in the tolerance to stressful conditions, including oxidative stress, antibiotic, inhibitors, and heavy metallic salts. To test the response of *hat1Δ* to various external stresses, we conducted the assays with following substances, including fluconzole, bialaphos sodium, hygromycin B, $CuSO_4$, SDS, sorbitol, and Congo red. Addition of fluconazole to CM plates did not cause a severe defect in growth, or strains only showed a susceptibility to a high concentration of this drug (Fig. 5). Treatment with bialaphos sodium can obviously inhibit conidiation and delay vegetative growth of strains (Fig. 5). On plates supplemented with 50 $\mu g\,ml^{-1}$ hygromycin B, 1.5 mM $CuSO_4$, or 0.07% (w/v) SDS (not shown), strains could hardly growth (Fig. 5). *hat1Δ* exhibited a hypersensitivity to 0.05% (w/v) Congo red and 2 M sorbitol, which

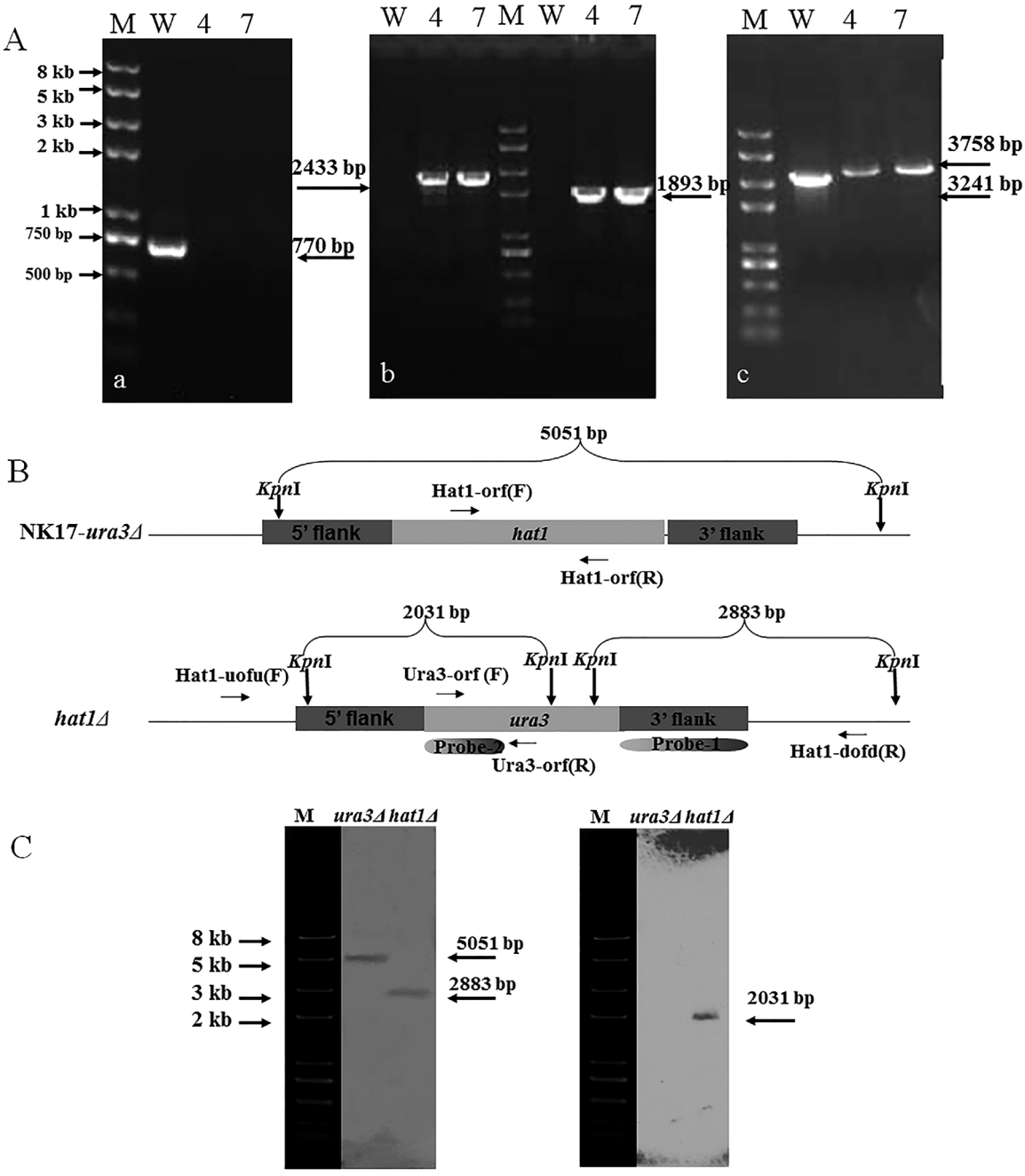

Figure 2. Deletion of *hat1* in NK17. (A) Confirmation of the presence of the *hat1* ORF with primer pair Hat1-orf(F)/Hat1-orf(R), a 770 bp *hat1* band was obtained for NK17-*ura3Δ*, but not for mutants; (a) Confirmation of the replacement of *hat1* by *ura3* marker through homologous recombination with pairs of primers, Hat1-uofu(F)/Ura3-orf(R) and Ura3-orf(F)/Hat1-dofd(R). Two bands, 2433 bp and 1893 bp, were acquired from *hat1Δ*, while no band was seen for NK17-*ura3Δ*; (b) Further confirmation of the disruption of *hat1* with primers Hat1-uofu(F)/Hat1-dofd(R), obtained a single 3241 bp band for NK17-*ura3Δ* and a sole 3758 bp band for *hat1Δ*; (c) Numbers 4 and 7 above the lanes refer to the analyzed *hat1Δ* mutants. The letters: W represents to the strain NK17-*ura3Δ*, M is molecular weight marker (Trans2K plusII, TransGen, China). (B) Schematic of *hat1* in NK17-*ura3Δ* (above) and *ura3* in *hat1Δ* (below). The primers used for PCR amplification, the probes used for Southern blot and the location of the recognition sites of *Kpn* I are indicated. (C) Southern blotting to confirm the deletion of *hat1*. Genomic DNAs from NK17-*ura3Δ* and *hat1Δ* strains were digested with *Kpn* I. The left panel was probed with probe-1 amplified by primers Hat1-down(F)/Hat1-down(R). Two different bands, a 5 kb band for NK17-*ura3Δ* strain and a 2.8 kb band for *hat1Δ* strain were respectively detected. The right membrane was probed with probe-2 amplified with primer pair Ura3-orf(F)/Ura3-orf(R), to generate a single 2 kb band in *hat1Δ* strain, while no bands were seen in NK17-*ura3Δ* strain.

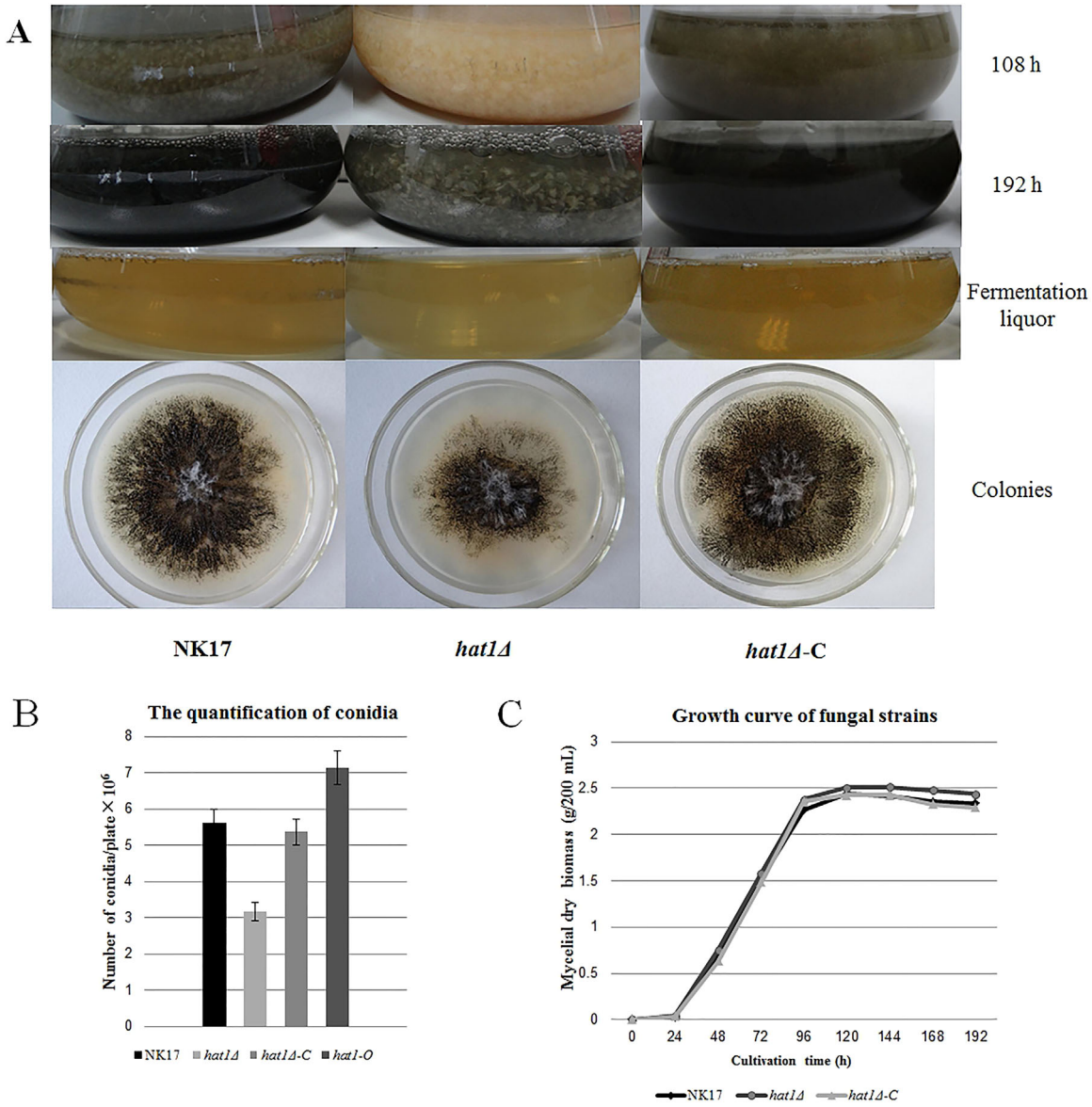

Figure 3. Phenotype characterization for *hat1*Δ. (A) Conidiation of the strains at 108 h and 196 h in PLB. Compared to the wild-type strain NK17 and complement strain *hat1*Δ-C, *hat1*Δ showed the delay of conidiation and formed obviously less conidia. (B) The quantification of conidia. The number of the conidia produced by NK17, *hat1*Δ, *hat1*Δ-C, and *hat1*-O was determined as $5.61 \pm 0.34 \times 10^6$ ($p < 0.01$), $3.17 \pm 0.25 \times 10^6$ ($p < 0.01$), $5.37 \pm 0.28 \times 10^6$ ($p < 0.01$), and $7.14 \pm 0.45 \times 10^6$ ($p < 0.01$), per plate, respectively. Triplicate PLA plates for each strain were incubated at 28 °C for 196 h and used for quantification. Error bars represent standard deviation. (C) The growth curve of NK17, *hat1*Δ, and *hat1*Δ-C stains when cultivated in PLB. Little difference in growth rate among strains was found. Values are the mean of three replicates.

significantly delayed its growth compared to NK17 and the complement *hat1*Δ-C (Fig. 5). This result suggests that the integrity or the biosynthesis of cell wall components in NK17 is impaired by the deletion of *hat1*.

Crucial roles of *hat1* in the production of secondary metabolites

Pathway-specific and global transcriptional regulators both exist in filamentous fungi to regulate the transcription of secondary metabolic pathways [36, 37]. With the purpose to identify the role of *hat1* in the production of SMs in NK17, we conducted HPLC profiling. Production of secondary metabolites usually commences late phase of the culture of microbes, and is commonly associated with conidiation in filamentous fungi [38]. We thus checked the time point of the production of SMs in our fungus and found that SMs were hardly detected with HPLC analysis in the culture before

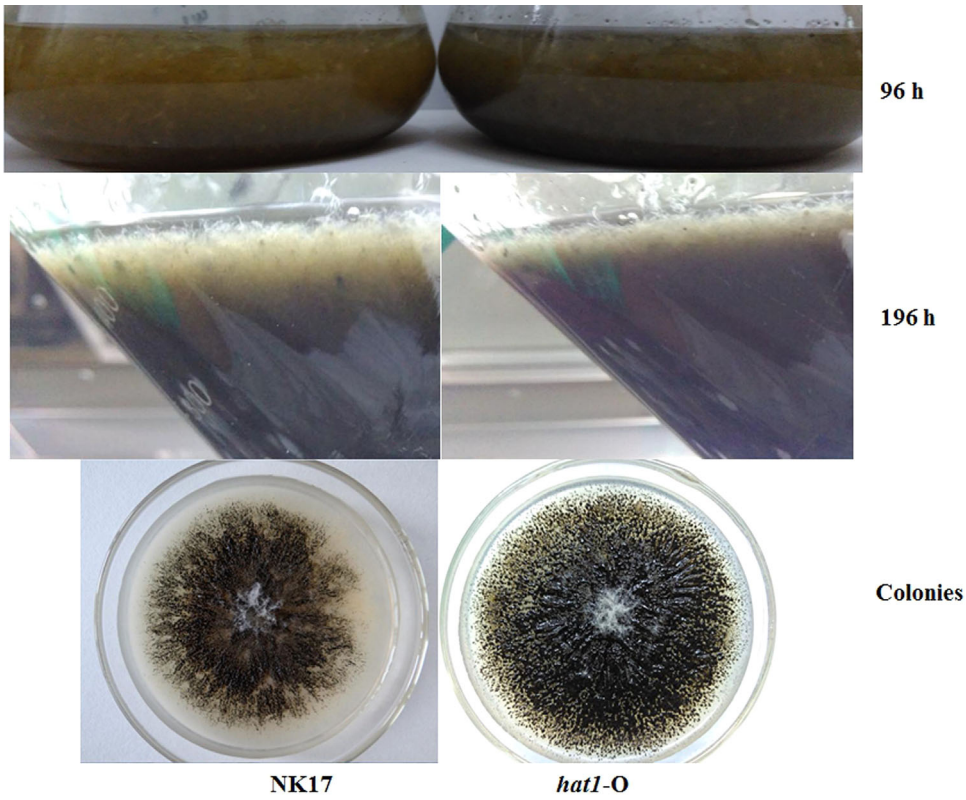

Figure 4. Phenotype characterization for *hat1*-O. Compared with NK17, overexpression of *hat1* (strain *hat1*-O) showed enhanced conidiation with earlier conidiation and the increased number of conidia.

96 h (Supporting Information Fig. S5). Small molecule products started to produce after 108 h of the culture (not shown) and reached the maximum quantity at around 196 h (Fig. 6A). This time point was in consistence with a stationary phase of NK17 growth (Fig. 3C). Based on this data, we did the HPLC profiling of the sample extract from the culture at 196 h.

The HPLC profiling (Fig. 6A and B) revealed that deletion of *hat1* resulted in a deficiency of most SM production. We have calculated the quantity of pestalotiollide B (PB) (Fig. 6A). Its production in NK17 was $6.69 \pm 0.36\,mg^{-1}$ [30], whereas in *hat1Δ*, PB was hardly detectable. The PB peak disappeared on the HPLC record (Fig. 6B). However, the pattern of SMs were totally restored when the wild-type *hat1* allele was reintroduced into *hat1Δ* (Fig. 6C and D). These results demonstrate that *hat1* functions as a global transcriptional factor in the biosynthesis of secondary metabolites in NK17. Surprisingly, the overexpression of *hat1* showed little impact on SMs production (Supporting Information Fig. S6), indicating that *hat1*-mediated regulation may have a ceiling effect on SMs biosynthesis.

Discussion

Fungi have a superb capacity to produce a wide range of secondary metabolites(SMs). SMs are generally regarded as dispensable for normal growth of an organism but play important roles, for example, as defense compounds [36] or signaling molecules [37], in ecological interactions. Genome mining efforts have revealed that the potential of fungi to produce SMs has been substantially underestimated as many of the gene clusters for SM biosynthesis are silent under standard cultivation conditions. Until now, some successful approaches based on molecular biology have been used to activate the silent gene clusters, such as overexpression of pathway-specific [39] or pleiotropic regulators [40], promoter exchange [41], and the generation of gene "knock outs" [42]. Strategies based on epigenetics open a new avenue for the elucidation of the regulation of secondary metabolism and cryptic natural products. Accumulating evidence supports the view that histone acetyltransferases, deacetylases, and methyltransferases proteins are active regulators for many if not all SM biosynthesis via likely modulating the chromatin structure.

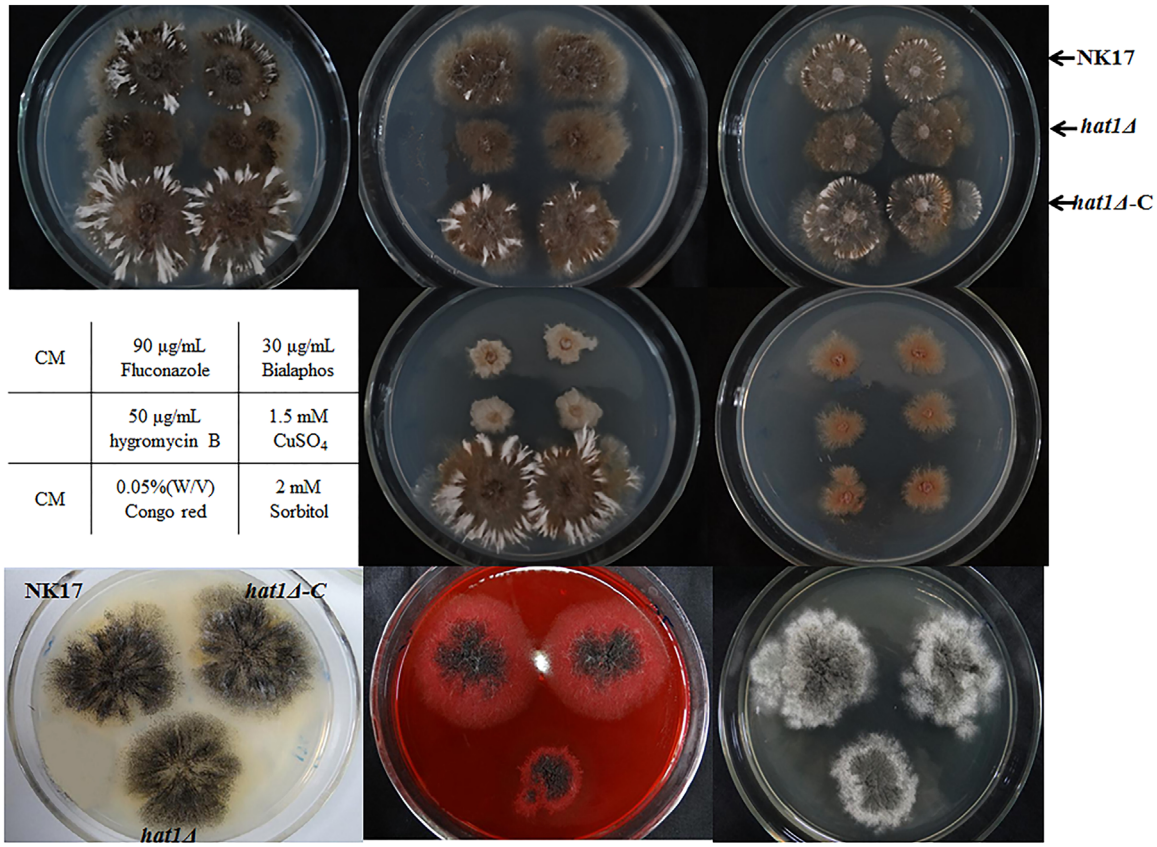

Figure 5. Susceptibility tests for fungal strains NK17, *hat1*Δ, and *hat1*Δ-C. Susceptibility tests on fluconazole, bialaphos sodium, hygromycin B, $CuSO_4$, and external oxidative stress, Congo red and sorbitol, on CM media. CM without any supplements served as control. The layout of strains on Congo red and sorbitol plates was shown on the control plate (CM). The layout of strains on other plates was indicated on the right, from up to down is the row of NK17, *hat1*Δ, and *hat1*Δ-C. The *hat1*Δ-C showed no inhibition on plate with hygromycin B in middle because of the presence of hygromycin B resistant gene as marker.

In this report, we explored the possible roles of histone acetyltransferases in secondary metabolism of filamentous fungus *P. microspora* (NK17). We identified a B-type histone acetyltransferase-encoding gene *hat1* and demonstrated it was a major player in the production of SMs in this filamentous fungus. The protein sequence alignment of Hat1 between NK17 and other filamentous fungi showed a high similarity and, in particular, Hat1 in NK17 had the common conserved N-terminal domain of Hat1 superfamily. Based on the sequence homology, we classified NK17 *hat1* to be a B-type HAT that was believed to acetylate newly synthesized free histones in cytoplasm for transport into the nucleus [43].

Although a null mutation of *hat1* in *Saccharomyces cerevisiae* conferred no observable phenotype change [21], the destruction of *hat1* in NK17 demolished SMs production and delayed conidiation, suggesting a diversity of functions of *hat1* in fungi. In yeast, *hat1* homolog was found to be associated with a second subunit, *hat2*, which was required for binding to histone H4 and contributed to substrate specificity, leading to forming a *hat1*/*hat2* complex [24]. We also knocked out a putative *hat2* in NK17. The mutant *hat2*Δ displayed a similar phenotype to *hat1*Δ, including a considerable defect in SMs, showing both of the subunits of the putative *hat1*/*hat2* complex were indispensable in our fungus (data not shown). According to the resulted phenotype of *hat1* disruption on SM biosynthesis suggests our *hat1*, a B-type HAT, functions as a global and essential regulator of chromosome structure associated with SM-related gene clusters in NK17.

Previous works demonstrate that Hat1 enzymes from a wide variety of eukaryotes have a remarkably conserved specificity for histone H4 [44, 45]. In *Aspergillus flavus*, histone H4 hyper-acetylation paralleled the activation of genes within the aflatoxin cluster [11]. In *A. nidulans*, Soukup et al. have examined four gene clusters (sterigmatocystin, penicillin, terrequinone, and orsellinic acid) for their chromatin marks and found that H4 acetylation played a role in its SM regulation [13].

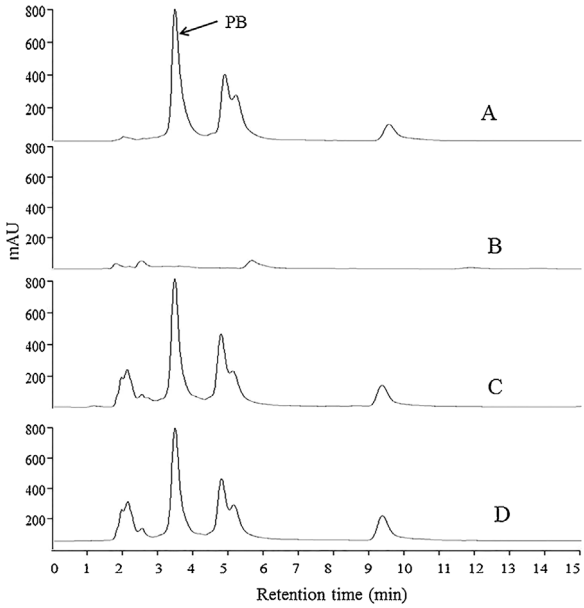

Figure 6. HPLC profiling for the secondary metabolite biosynthesis in the culture of NK17, *hat1*Δ and *hat1*Δ-C. Compared with NK17, *hat1*Δ showed a remarkably decreased, even undetectable level of the SMs. (A and B) PB (pestalotiollide B) a characterized SM in NK17. Production of SMs in *hat1*Δ-C was recovered with the complement of the wild-type *hat1* allele. (D) NK17 was analyzed as an internal control. (C) The HPLC profiling analysis was repeated for three times.

Based on this, we tend to speculate that the possible target of NK17 Hat1 protein is H4 histone. Additionally, the deletion of *hat1* significantly delayed conidiation and lowered the number of conidia on plate, but showed little effect on vegetative growth, suggesting that secondary metabolism is independent of vegetative growth in this fungus. And overexpression of *hat1* did enhance conidiation and increase the conidia number. This result further assured the function of *hat1* on conidiation. The remarkable defect of SMs accompanied by the delay of conidiation in *hat1*Δ indicates the probable relationship between SM biosynthesis and conidiation in this fungus.

Physiological effects of *hat1* on NK17 have been examined in this study. For instance, compared with the control, an obvious difference between NK17 and *hat1*Δ in sensibility to Congo red suggested the disruption of *hat1* had a significant role in the structure or integrity of cell wall of NK17, as cell wall mutants are more sensitive to Congo red which interferes with the construction and stress response of the cell wall [46]. While we have found out the significance of B-type HAT *hat1* in biosynthesis of SMs and cell wall integrity in NK17, our knowledge of the function of this type of HATs is still rudimentary. A few critical questions are remaining to be addressed before the mechanism of this B-type histone acetyltransferase modulating the production of SMs can be illustrated, for example, what gene clusters are affected by Hat1-mediated chromatin remodeling.

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Conflict of interest

The authors declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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