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# A PKS gene, *pks-1*, is involved in chaetoglobosin biosynthesis, pigmentation and sporulation in *Chaetomium globosum*

HU Yang, HAO XiaoRan, LOU Jing, ZHANG Ping, PAN Jiao & ZHU XuDong*

*State Key Program of Microbiology and Department of Microbiology, College of Life Sciences, Nankai University, Tianjin 300071, China*

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*Chaetomium globosum* is one of the most common fungi in nature. It is best known for producing chaetoglobosins; however, the molecular basis of chaetoglobosin biosynthesis is poorly understood in this fungus. In this study, we utilized RNA interference (RNAi) to characterize a polyketide synthase gene, *pks-1*, in *C. globosum* that is involved in the production of chaetoglobosin A. When *pks-1* was knocked down by RNAi, the production of chaetoglobosin A dramatically decreased. Knock-down mutants also displayed a pigment-deficient phenotype. These results suggest that the two polyketides, melanin and chaetoglobosin, are likely to share common biosynthetic steps. Most importantly, we found that *pks-1* also plays a critical role in sporulation. The silenced mutants of *pks-1* lost the ability to produce spores. We propose that polyketides may modulate cellular development via an unidentified action. We also suggest that *C. globosum pks-1* is unique because of its triple role in melanin formation, chaetoglobosin biosynthesis and sporulation. This work may shed light on chaetoglobosin biosynthesis and indicates a relationship between secondary metabolism and fungal morphogenesis.

**polyketide synthase (PKS), melanin, chaetoglobosin A, *Chaetomium globosum***

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*Chaetomium globosum* is widely found in soil, on plant debris, and in living plant hosts as either an endophyte or a pathogen. The fungus is also known as an agentive cause for human sick building syndrome (SBS) and allergy [1]. Some isolates are pathogenic to humans, causing invasive infection [2]. On the other hand, *C. globosum* has a broad range of applications in agriculture and industry. For instance, it has been used as a biocontrol agent against pathogenic microbes and even aphids [3,4]. Able to degrade plant biomass, *C. globosum* is potentially applicable for the making of biofuel from cellulignin materials [5]. In particular, tens of secondary metabolites have been identified from *C. globosum* [6,7]. Some of these may cause food contamination. Many others have strong bioactivity and are attractive for pharmaceutical research and drug development [8]. Chaetoglobosins are among the well-known secondary metabolites produced by this fungus [6,7,9]. This group includes mycotoxins that are considered to be cytochalasans that are capable of inhibiting the movement of tumor cells because of their ability to bind to actin filaments [10]. Chaetoglobosins were first identified in *C. globosum* in the 1970s, and were later identified in other fungi [11,12]. Chaetoglobosin A (CheA) is the most abundant secondary metabolite made by *C. globosum* and has been investigated for its strong cytotoxicity to tumor cells, antibacterial and antifungal activities [13]. The chemical structure of chaetoglobosin A consists of a polyketide backbone that is condensed from nine units of acetate/malonate, and has a tryptophan residue attached to the cyclized backbone [14,15].

In the past decade, many fungal genome projects have revealed a large number of polyketide synthase genes in this kingdom [16]. Molecular studies have also been performed

*Corresponding author (email: xudong82@nankai.edu.cn)

to determine the biosynthetic principle of important bioactive polyketides, such as aflatoxin [17–22]. Regulatory networks governing secondary metabolism have been gradually uncovered in several fungal species, for example, the central roles of the G protein-cAMP-PKA pathway and the LaeA and VeA protein complex have been determined [19,23–25].

In striking contrast, few molecular studies of *C. globosum* have been performed despite the importance of this fungus. In fact, few genes have been characterized in this fungus. We recently found that targeted disruption of genes by conventional homologous recombination was difficult in this fungus and to our knowledge, no case of *C. globosum* gene disruption has been reported in literature. Recently, RNA interference (RNAi) has emerged as a powerful tool for gene targeting in fungi [26–28]. Conventional RNAi machinery components, including the RNA-dependent RNA polymerase, Argonaut proteins and Dicers are conserved in some fungi, e.g., *Cryptococcus neoformans* [29], *Bipolaris oryzae* [30], *Aspergillus* and *Fusarium* Species [31] and *Cochliobolus sativus* [32]. However, its application in *C. globosum* has not been reported.

We report here the development of an RNAi method that was successfully used for the characterization of a polyketide synthase gene, *pks-1* (CHGG_00542), in *C. globosum*. The *C. globosum* CBS 148.51 genome project (http://www.broadinstitute.org/¬annotation/genome/¬chaetomium_globosum/Home.html and the NK102 genome project by our laboratory, unpublished data) ascertained that the RNAi machinery is present in this fungus. The putative RNA-dependent RNA polymerase, Argonaut proteins and Dicers are already defined in the genome of CBS 148.51 (CHGG_04734, CHGG_08996, CHGG_03638). According to the rational that mutants could be distinguished by mycelial color change, we chose a polyketide synthase gene (*pks-1*) as a target to make a knock-down mutant, as it shares similarity to PKSs involved in melanin formation. The molecular genetics of polyketide synthases involved in melanin biosynthesis has been studied in many fungi, including *Sordaria macrospora* [33], *Bipolaris oryzae* [34] and *Colletotrichum lagenarium* [35]. The *C. globosum pks-1* gene encodes a PKS that shares highest similarity to the melanin PKS from *Neurospora tetrasperma* (EGO61342.1, with 65% identity). We demonstrate in this study that *pks-1* is indeed involved in melanin formation. To our surprise, *pks-1* also plays a critical role in chaetoglobosin A biosynthesis, and in spore generation.

## 1 Materials and methods

### 1.1 Fungal strains and culture conditions

*C. globosum* NK102 (stock culture maintained in our laboratory) was used as the wild-type strain. Growth conditions were as described previously [6]. Media, PDA (potato dextrose agar) or PDB (PDA without agar) was used for routine culture of the fungus at 28°C, with shaking for liquid cultures.

### 1.2 Plasmid construction for RNA interference

The RNAi cassette was constructed in pSilent-1 (a kind gift from Dr. Song JinZhu, Harbin Institute of Technology, China), which has been used in a variety of fungi [26,27] (Figure 1C). Initially, we tested that the hygromycin B resistance gene (*hph*) functioned efficiently as a selection marker in *C. globosum*. The promoter used for RNA hairpin formation and for the expression of *hph* was PtrpC (Figure 1C). Plasmids were amplified in *E. coli* DH5α.

Based on the sequence of *C. globosum pks-1* (CHGG_00542), PCR primers PKS-ia/ib (Table 1), were designed to amplify the 320 bp fragment, pks-1a, to make the inverted

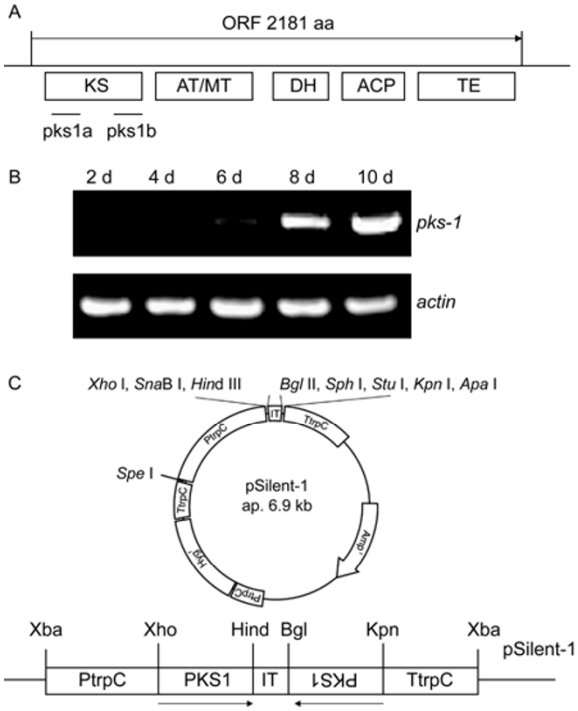


**Figure 1** A, Schematic depiction of PKS-1 domain arrangement: KS (β-ketoacyl synthase, amino acid 349–822), AT/MT (acyltransferase, 926–1224), DH (Dehydratase, 1341–1492), ACP (acyl carrier protein, 1667–1731), TE (thioesterase, 1939–2179). RNAi targeted regions on *pks-1* are denoted as pks-1a, which is located between nucleotides 366 and 685 (320 bp) (nucleotide 1 is start codon ATG), and pks-1b covering nucleotides 1847 to 2073 (227 bp) (for pks-1b RNAi, see Section 2.4 for details). B, Time-dependent expression of *pks-1*, assayed by reverse transcription PCR. Total RNA was isolated from cultures grown for 2–10 d in 500 mL Erlenmeyer flasks containing 200 mL PDB, shaking at 150 r $min^{-1}$ at 28°C. The lower panel shows the mRNA level of actin as control. C, Schematic depiction of RNAi cassette carrier vector, pSilent-1, and construction of the RNAi cassette. Arrows indicate direction of *pks-1* fragment. IT, intron 2 of cutinase (CUT) gene from *Magnaporthe oryzae*; PtrpC, promoter of *trpC* from *A. nidulans*; TtrpC, *trpC* terminator of *A. nidulans*. Restriction enzyme sites are also indicated.

**Table 1** PCR primers used in this study. Underlined sequences indicate restriction sites

| Primer name | Sequence (5′–3′) |
|---|---|
| PKS-ia(s) | ATTA CTCGAG GGTACC TTTTCTCGTCGGGCTTTGC |
| PKS-ia(as) | TCCGC AAGCTT AGATCT TCGGTGGACCAGATACTAC |
| PKS-ib(s) | ATAT CTCGAG GGTACC TTGACAATGATGCCGATGG |
| PKS-ib(as) | GGGCC AAGCTT AGATCT AACATCATGGGGATCAACT |
| Hyg(s) | ATGAAAAAGCCTGAACTCAC |
| Hyg(as) | GCAAAGTGCCGATAAACAT |
| qActin(s) | AACCGAGGCTCCCATCAAC |
| qActin(as) | TCACGGACGATTTCACGCTC |
| qPKS(s) | ATCTTTCCGCCTAACCCGA |
| qPKS(as) | GTCCTTCGTTTCTGGGTTGTC |

repeats in pSilent-1 (Figure 1A and C). pks1a was located at the 5′ end of *pks-1* (Figure 1A) and had little similarity to any other *C. globosum* gene to ensure that RNAi would not target any other gene. pks1a was digested by *Xho* I and *Hin*d III, or *Bgl* II and *Kpn* I for cloning in the inverted orientation in pSilent-1, resulting in pPKS-1a (Figure 1C). Another fragment, pks1b, was also used for interference in parallel to pks1a (see Section 2.4 and Figure 4B).

### 1.3 Transformation and selection for transformants

The wild-type *C. globosum* strain was transformed with uncut pPKS-1a by a PEG-mediated protoplast method as described by Turgeon *et al.* [36]. Transformants were selected on potato dextrose agar (PDA) containing 100 μg $mL^{-1}$ hygromycin B. To confirm transformants containing the silencing vector, the hygromycin B resistant cassette (200 bp) was amplified by PCR using primers hyg(s)/hyg(as) (Table 1) and PCR products were sequenced.

### 1.4 Southern blot analysis

To further confirm transformants, genomic DNA was extracted as previously described by Raeder and Broda [37], and Southern blot analysis was performed according to the method of Yang *et al.* [18]. DNA samples, digested with enzymes as indicated in the figure, were resolved on 0.8% agarose gels, and transferred onto Magmaprobe Nylon Transfer Membrane-$N^+$ (Osmonics, Minnetonka, MN, USA). Probes used were the hygromycin cassette from the pSilent-1, amplified using primers Hyg(s)/Hyg(as); the 320 bp *pks-1* fragment from pPKS-1a; or pSilent-1 linearized by *Xho* I. DNA labeling, hybridization, and detection were carried out according to instructions from the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche China, Shanghai, China).

### 1.5 RNA extraction and reverse transcription PCR (RT-PCR)

For RNA extraction, *C. globosum* was cultured on PDA at 28°C, then fresh mycelia plugs were taken from the edge of the culture and transferred into potato dextrose broth (PDB) at 28°C with shaking for 2–10 d. Total RNA was extracted from lyophilized and ground mycelium using a TRIzol Kit (Invitrogen, CA, USA). Total RNA was treated with RNase-free DNase (Takara, Dalian, China). First strand cDNA was synthesized using 1 μg of total RNA as template, in the presence of oligo (dT) primer and M-MLV reverse transcriptase according to the instructions of the M-MLV RTase cDNA Synthesis Kit (Takara, Dalian, China).

### 1.6 Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR was performed by Mastercycler PCR (Eppendorf, Hamburg, Germany) using SYBR green as a fluorescent reporter (BioRad, CA, USA) following the manufacturer's protocol. The expression of each gene of interest (Ct value) was normalized against actin mRNA. Primers used were qPKS(s)/qPKS(as) for *pks-1*, qActin(s)/qActin(as) for *actin* (Table 1). Reaction mixtures (20 μL) contained 10 μL of SYBR Green I PCR master mix (Roche China, Shanghai, China). PCR conditions were 94°C for 10 min, followed by 40 cycles of 94°C for 15 s, 59°C for 30 s, followed by a melting curve analysis. qRT-PCR was conducted in triplicate for each sample. Standard curves were created for each reaction using 4-fold serial dilutions. qRT-PCR data were analyzed using the $2^{-\Delta\Delta C_T}$ relative quantification method [38].

### 1.7 Detection of chaetoglobosin A by HPLC

*C. globosum* was cultured in 200 mL PDB with shaking at 180 r $min^{-1}$, 28°C for 8 d. The culture liquid was extracted with an equal volume of chloroform and methanol (10∶1, v/v). The organic phase was transferred to a vacuum evaporator and was concentrated until dry under reduced pressure at 55°C. The residue was dissolved in 2 mL methanol, resuspended and centrifuged at 12000 r $min^{-1}$ for 10 min. The supernatant was filtered through a 0.45 μm Millipore filter and subjected to HPLC.

A Kromasil C18 ODS column (4.6 mm×250 mm, AKZO Nobel, Gland, Switzerland) was used for HPLC (Angilent 1100, Angilent Technologies, CA, USA). The UV detection

wavelength was set at 227 nm. The flow rate for the samples was 1 mL $min^{-1}$. Standard chaetoglobosin A (Sigma, St. Louis, USA) served as control. For quantification of chaetoglobosin A, a standard curve was created using known concentrations of the standard sample.

## 2 Results

### 2.1 Time-dependent expression of a PKS-encoding gene *pks-1* in *C. globosum*

Annotation of the *C. globosum* CBS 148.51 genome project suggests more than a dozen polyketide synthase genes in the genome. One of them, *pks-1*, putatively encodes the largest PKS in the genome and shares homology to PKSs for melanin biosynthesis in other fungi. Full-length of *pks-1* was cloned from the *C. globosum* NK102 culture maintained in our laboratory and sequenced (GenBank ID: JX125042). The predicted synthase consists of five characteristic structural domains that feature the iterative fungal type I polyketide synthases, i.e., KS (β-ketoacyl synthase), AT/MT (acyltransferase), DH (dehydratase), ACP (acyl carrier protein) and a TE (thioesterase). These domains function supposedly in Claisen condensation and in the elongation of chaetoglobosin polyketide backbones [39–41] (Figure 1A). However, PKS-1 lacks ER (enoyl reductase) and KR (keto-reductase) domains.

Many genes or loci associated with secondary metabolism are silenced in fungal genomes [42]. Hence, we examined by reverse transcription PCR (RT-PCR), whether *pks-1* was expressed in *C. globosum*. RT-PCR indeed verified that *pks-1* was expressed in a culture-age dependent manner (Figure 1B). Expression of *pks-1* did not start until the 8th day and was in remarkable concomitance with the growth stationary phase and pigmentation of the fungus (Lou and Zhu, unpublished data). NK102 biomass was maximal on the eighth day and the mycelium started to turn dark on the seventh (data not shown). An actin gene, *actin*, was used as a standard that showed a nearly constant level of expression (Figure 1B, bottom panel). The RT-PCR results suggest that *pks-1* is an expressed gene and its expression is associated with fungal culture status.

### 2.2 Transformation of RNAi cassette and pigment-deficiency in transformants

An RNA interference expression cassette for *pks-1* was first constructed in plasmid pPKS-1a, as described in Section 1.2 (Figure 1C). The cassette was then transferred into NK102 by a protoplast PEG-mediated protocol. Forty hygromycin-resistant transformants (designated pA) were randomly picked and PCR amplification confirmed pPKS-1a was present in the transformants (PCR products were sequenced).

Most transformants exhibited phenotypic changes in pigmentation compared to wild-type NK102. Based on pigmentation intensity, the transformants were roughly sorted into three groups. Seven out of forty transformants (Group 1), e.g., pA6, maintained gray to dark mycelium on plates and were brown in culture, a phenotype close to the wild type (Figure 2A). The second group produced an ob-

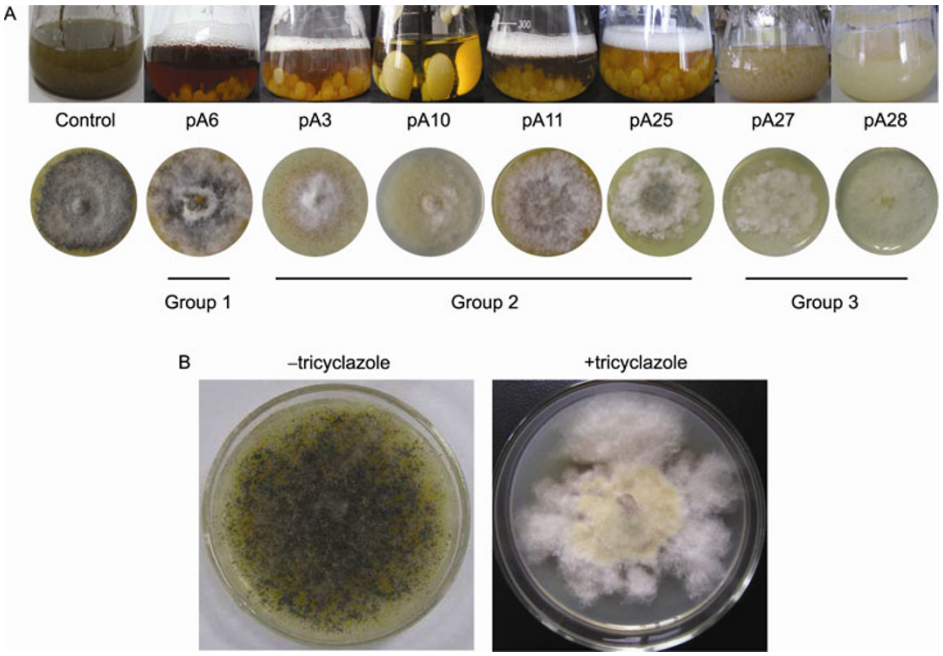


**Figure 2** Pigment-deficient phenotypes of *pks-1* knock-down strains of *C. globosum*. A, Upper panels show the color of liquid cultures of the mutants. Fungal strains were grown in 200 mL PDB at 28°C, 8 d. Bottom panels are plates of the transformants corresponding to the upper panels. The fungal strains were incubated on PDA with or without (Wt) hygromycin B (100 mg $L^{-1}$) for 7 d. B, Inhibition of *C. globosum* pigmentation by tricyclazole, on PDA plates with (right) and without (left) the drug (50 μg $mL^{-1}$), at 28°C for 7 d.

viously lighter mycelium (less pigment) than the wild type, but did not form completely albino colonies or colorless cultures by visual observation. This group included the majority of the transformants: 30 out of 40, for example, pA3, pA10, pA11 and pA25 in Figure 2A (the differences among transformants are best illustrated in the photographs of liquid culture). The 3rd group consisted of members displaying a complete melanin-deficient phenotype and formed albino mycelium or colorless cultures, e.g., pA27 and pA28. The group 3 pigment-deficient phenotype was similar to that of the fungus grown on plates containing tricyclazole (Figure 2B). Tricyclazole is a specific inhibitor of 1,3,6,8-tetrahydroxynaphthalene reductase and 1,3,8-trihydroxynaphthalen reductase [43], in the DHN biosynthetic pathway. Thus, our results suggest that RNAi efficiently knocked down expression of *pks-1* and resulted in melanin-deficiency in *C. globosum*. Therefore, *pks-1*, in part, is responsible for melanin production in this fungus.

### 2.3 Integration of RNAi cassette in the mutants

To demonstrate that melanin-deficiency in pA27 and pA28 did not result from a disruption of *pks-1*, Southern blotting was conducted. Genomic DNA was prepared from the seven transformants in Figure 2A, pA3, pA6, pA10, pA11, pA25, pA27 and pA28. A blot with undigested genomic DNA showed that pPKS-1a was integrated in the genome in all the transformants (mutants) (Figure 3A). In a second blot, DNA was digested with *Xba* I, and then probed with the *pks-1* fragment, pks1a. *Xba* I does not cut within the *pks-1* gene and a 9.2 kb *pks-1* band was detected in all the transformants (Figure 3B). In addition, *Xba* I digestion generates a 3.0 kb band that carries the entire interference cassette (Figure 1C) and this band was detected in all the transformants (Figure 3B). As control, pPKS-1a alone digested with *Xba* I gave the same 3.0 kb band (Figure 3B, lane P). These results confirmed that the native copy of *pks-1* was intact in the melanin-deficient transformants.

To determine the copy number of pPKS-1a in the transformants, Southern blots of genomic DNA digested with *Xho* I, which cuts pPKS-1a only once (Figure 1C), were prepared and hybridized with the original vector pSilent-1 (without *pks-1* fragments). Two bands were expected for a single insertion event. As shown in Figure 3C, only pA6 had a single copy of pPKS-1a inserted in the genome. Transformants pA25, pA28 and pA11 had three bands in the blot indicating that two copies of pPKS-1a were likely inserted in tandem at the same locus. The other three transformants pA3, pA10 and pA27 had multiple copies of pPKS-1a in the genome. No hybridization signal was visible for wild-type DNA (Wt in Figure 3C). These results indicate that copy number is not associated with RNAi outcome.

### 2.4 Decreased transcription of *pks-1* in the knock-down mutants

qRT-PCR was utilized to determine the transcription level of *pks-1* in the transformants. The mRNA level of *pks-1* was significantly decreased in all seven transformants (Figure 4A), ranging from approximately 11% to 61% of the control

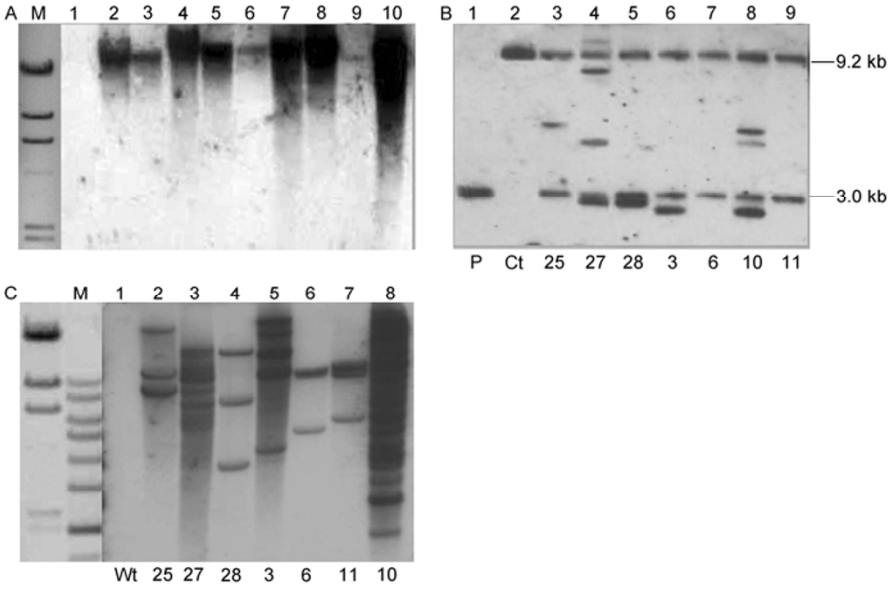


**Figure 3** Southern blot analysis of pPKS-1a interference vector integration. A, DNA samples were undigested and the blot was probed with the *hph* cassette from pSilent-1. M, *Hin*d III-digested λ DNA marker; lane 1, wild type; lane 2 through 8, randomly picked transformants; lane 9 and 10, two transformants with pSilent-1 only (blank plasmid without *pks-1* fragments). Approximately 5 μg DNA per lane was loaded on 0.8% agarose gels. B, DNA samples were digested with *Xba* I and probed with the 320 bp *pks-1* fragment in the RNAi cassette. The predicted 9.2 and 3.0 kb bands are indicated. Lane P, pPKS-1a cut with *Xba* I; Ct, the same control strain as in lane 10 in Figure 3A; 25, 27, 28, 3, 6, 10, 11 are transformants pA25, pA27 and so on. The numbers on the top of the membrane represent lanes. C, DNA samples were digested with *Xho* I and hybridized with linearized pSilent-1. Wt, the original wild type as a negative control.

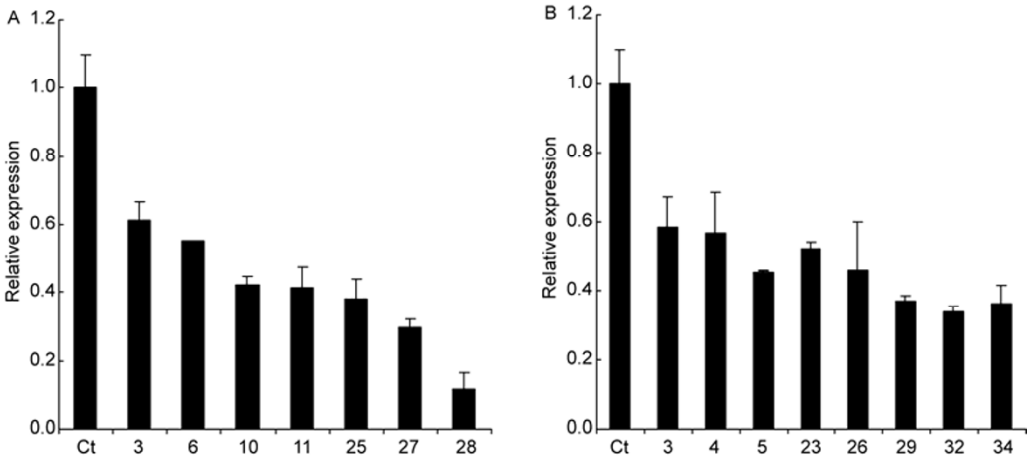


**Figure 4** Detection of mRNA levels in the *pks-1* knock-down strains using quantitative real-time PCR. A, qRT-PCR for pPKS-1a RNAi transformants (the numbers, 3, 6, 10, 11 25, 27 and 28, correspond to the transformants). Ct, a randomly picked control transformant with pSilent-1 only; *pks-1* cDNA was amplified using primers qPKS(s) and qPKS(as). *pks-1* transcripts were normalized against actin cDNA amplified with primers qActin(s) and qActin(as) (Table 1). B, qRT-PCR of pPKS-1b RNAi transformants (see Figure 1A and Section 2.4 for details).

(Ct in Figure 4A). Interestingly, transformants pA27 and pA28 in group 3, which formed albino mycelium, had the lowest level of *pks-1* mRNA, whereas members in group 1 (near wild-type pigmentation) had the highest levels of *pks-1* mRNA (pA3 and pA6 had 61% and 57% of the wild-type level, respectively), and group 2 transformants, pA10, pA11 and pA25, had intermediate levels (Figure 4A). Remarkably, the qRT-PCR results showed significant correlation between *pks-1* mRNA level and pigmentation providing further evidence that *pks-1* is responsible for melanin production, and that RNAi is effective for knocking down gene expression in *C. globosum*.

We conducted a second RNAi experiment on *pks-1* with a different *pks-1* fragment, pks1b (as indicated in Figure 1A), in plasmid pPKS-1b. All the experimental procedures for pPKS-1b were performed side by side with pPKS-1a. *pks-1* transcription in the resulting transformants (designated pB) was also measured by qRT-PCR. As anticipated, the level of *pks-1* transcript was decreased dramatically in all picked transformants, ranging from 34% to 59% of that in the control strain (Figure 4B).

### 2.5 Association of *pks-1* with chaetoglobosin A biosynthesis and sporulation

One purpose of the study was to identify PKS genes in chaetoglobosin A biosynthesis. Thus, we examined the production of chaetoglobosin A in the knock-down mutants using high-performance liquid chromatography (Figure 5, HPLC). Growth conditions for chaetoglobosin A biosynthesis were initially determined to be 200 mL PDB at 28°C for 8 d (Lou and Zhu, unpublished data). The concentration of chaetoglobosin A in all knock-down transformants was significantly reduced compared to control (Table 2, Figure 5 for pA28). In transformants pA25, pA27 and pA28, the concentration of chaetoglobosin A was 2.44, 4.83 and 2.94 mg $L^{-1}$, respectively, whereas the concentration in the Ct strain and wild type were 53.71 and 50.51 mg $L^{-1}$ respectively. This result clearly verifies that *pks-1* is not only involved in melanin biosynthesis, but is also involved in the biosynthesis of chaetoglobosin A.

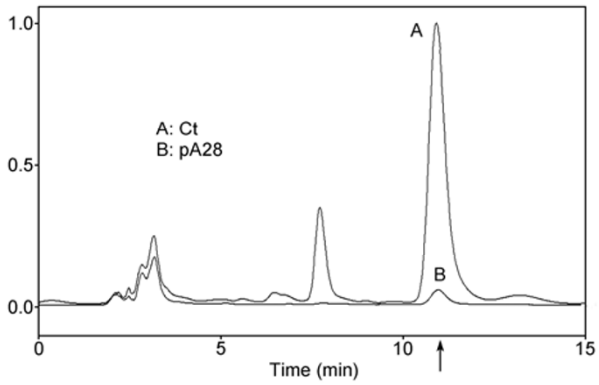


**Figure 5** HPLC for chaetoglobosin A production in *C. globosum* for the control (Ct) and *pks-1* knock-down strain, pA28. Arrow indicates the chaetoglobosin A peak.

**Table 2** Quantity of CheA in control (Ct) and *pks-1* knock-down strains

| Stain | Retention time (min) | CheA content (mg $L^{-1}$) |
|---|---|---|
| Wt | 10.973 | 50.51±8.79 |
| Ct | 11.012 | 53.71±4.34 |
| pA6 | 10.957 | 8.13±0.37 |
| pA25 | 10.967 | 2.44±0.28 |
| pA28 | 10.937 | 2.94±0.72 |
| pA27 | 10.943 | 4.83±0.55 |
| pA10 | 11.000 | 9.88±2.13 |
| pA3 | 10.947 | 16.14±3.69 |

We further found that *pks-1* was necessary for sporulation of *C. globosum*. In the knock-down mutants, few fruiting bodies (perithecia) and conidia were formed, while the wild type formed a great number of spores and perithecia (Figure 6). As expected, the phenotype of the Ct strain was similar to the wild type. Notably, microscopic observation showed mycelium of mutant pA28 to be much lighter than the wild type.

## 3 Discussion

We have previously encountered difficulty in achieving gene targeting by homologous recombination in *C. globosum*. Our failure to disrupt the single-copy *pks-1* gene, and also other genes, was likely because of the multi-ploidy property of the fungus's life cycle (unpublished data). Thus, an alternative technique, such as RNAi, is necessary to study gene function in this important fungus. Here, we have used RNAi to characterize a polyketide synthase gene, *pks-1*. Our results showed that the RNAi machinery functioned with high efficiency in *C. globosum*. Over 80% of the picked transformants (33 of 40 transformants pA and 32 out of 40 transformants pB) displayed a melanin deficiency (pigmentation) phenotype (Figure 2A). Interestingly, phenotypic outcome of the transformants was independent of PKS-1a copy number (Figures 2 and 3). Interference efficiency may depend on several factors, such as the integration site of PKS-1a, which will affect the production of hairpin RNA precursors of PKS-1a. In *N. crassa*, RNAi efficiency was less than 40% [44,45], while in the basidiomycete *C. neoformans*, efficiency was less than 10% [46]. In our study, qRT-PCR further revealed that all seven tested transformants with reduced pigmentation had decreased levels of *pks-1* mRNA (Figure 4A). The melanin-deficient mutants, pA27 and pA28, which formed albino mycelium on plates and under the microscope (Figures 2A and 6) had the lowest levels of *pks-1* mRNA. Southern blot analysis of these transformants excluded the disruption of *pks-1* itself and confirmed the ectopic insertion of the interference cassette in the genome (Figure 3). From these data, we conclude that *pks-1* is, in part, responsible for melanin biosynthesis in *C. globosum*. The gene *pks-1* encodes a polypeptide with 2181 amino acids which harbors five domains that are found in other fungal polyketide synthases (Figure 1A), suggesting that melanin biosynthesis in *C. globosum* is accomplished via the DHN pathway defined in various fungi [22,47,48]. To confirm this, tricyclazole, an inhibitor of the DHN pathway, was added to plates. This resulted in an absence of pigmentation (Figure 2B).

More importantly, RNAi revealed a critical role of *pks-1* in the biosynthesis of chaetoglobosin A. As shown by chromatography, chaetoglobosin A levels fell sharply in the silenced mutant pA28 (Figure 5). The concentration of chaetoglobosin A in the culture liquid was as low as 2.94 mg $L^{-1}$ which was only 5.8% of the wild type level (Table 2). RNAi does not completely inactivate transcription; therefore, it is understandable that a trace amount of chae-

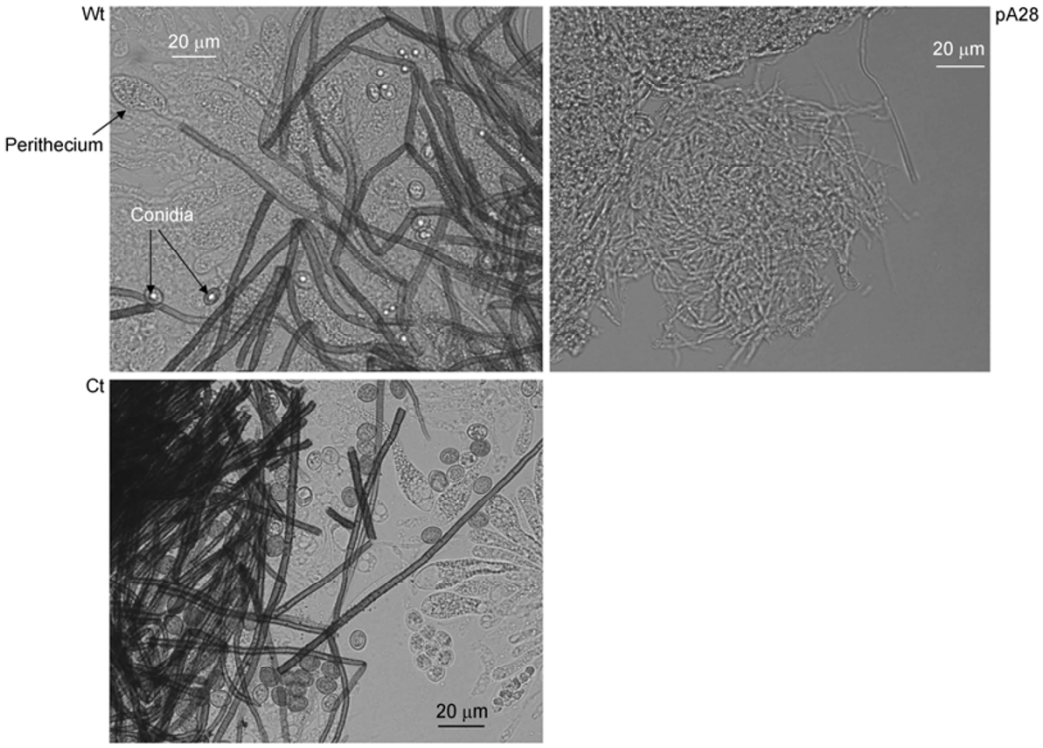


**Figure 6** Microscopic observation of sporulation in control (Ct) and *pks-1* knock-down strain, pA28. Wt, wild type; Ct, a randomly picked control transformant with pSilent-1 only.

toglobosin A remained in the mutants. It should be noted that *pks-1* is distinct in peptide sequence and domain architecture from the polyketide/non-ribosomal peptide hybrid synthetase (PKS-NRPS) gene *cheA* that is responsible for chaetoglobosin biosynthesis in *P. expansum* [41]. Only limited similarity over the KS, AT and DH domains is shared between PKS-1 and CheA. Extra domains, MT, ER, KR, and a PCP domain which adds tryptophan to the polyketide backbone in CheA, are missing in PKS-1. A TE domain in PKS-1, which is involved in the release of the polyketide chain, is not present in CheA [15]. We also searched the flanking regions of *pks-1*, but no comparable gene cluster was found in *C. globosum*. Chaetoglobosins consists of a polyketide backbone attached to tryptophan group [14,15]; therefore, PKS-1 alone can not complete the entire biosynthetic process. PKS-1 may also affect the biosynthesis of another secondary metabolite in *C. globosum* as shown by HPLC (Figure 5). A peak at approximately 7 min disappeared in pA28, indicating that PKS-1 provides a common precursor for other PKSs. In other words, PKS-1 may provide a precursor for chaetoglobosin and melanin biosynthesis. Our results demonstrate that a single PKS can generate diverse polyketide products and differential mechanisms for chaetoglobosin biosynthesis exist in fungi. Questions remain about chaetoglobosin biosynthesis in *C. globosum*, for example, an unknown non-ribosomal peptide synthetase that adds the tryptophan residue to the polyketide chain remains to be identified.

Another finding of this study was that *pks-1* is required for spore generation (probably for both conidia and ascospores) (Figure 6). Knock-down mutants of *pks-1* eliminated or reduced sporulation. In the albino mutant, pA28, which had the lowest level of *pks-1* mRNA, the capability to form spores was totally lost (Figure 6). This result indicates that secondary metabolism and reproductive differentiation may have direct crosstalk. A substantial body of evidence indicates that these processes are usually co-regulated by the G protein-cAMP-PKA pathway and the VeA-LaeA-VeB complex in fungi, in particular in *Aspergillus* spp. [19,23,25,49–51]. Secondary metabolites act as chemical indicators for the growth stage of fungi [49]. A previous study has shown that in *C. lagenarium*, melanin was critical for appressoria formation to penetrate plant hosts [52]. In *A. alternata*, melanin affects the size of conidia [53]. Earlier observations also suggested that an estrogenic mycotoxin produced by *F. graminearum* enhances its own perithecial production [54]. Butyrolactone I, produced by *A. terreus*, increases its hyphal branching, sporulation, and production of lovastatin [55]. However, to our understanding, direct evidence connecting secondary metabolism and development is still missing. Similar to our finding, a 6-methylsalicylic acid synthase (MSAS) gene, *fluP*, from *A. paraciticus* was essential for hyphae growth and sporulation, although it did not have a direct effect on aflatoxin production [56]. Based on these observations, it is intriguing to postulate that certain intracellular polyketides, perhaps as signaling molecules, may modulate other cellular processes such as sporulation and appressoria formation.

In this work, we established an RNAi protocol for *C. globosum*, which makes it possible to study gene function in this fungus. With RNAi, we demonstrated the roles of a polyketide synthase gene, *pks-1*, in both melanin and chaetoglobosin biosynthesis. Further, we found that *pks-1* has a critical role in asexual and sexual spore formation in *C. globosum*. No similar polyketide synthase has been documented to have such multiple roles. Our work may provide insight into the molecular basis of the mycotoxin chaetoglobosins and into the biosynthesis of other polyketides in *C. globosum*.

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