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Morphological changes induced by class III chitin synthase gene silencing could enhance penicillin production of *Penicillium chrysogenum*

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Abstract Chitin synthases catalyze the formation of β-(1,4)-glycosidic bonds between *N*-acetylglucosamine residues to form the unbranched polysaccharide chitin, which is the major component of cell walls in most filamentous fungi. Several studies have shown that chitin synthases are structurally and functionally divergent and play crucial roles in the growth and morphogenesis of the genus *Aspergillus* although little research on this topic has been done in *Penicillium chrysogenum*. We used BLAST to find the genes encoding chitin synthases in *P. chrysogenum* related to chitin synthase genes in *Aspergillus nidulans*. Three homologous sequences coding for a class III chitin synthase CHS4 and two hypothetical proteins in *P. chrysogenum* were found. The gene which product showed the highest identity and encoded the class III chitin synthase CHS4 was studied in detail. To investigate the role of CHS4 in *P. chrysogenum* morphogenesis, we developed an RNA interference system to silence the class III chitin synthase gene *chs4*. After transformation, mutants exhibited a slow growth rate and shorter and more branched hyphae, which were distinct from those of the original strain. The results also showed that the conidiation efficiency of all transformants was reduced sharply and indicated that *chs4* is essential in conidia development. The morphologies of all transformants and the original strain in penicillin production were investigated by light microscopy, which showed that changes in *chs4* expression led to a completely different morphology during fermentation and eventually caused distinct penicillin yields, especially in the transformants PcRNAi1-17 and PcRNAi2-1 where penicillin production rose by 27 % and 41 %, respectively.

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Introduction

Filamentous microorganisms such as fungi and *Streptomyces* are the most common microorganisms used for antibiotic production on an industrial scale. Traditionally, the production of β-lactam antibiotics has been improved through the development of better strains by a repeated mutation–selection approach, an example of which is the initial strain improvement program for the penicillin-producing strain *Penicillium chrysogenum*. However, as this method reaches its limits, it becomes necessary to look more closely at the functioning of the microorganisms to improve the fermentation process. With the advances in genetic engineering of β-lactam-producing microorganisms (Douma et al. 2010), it has become possible to use a more rational approach, often referred to as metabolic engineering, to strain improvement.

When grown in submerged culture, filamentous microorganisms exhibit different morphological forms, ranging from single hyphal elements (so-called disperse mycelia) over connected networks of hyphae up to distinct particles of biomass called pellets, depending on the culture conditions and the genotype of the strain (Papagianni 2004). Paul and Thomas (1996) formulated a very comprehensive mathematical morphological model describing growth, differentiation, and penicillin production in *P. chrysogenum*. The model is divided into four distinct regions on the basis of the activities and structures of the hyphal compartments, viz., actively growing (mainly apical) regions, nongrowing regions, vacuoles, and degenerated or metabolically inactive regions. Penicillin production is assumed to occur only at a nongrowing region next to an apical region. Similarly,

Zangirolami et al. (1997) divided the hyphae into three cell types: apical, subapical, and hyphal. Penicillin formation is assumed to take place in the subapical compartment and in the growing region of the hyphal compartment. All of the above reports suggested that penicillin secretion is closely correlated with hyphal extension rates and subtip growth. According to many reports, mycelial morphology is crucial to the process of fermentation due to the fact that it influences the rheology of the fermentation medium and thereby has a significant impact on mixing and mass transfer within the bioreactor. Agitation rates have been studied and are known to influence the physiological properties of the culture in addition to altering the morphology and penicillin production (Tucker and Thomas 1993; Shamlou et al. 1994; Makagiansar et al. 1993; Smith et al. 1990). The relationship between fungal morphology and process productivities has attracted interest from both academia and industry and attempts have been made to manipulate morphology to achieve maximal performance.

In recent years, there have been major advances in the tools available for metabolic engineering of morphology, such as developments in genomics (van den Berg et al. 2008), the increased number of suitable transformation vectors, and powerful image analysis tools that enable rapid quantification of fungal morphology. In particular, RNA interference, as one of the most fascinating areas in molecular biology, provides an exceptionally potent tool for determining how changes in morphology are related to the variable suppression of key genes. In this work, a silencing vector for class III chitin synthase gene *chs4* was constructed to clarify the roles of this gene in hyphal morphogenesis and subsequent penicillin yield in submerged fermentation of *P. chrysogenum*. Chitin synthases are membrane-bound proteins that catalyze the polymerization of GlcNAc using UDP-GlcNAc as a substrate to form chitin, the major component in the cell walls of most filamentous fungi. Chitin plays an important role in the structural rigidity and osmotic integrity of the wall and represents an important determinant of the shapes of hyphae and conidiophores. Fungal chitin synthases are divided into classes I to VII, according to the similarities in their amino acid sequences (Choquer et al. 2004). The functions of chitin synthases in filamentous fungi have been elucidated by gene disruptions or gene deletions. Deletion mutants of a class III chitin synthase encoding gene, *chsB*, in *Aspergillus nidulans* grew very slowly and formed extremely small colonies with highly branched hyphae, suggesting its important role in hyphal tip growth (Yanai et al. 1994; Borgia et al. 1996; Fukuda et al. 2009). Disruption of class III chitin synthase encoding genes in *Aspergillus fumigatus* caused growth defects similar to those of the *chsB* deletion mutant (Mellado et al. 1996). These findings suggest that through control of chitin synthases, it may be possible to metabolically engineer fungal morphology to optimize it for a given fermentation process to achieve appropriate viscosity and high productivity. However, there have been no relevant reports in *P. chrysogenum*, although the roles of chitin synthases in the morphogenesis of *Aspergillus* have been researched in detail. Understanding this process is essential if we are to consider optimization of penicillin production via morphological engineering.

Materials and methods

Strain and media

P. chrysogenum Pcoriginal (CCTCC AF2012008) was maintained on agar slants containing glycerol 7.5 gL^{-1}, yeast extract 3.0 gL^{-1}, sodium chloride 10.0 gL^{-1}, calcium sulfate 0.25 gL^{-1}, magnesium sulfate 0.005 gL^{-1}, potassium dihydrogen phosphate 0.006 gL^{-1}, copper sulfate 0.001 gL^{-1}, ammonium ferrous sulfate 0.0015 gL^{-1}, pH 6.8–7.0. For DNA or RNA extraction and protoplast preparation, cultures were grown in minimal medium (MM) for *P. chrysogenum* as described by Rowlands and Turner (1973). For penicillin production, fresh spores were incubated for 42–48 h at 220 rpm and 25 °C in 250 mL flasks with 40 mL seed medium containing corn steep liquor 67 gL^{-1}, sucrose 20 gL^{-1}, calcium carbonate 5 gL^{-1}, pH 5.8–5.9. The seed culture (1:10) was inoculated into 250-mL flasks with 30 mL fermentation medium [containing corn steep liquor 46.5 gL^{-1}, calcium carbonate 10 gL^{-1}, lactose 130 gL^{-1}, monopotassium phosphate 4 gL^{-1}, ammonium sulfate 4.5 gL^{-1}, sodium sulfate 1.5 gL^{-1}, corn oil 6.7 mLL^{-1}, ammonium phenylacetic acid (10 %) 3.3 mL L^{-1}, pH 5.8–5.9] under the same conditions for 220 h.

DNA isolation and polymerase chain reaction (PCR) amplification

Genomic DNA was prepared using the EZ spin column fungal DNA isolation kit (Sangon, China). Mycelia were grown at 25 °C and 220 rpm for 2 days in liquid MM. PCR amplification was carried out with *Taq* DNA polymerase according to the manufacturer's instructions (Sangon, China). Oligonucleotides chs4F (5′-CCATGCCATGGCGGTGC CTGTGGTGAAAT-3′) and chs4R (5′-CCATGCCATGGGC GACGCTGGGAGATAAA-3′) (sequences corresponding to the *Nco*I restriction sites are underlined) were used to amplify a 424-bp (from nucleotides 1,397 to 1,820) *chs4* fragment (Gene ID: 8315140) from genomic DNA.

Construction of silencing vector

The 424-bp *chs4* fragment was ligated to plasmid pJL43-RNAi (kindly donated by Professor Juan F. Martín) (Ullán

et al. 2008) that was digested with *Nco*I restriction enzyme to generate the pchs4-RNAi vector.

Transformation

Fungal transformation was performed by a polyethylene glycol (PEG)-mediated method as described by Crawford et al. (1995). For antibiotic selection, phleomycin was used in a concentration of 30 $\mu g mL^{-1}$.

RNA preparation and reversed transcript-PCR

Total RNA was extracted using TRIzol reagent (Sangon, China). For cDNA synthesis, RNA was treated with DNase I and used in the M-MuLV first strand cDNA synthesis kit (Sangon, China) using oligo(dT) primer according to manufacturer's instructions. For reversed transcript (RT)-PCR experiments, 1 μL of cDNA was amplified in a 20-μL reaction system using *Taq* DNA polymerase. The primers used were as follows: (1) for amplification of a 382-bp fragment from the *chs4* coding sequence, primers RTchs4F (5′-ATGGTTGTCCAGTCGTCCTA-3′) and RTchs4R (5′-TGGCGGTATTTGTGAAGC-3′) and (2) for amplification of a 322-bp fragment corresponding to the endogenous *actin* gene (Gene ID: 8307213), which was used as a control for the reaction, primers RTactinF (5′-ACAATGGTTCGGG TATGTG-3′) and RTactinR (5′-TTCTCACGGTTGA ACTTGG-3′). Agarose gel electrophoresis was used to detect PCR products.

Determination of conidiation efficiency and growth rate

Conidia were point inoculated on solid plates (SP medium) of the same composition as agar slants mentioned above and incubated at 25 °C for 9 days. Conidia produced by a colony were scraped thoroughly from the agar surface, suspended in 5 mL of water and then filtered through four layers of lens paper (45±12 μm pore, Sangon, China). After dilution, optical density of the conidia suspension at 560 nm was measured. At the same time, suspensions were smeared on plates and incubated until colonies appeared. The relationship between optical density and conidia number was graphed to make a standard curve. For determination of growth rates, colony diameters were determined. Data were taken from five colonies for each strain to determine the average and standard deviation of conidia number and colony diameter.

Sensitivity to Calcofluor white and Congo red

Mycelia were grown at 25 °C and 220 rpm for 2 days in liquid MM with glass beads. Cell densities were adjusted to the same OD_{600} value. Sensitivity to Calcofluor white (CFW, fluorescent brightener 28, Sigma-Aldrich, St. Louis, MO, USA) and Congo red (CR) was tested by spotting 10 μL and tenfold serial dilutions thereof on plates of SP medium supplemented with CFW (100 $\mu g mL^{-1}$) or CR (200 $\mu g mL^{-1}$) and incubating them for 9 days at 25 °C. To test if defects could be suppressed by osmotic stabilizer, solid plates containing 1.2 M mannitol were used.

Chitin content determination

Chitin was determined by a modified method based on that of Pessoni et al. (2005). Strains were grown in 50 mL of MM for 48 h at 25 °C. Mycelia were collected and finely ground in a mortar with liquid nitrogen. The powders were resuspended in 1 mL water. The suspensions were sonicated to ensure a complete breakage of cells, centrifuged for 5 min at 3,000*g* and then transferred into an oven set at 110 °C overnight to dry the biomass. The dried cell wall materials (approximately 2 mg) were wetted with 1 mL of 6 M HCl and the tubes shaken gently by hand to allow complete contact of the acid solution with the polysaccharides. The mixtures were left for 48 h at 90 °C. After removing the HCl by using a speed vacuum concentrator at 50 °C, dried samples were resuspended in 1 mL of water and centrifuged to remove insoluble material. The concentrations of glucosamine hydrochloride in the hydrolyzates were determined colorimetrically according to Chen and Johnson (1983). The dilute hydrolyzate solutions (1 mL) were added to 0.25 mL 4 % acetylacetone solution (4 %v/v acetylacetone in 1.25 M sodium carbonate) and mixtures were incubated at 90 °C for 1 h. After cooling in a water bath to room temperature, 2 mL of ethanol and 0.25 mL of Ehrlich reagent (1.6 g of *N*-*N*-dimethyl-*p*-aminobenzaldehyde in 60 mL of a 1:1 mixture of ethanol and concentrated HCl) were added and the absorbance was measured at 530 nm. Comparison with a standard curve of glucosamine hydrochloride (G4875, Sigma-Aldrich, St. Louis, MO, USA) at six concentrations (10, 20, 30, 40, 50, and 60 $mg mL^{-1}$) was used for calculation of the mycelium chitin content, as monomer equivalent *N*-acetyl-β-D-glucosamine expressed as micrograms of glucosamine hydrochloride per milligram dry weight of fungal mycelium.

Light microscopy

Samples from a shake flask culture were diluted to disperse the hyphal elements. The diluted samples were stained by lactophenol cotton blue for several minutes and observed using an Olympus TH4-200 microscopic imaging system (Olympus Optical Co. Ltd., Tokyo, Japan).

Scanning electron microscopy

Plugs (approximately 1 mm^3) were cut from the actively growing margins of colonies grown on solid medium using

a razorblade and fixed at 4 °C with 2.5 % (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After three buffer washes, samples were dehydrated in a graded series of increasing ethanol concentrations [30, 50, 70, 90, 100, and 100 % (v/v), 15 min each step]. After air drying, the fixed material was sputter-coated with gold for 180 s. The material was examined and photographed with a field emission scanning electron microscope (Sirion200, FEI, Hillsboro, OR, USA).

Penicillin assay

Penicillin titer was determined using the classical iodometric assay method according to the 2006 US Pharmacopeia and National Formulary (United States Pharmacopeial Convention 2006).

Results

Identification of a chitin synthase gene in *P. chrysogenum*

Given the close evolutionary relationships between *Penicillium* and *Aspergillus*, we used the BLAST tool in National Center for Biotechnology Information (NCBI) to find genes encoding chitin synthases in *P. chrysogenum* related to the chitin synthase genes of *A. nidulans*. Three homologous sequences, *chs4* (locus tag no. Pc13g15750), hypothetical protein (locus tag no. Pc13g06340), and hypothetical protein (locus tag no. Pc12g11730), which correspond to *chsB* (D21269), *chsA* (D21268), and *chsD* (U62895) in *A. nidulans*, respectively, were found with a percentage identity between 74 % and 78 %. The corresponding amino acid sequence identities were 87 %, 76 %, and 74 %, respectively. CHS4 (XP_002559970) that showed the highest identity was studied in detail. This protein has 915 amino acid residues and a calculated molecular mass of 101.6 kDa. Four conserved domains were found, that is, Chitin_synth_C (cd04190, C-terminal domain of chitin Synthase that catalyzes the incorporation of GlcNAc from substrate UDP-GlcNAc into chitin), Chitin_synth_1 (pfam01644, chitin synthase that is found commonly in chitin synthases classes I, II, and III), Chitin_synth_1N (pfam08407, the N-terminal domain of chitin synthase), and COG1215 (COG1215, glycosyltransferase that is probably involved in cell wall biogenesis).

Constructions for *chs4* gene silencing

A dual promoter system for RNA silencing in *P. chrysogenum* was developed. Plasmid pJL43-RNAi contains a phleomycin resistance gene (*ble*) and a dsRNA expression cassette (Ullán et al. 2008), which contains a *Nco*I cloning site that allows expression of DNA inserts under the control of two convergent promoters, the *A. nidulans gpdA* gene promoter and the *P. chrysogenum pcbC* gene promoter. To silence the *chs4* gene, pchs4-RNAi, which contains a 424-bp (from nucleotides 1,397 to 1,820) PCR product amplified from the *chs4* gene and cloned into the *Nco*I site of pJL43-RNAi, was constructed (Fig. 1).

RT-PCR analysis and colony observation

Protoplasts of *P. chrysogenum* were transformed with the integrative plasmid pchs4-RNAi. We obtained 33 different transformants. All transformants were then inoculated onto agar slants and incubated for 7 days at 25 °C. Twenty-two of them (66.7 %) showed a clear reduction in conidia formation. As shown in Fig. 2a, the randomly selected transformants PcRNAi1-15, PcRNAi1-17, PcRNAi2-1, and PcRNAi2-6 were unable to form conidia or only able to form a few conidia and, thus, appeared colorless. In contrast, the original strain (Pcoriginal) developed green conidia, suggesting that the pJL43-RNAi-based construct efficiently induced gene silencing of *chs4* at different levels in the different transformants. These phenotypic characterizations were confirmed by examining the expression of *chs4* mRNA by RT-PCR (Fig. 2b). Consistent with the phenotypic observation, *chs4* mRNA expression was very weak in all the strongly silenced transformants, whereas *chs4* mRNA was detected at lower levels than in wild type in the moderately silenced transformants.

Effects of *chs4* knockdown on growth and hyphal morphology

We analyzed the effects of *chs4* knockdown by comparing the radial growth rate on agar plates of silenced transformants with that of the original strain. Conidia of each strain were point-inoculated on solid medium and incubated at 25 °C for 9 days. The colony diameters were then

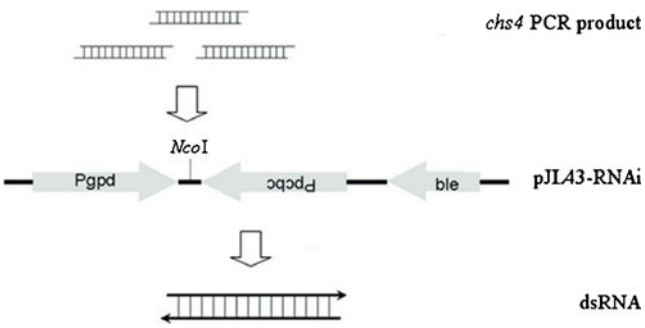

Fig. 1 Construction of the silencing vector pchs4-RNAi. A *chs4* fragment was amplified by PCR using a set of specific primers (chs4F and chs4R) and inserted into the *Nco*I site of pJL43-RNAi. DsRNAs are transcribed bidirectionally by the opposing promoters in the fungal cell. Transformants were selected by resistance to phleomycin. P*gpd A. nidulans gpdA* gene promoter, P*pcbc P. chrysogenum pcbC* gene promoter, *ble* phleomycin-resistant gene, *dsRNA* double-stranded RNA

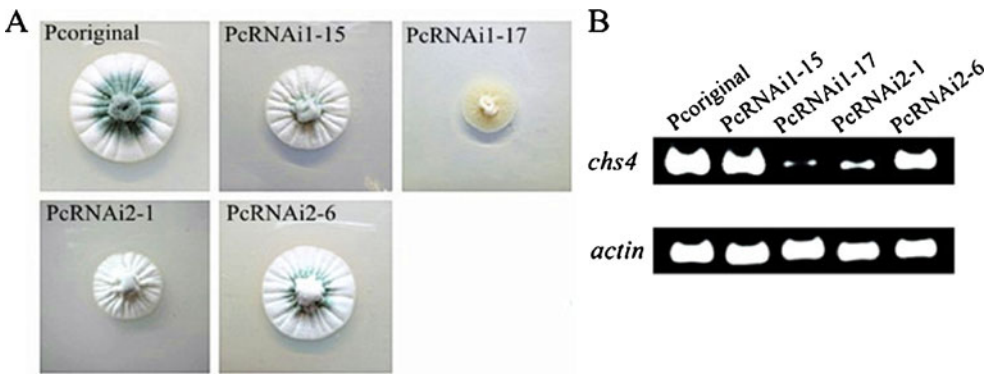

Fig. 2 *chs4* was targeted for silencing using the pchs4-RNAi vector. **a** All strains were point inoculated on plates of SP medium and incubated for 9 days. Conidiation of the original strain (Pcoriginal) is evidently larger than that of the mutants. **b** RT-PCR analysis of *chs4* mRNA expression in the *chs4*-silenced transformants. The *actin* gene was used as a control

determined (Fig. 3). All silenced transformants presented colonies much smaller than that of the original strain.

As chitin synthase catalyzes the formation of major components of the cell wall, we set out to determine the role of *chs4* in cell wall construction. Sensitivities to CFW and CR, compounds that interfere with the integrity of the cell wall by binding to cell wall polysaccharides, were determined using drop tests. All four *chs4* mutant strains showed increased sensitivity to both compounds as compared to the control strain. The CFW-hypersensitive phenotype could be suppressed by the addition of the osmostabilizer mannitol, suggesting a disruption of cell wall integrity in all mutants (Fig. 4).

Involvement of *chs4* in conidiation

We next examined the effect of *chs4* knockdown on conidiation by measuring the conidia formation of all strains. All four selected knockdown strains showed a dramatic reduction in the formation of conidia (Fig. 5). PcRNAi1-17 and PcRNAi2-1 did not conidiate. The reductions in conidiation efficiency of PcRNAi1-15 and PcRNAi2-6 were more moderate and were about 14 % and 48 % of that of the original strain, respectively. It is possible that the interference of *chs4* is incomplete in the latter two strains and that this incompleteness could relieve the conidiation defect to some extent. Conidia with abnormal morphology were microscopically visible in PcRNAi1-15 and PcRNAi2-6. In contrast to the spherical conidia in Pcoriginal, they formed smaller spores with fusiform (PcRNAi1-15) or oval (PcRNAi2-6) shape (Fig. 6).

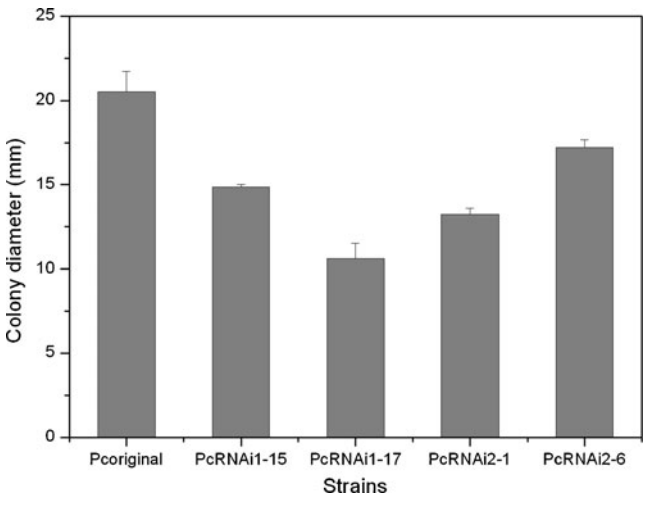

Fig. 3 Growth rates of the original strain and mutants. Strains were grown on plates of SP medium at 25 °C for 9 days. Colony diameters were measured. Data were taken from five colonies for each strain to determine the average and standard deviation

Chitin content of transformants

We next measured the chitin contents of *chs4* knockdown mutants grown in a liquid medium. Chitin contents in the cell walls of the mutants were decreased to different extents in comparison with that of the relevant wild type strain (Pcoriginal) (Fig. 7). PcRNAi2-6 contained about 95 % of the wild type chitin levels, whereas the chitin content of PcRNAi1-17 was 77 % of that of the wild type strain. The overall changes were not that significant, suggesting that the *chs4* product was responsible for the synthesis of relatively little chitin in vivo.

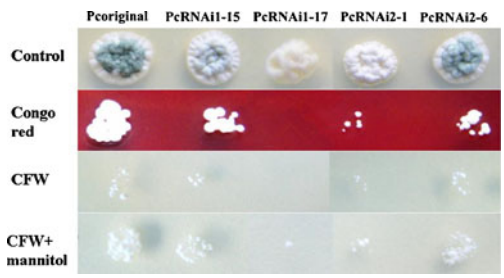

Fig. 4 *chs4* is involved in cell wall integrity. Ten microliters of equal concentration (OD_{600}=1) of growing cells were spotted on SP medium, respectively, containing Congo red (200 $\mu g mL^{-1}$) or CFW (100 $\mu g mL^{-1}$). Images were taken after 9 days of incubation at 25 °C. The transformants revealed a CFW-hypersensitive phenotype that could be suppressed by the addition of the osmostabilizer mannitol (1.2 M)

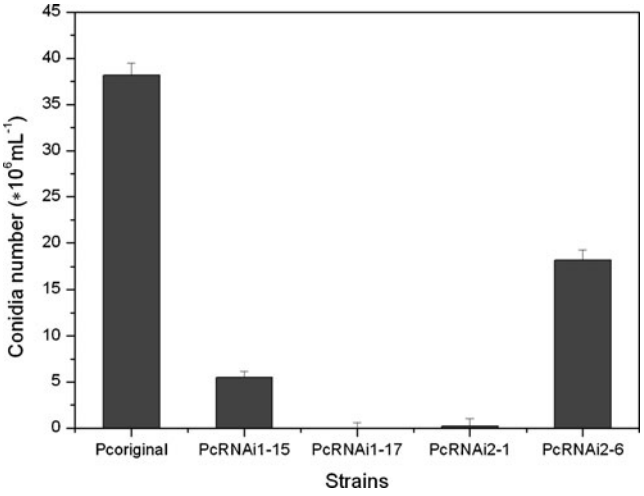

Fig. 5 Conidia formation efficiency of the original strain and mutants. Strains were point inoculated on plates of SP medium and incubated at 25 °C for 9 days. Data were taken from five colonies for each strain to determine the average and standard deviation

Changes of morphology and yield in penicillin fermentation process

To assess the effects of morphological changes on penicillin production, hyphal morphology in the seed and fermentation phases was observed. Figure 8 shows that, in the seed phase, the morphology of PcRNAi1-15 was almost the same as that of the original strain. Other transformants displayed severe defects in hyphal growth. PcRNAi1-17 produced stunted and bulging hyphae that had been proved to be connected with penicillin production (Luengo et al. 1986), whereas PcRNAi2-1 and PcRNAi2-6 formed short, thick, and highly branched phenotypes. Furthermore, the growth rates of PcRNAi1-17 and PcRNAi2-1 were much slower than the other strains (Fig. 9a). After entering the fermentation phase, loose clumps of mycelia were formed immediately by the agglomeration of hyphal elements in all strains. Pellets were formed rapidly with a dense core within the clumps in PcRNAi1-17 and PcRNAi2-1. The compactness of the agglomeration of hyphal elements decreased in the following order: PcRNAi1-17 > PcRNAi2-1 > PcRNAi2-6 ≥ Pcoriginal > PcRNAi1-15. Obviously, the diameters of pellets in PcRNAi1-17 were much bigger than in PcRNAi2-1 after 48 h in submerged fermentation. As the fermentation progressed, the pellets became more compact. In drastic contrast to the severe growth defect in the seed phase, PcRNAi1-17 and PcRNAi2-1 grew very fast in the fermentation period and finally surpassed the original strain (Fig. 9b). All transformants except PcRNAi1-15 exhibited enhanced penicillin yields, especially PcRNAi1-17 and PcRNAi2-1 which yields were 27 % and 41 % higher than that in the parent strain, respectively, indicating that compact pellets or highly branched morphology favored penicillin production (Fig. 9c).

Discussion

In fungi, conventional gene knockout by homologous recombination is traditionally used to identify gene functions. However, for filamentous fungi, the low frequency of homologous recombination due to integration by random ectopic recombination constrains its development (Maier et al. 2005; Kück and Hoff 2010). An alternative method for reduction of expression of a particular gene is the use of RNA interference. In filamentous fungi, two different RNA silencing vector types, namely, hairpin RNA (hpRNA)-expressing and opposing-dual promoter systems, have recently been used in different fungal species (Dang et al. 2011; Salame et al. 2011). Nakayashiki et al. (2005) developed the versatile vector pSilent-1 for ascomycete fungi, which carries a hygromycin resistance cassette and a transcriptional unit for hpRNA expression with multiple cloning sites and a spacer of an intron sequence. As the construction of this kind of vector requires two steps of oriented cloning, its applicability is limited to a small or moderate scale. To circumvent the limitations of the hpRNA expression vectors, Nguyen et al. (2008) demonstrated the applicability of

Fig. 6 Scanning electron micrographs of conidia of Pcoriginal and its transformants, PcRNAi1-15 and PcRNAi2-6. *Bars* represent 5 μm

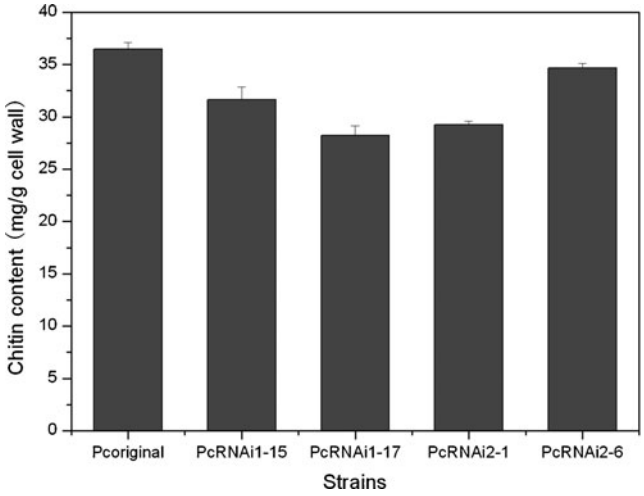

Fig. 7 Chitin contents of the original strain and *chs4* mutants. The *error bars* indicate the standard deviations of data from three independent cultures

implementing a dual-promoter RNAi system for large-scale elucidation of gene function in the plant pathogen *Magnaporthe oryzae*. Janus et al. (2009) used an inducible RNAi vector to generate silenced *P. chrysogenum* strains, which appeared colorless on xylose-containing medium. In this paper, we ligated a 424-bp fragment of *chs4* gene to the plasmid pJL43-RNAi containing a *Nco*I cloning site that allows expression of DNA inserts under the control of two convergent promoters to construct a silencing vector. After transformation into *P. chrysogenum*, dozens of mutants were obtained and several of them were randomly selected and studied in detail.

Previous studies have focused on gene knockout of chitin synthase genes. In *Neurospora crassa*, inactivation of a class III chitin synthase, Chs-1, leads to slow growth, aberrant hyphal morphology, and a decrease in chitin synthase activity (Yarden et al. 1991). In *A. fumigatus*, two genes encoding class III chitin synthases, *chsC* and *chsG*, have been identified. The *chsG* deletion mutant showed slow growth and defects in conidiation, and its hyphae were highly branched. The *chsC* deletion did not cause any phenotypic change. The *chsC chsG* double deletion mutant showed almost the same phenotype as the *chsG* single deletion mutant (Mellado et al. 1996). Yanai et al. (1994) concluded that the *chsB* gene is essential for hyphal growth in *A. nidulans* based on the observation that growth of a haploid *chsB* disruptant stopped immediately after the germination of conidia, with their hyphal tips swelling. Borgia et al. (1996) obtained *chsB* disruptants that grew very slowly with a high degree of branching and contained normal septa. The colonies did not form conidiophores and conidia and became brown after about 4 days of incubation. These changes are very similar to those seen with the knockdown strains generated in this study. By introducing silencing vectors into *P. chrysogenum*, we found that the *chs4* knockdown mutants exhibited severe growth defects, were highly branched, formed fewer conidia than the original strain, and, thus, appeared colorless. These results strongly suggest that class III chitin synthase is necessary not only for hyphal growth but also for conidiation.

In submerged fermentation, a particular morphological form may be favorable to achieve maximal performance. Take *Aspergillus niger*, for example, filamentous growth is preferred for pectic enzyme production, whereas the pelleted

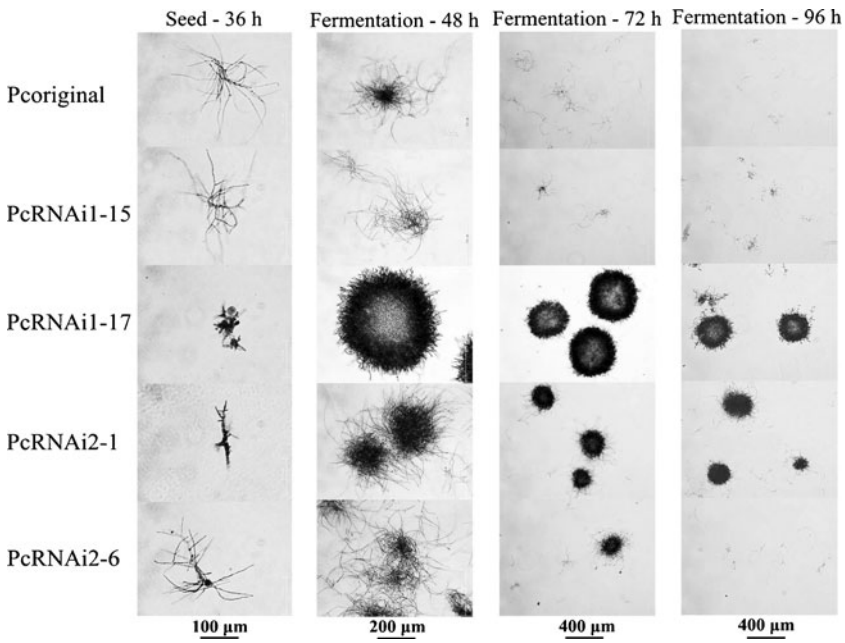

Fig. 8 Hyphal morphology of *P. chrysogenum* Pcoriginal and its transformants in submerged fermentation. Hyphae were collected from cultures in seed medium at 36 h of cultivation at 25 °C or in fermentation medium at 48, 72, and 96 h, stained with lactophenol cotton blue and observed under a light microscope

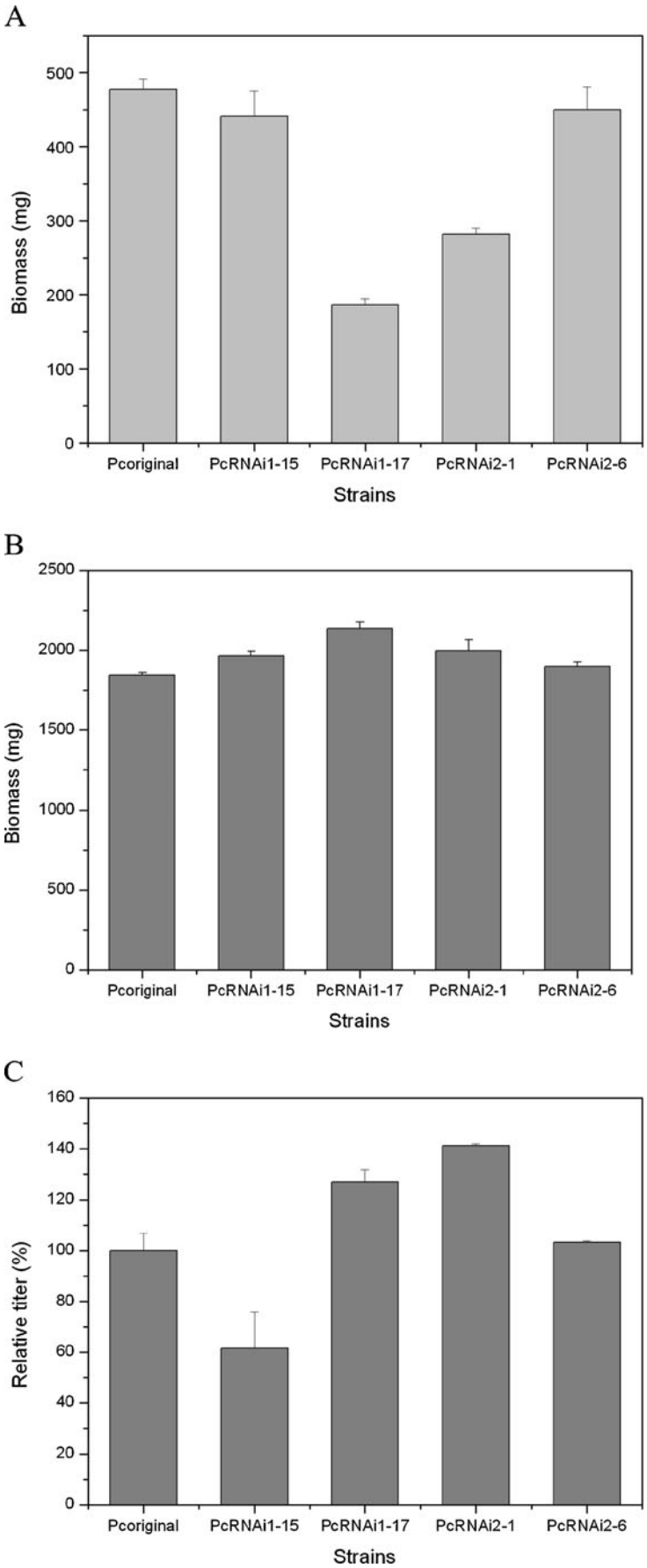

Fig. 9 Biomass and penicillin production in the submerged fermentations. **a** Mycelia from seed medium were collected at 45 h of cultivation, washed, and dried before determination of the biomass. **b** Dry biomass of mycelia collected from cultures at the end of fermentation. **c** After cultivation for 220 h in fermentation medium, penicillin yields were determined by the classical iodometric assay method. *Error bars* indicate the standard deviation of three determinations made from three independent cultures

form is in favor of citric acid production (Steel et al. 1954; Kristiansen and Bullock 1988). Generally, filamentous growth can lead to highly viscous broths with non-Newtonian, pseudoplastic flow behavior (Kristiansen and Bullock 1988), while pelleted growth exhibits low viscosities and approach Newtonian flow behavior (Chain et al. 1966). With increasing compactness of pellets, on the one hand, it was much easier to absorb nutrients due to a density effect, leading to a steady growth of the biomass. On the other hand, however, due to diffusional limitation of oxygen and nutrients, a possible severe drawback may be introduced in the interior of larger pellets, which can have a significant effect on both metabolism and product synthesis (Phillips 1966; Elmayergi et al. 1973; van Suijdam et al. 1980). Hence, it is generally considered that small pellets rather than large ones are suitable for filamentous fungal fermentations. In this paper, the morphological characteristics of the *P. chrysogenum* wild type strain Pcoriginal and its *chs4* silenced mutants in submerged fermentation were observed. Some of them grew filamentously, while others grew via small or large pellets. Penicillin production by the knockdown mutants was variable. The yield of one transformant was 41 % higher than in the original strain. These results suggest morphological changes may have a certain effect on penicillin production that may be attributed to the highly branched morphology and the significant decrease in viscosity that enhances the desirable mixing and mass transfer properties of the culture fluid considerably due to pellet formation. In particular, penicillin fermentation in all strains was performed under conditions that were optimal for the original strain. We believe that, by optimizing the fermentation conditions for the transformants, the penicillin yield will certainly be further improved.

In conclusion, since RNA interference can cause partial reduction (knockdown) instead of a complete loss (knockout) in gene expression, it promises to be a powerful tool for investigating the effects of an essential gene on the morphology of interest. It appears plausible that morphology has a significant role to play, influencing penicillin production either directly (tip number) or indirectly (by affecting mixing and mass transfer). Furthermore, comparative transcriptomics suggested an overrepresentation of genes encoding morphogenesis and developmental factors in a high penicillin producer, which was in line with the morphological differences between the two tested high and low penicillin producers (van den Berg et al. 2008), while the proteome analysis showed changes in the levels of several proteins involved in cell wall biosynthesis and/or integrity correlated well with the improved penicillin biosynthesis in the high producer strains (Jami et al. 2010). If all of the above results prove to be applicable to other fungal species and processes, manipulation of chitin synthases will represent a simple and inexpensive means of improving production during industrial filamentous fungal fermentations.

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