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ORIGINAL RESEARCH PAPER

Morphology engineering of *Penicillium chrysogenum* by RNA silencing of chitin synthase gene

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Abstract Chitin synthases, that catalyze the formation of chitin the major component of cell walls in most filamentous fungi, play crucial roles in the growth and morphogenesis. To investigate the roles of chitin synthase in *Penicillium chrysogenum*, we developed an RNAi system to silence the class III chitin synthase gene *chs4*. After transformation, mutants had a slow growth rate and shorter but highly branched hyphae. All transformants either were unable to form conidia or could form only a few. Changes in *chs4* expression could lead to a completely different morphology and eventually cause distinct penicillin yields. In particular, the yield of one transformant was 41 % higher than that of the original strain.

Keywords Chitin synthase · Morphology · Penicillin production · RNA interference

Introduction

Filamentous fungi are extensively used in industry since they are able to synthesize a large number of important products, including enzymes, antibiotics, organic acids and other industrially relevant compounds. Unlike bacterial unicellular production systems, a complex morphologic development needs to be faced in the case of filamentous fungi. Hyphal tip growth and branching result in different macroscopic appearances, ranging from freely dispersed mycelia, over connected networks of hyphae (so-called clumps) up to densely packed pellets. The fungal morphology plays a significant role on medium rheology, and thereby affecting the mixing and mass transfer within the bioreactor (Papagianni 2004), but also influences metabolite productivity, resulting in either lower specific growth rate (McIntyre et al. 2001), or enhanced enzyme production by strains with altered morphology (McCarthy et al. 2005). Therefore, it would be of great interest if one could devise a way of optimizing the hyphal morphology for mass production.

Since filamentous fungi have rigid cell walls that mainly comprise polysaccharides such as chitin and glucan, these polysaccharides are crucial for the morphogenesis of the filamentous fungi. Chitin synthases (EC 2.4.1.16) are membrane-bound proteins that catalyze the polymerization of GlcNAc using UDP-GlcNAc as a substrate. 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 (Yanai et al. 1994; Borgia et al. 1996; Fukuda et al. 2009). Disruption of *chsG* in *Aspergillus fumigatus* caused growth defects similar to those of the *chsB* deletion mutant (Mellado et al. 1996).

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These findings suggest that it may be possible to optimize the morphology for a fermentation process with appropriate viscosity and high productivity through control of chitin synthases. However, there have been no relevant reports in *Penicillium chrysogenum*. Understanding of this process is essential if we are to consider optimization of penicillin production via morphological engineering.

In the present study, we transformed a silencing vector containing class III chitin synthase gene, designated *chs4*, into *P. chrysogenum* and reported its effect on morphology and penicillin production during submerged cultivation.

Materials and methods

Construction of silencing vector and transformation

Genomic DNA was prepared using the EZ spin column fungal DNA isolation kit (Sangon, China). A 424-bp *chs4* fragment was amplified with *Taq* DNA polymerase using the primers as shown in Supplementary Table 1, and then introduced into plasmid pJL43-RNAi (kindly donated by Professor Juan F. Martín) which was digested with *Nco*I to generate the pchs4-RNAi vector. Fungal transformation was performed by a polyethylene glycol (PEG)-mediated method as described byCrawford et al. (1995). Transformant clones were selected by resistance to phleomycin (30 μg ml^{-1}).

RNA preparation and reversed transcript-PCR

Total RNA was extracted from mycelia during the seed stage using the Trizol reagent (Sangon, China). For cDNA synthesis, RNA was treated with DNase I and used in the M-MuLV first strand cDNA synthesis kit using Oligo(dT) primer (Sangon, China). For reversed transcript (RT)-PCR experiments, 1 μl of cDNA was amplified in a 20 μl reaction system using *Taq* polymerase and appropriate primers (Supplementary Table 1). *Actin* of *P. chrysogenum* was also

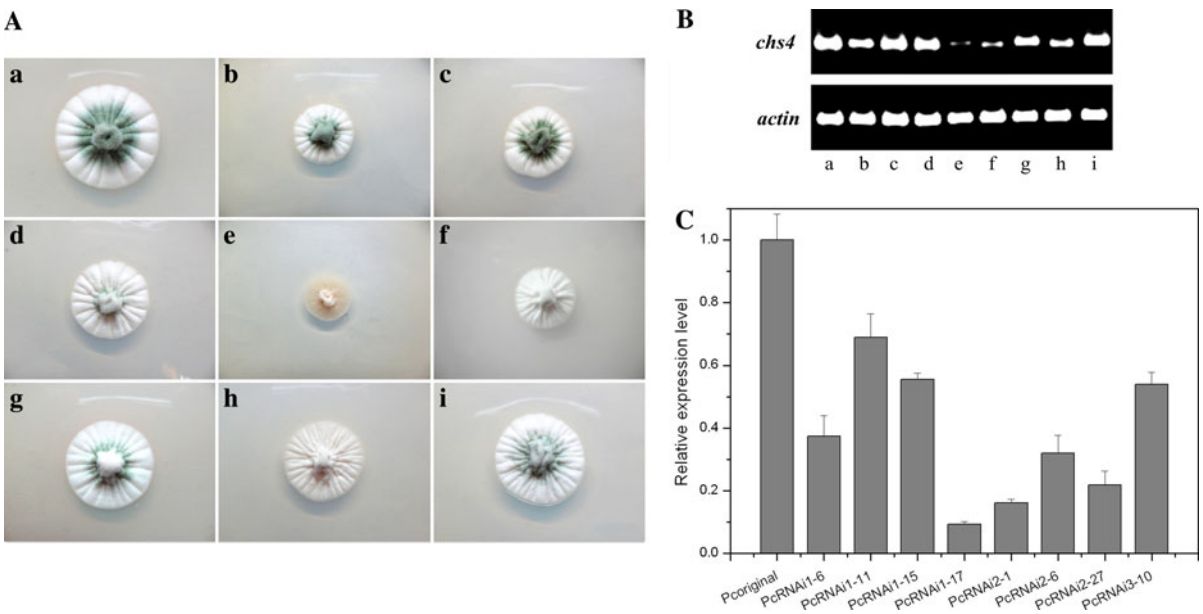

Fig. 1 *chs4* was targeted for silencing using the pchs4-RNAi vector. **A** All strains were point inoculated on plates and incubated for 9 days. Conidiation of the original strain (Pcoriginal) is evidently larger than that of the mutants. **B** RT-PCR analysis of *chs4* mRNA expression in the *chs4*-silenced transformants. The *actin* gene was used as a control. *a* Pcoriginal, *b*–*i* transformants, represent PcRNAi1-6, PcRNAi1-11, PcRNAi1-15, PcRNAi1-17, PcRNAi2-1, PcRNAi2-6, PcRNAi2-27, PcRNAi3-10, respectively. **C** qRT-PCR assay of *chs4* expression in Pcoriginal and its transformants. The *actin* gene was used as internal control and the ratio of change without treatment was standardized to 1. Mean and standard error were calculated with data from three biological replicates

amplified as a control. For quantitative real-time PCR (qRT-PCR), amplication of cDNA was carried out in StepOne real-time PCR system (Applied Biosystems, USA) using a SYBR Premix Ex Taq II (Perfect Real-time) kit (Takara, Japan). Specific primers for *chs4* and *actin* were used for mRNA quantification in each sample (Supplementary Table 1). All reactions were done in triplicate and no-template control was run for each primer pair. The amount of *chs4* mRNA relative to *actin* mRNA was calculated by comparative CT method using the relative expression function included in the StepOne v2.2 software package (Applied Biosystems, USA).

Determination of conidiation efficiency and growth rate

Conidia were point inoculated at the center of solid plates and incubated at 25 °C for 9 days. Conidia produced by a colony were scraped thoroughly from the agar surface, suspended in 5 ml water, filtered through four layers of lens paper and, after dilution,

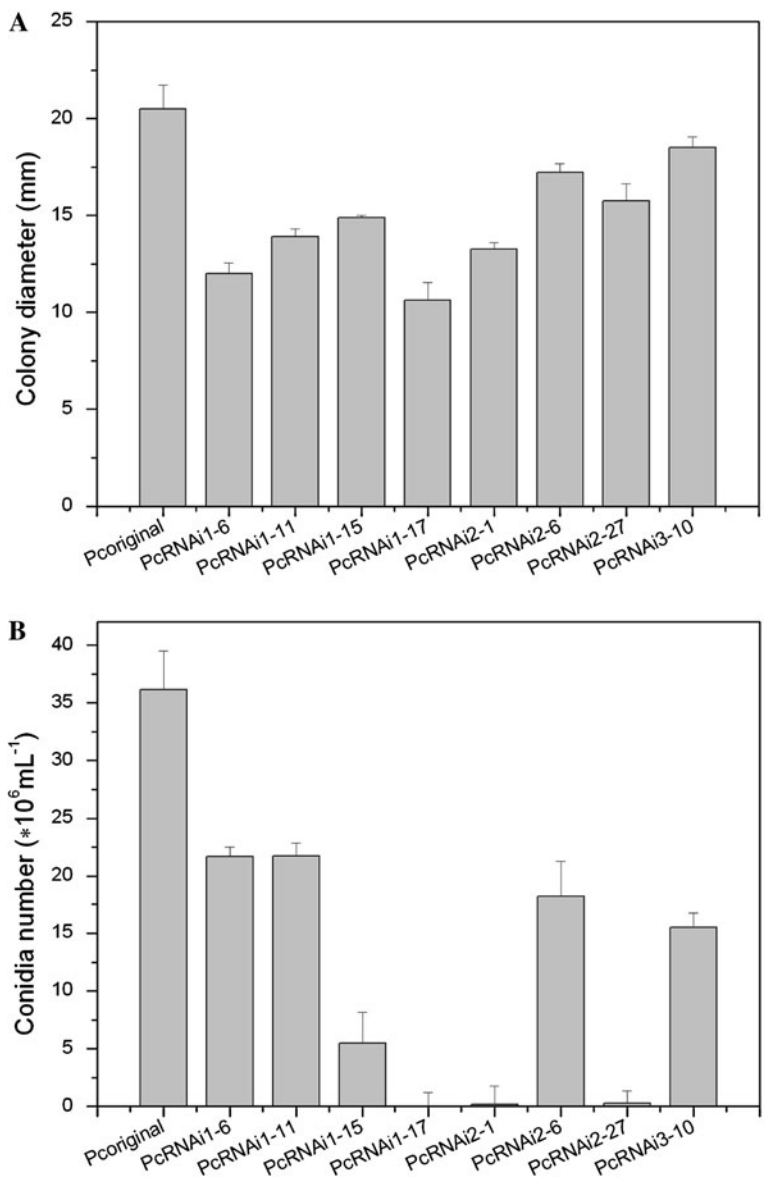

Fig. 2 Growth rates (**a**) and conidia formation efficiency (**b**) of Pcoriginal and its transformants. Data were taken from five colonies for each strain to determine the average and standard deviation

the OD_{560} was measured. At the same time, for counting the conidia, suspensions were smeared on plates and incubated until colonies appeared. The OD_{560} and conidia numbers were then correlated. For determination of growth rates, colony diameters were measured. Data were taken from five colonies for each strain to determine the average and standard deviation of conidia number and colony diameter.

Light microscopy

Samples from shake-flask culture were diluted to disperse the hyphal elements and stained by Lactophenol Cotton Blue and observed using microscopic imaging system (Olympus Optical Co Ltd, Tokyo, Japan).

Results and discussions

Constructions for *chs4* gene silencing

Deletion of class III chitin synthase-encoding genes leads to severe defects in most of the filamentous fungi. However, their detailed functions are unknown. Yanai et al. (1994) reported that growth of haploid *chsB* disruptant stopped immediately after the germination of conidia, with swollen hyphal tips. Borgia et al. (1996) obtained *chsB* disruptants with slow growth rate, highly branched hyphae and normal septa. To investigate the roles of CHS4 in *P. chrysogenum* morphogenesis, we developed an RNA interference system for the silencing of class III chitin synthase gene *chs4*. About 67 %

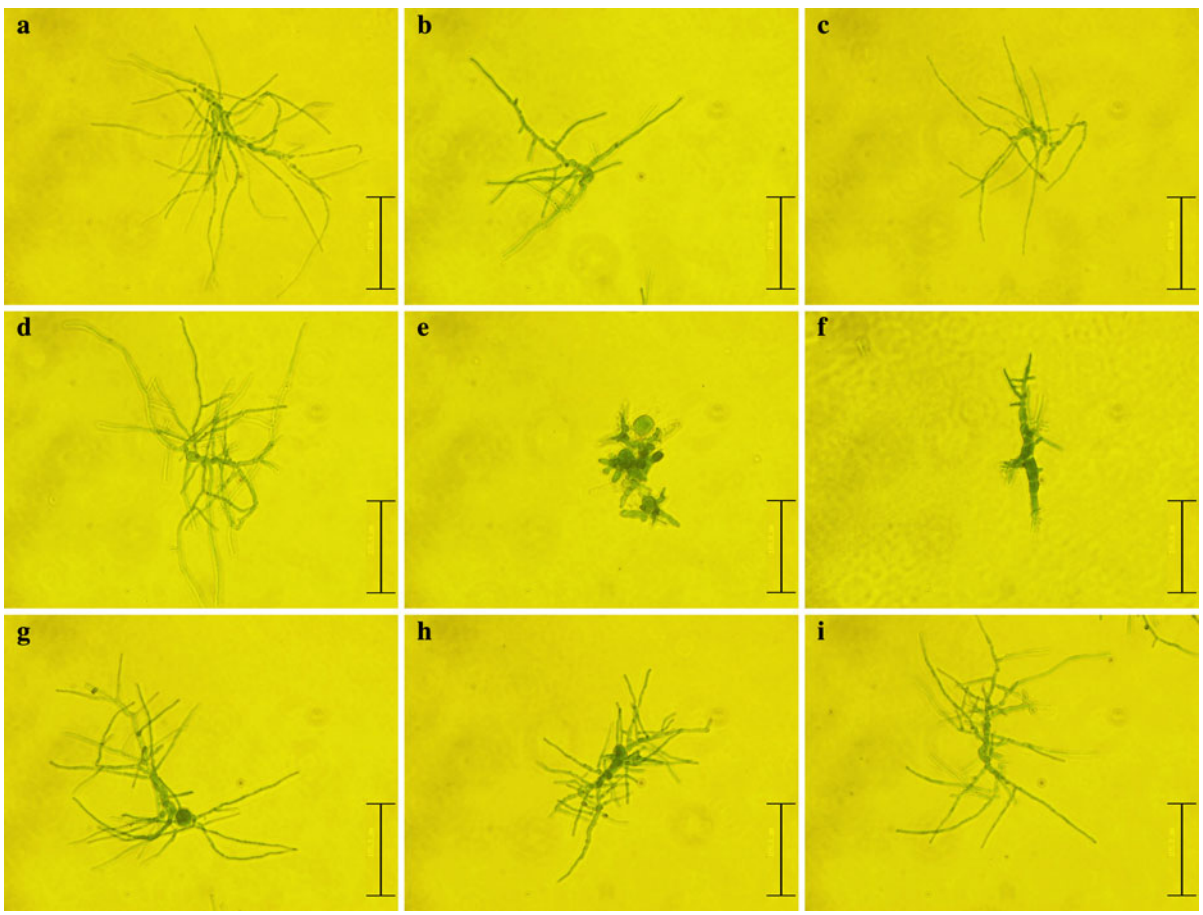

Fig. 3 Hyphal morphology of Pcoriginal and its transformants in seed culture at 36 h of cultivation at 25 °C. **a** Pcoriginal, **b**–**i** represent PcRNAi1-6, PcRNAi1-11, PcRNAi1-15, PcRNAi1-17, PcRNAi2-1, PcRNAi2-6, PcRNAi2-27 and PcRNAi3-10, respectively. *Bars* 100 μm

of the transformants showed a clear reduction in conidia formation. Figure 1A shows that colonies of the randomly selected transformants appeared colorless in sharp contrast to the green conidia of the original strain (Pcoriginal), suggesting that the pJL43-RNAi-based construct efficiently induced silencing of *chs4*. The transcript level of *chs4* in selected transformants was analyzed by semi-quantitative and real-time quantitative RT-PCR using *actin* gene as endogenous control. Consistent with the phenotypic observation, *chs4* mRNA expression was very weak in PcRNAi1-17 (91 % decrease), PcRNAi2-1 (84 % decrease) and PcRNAi2-27 (78 % decrease) while moderate in other transformants (Fig. 1B, C).

Involvement of *chs4* in growth and conidiation

Since chitin synthase catalyzes the synthesis of chitin, which is the major component of hyphal and conidial cell walls, a decrease in chitin synthase expression could affect hyphal growth and conidia development. 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. Just as in class III chitin synthase gene *chsB* repression in *A. nidulans*, all silenced transformants presented colonies much smaller than that of Pcoriginal, indicating the suppression effect of *chs4* silencing on hyphal growth (Fig. 2a). Figure 2b shows that, unlike the report of (Borgia et al. 1996) in which *chsB* gene

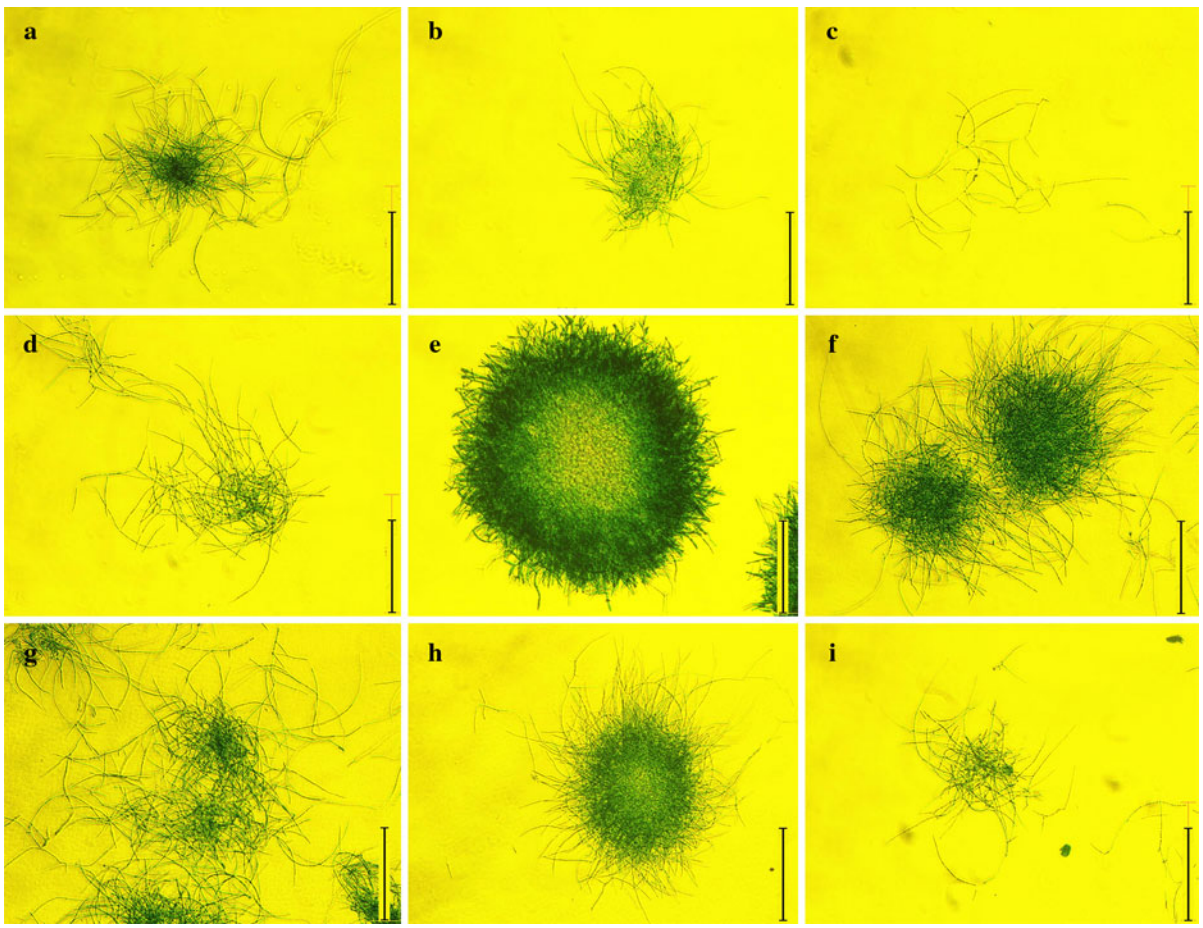

Fig. 4 Hyphae were collected from cultures in fermentation medium at 48 h of cultivation at 25 °C, stained with lactophenol cotton blue and observed under a light microscope. **a** Pcoriginal, **b**–**i** represent PcRNAi1-6, PcRNAi1-11, PcRNAi1-15, PcRNAi1-17, PcRNAi2-1, PcRNAi2-6, PcRNAi2-27 and PcRNAi3-10, respectively. *Bars* 200 μm

knockout strain did not conidiate, *chs4* silencing strains presented varied conidiation efficiency. PcRNAi1-17, PcRNAi2-1 and PcRNAi2-27 showed a drastic reduction, less than 1 % of the number produced by the original strain. PcRNAi1-15 could form only 15 % of the number of conidia produced by Pcoriginal. Conidiation efficiency of the rest was more moderate, varied from 43 to 60 % of that of the original strain, which could attributed to the partial reduction of *chs4* gene by RNA interference.

Changes of morphology and yield in submerged fermentation

According to the model described by (Paul and Thomas 1996) and Zangirolami et al. (1997), penicillin production is assumed to occur only at the non-growing region next to an apical region and in the growing region of the hyphal compartment, indicating that penicillin secretion was closely correlated with hyphal extension rates and sub-tip growth. Thus, we observed the hyphal morphology in liquid fermentation to better understand the effect of morphological changes on penicillin production.

At the seed phase, most of the mutants displayed severe defects in hyphal growth except PcRNAi1-15 and PcRNAi3-10 (Fig. 3). PcRNAi1-6 and PcRNAi1-11 appeared slower growth and shorter hyphae and branches. PcRNAi1-17 and PcRNAi2-1 produced stunted and bulging hyphae whereas PcRNAi2-6 and PcRNAi2-27 formed short, thick and highly branched phenotype. After entering the fermentation phase, loose clumps of mycelia were formed immediately by the agglomeration of hyphal elements in all strains (Fig. 4). Compactness of the agglomeration of hyphal elements decreased in the following order: PcRNAi1-17 > PcRNAi2-27 ≥ PcRNAi2-1 > PcRNAi2-6 ≥ Pcoriginal > PcRNAi1-6 > PcRNAi1-15 ≥ PcRNAi3-10 > PcRNAi1-11. Pellets then formed rapidly with a dense core in PcRNAi1-17, PcRNAi2-1 and PcRNAi2-27.

It is generally considered that a culture of pellets is much less viscous than a culture of disperse mycelia and could enhance the desirable mixing and mass transfer properties. However, a possible severe drawback may be introduced due to diffusional limitation of oxygen and/or other nutrients into the interior parts of the pellets (van Suijdam et al. 1980). Hence, it was generally considered that small pellets rather than large ones were suitable to filamentous−fungal fermentations. Figure 5 shows that four of the transformants produced less penicillin than Pcoriginal, which might be due to the slow growth rate or high viscosity. All the other transformants exhibited enhanced penicillin yields, especially PcRNAi1-17 and PcRNAi2-1 yield of which increased by 27 and 41 % respectively, indicating that the pellets or highly branched form may be favorable to penicillin production.

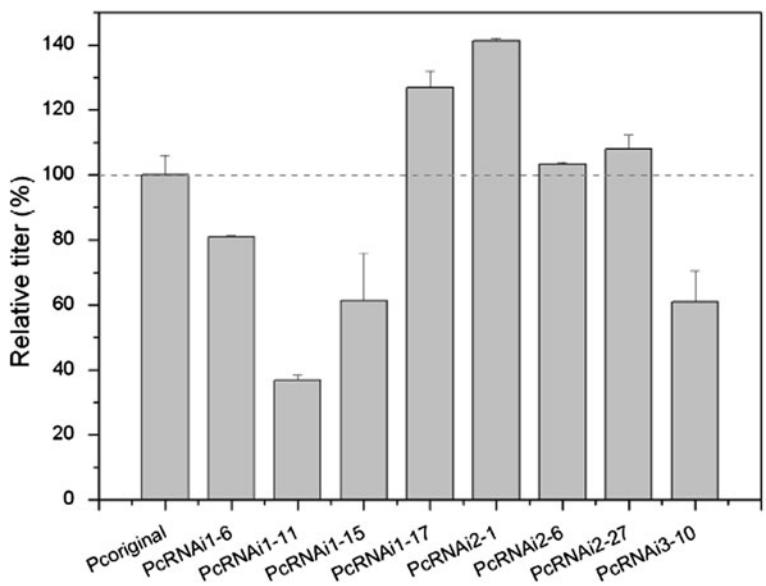

Fig. 5 Analysis of penicillin production in the culture broths of Pcoriginal and its transformants. Penicillin titer was determined using classical iodometric assay method according to the 2006 US Pharmacopeia and National Formulary (United States Pharmacopeial Convention 2006). *Error bars* indicate the standard deviation of three determinations made from three independent cultures

Conclusions

RNAi provides more detailed information compared to knockouts since RNAi causes only a partial reduction (knockdown) but not a complete loss (knockout) in gene expression, which makes it a powerful tool for investigating the effects of an essential gene on the morphology of interest. The results that silencing of *chs4* could cause various morphological changes and led to different penicillin yield will help convince that manipulation of chitin synthases will represent a simple way to improve production during industrial filamentous-fungal fermentations.

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