

## Down-Regulation of *sidB* Gene by Use of RNA Interference in *Aspergillus nidulans*

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### ABSTRACT

**Background:** Introduction of the RNA interference (RNAi) machinery has guided the researchers to discover the function of essential vital or virulence factor genes in the microorganisms such as fungi. In the filamentous fungus *Aspergillus nidulans*, the gene *sidB* plays an essential role in septation, conidiation and vegetative hyphal growth. In the present study, we benefited from the RNAi strategy for down-regulating a vital gene, *sidB*, in the fungus *A. nidulans*. **Methods:** The 21-nucleotide small interfering RNA (siRNA) was designed based on the cDNA sequence of the *sidB* gene in *A. nidulans*. Transfection was performed through taking up siRNA from medium by 6 hour-germinated spores. To evaluate the morphologic effects of siRNA on the fungus, germ tube elongation was followed. Moreover, total RNA was extracted and quantitative changes in expression of the *sidB* gene were analyzed by measuring the cognate *sidB* mRNA level by use of a quantitative real-time RT-PCR assay. **Results:** Compared to untreated-siRNA samples, a significant inhibition in germ tube elongation was observed in the presence of 25 nM of siRNA (42 VS 21  $\mu$ M). In addition, at the concentration of 25 nM, a considerable decrease in *sidB* gene expression was revealed. **Conclusion:** Usage of RNAi as a kind of post-transcriptional gene silencing methods is a promising approach for designing new antifungal agents and discovering new drug delivery systems. *Iran. Biomed. J. 18 (1): 55-59, 2014*

**Keywords:** *Aspergillus nidulans*, *sidB*, RNAi

### INTRODUCTION

RNA silencing is a comprehensive term that has been used to describe RNA interference (RNAi) in animals, post-transcriptional gene silencing in plants, and quelling in fungi, which are all similar forms of RNAi from mechanism aspect [1]. Introduction of RNA silencing machinery in fungi is recently considered to be one of the promising approaches in knocking down essential genes responsible for vital as well as virulence factors [2]. A homology-dependent gene silencing phenomenon termed “quelling” was first noted in the fungus *Neurospora crassa*. Quelling was recognized during attempts to increase the production of orange pigment expressed by the gene *all* of *N. crassa* [3]. RNAi mechanism is actually a natural process involves in using short double-stranded RNA molecules, named small interfering RNA (siRNA). In this mechanism,

double-stranded RNA or hairpin RNA is cleaved by RNaseIII-type enzyme called dicer into siRNA duplex of 21–26 nucleotides, which silence the expression of complementary target RNA by inducing RNA cleavage and subsequent reduction in protein expression levels [4-7]. RNAi has changed researcher’s conception in the field of gene function in many diverse model systems. Moreover, it has tangible applications for tissue/cell engineering and human therapeutics [8-10].

In fungi, cytokinesis or septation results in a cross-wall formation known as the septum. In *Schizosaccharomyces pombe*, it has been revealed that the septation initiation network is a kinase cascade which triggers the narrowing of the actomyosin ring and consequently the assembly of the septum [11, 12]. In *S. pombe* central players of septation initiation network, there are cascaded kinases of Cdc7p, Sid1p, and Sid2p. These kinases and their associated proteins exhibit dynamic localization patterns during the cell cycle

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[13]. It is strongly possible that a signaling network resembling the septation initiation network in fission yeast regulates septation in the filamentous fungi. Moreover, a similar signaling network, known as the mitotic exit network, has been characterized in *Saccharomyces cerevisiae* [14, 15].

Over the past 50 years, *Aspergillus nidulans* (*A. nidulans*) has been elevated to the status of a model organism and a number of methods have been developed to facilitate the efficient analysis of gene function in this filamentous fungus [16, 17]. Orthologues of Cdc7p and Sid2p, known as *sepH* and *sidB*, play an essential role in septation in *A. nidulans* [13, 18]. In addition to its essential function in septation and conidiation, *sidB* also played a role in vegetative hyphal growth [18]. In the present study, for the first time, we evaluated the utility of RNAi technology to inhibit the formation of vegetative hyphal growth in the filamentous fungus *A. nidulans* via down-regulating of *sidB* gene.

## MATERIALS AND METHODS

**Fungal strain and spore collection.** *A. nidulans* wild type strain PTCC 5014 was cultured on potato dextrose agar medium plates (Merck, Germany) and incubated at 37°C for 14 days. Fungal spores were collected on plates by surface scrapping of the colony with a pipette tip, suspended in distilled dH<sub>2</sub>O and filtered through glass wool to remove any remaining mycelia fragments. The spores were finally re-suspended in sterile dH<sub>2</sub>O at a concentration of  $1 \times 10^8$  spore ml<sup>-1</sup> using hemocytometry method.

**Small interfering RNA.** The 21-nucleotide siRNA was designed on the basis of the cDNA sequence of the *sidB* gene of *A. nidulans*. The sense strand siRNA having sequence (5'→3') GGGUGAGGAAUACGACUUUDTDT, antisense strand siRNA (5'→3') AAAGUCGUAUCCUCACCCDTDT and unrelated (negative control) siRNA sense sequence (5'→3') UACAUUUAACAGCGUAAAUGDTDT, antisense sequence (5'→3') CAUUUUACGCUGUAAAUGUADDTDT were synthesized. (Metabion, Germany). Sense/antisense annealing was performed in annealing buffer (30 mM HEPES-KOH [pH 7.4], 100 mM KCl, 2 mM MgCl<sub>2</sub>, and 50 mM NH<sub>4</sub>Ac) accordance to the procedure for siRNA annealing (<http://www.metabion.com/downloads/siRNAannealing.pdf>) to generate siRNA duplex with symmetric 2-nt 3' overhang.

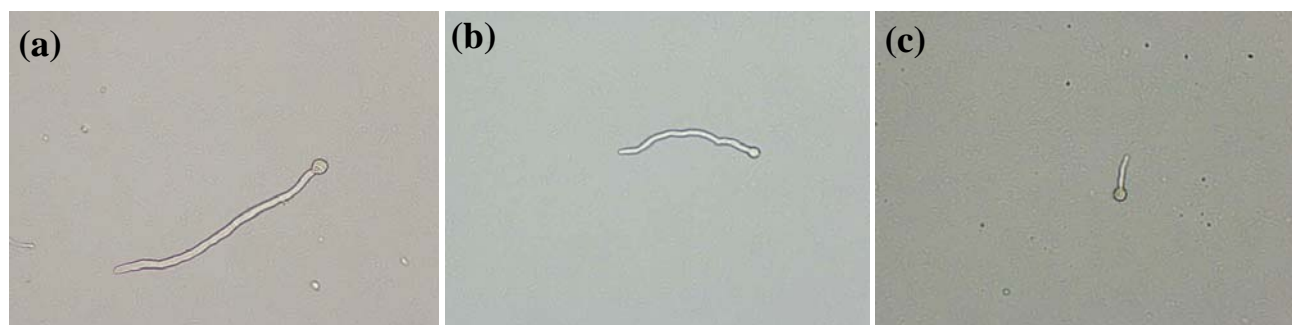
**Spore germination and germ tube growth.** Spores were germinated in potato dextrose agar medium at 1200 ×g at 37°C for 6 h, and then siRNA was added to get a final concentration of 25 nM [19], and finally

spores were incubated for another 12 h. Germinating spores were then fixed using 5% glutaraldehyde in order to calculate the spore germination percentage as well as length of germ tube. These experiments were repeated three times with three replicates in each experiment. Positive/negative controls (untreated/25 nM unrelated siRNA-treated samples) were also run along with the test samples.

**RNA extraction and quantitative real-time RT-PCR assay.** The cognate *sidB* mRNA was quantified in *A. nidulans* by quantitative real-time RT-PCR. Germinated spores were incubated for 18 h with siRNA, and then total RNA was extracted from both siRNA-treated (*sidB*-specific and unrelated siRNA) as well as untreated cells using GeneJET RNA Purification Kit (Fermentas, Germany) according to manufacturer's protocol. RNA concentrations and purity were determined via spectrophotometer methods (Eppendorf BioPhotometer, Germany). An equal amount of RNA (1 µg) was subjected to cDNA synthesis by using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany). The actin gene (*actA*) was used as endogenous reference gene. *sidB* and *actA* primers were designed on the bases of published sequences of the *sidB* (Accession No. BN001303), and *actA* (Accession No. BN001301 REGION: 1833364...1835708) genes in NCBI (<http://www.ncbi.nlm.nih.gov/pubmed/>). The sequences of forward and reverse primers have been shown in Table 1. Real-time RT-PCR was performed with a StepOne-Plus™ real-time PCR System (Applied Biosystems, USA) and SYBR® Premix Ex Taq™ II was used as a reagent specifically designed for real-time PCR using SYBR Green I. All PCR reaction mixtures contained 10 µl SYBR® Premix Ex Taq™ II (2×), 2 µl first strand cDNA, 0.4 µM each primer, 0.4 µl ROX Reference Dye (50×), and dH<sub>2</sub>O up to the final volume of 20 µl. The program for amplification was 95°C for 30 s as initial denaturation step, followed by 40-cycle PCR consisting of 95°C for 5 s and 60°C for 30 s. Negative controls (water as template) were included in each run. Expression of each investigated gene was normalized to the housekeeping *actA* gene and analyzed using StepOne Software v.2.0. *sidB* gene expression

**Table 1.** PCR primers for real-time RT-PCR analysis

Primer name	Sequence (5'-3')	RT-PCR product size (bp)
sidB_F	FAAgAgATggCTgAgCgTgAT	136
sidB_R	gTACCAAAgTCCTCggTTgg	
actA_F	gAAgTCCTACgAACTgCCTgATg	91
actA_R	AAgAACgCTgggCTggAA	



**Fig. 1.** Effect of *sidB*-specific siRNA on germ tube elongation in the fungus *A. nidulans*. The response of germinated spores to siRNA under *in vitro* conditions after 12 h of incubation. (a) untreated spores, (b) 25 nM unrelated siRNA-treated spores, (c) 25 nM siRNA-treated spores.

was normalized to the housekeeping gene *actA* and analyzed by using REST (2008 V2.0.7) software. The software uses the comparative Ct method ( $\Delta\Delta Ct$ ) to analyze data. Each experimental condition was performed in triplicate and each experiment was repeated twice on two different days for reproducibility.

## RESULTS

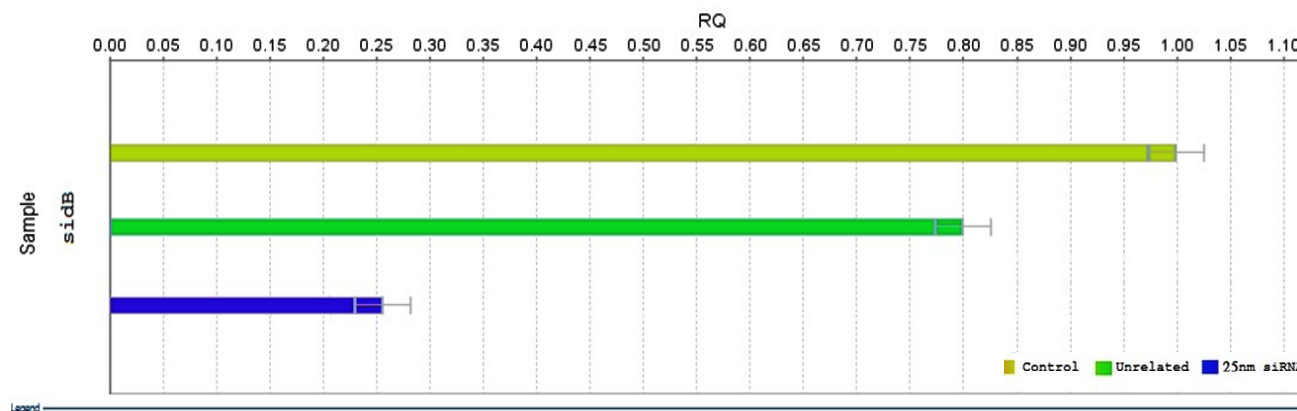
**Spore germination and germ tube growth.** After 12 h of incubation, the length of hypha was measured. Comparing with the control sample, a significant inhibition of hyphal elongation was revealed in 25 nM siRNA-treated germinated spores. About 50% of germinated spores were failed to be extended. In addition, the length of germ tubes in elongated spores was less than that in control samples. Figure 1 indicates the inhibition of hyphal elongation in siRNA-treated samples compared with untreated ones. Table 2 shows the inhibitory effect of *sidB* down-regulation on germ tube elongation.

**Table 2.** The inhibitory effect of *sidB* down-regulation on germ tube elongation in *A. nidulans* after 12 h incubation

Treatment	Spore germination (%)	Germ tube length ( $\mu\text{m}$ )
Positive control (untreated)	85	42 $\pm$ 0.97
25 nM unrelated siRNA	85	41 $\pm$ 0.76
25 nM gene-specific siRNA	62	21 $\pm$ 1.21

Data are presented as mean  $\pm$  SD, on the basis of three independent experiments

**Quantitative real-time RT-PCR assay.** *sidB* and *actA* mRNA levels were measured after a 18-h period of germinated spore incubation with both unrelated and *sidB*-specific siRNA. A positive control, untreated *A. nidulans* germinated spores, was included in each run of the experiment as well. Expression of each gene was indicated as ratio of expression relative to that of untreated logarithmic phase grown fungus. Based on obtained data, the relative quantification of *sidB* was calculated to be 0.25 after normalization of *sidB* gene expression to the housekeeping gene *actA* (1 for relative expression). Figure 2 indicates the relative



**Fig. 2.** Inhibitory effect of *sidB*-specific siRNA on *sidB* gene regulation. A significant decrease in *sidB* mRNA level was observed after the use of 25 nM gene-specific siRNA. From up to bottom: Rate of quantification for *sidB* gene expression in untreated sample, unrelated siRNA-treated sample and 25 nM gene-specific treated samples.

**Table 3.** Results for relative expression of the *sidB* gene using the  $\Delta\Delta C_t$  method (REST, 2008 V2.0.7)

Gene	Type	Reaction efficiency	Expression	Std. error	95% CI	P(H1)	Result
BetaAct	REF	1.0	1.000				
<i>sidB</i>	TRG	1.0	0.255	0.215 - 0.303	0.203 - 0.320	0.000	DOWN*
<i>sidB</i> (unrelated siRNA)	TRG	1.0	0.829	0.681 - 1.013	0.642 - 1.071	0.341	

\*Indicates down-regulation for *sidB* gene after treating with 25nM of gene-specific siRNA; Std. error, standard error; CI, confidence interval

quantification of *sidB* gene regulation. A significant decrease in *sidB* gene expression was observed after the use of 25 nM of *sidB*-specific siRNA ( $P < 0.05$ ). Table 3 shows the results from data analysis using REST software.

## DISCUSSION

Here, a novel approach was successfully applied to down-regulate the expression of *sidB* gene in *A. nidulans*, and the level of *sidB* mRNA was evaluated by taking advantages of real-time PCR approach. Evaluating of siRNA effectiveness is most often performed by quantifying target mRNA levels using real-time PCR under different conditions, since siRNA degrade their target mRNA. Apparently, different genes have different threshold requirements of knock down to obtain a biological phenotype [8-10]. Based on obtained data, it was revealed that the expression of the *sidB* gene was effectively decreased by using 25 nM of siRNA. Even this low concentration of *SidB*-specific siRNA had influence on phenotypic characteristics of the fungi. According to previous studies, the RNAi machinery does exist in this filamentous fungus, therefore, this effective system can be considered to be a new method for combating with *Aspergillus* pathogenesis [20, 21]. As the obtained results revealed, suppression of *sidB* gene, as a member of a gene group encoding proteins involved in mycelial septation as well as conidiation, which leads to inhibition of mycelial extension. Our findings represent a novel approach as a promising tool for designing new antifungal agents.

After the discovery of RNAi in 1998 [22], attempts were made to employ this technology for controlling gene expression in a variety of fungal species. Consequently, suppression of gene expression by a double-stranded RNA expressing plasmid or related-system has been shown in many fungal species, including Ascomycota, Basidiomycota and Zygomycota as well as the fungus-like Oomycota [23]. In certain species such as *A. nidulans*, *Magnaporthe oryzae*, *N. crassa*, and *S. pombe*, involvement of typical RNA silencing protein components such as

dicer in the silencing phenomena was shown. Moreover, biogenesis of siRNA was detected in *V. inaequalis* [24], *A. nidulans* [20], *M. oryzae* [25], *C. neoformans* [26], *A. fumigatus* [21], *N. crassa* [27], *H. capsulatum* [28], and *Candida albicans* [2, 29].

Janbon *et al.* [30] characterized the role of the RNAi pathway in regulation of many cellular processes in *C. neoformans* as a model system. Liu *et al.* [31] demonstrated the silencing effect of RNAi in *C. neoformans* on expression of genes involved in capsule synthesis and adenine biosynthesis. Rappleye *et al.* [32] reported the important role of  $\alpha$ -(1,3)-glucan in virulence of the fungus *H. capsulatum* through a plasmid-based RNAi system. In case of *A. nidulans*, Khatri and Rajem [19] explored the utility of RNAi as a tool for specific silencing of gene expression. In contrast to above researches which applied the vector-based methods for siRNA entrance, Khatri and Rajem [19] suggested that germinating spores are capable of taking up siRNA from the growth medium under *in vitro* conditions. In the present study, we also apply a simple, a rapid, and an effective method for siRNA to be up taken by growth medium. Although by using this method, the stability of siRNA is reported to be 18 h or more, the exact stability of siRNA in the target cells may be the issue to be concerned in future studies. Furthermore, it is strongly recommended to evaluate the effect of applied siRNA under *in vivo* condition.

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