CONSTRUCTION OF THE RNAi PLASMIDS TO SUPPRESS THE EXPRESSION OF CHITIN SYNTHASE-ENCODING GENES (*chs*) IN FUNGUS *Mucor lusitanicus*

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ABSTRACT

Mucormycosis is an emerging fungal infection caused by many species belonging to the order Mucorales. The lack of effective therapies to treat this disease is due to our limited understanding of its pathogenesis. Chitin is one of the fungal-specific components of the cell wall. In fungi, chitin synthase and chitin deacetylase are the key enzymes involved in chitin biogenesis. The RNAi mechanisms have been well-studied in the fungus *Mucor-lusitanicus*, a model organism for mucormycosis studies. The RNAi technique has been previously applied to study gene function. In this study, two chitin synthase-encoding genes (*chs1* and *chs2*) were identified using transcriptomic data. Approximately 1 kb of DNA fragments of the target genes were amplified and cloned into the pMAT1812 vector to generate the corresponding RNAi plasmids. Initial screening by colony PCR identified 3/10 and 1/10 potential colonies that might contain the RNAi plasmids. One plasmid for each clone was selected and named pAT63 and pAT64, corresponding to the *chs1* and *chs2* genes, respectively. The validation of these recombinant RNAi plasmids using different restriction enzymes (*Not*I - *Pvu*II and *Sal*I - *Pst*I for plasmids pAT63 and pAT64, respectively) showed the expected results. The DNA sequencing of the plasmids pAT63 and pAT64 using specific primers validated the results of the screening process. These RNAi plasmids can be used to suppress the expression of the target genes. The silencing mechanism of the target genes will be triggered by the expression of the RNAi plasmids. This approach allows us to study the function of the chitin synthase-encoding genes in this fungus.

Keywords: chitin, chitin synthase, cell wall, mucormycosis, *Mucor lusitanicus*.

INTRODUCTION

Mucormycosis is a rare but deadly fungal infection. It is an opportunistic disease, making it particularly perilous for patients with weakened immune systems, such as those with diabetes, organ transplants, AIDS, and COVID-19 (Marr *et al*., 2002). Mucormycosis is caused by various species in the order Mucorales (Richardson 2009; Steinbrink and Miceli 2021). The important pathogenic species belong to the genera *Rhizopus, Mucor, Lichtheimia, Apophysomyces, Rhizomucor,* and

Cunninghamella. Mucormycosis is one of the most difficult deep fungal infections to treat because the disease responds poorly to common antifungal drugs such as amphotericin B. There is no effective treatment for mucormycosis yet, primarily due to the limited understanding of the pathogenic mechanism (Lopez-Fernandez *et al.,* 2018).

Mucor lusitanicus CBS277.49 (formerly *Mucor circinelloides* f. *lusitanicus* CBS277.49) is a model fungus used for various studies, including the molecular mechanism of RNAi, carotene biosynthesis, lipid metabolism, and mucormycosis (Nicolás, Torres-Martínez, and Ruiz-Vázquez 2003). The mechanisms of RNAi in *M. lusitanicus* have been well characterized, encompassing both canonical and non-canonical pathways (Calo *et al.*, 2012; De Haro *et al.,* 2009; Trieu *et al*., 2015).

This fungus is also one of the agents that cause mucormycosis. Our study analyzes the structures and functions of some genes and gene families involved in controlling the filamentous phenotype and pathogenicity of this fungus, such as the gene families encoding myosin II and myosin V (Trieu *et al.*, 2017, 2022). These studies showed that the virulence of *M. lusitanicus* is closely related to some of its phenotypic characteristics. Specifically, growth rate, spore production, and yeast-like growth are signs that reduce the virulence of the fungus (Trieu *et al.*, 2017, 2022).

The fungal cell wall is the outer protective structure of the fungal cell. It is complex and consists of many different components (Garcia-Rubio *et al.*, 2020). Among them, chitin is one of the characteristic structures of the fungal cell wall and is related to the virulence of the fungus (Liu *et al*., 2023).

Chitin is a linear β (1-4) linked polymer of N-acetylglucosamine (GlcNAc) units, which is an important component of the fungal cell wall. It is synthesized at the cytoplasmic side of the cell membrane by membrane-bound chitin synthase enzymes (Lenardon, Munro, and Gow 2010). Therefore, we hypothesize that the genes involved in forming the structure of the fungal cell wall are closely related to the structure of the filamentous system as well as the pathogenicity of this fungus.

We identified two chitin synthase-encoding genes (*chs1*, ID 151786 and *chs2,* ID 85917) from 70 candidate genes that have canonical domain structures of chitin synthase in the *M. lusitanicus* genome (our unpublished data). In this study, we generated 2 silencing vectors to suppress their expression using the RNAi technique. These RNAi plasmids were verified using restriction enzymes and sequencing. They can be used to transform fungal protoplasts to initially screen their function in the generation of hyphal phenotypes as well as virulence in *M. lusitanicus.*

MATERIALS AND METHODS

Materials

The wild-type strain, *M. lusitanicus* R7B, and the plasmid pMAT1812 were obtained from the Department of Genetics and Microbiology, Faculty of Biology, University of Murcia, Spain. The *E. coli* DH5α strain was used to create competent cells for transformation experiments. The 9.5 kb plasmid pMAT1812, which includes two strong promoters (P*gpd1* and P*zrt1*) on both sides of the MCS region, was employed to produce the RNAi (dsRNA-expression) plasmids (Trieu *et al*., 2022).

Isolation of genomic DNA and plasmids

The i-genomic BYF DNA Extraction Mini Kit (Intron, Korea) was used to extract genomic DNA from the R7B strain. For isolating plasmids from E. coli strains, the bacterial cultures were grown overnight at 37 °C with shaking at 200 rpm. Plasmids were then isolated from bacterial cells using the DNA-spinTM Plasmid DNA Purification Kit (Intron, Korea), following the manufacturer's instructions.

PCR and electrophoresis

To amplify the approximately 1 kb fragments of the *Chs1* and *Chs2* gene, we conducted PCR reactions using a 2x PCR Master Mix Solution kit (Intron, Korea). The primer pairs used for *chs1* and *chs2* cloning were CHS1_F1 and CHS1_R1_NotI; CHS2_F1_SalI and CHS2_R1_SalI, respectively (Table 1). The PCR program included an initial denaturation step at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 20 seconds, annealing at 58 °C for 20 seconds, and extension at 72 °C for 1 minute and 20 seconds, with a final extension at 72 °C for 10 minutes.

All DNA products were purified using the MEGAquick-spin^{TM} plus Fragment DNA Purification Kit (Intron, Korea) following the manufacturer's recommendations. The DNA fragments in this study were visualized under UV light by using 1% agarose gel electrophoresis.

Table 1. The list of the primers used in this study.

Primers	Sequences (5´ - 3´)	Use
CHS1_F1	CCCAATGACTTTGCTGCCAGC	Silencing *chs1* gene
CHS1_R1_NotI	TAT<u>GCGGCCGC</u>GCAGTTCAAAGCACAGGATGCG	Silencing *chs1* gene
CHS2_F1_SalI	TTT<u>GTCGAC</u>TTCGGTGCCTCTGAAGTTCATGT	Silencing *chs2* gene
CHS2_R1_SalI	TTT<u>GTCGAC</u>GCAAGTCCATTGCCCTCAGC	Silencing *chs2* gene
peuka1	CATGAAGTGTGAGACATTGCG	Colony PCR, sequencing
PU	GT TGTAAAACGACGGCCAGT	Colony PCR, sequencing

Underlined regions indicated the recognition sites of restriction enzymes.

Cloning experiments

The cloning procedure followed a standard cloning protocol (Green, R and Sambrook 2012). In this study, two restriction enzymes, *Not*I and *Sal*I were used to clone the *chs1* and *chs2* genes, respectively. Approximately 1 kb of *Not*I or *Sal*I restriction fragments obtained from the PCR products were ligated into linear vector pMAT1812, which had been digested with *Not*I or *Sal*I, using T4 DNA ligase (Thermo Scientific). The ligation mixture included a linear vector to insert a DNA ratio of 1:3 (w/w), 1 µl 10X T4 DNA ligase buffer, 0.5 µl T4 DNA ligase, and H$_2$O up to a total volume of 10 µl.

The ligation mixtures were incubated for 15 minutes at room temperature. Subsequently, 5 µl of each was used to transform 50 µl of competent *E. coli* DH5α cells prepared using a cold MgCl$_2$ and CaCl$_2$ – glycerol solution. Transformation was performed using the heat shock method: cells were subjected to

42 °C for 45 seconds following a 20-minutes incubation on ice. After transformation, the cells were added to 1 mL of LB broth and incubated at 37 °C for 45 minutes to 1 hour, then spread onto LB agar plates supplemented with ampicillin (100 µg/ml).

Verification of recombinant plasmids

To identify the recombinant plasmids, ten *E. coli* colonies grown in LB supplemented with ampicillin were randomly selected for screening by the colony PCR method with peuka1 and PU primers (Table 1). The selected plasmids were extracted and confirmed using different combinations of restriction enzymes. Sequencing of the plasmids was conducted using peuka1 and PU primers, in accordance with, our previous studies (Le *et al.*, 2020; Trieu *et al.*, 2022).

RESULTS AND DISCUSSION

DNA extraction and amplification of the target genes

Genomic DNA was extracted from wild-type strain R7B using the i-genomic BYF DNA Extraction Mini Kit (Intron), which is designed for isolating genomic DNA from fungi. The extracted genomic DNA yielded satisfactory results for PCR reactions. Genomic DNA after the PCR reaction was tested using a NanoVue Plus UV-Vis spectrophotometer (GE Healthcare) resulting in a concentration of 500 ng/µl and a wavelength ratio of 260 nm/280 nm of about 1.7 to 2.0. The specificity of the PCR products (Figure 1) was confirmed by agarose gel electrophoresis.
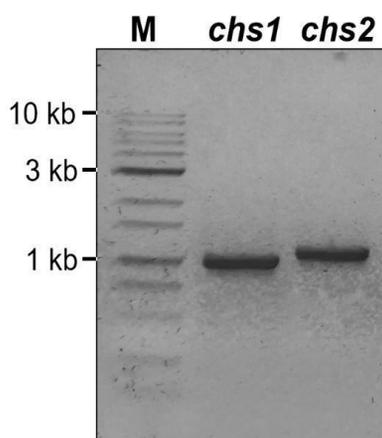
Figure 1. PCR results of 1 kb fragment from *chs1* and *chs2* genomic DNA of strain R7B. M: Sizer™ 1000 DNA Marker (Intron, Korea).

Construction of plasmid pMAT1812 harboring *chs1, chs2*

The 1kb PCR fragments, restricted by *Not*I or *Sal*I, were incorporated into the linear vector pMAT1812 at a ratio of approximately 4:1 using the T4 DNA ligase. The ligation mixture was then transformed into competent *E. coli* DH5α cells using the heat shock method. After incubation, the transformed *E. coli* DH5α cells were cultured on LB plates supplemented with ampicillin. We obtained approximately 50 colonies. The success rate of the ligation process was estimated to be 10-30% based

on the ratio of positive colony PCR results (Figure 2).

Colony PCR was performed to screen for positive colonies that contained expected plasmids. In this study, we adopted two primers, peuka1 and PU, which were located at the flanking positions of the MCS regions of the pMAT1812 vector. Therefore, the negative and positive results were approximately 2.5 kb and 3 kb fragments, respectively (Figure 2). The colony PCR results showed the expected band sizes, and confirmed that this method is effective in

identifying potential recombinant plasmids. Additionally, these plasmids were validated by using restriction enzyme digestion and Sanger sequencing (Figure 3).

For the recombinant gene *chs1*, positive results were observed in samples from lanes 1, 5, and 8, accounting for approximately 30%. For gene *chs2*, a positive result was observed in the sample from lane 1, which represents around 10%. The plasmids isolated from colonies carrying the *chs1* and *chs2* genes were named pAT63 (Figure 2A) and pAT64 (Figure 2B), respectively.
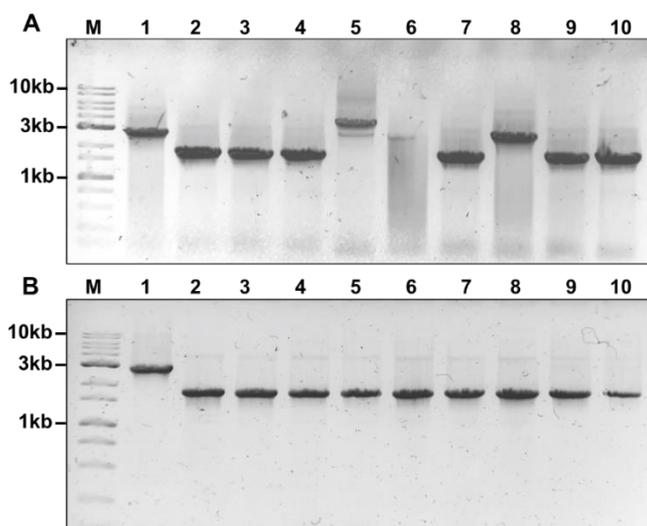
Figure 2. Results of the colony PCR to select the recombinant plasmids for *Chs1* gene **(A)** and *Chs2* gene **(B)**. Lanes 1 to 10: PCR results obtained from randomly selected colonies. M: SizerTM-1000 DNA Marker (Intron, Korea).

Confirmation of RNAi plasmids using restriction enzymes

Plasmids pAT63 and pAT64 were isolated to verify their structures. Plasmid pAT63 was digested with two different restriction enzymes, *Not*I and *Pvu*II. Plasmid pAT64 was digested with *Sal*I and *Pst*I (Figure 3).

As expected, the pAT63 recombinant plasmid generated 2 bands (9.5 kb and 1 kb)

when digested with *Not*I, and 3 bands (6.2 kb, 2.8 kb, and 1.5 kb) when digested with *Pvu*II, pAT64 plasmid generated 2 bands (9.5 kb and 1.1 kb) when digested with *Sal*I and 2 bands (6.2 kb and 4.4 kb) when digested with *Pst*I (Figure 3). The structure of the recombinant plasmids was validated using restriction enzymes, and the obtained results are consistent with those from the colony PCR selection (Figure 2).
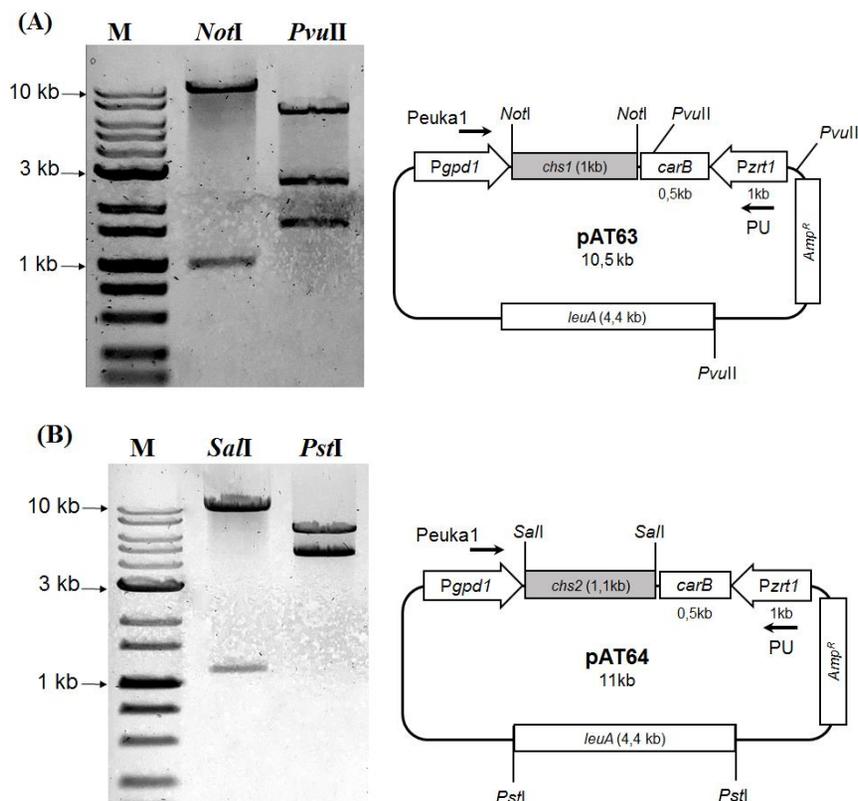
Figure 3. Validation of plasmids using restriction enzymes. **(A)** The recombinant plasmid pAT63 was validated using *Not*I and *Pvu*II restriction enzymes. **(B)** The plasmid pAT64 was checked by using *Sal*I and *Pst*I restriction enzymes. The diagrams show the expected architecture of the corresponding plasmids with the position and direction of the promoters (P*gpd1*, P*zrt1*), reporter gene (*carB*), selective markers (*leuA, amp^R*), insert sequences (*chs1, chs2*) and restriction recognition sites. M: Sizer™ 1000 DNA Marker (Intron, Korea).

Confirmation of RNAi plasmids by sequencing

To verify the sequencing of the plasmid, we sequenced plasmids pAT63 and pAT64 using the primer peuka1 (Table 1). The sequences obtained were compared using Clustal Omega (1.2.4) multiple sequence alignment. The result showed that the inserted fragments in plasmid pMAT1812 had the same sequence as the original DNA fragments obtained from *M. lusitanicus* genomic DNA. These findings confirmed that the utilization of colony PCR and restriction enzymes as selection methods is

effective in identifying recombinant plasmids, as previously demonstrated in our study (Le *et al.*, 2020). Taken together, the recombinant RNAi plasmids pAT63 and pAT64 were confirmed to successfully clone DNA fragments of the *chs1* and *chs2* genes, respectively. The RNAi plasmids generated from the pMAT1812 vector have been used for several previous studies. Those RNAi plasmids use 2 strong promoters flanking a fragment of the *carB* reporter gene and a polylinker region. The expression of the *carB* fragment generates the albino fungal mycelia, providing a simple method to validate the expression of the target gene

fragment (Le *et al.*, 2020; Trieu *et al.*, 2022). Therefore, the RNAi plasmids constructed in this study also provided tools for initial analyses of the biological function of the *chs1* and *chs2* genes in the morphogenesis and virulence of the human pathogenic fungus *M. lusitanicus*.

CONCLUSIONS

The experiment's results confirmed that we successfully obtained the recombinant plasmids pAT63 and pAT64, each containing a 1 kb fragment of the *chs1* and *chs2* genes, respectively, cloned into plasmid pMAT1812. These plasmids are now ready to be transformed into protoplasts of the wild-type *M. lusitanicus* fungal strain. This will help us investigate the role of the corresponding genes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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