Simplified and effective RNA interference and CRISPR-Cas9 systems for Cryptococcus neoformans

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Abstract

The 3,4-dihydroxyphenylalanine (DOPA) melanin is one of important virulence factors for Cryptococcus neoformans, which may trigger immune responses in the host. It is worth exploring the genetic function of C. neoformans, by which we may derive more antifungal strategies. Therefore, we established two systems that were constructed quickly and easily for the knock-down/knock-out of LAC1 gene: RNA interference (RNAi) and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9. The RNAi system used pSilencer 4.1-CMV neo plasmid and short hairpin RNA to realize the effective transcriptional suppression. The CRISPR-Cas9 system used the PNK003 vectors to obtain a stable albino mutant strain. The results of phenotype, qRT-PCR, Transmission Electron Microscope (TEM) and spectrophotometry were used to assess the ability of melanin production. As a result, the RNAi system displayed attenuation of transcriptional suppression when the transformants continuously passed on new plates. However, the transcriptional suppression of long loop in short hairpin RNA were more powerful and lasted longer. The CRISPR-Cas9 system constructed an albino strain completely without the ability to produce melanin. Considering the weakening of transcriptional suppression, we recommend using a long loop for the RNAi system and 1st or 2nd passage of knockdown strains for the subsequent studies. Besides, the different capacities of melanin production might be useful for exploring the linear relation between melanin and immunoreaction of the host. In addition, we recommend applying the PNK003 vectors to other serotypes of C. neoformans for quick screening of possible trait-regulating genes because of its easy construction and valid knockout effect.

1 Introduction

Cryptococcus neoformans is one of the significant human pathogens of basidiomycetes because it infects approximately 1 million individuals per year, with over 600,000 annual mortality attributable to the opportunistic pathogenic infection, resulting in almost one-third of AIDS deaths[1]. *C. neoformans* has two phenotypic characteristics, the capsule and the synthesis of melanin, which both protect the yeasts and induce host damage as virulence factors[2]. 3,4-dihydroxyphenylalanine (DOPA) melanin of *C. neoformans* is synthesized with the exogenous catecholamines, the entire process of which is catalyzed by laccase, predominantly encoded by the *LAC1*gene [3]. Recently, a study found that the recognition molecules of melanin in macrophages elicited metabolic reprogramming and associated inflammation, which contrasted with the inhibitory effects of melanin in previous studies [4, 5]. Therefore, we explored two systems that were constructed quickly and easily for the knock-down/knock-out of*LAC1* : RNAi and CRISPR-Cas9. These two systems are time-saving and convenient for quickly obtaining small amount of target strains or rudely screening possible trait-regulating genes at a one-time.

The DOPA melanin of *C. neoformans* impairs antifungal immune responses and clearance by weakening immunity response [6, 7] and lowering the accumulation of antimicrobial substances [8, 9]. DOPA melanin should anchor to chitin within the cell wall, but chitinase-inhibited *C. neoformans* leaked an amount of DOPA melanin, inducing robust inflammation in mice [10]; moreover, the immune response induced by the chitosan-deficient strain, including heat-killed cells of this strain, was sufficient to challenge a virulent wild-type (WT) strain [11]. This immune response contrasts with the inhibitory effects of DOPA melanin. But a known mechanism can explain the immune response: PRRs usually cause inflammation after PAMP recognition [12], which has been confirmed that 1,8-dihydroxynaphthalene melanin of *Aspergillus fumigatus* was recognized by melanin-sensing C-type lectin receptor of macrophages and induce inflammation mainly by activating glycolysis [4, 5]. However, further studies are needed to elucidate the specific mechanism of how DOPA melanin triggers the inflammation of immune cells. In this study, we used RNAi and CRISPR systems to obtain *LAC1* knock-down and knock-out strains, respectively, and the phenotypic effects in this study were obvious.

At first, RNAi was found to be a self-protective mechanism for numerous species (such as plants, animals, fungi, and protists) to prevent the interference by exogenous genes or endogenous transposon activation and movement[13-16]. An RNAse III-like endonuclease, Dicer, cleaves double-strands RNA (dsRNA) into 20–25bp pieces called small interference RNA (siRNA)[17]. These fragments are incorporated into Argonaute, the catalytic subunit of RNA-induced silencing complex, and guide to the complementary homologous mRNA to elicit mRNA degradation. Meanwhile, RNA-dependent RNA polymerases amplify additional dsRNA complementary to the target mRNA by siRNA primers, triggering more Dicer and Argonaute[17]. The mechanism of RNAi allows researchers to artificially trigger RNAi by dsRNA that has been either directly introduced into a cell or transcribed intracellularly from transfected vectors [18]. The dsRNA from vectors has no requirement for *in vitro* synthesis of dsRNA [19] and is widely used in many fungal species[20]. The intracellular transcription of dsRNA is usually done in two ways: the reverse complementary RNA strands pair to form dsRNA after being transcribed by two promoters in the opposite direction, and a single RNA strand consisting of reverse RNA oligonucleotides matches itself to form a small hairpin RNA (shRNA) after being driven by a single promoter [18, 19, 21, 22]. Both forming ways of dsRNA were previously used for the exploration of genic function (e.g., *CAP10* , *CAP59* , *ADE2*) of *C. neoformans* [18, 19, 22, 23]. The dsRNA from the former way is relatively inefficient in RNAi compared to hairpin RNA, perhaps due to the low efficiency of dsRNA formation [20]. However, there was little research on the interference by shRNA with *LAC1* . In this study, we successfully constructed a plasmid containing the target gene of *LAC1* , which could form a hairpin structure (shRNA) after transcription (Fig. 1A) and work effectively for RNAi.

CRISPR-Cas9 was firstly discovered as genome protection for bacteria from the genic invasion of viruses and plasmids, which was gradually applied to eukaryotic cells for gene editing, including gene knock-out, gene knock-in, repression or activation[24, 25]. A vector of CRISPR-Cas9 systems is mainly composed of *cas9* gene, single guide RNA (sgRNA, consist of 17-20 base pairs target sequences and tracrRNA sequences), selectable marker gene, and their respective operon(s)[24, 25]. After intracellular expression of the vector, Cas9, the type II RNA-guided endonuclease, is directed by sgRNA to the targeted gene with a 3-bp protospacer-adjacent motif (PAM) next to it. Then Cas9 introduces a double-strand break about 5bp away from the PAM, which will be repaired by nonhomologous end joining, usually resulting in insertions or deletions and even leading to frameshift of the reading frame and premature stop codons [26]. However, the expression of valid Cas9 protein and gRNA need specie-specific promoters, which is the main obstacle to applying CRISPR-Cas9 to *C. neoformans* , although the CRISPR-Cas9 system has proved effective for many yeasts and filamentous fungi [27-32]. Zhang ping and colleagues[33, 34] developed simplified all-in-one CRISPR-Cas9 vectors specific for *C. neoformans* : PNK003 for serotype A strain and PRH003 for serotype D strain. The difference between PNK003 and PRH003 vectors is present in their strains-specific promoters of gRNA and Cas9 expression cassettes. Meanwhile, the all-in-one vectors contain 'suicide' systems that can eliminate Cas9 and gDNA cassettes after gene editing, reducing the potential cytotoxicity of Cas9 endonuclease and conquering the difficulty of gene complementation. In this study, we used PNK003 for*LAC1* knock-out of serotype D strains (FIG. 2A) and found that promoters of serotype A strain can drive the expression of

gRNA and Cas9 cassettes in serotype D strain.

2 Material and methods

2.1 Strain, media and plasmids

Cryptococcus neoformans B-3501A (serotype D) was used as WT strains. YPDA (a complete medium containing 1% yeast extract, 2% peptone 2% glucose and 0.003% adenine sulfate dihydrate, with or without 2% agar) was for the routine growth of *C. neoformans*.DOPA medium contained L-dopa (0.2 g/L), L-asparagine (1 g/L), glycine (1 g/L), glutamine (1 g/L), $MgSO_4$ (2.5 g/L), KH_2PO_4 (4 g/L), vitamin B_1 (10 mg/L), glucose (1 g/L), with or without agar (20 g/L). For strain selection, G418 (100μg/mL) or hygromycin B (100μg/mL) was added to the YPDA and the DOPA medium. For the preparation of RNA extraction, the DOPA medium without L-dopa and glucose was named laccase-inducing medium. All cultures and plates were incubated at 30, and the liquid cultures needed to continue shaking at 150 rpm/min. The pSilencer 4.1-CMV neo plasmid was bought from Miao Ling Plasmid Platform, China. Supplementary Fig. 1 shows the map of pSilencer 4.1-CMV neo plasmid. The PNK003 vector (Genbank: MW938321) was gifted by professors Xudong Zhu and Ping Zhang. Supplementary Fig. 3 shows the map of PNK003 vector, and Supplementary sequence 3 shows the sequencing result. *E. coli* DH5α grew on LB medium (containing 0.5% yeast extract, 1% tryptone, 1% NaCl and 2% agar) with ampicillin to amplify and extract target vectors in this study. All the reagents were brought from Sinopharm Chemical Reagent Co., Ltd and Solarbio Science & Technology Co., Ltd.

Enzymes and primers

BspQI, HindIII-HF and BamHI-HF restriction endonucleases were obtained from New England Biolabs (Beijing, China). T4 DNA ligase and Taq DNA polymerases were obtained from Takara (Dalian, China). Yeast Transformation Kit was obtained from Coolaber (Beijing, China). Ezup column fungal genomic DNA extraction kits, DNA gel purification kits, primer synthesis and sequencing were from Sangon Biotech (Shanghai, China). EndoFree Plasmid Maxi Kit and SYBR qPCR Master Mix were purchased from Vazyme (Nanjing, China). Evo M-MLV RT Mix Kit with gDNA Clean for qPCR were purchased from Accurate Biotechnology (Hunan, China). The primers used in this study are listed in Supplementary Table 1.

2.3 Construction of recombinant vectors inserted with target gene segments from *LAC1* gene of *C. neoformans.*

2.3.1 Vector for RNAi

We used pS plasmid as the shRNA-expression vector. We used BLOCK-iT^{TM} RNAi Designer (*http://rnaidesigner.thermofisher.com/rnaiexpress/*) to choose some better-ranked sequences located in the CDS of the*LAC1* gene (Genbank: CNBG3550), which were blasted in NCBI to identify the most specific one as the target sequence for RNAi. We designed two different loops (5'-TTCAAGAGA and 5'-CTCGAG) linking two reverse target sequences to compose two types of double-strands shRNA genes, namely LAC1-RNAi-A-F/-R and LAC1-RNAi-B-F/-R (Supplementary Table 1). The designed single-strand shRNA genes were dissolved with ddH_20 (100 μM) and 2 μL of each were mixed with 5 μL 10 x PCR buffer and 41 μL ddH_20 at 95 for 5 min before cooling down at room temperature for 1 h. Two types of the double-strands shRNA genes hybridized respectively. The hybridized oligonucleotides contained sticky ends at 5' end of each strand. At the same time, the pSilencer plasmid was digested in a 20 μL system containing pS plasmid 6μL, 10 x buffer 2μL, HindIII 2 μL, BamHI 2 μL, ddH_20 8 μL. After digested, the hybridized oligonucleotides ligated to the long linear segments of the pS plasmid by using T4 DNA ligase at 16 for 16 h. The pS plasmid ligated with hybridized oligonucleotides (pS-LAC1-A and pS-LAC1-B, shown in Fig. 2A) and the empty pS plasmid were transformed into *E. coli* DH5α competent cells incubated on ampicillin-containing agar plates at 37 for 16 h. Finally, three random colonies were picked out for sequencing by primers M13-F and CMV-F to confirm the successful construction of the pS-LAC1 vectors (its sequencing was displayed in Supplementary Fig. 2, sequence 1 and 2).

2.3.2 Vector for CRISPR

The searching pattern of CRISPR target sites is set as 5'-GGX18NGG-3', 5'-GX19NGG-3' or 5'-X20NGG-3', where N and X is any base, NGG is the PAM sequence. In addition, CRISPR target sequences should align to the whole genome scale to choose the less off-target ones, improving the cleavage efficiency and specificity of CRISPR-Cas9 system [35]. Therefore, we designed the CRISPR target sequence of the *LAC1* gene as 5'- GGGCATGGTTTGCGGCAGCT-GGG-3' with the use of sgRNAcas9 target designing software[35]. Besides, we added 5' BspQI-compatible overhangs to the target sequence (LAC1-CRI-F) and its complementary sequence (LAC1-CRI-R) in order to ligate with the BspQI-digested PNK003 vector. The LAC1-CRI-F annealed with LAC1-CRI-R in a PCR thermocycler at 95 for 5 min, cooling down at room temperature for 1 h. Then, the hybridized oligonucleotides consisting of target sequences and 5' overhangs were diluted to 1:50 with ddH_20 and ligated into BspQI-digested PNK003 vectors (pre-digested at 50 for 20-30 min) at 4 for 4 h. The PNK003 vectors inserted with annealed *LAC1* target oligonucleotides (PNK-LAC1, shown in Fig. 2A) were transformed into *E. coli* DH5α competent cells incubated on ampicillin-containing agar plates at 37 for 16 h. Finally, three random colonies were picked out for the sequencing of the gRNA expression cassette by T3 universal primer (its sequencing was displayed in supplementary Fig. 4 and sequence 4).

2.4 Transformation and selection

We transformed the vectors into *C. neoformans* competent cells following the operating manual of the Yeast Transformation Kit. However, we prolonged the resuscitation time of cells transformed with orbicular vectors to 2-3 h. Every group was named by the plasmid that the cells transformed, namely group pS-LAC1-A, group pS-LAC1-B, group pS, and group PKN-LAC1.

After transformation, the selection for group pS-LAC1-A, pS-LAC1-B, and pS was addressed by YPDA broth with G418 (100 μg/mL) for 10 days, replacing fresh culture medium every 2-3 days. Then the yeast cells were maintained in YPDA broth with G418 (50 μg/mL) until harvested on day 14^{th}. After 14 days of selection, the cultures were centrifuged at 3000 rpm/min for 10 min, washed three times with PBS and re-suspended in 200 μl sterile ddH_20, which shook in 5ml laccase-inducing media for 1 h and subsequently prepared for the RNA extraction. In addition, we pipetted 30 μl cell suspension out of 200 μl re-suspension to plate on the DOPA agar at 30 for 7 days, aiming to observe the phenotype of every group. Then we plated the white colonies to new DOPA agars every 5 days to explore the stability of the RNAi effect.

After transformation and resuscitation, we washed the cells of group PNK-LAC1 three times with PBS and re-suspended them in 200 μl sterile ddH_20. Then the re-suspension was evenly coated on the DOPA agar with hygromycin B (100 μg/mL) and incubated at 30 for 7 days. Then we repeatedly streak the white colonies on DOPA agar every 5 days to select the stable albino mutant strains. To verify the transcription of *LAC1* , we randomly chose 3 colonies of the albino strains for RT- qPCR. Firstly, we amplified the colonies in 3 ml YPDA broths overnight and then in 10 ml YPDA till the log phase growth of yeast cells (OD_{600}=0.4-0.6). The cells were centrifuged at 3000 rpm/min for 10 min, washed three times with PBS and re-suspended in 10 ml laccase-inducing medium for one-hour shaking. Laccase-inducing cells were finally centrifuged, preparing for the RNA extraction.

2.5 qRT-PCR

2.5.1 RNA preparation

We collected the cells from one-hour laccase-inducing, added TRIzol to the cells pellet and mixed well to lyse the cells adequately. Then sequentially use chloroform, isopropanol and ethanol in proportion to extract total RNA.

2.5.2 cDNA synthesis and relative quantitative PCR

Total RNA was retro-transcribed by Evo M-MLV RT Mix Kit on Bio-Rad T100™ Thermal Cycler. The relative quantitative PCR was performed with SYBR qPCR Master Mix on Applied Biosystems 7500 Real-time PCR System. Data were analyzed using the 7500 software v2.0.6. The primers for qRT-PCR of RNAi groups and CRISPR-Cas9 cells are RNAi-QF/QR and CRISPR-QF/QR (listed in Supplementary Table 1).

18S ribosomal DNA (rDNA) was regarded as an internal reference for qRT-PCR, and its primers were listed as 18S-F/R in Supplementary Table 1.

2.6 TEM

The target cells were amplified in 5ml YPDA at 30 till about 10^8 cells of every group. Then we centrifuged to get the precipitations and incubated them in the DOPA medium at 30 for 3 days. Next, we centrifuged the cultures and fixed the cells with High Efficient Pre-fixed Electron Microscope Fixative Solution (Servicebio, Hubei, China) at room temperature for 2 h and 4 for transportation. The subsequent experimental procedure and image-capture of TEM were conducted by Servicebio Technology CO., LTD.

2.7 Laccase activity

We incubated the albino mutant, LAC1-knockdown and WT strains in 5ml YPDA at 30 overnight, washed the cells three times with PBS, and then transferred them to DOPA broth shaking for 3 days and 5 days. We quantified the laccase activity by the appearance of pigment in the culture supernatant with absorbance at 475nm (Thermo Scientific*Multiskan GO*).

Results

3.1 Successful construction of *LAC1* knock-down strain and its phenotypic stability

For gene knock-down, we used pSilencer 4.1-CMV neo plasmids to design two related vectors directing the transcription of shRNA, which contained the same 21–base pair double-stranded *LAC1* target sequence, and loops of nine (5'-TTCAAGAGA for group pS-LAC1-A) or six (5'-CTCGAG for group pS-LAC1-B) nucleotides. After chemical transformation and G418 selection in YPDA broth, a part cells of two experimental groups and a negative control (pS) group were prepared for qRT-PCR, and the other were plated on a DOPA agar for 7 days. We found that colonies of group pS-LAC1-A and pS-LAC1-B produced much less melanin than that of group pS (Fig. 1C), which was in accord with the qRT-PCR results of mRNA of *LAC1* ($p<0.01$, Fig. 1B). Therefore, shRNA containing either the long or short loops still conducted its interfering function. But we still observed a little brown pigment in the center of colonies of group pS-LAC1-A and pS-LAC1-B (Fig. 1C). When all the white-like colonies were passaged on DOPA agar every 5 days, the color of some colonies at the 3rd passage evenly changed to grey and even dark (Fig. 1D). There were 4 brown or black colonies of 39 in group pS-LAC1-A and 9 of 22 in group pS-LAC1-B (white arrows in Fig. 1D). Then we plated the whitest colony in Fig. 1D (belonging to group pS-LAC1-A) to a new DOPA agar for another 5 days (at the 4th passage), and we found its color also turned lightly grey (Fig. 1E).

3.2 Successful construction of *LAC1* knock-out strain and its phenotypic stability

For gene knock-out, we used the PNK003 vector gifted by Ping Zhang and Xudong Zhu, Beijing Key Laboratory of Genetic Engineering Drug and Biotechnology, College of Life Sciences, Beijing Normal University. PNK003 contains two reverse BspQI restriction sites between the U6 promoter of H99 (serotype A) and the gRNA component, allowing the hybridized target DNA with 5' overhangs to be conveniently introduced into the BspQI-cut plasmids. Apart from the simplified construction, the elimination of Cas9 and gDNA cassettes after gene editing is another highlight of the vector. In this study, we proved that the PNK003 could conduct the function of genic knockout in B-3501A (serotype D) (Fig. 2). We picked the white strains out of the DOPA agar containing hygromycin B (Fig. 2A) and passaged them by plate streaking on DOPA agar every 5 days to screen out the completely knock-out strains. Finally, we found that the albino mutant strains of group PNK-LAC1 were stable to maintain and pass on the albino phenotype (Fig. 2C, at the 5thpassage). Meanwhile, the qRT-PCR result of the albino strains was no amplification within 40 PCR cycles (Fig. 2D), indicating that PNK003 was also effective for gene editing in serotype D.

3.3 The ability to produce melanin of *LAC1*knock-down/knock-out strains

Furthermore, to assess the melanin production of LAC1-knockdown/knockout strains, we randomly chose and amplified a knock-down colony of group pS-LAC1-A at 1st passage and a stable albino colony of group PNK-LAC1, comparing them by the Transmission Electron Microscope (TEM) and the laccase activity. With

the reference TEM of Emma Camacho*et al.* ,[36], we could identify that, after grown in DOPA broth for 3 days, WT *C. neoformans* produced melanin granules of high density and almost spherical shape (Fig. 3A-B). And some of melanin granules had been transported to the cell wall (white arrows in Fig. 3B). However, the cells of group pS-LAC1-A produced much less melanin than the WT cells, and most importantly, the LAC1-knockdown cell hadn't yet transported the melanin granules to the cell wall after growing in DOPA for 3 days (Fig. 3C-D). The albino mutant cell produced no melanin granules (Fig. 3E-F), but some broadly polydispersed minute spherical L-DOPA particles resulted from auto-polymerization, which existed in the three types of cells (asterisk in Fig. 3). These minute L-DOPA particles were much smaller and had a lower density than melanin granules which can aggregate even up to 1 μm in diameter [36]. Besides, the capsule of LAC1-knockdown cell seemed thinner than that of WT and albino cells by rude evaluation (Fig. 3).

Laccase activity in LAC1-knockdown, albino and WT strains was quantified by incubating in DOPA broth for at least three days. Then the supernatant of all the cultures was quantified spectrophotometrically at 475nm [37]. After three-day incubation, the WT strains exhibited about 3-fold greater laccase activity ($p<0.01$) than the knock-down strains and about 7-fold greater ($p<0.01$) than the albino strains (Fig. 4B). When extending the incubation time to five days, the WT and knock-down strains demonstrated melanin accumulation, but the albino mutants did not. The WT strains exhibited more than 4-fold greater ($p<0.01$) laccase activity than the knock-down strains and about 40-fold greater ($p<0.01$) than the albino strains (Fig. 4B). Meanwhile, the laccase activity of knock-down strains became about 8-fold stronger ($p<0.01$) than the albino strains, whereas they were no statistical differences on the 3rd day. This observation was consistent with the phenotype (Fig. 4A) and TEM.

4 Discussion

We successfully constructed the time-saving and convenient systems of gene knockdown and knockout for *C. neoformans* by RNAi and CRISPR-Cas9. And the data in this study led to some interesting and useful conclusions.

We applied shRNA expression cassette ligating to pSilencer 4.1-CMV neo for RNAi in *C. neoformans* . It was found that shRNA containing either the long or short loops could both conduct its interfering function (Fig. 1). However, we found that some gray or even brown colonies appeared in both pS-LAC1-A and pS-LAC1-B groups with continuous passage, indicating that transcriptional interference mediated by siRNA would be gradually attenuated when passed on. The attenuation of transcriptional suppression might be related to variable expression of the transforming plasmids, possibly resulting from variable modification of the exogenous DNA expression[19]. The attenuated suppression also occurred in human cells: only about 50% of transfected clones still showed a significant reduction of target protein after 2 months [38]. More importantly, with continuously passed on 3 times, group pS-LAC1-A appeared to have a higher proportion of white-like colonies (35:39) compared to group pS-LAC1-B (13:22) (Fig. 1D). In addition, group pS-LAC1-A seemed to appear lower mRNA expression of *LAC1*gene compared to group pS-LAC1-B, although it wasn't statistically significant. Therefore, we inferred that the interfering effect of long loops might be more pronounced and longer lasting. RNAi in human cells also indicated that the size and nucleotide sequence of the loop had obvious influence to the interference, which was in accord with the results of this study [38]. In addition, other studies also used the shRNA with the same long loops to successfully conduct interference with*CAP10* gene of *C. neoformans* [22, 39]. And we further explored the phenotype of the *LAC1* knockdown strains and clarified at which passage the strains would present a visible attenuation of interfering effect.

For effectively genetic editing in CRISPR-Cas9 system,it is crucial to find the specie-specific promoters to initiate the expression of valid Cas9 protein and gRNA. However, our results might indicate that serotypes of the same species share the promoters of arbitrary serotype in the all-in-one CRISPR-Cas9 system. We successfully knocked out the*LAC1* gene of *C. neoformans* B3501A by PNK003, which was supposed to be a specific genetic editor for serotype A of *C. neoformans* [33]. It might be related to the flexibility of gene expression among closely related species [40, 41]. More importantly, the albino phenotype produced by the PNK003 vector containing*LAC1* target gene was stable even after continuous passage for more than 5 generations, with almost no mRNA expression of *LAC1*gene (Fig.2). These results indicated that the *LAC1*

knockout strain in this study was without the ability to produce melanin, that is, the albino phenotype was successfully constructed. Therefore, we inferred that the promoters of *CAS9* and sgRNA in the PNK003 vectors might apply to other serotypes of *C. neoformans* ,

By TEM and laccase activity assays, We found that the knockdown strain produced only a small amount of melanin, which cannot be delivered to the cell wall in a few days, while the albino strain produced even no melanin (Fig.3). Meanwhile, the laccase activity of knockdown strain was much lower than that of WT, but also with melanin-accumulation when incubated in DOPA broth (Fig.4). However, the albino strains had even no laccase activity and almost produced no melanin.

Based on this study, we recommend a rapid method to obtain genetic knock-down stains of *C. neoformans:* transfect strains with pSilencer 4.1-CMV neo plasmids that contain long loops and target genes and, more importantly, it is better to use 1^{st} or 2^{nd} passage of strains after transfection. Besides, the different capacities of melanin production that attributed to the attenuation of transcriptional suppression could be quantified spectrophotometrically, and applied to a linear exploration between melanin and immunoreaction of the host. And the stable genetic knock-out strains produced by PNK003 vectors could refer as a control group of no genetic expression. In addition, we recommended applying the PNK003 vectors to different serotypes of *C. neoformans* for quick screening of possible trait-regulating genes because of its easy construction and valid knockout effect, so it will be time-saving with no need to rebuild knockout vectors for different variants.

In addition, we transfected the competent yeast cells with vectors containing the *LAC1* gene by the Yeast Transformation Kit, whose principle is that the alkaline Li+ can enhance the permeability of the cell membrane, making the cells easier to absorb external DNA. Simultaneously, Polyethylene glycol in the kit protects the cells membrane from chemical damage of high-concentration lithium acetate, and carrier DNA protects the exogenous DNA from degradation by DNase of*C. neoformans* . The chemical transformation is easily accessible with no requirement for linearizing the plasmids, and electroporation can harm the cell membrane [42]. Therefore, we applied the chemical transformation to introduce exogenous DNA into *C. neoformans* . And the results in this study showed that the chemical transformation was effective but probably lowered the transfected efficiency. However, it was low-cost and easily accessible for rough screening of possible trait-regulating genes at a one-time.

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