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Short Communication

Generation of a synthetic binary plasmid that confers resistance to nourseothricin for genetic engineering of *Sporothrix schenckii*

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ABSTRACT

Some members of the *Sporothrix* genus can cause sporotrichosis, a worldwide distributed mycosis that affects several mammalian species, including human beings. *Sporothrix schenckii* and *Sporothrix brasiliensis* are the fungal species frequently associated with this disease, and the latter has gained significant interest because of the increased number of cases associated with transmission by cats. Despite the relevance of these organisms in the medical field, limited strategies for their genetic manipulation have been explored. Thus far, gene silencing using the hygromycin B resistance cassette is the sole strategy currently available to study these organisms. Here, we report the generation of a cassette that confers resistance to nourseothricin, which was successfully transferred from *Agrobacterium tumefaciens* to *Sporothrix* cells. Therefore, this can be used as a second selective marker to manipulate the genome of these organisms.

1. Introduction

Sporotrichosis is a mycosis that affects mammalian hosts, including human beings, and is caused by pathogenic species of the genus *Sporothrix* (Lopes-Bezerra et al., 2018; Lopez-Romero et al., 2011; Mora-Montes et al., 2015). These organisms have the ability to undergo dimorphism, going from a mold that grows in the environment to yeast-like cells upon infecting host tissues (Mora-Montes et al., 2015). Even though it is a worldwide distributed infection, most of the cases are concentrated in a handful of countries placed in America, Africa, Asia, and Oceania (Chakrabarti et al., 2015). There is currently a limited repertoire of tools available for genetic manipulation of these organisms, and this could explain the modest number of papers dealing with fundamental aspects of these pathogens (Mora-Montes et al., 2015). The genome sequence of both reference and clinical strains of *S. schenckii* are already available (Gomez et al., 2018; Teixeira et al., 2014), the *Agrobacterium tumefaciens*-mediated transformation was standardized (Lozoya-Pérez et al., 2018; Zhang et al., 2011), and the RNA interference methodology applied to study the relevance of genes in the biology of this organism (Rodriguez-Caban et al., 2011; Zhang et al., 2018). Moreover, we have identified *S. schenckii* genes of stable and constitutive expression that have been used as controls during the analysis of gene expression (Trujillo-Esquivel et al., 2017). Finally, the *ex vivo* model of interaction with human peripheral blood mononuclear cells, and the *in vivo* models of systemic and subcutaneous sporotrichosis in mice and the systemic infection in larvae of *Galleria mellonella* are currently available to study fungal fitness and virulence (Castro et al., 2013; Clavijo-Giraldo et al., 2016; Hachisuka and Sasai, 1981; Martinez-Alvarez et al., 2017).

Resistance to hygromycin B is currently the unique molecular marker for genetic manipulation in this organism (Rodriguez-Caban et al., 2011; Zhang et al., 2011; Zhang et al., 2018). This represents a limitation to developing new strategies for genetic manipulation of *S. schenckii*, such as single or multiple gene disruption and controlled expression of native or foreign genes. In addition, this organism has a naturally high tolerance to hygromycin B, which complicates the differentiation between transformed from non-transformed cells and involves the use of high concentrations of the compound to inhibit fungal growth (Lozoya-Pérez et al., 2018). To expand the molecular toolbox to study this organism, here we report the generation of a binary vector

Abbreviation: ORF, Open reading frame

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that can be transferred from *A. tumefaciens* to *S. schenckii* and confers resistance to the selective drug nourseothricin.

2. Materials and methods

2.1. Microorganisms and culture media

S. schenckii 1099-18 ATCC MYA 4821 (Castro et al., 2013), whose genome has been sequenced (Teixeira et al., 2014) was used in this study. Cells were incubated in solid YPD medium (1% [w/v] yeast extract, 2% [w/v] gelatin peptone, 3% [w/v] dextrose, 2% [w/v] agar), pH 4.5, at 28 °C for 6–9 days, and conidia were obtained by mechanical means as reported (Martinez-Alvarez et al., 2017).

2.2. Analysis of sensitivity to nourseothricin

Aliquots containing 1×10^6 conidia were spread on plates containing YPD, pH 4.5 with different concentrations of nourseothricin (Gold Biotechnology, USA) ranging from 5 to 200 μg mL^{-1} and incubated at 28 °C for 7 days. Fungal growth was also evaluated in YDP broth, pH 4.5 added with nourseothricin and cells were incubated at 28 °C for 2 days with reciprocal shaking at 200 rpm. Cell density was evaluated by measuring absorbance at 600 nm.

2.3. Generation of a binary vector with a nourseothricin resistance marker

We based the development of this new plasmid on the pCAMBIA3300 vector (CAMBIA, Canberra, Australia), since the pBGgHg vector, a derivative pCAMBIA1300 (Chen et al., 2000) has been recently used by our group to successfully transform *S. schenckii* (Lozoya-Pérez et al., 2018). The open reading frame (ORF) of *SAT1,* the gene encoding for nourseothricin resistance (Pierre et al., 2005) plus the *Agaricus bisporus* glyceraldehyde-3-phosphate dehydrogenase promoter (Pgpd) from pBGgHg vector were synthesized by Genewiz (USA) and cloned into the *Xho*I and *Eco*RI sites of pCAMBIA 3300, being this ORF transcriptionally regulated by Pgpd and the cauliflower mosaic virus polyadenylation signal (CaMV poly A signal), thus generating pCAMBIA-Nou. This vector contains the intact multicloning site and all the genetic elements for the transference of the plasmid to the fungal genome (Fig. 1).

The enhanced green fluorescent protein gene was isolated from the pBGgHg vector (Chen et al., 2000), by digesting with the *Sac*I and *Pst*I and this was cloned into the corresponding sites of the multicloning site of pCAMBIA-Nou, generating pCAMBIA-Nou-eGFP.

2.4. Agrobacterium tumefaciens-mediated transformation

The *A. tumefaciens* AGL-1 strain, transformed with the pCAMBIA-Nou vector was incubated overnight at 28 °C in LB broth (0.5 [w/v] yeast extract, 1% [w/v] gelatin peptone, and 1% [w/v] NaCl) added with 100 μg mL^{-1} ampicillin and 100 μg mL^{-1} kanamycin, cell density adjusted to 0.2, and a further incubation was performed in minimal medium (0.34 M K_2HPO_4, 0.16 M NaH_2PO_4, 0.37 M NH_4Cl, 0.24 $MgSO_4$, 0.04 M KCl, 1.8 mM $CaCl_2$, $FeSO_4$ 0.18 mM, pH 7.0 adjusted with 1 N HCl) supplemented with 200 μM acetosyringone (Sigma) for 4.5 h at 28 °C and reciprocal shaking (250 rpm). An aliquot containing 100 μL of these induced cells was co-incubated with 1×10^5 conidia contained in 100 μL, and were placed on a cellophane disk on top of a plate containing solid minimal medium and incubated 72 h at 28 °C. Then, the cellophane disk was placed on YPD, pH 4.5, supplemented with 40 μg mL^{-1} nourseothricin and 200 μM cefotaxime, and incubated for 3 days at 28 °C. Colonies growing in presence of nourseothricin were passed four times on solid medium supplemented with the drug, and then cells were stimulated three times to undergo dimorphism in YPD, pH 7.8 as described elsewhere (Lozoya-Pérez et al., 2018; Martinez-Alvarez et al., 2017).

2.5. Molecular characterization of transformant strains

Genomic DNA was isolated as reported (Robledo-Ortiz et al., 2012). Briefly, hyphae were grown in YPD broth, pH 4.5 overnight, frozen in liquid nitrogen, mechanically ground with mortar, resuspended in 200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% (*w/v*) SDS, 40 μg mL^{-1} RNase (Sigma), and incubated 1 h at 37 °C. Then, one volume of a phenol:chloroform (1:1) solution was added, thoroughly mixed with vortex, and the sample was centrifuged for 10 min at 8000 ×*g* and 4 °C. The liquid phase was saved, mixed with 0.5 volumes of neat isopropanol, and incubated for 4 h at −20 °C. The nucleic acid was washed with 70% (*v*/v) ethanol and used in PCR reactions to amplify the Pgpd plus the ORF that confers resistance to nourseothricin with the primer pair 5´-TAAGAGGTCCGCAAGTAGATT-3′ and 5´-TTA GGGGCAGGGCATGC-3′. Confirmation of the identity of isolated colonies upon transformation was established by amplifying a fragment of the encoding gene for the ribosomal protein L6 with the primer pair 5′-ATTGCGACATCAGAGAAGG-3′ and 5'-TCGACCTTCTTGATGTTGG-3′, as previously reported (Trujillo-Esquivel et al., 2017).

2.6. Fluorescent microscopy

Yeast-like cells were inspected under fluorescence microscopy in a Zeiss Axioscope-40 microscope and images were captured with an Axiocam MRc camera. In all cases, samples were exposed for 900 milliseconds to the laser beam.

3. Results and discussion

3.1. Sensitivity to nourseothricin by the wild-type strain

Natural resistance to streptothricin and nourseothricin is rare in bacteria, and even more difficult to find in members of the kingdom Fungi (Hamano et al., 2006). Therefore, we hypothesized that cells from *S. schenckii* would be naturally susceptible to the nourseothricin. As expected, the minimal inhibitory concentration for nourseothricin was at 30 μg mL^{-1}, and no paradoxical growth was observed when higher drug concentrations were used (Fig. 2), as reported for *Candida albicans* growing in presence of echinocandins (Moriyama et al., 2012). Similar results were observed when cells were grown in solid medium supplemented with nourseothricin (data not shown). Thus, it was likely that a gene conferring resistance to nourseothricin could be used as a selection marker during genetic manipulation of fungal cells. In addition, the minimal inhibitory concentration of this drug is lower than the concentration usually utilized for mutant selection when hygromycin B is used as a selective marker (Lozoya-Pérez et al., 2018; Zhang et al., 2011; Zhang et al., 2018).

3.2. Generation of pCAMBIA-Nou

We aimed the generation of a new binary vector for the *A. tumefaciens*-mediated transformation of *S. schenckii* cells, which harbors a new selection marker conferring resistance to nourseothricin. The new pCAMBIA-Nou vector (Fig. 1) was based on pCAMBIA3300 and contains the *SAT1* coding region under the control of *A. bisporus* Pgpd and CaMV poly A signal, the left and right borders to transfer DNA, an aminoglycoside phosphotransferase gene for transformed bacteria selection, the replication origin from pUC, and the encoding genes for RepA and StaA and the oriV from *Pseudomonas* pVS1 (Fig. 1).

3.3. Transformation of S. schenckii with pCAMBIA-Nou

Upon the *A. tumefaciens*-mediated transformation, the fungal cells were selected on YPD plates containing 40 μg mL^{-1} nourseothricin and cell growth was evident by the generation of colonies after 3 days of incubation at 28 °C (Fig. 3A). Control reactions where cells were only

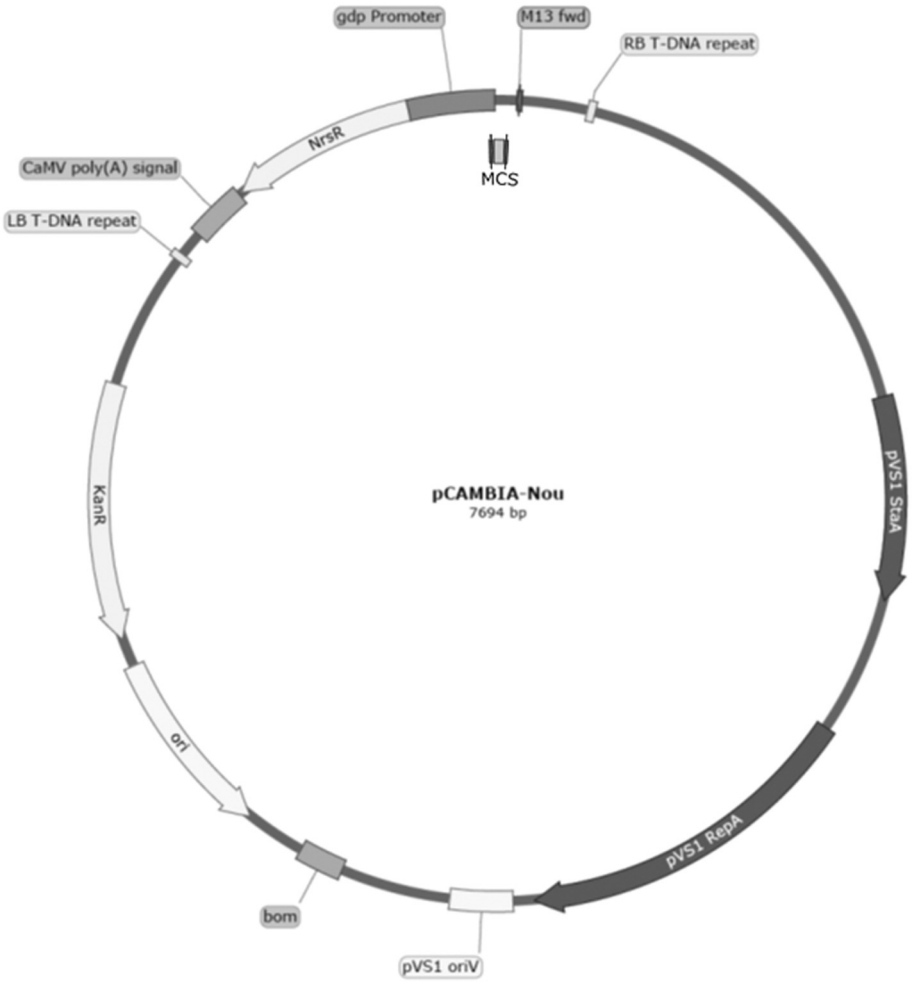

Fig. 1. Map of pCAMBIA-Nou. The Plasmid spans 7694 bp and contains the same multicloning site (MCS) of pCAMBIA3300 and all the genetic elements for Plasmid transference by *Agrobacterium tumefaciens*. It also contains the resistance cassette for kanamycin for Selection in bacteria and the nourseothricin resistance cassette (NrsR) for Selection in *Sporothrix schenckii*. The latter is composed by the open reading frame of *SAT1* under the control of *Agaricus bisporus gdp* promoter (gdp Promoter) and the cauliflower Mosaic virus polyadenylation signal (CaMV Poly A signal).

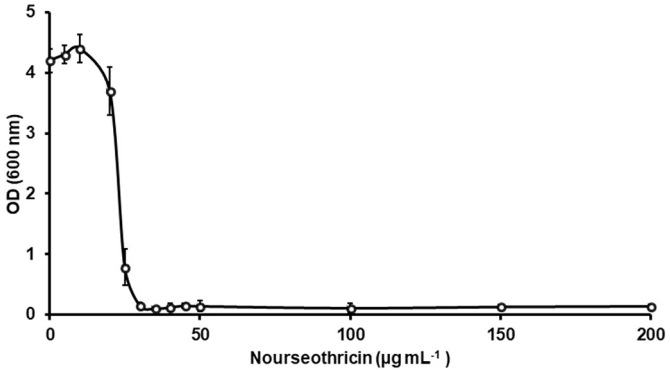

Fig. 2. Sensitivity of *Sporothrix schenckii* to nourseothricin. Aliquots containing 1×10^6 conidia were grown in YPD broth, pH 4.5 for 2 days at 28 °C with reciprocal shaking at 200 rpm, in presence of different concentration of nourseothricin, before reading the cell growth at 600 nm. The data represent means ± SD of three independent experiments assayed by duplicate. The minimal inhibitory concentration for nourseothricin was 30 μg mL^{-1}.

incubated with sterile deionized water did not show any fungal growth on plates, confirming that the cell growth was associated with the transference of the binary vector (Fig. 3A). After several passages on YPD supplemented with 40 μg mL^{-1} nourseothricin cells were disrupted, DNA isolated an used to demonstrate the presence of pCAMBIA-Nou within the genome of the transformant cells. Results in Fig. 3B showed the amplification of Pgpd plus the ORF that confers resistance to nourseothricin by PCR, confirming the transformation of *S. schenckii* with pCAMBIA-Nou. Moreover, PCR reactions aiming to amplify a fragment of the gene encoding for the ribosomal protein L6, which generates a specific 303 bp amplicon when *S. schenckii* genome DNA is used (Trujillo-Esquivel et al., 2017) confirmed these strains were indeed *S. schenckii* cells (Fig. 3B). Finally, when cell morphology was inspected under bright-light microscopy, normal cell morphology was observed (Fig. 3C), suggesting no gross changes in the cell biology of these organisms were associated with *A. tumefaciens*-associated transformation with pCAMBIA-Nou.

To further demonstrate the applicability of this plasmid in the genetic manipulation of *S. schenckii*, we cloned the gene encoding for the enhanced green fluorescent protein into pCAMBIA-Nou, generating pCAMBIA-Nou-eGFP, and used this construction to perform the transformation of wild-type cells as above described. As expected, cells transformed with pCAMBIA-Nou-eGFP, but not those containing pCAMBIA-Nou showed fluorescence when inspected by fluorescent microscopy (Fig. 4), showing pCAMBIA-Nou is a new tool for the incorporation of foreign genes into the *S. schenckii* genome.

In conclusion, here we report the generation of pCAMBIA-Nou, a binary vector with a new nourseothricin resistance cassette useful for selection of *S. schenckii* cells upon transformation. Since the current

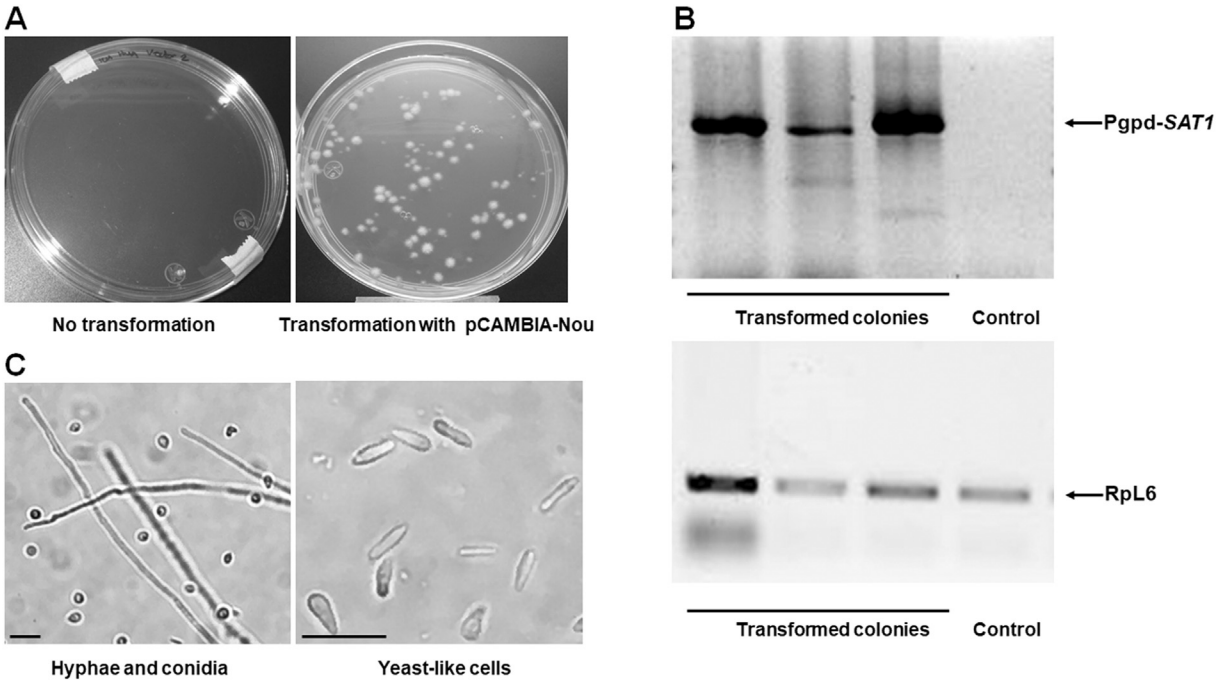

Fig. 3. *Agrobacterium tumefaciens*-mediated transformation of *Sporothrix schenckii* with pCAMBIA-Nou. A, Conidia were transformed with pCAMBIA-Nou and selected on YPD plates supplemented with 40 μg mL^{-1} nourseothricin. The No transformation control refers to mock transformation reactions using deionized water. B, Three randomly selected colonies showing resistance to nourseothricin were used for genomic DNA isolation and PCR reactions to amplify an 855 bp amplicon that corresponds to the open reading frame of *SAT1* under the control of *Agaricus bisporus gdp* promoter (Pgpd-*SAT1*). To confirm the cells were *S. schenckii*, a fragment of 303 bp corresponding to a fragment of the encoding gene for the ribosomal protein L6 (RpL6) was amplified by PCR. In both cases, the control lane refers to PCR reactions performed with genomic DNA from the wild-type strain 1099–18 ATCC MYA 4821. C, Cell morphology of transformed cells with pCAMBIA-Nou. Scale bar = 10 μm.

repertoire of molecular tools for genetic manipulation of this organism is limited, we anticipate this plasmid will be a relevant vector for genetic manipulation not only of this organism but other species or the same genus and molds compatible with the transcriptional elements that are controlling the *SAT1* ORF.

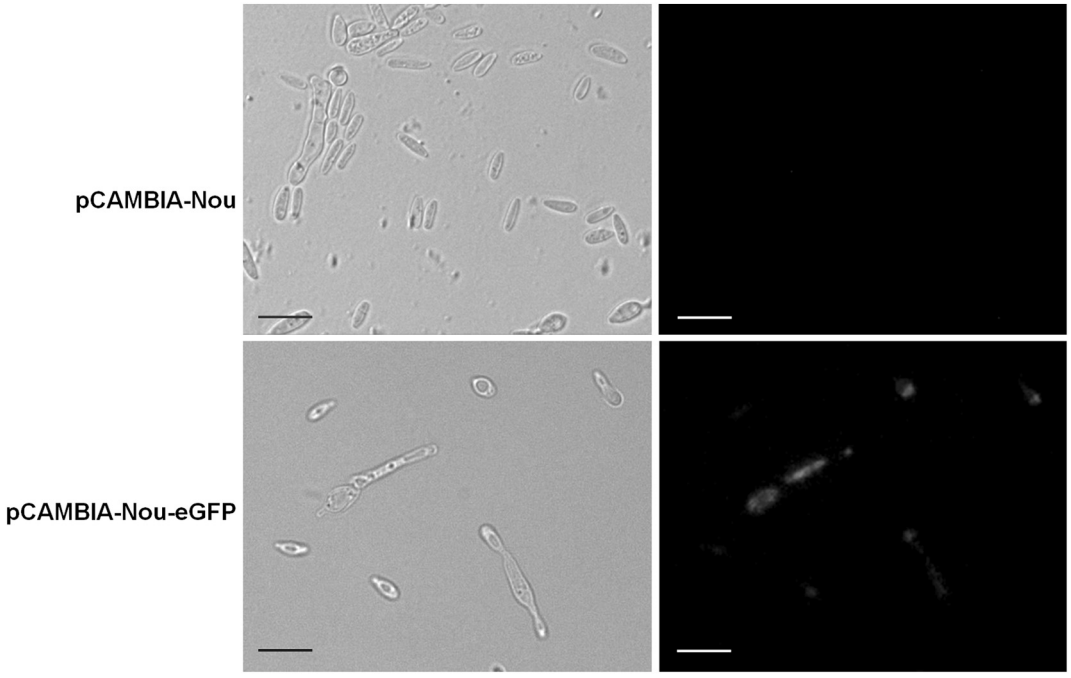

Fig. 4. Expression of the enhanced green fluorescent protein in *Sporothrix schenckii*. Conidia were transformed with either pCAMBIA-Nou or pCAMBIA-Nou-eGFP and after five monoconidial passages, cells were stimulated to undergo dimorphism and inspected by bright-field (left panels) or fluorescent microscopy (right panels). Scale bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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