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# Efficient insertional mutagenesis system for the dimorphic pathogenic fungus *Sporothrix schenckii* using *Agrobacterium tumefaciens*

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ABSTRACT

*Sporothrix schenckii* is a dimorphic pathogenic fungus that causes human and animal sporotrichosis globally. Here we developed and optimized an *Agrobacterium tumefaciens*-mediated transformation (ATMT) system of *S. schenckii* for insertional mutagenesis. The transformation efficiency reached more than 600 transformants per $10^6$ conidia. Using this protocol enabled us to obtain a large number of T-DNA insertional mutants within a short experimental period. Several mutants with altered phenotypes were obtained during the transformation experiments. The mutants displayed mitotic stability. Transferred DNA (T-DNA) flanking sequences were cloned by thermal asymmetric interlaced PCR (TAIL-PCR). Our results demonstrated that the ATMT system can be an effective tool for insertional mutagenesis in *S. schenckii*. This is the first report of a suitable mutagenesis system which may provide valuable mutants and information for both forward and reverse genetics research in the future for this medically important fungus.



## 1. Introduction

The dimorphic pathogenic fungus, *Sporothrix schenckii*, causes human and animal sporotrichosis, the most common subcutaneous disease throughout the world. In human, *S. schenckii* also causes two potentially fatal forms, pulmonary and disseminated sporotrichosis (Lopes-Bezerra et al., 2006). Recently, the incidence of sporotrichosis involving patients with acquired immunodeficiency syndrome (AIDS) has increased (Silva-Vergara et al., 2005).

*S. schenckii* is a temperature-dependent dimorphic fungus, existing in its nonpathogenic mycelial phase at temperature ranging from 25 °C to 30 °C and converting to its pathogenic yeast phase when the temperature is raised to 37 °C or infectious spores are inhaled by mammalian hosts (Lopes-Bezerra et al., 2006). Although many studies have focused on the rapid diagnosis, phylogenetic analysis and immune response to *S. schenckii*, the pathogenic mechanisms that underlie *S. schenckii* infection remain unclear owing to the lack of efficient genetic methods to generate mutants in this organism.

*Agrobacterium tumefaciens* is a Gram-negative plant pathogen, which has the ability to transfer a part of its DNA (transferred DNA or T-DNA), located on a tumor-inducing (Ti) plasmid, to its host genome at random sites. *A. tumefaciens*-mediated transformation (ATMT) has long been used for studying gene transfer and for tagging genes in plants (Machida and Yoshioka, 1992). Recently, several studies have reported the genetic manipulation of fungi using ATMT, providing new insights into fungal pathogenesis, pigmentation, sporulation and antibiotic resistance (Huser et al., 2009; Jeon et al., 2007; Michielse et al., 2009).

ATMT is an initiative bio-transformation system, with the principal advantage being that this bacterium can transform intact cells, such as conidia, mycelium, or even fruiting body, thereby eliminating the laborious process of protoplast preparation. Secondly, T-DNA integrates into the host genome in a non-sequence specific manner that is easy to generate a large number of mutants. Significantly, several methods have been described to isolate T-DNA tagged gene sequences (Jeon et al., 2000; Erster and Liscovitch, 2010, O'Malley et al., 2007; Singer and Burke, 2003). ATMT therefore offers an efficient tool for random insertional mutagenesis.

Here, we report the establishment of the ATMT system and an investigation into the important factors affecting the transformation frequency of *S. schenckii*. Highly efficient transformation method enabled us to obtain a large number of T-DNA insertional mutants within a short experimental period. The molecular analysis of transformants showed that insertion site flanking sequences could be identified by TAIL-PCR.

*Abbreviations:* ATMT, *Agrobacterium tumefaciens*-mediated transformation; T-DNA, transferred DNA; AS, acetosyringone; IM, induction medium; SM, selection medium; *hph*, phosphotransferase gene; TAIL-PCR, thermal asymmetric interlaced PCR; LB, left border; RB, right border.

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## 2. Materials and methods

### 2.1. Strains and plasmid

A clinical strain of *S. schenckii* was isolated from Jilin, north-east China, and was used as the parent strain for fungal transformation. This strain was cultured on potato dextrose agar (PDA) medium at 25 °C and stored as a monoconidial culture at −80 °C.

*A. tumefaciens* strains LBA4404, EHA105 and AGL-1 (Table 1) harboring the binary vector pBHt1 were cultured at 28 °C in Luria–Bertani (LB) medium. The vector pBHt1 carrying the bacterial hygromycin B phosphotransferase gene (*hph*), under the control of the *Aspergillus nidulans trp*C promoter, was used as a fungal selection marker (Mullins et al., 2001). Strains LBA4404 and EHA105 were obtained from the Culture Collection of Jilin University Mycology Research Center, China. Strain AGL-1 and plasmid pBHt1 were kindly gifted by Professor Zhonghua Wang (Fujian Agriculture and Forestry University).

### 2.2. A. tumefaciens-mediated transformation

The transformation procedure was based on a previously described protocol (Michielse et al., 2008; Zhang et al., 2008), with some modifications. Briefly, *A. tumefaciens* strains LBA4404, EHA105 and AGL-1 harboring pBHt1 were grown overnight in 10 ml of LB liquid medium supplemented with 20 μg/ml rifampicin and 100 μg/ml kanamycin at 28 °C while shaking (200 rpm). The culture (1.5 ml) was then centrifuged at 5000 rpm for 10 min and the pelleted cells were resuspended to an optimal density of $OD_{600\ nm}$ of 0.2–0.3 with induction medium (IM, described by Michielse et al., 2008) with 200 μM acetosyringone (AS) (IM + AS) or without AS (IM − AS).Then *A. tumefaciens* was pre-cultured for 8 h at 28 °C with gentle shaking at 100 rpm to an $OD_{600\ nm}$ of 0.6–0.8 in (IM + AS) or (IM − AS).

*S. schenckii* was incubated on a PDA slide for 7 d at 25 °C to sporulate. The conidia were scraped off from the fungal slide into 1 ml of sterile physiological salt solution and the concentration was determined using a hemocytometer. The conidia were then diluted to a final concentration of $5\times10^6$ conidia/ml in IM.

Sterile Hybond $N^+$ filters (0.45 μm pore, Amersham Pharmacia, USA) were placed on the IM + AS plates, and the induced *A. tumefaciens* cells were mixed with an equal volume of the conidial suspensions of *S. schenckii*. Then, 200 μl of the mixture was pipetted onto Hybond $N^+$ filters and the plates were incubated for varying lengths of time (24, 48 and 60 h) at 25 °C in the dark. Then the filters were transferred to a selection medium (SM: PDA containing 100 μg/ml hygromycin B and 200 μM of cefotaxime) to select for *S. schenckii* transformants while inhibiting the growth of *A. tumefaciens*. The plates were incubated for 5 d at 25 °C in the dark until colonies appeared.

### 2.3. Mitotic stability of transformants

The mitotic stability of the *S. schenckii* transformants was determined by analyzing the stability of hygromycin B resistance (Figueiredo et al., 2010; Zhong et al., 2007). Twenty randomly selected transformants were successively cultured on PDA plates without hygromycin B for five passages. Then the monoconidial cultures were transferred to PDA plates containing 100 μg/ml hygromycin B.

**Table 1**
*Agrobacterium tumefaciens* strains used in *A. tumefaciens*-mediated transformation of *Sporothrix schenckii*.

| Strain | Chromosome | Ti plasmid | Opine | Reference |
|---|---|---|---|---|
| EHA105 | C58 | pTiBo542DT-DNA | Succinamopine | Wirawan et al. (1993) |
| LBA4404 | Ach5 | pTiAch5 | Octopine | Heidekamp et al. (1983) |
| AGL-1 | C58 | pTiBo542DT-DNA | Succinamopine | Lazo et al. (1991) |

### 2.4. Molecular analysis of transformants

Putative hygromycin B resistance transformants of *S. schenckii* were cultured in potato dextrose broth (PDB) containing 100 μg/ml hygromycin B and 200 μM cefotaxime for five days at 25 °C with shaking (100 rpm). Mycelia were harvested by centrifugation at 12,000 rpm for 1 min, and then genomic DNA was extracted as previously described (Wang et al., 1998). PCR was performed using the phosphotransferase gene (*hph*) specific primers, *hph*-r and *hph*-f (Table 2), to detect the integration of T-DNA in the putative transformants (dos Reis et al., 2004).

Thermal asymmetric interlaced PCR (TAIL-PCR) was performed for the cloning of T-DNA flanking sequences from *S. schenckii* transformants. TAIL-PCR used two sets of primers that differed in their length and melting temperatures. One set of primers was the long, specific, and nested primer (LB1–LB3 and RB1–RB3) that was complementary to the known T-DNA left border and right border sequences, and had high melting temperatures. The other set of primers was the short, nonspecific, and arbitrary degenerate (AD) primer (AD1–AD4) that had low melting temperatures to create annealing sites within the unknown target sequences. Using these primer sets, T-DNA flanking sequences could be cloned and non-target products could be thermally controlled by one low-stringency PCR cycle, one high-stringency PCR cycle and one reduced-stringency PCR cycle (Mullins et al., 2001; Singer and Burke, 2003).The reaction products were sequenced using an ABI 3730XL DNA Sequencer (Invitrogen Co. Ltd., Beijing, China).

## 3. Results and discussion

### 3.1. The minimum inhibitory concentration of hygromycin B for S. schenckii

To determine the minimum inhibitory concentration of hygromycin B for the wild-type strain of *S. schenckii*, $1\times10^5$ conidia were inoculated onto PDA plates supplemented with hygromycin B at different concentrations (0, 50, 100, 150, 200 and 300 μg/ml). The results showed that *S. schenckii* conidia germination was inhibited on PDA medium containing ≥100 μg/ml hygromycin B. Therefore, 100 μg/ml was considered the minimum hygromycin B inhibitory concentration for the selection of *S. schenckii* transformants in the ATMT experiments (data not shown).

### 3.2. Optimization of the ATMT system for S. schenckii

It has been reported that T-DNA can be inserted not only into protoplasts but also into intact cells by ATMT (dos Reis et al., 2004).

**Table 2**
The primers used for PCR and TAIL-PCR.

| Primer name | Nucleotide sequence (5′ to 3′) |
|---|---|
| *hph*-r | 5′-CGACAGCGTCTCCGACCTGA-3′ |
| *hph*-f | 5′-CGCCCAAGCTGCATCATCGAA-3′ |
| LB1 | 5′-GGGTTCCTATAGGGTTTCGCTCATG-3′ |
| LB2 | 5′-CATGTGTTGAGCATATAAGAAACCCT-3′ |
| LB3 | 5′-GAATTAATTCGGCGTTAATTCAGT-3′ |
| RB1 | 5′-GGCACTGGCCGTCGTTTTACAAC-3′ |
| RB2 | 5′-AACGTCGTGACTGGGAAAACCCT-3′ |
| RB3 | 5′-CCCTTCCCAACAGTTGCGCA-3′ |
| AD1 | 5′-TGAGNAGTANCAGAGA-3′ |
| AD2 | 5′-AGTGNAGAANCAAAGG-3′ |
| AD3 | 5′-CATCGNCNGANACGAA-3′ |
| AD4 | 5′-CAAGCAAGCA-3′ |