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An efficient tool for random insertional mutagenesis: *Agrobacterium tumefaciens*-mediated transformation of the filamentous fungus *Aspergillus terreus*

Q1 Dongyang Wang, Dan He, Guangquan Li, Song Gao, Huiying Lv, Qiushi Shan, Li Wang *

Department of Pathogenobiology, Jilin University Mycology Research Center, Key Laboratory of Pathobiology, Ministry of Education, College of Basic Medical Sciences, Jilin University, Changchun 130021, China

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ABSTRACT

Agrobacterium tumefaciens-mediated transformation (ATMT) has been widely used in various organisms. In the current study, we developed a simple and efficient system for genetic transformation of the filamentous fungus *Aspergillus terreus* using ATMT. The transformation protocol was optimized for certain parameters to rapidly generate a library of Transferred DNA (T-DNA) insertion mutants of *A. terreus*. The presence of mitotically stable hygromycin resistance gene (*hph*) integration in the genome was confirmed by PCR, and T-DNA flanking sequences were cloned by thermal asymmetric interlaced PCR. The successful construction of the mutant library demonstrated the utility of the ATMT approach for future forward and reverse genetic studies in this important fungus.

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1. Introduction

Aspergillus terreus is a common saprophytic, filamentous fungus that is widespread in the environment. *A. terreus* produces a spectrum of secondary metabolites, such as itaconic acid (Shimi and El Dein, 1962), Q3 butyrolactone (Nitta et al., 1983) and emodin (Fujii et al., 1982). Consequently, *A. terreus* has been used extensively in industry as a fermentation strain. Alberts et al. (1980) isolated lovastatin from cultures of *A. terreus*. Lovastatin is an active inhibitor of cholesterol synthesis with hypolipidemic effects, and has been widely used as an antilipemic agent. Additionally, *A. terreus* also causes opportunistic infection in Q4 immune-compromised individuals (Lass-Flörl et al., 2005). Baddley et al. (2003) found that the percentage of *A. terreus* isolates relative to those of other *Aspergillus* species was significantly increased in the clinical cases at their institution, and the *A. terreus* isolates were frequently Q5 resistant to antifungal drugs (Baddley et al., 2003). However, few studies have focused on mutation breeding, and metabolite and pathogenesis-related genes of *A. terreus* (Barrios-González et al., 2008; Varga et al., 2003; Tevz et al., 2010; Vinci et al., 1991; Gressler et al., 2011) because of the lack of efficient genetic methods to generate mutants in this organism.

Agrobacterium tumefaciens is a gram negative plant pathogenic bacterium that causes crown gall in plants. *A. tumefaciens* is capable of transferring a piece of its tumor-inducing (Ti) plasmid DNA into host cells, where it is integrated into the host chromosome and expressed. Ti plasmid vectors have been developed to introduce target DNA sequences into plants, mammalian cells and several species of fungi (Krysan et al., 1999; Kunik et al., 2001; Michielse et al., 2005). Q6

Insertional mutagenesis techniques are considered to be efficient tools to investigate fungal gene functions (Campoy et al., 2003; Rodríguez-Tovar et al., 2005). The *A. tumefaciens*-mediated transformation (ATMT) system has been used widely as an effective tool for insertional mutagenesis (De, Groot et al., 1998; Sugui et al., 2005; Zhang et al., 2011). Studies on *Agrobacterium*-mediated fungal transformation demonstrated that the ATMT system has several advantages. First, the T-DNA can be randomly inserted in the host genome, typically as a single copy, and is mitotic stable (Covert et al., 2001; Morioka et al., 2006). Second, *Agrobacterium* can transform intact cells, such as conidia, mycelium, or even fruiting bodies (Michielse et al., 2005), thereby eliminating the tedious process of protoplast preparation. Therefore, the ATMT system offers an efficient tool for random insertional mutagenesis.

Here, we report the establishment of an ATMT system and an investigation into the important factors affecting the transformation frequency of *A. terreus*. The highly efficient transformation method enabled us to rapidly obtain a large number of T-DNA insertional mutants. The molecular analysis of the transformants showed that insertion site flanking sequences could be identified by thermal asymmetric interlaced PCR (TAIL-PCR).

* Corresponding author. Tel.: +86 431 85619486.
E-mail address: wli99@jlu.edu.cn (L. Wang).

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Table 1
Primers used in this study.

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′

Table 2
The effect of acetosyringone (AS) on the transformation efficiency of *A. terreus*. Pre: pre-cultivation period; Co: co-cultivation period; +AS: the presence of AS; −AS: the absence of AS;* transformant numbers (average ± standard error).

	Co − AS	Co + AS
Pre − AS	0*	60 ± 12
Pre + AS	15 ± 3	315 ± 35

2. Materials and methods

2.1. Strains and plasmids

A. terreus was used as a recipient strain for transformation. The fungus was isolated from Jilin, Northeast China, and grown in Potato Dextrose Agar (PDA) at 25 °C and stored at −80 °C.

A. tumefaciens strain AGL-1, harboring the binary vector pBHt1, was cultured at 28 °C in Lysogenic Broth (LB) medium. Vector pBHt1, carrying the bacterial hygromycin B phosphotransferase gene (hph) under the control of the *Aspergillus nidulans* trpC promoter, was used as a fungal selection marker (Mullins et al., 2001). Strain AGL-1 was kindly provided by Professor Zhonghua Wang (Fujian Agriculture and Forestry University). All strains were conserved at the Jilin University Mycology Research Center.

2.2. A. tumefaciens-mediated transformation

Before transformation, the minimum inhibitory concentration of hygromycin B for the wild-type strain of *A. terreus* was determined by transferring 100 μl 1×10^6 conidia/ml fungal cultures onto PDA plates supplemented with different concentrations of hygromycin B (0, 50, 100, 150, 200, 250 and 300 μg/ml).

The transformation procedure was based on a previously described protocol (Michielse et al., 2008) with some modifications. Briefly, *A. tumefaciens* strain AGL-1 harboring pBHt1 was grown overnight in 10 ml of LB liquid medium supplemented with 20 μg/ml rifampicin and 100 μg/ml kanamycin at 28 °C, with shaking (200 rpm). A 1.5-ml sample of the culture was centrifuged at 2400 ×*g* for 10 min and the pellets were resuspended at an optical density at 600 nm ($OD_{600\ nm}$) 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) (Table 2). *A. tumefaciens* was then pre-cultured at 28 °C with gentle shaking at 160 rpm to an $OD_{600\ nm}$ of 0.4, 0.6, 0.8 and 1.0 in IM(IM + AS) or (IM − AS).

A. terreus was incubated on a PDA slide for 7 d at 25 °C to induce sporulation. The conidia were scraped off the fungal slide into 1 ml of saline and the cell concentration was determined using a hemocytometer. The conidia were then diluted to a final concentration of 10^5, 10^6 or 10^7 conidia/ml in saline.

Sterile Hybond N^+ Filters (0.45 μm pore, Amersham Pharmacia, USA) were placed on (IM + AS) or (IM − AS) plates, and the *A tumefaciens* cells were mixed with an equal volume of the conidial suspensions of *A. terreus*. A 100-μl sample of the mixture was pipetted onto the Hybond N^+ Filters and the plates were incubated for varying lengths of time (24 h, 36 h, 48 h and 60 h) at different temperatures (22 °C, 25 °C and 28 °C) in the dark. The filters were then transferred to a selection medium (SM:PDA containing 200 μg/ml hygromycin B and 200 μM of cefotaxime) to select for *A. terreus* transformants while inhibiting the growth of *A. tumefaciens*. The plates were incubated for 3 d at 25 °C in the dark until colonies appeared.

2.3. Mitotic stability of the transformants

The stability of hygromycin B resistance was used to determine the mitotic stability of the *A. terreus* transformants (Figueiredo et al., 2010; Wang and Li, 2008). Twenty randomly selected transformants were cultured on PDA plates without hygromycin B for 7 d. Mycelia from the edge of the cultures were picked with a toothpick and grown on fresh PDA plates for another 7 d. After repeating this procedure five times, germinating mycelia from each transformant were transferred to PDA plates containing hygromycin B (200 μg/ml).

2.4. Molecular analysis of the transformants

Putative hygromycin B resistant transformants of *A. terreus* were cultured in potato dextrose broth (PDB) containing 200 μg/ml hygromycin B and 200 μM cefotaxime for 24 h at 25 °C with shaking (160 rpm). Mycelia were harvested by centrifugation at 12,000 rpm for 3 min, and their genomic DNA was extracted as previously described (Wang et al., 1998). PCR was performed using hygromycin phosphotransferase

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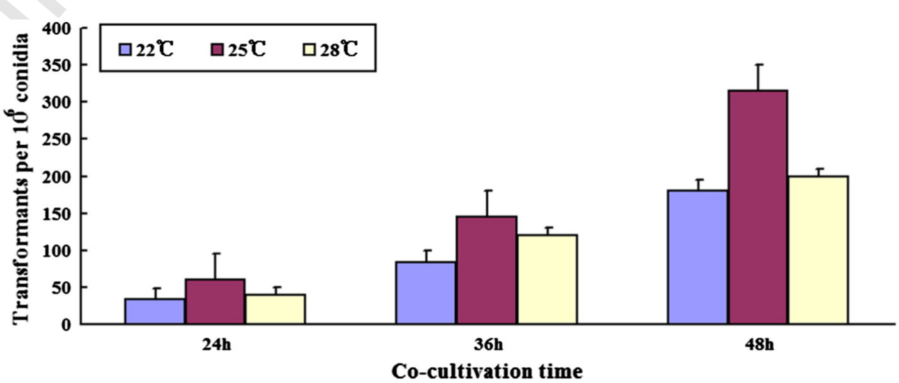

Fig. 1. The effect of different temperatures on co-culture period on transformation frequency.

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