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Agrobacterium tumefaciens-mediated transformation as a tool for insertional mutagenesis in the fungus *Penicillium marneffei*

Piyan ZHANG[a,*], *Bin XU*[b], *Yuezhu WANG*[b], *Yunqian LI*[c], *Zhen QIAN*[b], *Shengrong TANG*[b], *Shen HUAN*[b], *Shuangxi REN*[b]

[a]*Radiation Oncology Department, Thomas Jefferson University, Philadelphia, PA 19107, USA*
[b]*Chinese National Human Genome Center (CHGC) at Shanghai, Shanghai 201203, China*
[c]*Pathology Department and affiliated Nurse School, Guangxi Medical University, Nanning 530021, China*

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ABSTRACT

Penicillium marneffei is an opportunistic fungal pathogen of humans, causing respiratory, skin, and systemic mycosis in south-east Asia. Here we describe the transformation of *P. marneffei* with *Agrobacterium tumefaciens*, and the optimization of the transformation procedure. Transformations in different combinations between *A. tumefaciens* stains (LBA4404 and EHA105) and binary vectors (pCB309A, pBI129A, and pCaMBIA1312A) showed that EHA105/pBI129A were the most efficient partners. Southern blot analysis suggested that 87.5 % of transformants obtained with this protocol displayed single hybridization bands, indicating a single insert of T-DNA in each of the transformants. Unique hybridization patterns, along with thermal asymmetric interlaced PCR (TAIL-PCR) analysis of T-DNA insertion sites, suggested that *A. tumefaciens*-mediated transformation may be a powerful tool for insertional mutagenesis in *P. marneffei*. Several mutants with altered phenotypes were obtained during the construction of the mutant library, indicating the usefulness of the approach for functional genetic analysis in this important fungal pathogen.

Introduction

Penicillium marneffei, as the most important thermal, dimorphic fungus causing respiratory, skin, and systemic mycoses in south-east Asia, has not been well recognized since the first reported case of a natural human infection in 1973 (Woo *et al.* 2003; Kudeken *et al.* 1999). Typically, it infects humans afflicted with diseases involving cell-mediated immunity, such as Hodgkin's disease, patients with tuberculosis or acquired immunodeficiency syndrome (AIDS), and those undergoing corticosteroid therapy (Lobuglio & Taylor 1995). In fact, because of the increasing frequency with which it affects AIDS patients, it is considered as an 'AIDS defining pathogen'. The human immunodeficiency virus (HIV) pandemic, especially in south-east Asian countries, saw the emergence of the infection as an important opportunistic mycosis in immuno-compromised patients (Vossler 2001). Despite its medical importance, a large part of the ecology and epidemiology of *P. marneffei* remains unknown. Studies of this fungus at the molecular level have been limited (Woo *et al.* 2003).

Recently, several genes of *P. marneffei* involved in signal transduction pathways have been identified, mainly through

* Corresponding author. Tel.: +1 215 955 2044; fax: +1 215 955 2052.
E-mail address: piyan.zhang@jeffersonhospital.org

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the use of reverse genetics approaches involving the targeted disruption of candidate genes (Borneman et al. 2001; Boyce et al. 2005; Zuber et al. 2002). However, to fully understand the signal transduction pathways, cell growth control, and pathogenicity of the fungus, a genome-wide assessment of the role of such signal-regulated genes and virulence factors may be more valuable. This approach has been hampered by the lack of a highly efficient saturation mutagenesis system that can generate mutants.

Agrobacterium tumefaciens-mediated transformation (ATMT) of filamentous fungi has long been used for studying gene transfer and tagging genes in plants (Tinland 1996; Krysan et al. 1999). More recently, there have been many reports about successful transformation of fungi with *A. tumefaciens*, including yeast (Piers et al. 1996), plant pathogenic fungi (Covert et al. 2001), human and animal pathogenic fungi (Sullivan et al. 2002), mushrooms (Chen et al. 2000), and biocontrol fungi (Li et al. 2005). The main advantage of ATMT over plasmid-mediated transformation of fungi is that it avoids protoplast preparation. Furthermore, the genetic analysis of transformants and the identification of disrupted sequences are facilitated by the high efficiency of transformation and high percentage of single T-DNA insertion events. To analyse gene functions on a genome-wide scale, an efficient *A. tumefaciens*-mediated transformation system for *P. marneffei* was established. The molecular analysis of transformants of *P. marneffei* showed that ATMT is a suitable tool for insertion mutagenesis and subsequent identification of mutated genes in this fungus.

Materials and methods

Strains

Agrobacterium tumefaciens strains LBA4404 and EHA105 were kind gifts from Weihong Jiang (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) and Weiwen Kong (Fudan University), respectively. *A. tumefaciens* were grown in mannitol glutamate Luria salts medium (MG/L) supplemented with 100 $\mu g\,ml^{-1}$ kanamycin, 50 $\mu g\,ml^{-1}$ streptomycin, and 20 $\mu g\,ml^{-1}$ rifampicin to maintain plasmids (Piers et al. 1996).

Penicillium marneffei, isolated in Guangxi, south-west China, was maintained on Sabouraud medium, and stored as a monoconidial culture at −80 °C. The incubation period required to induce conidial formation was typically 10 d at 25 °C on LCS medium (1.5 % lactose, 0.25 % corn-steep, 0.5 % peptone, 0.4 % sodium chloride, 0.05 % magnesium sulphate, 0.06 % potassium dihydrogen phosphate, 0.0005 % ferric chloride, 0.0002 % cupric sulphate, 1.5 % agar). All specimens have been deposited in Ren's Laboratory of Bioinformation Department, Chinese Human Genome Center, Shanghai, 201203, China.

Plasmid constructions

The *PgpdA-ble-TtrpC* expression cassette was obtained by digestion of pAN8-1 with *Eco*RI and *Xba*I, or *Eco*RI and *Hin*dIII.

Table 1 – Plasmids used in this study

Plasmids	Size (kb)	Selection	Backbone	Reference
pCaMBIA1312A	12	*kan, ble*	pCaMBIA1304	Broothaerts et al. (2005)
pBI129A	15.5	*kan, ble*	pBI121	Chen et al. (2003)
pCB309A	7	*kan, ble*	pCB301	Xiang et al. (1999)

The fragments were inserted into appropriate sites of pCB301, pBI121 or pCaMBIA1304 to produce plasmids pCB309A, pBI129A and pCaMBIA1312A (Table 1). Primer pairs Pgfpgus1 (5′-GAAGCTTACCATGGTAGATCTGACTAG) and Pgfpgus2 (5′-CG AATTCTTCCCGACTACTAACATAG) were designed to amplify the *mgfp-gus* gene fragment and nos (nopaline synthase) 3′UTR from pCaMBIA1304 (CAMBIA, Canberra). The amplified 2.8 kb DNA fragment was digested with *Hin*dIII and *Eco*RI and inserted into pCB309A at appropriate restriction sites to produce pCB309A-gfpgus.

Fungal transformation

The transformation procedure was based on previous protocols (de Groot et al. 1998), with the following modification: *Agrobacterium tumefaciens* carrying binary vectors was grown overnight in MG/L liquid medium containing appropriate antibiotics at 28 °C with shaking. After dilution of *A. tumefaciens* cells to an $OD_{600\ nm}$ of *ca* 0.15–0.20 in induction medium (IM), which differs from MG/L in that 40 mM MES buffer (pH 5.3), 0.5 % glycerol (w/v) and 200 μM acetosyringone (AS) were added, the culture was incubated for 6 h at 28 °C in a reciprocating shaker. The harvested conidia of *Penicillium marneffei* were washed with sterile water two times, then incubated in fluid Sabouraud medium at 28 °C for 10–13 h. The pre-germinated spores were harvested by centrifugation at 2000 ***g*** for 10 min and resuspended in sterile water at a final conidia concentration of $5 \times 10^6\ ml^{-1}$.

Co-cultivation of *A. tumefaciens* and *P. marneffei* was performed as follows: bacterial cell cultures previous induced with 200 μM AS were mixed with an equal volume of the conidial suspension ($5 \times 10^6\ ml^{-1}$). Subsequently, the mixtures (100 μl per plate) were plated on nitrocellulose filters (0.45 μm pore and 90 mm diam) placed on co-cultivation medium plates (same as IM, except containing 5 mM instead of 10 mM glucose) and incubated at 28 °C for 4 d. Hereafter, the filters were transferred to Sabouraud plates containing 400 μM cefotaxime to kill the *A. tumefaciens* cells and 10 $\mu g\,ml^{-1}$ bleomycin to select for transformants. In order to confirm that transformation of the fungus by *Agrobacterium* was dependent on T-DNA transfer, a negative control was set in which the *vir* inducer AS was omitted.

Transformants visible 6 d later were transferred to LCS selection plates and incubated until conidiogenesis. To create monoconidial cultures, one germinating conidium from each transformant was picked and transferred to liquid Sabouraud medium supplemented with bleomycin (10 $\mu g\,ml^{-1}$). Finally, the monoconidial cultures were stored in 20 % glycerol at −80 °C until further analysis.

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Molecular analysis of transformants

Putative bleomycin-resistant transformants were cultured in liquid Sabouraud medium containing appropriate antibiotics for 4 d at 28 °C with shaking. Mycelia were harvested by centrifugation and mechanically disrupted by bead beating (FastPrep FP120; Qbiogene, USA) for 30 s at the max speed, and then, DNA was purified by phenol: chloroform extraction.

PCR analysis for the detection of the *mgfp-gus* gene in putative transformants was performed using the primer pairs Pgfpgus1 and Pgfpgus2. To determine the copy number of T-DNA, Southern blot analyses was performed. Genome DNA digested with *Eco*RI was size-fractionated on 0.7 % (w/v) agarose gel and transferred to positively charged nylon membranes. Preparation of digoxigenin-labelled probes, hybridization, and chemiluminescent detection were performed according to manufacturer's instruction (Roche, USA). The probe used to analyse the fate of transferred DNA was the 2.8 kb DNA fragment made by PCR amplification from vector pCB309A-gfpgus using Pgfpgus1 and Pgfpgus2 primers.

Cloning and sequencing of genomic DNA flanking T-DNA

A thermal, asymmetric, interlaced PCR (TAIL-PCR) was used for cloning genomic DNA flanking inserted T-DNA from 96 randomly selected transformants (Liu & Huang 1998). Genomic DNA from transformants selected was extracted and purified as described above. Degenerate primers (AD1, AD2, AD3, and AD4) and nested LB-specific primers (LSP1, LSP2, LSP3, and LSP4) are listed in Table 2. The reaction conditions and thermal cycling settings followed the protocol of Liu & Huang (1998). DNA amplifications were carried out using a PTC 200 thermal cycler (MJ Research, USA). The reaction products were analysed by using an ABI 377 DNA Sequencer (Perkin-Elmer, USA). The innermost primer LSP4 of T-DNA was used as a sequencing primer (Table 2).

Screening for phenotypic mutants

Penicillium marneffei transformants were visually scored for altered colony morphology (pigmentation and colony shape). Selected phenotypic mutants were successively cultured on Sabouraud plates without antibiotics for five generations to determine the stability of colony morphology. The transformants were then transferred to liquid Sabouraud medium containing 10 $\mu g\,\mu l^{-1}$ bleomycin and 400 μM cefotaxime. Finally, the genomic DNA of transformants was extracted to isolate and identify the flanking sequence of T-DNA insertion sites by Tail-PCR analysis.

Results

Agrobacterium tumefaciens*-mediated transformation of* Penicillium marneffei

Penicillium marneffei was found to be insensitive to hygromycin; therefore, we tested growth inhibition by inoculating 1×10^8 conidia per 90 mm plate with several concentrations of bleomycin: 0, 5, 10, 15, and 20 $\mu g\,ml^{-1}$. The results showed that *P. marneffei* could not grow even on plates containing Sabouraud medium plus 10 $\mu g\,ml^{-1}$ bleomycin, which suggested that bleomycin was appropriate as a selective agent for *P. marneffei*.

Various parameters were then assessed to optimize the transformation process with *Agrobacterium tumefaciens* strain EHA105 containing the binary vector pCB309A-gfpgus. Transformation rates were strongly influenced by the time that conidia were allowed to germinate. No transformants were obtained using non-germinated conidia. The optimal results with this strain and vector were achieved using 10–13 h old germlings and subsequently co-cultivation with strain EHA105 for 4–5 d in IM plates. Up to 82 transformants per 10^5 conidia of transformation were obtained. However, 3 d was found to be the minimal co-culture period to get fungal transformants per 90 mm IM plate. An increasing number of bleomycin-resistant colonies were obtained when the duration of the co-cultivation period was extended to 6 d. In addition, the number of transformants obtained per nitrocellulose disc generally increased with the number of conidia used. The number of conidia between 1×10^5 and 5×10^5 consistently yielded the highest number of transformants per disc, However, the transformation rate decreased dramatically at higher concentrations of conidia.

Using optimal transformation conditions, *A. tumefaciens* strains LBA4404 and EHA105 and three plasmids pBI129A, pCAMBIA1312A, and pCB309A were used for the introduction of a bleomycin resistant gene (*ble*) into *P. marneffei*. Three independent transformation experiments with each combination between two *Agrobacterium* strains and three plasmids were performed to compare their transformation efficiency (Fig 1). In a comparison between the two *A. tumefaciens* strains, EHA105 proved more efficient than LBA4404. Transformation with three binary vectors yielded different transformation

Table 2 – Sequences and characteristics of the primers used for TAIL-PCR

Primer name	Nucleotide sequence (5′ to 3′)	Tm (°C)	References
LSP 1	GAGAAAGTAGTGACAAGTGTTGGCC	62	This paper
LSP 2	TCCGTATTGTTGCATCACCTTCAC	60.2	This paper
LSP 3	CCTTCACCCTCTCCACTGACAG	63.8	This paper
LSP 4	CAAGAATTGGGACAACTCCAGTG	60.2	This paper
AD 1	NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT	Ca. 45	Liu & Huang (1998)
AD 2	NGTCGA(G/C)(A/T)GANA(A/T)GAA	Ca. 45	Liu & Huang (1998)
AD 3	NCAGCT(A/T)(G/C)TNT(A/T)GAT	-	Qin *et al.* (2003)
AD 4	NGTCGA(G/C)(A/T)CNT(A/T)CTA	-	Qin *et al.* (2003)

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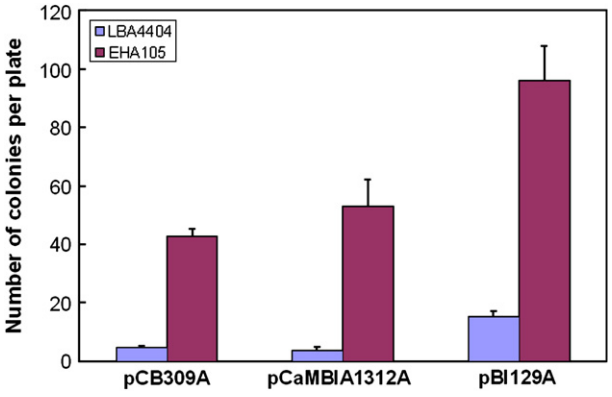

Fig 1 – Comparison of efficiency of *Penicillium marneffei* transformation with each combination of two *Agrobacterium* strains LBA4404 and EHA105 and three plasmids pBI129A, pCAMBIA130A, and pCB309A

rates in *P. marneffei*. Plasmid pBI129A resulted in the highest transformation efficiency, whereas pCB309A produced the lowest transformation efficiency.

Molecular analysis of transformants

In contrast to the situation described for *Agrobacterium*-mediated transformation of *Hebeloma* or *Fusarium* (Combier *et al.* 2003; Mullins *et al.* 2001), we found that the inclusion of AS during the 6 h growth of *A. tumefaciens* cells prior to co-cultivation was essential for the successful integration of T-DNA into the *P. marneffei* genome. We grew *A. tumefaciens* cells in IM, with and without, 200 μM AS for 6 h at 28 °C before co-cultivation with *Penicillium marneffei* conidia. After 4 d of co-cultivation the nitrocellulose filters, overlaid with the mixture of *A. tumefacien* cells and fungal conidia were transferred to Sabouraud plates containing bleomycin and cefotaxime to permit selection. With *A. tumefaciens* cells grown in the absence of AS (IM-AS), we obtained the same number of bleomycin-insistant colonies on selection plates as that with *A. tumefaciens* cells grown in the presence of AS (IM + AS). In subculture, all colonies remained resistant to bleomycin. However, using PCR and southern blotting analysis, we found that 92.5 % (37 out of 40) of colonies obtained with IM-AS had no exogenous gene insertion, which were, therefore, defined as false positives. However, with IM + AS only 12.9 % (six out of 47) were false positives (data not show).

To investigate the state of the T-DNA in the fungal transformants, we isolated total DNA from the above transformants and confirmed integration by PCR analysis. Genomic DNA was digested with *Eco*RI and hybridized with the probe derived from 2800 bp fragment made by PCR using primer pairs Pgfpgus1 and Pgfpgus2. Restriction nucleases *Eco*RI have no target site within the probe but have one site in the T-DNA sequence, which ensures that different T-DNA insertions yield different hybridization fragments on the blot (Fig 2). Southern analysis of *Eco*RI-cleaved DNA from the fungal transformants demonstrated that each possessed the inserted DNA fragment containing the *gfpgus* gene, 87.5 % (21 out of 24) of which displayed single hybridization bands indicating a single insert of T-DNA in each of these transformants (Fig 2). In addition, Southern analysis indicated that the frequency of single T-DNA integration events decreased as the concentration of AS was increased during the co-cultivation period. The addition of 200, 400 and 800 μM AS to co-cultivation IM plates resulted in, respectively, 85 % (17 out of 20), 65 % (13 out of 20), and 58.3 % (7 out of 12) of transformants containing a single insert of T-DNA per genome.

Determination of the flanking sequences in the transformants

TAIL-PCR was used to determine the sequences flanking the T-DNA insertion sites in 96 randomly selected transformants. Employing the AD primers with the LB-specific primers, LSP-1, 2, and 3, successful amplification of junction fragments was achieved in 70 out of the 96 transformants analysed. The sizes of the products ranged from 0.2 to 2.5 kb (Fig 3). In some transformants, more than one junction product was amplified, presumably due to the presence of multiple annealing sites of the AD primer in the vicinity of the inserted T-DNA. The flanking sequences were analysed by BLASTX searches. As a result of this study, a mutant library of *Penicillium marneffei* has been constructed. All flanking sequences for inserted sites have been identified using TAIL-PCR and subsequent sequencing. The primary analysis revealed about 20K insertion events occurred in 3215 protein-coding genes, which almost covered the whole genome of *P. marneffei*.

Screen for phenotypic mutants

Some phenotypic mutants were screened and identified on Sabouraud plates from our transformation experiments (Fig 4), including a colony producing a yellow pigment (B), a colony that lost the capacity to produce a characteristic red pigment (C), a rhizoid-like colony (D), a colony producing yellow conidia (E), a colony excreting mucus (F), a 'dwarf' colony (G), and a crateriform-like colony (H). All of them were significantly different to wild-type colony A (Fig 4). TAIL-PCR was performed to amplify the T-DNA border junctions of these mutants. Using T-DNA LB-specific primers along with primers AD1, 2, 3, and 4, all junction fragments of the seven mutants were successfully amplified and sequenced. The BLASTX algorithm results are listed in Table 3.

Discussion

This paper describes the use of *Agrobacterium tumefaciens*-mediated transformation for insertional mutagenesis of the dimorphic fungus *Penicillium marneffei*. Although ATMT has been developed for many filamentous fungi, the successful application of this technology remains far from routine in many species (Covert *et al.* 2001). This is the first report of *Agrobacterium*-mediated transformation of *P. marneffei* as a tool for insertional mutagenesis. We have optimized the transformation procedure in order to rapidly generate a large collection of transformants in which a single T-DNA has been integrated.

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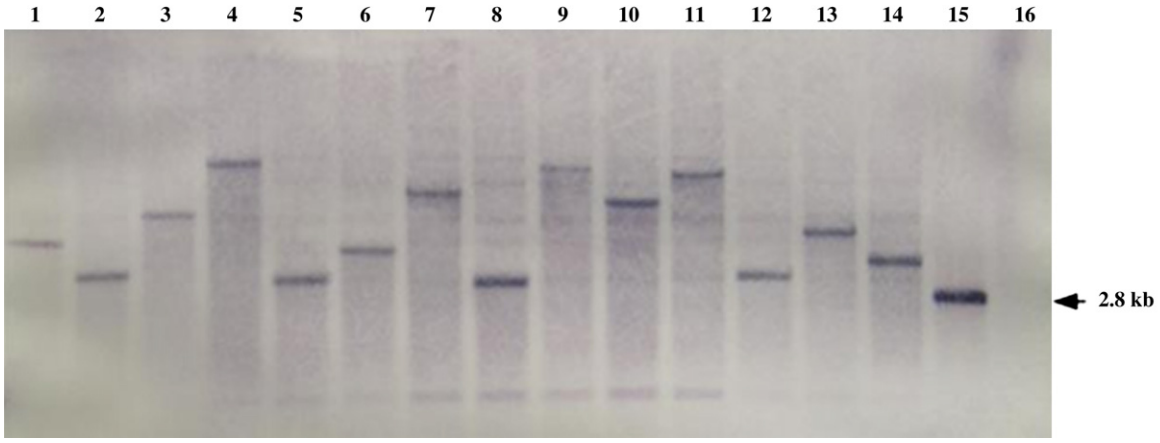

Fig 2 – Southern blot analysis of transformants of *Penicillium marneffei*. Genomic DNA was digested with *Eco*RI, which cut once in the T-DNA, run on a gel and transferred to a nylon membrane before probing with DIG-labelled 2.8 kb *mgfp-gus* gene fragments. Lanes 1–14, ATMT transformants; lane 15, 2800 bp *mgfp-gus* gene fragment as a positive control; lane 16, un-transformed wild-type strain as a negative control.

Various parameters affect the transformation efficiency in fungi, including the number and species of *A. tumefaciens* cells, the number of fungal spores, the co-cultivation temperature, the length of the co-cultivation period, etc. For *P. marneffei*, the transformation efficiency was strongly influenced by the time that conidia were allowed to germinate. Pre-germinated conidia were necessary for obtaining transformants during the co-cultivation period of 4–6 d, possibly because the intact conidia of *P. marneffei* could not germinate in time on IM plates during the given co-cultivation period. The effect of the number of conidia on transformation efficiencies has been discussed previously (Rogers *et al.* 2004). Interestingly, mixing $>7 \times 10^5$ conidia with a given concentration of *A. tumefaciens* per nitrocellulose discs, the number of transformants decreased dramatically. The same result has been observed in ATMT of *Coniothyrium minitans* (Li *et al.* 2005).

Transformations with different combinations of *A. tumefaciens* strains (LBA4404 and EHA105) and binary vectors (pCB309A, pBI129A, and pCaMBIA1312A) were investigated, showing that EHA105/pBI129A was the most efficient. It is clear that more transformants were obtained with the nopaline strain (EHA105), which was constructed as a super-virulent *A. tumefacien* strain (Hood *et al.* 1993), whereas transformations were less successful with octopine (LBA4404; Fig 1). Therefore, the use of a supervirulent strain of *A. tumefaciens* is essential to facilitate gene insertion into *P. marneffei*. Plasmids pCB309A, pBI129A, and pCaMBIA1312A were constructed from pCB301, pBI121, and pCaMBIA1304, respectively. The vectors pBI129A and pCaMBIA1312A designed in this study contain several useful features, including kanamycin-based selection in bacteria, high copy number plasmid replication, minimal extraneous DNA sequences, and stability of plasmids under non-selection conditions.

The plant phenolic AS induces expression of bacterial *vir* genes. Because AS is not produced by fungi, the additional of AS is essential for successful ATMT of these organisms. Contrary to previous reports showing that AS in IM is dispensable for the growth of bacterial cells prior to co-cultivation (Combier *et al.* 2003; Mullins *et al.* 2001), it was revealed in this study that IM + AS was essential for efficient ATMT of *P.*

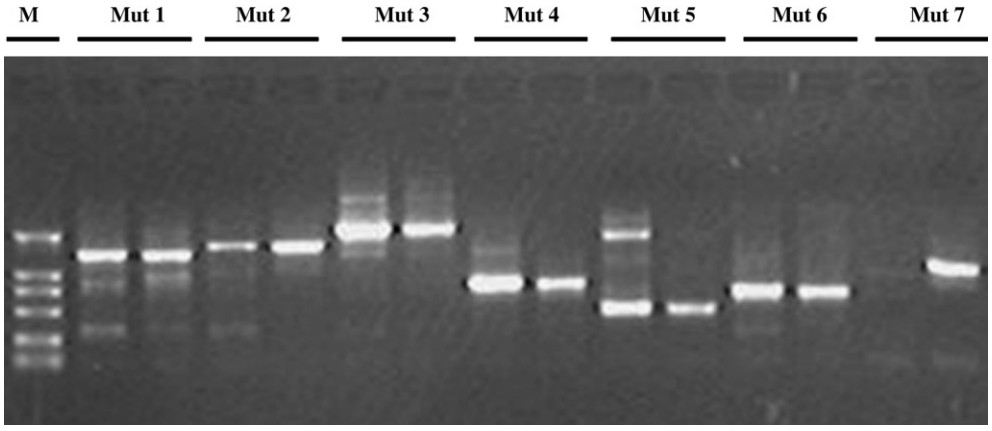

Fig 3 – Agarose gel analysis of TAIL-PCR products generated from seven randomly selected transformants. The products of the second and the third round amplification of each transformants were showed from right to left. Lane M, DL2000 (TaKaRa, Japan).

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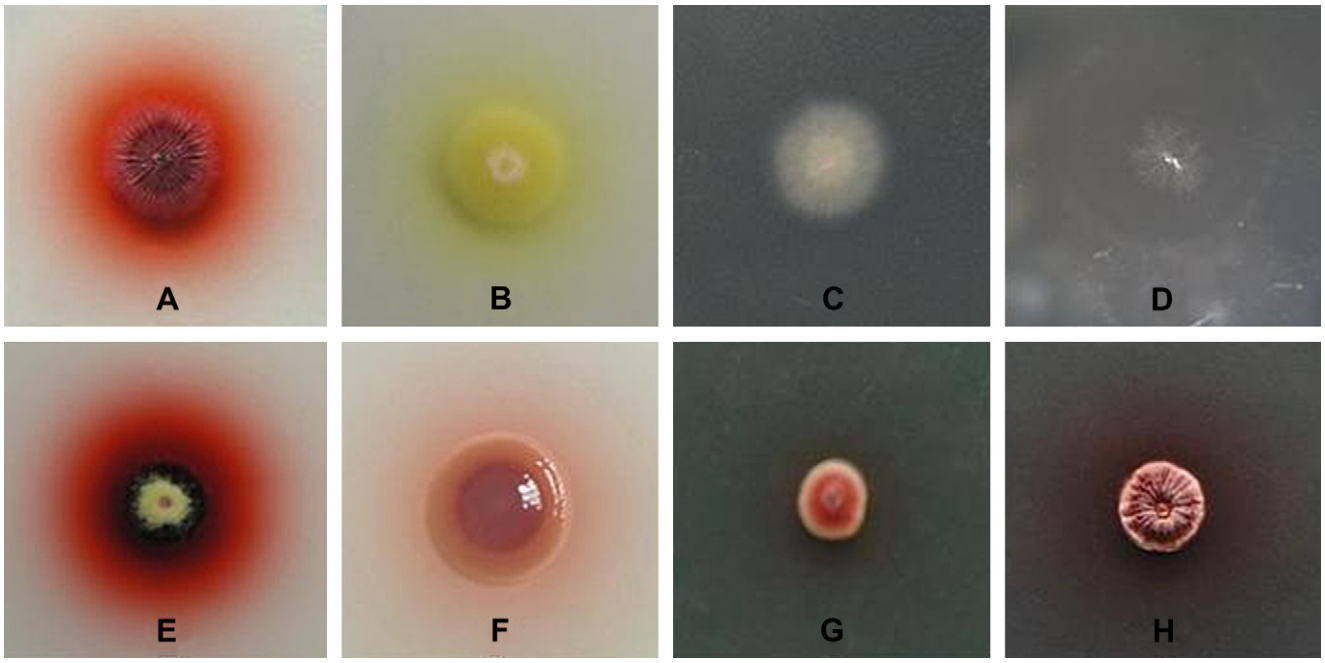

Fig 4 – Altered colony morphology of insertional mutants of *Penicillium marneffei*. All colonies were cultured on Sabouraud plates at 28 °C for 7 d. (A) Wild-type strain; (B–H) phenotype mutants selected from transformation experiments.

marneffei. With IM-AS, up to 92.5 % false-positive colonies were yielded by ATMT. Valuable insights into the molecular mechanisms of T-DNA transfer and integration may be obtained by further investigation regarding why so many false-positive colonies were produced with IM-AS.

For the construction of insertion mutant libraries, it is important that T-DNA insertion occurs as a single event, and that the site of integration is random throughout the genome. We showed that both single and multiple T-DNA integration can occur. However, a high proportion of single copy transformants were obtained by the protocol described in this paper. Southern analysis of 24 randomly selected transformants indicated that the *mgfp-gus* transgene sequences were integrated in all transformants, 87.5 % of which were single copy insertion (Fig 2). The variety of hybridization patterns observed suggests that integration randomly occurred at different sites in the genome. Additionally, by sequencing analysis of TAIL-PCR products, sequences flanking the insertion sites were different from each other. Preliminary analysis of 3215 protein-encoding genes hit during ATMT indicated that there were no significant 'insertion hotspots' in any particular region of the genome.

Seven interesting mutants with different colony morphologies were identified during ATMT (Fig 4). Sequence analysis of flanking insertion T-DNA of mutant B showed that B, C, D, E, G, and H phenotype alterations were involved in MFS monosaccharide transporter gene, citrinin polyketide synthase gene, thiamine-4 (*thi-4*) gene, a transcriptional regulator (ArsR family) gene, a putative cell division protein kinase (Ctk1), and a hypothetical protein gene, respectively (Table 3). Abundant mucus was produced over colony F. No homologue of the mutated gene was found by the BLASTX algorithm, so the nature of the pathway(s) affecting the synthesis and regulation of the mucus remains elusive and will be the subject of further study.

In this report, we have demonstrated a simple and efficient method for transforming *P. marneffei* by use of *A. tumefaciens*-

Table 3 – Results of the BLAST algorithm with T-DNA flanking sequences of mutants B–H from NCBI databases

Mutants	Accession no.	Description	E value
B	EAL88478	MFS monosaccharide transporter	8e-31
C	BAD44749	Citrinin polyketide synthase	1e-87
D	BAA21049	Thiamine-4	0
E	YP_023235	Transcriptional regulator, ArsR family	0
F	No significant similarity found		
G	EAL90692	Cell division protein kinase (Ctk1)	2e-68
H	EAA61734	Hypothetical protein AN7363.2	6e-39

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mediated DNA transfer. This approach could provide a tool of insertional mutagenesis gene tagging in this fungus, as has been done with other fungi. The addition of homologous sequences to the transforming DNA could in the future permit targeted gene disruption. In addition, it should be possible to adapt this relatively simple and useful methodology to a variety of other fungal species.

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