An improved Agrobacterium-mediated transformation system for the functional genetic analysis of Penicillium marneffei

Aksarakorn Kummasook*, Chester R. Cooper, Jr† & Nongnuch Vanittanakom*

*Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, and †Department of Biological Sciences, Youngstown State University, One University Plaza, Youngstown, Ohio, USA

We have developed an improved Agrobacterium-mediated transformation (AMT) system for the functional genetic analysis of Penicillium marneffei, a thermally dimorphic, human pathogenic fungus. Our AMT protocol included the use of conidia or pre-germinated conidia of P. marneffei as the host recipient for T-DNA from Agrobacterium tumefaciens and co-cultivation at 28°C for 36 hours. Bleomycin-resistant transformants were selected as yeast-like colonies following incubation at 37°C. The efficiency of transformation was approximately 123 ± 3.27 and 239 ± 13.12 transformants per plate when using 5 × 10⁴ conidia and pre-germinated conidia as starting materials, respectively. Southern blot analysis demonstrated that 95% of transformants contained single copies of T-DNA. Inverse PCR was employed for identifying flanking sequences at the T-DNA insertion sites. Analysis of these sequences indicated that integration occurred as random recombination events. Among the mutants isolated were previously described stuA and gasC defective strains. These AMT-derived mutants possessed single T-DNA integrations within their particular coding sequences. In addition, other morphological and pigmentation mutants possessing a variety of gene-specific defects were isolated, including two mutants having T-DNA integrations within putative promoter regions. One of the latter integration events was accompanied by the deletion of the entire corresponding gene. Collectively, these results indicated that AMT could be used for large-scale, functional genetic analyses in P. marneffei. Such analyses can potentially facilitate the identification of those genetic elements related to morphogenesis, as well as pathogenesis in this medically important fungus.

Keywords: Penicillium marneffei, dimorphic fungus, Agrobacterium-mediated transformation, fungal morphogenesis, random insertional mutagenesis

Introduction

Penicillium marneffei is the only dimorphic species of the genus Penicillium and is the etiological agent of penicilliosis marneffei, a disease involving the infection of the reticuloendothelial system, but one rarely noted prior to the acquired immune deficiency syndrome (AIDS) epidemic [1]. The fungus always disseminates in immunocompromised patients and, if untreated, results in death. The infection has become more prevalent in the endemic areas encompassing Southeast Asia and Southern China, paralleling the increased frequency of HIV infections in these regions [1–4].

A number of genetic studies involving P. marneffei have appeared since 2000 [2]. Although the P. marneffei genome has been sequenced and released recently [5], studies have yet to elucidate the functions of most genes in this fungus, particularly those that may be involved in pathogenesis. Therefore, developing high-throughput methods to determine the biological function of these genes is of great significance. One potential tool for this purpose is an Agrobacterium-mediated transformation (AMT) system that can cause large-scale, random mutagenesis [6].
Zhang and co-workers [7] recently described the use of AMT as a tool for the functional genetic analysis of *P. marneffei*. These investigators examined different combinations of *Agrobacterium tumefaciens* strains and binary vectors in transforming pre-germinated conidia of *P. marneffei*. Putative transformants were selected as bleomycin-resistant (bleR) colonies at 28°C, a temperature at which the mycelial phase of *P. marneffei* is normally formed. However, the most efficient of these experiments generated nearly 13% false positive transformants. Among confirmed transformants, 88% contained a single T-DNA copy insertion. Subsequent amplification of the genomic DNA flanking the T-DNA insertion site by a thermal, asymmetric, interlaced (TAIL) polymerase chain reaction (PCR) yielded products in only 73% of randomly selected, confirmed transformants. Nonetheless, sequencing of a selected number of these junction sites demonstrated the specific genes disrupted by the T-DNA insertion.

Although this previous study established the usefulness of AMT as a tool to genetically dissect *P. marneffei*, we sought to both improve its efficacy and isolate morphologically distinct mutants. Therefore, we developed an enhanced AMT protocol through improved co-cultivation and host cell conditions. Moreover, we employed a simple, robust inverse PCR method to efficiently identify nucleotide sequences flanking the T-DNA integrated regions. Using this more efficacious AMT protocol, we easily generated a number of *P. marneffei* mutants and identified their corresponding genetic defects. Hence, our enhanced AMT protocol may help provide insights into the molecular basis of both morphogenesis and virulence of this important fungal pathogen.

**Materials and methods**

**Strains and their maintenance**

The wild-type strain of *Penicillium marneffei* employed in our investigations (strain F4; CBS 119456) was maintained on malt extract agar (MEA; Oxoid, Hampshire, United Kingdom) at 25°C. Mutants generated from this isolate were maintained at 25°C on potato dextrose agar (PDA [Difco brand]; Becton-Dickinson, Franklin Lakes, New Jersey) containing 4 μg/ml bleomycin (Enzo Life Sciences, Farmingdale, New York). For long-term storage, mycelia of a given strain were suspended in 30% (w/v) sterile glycerol and frozen at –80°C.

*Agrobacterium tumefaciens* AGL1 [8], containing the plasmid pUPRSO [9], was maintained on Luria-Bertani agar supplemented with kanamycin (50 μg/ml) and rifampicin (25 μg/ml), then incubated at 25°C for 48 h. Several colonies from these plates were inoculated in liquid minimal medium (MM; 50 ml/250-ml flask) containing 50 μg/ml kanamycin and incubated at 28°C with shaking at 150 rpm for 24 h. Minimal medium (MM; final pH 7.5) contains the following reagents (all from Amresco), per liter: 10 ml of potassium-buffer pH 7.0 (200 g/l K ₂HPO ₄, 145 g/l KH ₂PO ₄); 20 ml of magnesium-sodium solution (30 g/l MgSO ₄·7H₂O, 15 g/l NaCl); 1 ml of 1% (w/v) CaCl₂·2 H₂O; 4 ml of 50% (w/v) glucose; 10 ml of 0.01% (w/v) FeSO₄·7H₂O; 5 ml of trace elements (100 mg/l MnSO₄·7H₂O; 100 mg/l CuSO₄·5H₂O; 100 mg/l H₂BO₃; 100 mg/l MnSO₄·H₂O; 100 mg/l Na₂MoO₄·2 H₂O); and 2.5 ml of 20% (w/v) NH₄NO₃. From this liquid MM culture, bacterial cells were collected by centrifugation and resuspended in induction medium (IM; final pH 5.3) to an optical density of 0.15 absorbance units at 600 nm. The composition of IM is the same as MM, but is additionally supplemented with 40 mM 2-(4-morpholino)ethanesulfonic acid (MES buffer; Amresco); 0.5% (w/v) glycerol; and 200 μM acetosyringone (AS; Sigma-Aldrich). The resulting bacterial suspension was incubated for 48 h at 28°C in a shaker bath (150 rpm) to pre-induce the virulence of *A. tumefaciens*. Finally, the pre-induced *Agrobacterium* culture was adjusted to an optical density of 0.25 at 600 nm (approximately 2.5 × 10⁶ cells/ml).

For transformation experiments, the experimental *P. marneffei* inocula consisted of a conidial suspension prepared from cultures grown on MEA for 7 days at 25°C. Following the scraping of surface growth and the suspension of mycelia in sterile saline, conidia were isolated by filtration through sterile glass wool [10]. For experiments requiring pre-germinated conidia, seven-day-old conidia were cultured in Sabouraud Dextrose Broth (SDB [Difco brand]; Becton Dickinson) at 25°C for 12 h in a shaking water bath operating at 150 rpm. Prior to their use in transformation experiments, suspensions of either non-germinated or pre-germinated conidia were prepared in IM at a concentration of 1 × 10⁶ per ml. Subsequently, equal volumes chromosomal markers for rifampicin and carbenicillin resistance, whereas the plasmid possesses the antibiotic resistance genes for kanamycin, neomycin, and bleomycin. For long-term storage, *A. tumefaciens* AGL1-pUPRSO was stored as a 15% (w/v) sterile glycerol suspension at –80°C.

**Agrobacterium-mediated transformation system**

The method of *Agrobacterium*-mediated transformation for use with *P. marneffei* strain F4 was slightly modified from that employed in *Trichoderma* species [9]. *Agrobacterium tumefaciens* AGL1-pUPRSO was streaked onto Luria-Bertani agar supplemented with kanamycin (50 μg/ml) and rifampicin (25 μg/ml), then incubated at 25°C for 48 h. Several colonies from these plates were inoculated in liquid minimal medium (MM; 50 ml/250-ml flask) containing 50 μg/ml kanamycin and incubated at 28°C with shaking at 150 rpm for 24 h. Minimal medium (MM; final pH 7.5) contains the following reagents (all from Amresco), per liter: 10 ml of potassium-buffer pH 7.0 (200 g/l K ₂HPO ₄, 145 g/l KH ₂PO ₄); 20 ml of magnesium-sodium solution (30 g/l MgSO ₄·7H₂O, 15 g/l NaCl); 1 ml of 1% (w/v) CaCl₂·2 H₂O; 4 ml of 50% (w/v) glucose; 10 ml of 0.01% (w/v) FeSO₄·7 H₂O; 5 ml of trace elements (100 mg/l MnSO₄·7H₂O; 100 mg/l CuSO₄·5H₂O; 100 mg/l H₂BO₃; 100 mg/l MnSO₄·H₂O; 100 mg/l Na₂MoO₄·2 H₂O); and 2.5 ml of 20% (w/v) NH₄NO₃. From this liquid MM culture, bacterial cells were collected by centrifugation and resuspended in induction medium (IM; final pH 5.3) to an optical density of 0.15 absorbance units at 600 nm. The composition of IM is the same as MM, but is additionally supplemented with 40 mM 2-(4-morpholino)ethanesulfonic acid (MES buffer; Amresco); 0.5% (w/v) glycerol; and 200 μM acetosyringone (AS; Sigma-Aldrich). The resulting bacterial suspension was incubated for 48 h at 28°C in a shaker bath (150 rpm) to pre-induce the virulence of *A. tumefaciens*. Finally, the pre-induced *Agrobacterium* culture was adjusted to an optical density of 0.25 at 600 nm (approximately 2.5 × 10⁶ cells/ml).

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of the particular conidial suspension and the pre-induced Agrobacterium suspension were mixed. From these mixtures, 100 μl (approximately 5 × 10⁶ conidia and 1.25 × 10⁶ bacteria) were spread onto cellophane sheets (purchased in retail stores) that had been placed on the surface of IM agar plates. IM agar plates differ from IM broth in that the former contains 1.5% (w/v) agar (Fisher Scientific, Pittsburgh, Pennsylvania), but half the amount glucose (5 mM). Control plates, containing either 50 μl of the pre-germinated or non-germinated conidial suspension and no Agrobacterium cells, were similarly prepared on IM agar. All plates were incubated at either 22°C or 28°C. After co-cultivation for varying lengths of time (24, 36, or 48 h), the cellophane sheets were transferred to brain heart infusion agar (BHA [Difco brand]; Becton Dickinson) containing 400 μg/ml cefotaxime (Sigma-Aldrich), and 4 μg/ml bleomycin. The plates were then incubated at 37°C for 4–10 days. The number of colonies on each plate was counted. More than 12,000 putative transformants, originally isolated as yeast-like colonies at 37°C, were transferred to PDA and cultured at 25°C for 4–5 days. The resulting colonies were initially screened for differences in their macroscopic appearance compared to the wild-type strain. Selected colonies were also assessed for microscopic morphological differences. Mutants of interest were further characterized with regard to their mitotic stability and particular genetic defect.

Molecular analysis of transformants

To extract genomic DNA from P. marneffei, one loop-full of conidia including hyphae was inoculated into 10 ml SDB in a 50 ml conical centrifuge tube and subsequently incubated at 25°C for 24 h in a shaking water bath operating at 150 rpm. The centrifuge tube was then transferred to a 37°C shaking water bath operating at 150 rpm and incubated for an additional 40 h. Genomic DNA was extracted from this culture as previously described [11].

To determine if bleR isolates contained integrated T-DNA, a duplex polymerase chain reaction (PCR) was performed using two pairs of primers (Table 1). One pair of primers, Gpda-F and Cyc1-R, was used to detect the bleomycin-resistance gene (ble), whereas a second pair of primers, ACT-exF and ACT-exR, was used as an internal PCR control to amplify a portion of the actin gene of P. marneffei. These two primer pairs, designed using Primer3 ([12]; http://frodo.wi.mit.edu/primer3/input.htm), are based upon the flanking nucleotide sequence of bleomycin-resistance gene from pUPRSO plasmid [9] and the nucleotide sequence of the P. marneffei actin-encoding gene (GenBank Accession No. FJ393547). The 50 μl PCR mixtures contained 100 ng of each DNA sample, 3 mM MgCl₂, 0.2 mM of each dNTP, 0.4 μM of each primer, and 1.25 Unit of Taq DNA polymerase (Qiagen, Valencia, California). Amplifications were performed using a MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, California) programmed as follows: 95°C for 5 min; 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min.

To determine the number of T-DNA copies in bleR transformants, Southern blot analysis was performed using the ECL Direct Nucleic Acid Labeling and Detection Kit (GE Healthcare Biosciences, Piscataway, New Jersey) in a manner similar to that previously described [10]. First, genomic DNA was digested with NdeI and KpnI (these restriction enzymes have no recognition site within the probe), then subjected to electrophoresis on 0.8% agarose gel, transferred onto a nylon membrane, and fixed by UV cross linking. A fragment of the ble gene, which was amplified by a PCR using the Gpda-F and Cyc1-R primers (Table 1), was used as a hybridization probe. The presence of multiple hybridization signals suggested that two or more T-DNA integration events occurred, whereas integration of a solitary T-DNA molecule was indicated by the observation of a single hybridization band.

To recover the genomic DNA sequences flanking the T-DNA integration site, an inverse PCR protocol was used. Intact genomic DNA (5 g) from mutants of interest was digested overnight with NdeI, XhoI, BamHI, or XbaI (FastDigest; Fermentas, Glen Burnie, Maryland). These restriction enzymes have no recognition site within the T-DNA vector. The digested DNA was ethanol precipitated [11] and dissolved in 8 μl of water prior to self-ligation using T4 DNA ligase (New England Biolabs, Ipswich, Massachusetts) in a total reaction volume of 10 μl incubated overnight at room temperature. One microliter of the ligation reaction was used as a template for a PCR using primers LBa1 and RB1 (Table 1). Amplification reactions were performed in a MJ Mini Personal Thermal Cycler (Bio-Rad) using 25 μl reaction volumes in accord with the vendor’s instructions of the Long Range PCR Kit (Qiagen). The parameters of the PCR were as follows: 93°C for 3 min; 35 cycles of 93°C for 15 s, 65°C for 30 s; 68°C for 6 min; and hold at 4°C. Following electrophoresis [10], the resulting PCR fragments were recovered from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen), then directly sequenced as previously described [13] employing primers LBb1 and RB2 (Table 1). Using Primer3 ([12]; http://frodo.wi.mit.edu/primer3/input.htm), primers RB1 and RB2 were designed based upon the T-DNA nucleotide sequence of the right border repeats in pUPRSO [9]. The resulting DNA sequences were analyzed using the BLAST ([14,15]; http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Geneious programs [16] to search for sequence similarities and gene identifications, particularly within the recently released P. marneffei genome database [5].

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Mitotic stability of transformants

To assess the mitotic stability of the integrated bleomycin-resistance marker, transformants were cultured on PDA without bleomycin for 10 generations. Subsequently, monoconidial cultures derived from the 10th generation of each transformant were cultured on PDA containing bleomycin (4 μg/ml). Those cultures retaining the bleomycin-resistant phenotype (bleR) were considered mitotically stable with regard to the integrated T-DNA.

Results

Agrobacterium-mediated transformation in P. marneffei

Growth of P. marneffei strain F4 was essentially completely inhibited on various media containing 4 μg/ml of bleomycin (data not shown). Therefore, this drug concentration was considered suitable for the selection of AMT-generated bleR colonies. Subsequent AMT was carried out with P. marneffei using the pUPRSO vector containing the ble gene as a selectable marker. The resulting transformants showed growth on BHA containing 4 μg/ml of bleomycin, whereas the wild type did not grow (Fig. 1). BHA was chosen as the base medium since it is a complete medium that enhances the efficient formation of the yeast phase.

To optimize our protocol for AMT in P. marneffei, conidia or pre-germinated conidia were used as starting material. In addition, two different temperatures of co-cultivation, as well as several periods of incubation were examined to assess their effect on transformation efficiency. Our experiments demonstrated that both conidia and pre-germinated conidia could be used as starting materials for AMT in P. marneffei as each generated bleR transformants. However, approximately a two- to three-fold higher transformation efficiency was obtained when using pre-germinated conidia (Table 2). A similar transformation efficiency was observed when pre-germinated conidia, co-cultivated with A. tumefaciens AGL1-pUPRSO at 28°C as compared with similar preparations incubated at 22°C (Table 2). No difference in number of transformants was obtained when comparisons were made between cells co-cultivated at 25°C and 28°C (data not shown). Experiments focused on the optimal duration of co-cultivation demonstrated that the highest transformation frequency and lowest background of bleR colonies were obtained after 36 h. By comparison, co-cultivation for 24 h generated fewer transformants and co-cultivation for 48 h produced a higher number of transformants. However, the latter co-cultivation period also resulted in significantly higher background growth of non-transformants (data not shown).

Table 1  Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ to 3′)</th>
<th>Amplification/sequencing target</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gpda-F</td>
<td>CAAGGTGCTTGGCCTCAAGTC</td>
<td>ble gene, 752 bp fragment</td>
<td>This paper</td>
</tr>
<tr>
<td>Cyc1-R</td>
<td>GCGTGAAGTGAAACGCTGCA</td>
<td>ble gene, 752 bp fragment</td>
<td>This paper</td>
</tr>
<tr>
<td>ACT-exF</td>
<td>CAACGCTCTGCATCTAACTGTC</td>
<td>P. marneffei actin gene, 450 bp fragment</td>
<td>This paper</td>
</tr>
<tr>
<td>ACT-exR</td>
<td>ATGACGGAAAGCTGGAAGAG</td>
<td>T-DNA flanking (left border) sequences</td>
<td>[35]</td>
</tr>
<tr>
<td>LBa1</td>
<td>TGGTTACAGTATGACCCACGCTGAAAGAG</td>
<td>T-DNA flanking (right border) sequences</td>
<td>This paper</td>
</tr>
<tr>
<td>LBb1</td>
<td>GCGTGGACCGCTTGGAGCAAC</td>
<td>T-DNA flanking (left border) nucleotides</td>
<td>[35]</td>
</tr>
<tr>
<td>RB1</td>
<td>TAGCTGATAGTGACCCCTAGCGGA</td>
<td>T-DNA flanking (right border) nucleotides</td>
<td>This paper</td>
</tr>
<tr>
<td>RB2</td>
<td>TCTGACGTATGCTTGCCTCA</td>
<td>T-DNA flanking (right border) nucleotides</td>
<td>This paper</td>
</tr>
</tbody>
</table>

Fig. 1  Selection of putative bleomycin-resistant transformants of Penicillium marneffei. Pre-germinated conidia cultivated in the absence of A. tumefaciens served as a control (a). Very little background growth was observed on the selection media (BHA containing 4 μg/ml bleomycin). After 36 h of co-cultivation, AMT-treated conidia developed on the selection media as individual yeast-like colonies (b), whereas 48 h of co-cultivation generates high background growth on the same medium (c).
Table 2 Average number of bleR transformants generated by Agrobacterium-mediated transformation of Penicillium marneffei.*

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Co-cultivation conditions (hours/temperature)</th>
<th>24 h</th>
<th>36 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conidia</td>
<td></td>
<td>3.67 ± 1.53</td>
<td>10.33 ± 2.08</td>
</tr>
<tr>
<td>Pre-germinated conidia</td>
<td></td>
<td>123 ± 3.27</td>
<td>239 ± 13.12</td>
</tr>
</tbody>
</table>

*Values represent averages from triplicate experiments in which 5 × 10^4 conidia per treatment were subjected to AMT. ND, not determined.

**Confirmation of ble gene integration**

A duplex PCR assay was developed to test for the integration of the ble gene into the genomes of the putative *P. marneffei* transformants. Primers ACT-exF and ACT-exR, as well as primers Gpda-F and Cyc1-R, which are specific for the actin and ble genes of *P. marneffei*, respectively, were employed. All transformants that were tested exhibited two distinct PCR products having the expected sizes for both an actin (450 bp) and ble (752 bp) gene fragment, which confirmed that genomic integration of the T-DNA occurred (data not shown). Additional confirmation was obtained by Southern blot analysis using NdeI- and KpnI-digested genomic DNA from the transformants and wild type (Fig. 2). Neither of these restriction enzymes have a recognition site within the ble gene of the T-DNA. Therefore, observation of a single hybridization signal would indicate the presence of a single T-DNA copy. Of the selected transformants examined in this manner, 19 of 20 (95%) had a single band when using either NdeI or KpnI. Only one transformant showed double integration of T-DNA (data not shown).

The mitotic stability of the integrated T-DNA was assessed with 20 randomly selected bleR transformants of *P. marneffei*. All maintained their bleR phenotypes after being subcultured for ten generations in the absence of bleomycin, i.e., all grew equally well in the presence of 4 μg/ml bleomycin as in its absence. This observation strongly suggests that the AMT-generated integration event was mitotically stable.

**Inverse PCR analysis of the T-DNA insertion junctions**

Inverse PCR was used to facilitate analysis of the flanking sequence of T-DNA insertion junctions among confirmed bleR transformants. Four enzymes (NdeI, XhoI, XbaI and BamHI) were selected, all of which lack recognition sites in the T-DNA region. After digestion with one of the above restriction enzymes, the flanking sequences were ligated, then amplified in a PCR using primers LBA1 and RB1. Of 20 DNA samples from different transformants, all generated amplification products following digestion by one or more of the above restriction enzymes. The resulting inverse PCR products ranged in size from 1.0–6.0 kb (Fig. 3). Specifically, NdeI-digested DNA produced PCR products in 8 of 20 samples tested, whereas 7 of 20 samples generated PCR products from XhoI-digested DNA. Use of XbaI and BamHI resulted in fewer positive PCR results (5 and 1 out of 20 samples, respectively). One sample produced PCR products from both XhoI- and XbaI-digested DNA.

Sequence analysis of 10 selected junctions derived by inverse PCR revealed that they contained sequences corresponding to either left border or right border of the T-DNA (data not shown). These flanking sequences possessed no homology among one another suggesting that T-DNA integration occurs at random positions in *P. marneffei* genome by a process of non-homologous recombination.

**Characterization of mutant phenotypes and corresponding mutations**

Approximately 12,000 transformants were originally selected as bleR, yeast-like colonies at 37°C. Subsequently, their colonial phenotypes were screened at 25°C. Several mutants of interest were selected and their flanking integration sites isolated using the inverse PCR protocol described above. These PCR products, representing a portion of the
sequences flanking the T-DNA integration sites, were then analyzed against the genome of *P. marneffei* and other fungi contained in the GenBank database (Table 3). Nucleotide analysis of one of the mutants of interest (strain I27) showed that the integration site perfectly matched with *stuA* in *P. marneffei* (Fig. 4) and its colony appearance was similar to that previously ascribed to a *stuA* mutant of *P. marneffei* [17]. In addition, microscopic examination of strain I27 found no metula or phialide formation during conidiation, which is consistent with the previous study (data not shown). Furthermore, we detected a *gasC* mutant (strain I132) among those we generated by AMT. This mutant was defective in the production of the characteristic red soluble pigment formed by the mycelial phase of *P. marneffei*. Again, the phenotype of this mutant strain is consistent with that previously described for a *gasC* mutant of *P. marneffei* [18]. Moreover, Southern blot analysis revealed only a single copy of T-DNA in the *stuA* and *gasC* mutants (strains I27 and I132, respectively, Fig. 2), indicating that integration of T-DNA occurred only at one site and within a specific gene. The majority of the remaining mutants characterized in this study also possessed T-DNA integrations within a specific gene (Table 3). However, in one mutant, the integration site was within the putative promoter region of a gene encoding a vacuolar ATP synthase subunit (strain I50), whereas integration of T-DNA in another mutant (strain I81) resulted in the deletion of an adjacent gene encoding a DUF907 domain protein.

**Discussion**

Insertional mutagenesis is a powerful tool for identifying new genes and their functions, but *Agrobacterium*-mediated transformation appears to be underutilized [6]. AMT has been successfully applied to a number of fungi to generate mutants by random integration of T-DNA. In this study, we sought to improve upon the only existing report of AMT in *P. marneffei* [7] by evaluating both co-

![Fig. 3 Identification of T-DNA insertion by inverse PCR. Genomic DNA extracted from AMT-derived transformants was digested with restriction enzymes (*Nde*I, *Xho*I, *Xba*I, or *Bam*HI), then subjected to inverse PCR. Selected results are shown that depict a single product generated from each AMT-derived transformant. Respectively, lanes 1–15 represent the results obtained from the following mutants strains digested with the indicated restriction enzyme: I6 (*Nde*I), I27 (*Nde*I), I81 (*Xho*I), 225E (*Nde*I), A30 (*Nde*I), I50 (*Nde*I), I140 (*Nde*I), AC4 (*Nde*I), I131 (*Xho*I), I133 (*Bam*HI), I231 (*Xho*I), 1205 (*Xba*I), I198 (*Xho*I), I132 (*Xho*I), and 1186 (*Xho*I). Lane 16 depicts the results using wild-type DNA digested with *Nde*I. Lane M contains a molecular size ladder (in kbp).](https://academic.oup.com/mmy/article-abstract/48/8/1066/955485)

**Table 3 Genetic defects in AMT-generated mutants of *Penicillium marneffei*.**

<table>
<thead>
<tr>
<th>Mutant strain no.</th>
<th>Phenotype at 25°C</th>
<th>T-DNA Integration Site</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A30</td>
<td>Dwarf colony</td>
<td>ARF GTPase activator (Glo3), putative</td>
<td>XM_002148215</td>
</tr>
<tr>
<td>I6</td>
<td>No conidial production</td>
<td>S-adenosylmethionine decarboxylase proenzyme</td>
<td>XM_002147750</td>
</tr>
<tr>
<td>I27</td>
<td>Abnormal conidiation and white colonies</td>
<td>Basic helix-loop-helix transcription factor StuA (<em>stuA</em> gene)</td>
<td>AF436076</td>
</tr>
<tr>
<td>I50*</td>
<td>Dwarf colony</td>
<td>Vacular ATP synthase subunit c</td>
<td>XM_002151369</td>
</tr>
<tr>
<td>I81**</td>
<td>Yeast-like colony</td>
<td>DUF907 domain protein</td>
<td>XM_002147624</td>
</tr>
<tr>
<td>I131</td>
<td>No secreted red pigment production</td>
<td>Short-chain dehydrogenase, putative</td>
<td>XM_002143175</td>
</tr>
<tr>
<td>I132</td>
<td>No secreted red pigment production</td>
<td>G protein alpha subunit (gasC)</td>
<td>AX170625</td>
</tr>
<tr>
<td>I133</td>
<td>Low numbers of conidia</td>
<td>DNA damage response protein Rtt109, putative</td>
<td>XM_002147785</td>
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<tr>
<td>I140</td>
<td>Dwarf colony</td>
<td>SAM domain protein</td>
<td>XM_002150150</td>
</tr>
<tr>
<td>I231</td>
<td>Fluffy colony</td>
<td>Protein kinase Yak1, putative</td>
<td>XM_002148368</td>
</tr>
</tbody>
</table>

*Integration of T-DNA was mapped to the putative promoter region of this gene.
**T-DNA integration on the S' end of this gene was accompanied by its apparent deletion.*

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cultivation and host cell conditions to enhance the efficacy of transformation.

One of the advantages of AMT is that various starting materials, such as spores, protoplasts, or germinated spores, can be used for the transformation in the fungi [6]. It has been shown that germination of spores is necessary for the transformation of Coccidioides immitis [19], Coniothyrium minitans [20], and P. marneffei [7]. Similarly, our AMT protocol showed that higher transformation efficiency was obtained when using pre-germinated conidia of P. marneffei, although we found that both germinated conidia and non-germinated conidia could be used as starting materials. Clearly, the conditions for AMT must be adjusted for the specificities of the fungal system being investigated. The differences in the optimal AMT conditions could depend on several factors, i.e., the strain of A. tumefaciens employed, the plasmids used, and efficiency and susceptibility of starting materials to A. tumefaciens. For example, a study of Cryphonectria parasitica found that the supervirulent A. tumefaciens A281 strain and its derivative (AGL-1) provided for greater efficiency than the A. tumefaciens LBA4404 strain [21]. This observation is further supported by the work of Zhang et al. [7] which compared the transformation efficiency of P. marneffei by A. tumefaciens strain LBA4404 to that of a supervirulent derivative, strain EHA105, with the latter distinctly more efficient than strain LBA4404.

In addition, several AMT studies have shown that each fungal system examined has an optimal combination of co-cultivation period and temperature by which a maximum number of transformants may be obtained [6]. The growth rate of the hosts and differences in their susceptibility to A. tumefaciens might account for these differences. With regard to co-cultivation temperature, 20°C to 37°C have been tested in different fungal systems. Typically, temperatures between 22°C and 25°C were found to be optimal [6]. In Zhang et al.’s prior study, the co-cultivation temperature employed in the AMT of P. marneffei was 28°C [7]. The authors did not report on any efforts to optimize the co-cultivation temperature in their AMT procedure. Therefore, we compared the transformation efficiency between co-cultivation at 22°C and 28°C and found a two-fold higher transformation efficiency when cells were co-incubated at 28°C (Table 2).

Another variable that affects the efficiency of AMT transformation is the length of the co-cultivation incubation period. In many fungi, more transformants can be obtained with longer periods of co-cultivation [6,22–28]. Zhang et al. reported that 3 days and 4–5 days were found to be the minimal and optimal co-cultivation period, respectively, to generate transformants of P. marneffei [7]. However, our particular protocol required only 24 and 36 h as the minimal and optimal co-cultivation periods, respectively. A prolonged co-incubation period (48 h) at a temperature of 22°C or 28°C led to increased fungal background growth (Fig. 1), thereby hampering the isolation of true bleR transformants.

A variable that we did not investigate was the affect of different filter types on transformation efficacy. Various filters, such as nitrocellulose, nylon, filter paper, cellophane sheets, and polyvinylidene difluoride, have been used...
successfully in the co-cultivation step of different AMT investigations. However, some nitrocellulose filters appeared to lower transformation efficiency [6,29,30]. Although nitrocellulose membranes were used previously for the AMT of P. marneffei [7], we demonstrated that the more cost-effective cellophane sheets could be used successfully to select and screen transformants much like a similar AMT study of *Trichoderma reesei* [31].

One popular application of AMT is to produce a collection of random insertional transformants that can be screened for phenotypes of interest [7,20,27,28,32–34]. The genomic DNA flanking the integrated T-DNA sequences can be rescued by inverse PCR or TAIL-PCR approaches and subsequently identified based on similarity to genome sequence databases. However, it is important that the transformants contain only a single copy of T-DNA so that the flanking regions from a lone integration event may be easily rescued. In our study, Southern blot analysis indicated that 95% of the transformants contained single copy integrations, thereby attesting to the efficacy of our AMT protocol. By comparison, Zhang and colleagues noted nearly equivalent single copy integration events (88%) [7].

Several methods have been described to isolate the chromosomal DNA flanking the T-DNA insertion site, e.g., plasmid rescue and PCR-based methods [6]. Although TAIL-PCR has been used for identifying T-DNA flanking sequences in *P. marneffei* [7], alternative methods can be employed. In our study, we demonstrated that inverse PCR is a simple, robust procedure that can be used for identifying the T-DNA integration sites. All samples that we tested yielded a product that could be isolated, whereas only 73% of transformants were reported by Zhang and colleagues to yield positive TAIL-PCR products [7]. Nonetheless, both TAIL-PCR and inverse PCR can be powerful tools for rapid and efficient recovery of flanking DNA sequences from AMT transformants of *P. marneffei*.

In the present study, we were particularly interested in the isolation of morphological mutants given the importance of dimorphism in the pathogenicity in *P. marneffei* [1,2]. We were successful in isolating a number of such mutants from a screen of 12,000 transformants, eight of which we describe in this report, in addition to two pigment secretion mutants (Table 3). These results attest to the general applicability of our AMT protocol for selecting developmental mutants. It is important to note, however, that our AMT-generated mutants were originally isolated at 37°C as yeast-like colonies, then screened for phenotypic differences at 25°C, a temperature that normally induces mycelial growth. Hence, the morphological mutants we describe here appear defective only in normal mycelial development, except for mutant strain I81 (see below), and may not provide significant insight into the dimorphism of *P. marneffei*. Nonetheless, we did isolate two mutants from our AMT experiments that grew as a mold at 37°C (data not shown). We are currently in the process of characterizing these mutants and the possible information they can provide regarding the molecular mechanisms of dimorphism in this fungus.

The T-DNA integration sites of ten mutants were identified using inverse PCR (Table 3). Comparison of these sequences indicated that integration was random. Furthermore, analysis of two integration sites indicated that they were within the coding regions of two previously described genes, *stuA* and *gasC*. Both mutants showed phenotypes similar to those described in prior reports [17,18]. These results not only confirmed that T-DNA integration events caused the dysfunction of these genes, but also validated the efficacy of our experimental protocol. Moreover, such observations further support the use of AMT for large-scale functional genetic analysis in *P. marneffei*. However, as indicated in our analyses, not all T-DNA integrations occur within the coding regions of a particular gene. Integrations in two of our AMT-generated mutants took place in the putative promoter regions of genes. One of these promoter-region integrations was accompanied by the deletion of the corresponding gene (strain 181). Hence, our analyses also demonstrate the importance of confirming the molecular nature of the mutant phenotype.

In summary, the results of the present investigations indicated that the optimal conditions for our AMT protocol included the use of pre-germinated *P. marneffei* strain F4 conidia and co-cultivation with *A. tumefaciens* AGL1-pUPRSO at 28°C for 36 h. A direct comparison of the efficacy of our AMT method to that of Zhang *et al.* [7] is not possible given the different parameters employed by each laboratory. Nonetheless, our results do demonstrate that the protocol described in this report provides a significantly enhanced method to potentially generate a library of mutants as demonstrated by transformation efficiency. Use of our AMT protocol in conjunction with additional or modified selection methods will greatly facilitate the search for genes critical to the morphogenesis and pathogenesis of this medically significant fungus.

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