Highly efficient transformation system for Malassezia furfur and Malassezia pachydermatis using Agrobacterium tumefaciens-mediated transformation

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Malassezia spp. are part of the normal human and animal mycobiota but are also associated with a variety of dermatological diseases. The absence of a transformation system hampered studies to reveal mechanisms underlying the switch from the non-pathogenic to pathogenic life style. Here we describe, a highly efficient Agrobacterium-mediated genetic transformation system for Malassezia furfur and M. pachydermatis. A binary T-DNA vector with the hygromycin B phosphotransferase (hpt) selection marker and the green fluorescent protein gene (gfp) was introduced in M. furfur and M. pachydermatis by combining the transformation protocols of Agaricus bisporus and Cryptococcus neoformans. Optimal temperature and co-cultivation time for transformation were 5 and 7 days at 19 °C and 24 °C, respectively. Transformation efficiency was 0.75–1.5% for M. furfur and 0.6–7.5% for M. pachydermatis. Integration of the hpt resistance cassette and gfp was verified using PCR and fluorescence microscopy, respectively. The T-DNA was mitotically stable in approximately 80% of the transformants after 10 times sub-culturing in the absence of hygromycin. Improving transformation protocols contribute to study the biology and pathophysiology of Malassezia.

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1. Introduction

Malassezia is a genus of yeasts that are characterized by their lipid dependence (Mayser and Gaitanis, 2010; Triana et al., 2015; Wu et al., 2015). It is part of the mycobiome of human skin that is rich in sebum production and also has been isolated from many other niches (Velegraki et al., 2015). Currently, 17 species have been defined based on phenotypic and molecular data (Honnavar et al., 2016; Puig et al., 2016; Wu et al., 2015). Dermatological diseases such as dandruff/seborrheic dermatitis, pityriasis versicolor, and atopic dermatitis in humans have been associated with Malassezia globosa, Malassezia restricta, Malassezia sympodialis and Malassezia furfur (Harada et al., 2015; Prohč et al., 2016; Velegraki et al., 2015; Wikramanayake and Borda, 2015), while Malassezia pachydermatis has been associated with otitis externa and dermatitis in dogs (Puig et al., 2016). In addition, M. furfur and M. pachydermatis have been related with bloodstream infections in patients who received parenteral lipid supplementation (Arendrup et al., 2009; Chrysanthou et al., 2001; Velegraki et al., 2015). The increasing interest in Malassezia as a pathogen urged the development of molecular tools for efficient transformation and genetic modification.

Agrobacterium tumefaciens-mediated transformation (AMT) is based on the capacity of this bacterial-plant pathogen to transfer DNA (T-DNA) into a host cell. This method combines the use of a binary vector system with a plasmid containing the T-DNA and a plasmid containing the virulence genes that are involved in the transfer of the T-DNA to the host (Michielse et al., 2008, 2005). This methodology was first described in fungi for Saccharomyces cerevisiae (Bundock and Hooykaas, 1996). Since then, it has been implemented successfully in yeasts and filamentous fungi including the pathogens Candida spp., Paracoccidioides brasiliensis, Cryptococcus neoformans, Coccidioides immitis, and Trichophyton mentagrophytes (Abuodeh et al., 2000; Leal et al., 2004; McClelland et al., 2005; Shi et al., 2015; Tempesta and Furlateno, 2007). Recently ATM was used to transform Malassezia and to inactivate genes by homologous recombination (Ianiri et al., 2016).

In this study, we have adapted AMT from the protocols reported for A. bisporus and C. neoformans (Chen et al., 2000; McClelland et al., 2005) to transform M. furfur and M. pachydermatis. We tested different co-cultivation parameters, including temperature and time. We used the hygromycin B phosphotransferase (hpt) gene as a selection marker and evaluated the use of GFP as a reporter protein in this yeast. The improvements we obtained when compared to the published transformation system (Ianiri et al., 2016) will enable molecular studies to reveal mechanisms underlying pathogenicity of Malassezia.

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2. Materials and methods

2.1. Strains and culture conditions

Frozen stocks of M. furfur CBS 1878 and M. pachydermatis CBS 1879 were reactivated for 4 to 5 days at 33 °C on modified Dixon agar (mDixon; 36 g L\(^{-1}\) mycosel agar [BD], 20 g L\(^{-1}\) Ox bile [Sigma-Aldrich], 36 g L\(^{-1}\) malt extract [Oxoid], 2 mL L\(^{-1}\) glycerol [Sigma-Aldrich], 2 mL L\(^{-1}\) oleic acid [Sigma-Aldrich], and 10 mL L\(^{-1}\) Tween 40 [Sigma-Aldrich]) (Boekhout, 2010; Guého et al., 1996). For liquid shaken cultures, Malassezia was grown in 150 mL Erlenmeyers at 180 rpm and 33 °C using 150 mL mDixon broth (36 g L\(^{-1}\) malt extract [Oxoid], peptone 6 g L\(^{-1}\) [Oxoid], 20 g L\(^{-1}\) Ox bile [Sigma-Aldrich], 2 mL L\(^{-1}\) glycerol [Sigma-Aldrich], 2 mL L\(^{-1}\) oleic acid [Sigma-Aldrich], and 10 mL L\(^{-1}\) Tween 40 [Sigma-Aldrich]).

To determine the minimum concentration of hygromycin B [Sigma-Aldrich] that abolishes yeast growth, 100 mL Malassezia suspension (10\(^{-3}\) yeast mL\(^{-1}\)) was incubated in triplicate for 7 days at 33 °C on mDixon agar supplemented with 6.25–100 μg mL\(^{-1}\) antibiotic. The minimal hygromycin B concentration was 25 and 50 μg mL\(^{-1}\) for M. furfur and M. pachydermatis, respectively. This assay was performed with each new hygromycin batch.

2.2. Transformation vectors

Plasmid pBHg (kindly provided by Peter Romaine, Pennsylvania State University) contains the gpt gene from Escherichia coli under the control of the A. bisporus glyceraldehyde-3-phosphate dehydrogenase (gpd) promoter (Chen et al., 2000). Vector pBH-GFP-ActPT was constructed to express the green fluorescent protein gene gfp from Aequorea victoria under the control of the regulatory sequences of the actin gene (act) of A. bisporus. To this end, primers 1 & 2 and 3 & 4 (Table 1) were used to amplify the act promoter and terminator, respectively. The products were cloned in pGEMT [Promega] and reamplified with primers 5 & 6 and 7 & 8. The fragments were cloned in PacI/AscI [Thermo scientific] digested pBHg-PA [Pelkmans et al., 2016] using In-Fusion cloning [Clontech], resulting in plasmid pBHg-ActPT that contains PacI and AscI sites between the act promoter and terminator. Gene gfp from Aequorea victoria [Entelechin GmbH] was amplified using primers 9 & 10, digested with PacI/Ascl and inserted in PacI/Ascl digested pBHg-ActPT, resulting in the 10,704 bp pBH-GFP-ActPT plasmid.

2.3. AMT of M. furfur and M. pachydermatis

The transformation procedure was adapted from protocols for transformation of the A. bisporus and C. neoformans (Chen et al., 2000; McClelland et al., 2005). Briefly, A. tumefaciens strain LBA100 was transformed with vectors pBHg and pBH-GFP-ActPT by electroporation applying 1.5 kV with capacitance set at 25 μF (Gene Pulser and Pulse Controller, Biorad, UK). Transformants were selected at 28 °C in Luria broth (LB) supplemented with 50 μg mL\(^{-1}\) kanamycin and 100 μg mL\(^{-1}\) hygromycin. After 2 days, transformants were transferred to minimal medium (Hooikaas et al., 1979) supplemented with 50 μg mL\(^{-1}\) kanamycin and grown overnight on a rotary shaker at 28 °C and 250 rpm to OD\(_{600}\) 0.6–0.8. Cells were collected by centrifugation for 15 min at 1248 g and resuspended in induction medium containing 200 μM acetylsyringone (AS) [Sigma Aldrich]. The bacterial suspension was incubated for 3 h at 19 °C with shaking at 52 rpm. Malassezia cells were harvested from liquid shaken cultures by centrifugation for 5 min at 2432 g, washed twice in milliQ H\(_2\)O with Tween 80 (0.1%), and suspended in induction medium at a density of 10\(^6\) cells mL\(^{-1}\). Equal volumes of yeast and A. tumefaciens cells were mixed and 20 mL of the mix was filtered through a 0.45 μm pore cellulose membrane [Millipore] using a 13 mm diameter syringe filter holder. The membrane filters were placed on co-cultivation medium with 200 μM (AS) and incubated at 19 °C, 24 °C, or 28 °C for 3, 5, or 7 days. The membranes were washed with 0.1% Tween 80 and transferred to mDixon agar containing 50 μg mL\(^{-1}\) hygromycin B, 200 μg mL\(^{-1}\) cefotaxin [Sigma Aldrich], 100 μg mL\(^{-1}\) carbenicillin [Sigma Aldrich], and 25 μg mL\(^{-1}\) chloramphenicol to select transformants. Individual colonies were transferred to a fresh selection plate. Experiments were performed in duplo using biological triplicates.

2.4. Fluorescence microscopy analysis

GFP fluorescence was monitored using a confocal microscope (Leica SPE-II) with 63 × ACS APO (NA = 1.30) oil objective. Fluorescence was detected using the spectral band 500–600 nm. The Fiji image processing package of ImageJ (www.fiji.sc) was used for image analysis and processing.

2.5. Molecular analysis and evaluation of mitotic stability

Genomic DNA of wild-type strains and transformants of M. furfur and M. pachydermatis was extracted as described (Grajales et al., 2009). Presence of the hygromycin cassette was analyzed by PCR using primers Hy-Fw & Hy-Rv (Table 1). Mitotic stability of 30 transformants was assessed by sub-culturing 10 times on mDixon agar without hygromycin followed by culturing in the presence of the antibiotic.

2.6. Statistical analysis

The number of transformants obtained at the different growth conditions was analyzed by two-factor ANOVA in order to assess the effect of temperature and days of incubation. Normality and homoscedasticity of the data was evaluated with R using the Shapiro–Wilk test and Bartlett’s test, respectively (R Development Core Team, 2013). The best condition for the transformation was determined using Student’s t-test between the means of the repeated experiments (R Development Core Team, 2013).

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Table 1

| Primer | Primer name | Sequence 5′−3′ |
|--------|-------------|---------------|
| 1      | Actin pmrF  | AAGCTTACCCAGACAAAGATGCCC |
| 2      | Actin pmrR  | CCAATGGTGTTCATGTCGTC |
| 3      | Actin trmF  | GCACTCCGGTATGCTGTTATGTTAGAATAAATAGATCTTGG |
| 4      | Actin trmR  | GAATCTACTACACCCAAAAACGGAGATTC |
| 5      | Act-Pr_F    | CCAGCGAGATCCGGTTAAAAGGCTAGGAGAG |
| 6      | pHg ActP_R  | AATAGAGAATCAGTCTCAATTCGGGCGCTTTGATTCCTGTCG |
| 7      | pHg Act_T_F | TCTGATGTTCATATAAGTTACGGCAGTATGTCGTTATG |
| 8      | Act-Ter_R   | CCGCGAATTGGCGGCGATCTACACTACCC |
| 9      | GFP-Fw      | CATGATGGCGCCGCGATACGGAGTATGAC |
| 10     | GFP-Rv      | CATGATGGCGCCGCGATACGGAGTATGAC |
| 11     | Hy-Fw       | GCCAGGTCGGCGGGCGCAAGGCTTGTAGTGGGAG |
| 12     | Hy-Rv       | CCTACCGAAAGATGTCGCGGCGGATCTGTGATA |

Primer used in this study.
3. Results

3.1. Effect of temperature and time of co-cultivation on transformation efficiency of M. furfur and M. pachydermatis

*Agrobacterium tumefaciens* containing the vector pBHg or pBH-GFP-ActPT was co-cultivated with *M. furfur* and *M. pachydermatis* at 19 °C, 24 °C, and 28 °C for 3, 5, and 7 days. Optimal co-cultivation time and temperature for transfer of pBHg was 5 and 7 days at 19 °C or 24 °C and for the GFP construct 5 days at 19 °C. Transformation efficiencies were 0.75–1.5% (Fig. 1A, B) and 0.6–7.5% (C, D) for *M. furfur* and *M. pachydermatis*, respectively.

3.2. Molecular analysis of the transformants and mitotic stability

Transformants were examined by PCR analysis to confirm integration of the T-DNA. PCR products of expected size of 1049 and 774 bp for the hpt and the gfp gene, respectively, were obtained from 30 out of 30 *M. furfur* and *M. pachydermatis* transformants (Fig. 2). In no case was a fragment amplified from the wild-type strains. Sequencing of the PCR

Fig. 1. Transformation efficiency of *M. furfur* (A, B) and *M. pachydermatis* (C, D) transformed with pBHg (A, C) and pBHg-GFP-ActPT (B, D) at 19 °C (blue boxes), 24 °C (green boxes), and 28 °C (red boxes) after 3, 5, and 7 days of co-cultivation with *A. tumefaciens*.

Fig. 2. Gel electrophoresis of *gfp* (A) and *hpt* (B) fragments amplified by PCR from *M. furfur* (lanes 1–10) and *M. pachydermatis* transformants (lanes 11–20). Wild-type *M. furfur* (WT1) and *M. pachydermatis* (WT2) were used as negative controls; vector pBHg and pBH-GFP-ActPT were used as positive control (PC), with M representing the molecular size marker (M).
products confirmed the presence of both genes in the Malassezia transformants. Microscopy showed GFP fluorescence in *M. furfur* and *M. pachydermatis* transformants with wild-type strains showing some background autofluorescence (Fig. 3).

A total number of 30 *M. furfur* and 30 *M. pachydermatis* transformants were 10 times subcultured on mDixon plates in the absence of hygromycin. Of these transformants, 80% were mitotically stable as shown by replating on hygromycin.

**Fig. 3.** Fluorescence microscopy analysis of *M. furfur* (A–F) and *M. pachydermatis* (G–L). Wild-type strains show low fluorescence (A–C, G–I) when compared to strains transformed with the *gfp* expression vector pBHg-GFP-ActPT (D–F, J–L).
4. Discussion

M. furfur and M. sympodialis were recently transformed using A. tumefaciens (Janiri et al., 2016). Here, A. tumefaciens mediated transformation (AMT) was optimized resulting in a highly efficient transformation system for M. furfur and M. pachydermatis.

Several changes in the AMT protocol were introduced to improve transformation efficiency. (i) A filtration step of the mixture of A. tumefaciens and Malassezia suspension was introduced instead of placing this suspension directly onto induction medium or onto a filter as is usually done (Janiri et al., 2016, 2011; Leal et al., 2004; McClelland et al., 2005; Michielse et al., 2008). Possibly, filtration facilitates the contact between the bacterial and yeast cells. (ii) Minimal medium was used as co-cultivation medium. Notably, Malassezia spp. was able to recover its growth after a co-cultivation period in this medium for 7 days despite the fact that these yeasts are lipid dependent. (iii) A concentration of 200 μM acetosyringone (AS) was used instead of 100 μM as was reported for basidiomycota yeast transformation (Janiri et al., 2016, 2011). This result is in line with previous work showing that high transformation frequencies are obtained when sufficient AS is present during Agrobacterium pre-culture and during co-cultivation (Michielse et al., 2005). (iv) A mixture of 10^5 bacterial cells mL^-1 and 10^-6 Malassezia cells mL^-1 resulted in the highest transformation efficiency. This ratio corresponds to 100 bacterial cells per yeast cell. A correct ratio of A. tumefaciens cells relative to fungal cells is important to avoid the bacterium to overgrow the fungus and to obtain optimal transformation efficiency (Michielse et al., 2008, 2005). (v) The optimal temperature and co-cultivation time were 5 and 7 days at 19 °C and 24 °C, respectively, for the two constructs that were tested. These co-cultivation temperatures agree with those of the yeasts C. neoformans and Candida albicans (McClelland et al., 2005; Tempesta and Furlateno, 2007) but not of P. brasiliensis that was most efficiently transformed at 28 °C (Leal et al., 2004). These differences have been evaluated with the growth rate of fungi and differences in their susceptibility to A. tumefaciens (Michielse et al., 2008).

An overall transformation efficiency of 0.75–1.5% and 0.6–7.5% was obtained for M. furfur and M. pachydermatis, respectively. These efficiencies are substantially higher than those reported for M. furfur and M. sympodialis (Janiri et al., 2016) or other yeast such as C. neoformans and P. brasiliensis that showed efficiencies of 0.2% and 0.0003%, respectively (Leal et al., 2004; McClelland et al., 2005). On the other hand, the transformation efficiency of C. albicans (Tempesta and Furlateno, 2007) was similar to our study.

The hygromycin resistance was mitotically stable as 80% of the transformants remained resistant after 10 times sub-culturing in the absence of the antibiotic. This was similar to other fungi and yeasts (Bernardi-Wenzel et al., 2016; Leal et al., 2004; Mora-Lugo et al., 2014). M. furfur transformants showed consistent high fluorescent signals using the act promoter of A. bisporus. Signals were lower in the case of M. pachydermatis but still sufficient for detection. These results and those obtained with the hpt gene show that regulatory sequences from A. bisporus are active in Malassezia.

5. Conclusions

In this study, a highly efficient Agrobacterium-mediated transformation system is described for M. furfur and M. pachydermatis. The efficiency would even enable a marker free transformation. GFP was shown to be expressed in Malassezia enabling localization and expression studies aimed to understand the life style of these fungi.

Conflicts of interest

No conflict of interest declared.
