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

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ABSTRACT

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*.

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/seborrhoeic 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; Prohic 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; Chryssanthou 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} Oxbile [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} Oxbile [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 μL *Malassezia* suspension (10^{-6} 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 *hpt* 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* [Entelechon GmbH] was amplified using primers 9 & 10, digested with *PacI*/*AscI* and inserted in *PacI*/*AscI* 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 *A. bisporus* and *C. neoformans* (Chen et al., 2000; McClelland et al., 2005). Briefly, *A. tumefaciens* strain AGL-1 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 (Hooykaas et al., 1979) supplemented with 50 μg mL^{-1} kanamycin and grown overnight on a rotatory shaker at 28 °C and 250 rpm to OD$_{600}$ 0.6–0.8. Cells were collected by centrifugation for 15 min at 1248g and resuspended in induction medium containing 200 μM acetosyringone (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 2432g, washed twice in milliQ H$_2$O with Tween 80 (0.1%), and suspended in induction medium at a density of 10^7 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} cefatoxin [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).

Table 1
Primers used in this study.

| Primer	Primer name	Sequence 5′–3′
1	Actin prmtr F	AAGCTTAGCCGAGAGAAGATGCCCC
2	Actin prmtr R	CCATGGTTTGTTATTCGTGTGTTCG
3	Actin trmntr F	GGATCCGCTGATGGTGCTTTATGATAAATAAAGTCCTTGGG
4	Actin trmntr R	GAATTCTACTACTACCCCCAAAACCGACATCATCC
5	Act-Pr_F	CCAGGGGGATCGTTAAAGCTTAGCCGAGAGAAG
6	pBHg_ActP_R	AATTAAGAATTCAGATCTCAATTGGGCGGCCCTTTGTTATTCGTGTGTTCG
7	pBHg_ActT_F	TCTGAATTCTTAATTAAGGATCCGCTGATGGTGCTTTATG
8	Act-Ter_R	CGCCGAATTGGCGCGGAATTCTACTACTACCCCC
9	GFP-Fw	CATGATGGCGCGCCATGACCATGATTACGCCAAGC
10	GFP-Rv	CATGATTTAATTAAGGATCCTTACTTGTACAGCTCG
11	Hy-Fw	GACAGGTCGAGGCGGGAAGCTTTAAGAGGTCCGCAAG
12	Hy-Rv	CGTACGCAAAGATGGTCGGGGGATCTGGATTTTAG
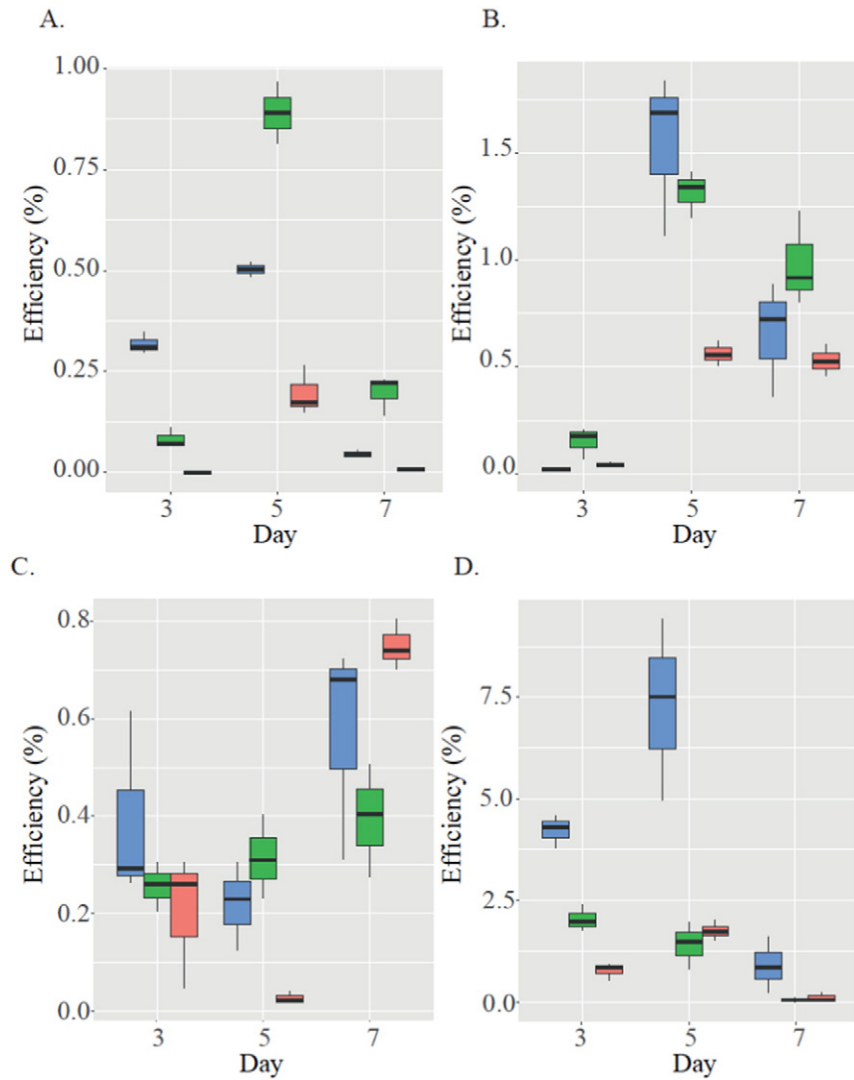
Fig. 1. Transformation efficiency of *M. furfur* (A, B) and *M. pachydermatis* (C, D) transformed with pBHg (A, C) and pBHg-GFP-ActPT (B, D) at 19 °C (blue boxes), 24 °C (green boxes), and 28 °C (red boxes) after 3, 5, and 7 days of co-cultivation with *A. tumefaciens*.

3. Results

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

A. 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
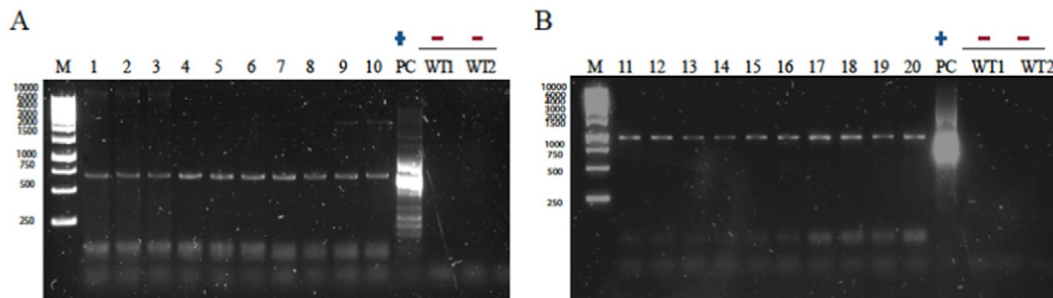

Fig. 2. Gel electrophoresis of *gfp* (A) and *hpt* (B) fragments amplified by PCR from *M. furfur* (lanes 1–10) and *M. pachydermatis* transformants (lanes 11–20). Wild-type *M. furfur* (WT1) and *M. pachydermatis* (WT2) were used as negative controls; vector pBHg and pBH-GFP-ActPT were used as positive control (PC); with M representing the molecular size marker (M).
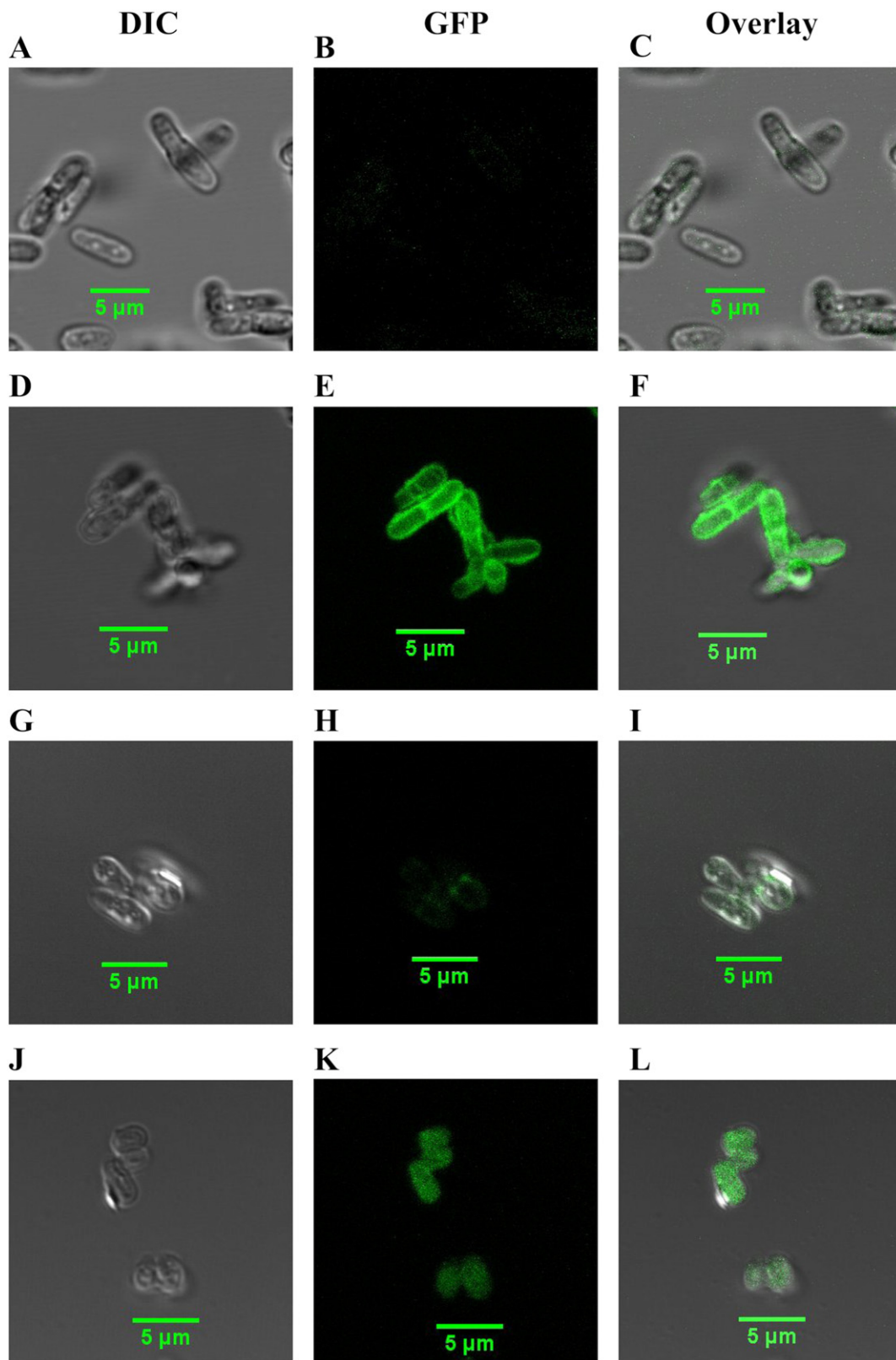
Fig. 3. Fluorescence microscopy analysis of *M. furfur* (A–F) and *M. pachydermatis* (G–L). Wild-type strains show low fluorescence (A–C, G–I) when compared to strains transformed with the *gfp* expression vector pBHg-GFP-ActPT (D–F, J–L).

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.

4. Discussion

M. furfur and *M. sympodialis* were recently transformed using *A. tumefaciens* (Ianiri 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 (Ianiri 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 (Ianiri 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 10^8 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 related 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* (Ianiri 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.

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