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Agrobacterium tumefaciens-mediated transformation of *Guignardia citricarpa*

J.G. Figueiredo [a,1], E.H. Goulin [a,2], F. Tanaka [a,2], D. Stringari [a,3], V. Kava-Cordeiro [a,3], L.V. Galli-Terasawa [a,4], C.C. Staats [b,5], A. Schrank [b,6], C. Glienke [a,*]

[a] UFPR, Department of Genetics, Curitiba, PR, Brazil
[b] UFRGS, Biotechnology Center, Porto Alegre, RS, Brazil

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ABSTRACT

Guignardia citricarpa, the causal agent of Citrus Black Spot, was successfully transformed via *Agrobacterium tumefaciens* with cassettes for gfp and bar expression. Transformation is essential to understand the role of genes during interaction between plants and its pathogens. Using a binary plasmid vector based in the pPZP201BK, both germinated conidia and physically fragmented hyphae of *G. citricarpa* were transformed. Eight independent transformants of *G. citricarpa* resistant to ammonium glifosinate displayed GFP fluorescence. The majority (93.75%) of the *G. citricarpa* transformants was mitotically stable and contained a single T-DNA copy ectopically integrated to the chromosome. This is the first report of *G. citricarpa* transformation and will allow future work on virulence determinants of the fungus and possibly its control.

1. Introduction

Guignardia citricarpa (anamorph: *Phyllosticta citricarpa*) is the causal agent of Citrus Black Spot (CBS), an A1 quarantine disease for countries of the European Union and for the USA (Wulandari et al., 2009). It affects practically all citrus species of economic importance, especially sweet oranges (*Citrus sinensis*). Currently, CBS is reported in Africa, Asia, Oceania and South America. In some of these countries, CBS is one of the most important fungal diseases. Two morphologically very similar *Guignardia* spp. are associated to citrus plants: *G. citricarpa* (anamorph: *Phyllosticta citricarpa*) and *G. mangiferae* (anamorph: *P. capitalensis*) (Baayen et al., 2002; Glienke-Blanco et al., 2002; Stringari et al., 2009). However, only *G. citricarpa* causes CBS (Baldassari et al., 2008) in spite of the fact that both *G. citricarpa* and *G. mangiferae* can be isolated from the leaves of citrus plants (Baldassari et al., 2008). Baldassari et al. (2008) showed that *G. mangiferae* can also be isolated from citrus fruit affected by CBS. Nevertheless, these isolates are opportunists and they are considered negative for pathogenicity (Baldassari et al., 2008).

In spite of the importance of the disease and the long time that CBS has been associated with *G. citricarpa*, there is still considerable lack of information about this pathosystem. The lack of an efficient transformation system for *G. citricarpa* is detrimental to the progress of the characterization of genes involved in the pathogenicity of this fungus.

Currently, transformation methods for filamentous fungi involve isolation of protoplasts and introduction of an exogenous DNA through electroporation, fusion by polyethylene glycol or calcium chloride treatment followed by heat shock. Intact cells can be transformed using biolistics (Armaleo et al., 1990). A strategy initially developed for plants uses *Agrobacterium tumefaciens* to transform filamentous fungi (Groot et al., 1998; Covert et al., 2001). *A. tumefaciens*-mediated transformation (ATMT) led to the further expansion of the range of fungal species that could be transformed (Michielse et al., 2005).

* Corresponding author. Universidade Federal do Paraná, Setor de Ciências Biológicas, Departamento de Genética, P.O. Box 19071-81531-990, Curitiba, PR, Brazil. Tel.: +55 41 33611562; fax: +55 41 33611793.
E-mail addresses: jo.gomesfigueiredo@gmail.com (J.G. Figueiredo), eduardo_goulin@yahoo.com.br (E.H. Goulin), fertanaka02@yahoo.com.br (F. Tanaka), danystringari@gmail.com (D. Stringari), vankava@ufpr.br (V. Kava-Cordeiro), lterasawa@ufpr.br (L.V. Galli-Terasawa), staats@cbiot.ufrgs.br (C.C. Staats), aschrank@cbiot.ufrgs.br (A. Schrank), cglienke@ufpr.br (C. Glienke).
[1] Universidade Federal do Paraná, Setor de Ciências Biológicas, Departamento de Genética, P.O. Box 19071, 81531-990 Curitiba, PR, Brazil. Tel.: +55 41 3611731; fax: +55 41 33611793.
[2] Universidade Federal do Paraná, Setor de Ciências Biológicas, Departamento de Genética, Centro Politécnico, s/ n°, P.O. Box 19071, 81531-990 Curitiba, PR, Brazil. Tel.: +55 41 3611731; fax: +55 41 33611793.
[3] Universidade Federal do Paraná, Setor de Ciências Biológicas, Departamento de Genética, Centro Politécnico, s/ n°, P.O. Box 19071, 81531-990 Curitiba, PR, Brazil. Tel.: +55 41 3611557; fax: +55 41 33611793.
[4] Universidade Federal do Paraná, Setor de Ciências Biológicas, Departamento de Genética, Centro Politécnico, s/ n°, P.O. Box 19071, 81531-990 Curitiba, PR, Brazil. Tel.: +55 41 3611772; fax: +55 41 33611793.
[5] Universidade Federal do Rio Grande do Sul, Centro de Biotecnologia, Laboratório de Biologia Molecular de Fungos Filamentosos, Avenida Bento Gonçalves, 9500, Bloco IV, Prédio 43421, Laboratório 205 Agronomia 91509-900, Porto Alegre, RS, Brazil, Caixa-Postal: 15005. Tel.: +55 51 33167767; fax: +55 51 33167309.
[6] Universidade Federal do Rio Grande do Sul, Departamento de Biologia Molecular e Biotecnologia, Centro de Biotecnologia, Campus do Vale UFRGS, Av. Bento Gonçalves, 9500 Agronomia, 91501970 Porto Alegre, RS, Brazil. Tel.: +55 51 33166071; fax: +55 51 33167309.

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The development of a reliable and efficient transformation system for *G. citricarpa* is important for the elucidation of gene function related to pathogenicity and virulence, in turn opening up the possibility of targeted molecular improvements for the purpose of biological control.

In the present study we report for the first time an efficient protocol for the ATMT of *G citricarpa*. This transformation approach represents a useful tool for genomic studies in *G. citricarpa*.

2. Methods

2.1. Strains and growth conditions

The *G. citricarpa* LGMF06 wild-type strain was isolated from CBS lesions in São Paulo, Brazil, and tested for pathogenicity according to Baldassari et al., 2008. Strains were stored in Citrus Medium Fabris–Nishimura (CFN) (28 $g l^{-1}$ of citrus leaves, 20 $g l^{-1}$ glucose, 15 $g l^{-1}$ agar, adjusted to pH 5.8 with HCl) and are currently part of the biological bank of the Laboratory of Microorganisms (LabGeM) at the Federal University of Paraná, Curitiba, Paraná, Brazil. *A. tumefaciens* strain EHA 105 harboring the appropriate binary vectors (Fig. 1) *A. tumefaciens* EHA105 strain was a kind gift from G. Pasquali (Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul) and was grown at 28 °C and maintained in Luria–Bertani medium (10 $g l^{-1}$ tryptone, 5 $g l^{-1}$ yeast extract, 5 $g l^{-1}$ NaCl).

2.2. A. tumefaciens-mediated transformation

A. tumefaciens- mediated transformation of *G. citricarpa* LGMF06 was carried out as previously described (Covert et al., 2001; Reis et al., 2004; Staats et al., 2007) with modifications. *A. tumefaciens* strain EHA-105 harboring the appropriate binary vectors (Fig. 1) was grown at 28 °C for 24 h at 180 rpm in liquid LB medium supplemented with 50 μg kanamycin ml^{-1}. The culture was diluted to OD660 of 0.15 in 20 ml of IM medium amended with 200 μmol l^{-1} acetosyringone (AS). Cells were grown under the same conditions to reach OD660 of 0.6. *G. citricarpa* (LGMF06) was grown on CFN medium, pH 5.8 at 28 °C for 7–10 days; conidia and mycelium were harvested from the growing colonies and placed in tubes containing 2 ml of saline solution. After homogenization, the solution was incubated at 40 °C for 5 min. Co-cultivation between *A. tumefaciens* and conidia and mycelia of *G. citricarpa* was performed by adding 100 μl of bacterial culture to 100 μl of fungal conidia suspension (1×10^6 conidia ml^{-1}). The mix was plated onto membranes of cellulose (90-mm diameter) on a co-cultivation medium (IM) (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.7 mM $CaCl_2$, 9 mM $FeSO_4$, 4 mM NH_4SO_4, 10 mM glucose, 40 mM 2-[N-morpholino] ethanesulfonic acid, pH 5.3, 0.5% glycerol (w/v),) with or without 200 μmol l^{-1} AS. Following co-cultivation at 28 °C for 48 h, 5 ml of molten CM agar (same as IM, except containing 5 mM instead of 10 mM glucose) and Citrus FN supplemented with 100 μg ammonium glufosinate ml^{-1} and 200 μg cefatoxim ml^{-1} were poured over the plates as an overlay. Molten CM agar was used to select fungal transformants, and CFN was supplemented with 100 μg ammonium glufosinate ml^{-1} and 200 μg cefatoxim ml^{-1} to inhibit A. tumefaciens growth. Each putative transformant was transferred to CM or CFN agar along with 100 μg ammonium glufosinate ml^{-1} and incubated as described.

2.3. Transgene stability in the G. citricarpa

All transgenic isolates from agro-transformed *G. citricarpa* were propagated on M-100 (10 $g l^{-1}$ glucose, 3 $g l^{-1}$ KNO_3, 62,5 ml^{-1} M-100 Salt Solution, 1,5% agar) or CFN medium in absence of ammonium glufosinate. Plates were incubated at 28 °C for 7 days. This procedure was repeated three times. Fungal colonies were then transferred to selective media and their growth rate evaluated.

2.4. Expression analysis of reporter GFP

The green fluorescence emission associated with GFP was detected using a Leica UV microscope (DMKLB or MZFL111) with the following filter settings: 488 nm excitation and 515 nm emission. Images were recorded and processed using Picasa 3.1.0 software.

2.5. PCR assays

For the detection of the transgenes in *G. citricarpa* by PCR, total DNA was extracted from mycelium grown on CFN medium for three days at 28 °C. The mycelium was harvested, lyophilized for 24 h and ground with mortar and pestle under liquid nitrogen. Genomic DNA was obtained according to the methods described by Raeder and Broda (1985), and modified by Glienke-Blanco et al. (2002). The reaction of 49 putative transformants was performed on a thermocycler (Mastercycler, Eppendorf) using primers barR (TCAGATCTCGACGGG) and barF (ATGAGCGAACGACGC). The reaction was carried out using 50 ng of *G. citricarpa* total DNA, 3 mM $MgCl_2$, 4 μM of each primer, 0.2 mM of each dNTP (Invitrogen®), 1 × PCR buffer, and 1.5 units of Taq polymerase (Invitrogen®). PCR conditions were as follows: 94 °C, 30 s; 60 °C, 30 s and 72 °C, 45 s, for 30 cycles. PCR products were resolved by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light

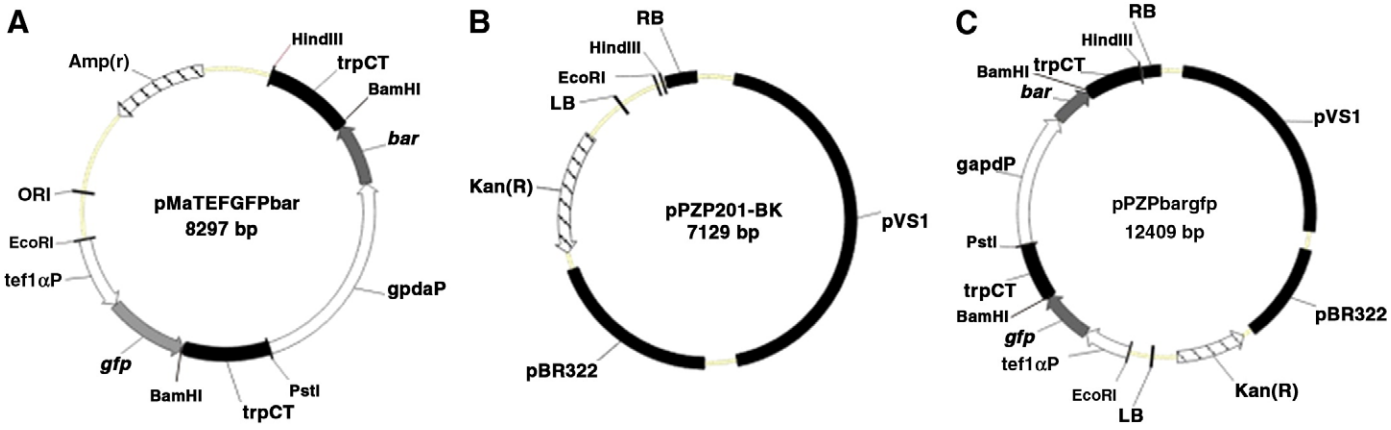

Fig. 1. Construction of pPZPbargfp. The gfp and bar expression cassette was obtained by subcloning an EcoRI–HindIII restriction fragment from pMaTEFGFPbar (A — Nakazato et al., 2006) into the binary vector pPZP201-BK (B — Covert et al., 2001). The pPZPbargfp (C) plasmid T-DNA contains an ammonium glufosinate resistence gene (bar) expression cassette (*Aspergillus nidulans* gpdA promoter and trpC terminator) and a gfp expression cassette (*Metarhizium anisopliae* tef promoter and *A. nidulans* trpC terminator between its left and right borders.

Table 1
Agrobacterium tumefaciens-mediated transformation of *G. citricarpa* LGMF06 to ammonium glufosinate resistance, mitotic stability and PCR analysis.

	G. citricarpa	LGMF06
Number of transformants*	M-100	CFN
+AS	14	16
−AS	00	00
Percentage of mitotically stable transformants	42.85	93.75
PCR analysis with amplification of gene bar	6	15
Percentage of putative tranformants confirmed by PCR	85.7	93.75

*AS: acetosyringone. (+AS) AS present medium; (−AS) AS-absent medium.

(Ultraviolet Benchtop transilluminators) and photographed (Digi doc it software).

2.6. Southern blot analysis

Purified genomic DNA (10 μg) of individual transformants and wild-type strain were digested with BamHI, EcoRI or PstI, resolved on 1% agarose gel and capillary blotted onto Amersham Hybond-N+ membrane (Buckinghamshire, UK) using standard techniques (Sambrook and Russel, 2001). Probe labeling, hybridization, stringency washes and detection were carried out under conditions recommended by Amersham Genes Images Alkphos Direct Labeling CPD Star Detection kit (GE Healthcare, USA). Using a 600 bp DNA probe obtained of PCR product with specific primers barR and barF.

3. Results

In order to determine the minimum inhibitory concentration *G. citricarpa* mycelium and conidia were grown on PDA at concentrations 50, 75, 100, 150 and 200 μg ammonium glufosinate ml^{-1}. The concentration of 100 μg ammonium glufosinate ml^{-1} was found to completely suppress growth (data not shown) and was used to select the transformants.

A. tumefaciens harboring the pPZPbargfp binary plasmid efficiently transferred T-DNA to cells as shown by active fungal cell growth on selective culture medium. Ammonium glufosinate-resistant mycelia cells were obtained only when the bacterial culture was induced with acetosyringone (Table 1). The confirmation of transformation was done by PCR, in which an amplicon of 550 bp, representing the bar gene sequence, was obtained (Fig. 2, panel A) using as template DNA extracted from seven day old cultures of the transformants and wt strain. All *G. citricarpa* LGMF06 transformants recovered from CFN medium and 85.7% transformants from M-100 medium yielded the predicted amplicon (Table 1). As shown by control assays, the wild-type strain failed to amplify the bar gene.

Genetic stability of transgenic *G. citricarpa* was evaluated by propagating fungi without selective pressure for 3 months, and then grown in the presence of ammonium glufosinate (100 μg ml^{-1}). Detection of the transgene in previously transformed cells failed in only one out of sixteen tested fungal colonies on CFN medium (Table 1) indicating a high mitotic stability. However, the most important factor observed in CFN medium was the transformant's growth rate. In M-100 medium, colonies had reached a size of approximately 1 cm in diameter after 21 days, while those grown in CFN medium reached the same size in approximately 5–7 days (data not shown). The high growth rate exhibited by transformants in CFN medium made it possible to obtain a more efficient mycelia production for both total DNA extraction and maintenance.

These transgenic isolates were then tested for the presence of the T-DNA insertion by Southern blot analysis. Genomic DNA was digested with BamHI, EcoRI or PstI restriction enzymes (Fig. 2B, C, D) and blots were probed with a fragment of the 600 bp bar gene generated by PCR of the pPZPbargfp binary plasmid. As shown in Fig. 2, panels B, C and D,

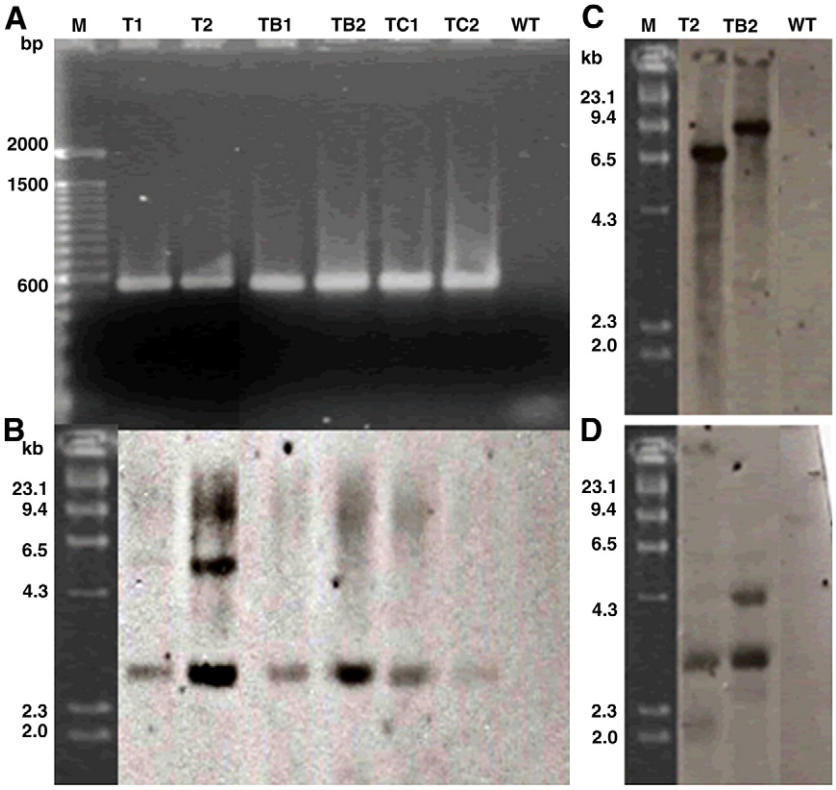

Fig. 2. Detection of *G. citricarpa* transformants. Analysis of wild-type strain LGMF06 (wt) and *G. citricarpa* transformants obtained by ATMT. (A) bar gene amplicon of wt and 6 independent transformants. (B, C, D) Southern blot analysis performed on the wt and 8 independent transformants. Genomic DNA was digested with BamHI (B), EcoR1(C) or Pst1 (D) and hybridized with bar gene probe. (B) Lanes 2–8 (T1, T2, TB1, TB2, TC1 and TC2): Putative transformants obtained on M-100 medium. (C and D) T2 and TB2 transformants obtained on M-100 medium. M, molecular size markers: 100 bp DNA Ladder (A) or HindIII digest of lambda DNA (B, C and D) (Invitrogen Life Technologies, Carlsbad, CA, USA) is indicated on the left.

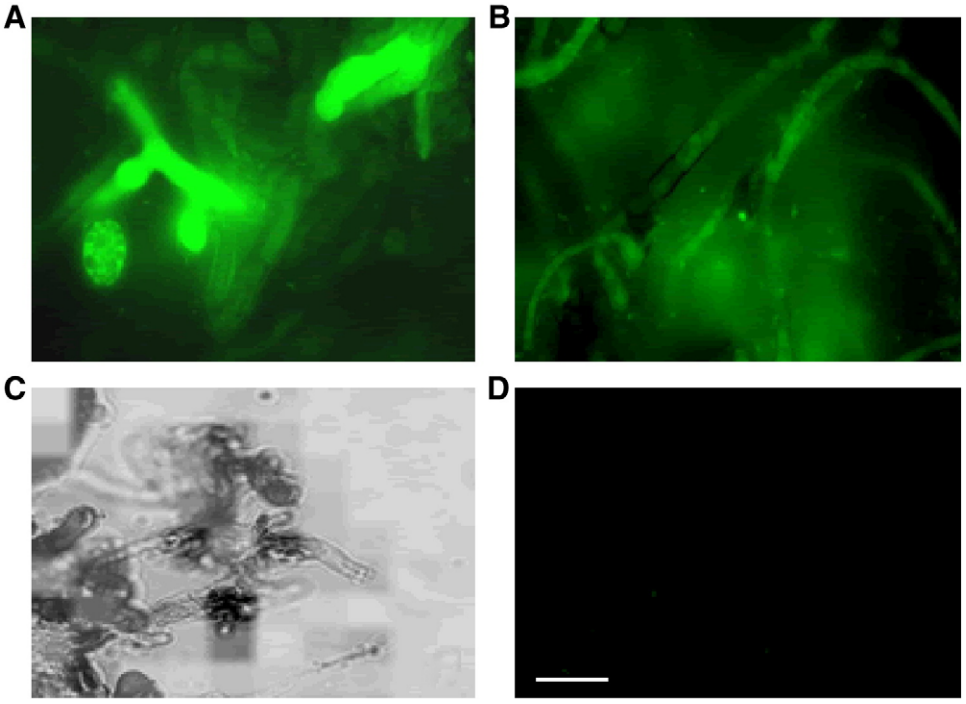

Fig. 3. GFP fluorescence from *G. citricarpa* transformants. Fluorescence of *G. citricarpa* LGMF06 transformant obtained after ATMT with pPZPbargfp (A, B), showing spores and mycelia under green light excitation. Bright-field and fluorescence microscopic images of wild-type *G. citricarpa* mycelia (C, D). Scale bar — 10 μm.

bands of different sizes were detected in independent transformants, indicating random insertion of the T-DNA into the fungal genome. We observed a complex integration pattern of T-DNA in some transformants. Based on hybridization signals of T2 DNA digested with BamHI (Fig. 2B), at least two copies of the T-DNA are integrated in the genome of the transformant. One of the copies possibly harbors a truncated transformant DNA that eliminates the BamHI site in the T-DNA that renders a higher size hybridization signal than the fragment flanked by the BamHI sites within the T-DNA. A multiple integration pattern is corroborated by a more intense hybridization signal in the blots from T2 DNA digested with EcoRI and PstI (Fig. 2C and D, respectively).

Transgenic fungi obtained by Agro-transformation were also analyzed to determine GFP expression and its accumulation in mycelia (Fig. 3). Wild type and transformed mycelium were examined using Epifluorescence microscopy. GFP-associated green fluorescence was present in mycelia and conidia (Fig. 3, panel A and B). No green fluorescence was detected in wild-type *G. citricarpa* (Fig. 3, panel D).

4. Discussion

ATMT has been successfully applied to a variety of different fungal species and systems during the last decade due to its technical simplicity and efficiency (Michielse et al., 2005). The main objective of this work was to establish an efficient system for *A. tumefaciens*-mediated transformation (ATMT) for *G. citricarpa*. An efficient transformation system is an essential tool for gene manipulation and to study the functional genomics of *G. citricarpa*, which is an important pathogen of citrus worldwide. This is the first report of *G. citricarpa* DNA-transformation. An optimized Agrobacterium-mediated transformation protocol for *G. citricarpa* was developed after some modifications. First, conidia of *G. citricarpa* LGMF06 strain were submitted to 40 °C for 5 min before co-cultivation. Such procedure increased the germination rate facilitating the interaction between the bacteria and fungal germlings. Second, the culture medium pH was changed to 5.8, since this is the optimum pH for *G. citricarpa* growth. And third, after co-cultivation between *G. citricarpa* and *A. tumefaciens*, selective medium was added to the membranes instead of transferring them to the selective medium. These changes significantly contributed for the success of agro-transformation.

The transformant mycelia cells were obtained only when the bacterial culture was induced with acetosyringone — AS (Table 1). This is consistent with earlier reports that show the essential role of AS for fungal transformation (Michielse et al, 2008; Wang and Li, 2008; Yang et al, 2007; Reis et al., 2004; Mullins and Kang, 2001; Chen et al., 2002; Groot et al., 1998). Given the requirement of AS for *A. tumefaciens* to transform both plants and fungi, it was speculated that A. tumefaciens uses a similar mechanism to transform in both systems (Groot et al., 1998).

Despite frequency of transformation to be low (14–16 transformants/10^6 spores) the transformation system mediated by *A. tumefaciens* may prove to be a powerful tool to achieve filamentous fungi transformation and functional genomics studies, due to the simplicity of T-DNA integration and genetic stability of the transformants. Considering the efficiency of the ATMT protocol described, this approach would represent a useful way to study genetics in *G. citricarpa* regarding gene knock-out and analysis of genes with known functions (Duarte et al, 2007; Massart and Jijakli, 2007).

Mitotic stability analysis showed that the transformants tested remained mitotically stable, maintaining their resistance conferred by the bar gene after being cultured in the absence of ammonium glufosinate. This mitotic stability is consistent with T-DNA integration into chromosomal DNA and compares favorably with those obtained for several filamentous fungi using AMT (Covert et al., 2001; Rho et al., 2001; Fitzgerald et al., 2003; Meyer et al., 2003; Reis et al., 2004). In addition, the transformants were able to express GFP fluorescence as a result of stable mRNA synthesis and translation.

Therefore we show here the feasibility of ATMT transformation protocol to the important citrus pathogen *G. citricarpa* that opens the possibility of gene function studies to determine pathogenicity determinants.

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