A survey on optimization of *Agrobacterium*-mediated genetic transformation of the fungus *Colletotrichum gloeosporioides*

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Abstract

The fungus *Colletotrichum gloeosporioides* is the causative agent of anthracnose disease of many tropical, subtropical and temperate fruits, and a microbial source of the anticancer drug, Taxol. Here, we introduce an optimized *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocol for genetic manipulation of this fungus using *hph* and *gfp*-tagged *hph* genes as selection markers. Results showed that falcate spores can be easily used instead of protoplasts for transformation. Several experimental parameters were shown to affect transformation efficiencies, among which the length of co-cultivation, the ratio of fungal conidia to bacterium during co-cultivation, the kind of membrane during co-cultivation, and the kind of fungal growth medium during transformants selection, showed the highest influences on ATMT frequencies. Results indicated that the optimal ATMT of *C. gloeosporioides* was achived after 3 days of co-cultivation, at 10^7 per ml fungal conidia, via the use of Fabriano 808 filter paper and Czapek's culture medium. Successive subculturing of transformants on selective and non-selective media demonstrated the stable expression of transgens, and subsequent PCR based analyses of transformants revealed the presence (100%) of transferred genes. Flourescence microscopy analyses showed a punctuate pattern for localization of an expressed Gfp-tagged Hph protein inside fungal hyphae. The optimized ATMT protocol generated mutants that showed different phenotypes based on their vegetation and pigmentation. This suggests the possible applicability of this technique for functional genetics studies in *C. gloeosporioides*, through insertional mutagenesis.

Keywords**:** *Colletotrichum gloeosporioides; Agrobacterium tumefaciens;* ATMT*;* Genetic transformation*;* Insertional mutagenesis

Introduction

Colletotrichum is one of the most common and important genera of filamentous fungi, that cause post-harvest rots, anthracnose spots, and blights of aerial plant parts. Members of this genus cause major economic losses, especially in fruits, vegetables, and ornamentals (Damm et al., 2010). The plant pathogenic fungus *Colletotrichum gloeosporioides* (Penz) Penz & Sacc in Penz,, is the causal agent of anthracnose on many tropical, subtropical and temperate fruits (Waller, 1992; Freeman and Shabi, 1996), especially in *Citrus* species, including oranges, tangerines, navel oranges, and grapefruits. Post-harvest problems caused by *C. gloeosporioides* are particularly prevalent in the tropics, where they are often a significant factor in limiting export (Fitzell and Peak, 1984). The economic cost of cryptic infections caused by *C. gloeosporioides* is about 25% greater than that reported for field losses (Jeger and Plumbley, 1988). Accordingly *C. gloeosporioides* has been grouped among the most important post-harvest pathogens.

In addition to its considerable detrimental economic importance, recently it has been shown that endophytic *C. gloeosporioides*, apparently nonpathogenic, is a source for production of secondary metabolites, with anticancer property (Nithya, and Muthumary, 2009). Currently, discovery and strain improvement of secondary metabolite producing fungi for industrial fermentation have gained significant interest worldwide (Zhou et al., 2010). Hence, there will be a new potential for Taxol production using improved strains of *C. gloeosporioides* in future.

Further, the *Colletotrichum* fungi are highly significant as experimental models for study of many aspects of fungal biology like development, infection process, host resistance, signal transduction, and the molecular biology of plant-pathogen interactions (The *Colletotrichum* genome database). However, very little information is

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available on the molecular mechanisms regulating varied pathogenicity life styles and secondary metabolite productions in these fungi and the basic tools required are only beginning to be developed by various groups.

Currently, *Agrobacterium tumefaciens*-mediated transformation (ATMT) is a powerful method for large-scale random mutagenesis, and efficiently targeted gene disruption in some fungi, based on the transfer of the T-DNA into the recipient fungal genome (Soltani et al., 2008; Soltani et al., 2009). This technique has been shown to be applicable to many filamentous fungi (Michielse et al., 2005; Soltani et al., 2008). From the first published paper on ATMT of filamentous fungi including *C. gloeosporioides* (de Groot et al., 1998), ATMT has been established as a genetic analysis tool for several other *Colletotrichum* species, i.e. *C. lagenarium* (Tsuji et al., 2003), *C. trifolii*.(Takahara et al., 2004), *C. graminicola*. (Flowers and Vaillancourt, 2005), *C. acutatum* (Talhinhas et al., 2008), *C. higginsianum* (Ushimaru et al., 2010) and *C. sansevieriae* (Nakamura et al., 2012). However, various parameters which might influence ATMT frequency of *C. gloeosporioides* have not been explored yet. A reliable insertional mutagenesis system for *C. gloeosporioides* is highly important for discovering genes involved in the pathogenesis or genes involved in the production of the anticancer compound Taxol by this species. Here, using both *hph* and *gfp*-tagged *hph* selection markers, we aimed at optimizing ATMT protocol for the efficient transformation of *C. gloeosporioides*. We further showed that this optimized ATMT resulted in producing mutants showing different phenotypic characteristics.

Materials and Methods

Fungal and bacterial strains and growth media

Colletotrichum gloeosporioides wildtype strain JSN-1389, which was isolated as a plant pathogen from Citrus species in Iran, was used as the model. Fungus strain was maintained on potato dextrose agar (PDA) medium (Merck, Darmstadt, Germany) at 28ºC. *Escherichia coli* strain XL1-blue (Stratagene) was used as a host for gene manipulations and *Agrobacterium tumefaciens* strain LBA1100 (Bundock et al., 1995) as a T-DNA donor for fungal transformation. The binary vectors pTAS10 (de Groot et al., 1998) and pBin-GFP-hph (O'Connell et al., 2004) were transferred to this strain to yield *A. tumefaciens* pSDM2312 (de Groot et al., 1998) and pBSY90 strains (this study), respectively. The *Agrobacteria* and *E.coli* strains were maintained on Luria–Bertani (LB) media (Sambrook et al., 1989) at 28ºC and 37ºC, respectively.

Fungal resistance to Hygromycin B

C. gloeosporioides JS-1389 was grown on Czapek's medium at 0, 50, 100, 150, 200, 250, 300 µg/ml hygromycin B (Sigma-Aldrich). The zone of hyphae growth of the wildtype fungus was checked daily until the colony covered the whole petri plate.

Fungal transformation

C. gloeosporioides JS-1389 was transformed using the ATMT protocol according to the method described previously (de Groot et al., 1998) as follow, with minor modifications to explore optimal conditions. Fresh *A. tumefaciens* carrying a binary vector was grown on LB medium containing 50 µg/ml kanamycin, at 28ºC overnight. The day after, it was transferred to the induction medium (IM; Bundock et al., 1995) containing 200 µM acetosyringone (AS) (Sigma–Aldrich) and grown for 6 hours. *C. gloeosporioides* JS-1389 was grown on PDA medium for 20 to 30 days to obtain a high number of conidia. 60 µl of agrobacterial suspension (OD_{620}=0.5) was mixed with 60 µl of fungal conidia (both 10^6 and 10^7 per mL). A 100 µl aliquot of the mixture was spread over Fabriano 808 or Whatman 41 (Roche Chemicals, Mannheim, Germany) filter papers on IM containing 200 µM acetosyringone. After incubation at 22ºC for 2 to 3 days, the filter papers were transferred onto PDA (for hygromycin B resistance selection) or Czapek's (for GFP-hygromycin B expression selection) selection medium containing 200 µg/ml cefotaxime (Duchefa, Netherlands) to kill the agrobacterial cells, and 100 µg/ml hygromycin B (Sigma-Aldrich) to select for fungal transformants. Stability of hygromycin resistance of transformants was tested by subculturing them five times on Czapek's media containing 100 µg/ml hygromycin B. Then, transformants were maintained on PDA. *C. gloeosporioides* JS-1389 conidial suspention, not co-cultured with *A. tumefaciens* cells but handled as described above, served as negative control. Genetic transformations of hygromycin-resistant fungal colonies were confirmed by genomic DNA analysis using PCR and fluorescence microscopy for the Gfp-tagged Hph.

Isolation of Genomic DNA

To extract DNA for Polymerase chain reaction (PCR) assays, transformants were grown on PD broth medium at room temperature for 10-15 days. A 2-5 mg mycelia of each fungal transformant was

filtered through sterile filter paper, frozen in liquid nitrogen, and grounded to a fine powder. Then DNA was extracted by the CTAB method (Zhang et al., 1996). Primers *hph*-F (5′-GCTGCGCCGATGGTTTCTACA-3′) and *hph*-R (5′-GCGCGTCTGCTGCTCCAT-3′) (Flowers,and Vaillancourt, 2005) were used to amplify a 544 bp *hph* fragment. PCR was performed with 5 µL template DNA, 1 µM each primer and Taq PCR Mix (Cinnagene) in a final volume of 25 µL. Thermocycler was programmed for one cycle of 5 min at 94 ºC, 30 cycles of 1 min at 94ºC, 1 min at 58ºC, 2 min at 72ºC, and a final cycle of 10 min at 72ºC.

Microscopy for *gfp*-tagged Hph Expression

GFP expression in the *C. gloeosporioides* transformants obtained with *A. tumefaciens* pBSY90 carrying pBin-*GFP-hph* binary vector was assessed by fluorescence microscopy. Actively growing hyphae from hygromycin resistant cultures, grown on Czapek's medium, were observed under ultraviolet light (excitation at 395–475 nm) on a Fluorescence Microscope (Bel Engineering, Italy) at 40× magnification. Wildtype isolate JS-1389 was used as the control.

Results

Hygromycin B sensitivity of *C. gloeosporioides*

In the only reported ATMT of *C. gloeosporioides*, selection of hygromycin resistant fungal transformants was performed on PDA medium as described by de Groote et al., (1998). In our experiments, addition of hygromycin B to PDA selection media resulted in variable observations. Hence, the Czapek's medium was used alternatively. The activity of hygromycin B in Czapek's medium was consistent and reliable. Consequently, inhibition of vegetation of *C. gloeosporioides* JS-1389 was assessed by growing the fungi on Czapeck's medium supplemented with hygromycin B in different concentrations, i.e. 0, 50, 100, 150, and 200 µg/ml. Growth was totally inhibited on Czapeck's medium containing 100 µg hygromycin/ml. Therefore, that concentration was considered for the selection of resistant colonies in our ATMT experiments.

Effects of experimental parameters on transformation efficiency

Transformation efficiencies were compared in experiments, in which acetosyringone (AS) was omitted from the liquid IM and the IM co-cultivation media. In agreement with most previous studies (Gouka et al., 1999; Malonek and Meinhardt 2001), inclusion of AS in the IM media was essential for the transformation of *C. graminicola*, since in the absence of AS during co-cultivation, no transformants were formed (data not shown). Co-cultivation of *C. gloeosporioides* JS-1389 conidia with *A. tumefaciens* in the presence of AS led to the formation of hygromycin-resistant fungal colonies. The transformation frequency was in the range of 70 to 120 transformants per 60 µL of 10^6 to 10^7 conidia. The average numbers of hygromycin-resistant transformants in two experiments under different conditions are shown in Table 1.

From the number of transformants produced with a given set of parameters in two experimental replications, we could conclude that some parameters had a positive effect on transformation efficiencies. A total number of 10^6 or 10^7 per mL conidia from *C. gloeosporioides* JS-1389 were co-cultivated with *A. tumefaciens* cells. As seen in Table 1, increasing the conidial concentration from 10^6 to 10^7 per mL increased ATMT in general. The previous study on ATMT of *C. gloeosporioides* has shown that 10^6 conidia per mL could result in a variable number of 50 to 130 hygromycin resistant transformants on nitrocellulose filters (de Groot *et al.*, 1998). Our data indicates that ATMT efficiency could be improved (10 to 30%) by using 10^7 conidia per mL. So, a higher number of conidia results in a higher ATMT frequency.

It is also shown that ATMT of *C. gloeosporioides* could be achieved upon 2 days of co-cultivation (de Groot et al., 1998). Here, *C. gloeosporioides* JS-1389 conidia were co-cultivated with *A. tumefaciens* cells for 2 and 3 days. As shown in Table 1, transformation efficiency was increased, 11 to 24%, after a longer (3 days) co-cultivation period. However, on the day 3, because of excessive growth of fungus and bacteria, selection of transformants was not facile.

Another experimental parameter was the choice of co-cultivation membrane. ATMT protocols usually make use of nitrocellulose filters. The only report on ATMT of *C. gloeosporioides* has introduced the efficiency of nitrocellulose filters (de Groot et al., 1998). As it is shown in Table 1, in our experiments the kind of filter paper have a relevant effect on the improvement of transformation efficiency, regardless of other parameters. Here, *C. gloeosporioides* transformants were recovered from both the Fabriano 808 and Whatman 41 membranes. Significantly, co-cultivation of Agrobacterium-*Colletotrichum* on Fabriano 808 membrane increased transformation efficiencies from 2 to 20% (Table 1).

As seen, transformation efficiencies obtained by pSDM2315 versus pBSY90 binary vector, in the same *A. tumefaciens* strain, were not significantly different. This indicates that *A. tumefaciens* LBA110 regardless of containing which plasmid , produces a similar number of transformants (Table 1). So, the binary vectors did not account for the variations we saw in transformation efficiencies.

Transformant stability

An assessment of the mitotic stability of 24 randomly selected transformants showed that they all maintained their hygromycin resistance after being sub-cultured for five generations in the presence and two generations in the absence of hygromycin on Czapeck's medium (data not shown). All 24 transformants grew when transferred onto selection media, and retained *Gfp* expression. These results demonstrated that the ATMT transformants were mitotically stable.

Confirmation of the presence of *hph* gene in genomic DNA of fungal transformants

Twenty-four transformants, which had been proved to be resistant to hygromycin B at 100 µg/ml and to retain their mitotic stability, were selected and designated in MY1 to MY24. Genomic DNA from the 24 transformants were tested for the presence of the *hph* gene by PCR using specific primers *hph*-F and *hph*-R (Fig. 1). The expected 544-bp PCR products were all detected from the 24 transformants (100%). *hph* gene product was not detected with untransformed *C. gloeosporioides* genomic DNA (Fig. 1).

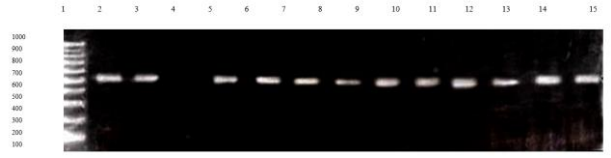

Figure 1. PCR amplification of *hph* selection marker gene (544-bp) in mitotically stable transformants (No.5-14) of *C. gloeosporioides* obtained by *A. tumefaciens* strain pSDM2315 (lanes:5-9), and by *A. tumefaciens* strain pBSY90 (lanes:10-14). Lanes 2 and 3 include positive controls (from binary vectors pTAS10, and pBin-*GFP-hph*). Lane 4 represents negative control. DNA ladder: 1000 bp ladder (Cinnagene). The observed PCR bands accord to 544 bp, as expected.

Fluorescence microscopy

To determine the stable Gfp-tagged Hph expression inside the *C. gloeosporioides* transformants, fluorescence microscopical analyses were performed on actively growing hyphae from Czapek's-hygromycin cultures. Seven out of 24 hygromycin-resistant isolates were randomely selected for fluorescence microscopy. Cells expressing a Gfp-tagged Hph protein revealed a punctuate localization pattern of this protein throughout the cell (Figure 2).

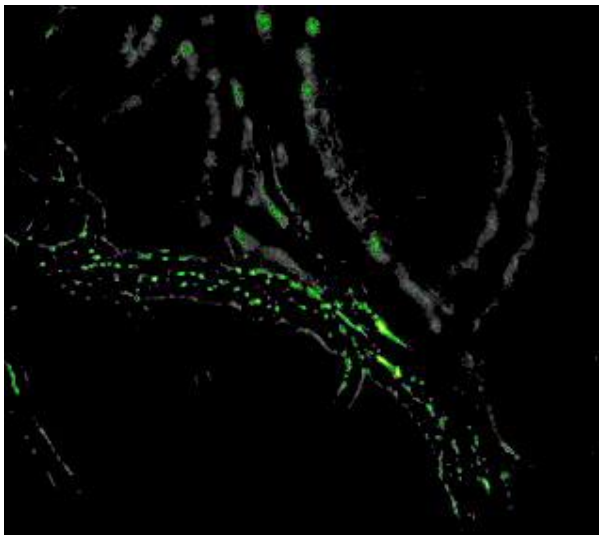

Figure 2. Gfp expression in a representative hygromycin-resistant transformant's hyphae of *C. gloeosporioides*, after ATMT with pBSY90.

Phenotypic characteristics of transformants

Seven hygromycin-resistant mutants of *C. gloeosporioides*, mycelia of which showed fluorescence illumination under microscopy experiments, were phenotypically different than their wildtype isolate *C. gloeosporioides* JSN-1389. It was observed that the rate of growth and the conidiation of transformants were increased in compared to their parental isolate (Figure. 3). Analysis of variance confirmed that vegetation of the transformants significantly differ from their parental isolate at ($P \leq 0.01$, not shown). Moreover, the color and the form of the fungal colonies on PDA plates had been changed (Figure. 3).

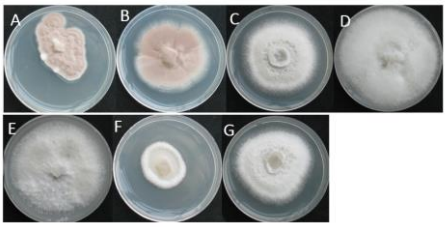

Figure 3. Different range growth and morphology/pigmentation of six *C. gloesporioides* transformants (A-F) as compared to their parental wild type (G) on PDA plates 12 days after incubation at 25ºC

Discussion

Colletotrichum gloeosporioides is of special importance in phytopathology, and more recently in pharmacology for its ability to produce anticancerous metabolites (Nithya and Muthumary, 2009). *Colletotrichum* species have haploid genomes which facilitates molecular genetic approaches, such as gene targeting and insertional mutagenesis. *C. gloeosporioides* genome is not sequenced yet. For functional genetics of this fungus in order to discover the genes involved in the pathogenesis, or the genes involved in the production of the anticancer compound, Taxol, by this species, a reliable insertional mutagenesis system is highly important. Restriction Enzyme-Mediated DNA Integration (REMI) and Polyethylene Glycol (PEG) genetic transformation protocols have several drawbacks for fungal transformation, but *A. tumefaciens*-mediated transformation has several advantages over these methods (Michielse et al., 2005; Soltani et al., 2008) such as stable transformants with a single-copy integrated DNA.

Agrobacterium tumefaciens-mediated transformation of several *Colletotrichum* species has been reported before (de Groot et al., 1998; Tsuji et al., 2003; Takahara et al., 2004; Flowers and Vaillancourt, 2005; Talhinhas et al., 2008; Ushimaru et al., 2010; Nakamura et al., 2012). Here, we aimed at exploring the optimal conditions for *A. tumefaciens*-mediated transformation of *C. gloeosporioides* using *hph* and *gfp* genes as selection markers, as well as initial assessment of possibility of ATMT for insertional mutagenesis of this fungus. Results showed that for ATMT falcate spores can be used instead of protoplasts. Several experimental parameters were shown to affect transformation efficiencies, i.e. the length of co-cultivation, the ratio of fungal conidia to bacterium during co-cultivation, the kind of membrane during co-cultivation and the kind of fungal growth medium during transformant selection showed the highest influences on ATMT frequencies. Our results indicate that the optimal ATMT of *C. gloeosporioides* is achieved after 3 days of co-cultivation, at 10^7 per mL fungal conidia, via the use of Fabriano 808 filter paper and Czapek's culture medium. It was already shown that after 2 days of co-cultivation of *A. tumefaciens* with 10^6 per mL *C. gloeosporioides* conidia could result in a variable number of 50 to 130 hygromycin resistant transformants on nitrocellulose filters (de Groot et al., 1998). Here, it is shown that fabriano filters, and Czapek;s medium have improved the reliability of the protocol. Moreover, successive subculturing of transformants on selective and non-selective media demonstrated the stable expression of transgens as already seen for ATMT (Soltani et al., 2008). PCR analysis revealed the presence of transferred genes, and flourescence microscopy showed the expression of Gpf-tagged Hph protein inside the fungal hyphae. This finding suggests a possibility for subcellular localization of fungal Gfp-tagged proteins. The obtained insertional mutants varied in their growth rate, conidiation, color and shape, as compared with their parental wildtype isolate. This suggests the applicability of this technique for functional genetic analysis of *C. gloeosporioides* through insertional mutagenesis. Further research on the molecular mechanisms regulating varied pathogenicity life styles and secondary metabolite productions in *C.*

Table.1. Effect of different parameters (time, paper, number of conidia) during co-cultivation at 22ºC, as well as the *A.tumefacience* strains used for ATMT on *Colletotrichum gloeosporioides* JS-1389 on the number of hygromycin-resistant transformants.

Co-cultivation parameters			*A.tumefaciens* strain	
Conidia cell/mL	membrane	Days	pSDM2315	pBSY90
			number of hygromycin-resistant transformants per 60 µL conidia	
1×10^6	F	2	80	77
1×10^7	F	2	96	89
1×10^6	w	2	71	70
1×10^7	w	2	88	79
1×10^6	f	3	98	85
1×10^7	f	3	119	111
1×10^6	w	3	83	83
1×10^7	w	3	99	92

f) Fabriano 808, w) Watman 41
Data are averages of 2 independent experiments.

gloeosporioides will shed light on the hidden secrets of this fungus.

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