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Agrobacterium tumefaciens-mediated transformation of *Trichoderma viridescens*

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Abstract: Fungi have been subjected to genetic engineering in various ways. *Agrobacterium tumefaciens*-mediated transformation (AtMT) is an important method for the genetic manipulation of different fungal species. Here, gene transfer to *Trichoderma viridescens* was performed and optimized using *A. tumefaciens* strain pSDM2315. Also, the effect of different temperatures on the growth and conidiation rates of the wild-type and transformed fungi was investigated. The results indicated that the best conditions for maximum transformation in *T. viridescens* were the combination of one day of incubation, 28˚C, pH 5.0, and a concentration of 10^7 conidia mL^{-1}. The results of gene transfer and stable expression of transgenes were confirmed using sequential culture in selective media and PCR. Moreover, the mycelial growth of transformed fungi at different temperatures did not show an obvious difference from the wild-type, but the mutants produced different numbers of conidia. This indicates the potential of AtMT for functional mutagenesis and physiological studies in *T. viridescens*.

Keywords: *hph*, hygromycin B, *T. viridescens*, conidiation, AtMT

INTRODUCTION

Fungi are one of the main decomposers in nature and are considered very important organisms due to the production of various enzymes. *Trichoderma* species exist in all parts of the world and are found in the soil, on rotting wood, and on vegetables. *Trichoderma* species are among the dominant organisms of soil microflora and have spread in different habitats. They are usually saprophytic and have little nutritional needs and can grow quickly and produce conidia. The optimal growth temperature for most *Trichoderma* species is around 25-30 ˚C (Zafari, 2004). As decomposers in the soil, *Trichoderma* species are useful for the entire ecosystem and are probably effective in soil fertility. These fungi are also able to produce hydrolytic enzymes, especially cellulase, biochemical substances, and antibiotics.

One of the microorganisms that are important in the field of genetic modification is *Agrobacterium tumefaciens*, which has provided many possibilities for the genetic modification of eukaryotic cells (Soltani et al., 2008; Hooykaas et al., 2018). *Agrobacterium tumefaciens*-mediated transformation (AtMT) was first developed for plants, and then successfully used for the transformation of yeast and fungi (de Groot et al. 1998; Covertet et al. 2001; Malonek and Meinhardt, 2001; Mullins et al. 2001; Sharma et al., 2010; Soltani et al., 2009), because in comparison with other methods the AtMT is more efficient and relatively easier to apply. Usually, there is similarity for AtMT in different organisms, but for obtaining optimal transformation efficiency the optimal conditions should be explored in each organism. In addition to DNA, *Agrobacterium* can transfer proteins to host organisms, too (Michielse et al. 2005a; Soltani et al. 2008; Hooykaas et al., 2018).

During the past several decades, it is shown that conidiogenesis in *Trichoderma* can be stimulated by several environmental factors such as light, nutritional ability, and various types of stress such as drying and space limitation. Indeed, in comparison with the wild-type parents, mutants of the *Trichoderma reesei* have shown significant differences in the karyotype (Mantyla et al., 1992). So far, *Trichoderma atroviride*, *T. harziamum*, and *T. reesei* are transformed using AtMT (De Groot et al., 1998; Qian et al., 2007; Zeilinger, 2004; Zhang et al., 2006; Zhong et al., 2007).

Several factors affect the rate of AtMT during the co-cultivation of fungi and *Agrobacterium*. These include the ratio between *A. tumefaciens* and fungal cells, temperature of incubation, duration of co-cultivation, pH, kind of filter, and concentration of acetosyringone. It seems that there is a unique set of parameters specific to each fungal species that enables the optimal transfer

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of T-DNA during AtMT. The diversity seen in co-cultivation factors indicates the need for optimization of AtMT for every species. In terms of efficiency, AtMT of *T. reesei* yielded 240 transgenes per 10^7 conidia mL^{-1} (De Groot et al., 1998). Also, in *T. atroviride*, the highest transformation was obtained by using 10^7 conidia mL^{-1} (Zeilinger, 2004). Other researchers have shown that in *T. atroviride* strain T23, about 30 to 50 transformations were obtained from every 10^7 conidia mL^{-1}, while 1 to 5 transformations were obtained for 10^6 conidia mL^{-1}, and no transgenic colonies appeared using 10^5 conidia mL^{-1} (Sun et al., 2009).

The temperature of co-cultivation also significantly affects the transformation efficiency, and according to the data, a temperature between 22 and 25 ˚C is the most suitable (Bundock et al. 1995; Abuodeh et al. 2000; Zwiers and De Waard 2001). ; Combier et al. 2003; Gardiner and Howlett 2004; Idnurm et al. 2004; Michielse et al. 2004; Almeida et al. 2007). The incubation period for conidia in *T. atroviride* is reported to be at least 24 hours (Zeilinger, 2004). The appropriate pH, which leads to the highest rate of fungi transformation, is between 5.3 and 5.0 (Soltani, 2009), depending on the used *Agrobacterium* strain (Soltani et al. 2008).

Until now, paper, cellulose, Hybond N, or Hybond N+ nitrocellulose, Fabriano 808, and nylon filters are used as the co-cultivation substrates (Michielse et al., 2005; Yousefi-pour et al., 2013). Also, it is found that increasing the concentration of acetosyringone during co-cultivation, up to 1 M, increases the number of transgenes, and a high transgenic rate is obtained only when a sufficient amount of acetosyringone is added to the co-cultivation medium (Combier et al. 2003; Leclerque et al., 2004).

In this research, gene transfer to *T. viridescens* using *A. tumefaciens* was investigated and optimized. Furthermore, after transformation, the differences in growth rate and conidiation rate in wild-type and transgenic *T. viridescens* were investigated at different temperatures.

MATERIALS AND METHODS

Trichoderma viridescens, which was previously identified by molecular methods in our group, was employed (Nazmi, 2015; Zafari, 2015). Strains of *A. tumefaciens* LBA1100 (as the control without binary vector) and *A. tumefaciens* 2315 pSDM (as strain with binary vector pTAS10 carrying *hph* reporter gene) were obtained from the Department of Molecular and Developmental Genetics, Institute of Biology, Leiden University, The Netherlands.

Sensitivity test of *T. viridescens* to the antibiotic hygromycin B

To determine the sensitivity of *T. viridescens* to hygromycin B, doses of 100, 150, 200, 250, and 300 µg mL^{-1} were applied. The lowest concentration that prevented the growth of the fungus was determined for use in the selective medium.

A. tumefaciens*-mediated transformation of *T. viridescens

For AtMT of *T. viridescens*, *Agrobacterium* strains pSDM2315 and LBA1100 were cultured for 14-16 hours on a shaker incubator, at 120 rpm and 28 ˚C. Two mL of each overnight-grown bacterial strains LBA1100 and pSDM2315 were poured into separate microtubes and centrifuged at 13,000 g for 5 minutes. Then the supernatants were discarded and 1 mL of fresh liquid IM medium was added. IM medium contained K (a mixture of 1.25 M KH_2PO_4 and 1.25 M K_2HPO_4 with pH = 4.8), M-N (a mixture of 30 g/L $MgSO_4.7H_2O$ and 15 g/L NaCL), Microspores (a mixture of 100 mg L^{-1} of each of Na_2MoO_4, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5\ H_2O$ and H_3BO_3), Fe^{2+} stock (100 mg L^{-1} $FeSO_4.7H_2O$), Ca^{2+} stock (10 g L^{-1} $CaCL_2.2\ H_2O$), NH_4^+ stock (200 g L^{-1} NH_4NO_3), MES (195.2 g L^{-1} 2 [n-morpholine] ethanesulfonic acid) (Sigma) pH=5.5, and glucose 200 g L^{-1}. Then the microtubes were homogenized by vortex and centrifuged for 5 minutes at 13,000 rpm. Supernatants were removed and 1 mL of freshly prepared liquid IM was added to the precipitates. This process was repeated twice and finally, 5 ml of fresh liquid IM and 5 mL of acetosyringone were added to the precipitate. The media were placed in a shaker incubator for 6 hours at 28 ˚C, 120 rpm. Then 1 mL of their contents was taken and centrifuged at 13000 g for 5 minutes, the supernatant solutions were discarded, and 1 mL of freshly prepared liquid IM was added to the precipitates and homogenized with a vortex until used in the co-cultivations (Hooykaas et al. 2006).

Preparation of *T. viridescens* conidia for co-cultivation

To produce conidia, the fungus was cultivated on the PDA medium and kept at temperatures of 10, 15, 20, 25, and 30 ˚C during 12 hours of darkness and 12 hours of light. After the emergence of conidia, *T. viridescens* conidia were counted using slide hemocytometry and the concentrations of 10^6 and 10^7 conidia per milliliter were prepared.

Co-cultivation of *A. tumefaciens* and *T. viridescens*

One hundred µL of *T. viridescens* conidium suspension with concentrations of 10^6 and 10^7 conidia per mL, and 100 mL of *A. tumefaciens* suspension (OD600=0.5) were mixed and placed on solid IM media containing Whatman filter paper No.1. Also, depending on the volume of the culture medium, the appropriate volume of acetosyringone was added and incubated at 28 ˚C for 24 hours. After the emergence of transgenic colonies in the selective medium, the growths of which were confirmed by microscopy and their visibility on the filter, the colonies were counted and several transgenic colonies were randomly selected. The colonies were screened on the selective medium, containing the substrate of the reporter gene - hygromycin B - that prevents the growth of the wild-type fungus and acts as a selective agent for the transgenic fungi.

To ensure the transfer of the gene and its stabilization in the genome and to determine the stability of its transfer, all the transgenic fungi were successfully transferred three times to the culture medium containing hygromycin B, and the resistant colonies were used for growth rate and conidiation studies.

DNA extraction from transgenic and wild-type fungi

Transgenic and wild-type isolates were cultured in Potato-dextrose-broth (PDB) medium and incubated at 27 °C, 120 rpm. Then, DNA was extracted using the modified protocol of Chen et al. (1998).

PCR to investigate gene transfer to fungi

To confirm the *hph* gene transfer to *T. viridescens*' resistant colonies to hygromycin B, PCR was performed on the genomic DNA of seven randomly selected transgenic fungi. Specific primers of the *hph* gene, i.e. hph-F (5′-GCTGCGCCGATGGTTTCTAC A-3′) and hph-R (5′-GCGCGTCTGCTGCT CCAT-3′) were used to amplify a 544 bp fragment from the *hph* gene (Flowers and Vaillancourt, 2005). PCR conditions were set up as reported before (Yousefi-pour et al., 2013). For gel electrophoresis, the PCR products were run in Agarose gel (1%) and detected by Ethidium Bromide (0.5 µg/mL).

Investigating the growth and conidiation of wild-type and transgenic fungi

After the molecular confirmation of the *hph* gene transfer to *T. viridescens*, the wild-type and transgenic isolates were cultured on the PDA medium and at different temperatures of 10, 15, 20, 25, and 30 °C, under the same conditions. A photoperiod of 12 hours of darkness and 12 hours of light was applied for fungal growth. The fungi were inspected daily for radial growth and conidium formation rates.

RESULTS

Hygromycin B sensitivity of *T. viridescens*

Concentrations of 100, 150, 200, 250, and 300 µg mL^{-1} hygromycin B were investigated on *T. viridescens*. The result showed that the fungus started to grow in the medium without antibiotics after 24 hours and filled the entire Petri plate at a temperature of 25 to 28 °C after 4 days. The lowest concentration of hygromycin that inhibited the growth of the fungus was 150 µg mL^{-1}, which was applied to the selective medium (Fig 1).

Optimum conditions for co-cultivation of *A. tumefaciens* and *T. viridescens*

The transgenic colonies were obtained in 24 hours with a concentration of 10^7 conidia per mL, a temperature of 28 °C, and the presence of acetosyringone. The results of the PCR reaction (Fig 2) and the subsequent cultures of the colonies on the mediums containing hygromycin B, indicated the stable expression of the *hph* gene in the transgenic fungi.

Optimum conditions for AtMT in *T. viridescens*

In this research, the density of conidia used for transformation was determined as 10^6 and 10^7 conidia mL^{-1}. The results showed that the highest transgenic numbers were obtained by 10^7 conidia per mL^{-1}. The number of transgenic cells at a concentration of 10^7 conidia mL^{-1} was 186, and at 10^6 conidia mL^{-1} was 140. The results also showed that *T. viridescens* transgenics, obtained at a concentration of 10^7 conidia mL^{-1}, grew in a shorter period (8 days), but at a concentration of 10^6 conidia mL^{-1} they grew in 12 days.

Conidiation in *T. viridescens'* wild-type and transformed isolates at different temperatures

At the investigated temperatures (10, 15, 20, 25, and 30 °C) there was no significant difference between the average mycelial growth rates of the wild-type and transgenic *T. viridescens* isolates. However, they produced different numbers of conidia at different temperatures (Table 1), so that at 10 °C no conidia were formed in wild-type and transgenic isolate of *T. viridescens* (M2 isolate). At 15°C, the wild-type isolate and at the temperature of 20 and 30°C, the transgenic isolate produced more conidia. At 25 °C, wild-type and transgenic isolates produced the same number of conidia. These changes in the rate of conidiation are possible indications of functional mutations in the genome of *T. viridescens.*

T**able 1.** Conidiation rate in wild-type and transformed mutants of *T. viridescens* (produced by AtMT) at different temperatures

Tm (°C)	Wild-type		Transformed mutants	
	Growth period (Day)	Number of conidia	Growth period (Day)	Number of conidia
10	30	0.0	30	0.0
15	7	2.2×10^6	8	2.5×10^5
20	4	1.6×10^7	5	7.0×10^7
25	3	1.0×10^8	3	1.0×10^8
30	3	3.0×10^7	3	1.0×10^8

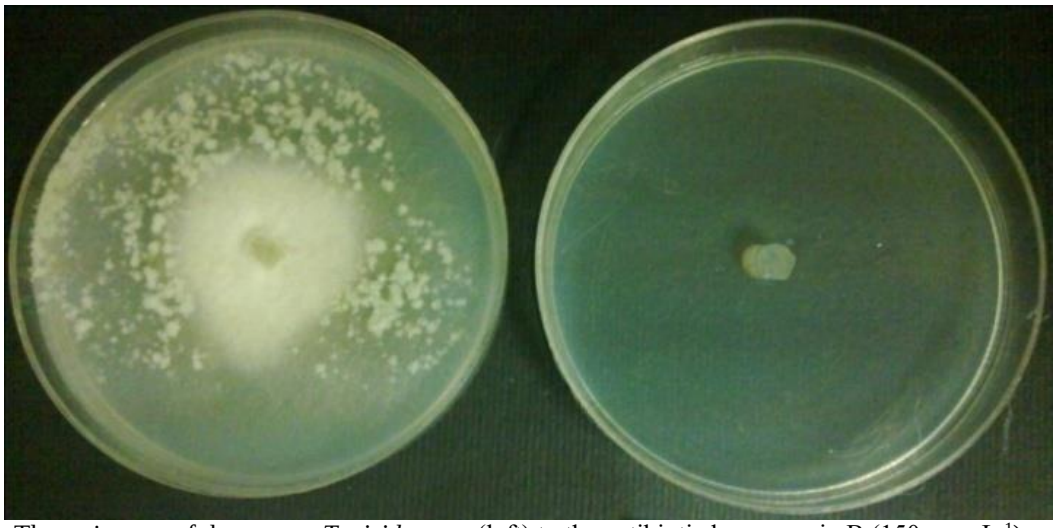

Fig.1. The resistance of the mutant *T. viridescens* (left) to the antibiotic hygromycin B (150 µg mL^{-1}) compared to the wild-type (right) at 25°C, and a photoperiod of 12 hours darkness and 12 hours light.

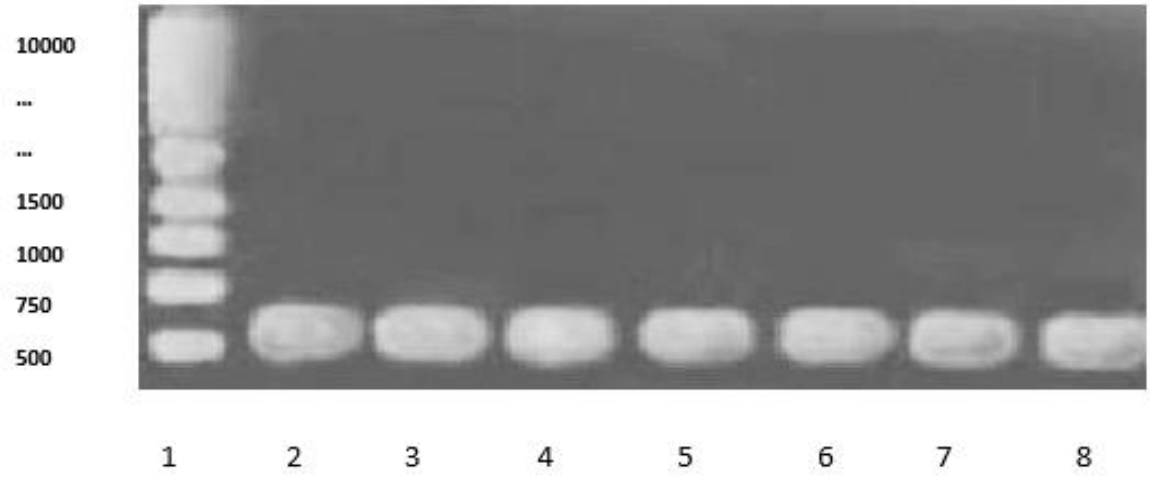

Fig. 2. PCR amplification of *hph* selection marker gene (544-bp) in mitotically stable transformants (No.2-8) of *T. viridescens* obtained by *A. tumefaciens* strain pSDM2315. DNA ladder: 10000 bp ladder.

DISCUSSION

Functional genetics of fungi needs reliable insertional mutagenesis systems. Two such genetic transformation methods have been in use, i.e., Restriction Enzyme Mediated DNA Integration (REMI) and Polyethylene Glycol (PEG), although with several drawbacks (Michielse et al., 2005). However, because of its advantages, *A. tumefaciens*-mediated transformation (AtMT) has shown priority to those methods over the last two decades (Michielse et al., 2005; Soltani et al., 2008; Yousefi-pour et al., 2013). The most suitable advantage of AtMT in fungi is transformants that show stability and carry single-copy integrated DNA. Thus, so far, a wide range of yeasts and fungi are transformed by the AtMT (Soltani et al., 2008; Hooykaas et al., 2018). In 1998, De Groot et al. used the AtMT method for gene transfer to

filamentous fungi and showed that the transgenic rate by *A. tumefaciens* increased significantly compared to the previous methods. Gene transfer by the AtMT method in *T. atroviride* (Zeilinger, 2004), *T. harziamum* (Qian et al., 2007), and *T. reesei* (Zhong et al., 2007) has been achieved. The results of their work also confirmed that the AtMT can efficiently increase the rate of transformation in different species of *Trichoderma* fungi. To the best of our knowledge, *T. viridescens* has not been transformed by the AtMT yet. Therefore, in this research, gene transfer by AtMT to *T. viridescens* was shown, and the results were confirmed by PCR and multiple subcultures in screening culture mediums. This further indicates the stability of transferred genes' expression, which is in accordance with former reports (Soltani et al., 2008). Indeed, within 24 hours co-cultivation of *A. tumefaciens*-*T. viridescens* with, 186 transformants appeared. It is reported that with a conidia concentration of 10^7 mL^{-1}, or 90-110 transformants in *T. harzianum* (Yang et al., 2011), 240 in *T. reesi*, and 30-50 in *T. atroviride* (Sun et al., 2009; Zeilinger, 2004) are achieved. This indicates that the AtMT is an efficient tool for the genetic transformation of *T. viridescens*. Moreover, although the AtMT did not affect the growth rate, different numbers of conidia were produced which can indicate a functional mutation in the genome of *T. viridescens*. Indeed, the results showed that the AtMT of *T. viridescens* affected conidium formation at higher temperatures, and resulted in more conidium production compared to the wild-type parent. These indicate the possible usefulness of the AtMT for functional mutagenesis and physiological studies in *T. viridescens.*

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تراریخت ژنتیکی *Trichoderma viridescens* به واسطه *Agrobacterium tumefaciens*

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بخش بیماری‌شناسی گیاهی، گروه گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه بوعلی‌سینا، همدان

چکیده: قارچ‌ها تاکنون به روش‌های مختلفی تحت دستورزی ژنتیکی قرار گرفته‌اند. انتقال ژن به واسطه‌ی *Agrobacterium tumefaciens* (AtMT) روش مهمی برای تراریخت گونه‌های مختلف قارچ‌ها است. در پژوهش حاضر, انتقال ژن به قارچ *T. viridescens* با استفاده از *A. tumefaciens* pSDM2315 انجام و بهینه سازی شد. همچنین اثر دماهای مختلف بر سرعت رشد و میزان کنیدیوم‌زایی تیپ وحشی و تراریخته‌ی قارچ مذکور بررسی گردید. نتایج بیانگر آن بودند که برای داشتن حداکثر تراریختی در گونه‌ی *T. viridescens* بهترین شرایط, ترکیب یک روز هم‌کشتی، دمای ۲۸ درجه‌ی سانتی‌گراد، اسیدیته ۵ و غلظت کنیدیومی 10^7 کنیدیوم بر میلی‌لیتر می‌باشد. نتایج انتقال ژن و بیان پایدار تراژن‌ها با استفاده از کشت متوالی در محیط‌های غربال و واکنش زنجیره‌ای پلیمراز به تایید رسید. رشد میسلیومی در دو جدایه‌ی تیپ‌وحشی و تراریخته در دماهای مختلف تحت بررسی، اختلاف چندانی نداد, اما جدایه‌ی تراریخته در دماهای مختلف تعداد متفاوتی کنیدیوم تولید کرد. این یافته، بیانگر پتانسیل روش انتقال ژن آگروباکتریومی برای جهش‌زایی و بررسی‌های فیزیولوژیک در *T. virdescens* می‌باشد.

کلمات کلیدی: ژن *hph*، هایگرومایسین بی، *T. viridescens* ، کنیدیوم‌زایی، تراریخت اگروباکتریومی

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