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AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF *TRICHODERMA HARZIANUM*

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ABSTRACT

The development of a transformation system is a significant technical hurdle in the study of many filamentous fungi. *Agrobacterium tumefaciens* has been shown to transform yeasts, fungi and even human cells. By using this transformation system, filamentous fungus *Trichoderma harzianum* was successfully transformed with an efficiency of 60-110 transformants per 10^7 spores. PCR and Southern analysis showed that the T-DNA was integrated into the genome. The transformants contained a single copy that was stable through mitotic cell division. The tranformation system mediated by *Agrobacterium tumefaciens* may prove to be a powerful tool for filamentous fungi transformation and also for functional genomic study due to its high transformation frequency, simplicity of T-DNA integration, and genetic stability of transformants. These findings should facilitate future study of *T. harzianum* and stimulate wider use of this valuable transformation method in fungal research.

KEYWORDS: *Agrobacterium tumefaciens*, genetic transformation, *Trichoderma harzianum*, hygromycin resistance

1. INTRODUCTION

The ability to transform an organism is an important experimental tool since it can be used to test the function of cloned genes. *Agrobacterium tumefaciens* is a soil bacterium that is widely used to transform plants [1]. For the genetic transformation of filamentous fungi, several methods are available. Most filamentous fungi are transformed either by incubation of protoplasts with polyethylene glycol, or by electroporation of intact cells [2]. However, applying these techniques to fungi can be very time-consuming with no guarantee of success. Even if transformation is achieved by these methods, the efficacy of the process is very low [3-5], limiting the utility of the system. Species of *Trichoderma* are commercially applied as biological control agents against plant fungal pathogens based on different mechanisms, such as the production of antifungal metabolites, competition for space and nutrients, and mycoparasitism [6]. An established method for plant transformation by *A. tumefaciens* has been further developed for use in yeast, and a number of filamentous fungi. [7-11]. Therefore, the purpose of this study was to determine if *T. harzianum* could be transformed by *A. tumefaciens*. This report describes the transformation of *T.*

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harzianum by *A. tumefaciens*-mediated transformation system. A transformation system for the *T. harzianum* would greatly facilitate studies of anti-pathogenicity in this organism.

2. MATERIALS AND METHODS

2.1 Strains and plasmids

Agrobacterium tumefaciens AGL-1 was kindly provided by Prof. Chu Chengcai (Institute of Genetics and Developmental Biology, the Chinese Academy of Sciences), China. *Trichoderma harzianum* T88 from Hebei Agricultural University, China. Plasmid pCAMBIA1301 was from our laboratory (Figure 1).

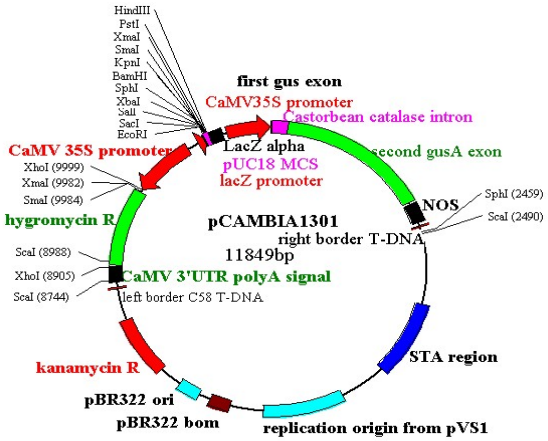

Figure 1 The map of pCAMBIA1301

2.2 Preparation of *A. tumefaciens* cells

Plasmid pCAMBIA1301 was transformed into *Agrobacterium* strain AGL-1 by cold and melt method. Start liquid culture of *Agrobacterium* strain AGL-1 by inoculating 7 mL LB (containing 50μg/mL kanamycin, 50 μg/mL Streptomycin) with a bit of frozen glycerol stock on the tip of a sterile toothpick. Incubate the tube at 250 r/min, 29℃ overnight (16-20h). Read A_{660} of the Agrobacterium culture in the next morning. Dilute the cells with MM medium (containing 200μmol/L Acetosyringone, AS) to achieve an A_{660} of 0.15.The final volune should equal 20mL.Grow the Agrobacterium cells for approximately 4 h at 29, 250 r/min. Final A_{660} may arrange from 0.6 to 0.8.Get AGL-1 50μL、100μL、150μL、200μL、250μL, centrifuge 40000 r /min, 5 min, 100μL MMliquid medium to resolve the AGL-1 to prepare for transformation.

2.3 Preparation of spores of *T. harzianum*

Shortly before *Agrobacterium* cells grew, use 5 mL sterile water to harvest *T. harzianum* spores from 1 week old cultures on potato dextrose agar. Determine the spore concentration by counting with a haemocytometer. Dilute the spore suspension with MM medium to 10^5-10^6 spores/mL.

2.4 Transformation of *T. harzianum*

Mix 100µL of diluted spores with 100µL of *Agrobacterium* cell (A660=0.6-0.8) and spread the mixture evenly on MM medium (200µmol/L AS or no AS) plates. Incubate the plates at 27℃ for 12h~60 h. After co-cultivation, re-plate M-100 medium (containing 200µg/mL hygromycin and 300µg/mL Cefotaxime) on the MM plates. Putative transformants should be visible after 5-7d.

2.5 Southern blot analysis of fungal transfromants

Genomic DNA from *T. harzianum* was isolated. Hygromycin primer: hphR: 5'-TTCGATGTAGGAGGGCGTGGAT-3', hphF: 5'- CGCGTCTGCTGCTCCATACAAG-3, Hygromycin PCR reaction: 94℃ 4min, 94℃45s, 60℃ 1min, 72℃ 1.5min, 35 cycles, 72℃ 10min. The hygromycin of pCAMBIA1301 was used to make probes. DNA gel blot, labeling reactions and hybridization were carried out as described by DIG High Prime DNA Labeling and Detection Starter Kit (Roche Diagnostics Corporation).

2.6 Mitotic stability of the transformant

To determine the stability of the transformant, the transformant was cultured on PDA without hygromycin B. Following colonization of each plate, a mycelial plug was taken from the edge of the culture and transferred onto fresh PDA. This procedure was repeated ten times. Resistence of these monoconidial cultures to hygromycin B was tested by growing them on PDA containing hygromycin B.

3. RESULTS

3.1 The original sensitivity level of *T. harzianum* to hygromycin

The result showed that Hygromycin inhibited the growth of *T. harzianum* obviously when its concentration was up to 100µg/mL (Table 1). The fungus cannot survive when the concentration was up to 150µg/mL.

Table 1 Inhibition efficiency of Hygromycin on *T .harzianum* growth

c (Hygromycin) /µg.mL^{-1}	0	50	75	100	125	150
1	0	38.97	42.58	66.25	88.74	100
2	0	24.25	31.25	48.28	91.56	100
3	0	26.32	38.54	56.12	93.72	100
$\bar{x}$	0	30.05	37.45	56.88	91.34	100
S_{n-1}	0	10.41	7.32	6.47	1.09	0

3.2 The impact of acetosyringone induction on transformation

Co-culture of *T. harzianum* and *A. tumefaciens* on IM plates without AS failed to grow. However, Co-culture of *T. harzianum* and *A. tumefaciens* on IM plates with 200µM AS succeeded to grow (Table 2).

Table 2 The impact of different AS treatment on the transformation rate

	Colonies / plate
Co-culture on IM + 200μM AS	62~110
Co-culture on IM without AS	0

3.3 The impact of A. tumefaciens initial volume for transformation

The amount of colonies was significantly increased with extension of co-cultured concentration, and it reached the peak at the co-cultured concentration 100μL.

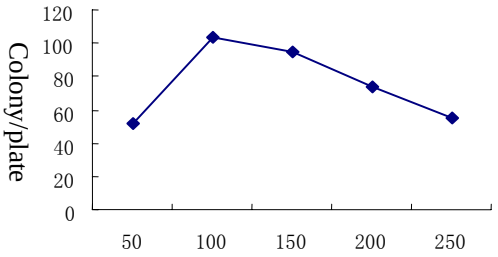

Figure 2 Effect on transformation efficiency of *A. tumefaciens* initial volume

3.4 The impact of co-cultivation time for transformation

After co-cultivation of *T. harzianum* and *A. tumefaciens* on IM plates with 200μM AS for 12 h, there were no transformant on plate. Until 24 h, there were a few transforamants on plate. At 48 h there was the largest number of transformants on plate (Figure 3).

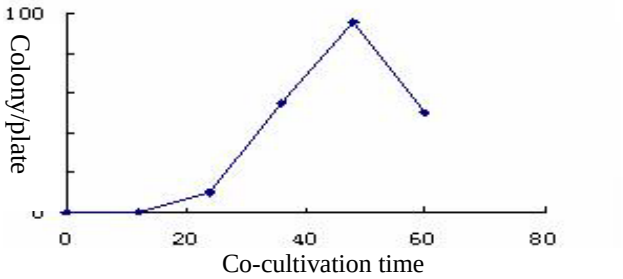

Figure 3 The impact of co-cultivation time for transformation

3.5 Screening of fungal transformants

After 5-7 d plating on MM seclecting medium, many putative transformants were visible. When inoculating these transformants to new plate containing 200μg/mL Hygromycin, they grew well even when hygromycin was up to 300μg/mL. The results showed the transformation efficiency was 70-110 transformants per 10^7 spores.

3.6 Molecular biological analysis of the tranformants

DNA gel blots indicated that pCAMBIA1301 gene was integrated into the genome of *T.harzianum* as a single copy (Fig.4). The pCAMBIA1301 transformation was successful.

W: wild strain；1: pCAMBIA1301 transformant

Figure 4 Southern blotting of transformant pCAMBIA1301 for hygromycin resistance

3.7 Mitotic stability of the transformants

The mitotic stability of the transformant was tested by growing them on PDA plates without selection. After successively repeating 10 generations, we inoculated these transformants to PDA plates containing 200μg/mL hygromycin. The transformant continued to grow up well. This showed that it was mitotically stable.

3.8 General discussion

AS is necessary for *A. tumefaciens*-mediated transformation of *T. harzianum* like other *A. tumefaciens*-mediated transformation of fungi and plants, especially at the co-culture stage. In this study there is no positive transformant without AS. This indicated that the induction of vir gene is necessary for transferring the T-DNA to *T. harzianum. A. tumefaciens* need time to infect spores of *T. harzianum* for T-DNA integration. So co-culture time is important to *Agrobacterium tumefaciens*-mediated transformation of *T. harzianum*. If co-culture time is shorter, *A. tumefaciens* doesn't finish the process of infecting spores of *T. harzianum*. If co-culture time is longer, it' doesn't faciliate to express of insert external gene. And Over-product none transformants compete for the nutriments with the transformants. In this study the best co-culture time is 48h for *A. tumefaciens*-mediated transformation of *T. harzianum*. Certainly the best co-culture time may be different for different *A. tumefaciens* and fungi.

Gao et al successfully transformed the T-DNA into the *Chaetomium globosum* mediated by *Agrobacterium tumefaciens* [12-14]. *A. tumefaciens*-mediated transformation system has the advantages with simple operation, high transformation efficiency and easy integration. *T .harzianum* was transformed in this study using this transformation system with an efficiency was 60-110 tranformants/10^7 spores. The transformation efficiency was higher than that of previous transformation of protoplast mediated by PEG, thus overcoming the hurdle confronted by conventional transformation protocols. *A. tumefaciens*-mediated transformation of *T .harzianum* provided a new technique to the genetic modification of *T .harzianum*. The tranformation system mediated by *A. tumefaciens* may prove to be a powerful tool for the filamentous fungi transformation and functional genomic study with its high transformation frequency, simplicity of T-DNA integtation and genetic stability of the transformants. These findings should facilitate

future study of *T. hazianum* and stimulate wider use of this valuable transformation method in other fungal transformation.

4. CONCLUSIONS

A. tumefaciens-mediated transformation of *T .harzianum* was a useful transformation method for the study of many filamentous fungi. This provided a new technique to the genetic modification of *T .harzianum*.

5. ACKNOWLEDGEMENTS

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