Current Microbiology Vol. 52 (2006), pp. 469–472
DOI: 10.1007/s00284-005-0402-6

Current Microbiology
An International Journal

Efficient Genetic Transformation System for the Ochratoxigenic Fungus *Aspergillus carbonarius*

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Received: 31 October 2005 / Accepted: 14 December 2005

Abstract. *Aspergillus carbonarius* is a potent ochratoxin A producer that has been found in products such as grapes, coffee, spices, and cocoa. Ochratoxin A has nephrotoxic effect, and it has been classified as a possible carcinogenic substance for humans. Here we describe for the first time a transformation system for *A. carbonarius*, providing an important step towards its genetic manipulation. Conidia were transformed to acquire hygromycin B resistance using the AGL-1 strain of *Agrobacterium tumefaciens*. Genetic transformation was evaluated growing *A. tumefaciens* cells in induction medium containing or not acetosyringone prior to co-cultivation. The mean transforming efficiencies in IM+AS and IM-AS conditions were 62.2 and 44.5 transformants per 10^5 conidia, respectively. The *hph* gene was random integrated into the genome of *A. carbonarius*. Fungal sequences flanking the insertion site could be amplified by TAIL-PCR.

Aspergillus carbonarius is a potent ochratoxin A (OTA) producer that has been found in products such as grapes, coffee, spices, and cocoa [1, 2, 4, 8, 13, 16]. OTA has nephrotoxic effect and it has been classified as a possible carcinogenic substance for humans. This mycotoxin was originally described as a secondary metabolite of *Aspergillus ochraceus* strains [22]. Nowadays, *A. carbonarius* is recognized as one of the main OTA-producing species in tropical countries [20].

The genetics of the *A. carbonarius* is poorly studied, and currently no transformation system has been developed for this species. The generation of *Aspergillus* species transformants has been achieved using two alternative strategies. The strategy denoted ''direct DNA transfer'' is a group of unrelated techniques, which includes methods such as transformation of protoplasts mediated by polyethylene glycol [5], particle bombardment [10], and electroporation [23]. The other strategy exploits the ability of *A. tumefaciens* to transfer a part of its DNA (T-DNA) into the fungal genome [3]. Recently, the *Agrobacterium*-mediated transformation (AMT) has been considered advantageous over ''direct DNA transfer'' because it generates high percentage of transformants, mostly with one single foreign-DNA copy integrated at random sites in fungal genome [6, 11, 15, 21]. These attributes make the AMT method an important tool to generate insertional mutants, and ultimately to isolate genes tagged by the transforming DNA [14].

Here we report for the first time the genetic transformation of *A. carbonarius*, thereby providing an important step towards the genetic manipulation of this ochratoxigenic species.

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Materials and Methods

Strains and plasmid. The ITAL99 strain of *A. carbonarius* was isolated from Brazilian coffee-bean samples by M. H. Taniwaki (Instituto de Tecnologia de Alimentos, Campinas, Brazil) who kindly provided it to our laboratory. *A. tumefaciens* AGL-1 strain was generously given by C. P. Romaine (Pennsylvania State University, PA). The pPK2 binary vector [7] that contains the T-DNA borders harboring the hygromycin B (Hyg B) resistance gene driven by the *Aspergillus nidulans gpd* promoter and *trp*C terminator was kindly provided by S. Covert (University of Georgia, Athens, GA).

Nuclei staining. The conidium staining was performed according Tanaka et al. [19]. Briefly, conidia were attached to a coverslide by using one drop of albumin solution. The material was fixed by using ethanol-acetic acid (3:1 v/v) for 30 min at room temperature. The slide was dried, washed once in 95% ethanol and once in 70% ethanol, and then treated with 1 N HCl at 60°C for 12 min. The coverslide was washed in water, treated with Giemsa stain (0.07% Giemsa in 50 mM phosphate buffer, pH 6.9, 3% glycerol, 3% methanol) for 30 min, and again rinsed in water. The stained nuclei in conidia were viewed by light microscopy.

***Agrobacterium tumefaciens*-mediated transformation.** Cells of the *A. tumefaciens* AGL-1 carrying the binary vector (pPK2) were grown at 28°C for 48 h in minimal medium supplemented with kanamycin (50 μg mL^{-1}) and streptomycin (50 μg mL^{-1}). The bacterial cells were diluted to OD660 = 0.15 in induction medium (IM) (10 mmol L^{-1} K_2HPO_4, 10 mmol L^{-1} KH_2PO_4, 2.5 mmol L^{-1} NaCl, 2 mmol L^{-1} $MgSO_4$, 0.7 mmol L^{-1} $CaCl_2$, 9 μmol L^{-1} $FeSO_4$, 4 mmol L^{-1} NH_4SO_4, 10 mmol L^{-1} glucose, 40 mmol L^{-1} 2-[*N*-morpholino] ethanesulfonic acid, pH 5.3, 0.5% glycerol) [9], both in the presence (IM+AS) or absence (IM-AS) of 200 μmol L^{-1} acetosyringone (AS). The cells were grown for additional 9 h before mixing them with an equal volume of a conidial suspension from the ITAL99 strain (10^6 per mL each). This mix (200 μL) was spread onto nitrocellulose filters (0.45 μm pore and 90 mm diameter, MFS-Japan) that were placed on agar plates containing the co-cultivation medium (same as IM + AS, but containing 5 mmol L^{-1} instead of 10 mmol L^{-1} of glucose). After co-cultivation at 28°C for 36 h, the membranes were transferred to M-100 plates (55 mmol L^{-1} glucose, 30 mmol L^{-1} KNO_3) plus 6.2% v/v mineral solution (117 mmol L^{-1} KH_2PO_4, 28 mmol L^{-1} Na_2SO_4, 107 mmol L^{-1} KCl, 8 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 9 mmol L^{-1} $CaCl_2$, 7.8 μmol L^{-1} H_3BO_3, 5.6 μmol L^{-1} $MnCl_2 \cdot 4H_2O$, 2.3 μmol L^{-1} $ZnCl_2$, 1.3 μmol L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 2.9 μmol L^{-1} $FeCl_3 \cdot 6H_2O$, 12.8 μmol L^{-1} $CuSO_4 \cdot 5H_2O$) and 1.5% agar [18] that had hygromycin B (75 μg mL^{-1}) as the selection agent for fungal transformants, and cefoxitin (150 μg mL^{-1}) to inhibit growth of *A. tumefaciens* cells. After incubation for 3 to 4 days at 28°C, the number of hygromycin-resistant colonies was counted. Higher concentration of conidia (10^7 per mL) was also analyzed; however, it resulted in too much fungal growth during co-cultivation, which makes the subsequent isolation of single transformants difficult.

Assay for mitotic stability of transformants. To determine the mitotic stability, 100 randomly selected *A. carbonarius* transformants were cultured on M-100 devoid of hygromycin B. After they were grown, conidia of each of the transformants were picked up onto fresh M-100. This procedure was repeated 10 times. Then, conidia of each of the transformants were picked up onto M-100 containing hygromycin B (75 μg mL^{-1}).

Genomic DNA extraction. Total genomic DNA extraction was performed using DNAzol (Invitrogen Life Technologies, USA) according to the manufacturer's recommendation.

PCR analysis. The primer pair *hph*1 (5′-TTCGATGTAGGAGGG CGTGGAT-3′) and *hph*2 (5′-CGCGTCTGCTGCTCCATACAAG-3′) was used in polymerase chain reaction (PCR) analysis for amplifying the *hph* fragment in putative transformants. The cycling conditions were as follows: an initial denaturation step (95°C, 2 min), 35 cycles of denaturation (92°C, 45 s), annealing (60°C, 1 min), and elongation (72 °C, 1.5 min), and at the end a final elongation pace (72°C, 5 min).

Southern analysis. To identify the number of the copies of the foreign integrated DNA in the transformants' genome, DNA digestion was performed using *Sst*I because it cuts the T-DNA once, outside of the *hph* gene. When the fragment of *hph* gene is used as probe, single-hybridizing bands will be indicative of single copy integration of the expression cassette. Standard procedures described by Sambrook and Russel [17] were used for restriction endonuclease digestion, agarose-electrophoresis, and transference onto nylon membranes. As a probe a fragment of the hygromycin gene (0.6 kb) was used. This fragment was obtained by PCR using the primer pair *hph1* and *hph2*. DNA probe labeling and hybridization were performed using a digoxigenin hybridization system (Roche, Mannheim, Germany) according to the manufacturer's recommendation.

TAIL-PCR and DNA sequencing. Genomic DNA from four transformants was used as template in TAIL-PCR reactions. AD1-degenerated primers and RB-specific primers, reaction conditions, and thermal cycling settings were identical to those used by Combier et al. [6]. PCR products resulting from tertiary PCR reaction that was approximately 100 bp shorter than that obtained after the secondary PCR reaction were excised from the gel and purified using the CONCERT Rapid Gel Extraction System (Gibco BRL, Germany). The resulting DNA was sequenced using the DYEnamic ET dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Inc.) on MegaBACE 1000. Sequence comparison was performed using BLAST tools available on the NCBI website (http://www.ncbi.nlm.nih.gov).

Results and Discussion

A. carbonarius is an OTA-producing fungus, with little known molecular biology. We aimed to devise a transformation method for this species using a cassette for hygromycin resistance. A prerequisite for the use of hygromycin resistance gene as a selection marker is the sensitivity of the host strain to this drug. Therefore, the sensitivity of *A. carbonarius* to hygromycin (Hyg B) was tested by plating 10^5 conidia on agar plates containing M-100 plus different concentrations of Hyg B. Growth was totally inhibited on plates containing at least 50 μg mL^{-1} Hyg B. For selecting Hyg B transformants, 75 μg mL^{-1} Hyg B was enough to prevent growth of untransformed colonies.

Co-cultivation of *A. carbonarius* with *A. tumefaciens* harboring pPK2 binary vector onto co-cultivation medium (IM + AS) resulted in hygromycin-resistant colonies after 4–5 days after the transference to the selection medium. In absence of AS during the co-cultivation, no resistant colony was found. The frequency of resistant colonies ranged from 23 to 101 per 10^5 conidia, in four independent experiments (Table 1). The AMT frequency obtained in our study was similar to that achieved for *Aspergillus awamori* (20 to 90 transformants per 10^5 conidia) and much higher than that described for *Aspergillus niger* (5 transformants per 10^7 conidia), both reported by De Groot et al. [9]. To confirm the presence of the *hph* gene in the hygromycin-resistant colonies, 28 putative transformants were screened by PCR analysis. Using *hph*1 and *hph*2 oligonucleotide primers, the expected 600-bp fragment was

Table 1. Number of hygromycin-resistant *Aspergillus carbonarius* colonies after transformation in four different experiments[a]

	Number of Hyg B^r colonies obtained per 1×10^5 conidia after co-cultivation with acetosyringone[a]			
Condition	I	II	III	IV
IM-AS condition[b]	23	74	52	29
IM+AS condition[b]	25	101	59	64

[a]Data derived from four trials.
[b]Acetosyringone added (IM+AS condition) or not (IM−AS condition) in pretreating of bacterial cells prior to co-cultivation.

detected in all transformants, which was not amplified from untransformed DNA (data not shown). The genetic stability of the transformants was high (92%).

Transformants were subjected to Southern analysis. Figure 1 shows the results of 16 out of 28 transformants analyzed. Random integration of the *hph* gene into the *A. carbonarius* genome was observed. Some 62% of the transformants derived from the IM+AS condition possessed a single insert of exogenous DNA, whereas 87% of that generated by cell growth in the IM-AS condition had a single T-DNA insert. According to the literature [14], the need of AS during co-cultivation for fungal-transformant generation is unambiguous; however, for unknown reasons, the addition of AS to the *Agrobacterium* preculture has been reported to result in either a decrease or an increase in single-copy T-DNA integration.

The high percentage of transformants with a single copy integrated at random into the host genome in a non-sequence-specific manner makes this transformation method a valuable tool for mutational analysis in *A. carbonarius* by insertional mutagenesis. The most important advantage of this strategy over traditional methods, such as physical or chemical mutagenesis, is that the mutated gene is tagged by transforming DNA and can be subsequently identified revealing its function.

The loss-of-function allele usually is recessive. In the screening of mutants for a desirable phenotype, multinucleated conidia hamper its recognition. For this, the nuclei number per conidium of the *A. carbonarius* (strain ITAL99) was assessed by Giemsa staining. The number of nuclei per conidium varied from five to seven. In order to analyze the segregation of the *hph* gene, asexual spores obtained directly from nitrocellulose filters were cultivated on both selective and nonselective medium. The number of colonies on nonselective medium was notably higher than that obtained on selective medium, denoting a heterokaryotic state after transformation. However, after a second round of selection the secondary transformants were homokaryons. The elimination of the heterokaryotic state after only one round of replication on selective medium may allow the identification of recessive mutants.

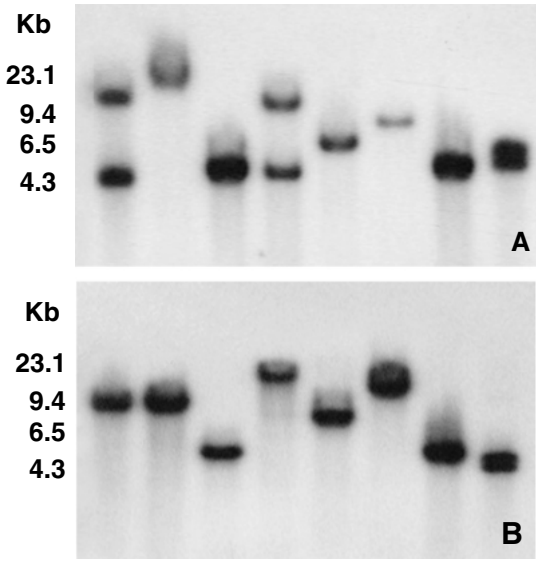

Fig. 1. Southern analysis of *Aspergillus carbonarius* transformants. (A) The transformants were obtained with pretreating the bacterial cells with AS prior to co-cultivation. (B) The transformants were obtained with pretreating the bacterial cells without AS prior to co-cultivation. The molecular masses of marker λ/*Hin*dIII are indicated in kb on the left. Genomic DNA was restricted with *Sst*I and electrophoresed on a 0.8% agarose gel. For hybridization, a 0.6-kb fragment of *hph* gene labeled with digoxigenin was used.

To test whether T-DNA insertion provides a suitable tool for gene identification in *A. carbonarius*, we investigated four randomly selected transformants. The thermal asymmetric interlaced PCR (TAIL-PCR) [12] methodology was used for amplifying the genomic DNA fragment flanking the site of the T-DNA insertion. Using T-DNA RB-specific primers and degenerated primer AD1, we successfully amplified junction's fragments in three transformants. In closing, this study described the genetic transformation of *A. carbonarius* and showed that AMT can be considered a promising tool for generating of *A. carbonarius* insertional mutants.

ACKNOWLEDGMENTS

Grants and fellowships from the following Brazilian institutions supported this work: Fundação Araucária and Conselho Nacional de Pesquisa (CNPq).

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