International Journal of Food Microbiology 148 (2011) 135–140

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

# Development of a green fluorescent tagged strain of *Aspergillus carbonarius* to monitor fungal colonization in grapes

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ARTICLE INFO

*Article history:*
Received 16 March 2011
Received in revised form 20 May 2011
Accepted 24 May 2011
Available online 30 May 2011

*Keywords:*
*Aspergillus carbonarius*
ATMT
Green fluorescent protein
Confocal microscopy
Ochratoxin production
Grapes
Wine

ABSTRACT

An enhanced green fluorescent protein has been used to tag an OTA-producing strain of *Aspergillus carbonarius* (W04-40) isolated from naturally infected grape berries. Transformation of the fungus was mediated by *Agrobacterium tumefaciens*. The most efficient transformation occurred when the co-cultivation was done with $10^4$ conidia due to higher frequency of resistance colonies (894 per $10^4$ conidia) and lower background obtained. To confirm the presence of the *hph* gene in hygromycin resistant colonies, 20 putative transformants were screened by PCR analysis. The *hph* gene was identified in all the transformants. Variation on the expression levels of the eGFP was detected among the transformants and 50% of them appeared bright green fluorescent under the microscope. Microscopic analysis of all the bright fluorescent transformants revealed homogeneity of the fluorescent signal, which was clearly visible in the hyphae as well as in the conidia. eGFP expression in *A. carbonarius* was shown to be stable in all transformants. Confocal Laser scanning microscopy images of grape berries infected with the eGFP transformant demonstrated fungal penetration into the berry tissues. OTA production was importantly increased in the eGFP transformant in comparison with the wild type strain and pathogenicity on grape berries was slightly decreased after four days of inoculation. However, no differences in virulence were found after seven days of inoculation, thus allowing utilization of this eGFP mutant for *in situ* analysis of *A. carbonarius* infection of grape berries. To our knowledge, this is the first report describing the construction of a GFP-tagged strain belonging to *Aspergillus* section *Nigri* for monitoring *Aspergillus* rot on grape berries.



## 1. Introduction

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, carcinogenic, immunotoxic, genotoxic and teratogenic effects (Creppy, 1999; Kuiper-Goodman and Scott, 1989; Petzinger and Ziegler, 2000; Pfohl-Leszkowicz and Manderville, 2007). Grapes and wines have been reported among the food commodities contaminated by OTA with the greatest OTA intake, second only to cereals (Bau et al., 2005; Bellí et al., 2004). Several studies in Europe, South America, Australia and the North of Africa have determined that OTA is produced during infection of grapes in vineyards by mycotoxigenic strains of *Aspergillus* species belonging to section *Nigri* (Battilani et al., 2006; Chulze et al., 2006; Leong et al., 2006; Martínez-Culebras et al., 2009; Oueslati et al., 2010). It is now widely accepted that OTA contamination of wine and other grape products is mainly due to *Aspergillus carbonarius*. Although *A. carbonarius* is less common than other black *Aspergillus* species, such as *Aspergillus niger* and *Aspergillus tubingensis*, it is the main species responsible for OTA in grapes because almost all strains are high OTA producers (Martinez-Culebras and Ramon, 2007; Perrone et al., 2007).

Soil and vine trash on soil are the primary sources of *A. carbonarius* in vineyards. Wind-borne spores from the soil are deposited onto the surface of vines, including berry surfaces. Black *Aspergillus* species appear to be secondary invaders that infect grapes only after they have been damaged by pre-harvest rain, mechanical impacts, insects and other fungal pathogens such as *Botrytis cinerea*. However, several studies have reported the ability of *A. carbonarius* strains to colonise and penetrate berries even without skin damage in artificially inoculated grapes (Battilani et al., 2004; Bellí et al., 2007). The way *A. carbonarius* penetrates the fruit in undamaged grapes is still unknown. Additionally, the presence of OTA in healthy berries has been reported by Serra et al. (2006), which suggests that *A. carbonarius* may infect berries at earlier stages of berry development. Actually, the study of Kazi et al. (2008) showed that berry infections can be established as early as flowering and persist in berries until harvest. These studies highlighted the need to study more deeply the entrance of *A. carbonarius* into berries, whether it is through the stigma, pedicels, natural openings or by direct penetration of the cuticle.

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doi:10.1016/j.ijfoodmicro.2011.05.021

The use of fungal transformants expressing cytoplasmic fluorescent proteins has greatly enhanced the ability to visualize and analyze the colonization and infection processes. The majority of studies utilising this technology in fungi have used modified forms of the green fluorescent protein (GFP) (Chalfie et al., 1994), such as enhanced green fluorescent protein (eGFP) (Cormack et al., 1996), which confer higher levels of fluorescence without major effects on fungal growth or pathogenicity. Although a large number of GFP expression vectors have been developed for most important fungal pathogens (Czymmek et al., 2004; Lorang et al., 2001), GFP tagging of an ochratoxigenic fungus has only been described for *Penicillium nordicum* (Schmidt-Heydt et al., 2009). GFP tagging of a specific fungal strain depends on both the development of an efficient transformation protocol and the stable expression of the *gfp* gene in the fungus under natural environmental conditions.

The genetics of the *A. carbonarius* is poorly studied and currently only one study has reported the transfer of exogenous genes to *A. carbonarius*. Morioka et al.(2006) transformed a coffee isolate of *A. carbonarius* with the hygromycin phosphotransferase gene (*hph*) using *Agrobacterium tumefaciens*-mediated transformation (ATMT). ATMT has been considered advantageous over "direct DNA transfer" because it generates a high percentage of transformants. On the other hand, GFP tagging of *A. carbonarius* has not been reported yet. In this article, we describe an ATMT protocol for the efficient expression of the eGFP in *A. carbonarius* in order to facilitate microscopic visualisation of the infection process of grapes of this ochratoxigenic species.

## 2. Materials and methods

### 2.1. Strains and culture conditions

The OTA-producing *A. carbonarius* strain W04-40 was isolated from a Spanish vineyard by Martínez-Culebras and Ramon (2007) and deposited in the Institute of Agrochemistry and Food Technology of the Spanish National Research Council (IATA-CSIC). *A. tumefaciens* AGL-1 strain was kindly provided by L. Peña (Instituo Valenciano de Investigaciones Agrarias, Valencia, Spain)

*A. carbonarius* was grown on Petri dishes containing Malt Extract Agar (MEA) medium in the dark at 30 °C for 6 days to achieve conidia production. Conidia were collected with a sterile solution of 0.005% (v/v) Tween 80 (J.T. Baker, Deventer, Holland) and were adjusted to $10^6$ conidia/mL using a haemocytometer. 100 μL of the conidial suspension was homogeneously spread on Petri dishes containing Czapeck Yeast Extract Agar (CYA) medium and sub-cultured in the dark at 30 °C. Routinely growth of transformed strains was performed on media containing 100 μg/mL of hygromycin B (Hyg B; Invivogen, San Diego, USA).

### 2.2. Construction of plasmid pRFHUE-eGFP

The enhanced green fluorescent protein encoding gene (*egfp*) was amplified from plasmid pEGFP-C3 (Clontech, Mountain View, USA) with primers EGFP03 (5′-GGACTTAAUGGTGAGCAAGGGCGAG-GAGCTGT-3′) and EGFP04 (5′-GGACTTAAUGGTGAGCAAGGGCGAG-GAGCTGT-3′), which incorporate a uracil near the 5′ end, and the high fidelity Pfu Turbo HotStart DNA Polymerase (Agilent Technologies, Santa Clara, USA). Cycling conditions consisted of an initial denaturation step at 94 °C for 2 min, 30 cycles of 94 °C for 2 min, 58 °C for 30 s and 72 °C for 1 min and a final elongation step at 72 °C for 10 min. The amplified fragment was cloned into the plasmid vector pRF-HUE (Frandsen et al., 2008), a binary vector designed to be used with the USER friendly cloning technique (New England Biolabs). Digestion of this plasmid with the restriction enzyme *Pac*I followed by treatment with the nicking enzyme *Nt.Bbv*CI generates 9-nucleotide long 3′ single-stranded ends that are complementary to those present in the PCR-amplified fragment. DNA insert and treated vector were mixed together and treated with the USER (uracil-specific excision reagent) enzyme (New England Biolabs), which excises the uracil residues originating a single-stranded end PCR fragment that can anneal to the one generated in the vector, to obtain plasmid pRFHUE-eGFP. An aliquot of the mixture was used to transform chemical competent *E. coli* XL1-Blue cells. Kanamycin resistant transformants were screened by PCR. Proper fusion was confirmed by DNA sequencing. Then, plasmid pRFHUE-eGFP was introduced into electrocompetent *A. tumefaciens* AGL-1 cells.

### 2.3. Agrobacterium tumefaciens-mediated transformation of A. carbonarius

*A. tumefaciens* AGL-1 carrying plasmid pRFHUE-eGFP was grown at 28 °C for 72 h in Luria Bertani (LB) agar supplemented with kanamycin (50 μg/mL), rifampicin (20 μg/mL) and carbenicillin (75 μg/mL). A single colony was used to inoculate a starter culture of 5 mL of LB medium containing the aforementioned antibiotics then incubated for 24 h. Bacterial cells were centrifuged, washed with induction medium (IM) (10 mM $K_2HPO_4$, 10 mM $KH_2PO_4$, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.6 mM $CaCl_2$, 9 μM $FeSO_4$, 4 mM $(NH4)_2SO4$, 10 mM glucose, 40 mM 2-[N-morpholino] ethanesulfonic acid, pH 5.3, 0.5% glycerol) (De Groot et al., 1998) and diluted in the same medium amended with 200 μM acetosyringone (IMAS) to an OD600 = 0.15. Cells were grown at 28 °C and 200 rpm until they reached an OD600 of 0.5–0.75. An equal volume of this culture was mixed with a conidial suspension of *A. carbonarius*. Three conidial suspension concentrations were tested ($10^7$, $10^6$ and $10^5$ conidia per mL). 100 μl of these mixtures were spread onto cellulose nitrate membrane filters (0.45 μm pore and 47 mm diameter, Albet, Dassel, Germany) which were placed on agar plates containing the co-cultivation medium (same as IMAS, but containing 5 mM instead of 10 mM of glucose). After co-cultivation at 26 °C for 40 h, the membranes were transferred to CYA plates containing Hyg B as the selection agent for fungal transformants, and cefotaxime (200 μg/mL) to inhibit growth of *A. tumefaciens* cells. Although hygromycin sensitivity to *A. carbonarius* has been previously reported by Morioka et al. (2006), a prerequisite for the use of hygromycin resistance gene as a selection marker is to determine the sensitivity of the host strain to this drug. *A. carbonarius* did not grow at concentrations equal to or higher than 50 μg/mL. For selecting Hyg B transformants, 100 μg/mL Hyg B was enough to prevent growth of untransformed conidia. Hygromcycin resistant colonies appeared after 3 to 4 days of incubation at 30 °C.

### 2.4. Genomic DNA extraction

All strains were grown on MEA medium for 4 days. Mycelium was collected from the plates, frozen in liquid nitrogen and ground to a fine powder. DNA extractions were performed using 100 mg of powder and the commercial EZNA Fungal DNA kit (Omega Bio-Tek, Doraville, USA) according to the manufacturer's instructions.

### 2.5. PCR analysis

Primers Hmbr1 (5′-CTGATAGAGTTGGTCAAGACC-3′) and Hmbf1 (5′-CTGTCGAGAAGTTTCTGATCG-3′) were used in PCR analysis to amplify a fragment of the *hph* gene in putative transformants. The cycling conditions were as follows: an initial denaturation step (95 °C, 2 min), 35 cycles of denaturation (95 °C, 30 s), annealing (62 °C, 45 s), and elongation (72 °C, 1 min), and a final elongation step (72 °C, 10 min).

### 2.6. Determination of cultural characteristics

For growth assessment, CYA plates were inoculated centrally with 5 μl of conidia suspensions ($10^6$ conidia/mL) from the wild-type strain