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Constitutive expression of fluorescent protein by *Aspergillus* var. *niger* and *Aspergillus carbonarius* to monitor fungal colonization in maize plants

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ABSTRACT

Aspergillus niger and *Aspergillus carbonarius* are two species in the *Aspergillus* section *Nigri* (black-spored aspergilli) frequently associated with peanut (*Arachis hypogea*), maize (*Zea mays*), and other plants as pathogens. These infections are symptomless and as such are major concerns since some black aspergilli produce important mycotoxins, ochratoxins A, and the fumonisins. To facilitate the study of the black aspergilli–maize interactions with maize during the early stages of infections, we developed a method that used the enhanced yellow fluorescent protein (eYFP) and the monomeric red fluorescent protein ($mRFP_1$) to transform *A. niger* and *A. carbonarius*, respectively. The results were constitutive expressions of the fluorescent genes that were stable in the cytoplasms of hyphae and conidia under natural environmental conditions. The hyphal *in planta* distribution in 21-day-old seedlings of maize were similar wild type and transformants of *A. niger* and *A. carbonarius*. The *in planta* studies indicated that both wild type and transformants internally colonized leaf, stem and root tissues of maize seedlings, without any visible disease symptoms. Yellow and red fluorescent strains were capable of invading epidermal cells of maize roots intercellularly within the first 3 days after inoculation, but intracellular hyphal growth was more evident after 7 days of inoculation. We also tested the capacity of fluorescent transformants to produce ochratoxin A and the results with *A. carbonarius* showed that this transgenic strain produced similar concentrations of this secondary metabolite. This is the first report on the *in planta* expression of fluorescent proteins that should be useful to study the internal plant colonization patterns of two ochratoxigenic species in the *Aspergillus* section *Nigri*.

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1. Introduction

The *Aspergillus* subgenus *Circumdati* section *Nigri* (black aspergilli) is an important group of fungal species because of their worldwide distribution and their positive impact in the biotechnological industry. *Aspergillus niger* is widely used in industrial processes and has been granted the Generally Recognized As Safe status by the U.S. Food and Drug Administration (Schuster et al., 2002). Conversely, some species within this section are frequently linked to negative impacts in agriculture where they are known as pre and post-harvest plant pathogens, causing a wide range of plant diseases in different hosts, including: grape, onion, garlic, peanut, maize, coffee, fruits and vegetables (Lorbeer et al., 2000; Magnoli et al., 2006; Rooney-Latham et al., 2008; Waller et al., 2007). Recent evidence showed that some black aspergilli are able to colonize plant hosts as symptomless endophytes (Palencia et al., 2009; Wani et al., 2010). Symptomless endophytic states occur in plants characterized as balanced symbionts in that the fungus and plant host are highly compatible. The ingestion of contaminated crop products is a potential threat to humans and animal health since some black aspergilli, especially *A. niger* and *Aspergillus carbonarius*, are known to produce toxic secondary metabolites.

The species of the *Aspergillus* section *Nigri* isolated from natural substrates including peanut, maize, and grape are able to produce and accumulate mycotoxins such as ochratoxins and fumonisins (Astoreca et al., 2007a, 2007b; Frisvad et al., 2007; Mogensen et al., 2009). Ochratoxin A is nephrotoxic, teratogenic, immunosuppressive, and classified as a potential carcinogen in humans by the International Agency on Research on Cancer (IARC, 1993). Ingestion of staple foods contaminated with elevated levels of ochratoxin a by humans is associated with Balkanic Endemic Nephropathy, a chronic condition that leads to kidney failure (Vrabcheva et al., 2004). Ochratoxin A was originally described as a mycotoxin produced by *Aspergillus ochraceus*; however since *A. niger* was reported as ochratoxin A producer (Abarca et al., 1994), other black aspergilli are reported to produce this secondary metabolite. Recent studies also reported that *A. niger* strains are able to produce fumonisins, another group of mycotoxins described as secondary metabolite produced by filamentous fungi in the genus *Fusarium* (Logrieco et al., 2010; Varga et al., 2010). In humans, the ingestion of contaminated crop grains with fumonisins has been strongly associated with esophageal cancer and neural tube defects in areas where maize

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is a dietary staple (Gelineauvan Waes et al., 2009; Shephard et al., 2000). The production of ochratoxins and fumonisins by black aspergilli presents a potential food safety problem for human and animal health because the fungal species within this group can infect important crops such as maize, grape, and peanut.

The visualization of plant–pathogen interactions has been facilitated by using molecular markers such as the green fluorescent protein (GFP) isolated from *Aequorea victoria* (Prasher et al., 1992), especially for dissecting the endophytic-plant host associations for bacteria and fungi. Recent studies showed the efficient integration of the GFP gene into fungal endophytes, including the slow-growing endophytic fungus *Undifilum oxytropis* (Mukherjee et al., 2010), the natural root endophyte *Fusarium equiseti* (Macia-Vicente et al., 2009), and the wide host range fungus *Muscodor albus* (Ezra et al., 2010). The success of GFP as live imaging marker for fungal cells has increased the interest of many researchers to develop and optimize vectors to express fluorescent proteins with different excitation and emission wavelengths, including the enhanced yellow fluorescent protein (eFYP), and the monomeric red fluorescent protein ($mRFP_1$) (Andrie et al., 2005). The main advantage of using fluorescent markers to monitor microbe–plant associations over traditional techniques relies on the fact that fluorescent proteins do not require preparatory steps, which might affect the structure of living cells. A major aim of this study was to develop an efficient protoplast-based transformation system for the black aspergilli using *A. niger* and *A. carbonarius* as examples. Other objectives were to determine maize–aspergilli *in planta* interactions and to compare the production of ochratoxin A by transformed and wild type strains as an indicator of genetic alterations of the transformants.

2. Materials and methods

2.1. Black aspergilli strains and plasmids

The two black-spored species used for genetic transformation were provided by Maren Klich, USDA-ARS, Southern Regional Research Center (SRRC), New Orleans, LA. The origins of *A. niger* var. *niger* SRRC 13 (=NRRL 2042, National Research Laboratory, USDA-ARS, Peoria, IL) and *A. carbonarius* SRRC 2131 (=FRR 639, Food Research in North Ryde, New South Wales, Australia) have been described earlier (Palencia et al., 2009). The plasmids used for genetic transformation included pCA45, expressing enhanced yellow fluorescent protein (eYFP), and pCA51, expressing the monomeric red fluorescent protein ($mRFP_1$) (Andrie et al., 2005). These plasmids were provided by Lynda Ciuffetti, Department of Botany and Plant Pathology, Oregon State University. The plasmids pCA45 and pCA51 contained the hygromycin resistance gene (*hph*) as selective marker and the *Tox A* promoter from *Pyrenophora tritici-repentis* to drive the constitutive expression of eYFP and $mRFP_1$ (Carroll et al., 1994).

2.2. Culture and protoplasting conditions

The fungi were stored at −80 °C in a 0.01% Tween 80, 15% glycerol solution. Potato dextrose agar slants were inoculated with a 10-μl loop sample of *A. niger* SRRC 13 and *A. carbonarius* SRRC 2131 stock solutions and incubated at 25 °C for 10 days. Spore suspensions were prepared by adding 10 ml of sterile 0.01% Tween 80 solution to the PDA slants and filtering the resulting suspension through sterile cheesecloth. Fungal suspensions were concentrated by centrifugation at 3000 rpm for 10 min. Protoplasts were generated using a modified method described in Szewczyk et al. (2007). Briefly, a suspension of 1×10^8 spores/ml was added to 20 ml of complete medium consisting 500 ml of autoclaved distilled water containing: 1.0 g $NaNO_3$, 1.25 g N-Z case, 0.5 g yeast extract, 15 g sucrose, 0.5 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.25 g, 0.1 ml trace elements, and 5 ml of a vitamin solution (Szewczyk et al., 2007). This spore solution was incubated at 30 °C and 200 rpm for 16 h in an incubator shaker (Innova 4300, New Brunswick Scientific, Edison, NJ). After incubation, the hyphal mat was collected using sterile cheesecloth and aseptically transferred to 8 ml of a protoplasting solution and incubated in a rotary incubator at 80 rpm for 4 h at 30 °C. The protoplasting solution was prepared by mixing 10 ml of a citric acid buffer (1.1 M KCl, 0.1 M citric acid pH 8) with 1.34 g of Vinoflow enzyme (Gusmer Enterprises Inc., Napa, CA). This was followed by a filtration step using an Acrodisc 0.2 μm HT Tuffryn membrane syringe filter (Pall Corporation, Ann Arbor, MI).

Protoplasts were harvested by a slow addition of 20 ml of a cold 1.2 M sucrose solution and centrifuged at 3400 rpm for 20 min. After forming an interface, protoplasts were transferred to a sterile 50 ml conical tube and suspended in 40 ml pre-chilled sorbitol–tris–calcium (STC) buffer: 1.2 M sorbitol, 50 mM $CaCl_2 \cdot 2H_20$, and 10 mM Tris–HCl pH 8.0. The suspension was centrifuged at 2500 rpm for 15 min at room temperature. The concentration of the protoplast suspension was determined by using a hemocytometer (Reichert Scientific Instruments, Buffalo, NY).

2.3. Transformation of black-spored Aspergillus species

Genetic transformation of *A. niger* SRRC 13 and *A. carbonarius* SRRC 2131 was performed using a modified polyethylene glycol (PEG) mediated transformation method previously described (Glenn et al., 2008). Briefly, 10 μg of DNA plasmid vectors was separately mixed with 100 μl of STC buffer, 100 μl protoplast solution (1×10^7 protoplasts), and 50 μl of 30% PEG (Sigma Aldrich, Milwaukee, WI). The mixture was incubated for 20 min at room temperature, followed by the addition of 2 ml of 30% PEG. Finally, the resulting solution was mixed with a 2 ml-aliquot of STC buffer and 36 ml of molten overlay medium (0.22 g yeast extract, 0.22 g casein enzymatic hydrolysate, 2.2 g agarose in 110 ml of distilled deionized water). A 5-ml aliquot of the mixture was poured over 20 ml of solidified regeneration medium (2.0 g yeast extract, 2.0 g casein enzymatic hydrolysate, 32 g agar, 1.6 M sucrose) in 100 mm diameter Petri dishes. Dishes were incubated at 30 °C for 48 h, during which time single colonies were visible on the regeneration medium. Because both plasmid vectors carry the *hph* gene, putative fluorescent transformants were selected after overlaying cultures with10 ml of 1% water agar amended with hygromycin B (Roche Diagnostics, Indianapolis, IN). Two concentrations of hygromycin B were used: 300 μg/ml for *A. niger* yellow fluorescent transformants; and 150 μg/ml for *A. carbonarius* red fluorescent transformants. Mitotic stability of fluorescent transformants was monitored by transferring transformants to non-selective medium. The yellow and red fluorescent transformants were subcultured on PDA slants without the selective pressure of hygromycin B for five generations.

2.4. DNA manipulation and molecular biology analysis

Plasmids, pCA45 and pCA51, were shipped and recovered as described by Rosman and Miller (1990). The recovered plasmids were transformed into TOP10 electrocompetent *E. coli* strain (Invitrogen, Carlsbad, CA) by electroporation, following the manufacturer's instructions. Individual putative transformed *E. coli* colonies were picked and propagated in Luria–Bertani medium amended with ampicillin (50 μg/ml) as selective agent. Transformed *E. coli* cells resistant to ampicillin were stored at −80 °C in a 15% glycerol solution. For high DNA yields, the DNA extraction for fluorescent expression plasmid vectors was performed using a QIAGEN large-construct kit (Qiagen, Valencia, CA). Putative *A. niger* and *A. carbonarius* transformants were preliminarily screened by PCR using the rapid method of Zhang et al. (2010). Briefly, putative transformants resistant to hygromycin were individually inoculated onto PDA dishes and incubated at room temperature in darkness. After 3 days, hyphal tissue was aseptically transferred using sterile toothpicks into 100 μl of sterile water in a 1.5-ml centrifuge tube. The fungal suspension was vortexed and centrifuged at 10,000 ×*g* for 1 min to remove PCR inhibitors. Water was discarded and the pellet