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GFP sheds light on the infection process of avocado roots by *Rosellinia necatrix*

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ABSTRACT

In order to monitor *Rosellinia necatrix* infection of avocado roots, we generated a plasmid vector (pCPXHY1eGFP) constitutively expressing EGFP and developed a protoplast transformation protocol. Using this protocol, four *R. necatrix* isolates were efficiently transformed and were shown to stably express EGFP homogeneously while not having any observable effect on pathogenicity. Confocal laser scanning microscopy (CLSM) images of avocado roots infected with the highly virulent isolate CH53-GFP demonstrated that fungal penetration of avocado roots occurs simultaneously at several random sites, but it occurs preferentially in the crown region as well as throughout the lenticels and in the junctions between epidermal cells. Not only were *R. necatrix* hyphae observed invading the epidermal and cortical root cells, but they were also able to penetrate the primary and secondary xylem. Scanning electron microscopy (SEM) images allowed detailed visualisation of the hyphal network generated by invasion of *R. necatrix* through the epidermal, cortical and vascular cells, including hyphal anastomosis and branching points. To our knowledge, this is the first report describing the construction of GFP-tagged strains belonging to the genus *Rosellinia* for monitoring white root rot using CLSM and SEM.
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1. Introduction

The most important diseases affecting avocado (*Persea americana* Mill) orchards are *Phytophthora* root rot, caused by the oomycete *Phytophthora cinnamomi,* and white root rot, caused by the ascomycete fungus *Rosellinia necatrix*. While *P. cinnamomi* affects avocado orchards worldwide, infections caused by *R. necatrix* result in considerable damage to avocado trees inherent to the Mediterranean area, but primarily located in Israel and Spain. In addition, *R. necatrix* has been recognised to cause losses in many economically important crops and to cause the destruction of both ornamental and fruit trees, including apple trees in Japan and India, Japanese pear trees in Japan, and some tropical and subtropical species such as the mango tree (Sztejnberg and Madar, 1980; Hoopen and Krauss, 2006). Affected trees show rotting of the roots and are characterised by a yellowing of the leaves that eventually wilt and ultimately result in the death of the tree within a few weeks after the appearance of the first foliar symptoms.

Currently, there is little available information regarding the mechanism of infection of fruit tree roots by *R. necatrix* that go beyond pathologic-anatomical observations and light microscopy visualisation of infected roots grown under axenic conditions. In this sense, fungal invasion of young mulberry tree roots has been reported to take place by boring and dissolving cork cell and, on rare occasions, by wedging them. Alternatively, invasion of adult roots into the inner tissues appears to occur primarily through the suberized closing layers of the lenticels, generally as hyphal strands (Sakurai, 1952). In addition, penetration of *R. necatrix* into apple roots has been reported to occur in various phases, each involving different forms of hyphal aggregates (Tourvieille de Labrouhe, 1982). Nevertheless, a detailed description of the infection process of *R. necatrix* has not been reported to date for avocado or any other fruit tree.

A valuable tool to study the behavior of microbes in their natural environments, such as in soil, in a living plant, or in an animal host, is the use of reporter auto-fluorescent proteins (AFPs). In contrast to other reporters that depend on cofactors or additional substrates for activity, it is possible to visualise AFP expression in vivo within individual cells or throughout the entire organism interacting with their hosts. The vast majority of studies utilising AFP technology in fungi have used modified forms of the green fluorescent protein (GFP) (Chalfie et al., 1994), such as SGFP (Chiu et al., 1996) and EGFP (Cormack et al., 1996), which confer higher levels of fluorescence in filamentous fungi with no obvious effects on fungal growth or pathogenicity. Although a large number of GFP expression vectors have been developed for all major classes of filamen-

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tous fungi (for a review, see Lorang et al., 2001), GFP tagging of a specific fungal strain largely depends on both the development of efficient transformation protocols and the stable expression of the GFP gene in the fungus under the environmental conditions to be studied.

A number of transformation systems have been developed for filamentous fungi, including plant pathogens. However, to the best of our knowledge, only two studies have reported the transfer of exogenous genes to *R. necatrix*. Kanematsu and co-workers (2004) reported the transformation of *R. necatrix* protoplasts with plasmids pSH75 (Kimura and Tsuge, 1993) and pAN7.1 (Punt et al., 1987). In addition, *Agrobacterium tumefaciens*-mediated transformation of *R. necatrix* has also been reported (Aimi et al., 2005). Nevertheless, although hygromycin B (HygB) resistance was used as the selectable marker, a method that is widely used in fungi and is conferred by the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*), low transformation efficiency was reported in both of the aforementioned transformation protocols. Importantly, GFP tagging of *Rosellinia* spp. has not been reported. Therefore, the aim of this study was to develop a transformation system for *R. necatrix*, with hygromycin B resistance as the selectable marker and using EGFP as a visual marker, in order to facilitate microscopic visualisation of the infection process of avocado roots by this plant pathogen.

2. Material and methods

2.1. Fungal isolates and culture conditions

Fungal strains and plasmids used in this study are listed in Table 1. Fungal strains were grown at 25 °C on potato dextrose agar (PDA; Difco Laboratories, Detroit). Micellial growth on different hygromycin B (HygB) concentrations (10, 20, 40, 50, 75, and 100 μg/ml) was tested on PDA solid medium for all *R. necatrix* wild-type strains include in this study. None of the strains grew at concentrations equal or higher than 50 μg/ml. Routinely growth of transformed strains was performed on media containing 50 μg/ml of HygB.

2.2. Construction of plasmid pCPXHY1eGFP to express GFP in R. necatrix

The plasmid pCPXHY1eGFP, which contains the *egfp* gene and the *hph* gene that, respectively, encode for the enhanced green fluorescent protein and a selectable marker that confers resistance to HygB, was constructed as described below. The complete *egfp* open reading frame (Takano et al., 2001) was amplified by PCR using the plasmid pGLEGFP (Takano, Y., Kyoto University) as a template and the primers eGFPF-Sac (5′-GAGCTCGCCACCATGGTGAGC AAG-3′) and eGFPR-Stu (5′-GAGGCCTCTTTACTTGTACAGCTC-3′). The amplified fragment was digested with *Sac*I and *Stu*I and cloned into the *Sac*I/*Stu*I-digested pCPXHY1 (Craven et al., 1993) resulting in the plasmid pCPXHY1eGFP. Therefore, this vector contains the *egfp* gene under the control of the P*gpd* promoter and the T*gpd* terminator of the *Aspergillus nidulans gpd* (glyceraldehydes-3-phosphate dehydrogenase) gene and the *hph* gene flanked by the *A. nidulans* P*trp*C promoter and T*trp*C terminator (Cullen et al., 1987) in a pUC19 background (Fig. 1).

2.3. Isolation and transformation of R. necatrix protoplasts

Protoplast generation from *R. necatrix* was performed as described previously by Kanematsu and co-workers (2004) with slight modifications. Briefly, mycelia that were grown in potato dextrose broth (PDB; Difco Laboratories, Detroit) for 7 days at 25 °C were subsequently homogenised in 120 ml of fresh PDB using a Waring blender at 7000 rpm for 45 s. Aliquots of 10 ml of the mycelia suspension were added to 12 replicate flasks containing 30 ml of PDB and incubated for 2 days at 25 °C. Young mycelia were re-suspended gently in a filter-sterilized enzyme–osmoticum mixture that contained 0.6 M mannitol osmotic solution, 0.4% of an enzyme–osmoticum mixture containing zymolyase 100T and 1.5% lysing enzymes (Sigma–Aldrich, St. Louis).

For the transformation, 10 μg of the corresponding plasmid vector was gently mixed with the protoplast suspension in a Falcon tube and placed on ice for 30 min. Following the addition of 500 μl of PEG solution [PEG 4000], the protoplast suspension was gently mixed and incubated at 20 °C for 20 min. Each tube received 700 μl of regeneration broth (PDB supplemented with 0.5 M glucose) and was incubated at 25 °C for 7 days. Aliquots containing 300 μl of protoplasts suspension were plated on 10 ml of YCDA (0.1% yeast extract, 0.1% casein hydrolysate, 0.5 M glucose and 1.5% agar in a 9 cm Petri dish) and incubated at 25 °C in the dark for 2 weeks. Selection of transformants was carried out by overlaying PDA regeneration plates with 10 ml of PDA medium supplemented with HygB 80 μg/ml.

2.4. Recombinant DNA techniques

Overall, basic molecular biology techniques were performed following standard methods (Sambrook and Russel, 2001).

Total DNA was extracted from *R. necatrix* isolates pre-grown on PDA plates for 7 days in the dark at 25 °C. Small blocks containing mycelia were excised from the colony and transferred onto a PDA plate containing a cellulose membrane. After incubation at 25 °C for 5 days, genomic DNA was extracted as described by Nakayashiki et al. (1999). PCR amplification of the *hph* gene was performed for detection purposes using primers *hph*F (5′-GACCTATTGCA TCTCCCGCCGTG-3′) and *hph*R (5′-TCGCCTCGCTCCAGTCAATG-3′), which amplified a 400 bp sequence. The PCR amplification conditions included an initial denaturing step at 95 °C for 10 min and preceded a three-step cycle of 94 °C for 1 min, 56 °C for 30 s and 72 °C for 1 min, repeated for a total of 35 cycles.

Table 1
R. necatrix strains used in this study.

Name	Virulence[a]	Host	Country (province) of isolation	Year of isolation	No. of transformants[b]	References
CH10	V	Avocado	Spain (Granada)	1986	0	López et al. (2008)
CH12	AV	Avocado	Spain (Granada)	1988	20	López et al. (2008)
CH53	V	Avocado	Spain (Granada)	1991	1	López-Herrera and Zea-Bonilla (2007)
CH29	AV	Avocado	Spain (Malaga)	1990	1	This study
CH124	V	Avocado	Spain (Malaga)	1995	0	This study
CH320	V	Avocado	Spain (Malaga)	2001	0	López-Herrera and Zea-Bonilla (2007)
W37	V	Japanese pear	Japan (Chiba)	1994	5	Kanematsu et al. (2004)

[a] Virulence of *R. necatrix* isolates in the host of isolation. V, virulent; AV, avirulent.
[b] Number of transformants obtained for plasmid pCPXHY1eGFP.

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