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Agrobacterium-mediated transformation of *Guignardia citricarpa*: An efficient tool to gene transfer and random mutagenesis

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ABSTRACT

Guignardia citricarpa is the causal agent of Citrus Black Spot (CBS), an important disease in Citriculture. Due to the expressive value of this activity worldwide, especially in Brazil, understanding more about the functioning of this fungus is of utmost relevance, making possible the elucidation of its infection mechanisms, and providing tools to control CBS. This work describes for the first time an efficient and successful methodology for genetic transformation of *G. citricarpa* mycelia, which generated transformants expressing the gene encoding for the *gfp* (green fluorescent protein) and also their interaction with citrus plant. Mycelia of *G. citricarpa* were transformed *via Agrobacterium tumefaciens*, which carried the plasmid pFAT-gfp, contains the genes for hygromycin resistance (*hph*) as well as *gfp*. The optimization of the agrotransformation protocol was performed testing different conditions (type of membrane; inductor agent concentration [acetosyringone – AS] and cocultivation time). Results demonstrated that the best condition occurred with the utilization of cellulose's ester membrane; 200 μM of AS and 96 h as cocultivation time. High mitotic stability (82 %) was displayed by transformants using Polymerase Chain Reaction (PCR) technique to confirm the *hgr* gene insertion. In addition, the presence of gfp was observed inside mycelia by epifluorescence optical microscopy. This technique easy visualization of the behaviour of the pathogen interacting with the plant for the first time, allowing future studies on the pathogenesis of this fungus. The establishment of a transformation method

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for *G. citricarpa* opens a range of possibilities and facilitates the study of insertional mutagenesis and genetic knockouts, in order to identify the most important genes involved in the pathogenesis mechanisms and plant–pathogen interaction.

Introduction

Guignardia citricarpa Kiely (anamorph: *Phyllosticta citricarpa* McAlpine), an Ascomycete fungus, is the causal agent of Citrus Black Spot (CBS) disease in citrus plants. The most important symptoms caused by this disease are lesions on the rind of citrus fruits, which cause no internal decay (Kotzé 1981; Snowdon 1990; Smith *et al.* 1997), but fruits are depreciated in the fresh fruit market. Also, during fruit development, heavy infection close to the pedicel may induce premature fruit drop (Kotzé 1981; Smith *et al.* 1997) resulting in serious productivity losses.

Besides Brazil, the disease is present in countries like Argentina, Peru, Uruguay, South Africa, Mozambique, Kenya, Zambia, Zimbabwe, Japan, China, Hong Kong, Indonesia, Philippines, Taiwan, Australia, New Zealand (EPPO 2006), and recently, was reported at the United States of America (Adaskaveg *et al.* 2010; Schubert *et al.* 2010). This report was also investigated and confirmed by the Department of Agriculture of the United States (Adaskaveg *et al.* 2010). This disease has not been reported in North America thus far, marking a major spread of this pathogen in geographical context (Smith 2006).

At the European Union (EU), CBS is classified as A1 quarantine disease, which means that is not present in its member countries. Aiming to prevent the introduction of pathogens, they have a strong regulation that restricts the importation of citrus (Bonants *et al.* 2003). Detection of CBS disease in a single fruit prevents the landing of the good ones.

At CBS epidemic phase, *G. citricarpa* produces sexual and asexual spores. Pseudothecia along with ascospores are produced exclusively in decomposing leaves on the orchard floor (McOnie 1965; Kotzé 1981). Ascospores are released during rainfall events and are dispersed by wind. On the leaf surfaces in the presence of free water, ascospores infect the host by direct penetration and form a mycelial mass in the subcuticular region. The fungus then remains quiescent until the leaves fall or the fruit begins to ripen (Kotzé 2000).

Pycnidia with conidia are produced in hard spot and freckle spot fruit lesions as well as on dead branches and leaf litter prior to pseudothecia formation. When the pycnidia are mature, the conidia emerge from their ostiole covered by a mucilaginous substance and, in contact with water conidia are dispersed by splashing or being washed off by rain to nearby susceptible tissues, where new infections may occur (Kotzé 2000).

The underlying molecular mechanisms responsible for the complex symptomology have not yet been studied for *G. citricarpa*. Identification of plant infection and pathogenesis genes will enable us to address the key virulence aspects for this fungus and provide a foundation for better strategies to manage CBS. Analysis of gene functions can be accomplished *via* random mutagenesis or reverse genetic approaches. In this way, fungal transformation with heterologous DNA may result in random integration of the new genes into the fungal genome causing gene disruption as an insertional mutagenesis. The most important advantage of insertional mutagenesis over chemical or radiation mutagenesis is that the disrupted genes are tagged by the transforming DNA (T-DNA), which can be used to identify the disrupted genes (Sugui *et al.* 2005). Although classical genetic studies, such as parasexual and sexual recombination, had not been developed for *G. citricarpa*, functional genes related to plant infection, colonization, and pathogenesis can be identified through gene disruption by *Agrobacterium tumefaciens*-mediated transformation (ATMT), allowing the identification of genes related to these functions.

Many pathogenic fungi species and oomycetes have been already genetically transformated by this technique (Michielse *et al.* 2005; Lacroix *et al.* 2006). These results allow the study of the pathogenic behaviour of these fungi, such as the interaction with the plant, facilitating the comprehension of the pathogenic system, and the searching for control methods of disease. The plant–fungus interaction can also be studied by different approaches by microscopy techniques using reporter genes such as gene for green fluorescent protein (*gfp*) of the jellyfish *Aequorea victoria* (Lorang *et al.* 2001). The ATMT allow this kind of insertion as tool of study, and has been widely used for gene transfer and as a tool for insertional mutagenesis (Abdudeh *et al.* 2000; Blaise *et al.* 2007).

Michielse *et al.* (2005) describe different factors as determinants of processing efficiency, among them, the strain of *Agrobacterium*, concentration of acetosyringone (AS), conditions for cocultivation, and substrate composition.

Here we report the transformation of *G. citricarpa* mediated by *A. tumefaciens* for insertional mutagenesis, gene disruption. Also, we used a gfp tagged strain to study fruit colonization, showing that this is an important strategy to understand the pathogenesis of this fungus in citrus plants.

In this work, our major aims were:

1. To develop and optimise protocol for transformation of *G. citricarpa*
2. Evaluate transformation efficiency at different conditions of:
   a. Filter type
   b. Concentration of AS – bacterial virulence inductor agent
   c. Cocultivation period
3. To study the ability of *G. citricarpa* transformants to colonize the tissues of citrus fruit by microscopy technique
4. To check transformants for their infectiveness
5. To analyse enzymatic production modifications in transformants

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