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A highly efficient *Agrobacterium tumefaciens*-mediated transformation system for the postharvest pathogen *Penicillium digitatum* using DsRed and GFP to visualize citrus host colonization

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ABSTRACT

Penicillium digitatum is a major postharvest pathogen of citrus crops. This fungus broadly spreads worldwide and causes green mold disease, which results in severe losses for citrus production. Understanding of the citrus infection by *P. digitatum* may help develop effective strategies for controlling this pathogen. In this study, we have characterized a virulent strain of *P. digitatum* isolated in Vietnam and established a highly efficient *Agrobacterium tumefaciens*-mediated transformation (ATMT) system for this fungal strain with two newly constructed binary vectors. These binary vectors harbor dominant selectable markers for hygromycin or nourseothricin resistance, and expression cassettes for the red fluorescent protein (DsRed) or the green fluorescent protein (GFP), respectively. Using the established ATMT system, the transformation efficiency of the Vietnamese strain could reach a very high yield of 1240 ± 165 transformants per 10^6 spores. Interestingly, we found that GFP is much better than DsRed for *in situ* visualization of citrus fruit colonization by the fungus. Additionally, we showed that the transformation system can also be used to generate T-DNA insertion mutants for screening non-pathogenic or less virulent strains. Our work provides a new platform including a virulent tropical strain of *P. digitatum*, an optimized ATMT method and two newly constructed binary vectors for investigation of the postharvest pathogen. This platform will help develop strategies to dissect molecular mechanisms of host-pathogen interactions in more detail as well as to identify potential genes of pathogenicity by either insertional mutagenesis or gene disruption in this important pathogenic fungus.

1. Introduction

Citrus is considered as one of the most economically important crops in the world and harvested citrus fruits are usually stored for fresh consumption. During this storage period, the fruits are always in contact with environmental factors including fungal pathogens (Marcet-Houben et al., 2012; Palou, 2014). *Penicillium digitatum* is the most destructive postharvest pathogen causing green mold in citrus. This fungal pathogen can spread widely by dispersal of a large number of airborne asexual spores (conidia) produced on the surface of infected fruits. The fungus infects host fruits through the wounded peel and the disease can occur in different stages of citrus production including in fields, in storage houses or in commercial supply chains on the market. The growth of *P. digitatum* as white mycelium can be seen on citrus peel and the mold then turns olive color due to fungal sporulation (Lopez-Perez et al., 2015; Marcet-Houben et al., 2012; Palou, 2014). Understanding of the infection process of the fungus will help develop efficient strategies for controlling this pathogen. Until now although some genes involved in pathogenicity of *P. digitatum* have been identified (Harries et al., 2015; Lopez-Perez et al., 2015; Ma et al., 2016; Vilanova et al., 2016; Wang et al., 2016; Zhang et al., 2013b; Zhang et al., 2013c), molecular mechanism of the host citrus infection by the fungus remains unclear and needs to be further clarified. Therefore, provision of new tools and optimized methods for studies of *P. digitatum* will help researchers of the field perform specific experiments conveniently to understand more about the pathogenicity of this fungus.

Currently, there are two common methods for genetic transformation of filamentous fungi including *Agrobacterium tumefaciens*-mediated transformation (ATMT) and polyethylene glycol (PEG)-mediated protoplast transformation (de Groot et al., 1998; Michielse et al., 2005;

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Mullins et al., 2001). *A. tumefaciens*, a soil-borne bacterium, has the ability to transfer a DNA fragment (T-DNA) from its Ti (tumor inducing) plasmid into fungi. The T-DNA integration into the fungal genomes occurs randomly (Michielse et al., 2005). The most advantageous feature of the ATMT method is the direct use of fungal spores as the transformation material and therefore it avoids protoplast preparation, which is time-consuming and requires a tricky protocol with multiple complicated steps. When compared to other transformation methods, ATMT represents a simple method with high transformation efficiency and the obtained transformants possess stable T-DNA integrations (Michielse et al., 2005; Weld et al., 2006). For genetic transformation of filamentous fungi, the red fluorescent protein (DsRed) gene from the reef coral *Discosoma* sp. and the green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* are usually used as model marker genes (Eckert et al., 2005; Lorang et al., 2001; Mikkelsen et al., 2003). These genes have also been indicated to be useful fluorescent reporters for investigation of host-fungal interactions (Helber and Requena, 2008; Lu et al., 2004; Maor et al., 1998; Nizam et al., 2010; Oren et al., 2003; Paparu et al., 2009; Reusche et al., 2014; Sesma and Osbourn, 2004; Wu et al., 2016).

Recently, the ATMT method has been applied successfully to the citrus postharvest pathogen *P. digitatum* using hygromycin as a selection agent and the *GFP* as a reporter gene (Buron-Moles et al., 2012; Wang and Li, 2008). However, this method achieved the transformation efficiency of only 60 transformants per 10^6 spores (Wang and Li, 2008). In this study, we have isolated a new strain of *P. digitatum* and established a highly efficient ATMT system to express the fluorescent reporter genes *DsRed* and *GFP* in this fungal strain. With our optimized transformation system, we could obtain 1240 ± 165 transformants for 10^6 spores and the resultant GFP-tagged strains exhibited a good fluorescent signal for visualization citrus fruit colonization of the pathogenic fungus.

2. Materials and methods

2.1. Microbial strains

Escherichia coli DH5α (Grant et al., 1990) was used for plasmid propagation and the *A. tumefaciens* AGL1 (Lazo et al., 1991) was used for genetic transformation of the filamentous fungus *P. digitatum*. The newly isolated *P. digitatum* PdVN1 strain as a pure spore suspension containing 20% glycerol of final concentration was frozen in liquid nitrogen and stored in a freezer of − 80 °C.

2.2. Isolation and identification of a new P. digitatum strain

A rotten orange fruit with the symptom of green mold collected from a local market in Hanoi was used for isolation of *P. digitatum*. Some fungal spores from the decayed orange peel were picked with a sterile inoculating loop and resuspended in sterile water. This suspension was diluted and spread on potato dextrose agar (PDA) plates supplemented with chloramphenicol (100 μg/ml) to inhibit the growth of contaminated bacteria. The plates were incubated at 25 °C for 4–5 days to obtain fungal colonies. The dark yellowish-green (olive color) colonies were purified and examined for conidiophore structures under microscopy. In the next step, a *P. digitatum*-like isolate was selected and grown on the PDA plates at 25 °C for 4–5 days for harvesting fungal spores (Nguyen et al., 2016). The concentration of the obtained spore suspension was adjusted to 10^6 spores/ml. The virulence of the fungal isolate was tested on orange fruits *in vitro*. The spore suspension (10 μl) was inoculated to artificial wounds on the peel of orange fruits. The fruits were then incubated in sterile plastic boxes covered with a plastic foil at 25 °C until 6 days. For the negative control (mock), the sterile distilled water was used instead of the fungal spores.

For accurate identification of this pathogen, the rDNA internal transcribed spacer (ITS) of the fungus was analyzed. The fungal isolate was grown in potato dextrose broth (PDB) medium at 25 °C, 200 rpm for 4 days and the mycelium was harvested by filtration of the culture through Miracloth (Calbiochem, Darmstadt, Germany). Extraction of genomic DNA from the fungal mycelium was carried out as previously reported (Nguyen et al., 2016). The ITS region of rDNA was amplified from fungal genomic DNA by PCR using the universal primer pair ITS1/ITS4 (White et al., 1990) and Phusion® high-fidelity DNA polymerase (Thermo Scientific, Massachusetts, USA). The obtained PCR product was purified using Wizard® SV Gel and PCR Clean-Up (Promega, Madison, USA) and sequenced by 1st Base company (Singapore). The obtained ITS sequence of the PdVN1 strain was examined with the BioEdit 7.2 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and comparatively analyzed with the GenBank database using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The related fungal ITS sequences were extracted from the GenBank database for constructing the phylogenetic tree using the MEGA6 software (Tamura et al., 2013) with the neighbor-joining method and 1000 bootstrap replicates.

2.3. Testing of antibiotic sensitivity

The fungal strain was grown on the PDA medium supplemented with hygromycin or nourseothricin at different concentrations (0, 25, 50, 100 μg/ml). The plates were incubated at 25 °C for 3 days to examine the growth of the fungus. The antibiotic concentration, which inhibits completely fungal growth, was used for genetic transformation of the fungus.

2.4. PCR amplification

DNA fragments were amplified by PCR with specific primer pairs (Table 1) using Phusion® high-fidelity DNA polymerase (Thermo Scientific, Massachusetts, USA). The PCR procedure included parameters as follows: 94 °C (3 min); 35 cycles of 94 °C (30 s), 58 °C (30 s), 72 °C (30 s to 1 min); 72 °C (10 min). The obtained DNA products were purified with Wizard® SV Gel and PCR Clean-Up (Promega, Madison, USA) following the manufacturer's instruction. For screening the plasmids in

Table 1
Primers used in this study.

Name	Sequence (5′–3′)	Target DNA sequence	Product size (bp)	Reference
ITS1	TCCGTAGGTGAACCTGCGG	ITS of rDNA in *P. digitatum*	586	White et al. (1990)
ITS4	TCCTCCGCTTATTGATATGC			
DsRed-F	AACTCGAGCACGTGCTTAAGGATATCATGGCCTCCTCCGAGG	*DsRed* gene	729	Nguyen et al. (2016)
DsRed-R	AAGGATCCCCGCGGGAGCTCGATATCCTACAGGAACAGGTGGTGGC			
GFP-F	ATGGTGAGCAAGGGCGAG	*GFP* gene	720	Nguyen et al. (2016)
GFP-R	TCACTTGTACAGCTCGTCCATGC			
NAT-F	GGTTAATTAACAACTGATATTGAAGGAGCA (*Pac*I)	Nourseothricin resistance gene (*NAT*) under the control of the *A. nidulans trpC* promoter	970	This study
NAT-R	GGACTAGTGCTTTGGTTTAGGGTTAGG (*Spe*I)			
HPH-F	CCGGTGACTCTTTCTGGC	Hygromycin resistance gene (*HPH*) under the control of the *A. nidulans gpdA* promoter	1890	This study
HPH-R	CTATTCCTTTGCCCTCGG			