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Differential contribution of the two major polygalacturonases from *Penicillium digitatum* to virulence towards citrus fruit

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ABSTRACT

The fungus *Penicillium digitatum* is the causal agent of the citrus green mould, the major postharvest diseases of citrus fruit. Lesions on the surface of infected fruits first appear as soft areas around the inoculation site, due to maceration of fruit. The macerating activity has been associated with pectinases secreted by the fungus during infection. In order to evaluate the contribution to virulence and macerating activity of the two major polygalacturonases (PGs) secreted by *P. digitatum*, we have obtained and characterized mutants lacking either *pg1* or *pg2*, the genes encoding PG1 and PG2, respectively. Disease incidence of deletants in either gene was not different from that of the parental strain or ectopic transformants. However, disease progressed more slowly in deletants, especially in those lacking the *pg2* gene. The lesions originated by the Δ*pg2* deletants were not as soft and the pH was not as acid as those originated by either the wild type strain or the ectopic transformants. Total PG activity in the macerated tissue was also lower in fruits infected with the Δ*pg2* deletants. Interestingly, the macerated tissue of oranges infected with Δ*pg2* deletants showed around 50% reduction in galacturonic acid content with respect to lesions caused by any other strain.

1. Introduction

Green mould rot, caused by *Penicillium digitatum*, is the most common postharvest disease affecting citrus fruit in Spain (Tuset, 1987) and all production areas characterized by low summer rainfall (Eckert and Eaks, 1989). This pathogen may invade the fruit during the preharvest period through injuries occurred in the field, or/and in the packinghouses during storage and shelf-life periods. *P. digitatum* is a specialist pathogen that under natural conditions infects citrus fruit uniquely, although previous works demonstrated that it can infect overripe apple tissues (Buron-Moles et al., 2012; Vilanova et al., 2012; Vilanova et al., 2014). The use of synthetic fungicides has been the standard procedure to control this pathogen (Harding, 1972). However, these chemical treatments have several disadvantages, such as the persistence of the residues on the treated fruit, increase of the pathogen-resistant strains, as well as health and environmental problems (Bus, 1992). New approaches for designing new and safer control strategies would benefit from the knowledge of the molecular mechanisms underlying the pathogenesis of *P. digitatum*.

P. digitatum is a necrotrophic wound pathogen that requires preexisting injured fruit peel to penetrate the plant tissue (Kavanagh and Wood, 1967). Necrotrophs kill host cells by means of toxic molecules, which can be either host-specific, as tentoxin, or nonhost-specific toxins, as AM toxin, and lytic enzymes. However, the ultimate purpose of a necrotroph is not to kill its host, but to discompose the plant tissue and utilize the host-derived nutrients for its own growth (Zhang and Van Kan, 2013). During infection, necrotrophic plant pathogens macerate the host tissue by secreting significant amounts of carbohydrate-active enzymes (CAZYmes) that contribute to the degradation of plant cell wall polymers to obtain the nutrients required for its development (Zhao et al., 2013). Among these CAZYmes special attention has been paid to those involved in pectin degradation. Pectin is the collective name for a complex of polysaccharides that constitute the major carbohydrate type in the middle lamella (Jayani et al., 2005; Caffall and

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Table 1
Primers used in this study.

Name	Sequence (5′ to 3′)	Purpose
pg1-O1	GGTCTTAAUGCCCCACTGGTCGATCCTAACCTTCCA	Amplification of the upstream region of *pg1*
pg1-O2	GGCATTAAUTGGGGGTTGACGCTTGCATAACAGAGC	Amplification of the upstream region of *pg1*
pg1-A3	GGACTTAAUGCCAGCGATCAAATGGTGAACACCAAAC	Amplification of the downstream region of *pg1*
pg1-A4	GGGTTTAAUAAGCGTCTGCGTGGTGGTGTGCAGT	Amplification of the downstream region of *pg1*
pg2-O1	GGTCTTAAUTGCGTGGTCTGTGGGGTGGTCGTTT	Amplification of the upstream region of *pg2*
pg2-O2	GGCATTAAUTGGGTGCCGGTGTTCAATCCAGTCA	Amplification of the upstream region of *pg2*
pg2-A3	GGACTTAAUTTTGACTCCTTGCTGGCCGGGCTTG	Amplification of the downstream region of *pg2*
pg2-A4	GGGTTTAAUTCCGCTCGTGAACAGGAGCACGTTG	Amplification of the downstream region of *pg2*
RF-1	AAATTTTGTGCTCACCGCCTGGAC	Analysis of plasmid constructs
RF-2	TCTCCTTGCATGCACCATTCCTTG	Analysis of plasmid constructs
RF-5	GTTTGCAGGGCCATAGAC	Analysis of plasmid constructs
RF-6	ACGCCAGGGTTTTCCCAGTC	Analysis of plasmid constructs
HMBF1	CTGTCGAGAAGTTTCTGATCG	Amplification of the hygromycin B resistance marker
HMBR1	CTGATAGAGTTGGTCAAGACC	Amplification of the hygromycin B resistance marker
pg1-F7	AAGCTCGATGGAATAGCTT	Detection of double homologous recombination at the *pg1* locus
pg1-R7	CCCAGTAAAAGGACATGC	Detection of double homologous recombination at the *pg1* locus
pg1-F8	AAAGAAGAAGCCCAAGTTCT	Detection of *pg1*
pg1-R8	AGCTACCGTTACCGCAGAGA	Detection of *pg1*
pg2-F7	ATGCTATTGGTTCTTTCCTC	Detection of double homologous recombination at the *pg2* locus
pg2-R7	TCCCTCCGTAAACTAAACAA	Detection of double homologous recombination at the *pg2* locus
pg2-F8	TCGATGGCGCTAAGGAGCTTACT	Detection of *pg2*
pg2-R8	CTCGGCACACAGAATGTA	Detection of *pg2*
pg1-F9	CGGACGGAGTAGATCTCACAACT	Determination of T-DNA copy number in *pg1* transformants
pg1-R9	CCTGCGCTAACATCCTCATGAAAC	Determination of T-DNA copy number in *pg1* transformants
pg2-F9	CCTCGTGGTGCTTGTACCTTCTC	Determination of T-DNA copy number in *pg2* transformants
pg2-R9	TCAGGGTAATGGTCGAGCAAGC	Determination of T-DNA copy number in *pg2* transformants
betatubPDIG1	CGATGGCGATGGACAGTAAGTTT	Determination of T-DNA copy number in *pg1* and *pg2* transformants
betatubPDIG2	TTGGTTCGTGGTCGTTGTACTCA	Determination of T-DNA copy number in *pg1* and *pg2* transformants

Mohnen, 2009). The most abundant type of pectin is homogalacturonan, a linear polymer of α-1,4-linked D-galacturonic acid, which can be modified by acetylation and methyl-esterification. Other pectins include rhamnogalacturonan I and II, and xylogalacturonan. Enzymes involved in the degradation of the pectin backbone include polygalacturonases (PGs), pectate and pectin lyases (PLs), rhamnogalacturonases and rhamnogalacturonase lyases (recently reviewed by Ramoni and Seiboth (2016)). Depending upon the pattern of action (random or terminal) polygalacturonases (PGs) are termed as endo- or exo-enzymes, respectively. Endo-PGs are widely distributed among fungi, bacteria and many types of yeast whereas, in contrast, exo-PGs occur less frequently (Jayani et al., 2005).

PGs play a critical role in pectin degradation by fungal pathogens and they hydrolyse the polygalacturonic acid chain across the oxygen bridge (Jayani et al., 2005). PG activity has been detected in decayed tissue and has been implicated as a virulence factor in several soft rot diseases (Reignault et al., 2008). In some pathogens, the disruption of PG genes reduced virulence, which suggests that this enzyme is a significant virulence factor in several plant-infecting fungi (Scott-Craig et al., 1990; Shieh et al., 1997). However, in several other cases, disruption of cell wall-degrading enzymes caused only partial or no reduction in pathogenicity, suggesting that not all enzymes produced by the pathogen are required for pathogenicity (Scott-Craig et al., 1990). However, few studies on *P. digitatum*'s cell walls degrading enzymes (CWDEs) encoding genes as virulence factors have been conducted so far (López-Pérez et al., 2015; T. Zhang et al., 2013; T.Y. Zhang et al., 2013). T.Y. Zhang et al. (2013) have shown that a *P. digitatum* mutant lacking the polygalacturonase PG2 was able to infect citrus fruits, although it was less virulent than the parental strain. A similar phenotype has been described for *P. digitatum* mutants lacking the pectin lyase PL1, which showed reduced virulence (López-Pérez et al., 2015).

In a recent work, the importance of CWDEs in the virulence of *P. digitatum* was highlighted because they constituted the second most abundant group of genes in a library containing up-regulated fungal genes during the infection of oranges (López-Pérez et al., 2015). The genome of *P. digitatum* is enriched in two families involved in pectin degradation, when compared to *P. chrysogenum*, a closely related but not pathogenic species. Thus, *P. digitatum* possesses eight polygalacturonases and rhamnogalacturonases belonging to family GH28 and three pectin methylesterases belonging to family CE8 (Marcet-Houben et al., 2012).

In order to clarify the role of *P. digitatum*'s PGs in pathogenicity, different strategies including physiological, biochemical and molecular investigations should be performed. In this work, we have compared the role in virulence of the two major PGs in *P. digitatum* by obtaining and characterizing in the same genetic background knockout mutants for the genes *pg1* and *pg2*, to provide evidence that these genes play a different role during pathogenesis on orange fruit.

2. Materials and methods

2.1. Fruits

'Valencia' orange fruits (*Citrus sinensis* L. Osbeck) were harvested from a commercial orchard in Tortosa (Catalonia, Spain) and processed the same day. Fruits were selected for uniform size, without physical injuries or apparent infections. Once the fruit arrived at the laboratory, they were surface-disinfected with a 10% commercial bleach solution for 1 min, rinsed with tap water and allowed to dry at room temperature. Colour index, firmness, soluble solids and acidity were determined as quality parameters following standard procedures (Vilanova et al., 2013).

2.2. Fungal strains and culture conditions

Conidial suspensions from *Penicillium digitatum* Sacc. isolate Pd1 (CECT20795; Marcet-Houben et al., 2012) were prepared by adding 5 mL of sterile water with 0.01% (w/v) Tween-80 over the surface of seven- to 10-day-old cultures grown on potato dextrose agar medium (PDA; 200 mL boiled potato extract, 20 g dextrose, 20 g agar and 800 mL water) and rubbing the surface of the agar with a sterile glass rod. Conidia were counted in a haemocytometer and diluted to 10^6 conidia mL^{-1} inoculum concentration.