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Differential expression of pectolytic enzyme genes in *Xanthomonas citri* subsp. *citri* and demonstration that pectate lyase Pel3 is required for the formation of citrus canker

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ABSTRACT

Bacterial canker, caused by *Xanthomonas citri* subsp. *citri* (Xcc), is one of the most destructive diseases of citrus. The pectolytic enzymes produced by phytobacteria are important virulence factors involved in tissue maceration, electrolyte loss and cell death of host plants. In this study, the promoter activity of the pectolytic enzyme genes *pel1*, *pel2*, *pel3*, *pglA*, and *peh-1* were investigated in Xcc XW19 strain using the β-glucuronidase (GUS) gene as a reporter. GUS activity expressed under the control of the *pel1*, *pel3*, *pglA*, and *peh-1* gene promoters positively correlated with bacterial growth. These gene promoters displayed high GUS activity in the presence of sodium polypectate. In addition, the four genes were induced in XVM2 minimal medium. However, only *pel1* was subjected to catabolite repression by glucose. GUS activity was significantly enhanced in the XW19-derived reporter strains after they were inoculated into the leaves of Mexican lime and grapefruit, suggesting the involvement of the *pel1*, *pel3*, *pglA*, and *peh-1* genes in XW19 pathogenesis. The *pel3* promoter produced the highest GUS activity under all test conditions, whereas no GUS activity was detected using the *pel2* promoter *in vitro* and *in planta*. In comparison with wild type Xcc, a *pel3* mutant generated from Xcc XW19 using unmarked mutagenesis displayed reduced growth and induced smaller canker lesions on the leaves of Mexican lime, demonstrating that Pel3 of Xcc strain XW19 is a virulence factor.

1. Introduction

Many phytobacteria are capable of producing pectolytic enzymes that are responsible for the degradation of pectin and the deposition of plant cell walls, causing tissue maceration, electrolyte loss, and cell death of host plants (Collmer and Keen, 1986). Pectolytic enzymes can be classified into several groups based on the types of substrates and the modes of action on pectin substances. Among them, pectate lyase (EC 4.2.2.2) and polygalacturonase (EC 3.2.1.15) are the major pectolytic enzymes that degrade pectic substances, in which the methoxyl group on C-6 of polymethylgalacturonate is removed by pectin methylesterase (PEM, EC 3.1.1.11) (Huang, 2001). Pectate lyase splits α-(1→4) glycosidic bonds of polygalacturonate to produce unsaturated oligosaccharides. Polygalacturonase cleaves α-(1→4) glycosidic bonds of polygalacturonate by hydrolysis to form saturated oligosaccharides (Collmer and Keen, 1986; Hugouvieux-Cotte-Pattat et al., 1996; Huang, 2001). The ability to disrupt plant cell wall by pectolytic enzymes is important for the pathogenesis of several plant pathogenic bacteria, including members of *Pectobacterium*, *Dickeya*, and *Pseudomonas* (Mount et al., 1970; Liao, 1989; Hugouvieux-Cotte-Pattat et al., 1996). Annotation of the sequenced genome of *Dickeya dadantii*, the causal agent of soft rot, identified at least seven pectate lyase-coding genes and one each of the genes encoding polygalacturonase, pectin methylesterase, and pectin lyase (Hugouvieux-Cotte-Pattat et al., 1996). The citrus canker bacterium *Xanthomonas citri* subsp. *citri* (Xcc) has three pectate lyase- and two polygalacturonase-coding genes (da Silva et al., 2002). Expression of the pectolytic enzyme genes in bacteria are affected by growth phase, temperature, osmotic pressure, catabolite repression, and nitrogen starvation (Hugouvieux-Cotte-Pattat et al., 1992). Pectate lyase is one of the major virulence factors in *Pseudomonas viridi-*

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flava, causing postharvest diseases of many crops (Liao et al., 1988). Moreover, pectate lyase has been shown to be required for the pathogenicity of *P. fluorescens* and *P. syringae* pv. *lachrymans* (Liao, 1991; Bauer and Collmer, 1997).

Certain pectolytic, yellow-pigmented strains of *Xanthomonas campestris* pv. *campestris*, *X. campestris* pv. *glycines*, and *X. campestris* pv. *vesicatoria* induce soft rot symptoms and cause problems on postharvest vegetables and fruits. These pathogenic bacteria are capable of producing pectolytic enzymes and causing maceration of potato tubers and green pepper (Starr and Nasuno, 1967; Liao and Wells, 1987; Beaulieu et al., 1991). The cotton blight pathogen, *X. campestris* pv. *malvacearum*, produces extracellular pectate lyases and also causes soft rot in potato tubers (Liao et al., 1996). However, *X. campestris* pv. *vesicatoria* XV56 mutants defective for pectate lyase exert wild-type virulence and growth in host plants, indicating that pectate lyase is not essential for virulence in this species (Beaulieu et al., 1991). In *X. campestris* pv. *campestris*, the expression of pectate lyase (*pelA1*), polygalacturonase (*pehA*), and pectin methylesterase (*pmeA*) is regulated by Clp and RpfF, subjected to catabolite repression, and repressed under conditions of oxygen limitation, high osmolarity, and nitrogen starvation (Hsiao et al., 2008, 2009, 2011). These pectolytic enzymes play no roles on promoting bacterial growth or symptom development in black rot disease (Dow et al., 1989; Hsiao et al., 2009, 2011). However, two polygalacturonases (*pghAxc* and *phgBxc*) of *X. campestris* pv. *campestris*, regulated by the type III secretion system regulators (HrpX and HrpG) and the global regulator Clp, are required for full virulence in the ecotype Kendle of *Arabidopsis thaliana* (Wang et al., 2008). It appears that the roles of pectolytic enzymes in xanthomonads and their interactions with plants vary considerably among different species, indicating the complexity of expression regulation of pectolytic enzyme genes.

Citrus canker, caused by *X. citri* subsp. *citri* (*ex* Hasse, 1915) (Gabriel et al., 1989; Schaad et al., 2006) (syn. *X. axonopodis* pv. *citri*), is one of the most destructive diseases in citrus production areas worldwide. Citrus canker is characterized by erumpent lesions on fruit, foliage, and young stems of susceptible citrus cultivars (Civerolo, 1984; Gottwald et al., 2002). The pectate lyase encoded by the *pel1* gene of the A (Asiatic)-type strain Xcc XW19 (Taiwan strain) has been genetically characterized to be involved in the formation of the water-soaked margins of canker lesions (Lin et al., 2010). In addition to *pel1* (XAC3562), two pectate lyase genes (XAC2373 and XAC2986) and two polygalacturonase genes (XAC2374 and XAC0661) were found in the complete genome of *X. citri* subsp. *citri* strain 306 (Xcc 306) (da Silva et al., 2002) whose nucleotide sequences are nearly identical to the homologous genes of Xcc XW19. However, their functions as pectolytic enzymes in the strains of Xcc 306 and XW19 remain uncharacterized. In addition, the conditions under which the corresponding genes are expressed are unknown. In this study, the expression patterns of three pectate lyase-coding genes, *pel1*, *pel2*, and *pel3*, and two polygalacturonase-coding genes, *pglA* and *peh-1*, which are respectively identical to Xcc 306 annotated genes XAC3562, XAC2373, XAC2986, XAC2374, and XAC0661, were investigated in Xcc XW19 using β-glucuronidase (GUS) as a reporter. Because GUS activity is not detectable in higher plants or most bacteria (Jefferson et al., 1987), GUS is an ideal reporter to investigate the expressions of *pel1*, *pel2*, *pel3*, *pglA*, and *peh-1* under different environmental conditions. The GUS-encoding gene *uidA* was transcriptionally fused with each of the *pel1*, *pel2*, *pel3*, *pglA*, and *peh-1* gene promoters, and the resultant reporter plasmids were transformed into XW19 to produce GUS reporter strains that were used throughout this study. Based on the level of GUS activity, we selected *pel3* for further genetic characterization and showed that *pel3* is required for bacterial virulence in citrus plants.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and DNA manipulations

The bacterial strains and plasmids used in this study are listed in Table 1. *Xanthomonas citri* subsp. *citri* XW19 was collected from a diseased grapefruit (*Citrus paradisi*) in Chiayi, Taiwan, and has been previously characterized (Wu et al., 1989). Xcc XW19r strain was a spontaneous mutant derived from XW19 growing on medium containing 40 ppm rifampicin. *Xanthomonas* strains were cultured on yeast extract-peptone-dextrose agar (YPDA) medium [0.7% (w/v) yeast extract, 0.7% (w/v) bactopeptone, 0.7% (w/v) dextrose, and pH 7.2] (Vernière et al., 1991) at 30 °C for 3 days. *Escherichia coli* strain DH10 B and its derived strains used to plasmid propagation were cultured on Luria-Bertani (LB) medium (Sambrook et al., 1989) at 37 °C. Assays for promoter activity were performed by growing bacterial strains in XVM2 medium commonly used for culturing xanthomonads [20 mM NaCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4, 0.01 mM $FeSO_4$, 10 mM fructose, 10 mM sucrose, 0.03% (w/v) casamino acids, and pH 6.7] (Wengelnik et al., 1996) or in XOLN medium containing 0.07% K_2HPO_4, 0.02% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.01% $MgCl_2{\cdot}6H_2O$, 0.001% $FeSO_4{\cdot}7H_20$, 0.0001% $MnCl_2$, 0.0625% yeast extract, and 0.0625% tryptone (pH 7.15) (Fu and Tseng, 1990). Glycerol, 2% (v/v), glucose, 2% (w/v), polygalacturonate, 0.5% (w/v) (Sigma-Aldrich, St. Louis, USA), or sodium polypectate, 0.5% (w/v) (NaPP, HP Bulmer, Plough Lane, UK) was added into medium as the sole carbon source. Antibiotics, including gentamicin (Gm, 20 $\mu g ml^{-1}$), kanamycin (Km, 50 $\mu g ml^{-1}$), and rifampicin (Rif, 20 $\mu g ml^{-1}$), were added into medium after autoclaving. Bacterial DNA was extracted according to the method described by Chen and Kuo (1993). DNA fragments and plasmids were purified using the PCR Clean-Up & Gel Extraction kit and Plasmid miniPREP kit (GeneDireX, Nevada, USA), respectively. Searching for consensus sequence in the promoter region was performed using a Vector NTI® program (Invitrogen, Carlabad, CA, USA).

2.2. Construction of transcription reporter plasmids

The upstream regions of the *pel1*, *pel2*, *pel3*, *pglA*, and *peh-1* genes used for promoter activity assays were amplified by PCR using a high fidelity Taq Plus DNA polymerase (Bio Basic Inc., Ontario, Canada) with primers containing restriction endonuclease cutting sites to facilitate further manipulations (Table 2). Each of the amplified products (ca. 500 bp) was cloned into the pCR II-TOPO cloning vector (Invitrogen) and confirmed by sequencing. A *Bam*HI or *Bam*HI-*Xba*I fragment was excised and transcriptionally fused with a promoterless *uidA* gene in pNCHU1842 carrying a Km resistance gene (Table 1) to generate pUBC6 (P*pel1*::*uidA*), pUBC7 (P*pel2*::*uidA*), pUBC8 (P*pel3*::*uidA*), pUBC9 (P*pglA*::*uidA*), and pUBC10 (P*peh-1*::*uidA*). The resultant plasmids were introduced into Xcc XW19 by electroporation (GenePulser, Bio-Rad, Hercules, CA, USA) according to the method described by Oshiro et al. (2006). Xcc XW19 strains carrying the constructed reporter plasmids pUBC6, pUBC7, pUBC8, pUBC9, and pUBC10 were named P19-1, P19-2, P19-3, P19-4, and P19-5, respectively. A P19-6 strain used as a negative control was created by transforming pNCHU1842 into Xcc XW19 strain.

2.3. Construction of the pel3 gene deletion mutation and complementation

The *pel3* gene was mutated by homologous recombination using an unmarked mutagenesis strategy (Fig. 1a). Primers pel3u-f and pel3u-r were used to amplify the upstream flanking region of *pel3* and primers pel3d-f and pel3d-r were used to amplify the down-