

# Phenotypic characterization of mutants of the citrus pathogen *Colletotrichum acutatum* defective in a PacC-mediated pH regulatory pathway

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## Keywords

cell wall-degrading enzymes; cutinase; lithium; toxicity.

## Introduction

*Colletotrichum acutatum* is a fungal phytopathogen causing disease in a wide range of plants. Key lime anthracnose (KLA) and postbloom fruit drop (PFD) of citrus are caused by two distinct *C. acutatum* pathotypes (Agostini *et al.*, 1992). The KLA pathotype infects all parts of the Key lime, causing anthracnose symptoms; it is also pathogenic to the flower petals of sweet orange, inducing young fruit abscission, as does the PFD pathotype. The mechanisms involved in young fruit abscission are postulated to be associated with a series of physiological modifications caused by an imbalance of plant hormones and/or growth regulators (Lahey *et al.*, 2004).

A mutant partially defective in Key lime pathogenicity was obtained previously by *Agrobacterium*-mediated mutagenesis; disruption of the *PacC<sup>KLAP2</sup>* gene, encoding a polypeptide resembling the pH-regulated PacC transcription factors of many filamentous fungi, was identified as being

## Abstract

Environmental pH plays an important role in the growth and differentiation of microorganisms. In fungi, one well-studied pH-response pathway is controlled by the transcriptional regulator PacC. The *PacC<sup>KLAP2</sup>* gene of the citrus pathogen *Colletotrichum acutatum* is required for virulence. Here, the phenotypes of *C. acutatum* mutants obtained by targeted disruption of *PacC<sup>KLAP2</sup>* are characterized. The *PacC<sup>KLAP2</sup>* null mutants displayed hypersensitivity to a wide range of compounds but were more tolerant than wild type to cell wall-degrading enzymes (CWDEs). The null mutants have lower cell-wall chitin content as well as lower cellulase, cutinase, xylanase, and catalase activities, but markedly increased pectolytic activities. Expression of the genes encoding endo-polygalacturonase and cellulase is higher in the null mutants compared with wild type, whereas expression of the gene for cutinase is almost completely abolished, suggesting that cutinase and other CWDEs may play a role in fungal pathogenicity.

responsible for this deficiency. *PacC<sup>KLAP2</sup>* is required for full virulence on both Key lime leaves and citrus flower petals (You *et al.*, 2007). In *Aspergillus nidulans*, the response to ambient pH is controlled by a global transcription factor, PacC, which up-regulates numerous alkaline-expressed genes and down-regulates acid-expressed genes; these include genes encoding xylanases, permeases, polygalacturonases, proteases, and phosphatases in various fungi (Denison, 2000; Arst & Peñalva, 2003). PacC also plays diverse roles in the biosynthesis of fungal toxins and secondary metabolites and regulates genes involved in fungal development and virulence (Peñalva & Arst, 2004).

In the present study, the mutant phenotypes resulting from the disruption of the *PacC<sup>KLAP2</sup>* gene in the citrus pathogen *C. acutatum* was further investigated and evidence is provided to support the role of this PacC-like transcription factor as a global regulator in fungi. The results also suggest that secretion of cell wall-degrading enzymes

(CWDEs) under  $PacC^{KLAP2}$  control may play a role in the infection process of *C. acutatum*.

## Materials and methods

### Fungal strains and culture conditions

The wild-type KLA207 strain of *C. acutatum* J.H. Simmonds was isolated from infected leaves and has been described elsewhere (Chung *et al.*, 2002). The  $PacC^{KLAP2}$  disruption mutants (D3, D4, and D5) and the strains C26 and C27 expressing a functional  $PacC^{KLAP2}$  were created in a previous study (You *et al.*, 2007). The pH of media was adjusted with 0.1 M sodium phosphate buffer as appropriate. Assays for hypersensitivity were conducted on regular potato dextrose agar (PDA; Difco, pH 5.6) plates or a complete medium (CM) containing 1 mg  $Ca(NO_3)_2 \cdot 4H_2O$ , 0.2 mg  $KH_2PO_4$ , 0.25 mg  $MgSO_4 \cdot 7H_2O$ , 0.15 mg NaCl, 10 g glucose, 1 g yeast extract, and 1 g casein hydrolysate  $L^{-1}$ . Sensitivity was calculated as a cumulative percentage of the growth of wild type and the  $PacC^{KLAP2}$  null mutants grown on the same plate.

### Miscellaneous assays for enzymatic activities

Extracellular activities of CWDEs were determined by measuring the amounts of reducing sugar released from 1% polygalacturonic acid (PGA), 1% citrus pectin, 0.5% carboxymethyl-cellulose (CMC), or 0.5% xylan (hemicellulose), and reacted with dinitrosalicylic acid (DNS) reagent under alkaline conditions (Bailey *et al.*, 1993). The regression line and correlation coefficient ( $r^2 > 0.98$ ) were established using glucose as a standard. One unit of enzyme activity is defined as that required to liberate 1  $\mu$ mol of glucose from the substrate per minute. Fungal isolates were grown for 7 days on modified CM (Chen *et al.*, 2005) containing a polysaccharide as the sole carbon source. Two 6-mm agar plugs bearing fungal mycelia were placed in 0.1 M sodium acetate buffer (pH 5.0) containing 0.5% CMC or xylan, or in Tris buffer containing 1% PGA or citrus pectin (pH 4.5 and 7.6). After incubation at 50 °C for 1 h, an equal volume of DNS reagent was added and boiled at 95 °C for 5 min and measured at  $A_{540\text{ nm}}$ .

Extracellular cutinase activities were determined by formation of a yellow color after reaction with 5 mM *para*-nitrophenyl butyrate (PNPB) dissolved in 50 mM potassium phosphate (pH 5.0) for 1 h and measured at  $A_{405\text{ nm}}$  (Stahl & Schäfer, 1992). Fungal isolates were grown on CM containing 0.1% 16-hydroxyhexadecanoic acid (HHDA) (dissolved in 1% sodium acetate) for 5 days for induction before the enzymatic assays. One unit of cutinase released 1  $\mu$ mol *p*-nitrophenol  $min^{-1}$ .

Cellular catalase activities were determined by measuring the decomposition of  $H_2O_2$  with a colorimetric reagent

(0.25 mM 4-aminoantipyrine and 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid in 150 mM potassium phosphate, pH 7.0) (Johanson & Borg, 1988). Protein extracts were obtained by grinding fungal mycelia in liquid nitrogen, soaking in ice-cold 50 mM potassium phosphate buffer (pH 7.0), and collecting the supernatant after centrifugation at 10 000 g for 15 min. Protein concentration was determined by a protein assay kit (Bio-Rad). Briefly, 32  $\mu$ g of fungal proteins were mixed with 3%  $H_2O_2$ , incubated at room temperature for 5 min, and stopped by adding 15 mM sodium azide. The reactions were then treated with 1 U horseradish peroxidase (Sigma) and a colorimetric reagent for 15 min to produce a red quinoneimine dye [*N*-(4-antipyril)-3-chloro-5-sulfonate-*p*-benzoquinone-monoimine], and measured at  $A_{520\text{ nm}}$ . One unit of catalase is defined as that required to consume 1  $\mu$ mol  $H_2O_2$   $min^{-1}$ . Peroxidase activities were estimated using 3,3-diaminobenzidine tetrahydrochloride (DAB) as a chromogen as described (Hsu & Soban, 1982). Protein crude extracts were mixed with 0.05 M Tris-HCl (pH 7.6) containing 50 mg DAB and 2% cobalt chloride for 5 min, reacted with 3% of  $H_2O_2$ , and measured at  $A_{488\text{ nm}}$ .

### Determination of chitin and indole derivatives

Chitin content was purified from the fungal cell wall and quantified by measuring the acid-released glucosamine from chitin using *p*-dimethylaminobenzaldehyde as a chromogen (Selvaggini *et al.*, 2004). The reaction was measured at  $A_{520\text{ nm}}$ , and the quantity of glucosamine was determined by reference to a regression line established using pure glucosamine as a standard. Indole derivatives were purified with ethyl acetate from fungal cultures and analyzed by thin-layer chromatography as described previously (Chung *et al.*, 2003b).

### Sensitivity of CWDEs and generation of fungal protoplasts

Assays for sensitivity to driselase,  $\beta$ -D-glucanase,  $\beta$ -glucuronidase, and lyticase were determined by the number of protoplasts released from fungal isolates over time. Enzymes were dissolved in an osmotic buffer for fungal protoplasts as described previously (Chung *et al.*, 2002). The formation of protoplasts was determined at 1-h intervals by microscopy. Unless otherwise specified, all treatments were performed at least three times with multiple replicates.

### Molecular techniques

Fungal DNA was isolated as described previously (Chung *et al.*, 2002). Part of the endo-polygalacturonase (endo-PG) gene was amplified and labeled with primers capg1 (5'-accacccttggtcactcaaggagtg-3') and capg2 (5'-acccggaccacttcagttgg-3'). Primers cut1-F (5'-caaatgaagtctctcagc-3')

and cut1-R (5'-gacgccttgatctggttct-3') were used to amplify and label the cutinase gene. DNA probes used for the genes encoding pectin lyase and endo- $\beta$ -1,4-glucanase (cellulase) were amplified and labeled from the genomic DNA of *A. nidulans*. Primers EBG1 (5'-tggttgccgtagcggctgt-3') and EBG2 (5'-atacgaatgccatccgtcg-3') were used to amplify a cellulase gene (accession no. DQ490496); primers PLA1 (5'-gaccacttctctgtttccc-3') and PLA2 (5'-gctgaggaaatcggtgtcgg-3') were for the pectin lyase gene (accession no. DQ490478). The hybridization probe incorporating digoxigenin-11-dUTP nucleotides (Roche Applied Science) was labeled by PCR. Fungal RNA was purified with TRIZOL reagent (Invitrogen) and denatured in a formaldehyde-containing agarose and buffer solution, blotted onto a positively charged nylon membrane, and hybridized to a PCR-generated DNA probe as appropriate (Chung *et al.*, 2003a). Hybridization of total RNA of *C. acutatum* to the *A. nidulans* probes was carried out at 40 °C and posthybridization washing was performed at low stringency.

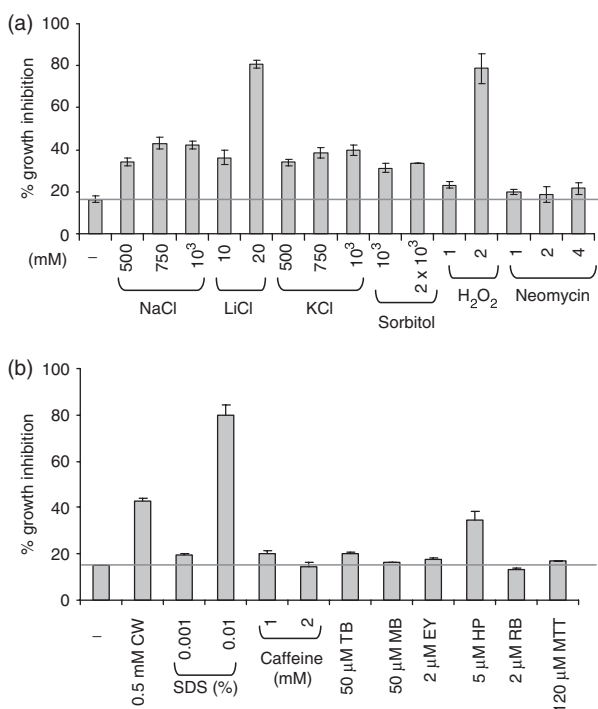
## Results

### Increased sensitivity to ions and hydrogen peroxides

The *PacC<sup>KLAP2</sup>* null mutants exhibited 18% growth reduction on nonbuffered medium compared with wild type (Fig. 1). Growth reduction > 18% was considered as being hypersensitive to the compound tested. Addition of NaCl, LiCl, or KCl to the medium resulted in growth inhibition of the *PacC<sup>KLAP2</sup>* disruption mutants. Growth reduction was observed when each of the *PacC<sup>KLAP2</sup>* null mutants was cultured in medium impregnated with sorbitol (Fig. 1a). However, the null mutants did not display significant growth reduction when mannitol or sucrose was used as the sole carbon source (data not shown). The null mutants were hypersensitive to H<sub>2</sub>O<sub>2</sub> at 2 mM and slightly sensitive to neomycin (Fig. 1a). The mutants were also hypersensitive to calcofluor white, sodium dodecyl sulfate (SDS), and hematoporphyrin (Fig. 1b), but exhibited weak sensitivity to caffeine or toluidine blue. The null mutants remained virtually insensitive to the Ca<sup>2+</sup>/calmodulin inhibitors chlorpromazine, lanthanum, nicardipine, trifluoperazine, or verapamil at 100 mM (data not shown), and remained unchanged in their sensitivity to thiazolyl blue tetrazolium bromide (MTT) or to the singlet oxygen-generating photosensitizers eosin Y, methylene blue, or rose Bengal (Fig. 1b).

### Reduced sensitivity to CWDEs

The *PacC<sup>KLAP2</sup>* null mutants are less sensitive than wild type to the effects of lytic enzyme, driselase,  $\beta$ -D-glucanase, and  $\beta$ -glucuronidase, as evidenced by the release of fewer proto-

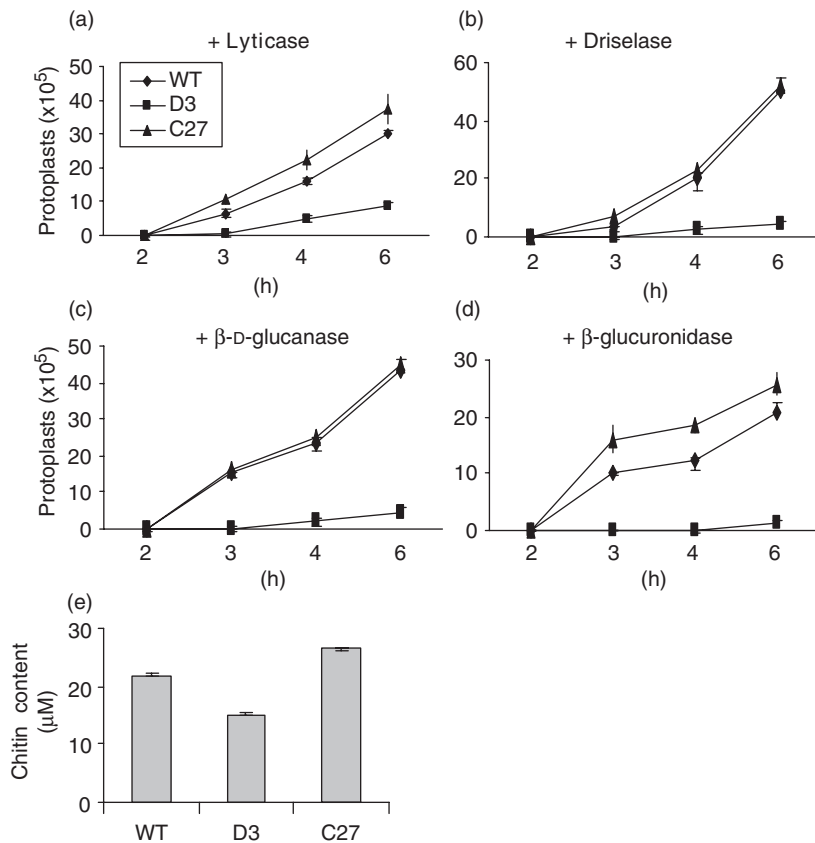


**Fig. 1.** (a) and (b) Phenotypic characterization of the *PacC<sup>KLAP2</sup>* null mutant (D3) and wild type of *Colletotrichum acutatum* in response to various compounds at concentrations as indicated. Fungal isolates were grown on nonbuffered PDA (pH 5.6) with or without a supplement at 28 °C for 5 days and the radial growth of fungal colonies was measured. The *PacC<sup>KLAP2</sup>* null mutants showed reduced growth by about 18% relative to wild type on regular medium, representing a basal line (indicated by a horizontal line) for chemical sensitivity. Sensitivity (percentage growth reduction) was calculated as a cumulative percentage of growth of wild type and the *PacC<sup>KLAP2</sup>* null mutant grown on the same plate and compared with the basal line. The data shown are the mean and SE of two experiments with at least five independent replicates. CW, Calcofluor white; EY, eosin Y; HP, hematoporphyrin; MB, methylene blue; MTT, thiazolyl blue tetrazolium bromide; RB, rose Bengal; SDS, sodium dodecyl sulfate; TB, toluidine blue.

plasts from the mutants over time (Fig. 2a–d). The complementation strain (C27), however, released slightly higher numbers of protoplasts than did wild type after treatment with the CWDEs, exemplified by lyticase and  $\beta$ -glucuronidase (Fig. 2a–d). The overall chitin content was slightly lower in the D3 mutant (Fig. 2e).

### Alterations to hydrolytic enzyme activities

The extracellular activities of cellulase, cutinase, xylanase, pectinase, and polygalacturonase were evaluated and compared among wild type, the D3, and C27 strains. The D3 disruptant exhibited lower cellulase and cutinase activities compared with those of wild type and the complementation strain (Fig. 3a and b). The D3 mutant had higher pectinase and polygalacturonase activities under both acidic and



**Fig. 2.** (a–d) Production of protoplasts over time by wild type (WT), the  $PacC^{KLAP2}$  null mutant (D3), and the genetically complemented strain (C27) of *Colletotrichum acutatum* after exposure to CWDEs: (a) lyticase, (b) driselase, (c)  $\beta$ -D-glucanase, and (d)  $\beta$ -glucuronidase. Fungal mycelia were harvested from 5-day-old cultures and subjected to CWDE digestion in an osmotic buffer at 28 °C. The release of protoplasts was determined with a hemocytometer by microscopy. (e) Determination of chitin content in the fungal cell wall. Chitin was purified and quantified by measuring the acid-released glucosamine from chitin using  $p$ -dimethylamino-benzaldehyde as a chromogen at  $A_{520\text{nm}}$ . Each point represents the mean number of protoplasts released  $\pm$  SE from two independent experiments with at least three replicates.

alkaline conditions (Fig. 3c and d), consistent with previous findings (You *et al.*, 2007). Xylanase activity was significantly lower in the D3 mutant (Fig. 3e). The D3 mutant had lower catalase activity (Fig. 3f), whereas there was no significant difference in peroxidase activity (data not shown).

### Differential gene expression

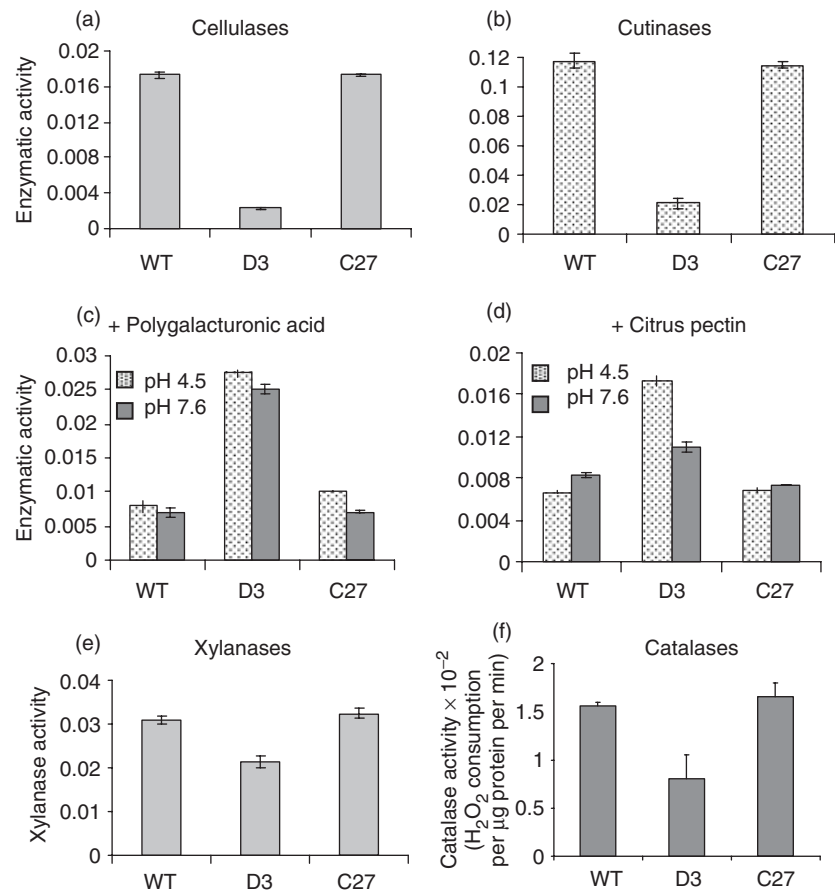
Northern blot analysis revealed that the 1.7-kb transcript of the endo-PG coding gene was barely detectable when wild type was grown on PDA medium (pH 5.6), whereas the null mutants accumulated higher levels of the transcript (Fig. 4a). Wild type accumulated the endo-PG gene transcript when grown on noninduced and nonbuffered CM (Fig. 4b and c). The endo-PG gene transcript was completely undetectable in wild type or the C27 complementation strain grown on CM containing polygalacturonic acid (PG) at either pH 4.5 or 7.6. Both the D3 and D4 null mutants, grown on CM impregnated with PG, had higher levels of the endo-PG gene transcript at either pH level (Fig. 4b and c). When grown on CM supplemented with PG at pH 7.6, the D3 mutant, but not the D4 mutant, accumulated abundant endo-PG transcript (Fig. 4c).

Expression of the pectin lyase (PLA) gene in wild type appeared to be similar when grown on medium with or

without citrus pectin, and at pH 4.5 or 7.6 (Fig. 5a and b). The  $PacC^{KLAP2}$  null mutants accumulated the pectin lyase gene transcript to a slightly, but not significantly, higher level than that of wild type. Expression of the endo- $\beta$ -1,4-glucanase (cellulase) gene in wild type was induced by CMC (Fig. 5c). Accumulation of this gene transcript was slightly but not significantly higher in the null mutants compared with wild type and the C27 complementation strain. Expression of the cutinase (*CUT1*) gene appeared to be induced by HHDA (Fig. 5d). On CM impregnated with HHDA, the C27 complementation strain accumulated the cutinase gene transcript to a level comparable to that of wild type. Expression of the cutinase gene was nearly abolished in the  $PacC^{KLAP2}$  disruption mutants.

### Discussion

The *C. acutatum*  $PacC^{KLAP2}$  gene was originally identified from a pathogenicity-deficient mutant induced by *Agrobacterium* T-DNA insertion (You *et al.*, 2007).  $PacC^{KLAP2}$ , a PacC ortholog, acts as an alkaline-responsive regulator in *C. acutatum*. In fungi, regulation of gene expression by ambient pH is primarily mediated by a signal transduction cascade involving at least six conserved gene products and



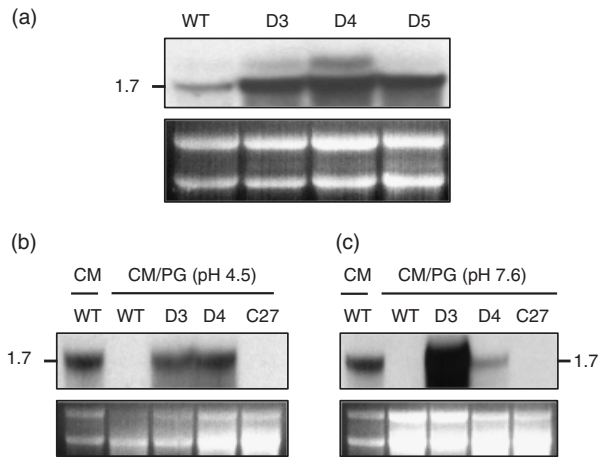
**Fig. 3.** Determination of extracellular enzyme activities: (a) cellulases, (b) cutinases, (c) pectolytic activities (mainly pectin lyase, pH 4.5 and pectin methyl esterase, pH 7.6), (d) pectolytic activities (primarily endo- and exo-PGs, pH 4.5 and pectate lyase, pH 7.6), (e) xylanases, and (f) cellular catalases by wild type (WT), the *PacC*<sup>KLAP2</sup> null mutant (D3), and the genetically complemented strain (C27) of *Colletotrichum acutatum*. Fungal isolates were grown on PDA with inducers [0.5% CMC; 0.1% HHDA, 1% PG, 1% citrus pectin, or 0.5% xylan (hemicellulose)] as appropriate for 5–7 days and enzymatic activities were measured by spectrophotometry after reaction with appropriate chromogens. Each column represents the mean value of enzymatic activity  $\pm$  SE from two independent experiments with at least three replicates. Details in measuring enzymatic activities are described in the text.

one transcriptional regulator, PacC (Negrete-Urtasun *et al.*, 1999). Genetic inactivation of the PacC gene in a fungal strain often results in multiple phenotypes. PacC has also been reported to regulate the biosynthesis and secretion of numerous toxins and biologically active secondary metabolites (Peñalva & Arst, 2004).

In the present study, it was shown that disruption of *PacC*<sup>KLAP2</sup> in *C. acutatum* also leads to multiple alterations in physiological and developmental processes. All *PacC*<sup>KLAP2</sup> null mutants recovered were found to be hypersensitive to or display an elevated sensitivity to Na<sup>+</sup>, K<sup>+</sup>, and Li<sup>+</sup> ions, sorbitol, hydrogen peroxide, calcofluor white, SDS, hemo- porphyrin, neomycin, caffeine, toluidine blue, and eosin Y. Many of these compounds are recognized cell wall-compromising agents or have destructive effects on the integrity of cell walls and/or membranes. It is postulated that disruption of *PacC*<sup>KLAP2</sup> gene function may have inhibitory effects on membrane proteins involved in proton fluxes and ion uptake. For example, the toxic effect of the ions may simply be due to a malfunction of Na<sup>+</sup>, K<sup>+</sup>, and Li<sup>+</sup> influx/efflux systems (Jia *et al.*, 1992; Watanabe *et al.*, 2005). Thus, *PacC*<sup>KLAP2</sup> might directly or indirectly regulate ion transporters. A functional link between pH signaling

and expression of the gene encoding a P-type Na<sup>+</sup>-ATPase has recently been shown in *Fusarium oxysporum* (Caracuel *et al.*, 2003). Lithium has multiple inhibitory effects on diverse enzymes in cells (Klein & Melton, 1996). The lithium tolerance might be associated with a mitogen-activated protein kinase (MAPK) cascade as suggested from *Eurotium herbariorum*, a common fungal species in the Dead Sea (Jin *et al.*, 2005). An increased sensitivity to H<sub>2</sub>O<sub>2</sub> and SDS in the *PacC*<sup>KLAP2</sup> null mutants might be, at least in part, attributed to lower catalase activity and/or damage to membranes.

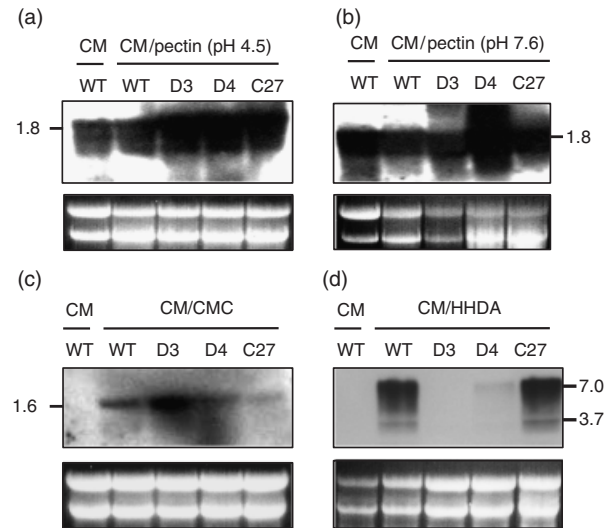
The *PacC*<sup>KLAP2</sup> null mutants are hypersensitive to calcofluor white, which can specifically bind to chitin and interrupt chitin biosynthesis, thereby disrupting cell wall integrity (Hill *et al.*, 2006). Indeed, the *PacC*<sup>KLAP2</sup> null mutants have a lower chitin content than wild type, implying that *PacC*<sup>KLAP2</sup> may partially regulate expression of the chitin synthase-encoding genes. Impairment of *PacC*<sup>KLAP2</sup> gene function apparently interferes with cell wall biosynthesis, thereby resulting in structural deformations. However, severe abnormalities of cell wall integrity are not supported by the unexpected finding that the *PacC*<sup>KLAP2</sup> null mutants liberated fewer protoplasts upon exposure to lyticase and other CWDEs compared with wild type.



**Fig. 4.** Differential expression of the endo-PG gene in wild type (WT), *PacC<sup>KLAP2</sup>* null mutants (D3, D4, and D5), and a genetically complemented strain (C27) of *Colletotrichum acutatum*. Fungal isolates were grown on (a) PDA (pH 5.6), (b) CM with or without PG at pH 4.5, or (c) at pH 7.6 for 5 days. Total RNA was purified, electrophoresed in formaldehyde-containing gels, blotted on to nylon membranes, and hybridized to an endo-PG gene probe. The sizes of hybridizing bands are indicated in kilobase pairs (kb). Ethidium bromide-stained rRNA is shown to indicate the relative loading of the samples.

The *PacC<sup>KLAP2</sup>* null mutants are hypersensitive to hematoporphyrin. Hematoporphyrin is a photosensitizing compound that generates singlet oxygen ( $^1O_2$ ) after irradiation by visible light and is used in photodynamic therapy for various cancers (Athar *et al.*, 1989). Singlet oxygen is extremely toxic to various cell components (Daub *et al.*, 2005). Surprisingly, the *PacC<sup>KLAP2</sup>* null mutants showed little or no sensitivity to other singlet oxygen-generating compounds. Thus, the toxicity of hematoporphyrin may not be primarily due to the production of  $^1O_2$ . Hematoporphyrin can inhibit the mitochondrial proton-translocating ATPase, an enzyme essential for coupling electrochemical proton gradients to the formation of ATP (Perlin *et al.*, 1985).

Pathologically, the *PacC<sup>KLAP2</sup>* null mutants are apparently delayed in penetration and lesion formation on both Key lime leaves and flower petals (You *et al.*, 2007). Pathogenicity and lesion development were restored by wounding before inoculation and by introducing a functional copy of the *PacC<sup>KLAP2</sup>* gene, suggesting a requirement for CWDEs in fungal penetration. The *PacC<sup>KLAP2</sup>* null mutants produced pronounced lipolytic and pectolytic activity but no detectable proteinase activity relative to wild type and the genetically complemented strains (You *et al.*, 2007). Thus, the lipolytic and pectolytic enzymes may not play a critical role in the pathogenicity of *C. acutatum*. Accumulation of the endo-PG-coding gene transcript appeared to be higher in the *PacC<sup>KLAP2</sup>* null mutants, whereas accumulation of the



**Fig. 5.** Differential expression of the pectin lyase gene (a and b), the cellulase gene (c), and the cutinase gene (d) in wild type (WT), *PacC<sup>KLAP2</sup>* null mutants (D3 and D4), and a genetically complemented strain (C27) of *Colletotrichum acutatum*. Fungal isolates were grown on CM with or without citrus pectin at pH 4.5 (a) or pH 7.6 (b), or containing CMC, or HHDA (a cutin monomer) for 5 days.

pectin lyase-coding gene transcript remained constitutive among isolates at either pH 4.5 or 7.6. In addition to CWDEs, the reduction of fungal pathogenicity paralleled the decreases in proteinase activity (You *et al.*, 2007), suggesting that proteolytic enzymes might be involved in fungal penetration and colonization. Many *Colletotrichum* spp. are capable of producing proteases, whereas a role for protease in pathogenicity or virulence has not yet been rigorously determined at the molecular level. A requirement for extracellular protease activity in pathogenicity has been reported in other fungi and bacteria of plants (Walton, 1994; Reddy *et al.*, 1996; Redman & Rodriguez, 2002).

Many phytopathogenic fungi produce a variety of CWDEs that can synergistically degrade the polysaccharides of cell walls to acquire nutrients. Thus, production of CWDEs is coordinately regulated by environmental pH and the levels of carbon and/or nitrogen sources (Aro *et al.*, 2005). The role of CWDEs in host-pathogen interactions has been scrutinized (Mendgen *et al.*, 1996), and yet the actual requirement for extracellular enzymes during fungal pathogenesis remains controversial for different fungal species. In this study, it was observed that the *PacC<sup>KLAP2</sup>* null mutants displayed a marked reduction of cellulase and cutinase activities. Thus, it is speculated that those hydrolytic enzymes might play important roles in the pathogenicity of *C. acutatum* to citrus. Those enzymes are often encoded by multiple genes and differentially regulated in filamentous fungi (Apel-Birkhold & Walton, 1996; MacCabe

*et al.*, 1998; Lev & Horwitz, 2003). Northern blot analysis of fungal RNA hybridized to a cutinase-coding gene (*CUT1*) probe revealed that wild type and the complementation strains accumulated abundant *CUT1* transcripts. The *PacC*<sup>KLAP2</sup> mutants failed to accumulate the *CUT1* gene transcript, indicating that *PacC*<sup>KLAP2</sup> regulates expression of *CUT1*. However, expression of the cellulolytic enzyme-encoding gene was relatively higher in the *PacC*<sup>KLAP2</sup> null mutants compared with that of wild type, which is inconsistent with the observed marked decrease in cellulolytic activity in the mutants (Fig. 4a); however, this discrepancy is likely due to the multiple copies of cellulase-encoding genes in *C. acutatum*. The requirement for cutinases, cellulases, and xylanases in the pathogenicity of *C. acutatum* remains uncertain. Further analysis by identifying and characterizing the targeted disruption mutants of the respective genes should provide conclusive evidence for a role of CWDEs in the pathogenicity of *C. acutatum*. In summary, the present data highlight that ambient pH regulation mediated by *PacC*<sup>KLAP2</sup> is essential for controlling salt tolerance, the integrity of cell walls and membranes, the production of hydrolytic enzymes, and a virulence determinant in *C. acutatum*, consistent with the global regulatory role of *PacC*.

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