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The pH signaling transcription factor PacC is required for full virulence in *Penicillium digitatum*

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Header: *P. digitatum pacC* ortholog is involved in pathogenicity

Abstract: *Penicillium digitatum* is the most important postharvest pathogen of citrus fruit. Along disease progression the infected citrus peel tissue is acidified due to the accumulation of organic acids. So far, relatively little is known about the environmental factors that regulate pathogenicity in this fungus. In this study, the role of the pH signaling transcription factor PacC in the pathogenesis of *P. digitatum* was investigated. We identified the *pacC* ortholog (*PdpacC*) in *P. digitatum* and found that its transcript levels were elevated under alkaline conditions (pH≥7) *in vitro*, as well as during the infection of citrus fruits o in spite of the low pH (about 3.0 to 3.5) of the macerated tissue. Na^+ and pectin also induced the expression of *PdpacC*. Disruption of *PdpacC* resulted in impaired mycelial growth under neutral or alkaline

pH conditions and on synthetic medium supplemented with pectin as the sole carbon source, and attenuated virulence towards citrus fruits. Introducing the full length of *PdpacC* into the Δ*PdpacC* mutant restored all these phenotypes. The expression of the polygalacturonase gene *Pdpg2* and pectin lyase gene *Pdpnl1* in *P. digitatum* was up-regulated in the wild-type strain but not or weakly up-regulated in the Δ*PdpacC* mutant during infection. Disruption of *Pdpg2* also resulted in attenuated virulence of *P. digitatum* towards citrus fruits. Collectively, we conclude that PdPacC plays an important role in pathogenesis of *P. digitatum via* regulation of the expression of cell wall degradation enzyme genes, such as *Pdpg2* and *Pdpnl1*.

Introduction

Green mold, caused by *Penicillium digitatum* (Pers.:Fr) Sac., is the most destructive postharvest disease of citrus fruit and is responsible for up to 90% of the total losses during postharvest packing, storage, transportation and marketing (Eckert and Eaks 1989). *P. digitatum* is a typical necrotrophic pathogen that requires a pre-existing wound in the peel of the fruit to be allowed to penetrate successfully. During the infection, *Penicillium* spp. macerates the host tissue by producing significant amounts of hydrolytic enzymes, especially abundant for polygalacturonases (PG) (Marcet-Houben et al. 2012; Sánchez-Torres and González-Candelas 2003; Yao et al. 1996). Another important characteristic for *Penicillium* spp. is that they are able to acidify the infected tissue by secretion of organic acids, which have been reported to be a virulent factor in these fungi (Prusky et al. 2004; Barad et al. 2012).

The extracellular pH has been widely demonstrated to be a critical signal for virulence in plant pathogenic fungi. Previous reports indicated that during infection some plant pathogens, like *Colletotrichum* spp. and *Alternaria alternata*, alkalinize their host tissue by producing ammonia (Eshel et al. 2002; Prusky et al. 2001), whereas other pathogens, like *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Phomopsis mangiferae* and *Penicillium* spp., acidify the infected tissues by producing organic acids and/or utilizing ammonia (Barad et al. 2012; Davidzon et al. 2010; Prusky et al. 2004; Rollins and Dickman 2001; Verhoeff et al. 1988). By modulating the pH at the colonized tissue, plant pathogens bring the environmental conditions close to the optimum for the production of hydrolytic enzymes and secondary metabolites, ensuring an optimal pH for fully performing the physiological functions of hydrolytic enzymes, etc., thereby enhancing their virulence (Eshel et al. 2002; Miyara et al. 2010; Miyara et al. 2012; Prusky et al. 2001; Prusky et al. 2004; Prusky and Yakoby 2003).

Sensing and transduction of ambient pH in fungi is mediated via a conserved PacC/Rim101 signaling cascade, which is constituted by PalA, PalB, PalC, PalF, PalH, PalI and PacC (Peñalva and Arst 2004; Peñalva et al. 2008). PacC, the terminal component of the pH signaling pathway, is a transcription factor containing 3 Cys2His2 zinc finger DNA binding domains. The full-length PacC polypeptide is cleaved by two successive proteolytic cleavages to produce a shorter and functional polypeptide (Hervás-Aguilar et al. 2007; Orejas et al. 1995). The functional form of PacC is translocated into the nucleus, where it regulates the expression of PacC-dependent genes, which are involved in numerous physiological functions, including growth, differentiation and virulence, in several fungal pathogens of humans, plants, insects and fungi (Espeso and Arst 2000; Peñalva and Arst 2002, 2004; Suarez and Penalva

1996; Tilburn et al. 1995; Trushina et al. 2013; You et al. 2007).

The role of PacC regulating pathogenicity has been shown in several plant pathogens. In *Colletotrichum acutatum*, the *pacC* ortholog is essential for virulence on citrus (You et al. 2007). In contrast to the wild type, the *pacC* disrupted mutants of the *S. sclerotiorum* were less virulent on *Arabidopsis* and tomato (Rollins 2003). Oppositely, in *Fusarium oxysporum*, a vascular wilt pathogen, a constitutive-active *pacC* mutant was found to be less virulent than the wild type strain on tomato (Caracuel et al. 2003b). Although the effect of PacC on virulence seems to be dependent on the pathogen, the regulation mechanisms in different pathogens are mainly via controlling the production of virulence factors. Nevertheless, although previous studies have indicated that hydrolytic enzymes, especially polygalacturonases (PG), and organic acids play a role on pathogenesis in postharvest pathogens of the genus *Penicillium* (Barad et al. 2012; Prusky et al. 2004; Sánchez-Torres and González-Candelas 2003; Yao et al. 1996), the role of PacC in the regulation of virulence has not been demonstrated in this important group of fungal pathogens. In the present work, the *pacC* ortholog of *P. digitatum* has been isolated and its role in virulence has been investigated. Our results indicate that PdPacC in *P. digitatum* is a positive regulator of virulence, and this effect is at least partially mediated *via* the regulation of the expression of the polygalacturonase encoding gene *Pdpg2*.

Materials and methods

Culturing of *P. digitatum*

The wild-type strain Pd01 of *P. digitatum* used in this study was isolated in Zhejiang

89 province as described previously (Zhu et al. 2006) and deposited in the CBS-KNAW Fungal
90 Biodiversity Centre, Utrecht, the Netherlands (accession number CBS130525). The wild-type
91 and mutant strains were stored as conidial suspensions in 20% glycerol at -80 °C. For this
92 study, all strains were recovered on PDA (extract of 200 g potato boiled water, 20 g dextrose,
93 and 13 g agar per liter). Conidia were obtained from 7 to 10 days' old cultures by scraping
94 them with a sterile spatula, resuspended in sterile water, counted with a haematocytometer
95 and adjusted at the desired concentration. Mycelium was obtained by inoculating 5 μl (1.0
96 $\times 10^6$ conidia/ml) in 150 ml liquid PDB (PDA without agar). Liquid cultures were incubated
97 on a rotary shaker at 160 rpm and 25 °C.

98 **Isolation of the *pacC* ortholog from *P. digitatum***

99 The *P. digitatum pacC* ortholog, designated *PdpacC*, was amplified from the genomic DNA
100 of *P. digitatum* by PCR using the specific primers PdpacCC-F and PdpacCC-R (Table 1). The
101 primers were designed according to genomic sequence data of *P. digitatum* (Marcet-Houben
102 et al. 2012) and the *pacC* gene sequence from *P. chrysogenum* (GenBank, accession No.
103 U44726). The amplified fragment was cloned into the vector pMD18-T (TaKaRa Biotech. Co.,
104 Dalian, China) and the absence of polymerase-derived errors was checked by DNA
105 sequencing.

106 **Construction of *PdpacC* and *Pdpg2* disruption plasmids**

107 The *PdpacC* disruption vector was constructed by inserting the two flanking sequences of
108 *pacC* into the up- or downstream sides of the hygromycin resistance gene (*hph*) in the vector
109 pTFCM (Wang and Li 2008) (Fig. 1A). The primers used to amplify the homologous arms
110 were synthesized according to the genomic sequence of *P. digitatum* (Marcet-Houben et al.

2012). Briefly, a 0.7 kb DNA fragment containing the 3' downstream flanking sequence of *PdpacC* was amplified from *P. digitatum* genomic DNA by PCR using primers PdpacCA and PdpacCB (Table 1). After digestion with *Xho*I and *Spe*I restriction enzymes, the PCR fragment was inserted into the *Xho*I/*Spe*I site of pTFCM to generate the plasmid pTFCM-3'. Subsequently, a 0.7 kb fragment representing the 5' upstream flanking sequence of the *PdpacC* gene was PCR amplified using primers PdpacCC and PdpacCD (Table 1). After digestion with *Sac*I and *Kpn*I restriction enzymes, the PCR fragment was inserted into the *Sac*I/*Kpn*I site of the plasmid pTFCM-3' to generate the *PdpacC* disruption plasmid pTFCM-*PdpacC*-del, in which the region spanning from the residue P211, located downstream of the zinc finger, to the A397 residue was replaced by *hph* (Fig. 1A). Thereafter, the plasmid pTFCM-*PdpacC*-del was transformed into *Agrobacterium tumefaciens* strain AGL-1 by electroporation following the method described by Wang and Li (2008).

The *Pdpg2* disruption vector was constructed using an identical strategy to that described for the plasmid pTFCM-*PdPacC*-del. Briefly, a 0.9 kb DNA fragment containing the 3' downstream flanking sequence of *Pdpg2* was PCR amplified from *P. digitatum* genomic DNA using primers Pdpg2C and Pdpg2D (Table 1). Subsequently, a 0.75 kb fragment representing the 5' upstream flanking sequence of the *Pdpg2* gene was PCR amplified using primers Pdpg2A and Pdpg2B (Table 1). The resulting *Pdpg2* gene deletion plasmid, pTFCM-*Pdpg2*-del, was transformed into *A tumefaciens* strain AGL-1 by electroporation.

Transformation of *P. digitatum*

To obtain a *PdpacC* disruption mutant, the *A. tumefaciens*-mediated transformation was performed as described previously (Wang and Li 2008). Briefly, the *A. tumefaciens* strain

AGL-1 containing the plasmid pTFCM-*PdPacC*-del was grown in minimal medium (MM) (Hooykaas et al. 1979) supplemented with kanamycin (50 μg/ml) for two days at 28 °C. Cells were diluted to an OD_{600}=0.15 in induction medium (IM) containing 200 μM acetosyringone (AS). After 6 h of incubation, the *A. tumefaciens* cells were mixed with an equal volume of conidial suspension of the wild type *P. digitatum* Pd01 (1×10^6 conidia/ml). A 200 μl mixture was spread onto a nylon membrane that was placed on an IM plate containing 200 μM AS. After co-cultivation at 25 °C for 2 days, the nylon membrane was transferred to a PDA medium containing 75 μg/ml hygromycin B and 50 μg/ml cefoxitin to select for fungal transformants and to kill *A. tumefaciens* cells. After 3 to 4 days of incubation, individual colonies of *P. digitatum* were transferred to PDA plates containing 75 μg/ml hygromycin B.

Complementation of the *PdpacC* gene

A DNA fragment including the full genomic sequence of *PdpacC* as well as its promoter (1391 bp upstream of the start codon) and terminator (55 bp downstream of the stop codon) regions was PCR amplified using primers PdpacCC-F and PdpacCC-R (Table 1). After digestion with *Eco*RI, the fragment was cloned into the plasmid pCA-Sur (Yan et al. 2011) to obtain the complementation plasmid pCA-Sur-*PdpacC*. Transformation of the *P. digitatum* Δ*PdpacC* deletant with plasmid pCA-Sur-*PdpacC* was conducted as described above except that chlorimuron-ethyl (800 μg/ml) was used as a selection agent.

pH measurement

The pH value of liquid PDB or SM was measured directly with a micro pH electrode Model 9810BN (Orion, Beverly, MA) in 1- to 3-ml aliquots sampled at indicated times after fungal incubation. Four replicates were tested for each treatment. The pH at the inoculation

sites in the peel of citrus fruits was measured by inserting the micro pH electrode directly into the mesocarp at different times after inoculation. 9 citrus fruits were tested for each measurement (at least 30 measurements).

Analysis of *PdpacC* expression in *P. digitatum*

Total RNA extraction and first strand cDNA synthesis were conducted as described previously (Wang et al. 2012). Specific primers PdpacC-qF and PdpacC-qR (Table 1) were used for qRT-PCR using a 7300 Real Time PCR system (ABI, USA). Real time PCR was carried out using the SYBR Premix Ex TaqTM (Perfect Real Time) kit (TaKaRa Biotech. Co., Dalian, China). The thermal cycling conditions were 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 31 s. Expression of the *P. digitatum* γ-actin gene (GenBank, AB030227), determined using primers Actin-qF and Actin-qR (Table 1), was used as an internal control. During the validation experiment, PCR efficiency for each gene was calculated using 10-fold serial cDNA dilutions. The relative quantification of the target gene in comparison with the internal control γ-actin was calculated according to the formula $Ratio=[(E_{target})^{\Delta Ct\ target\ (control-sample)}]/[(E_{reference})^{\Delta Ct\ reference\ (control-sample)}]$ (Pfaffl et al. 2002).

To determine the effect of ambient pH on *PdpacC* expression, about 0.1 g fresh weight of mycelium from 4-day-old *P. digitatum* cultures grown in PDB was transferred to PDB medium, in which the pH was adjusted to 3, 4, 5, 6, 7 and 8, respectively, using 0.1 mol/l citrate-phosphate buffer, and the cultures were incubated on a rotary shaker at 160 rpm, 25 °C for 12 h. Mycelia were harvested by filtration through 3 layers of cheesecloth, washed with double distilled water (ddH_2O), frozen in liquid nitrogen, and stored at -80 °C until RNA isolation. Mycelium of the wild type *P. digitatum* grown in non-buffered PDB (pH 6.32) was

177 used as the control. Three replicates were set for each treatment and the experiment was
178 repeated three times.

179 The expression dynamics of *PdpacC* during infection of citrus fruits was assayed using
180 qRT-PCR. Healthy, non-wounded mature citrus fruits (*Citrus unshiu)* were wounded by
181 pinpricking the fruits with a bunch of 5 needles (2 to 3 mm in depth). Three microliters of a
182 conidial suspension (10^6 conidia/ml) was inoculated onto each wound as described previously
183 (Zhang et al. 2013). Fifty mg of citrus peel tissue at the inoculation site of each fruit was
184 harvested for RNA isolation at 12, 24, 48, 72 and 96 h post inoculation. Three replicates were
185 set for each treatment. The experiment was repeated three times.

186 The effect of Na^+ on *PdpacC* expression was determined by adding NaCl to a final
187 concentration of 0.5 mol/l to 4-day-old *P. digitatum* cultures grown in PDB and followed by
188 additional incubation on a rotary shaker set at 160 rpm for 12 h before mycelium harvesting
189 and RNA extraction. The accumulation of *PdpacC* RNA was determined by qRT-PCR, and
190 the accumulation of *PdpacC* RNA in mycelium grown in PDB was used as the control.

191 The effect of pectin on *PdpacC* expression was determined by transferring 4-day-old *P.*
192 *digitatum* cultures to a synthetic medium SM (DiPietro and Roncero 1996) without agar
193 supplemented with 1% citrus pectin (Sigma, St. Louis, MO, USA) as the sole carbon source
194 (pH 6.3 at beginning). The cultures were then grown on a rotary shaker at 25 ºC and 160 rpm
195 for 60 h before mycelium harvesting and RNA extraction. Cultures grown in liquid SM
196 supplemented with 1% glucose (pH 6.3 at beginning) were used as the control. The
197 expression of *PdpacC* was determined by using qRT-PCR. Three replicates were set for each
198 treatment, and the experiment was repeated three times.

Expressions of CWDE genes during infection

Based on the annotation of the genomic *P. digitatum* DNA sequence (Sun et al., unpublished), several cell wall degradation enzyme (CWDE) genes, including polygalacturonase (*Pdpg1*, Genbank accession No.AB015286, *Pdpg2*, Genbank accession No. JX298854, *Pdexpg1*, Genbank accession No. JX495169, *Pdexpg2*, Genbank accession No. JX298856), pectin lyase (*Pdpnl1*, Genbank accession No. JX298853, *Pdpnl2*, Genbank accession No. JX495170) and xylanase (*Pdxy1*, Genbank accession No. JX298855, *Pdxy2*, Genbank accession No. JX495171) were chosen for dynamics analysis of RNA accumulation in the wild-type and the Δ*PdpacC P. digitatum* strains during the infection through qRT-PCR. The primers used in this study were listed in Table 1. The inoculated tissues (50 mg) were harvested at 12, 24, 48, 72 and 96 h post inoculation and used for RNA isolation. The wild type strain grown in PDB was used as the control. Three replicates were set for each treatment and the experiment was repeated three times.

Comparison of radial growth among the wild-type and *PdpacC* mutant strains

To determine the role of *PdpacC* in the response to NaCl or KCl stress in *P. digitatum*, mycelial plugs of the wild type, Δ*PdpacC* and complementation mutant (CP*PdpacC*) strains of *P. digitatum* were prepared as described previously (Zhang et al. 2008), then were transferred onto PDA plates supplemented with or without 1.0 mol/l NaCl or KCl. To determine the role of *PdpacC* in the response to different pH conditions in *P. digitatum*, mycelial plugs were transferred onto PDA plates buffered at pH 4, 6 or 8 with 0.1 mol/l citrate-phosphate buffer. To determine the expression pattern of *PdpacC* in the presence of pectin as the sole carbon source, mycelial plugs were transferred onto SM supplemented with

1% glucose or 1% pectin as the sole carbon source. The colony diameters of the tested strains of *P. digitatum* were measured after 7 days (10 days for SM) of incubation (25 °C). Three plates were used per strain and the experiment was repeated twice.

Virulence assay

To investigate the function of *PdpacC* on *P. digitatum* virulence, citrus fruits (*C. unshiu*) were wounded with a bunch of 5 needles (2 to 3 mm in depth) as described previously (Wang et al. 2012). Conidial suspensions (10^6 conidia/ml) of the wild type, Δ*PdpacC* and CP*PdpacC* mutants were collected from 7-day-old PDA plates. Three µl of the conidial suspension was added onto each wounded site. The inoculated fruits were incubated at room temperature and disease symptoms were observed daily. The virulence of the wild type and each mutant was determined by measuring the lesion size. Thirty fruits were used for each strain and the experiment was repeated twice. Virulence assay of the Δ*Pdpg2* mutant was conducted as described above.

Data analysis

Data obtained in this study were analyzed using analysis of variance (ANOVA) and the least significant test along with the Data Processing System as described by Tang and Feng (2007).

Results

Cloning of the *P. digitatum's PdpacC* gene

A 3431 bp fragment of the *PdpacC* was amplified using specific primers PdpacCC-F and PdpacCC-R (Table 1). Sequencing results indicates that the coding sequence of *PdpacC* is 1985 bp in length and has an intron (59 bp) at nucleotide position 227 to 285. The nucleotide

sequence of the *PdpacC* has been deposited in the GenBank with accession number JX298852. This sequence differs in only two nucleotide from the genomic sequences of the two *P. digitatum* Spanish strains recently published (Marcet-Houben et al., 2012), which result in a G to D change at amino acid 636. *PdpacC* is predicted to encode a protein of 641 amino acids (aa). The protein PdPacC shares high sequence identity with the PacC orthologs reported in other fungi, including *Aspergillus nidulans* (62%, GenBank accession No. EAA63426), *A. niger* (69%, accession No. CAA67063), *P. chrysogenum* (91%, accession No. CAP94266), *S. sclerotiorum* (42%, accession No. ACO55072) and *F. oxysporum* (43%, accession No. AAM95700). PdPacC contains three C2H2-type zinc finger motifs at its N-terminal region (amino acid residues L57 to H145) and the amino acid sequence of the zinc finger domain is 100% identical to those identified in the above mentioned fungi. The other conserved region observed in PdPacC is located between residues L461 and P483, previously reported as a protease recognition region (Díez et al. 2002). The nucleotide sequence upstream of the start codon (-222 to -723) contains four repeats of the canonical PacC binding site (three repeats are 5'-GCCAAG-3' and one is 5'-GCCAGG-3') (Tilburn et al. 1995).

pH dynamics at inoculated citrus peel

At the beginning of the experiment (0 h after inoculation), the pH value of the citrus peel was 4.64± 0.13. Maceration symptoms appeared at the inoculation sites at 24 h after inoculation. At this moment, the pH value at inoculated sites decreased to 3.83± 0.14. Then, it further decreased and reached a value of 3.33± 0.12 at 48 h after inoculation. Thereafter, the pH remained relative stable (Table 2). In contrast, the pH values in non-infected tissue remained stable in a pH range from 4.61 to 4.70 at all time points (data not shown).

Expression of *PdpacC*

The effect of pH on the expression of *PdpacC* was evaluated by growing the mycelium in PDB buffered at different pHs for 12 h. In contrast to the expression of *PdpacC* in mycelium grown in un-adjusted pH PDB (pH=6.32 after 12 h), the transcription levels of *PdpacC* in mycelium grown at pH 3, 4, 5 and 6 were significantly lower (ratio＜1) than those determined at pH 7 and 8 (ratio＞1) (Fig. 2A), indicating that *PdpacC* is an alkaline pH-induced gene *in vitro* (pH≥7).

During infection of citrus fruits, transcription of *PdpacC* was detected at 12 h post inoculation, at which time the conidia were germinating. The mRNA accumulation of *PdpacC* increased and reached a peak at 48 h post inoculation, then decreased and kept a stable level later on (Fig. 2B), indicating that *PdpacC* is involved in the infection of citrus fruits by *P. digitatum*. Our results also showed that the expression of *PdpacC* is induced by NaCl, being the expression of *PdpacC* in Na^+-added PDB 2.6 fold higher than that in non-Na^+-added PDB (Fig. 2C).

The pH of SM amended with glucose or pectin as the sole carbon source was about 6.3 at the beginning of the experiment, and decreased to 2.9 (glucose as sole carbon source) and 3.1 (pectin as sole carbon source), respectively, after 60 h of incubation. Interestingly, in contrast to the mycelium grown in medium using glucose as sole carbon source, the expression of *PdpacC* was about 20-fold higher when grown in medium using pectin as the sole carbon source (Fig. 2D). This result suggests that *PdpacC* is involved in the utilization of pectin.

Generation of *PdpacC*-disruption and -complementation mutants

PdpacC disruption mutants (Δ*PdpacC*) were generated by homologous recombination (Fig.

1A). The disrupted *PdpacC* allele lacks a fragment spanning from the residue P211, located downstream of the zinc finger, to the A397 codon, which includes the regions of nuclear localization signal (You et al. 2007) and the proteolytic processing site for final activation (Díez et al. 2002). The resulting 160 hygromycin-resistant transformants were subjected to PCR identification with primers PdpacCjd-F and PdpacCjd-R. A 1.3 kb fragment in the wild type Pd01 was substituted by a 2.8 kb recombinant fragment in the deletion transformants (Fig. 1B). Two deletion transformants were further subjected to Southern blot analysis using a probe specific to the 3'region of *PdpacC* (Fig. 1A). The results confirmed that additional ectopic integration of the replacement cassette did not occur in these transformants (Fig. 1C). The growth phenotypes of Δ*PdpacC*A and Δ*PdpacC*B were identical during initial assays, thus Δ*PdpacC*A was chosen for further analysis and abbreviated as Δ*PdpacC*.

Twelve putative complementation mutants (CP*PdpacC*) were initially selected on medium containing chlorimuron-ethyl (800 μg/ml). The insertion of *PdpacC* in the CP*PdpacC* mutants was confirmed by PCR (data not shown) followed by Southern blot analysis (Fig. 1C). One of the mutants with a single insertion of *PdpacC* (Fig. 1C) was then chosen for further studies.

The *Pdpg2* disruption mutant was created following the same strategy. Forty five hygromycin-resistant transformants were obtained. PCR and Southern blot analysis indicated that a Δ*Pdpg2* transformant contained a disrupted *Pdpg2* without additional ectopic insertions of the replacement cassette (SFig. 1B and C). This mutant was then chosen for further studies.

Disruption of *PdpacC* impairs the tolerance to Na^+ or K^+ stress

To determine the role of *PdpacC* in response to Na^+ or K^+ stress, mycelium plugs of the wild-type strain, Δ*PdpacC* and CP*PdpacC* mutants were placed on PDA media supplemented

with 1.0 mol/l NaCl or KCl. Results showed that the radial growth of the three strains were identical on PDA medium (start pH=6.32, Fig. 3 and SFig. 2), indicating that *PdpacC* is dispensable for mycelial growth of *P. digitatum* under non stress conditions. The radial growth of the wild-type *P. digitatum* on PDA medium supplemented with 1.0 mol/l NaCl or KCl was moderately reduced, whereas the growth of the Δ*PdpacC* mutant was severely reduced (Fig. 3 and SFig. 2). This growth defect was partially restored in the CP*PdpacC* mutant. However, the radial growth rates of the wild-type strain, Δ*PdpacC* and CP*PdpacC* mutants on PDA supplemented with 1.0 mol/l $CaCl_2$ or $MgCl_2$ were not significantly different (date not shown). These results suggest that the *PdpacC* is required for *P. digitatum* in response to Na^+ or K^+ stress but not to Ca^{2+} or Mg^{2+} stress.

***PdpacC* is required for mycelial growth at alkaline pH and full utilization of pectin**

Radial growth of the wild-type strains, Δ*PdpacC* and CP*PdpacC* mutants were determined by culturing them separately on PDA buffered at pH 4, 6 and 8. Results showed that the radial growth of the Δ*PdpacC* mutant on PDA was similar to that of the wild-type at pH 4 (Fig. 4). Contrasting to the growth at pH 4, the radial growth of both mutant and wild type strains at pH 6 and 8 was impaired (Fig. 4), indicating that the conditions of neutral or alkaline pH do not favor the myceilal growth of *P. digitatum*. However, growth reduction was more pronounced in the Δ*PdpacC* mutant at pH 6 and 8 in comparison to the wild-type strain (Fig. 4). Normal growth was recovered in the CP*PdpacC* mutant, indicating that *PdpacC* is required for mycelial growth of *P. digitatum* at alkaline conditions.

The radial growth of the wild-type strain, Δ*PdpacC* and CP*PdpacC* mutants on SM supplemented with glucose as the sole carbon source were similar. However, the radial growth

of the Δ*PdpacC* mutant was reduced by 57.7% compared to the wild type when grown on SM supplemented with pectin as the sole carbon source (Fig. 5 and SFig. 3). Restoring *PdpacC* in the Δ*PdpacC* mutant recovered almost completely the phenotype of the wild type strain (Fig. 5 and SFig. 3), indicating that *PdpacC* plays an important role in pectin utilization.

PdpacC is required for full virulence in _P. digitatum_

The role of *PdpacC* on *P. digitatum* virulence was investigated by inoculating citrus fruits with the wild-type, Δ*PdpacC* or CP*PdpacC* mutants. Maceration symptoms were observed on all inoculated fruits at 24 h post inoculation, but the symptoms in the fruits inoculated with the Δ*PdpacC* mutant developed much more slowly than in those inoculated with the wild-type *P. digitatum* strain. The average diameter of the macerated lesions in citrus fruits inoculated with the wild-type strain was about 4.8 cm at 4 days post inoculation, whereas that of the Δ*PdpacC* mutant-inoculated citrus fruits was about 2.9 cm (Fig. 6A and B). The reduction in virulence of the Δ*PdpacC* mutant was reversed when *PdpacC* was introduced into Δ*PdpacC*, as shown in Fig. 6A and B. The average diameter of the lesions induced by CP*PdpacC* was comparable to that of the wild-type *P. digitatum*. These results indicate that *PdpacC* is required for full virulence in *P. digitatum*.

PdpacC regulates the expressions of _Pdpg2_ and during infection

Expressions of several *CWDE* genes, including polygalacturonases *Pdpg1* (AB015286) and *Pdpg2* (JX298854), *Pdexpg1* (JX495169) and *Pdexpg2* (JX298856), pectin lyases *Pdpnl1* (JX298853) and *Pdpnl2* (JX495170), and xylanases *Pdxy1* (JX298855) and *Pdxy2* (JX495171), during the infection of the wild-type and Δ*PdpacC* mutant of *P. digitatum* were evaluated through qRT-PCR. Results showed that during infection of citrus fruits, the

353 expression of *Pdpg2* and *Pdpnl1* in Δ*PdpacC* were different from that in the wild-type strain.
354 The expression of *Pdpg2* in both wild-type and Δ*PdpacC* strains was detectable at 12 h post
355 infection, and significantly up-regulated in the wild type at 24, 48 and 72 h post inoculation.
356 In contrast, the expression of *Pdpg2* in Δ*PdpacC* was not or weakly up-regulated at the
357 corresponding time points (Fig. 7A). The accumulated RNA of *Pdpg2* in the wild-type
358 strain-infected tissue was about 30 fold higher than in the Δ*PdpacC*-infected tissue at 72 h
359 post inoculation. In addition, the expression of *Pdpnl1* was also induced to a higher level in
360 the wild-type strain than in the Δ*PdpacC* mutant (Fig. 7B). The expression patterns of the
361 other *CWDE* genes tested in this study were similar between the Δ*PdpacC* mutant and the
362 wild-type strain (data not shown). These results indicated that *PdpacC* is involved in the
363 regulation of both *Pdpg2* and *Pdpnl1* during infection of citrus fruits.
364
365 **Disruption of the *Pdpg2* resulted in attenuated virulence to citrus**
366 To investigate the role of *Pdpg2* on virulence, a *Pdpg2* disruption mutant (Δ*Pdpg2)* was
367 obtained following the same method described previously for *PdpacC*. Pathogencity tests
368 indicated that decay development in citrus fruits inoculated with the Δ*Pdpg2* mutant was
369 slower than that in fruits inoculated with the wild type *P. digitatum* (Fig. 8A and B). The
370 diameter of the macerated lesions caused by the Δ*Pdpg2* mutant was about 30% smaller than
371 that caused by the wild type *P. digitatum* after 4 days of inoculation (Fig. 8A and B),
372 indicating that the *Pdpg2* is involved in *P. digitatum*'s virulence.
373

Discussion

For adaptation to a variable pH environment, fungi have developed a complex pH signaling cascade by which they are able to sense and respond to extra-cellular pH changes. The pH signaling system has been extensively studied in *A. nidulans* and is known to be conserved in fungi (Lamb et al. 2001; Nobile et al. 2008; Peñalva et al. 2008). PacC, a zinc finger transcription factor, the terminal component of the pH signaling cascade, is responsive to extra-cellular pH changes and mediates the activation or repression of an array of pH-responsive genes (Andersen et al. 2009; Trushina et al. 2013). The roles of PacC on the regulation of growth, differentiation and virulence, as well as the production of secondary metabolites, have been studied in several fungi (Caracuel et al. 2003a, b; Merhej et al. 2011; Rollins 2003; Suarez and Penalva 1996; Trushina et al. 2013; You et al. 2007; Zou et al. 2010). In this study, we have characterized the *A. nidulans pacC* ortholog in *P. digitatum* (*PdpacC*), the most important postharvest pathogen of citrus, and revealed that *PdPacC* is required for the full utilization of pectin and its full virulence towards citrus fruits, most likely by the regulation of the expression of the polygalacturonase *Pdpg2* and the pectin lyase *Pdpnl1* genes. In addition this study also indicated that *PdpacC* participates in the response to Na^+ and K^+ stresses, and is required for mycelial growth of *P. digitatum* at alkaline conditions.

In *A. nidulans*, the full-length PacC polypeptide is processed by two successive proteolytic cleavages and yields a shorter, functional PacC at alkaline pH. The processed and functional PacC is an activator of alkaline-expressed genes and repressor of acid-expressed genes (Orejas et al., 1995; Penalva and Arst, 2002; Penalva et al., 2008). In this study we found that neutral or alkaline pH conditions did not favor the mycelial growth of *P. digitatum* (Fig. 4),

but induced the expression of *PdpacC* (Fig. 2A). Disruption of *PdpacC* impaired the mycelial growth of *P. digitatum* at neutral or alkaline conditions, indicating that *PdpacC* is an alkaline pH-induced gene and is required for *P. digitatum* growth at alkaline conditions *in vitro*. This result agrees with previous reports in other fungi, such as *A. nidulans* (Tilburn et al. 1995), *S. sclerotiorum* (Rollins 2003), *F. oxysporum* (Caracuel et al. 2003b), *C. rosea* (Zou et al. 2010), *F. graminearum* and *T. virens* (Merhej et al. 2011; Trushina et al. 2013). Previous studies indicated that PacC binds to the consensus sequence "GCCARG'' (Espeso et al. 1997; Tilburn et al. 1995). This consensus sequence is present in the promoter of *PdpacC* itself, suggesting that PdpacC can activates its own transcription at neutral or basic pH.

The expression dynamics of *pacC* during pathogenic fungal infection has been rarely investigated. The PacC ortholog in the nematophagous fungus *C. rosea* was up-regulated during the early stage of its infection of nematode whenthe pH was higher than 5 (Zou et al. 2010). In mature citrus fruits, the pH value of the peel is usually between 4.5 to 5.0, and decreases to 3.0 to 3.5 after infection by *P. digitatum* (Prusky et al., 2004, Table 2). Contrary to the expression pattern observed *in vitro*, the expression of *PdpacC* was definitely up-regulated during infection of citrus fruits regardless of the low pH (Fig. 2B). This result suggests that in addition to the well-known alkaline pH induction, alternative signal pathways that up-regulate or de-repress the *PdpacC* expression have been evolved in *P. digitatum*, thereby up-regulating the expression of *pacC*-dependent genes, such as *Pdpg2* and *Pdpnl1*, and allowing *P. digitatum* to adapt to acidic niches. Although other signaling pathways that trigger *PdpacC* overexpression during citrus fruit infection are unknown, our results showed that under *in vitro* conditions there was a clear up-regulation of *PdpacC* in SM medium when

418 pectin was added as the sole carbon source, up-regulation that was not observed in glucose
419 supplemented SM medium, although the pH in both instances was about 3.0 (Fig. 2D). This
420 result indicates that pectin triggers the induction or de-repression of *PdpacC.* Moreover, the
421 impaired utilization of pectin in the Δ*PdpacC* mutant (Fig. 5) suggests that *PdpacC* is
422 required for full utilization of pectin by *P. digitatum*.

423 PacC plays an important role in the regulation of virulence *via* programming the expression
424 or secretion of virulent factors such as hydrolytic enzymes, toxins and oxalic acid in different
425 plant pathogenic fungi. In *C. acutatum*, *C. gloeosporiodes* and *S. sclerotiorum*, PacC regulates
426 the virulence in a positive manner (Miyara et al. 2008; Rollins 2003; Rollins and Dickman
427 2001; You et al. 2007), whereas in *F. oxysporum* and *F. graminearum* PacC is a negative
428 regulator of virulence (Caracuel et al. 2003b; Merhej et al. 2011). In *C. gloeosporioides* the
429 decreased virulence in a *CgpacC* disrupted mutant was associated with impaired expression of
430 a pectate lyase (*PELB*) (Miyara et al. 2008). In *S. sclerotiorum* the decreased virulence in a
431 disrupted *pacC* mutant was associated with the impaired production of oxalic acid and the
432 shifting of endopolygalacturonase (*pg1*) expression to higher ambient pH (Rollins 2003). In
433 contrast, the increased virulence in the *F. oxysporum pacC*$^{+/-}$ loss-of-function mutant was
434 related to the increased expression of two endopolygalacturonase genes, *pg1* and *pg5*
435 (Caracuel et al. 2003b).

436 The results presented in this study showed that disruption of the *PdpacC* significantly
437 decreased the virulence of *P. digitatum* on citrus fruits (Fig.6). This lower virulence of the
438 Δ*PdpacC* mutant could not be attributed to a lower fitness of the mutant because the mycelial
439 growth of the Δ*PdpacC* mutant at acidic pH was similar to that of the wild type strain (Fig. 4).

However, our study showed that one of endopolygalacturonase gene (*Pdpg2*) and one pectin lyase gene (*Pdpnl1*) were up-regulated during the infection of citrus fruit in the wild-type but not, or weakly up-regulated, in the Δ*PdpacC* mutant (Fig. 7), suggesting that *Pdpg2* and *Pdpnl1* are *PdpacC*-responsive genes, and the decreased virulence in the Δ*PdpacC* mutant is associated with the impaired expression of *Pdpg2* and *Pdpnl1*. We have provided evidence that disruption of *Pdpg2* led to a decreased the virulence towards citrus fruits (Fig. 8A and B), further indicating that *Pdpg2* is a virulent factor for *P. digitatum*, and its expression is regulated by *PdpacC*. Pectin is the major component of the citrus peel (Mahmood et al. 1998). and the participation of endopolygalacturonases and pectin lyases in pathogenesis of *P. digitatum*, a typically necrotrophic pathogen, is expected. Overall, our results lead us to hypothesize that *PdpacC* is involved in pathogenesis of *P. digitatum via* regulation the expression of *CWDE* genes that are required for degradation of pectin, the major component of citrus peel.

The pH- and PacC-dependent genes were recently identified in *Trichoderma virens* by comparing the transcriptomes of wild type and *pacC* mutant cultures exposed to high or low pH (Trushina et al. 2013). The attenuation of virulence (Fig. 8A and B) in the Δ*Pdpg2* mutant was lower than that observed in Δ*PdpacC* (Fig. 6A and B) and could not account for the attenuation of virulence observed upon the loss of *PdpacC*. This difference in virulence berween Δ*Pdpg2* and Δ*PdpacC* suggests that other PdpacC-dependent genes are probably involved in virulence, and *Pdpnl1* is probably one of them. Comparison of the transcriptomes of the wild-type and the Δ*PdpacC* mutant of *P. digitatum* at acid conditions or during infection of citrus fruit would contribute to the identification of other genes or pathways

involved *PacC*-dependent pathogenesis.

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Legends to figures

Fig. 1 Construction and identification of *P. digitatum PdpacC* null mutants (Δ*PdpacC)*. **A**. *PdpacC* and hygromycin resistance cassette (*hph*) are denoted by large black and white arrows, respectively. Annealing sites of PCR primers are indicated with small arrows. **B**. Identification of Δ*PdpacC* mutants by PCR using primers PdpacCjd-F and PdpacCjd-R (Table 1). A 1.3 kb fragment of wild-type strain was replaced by a 2.8 kb in Δ*PdpacC* mutants. **C**. Southern blot analysis of genomic DNAs isolated from wild-type *P. digitatum*, Δ*PdpacC*A, Δ*PdpacC*B and CP*PdpacC* mutants. Ten μg of genomic DNA was digested with *Kpn*I and detected using a probe (the probe line shown in panel A) specific to the 3' region of *PdpacC*.

Fig. 2 Expression profiles of *PdpacC* in *P. digitatum*. The relative expression was calculated as fold changes ($2^{-\Delta\Delta Ct}$) using the *P. digitatum* γ-actin as the reference gene. Bars represent the average fold-change plus the calculated standard error calculated from 3 independent samples. **A**. 4-day-old mycelium was transferred to PDB buffered at different pH values and grown for another 12 h at 160 rpm and at 25 °C. The wild-type *P. digitatum* grown in unbuffered PDB was used as the control. **B**. Citrus fruits were inoculated with *P. digitatum* and incubated at room temperature. The tissue around the inoculation site was harvested at the indicated time points post inoculation. Wild-type *P. digitatum* grown in PDB was used as the control. **C**. 4-day-old mycelium was transferred to PDB supplemented with 0.5 mol/l NaCl for 12 h at 160 rpm and at 25 °C. Wild type *P. digitatum* grown in PDB was used as the control. **D**. Four-day-old cultures of the wild type and the Δ*PdpacC* mutant were transferred to SM supplemented with 1% pectin as the sole carbon source. Total RNAs were isolated from mycelium after 60 h of incubation at 160 rpm and at 25 °C. Wild-type of *P. digitatum* grown

624 in SM medium supplemented with 1% glucose as the only carbon source was used as the
625 control.

626

627 **Fig. 3** Effect of Na^+ and K^+ stress on growth of *PdpacC* mutants and the wild-type *P.*
628 *digitatum*. 7-mm-diameter mycelial plugs of the wild type and mutant cultures were placed
629 onto PDA supplemented with the 1 mol/l NaCl or KCl and incubated for 7 days at 25 °C. Bars
630 represent the average diameters plus the standard errors calculated from 3 independent
631 colonies. Different letters indicate significant differences between samples (P =0.05).

632

633 **Fig. 4** Growth assay of the wild-type *P. digitatum* and the *PdpacC* mutants at different pH
634 conditions. Mycelial plugs (7-mm-diameter) of the wild-type strain and the *PdpacC* mutants
635 were placed on PDA buffered at pH 4, 6 and 8, and incubated for 7 days at 25 °C. Bars
636 represent the average diameters plus the standard errors calculated from 3 independent
637 colonies. Different letters indicate significant differences between samples (P =0.05).

638

639 **Fig. 5** Growth assay of the wild-type strain and the *PdpacC* mutants of *P. digitatum* on SM
640 with 1% glucose or 1% pectin as the sole carbon sources. Mycelial plugs (7-mm-diameter) of
641 the wild type and the *PdpacC* mutants were placed on SM and incubated for 10 days at 25 °C.
642 Bars represent the average diameters plus the standard errors calculated from 3 independent
643 colonies. Different letters indicate significant differences between samples (P =0.05).

644

645 **Fig. 6** Virulence assay of the wild-type strain and the *PdpacC* mutants of *P. digitatum* on
646 citrus fruits. **A**. Citrus fruits (*Citrus unshiu*) were inoculated with conidial suspensions (10^6

647 ml^{-1}) prepared from the wild type and the mutants and incubated for 4 days at room
648 tempreature. **B**. Diameters of the lesions were measured at 4 day post inoculation. Bars
649 indicate the average diameters plus the standard errors calculated from 30 independent lesions.
650 Different letters indicate significant differences between samples (P =0.05).
651
652 **Fig. 7** Relative expression of *Pdpg2* (A) and *Pdpnl1* (B) in the wild-type strain and the
653 Δ*PdpacC* mutant of *P. digitatum* during infection of citrus (*Citrus unshiu*) fruits. Fifty mg of
654 peel tissue was harvested from each inoculated site at the times indicated. The relative
655 expression was calculated as fold changes ($2^{-\Delta\Delta Ct}$) using the *P. digitatum* γ-actin as the
656 reference gene and the wild-type *P. digitatum* grown in PDB as the control. Bars represent the
657 average fold-changes plus the calculated standard errors calculated from 3 independent citrus
658 fruits (P =0.05).
659
660 **Fig. 8** Virulence assay of the wild-type and the Δ*Pdpg2* mutant of *P. digitatum*. **A**. Citrus
661 (*Citrus unshiu*) fruits were inoculated with conidial suspensions (10^6 ml^{-1}) prepared from the
662 wild-type and the Δ*Pdpg2* mutant of *P. digitatum* and incubated at room temperature for 4
663 days. **B**. Diameters of the lesions were measured at day 4 post inoculation. Bars represent the
664 average diameters plus the standard errors calculated from 30 independent lesions. Different
665 letters indicate significant differences between samples (P =0.05).
666
667
668

669 Table 1. Primers used in this study

Name	Sequence (5' to 3')[a]	Purpose
PdpacCC-F	GGAATTCCGGGAAGAGGGAGGAGATGGG	PCR primers used to amplify full *PdpacC* including 1391 bp upstream and 55 bp downstream of the coding region
PdpacCC-R	GGAATTCCACATGTCGTTACGGCGTAAATC	
PdpacCjd-F	CCGAGCATGAAGCATCCTGACA	PCR primers used to identify transformants of *PdpacC*
PdpacCjd-R	TGGAGTTGGAGAAGCCGACGAG	
PdpacC-qF	AACTGCGGCGGTGAACAA	PCR primers used to amplify the *PdpacC* gene in qRT PCR assay
PdpacC-qR	CGAATGTGGGAGGTGATGTGAT	
PdpacCA	CCGCTCGAGCCCATCCTCGTCTTCATCCACA	PCR primers used to amplify downstream fragments of *PdpacC* (probe)
PdpacCB	GACTAGTCGCACAGTCATACACCTCCAACCG	
PdpacCC	CCGAGCTCGGCGGGTTGGTGGCTGCCTGAGGTT	PCR primers used to amplify upstream fragment of *PdpacC*
PdpacCD	GGGGTACCCCTCTAGGCATTCCGCCAATTCCAG	
Actin-qF	TCCACTACTGCCGAGCGTGAAAT	PCR primers used to amplify the reference gene γ–actin in qRT PCR assay
Actin-qR	CCGCCAGACTCAAGACCAAGAAC	
pg2-qF	GAAGGAACCACCACTTTCGG	PCR primers used to amplify the *Pdpg2* gene in qRT PCR assay
pg2-qR	TCAGGCTGTGAGCGTAGAAGAA	
Pdpg2A	CCCAAGCTTGCACTCGACAGGTGGGTAAAA	PCR primers used to amplify upstream fragment of *Pdpg2*
Pdpg2B	CCGAGCTCGCTTCTGGATAGGACAAGGCA	
Pdpg2C	GACTAGTCGAAGGAACCACCACTTTCGG	PCR primers used to amplify downstream fragments of *Pdpg2*
Pdpg2D	CCGCTCGAGGGACCAGTTGGAGCAAGCAC	
Pdpg2jd-F	TTGTGGCTGGGCTTTGGT	PCR primers used to identify transformants of *Pdpg2*
Pdpg2jd-R	ATCTTGCCGCCGTTGGTT	

670 a The underlined sequences indicate restriction sites in the primers.

671

672

673 Table 2. pH values at the inoculated sites in the peel of *C. unshiu* fruits

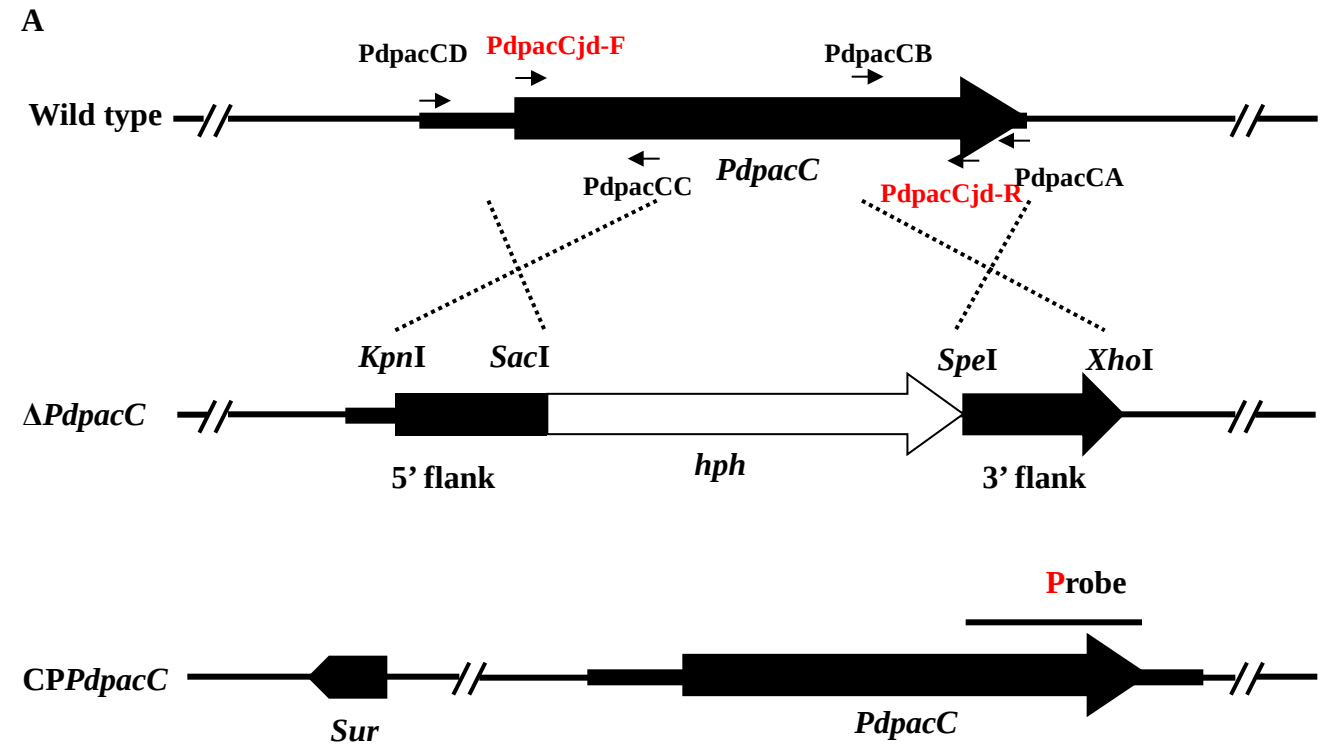
Time (h)*	0	12	24	48	72	96
pH	4.64 ±0.13	4.61 ±0.18	3.83 ±0.14	3.33 ±0.12	3.31 ±0.23	3.22 ±0.15

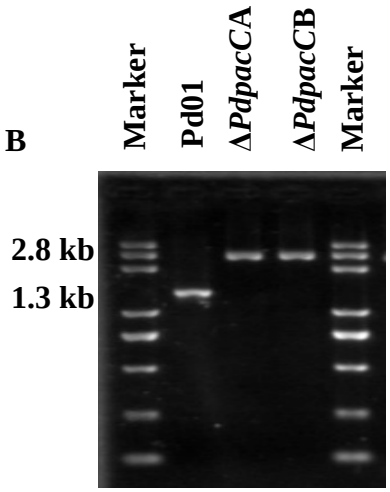
674 * Hours after inoculation. Average pH values ± standard error. pH was measured by inserting the micro combination pH
675 electrode Model 9810BN (Orion, Beverly, MA)directly into the mesocarp. All measurements were repeated on 9 fruits (at
676 least 30 measurements).

677

678

Fig. 1


A
PdpacCD
PdpacCjd-F
PdpacCB
Wild type
PdpacC
PdpacCC
PdpacCjd-R
PdpacCA
KpnI
SacI
SpeI
XhoI
ΔPdpacC
5' flank
hph
3' flank
Probe
CPPdpacC
Sur
PdpacC


B
Marker
Pd01
ΔPdpacCA
ΔPdpacCB
Marker
2.8 kb
1.3 kb

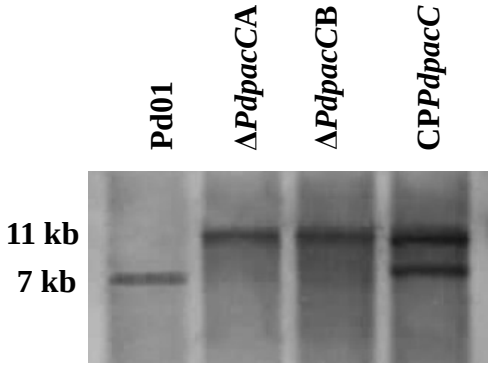

C
Pd01
ΔPdpacCA
ΔPdpacCB
CPPdpacC
11 kb
7 kb

Fig. 2

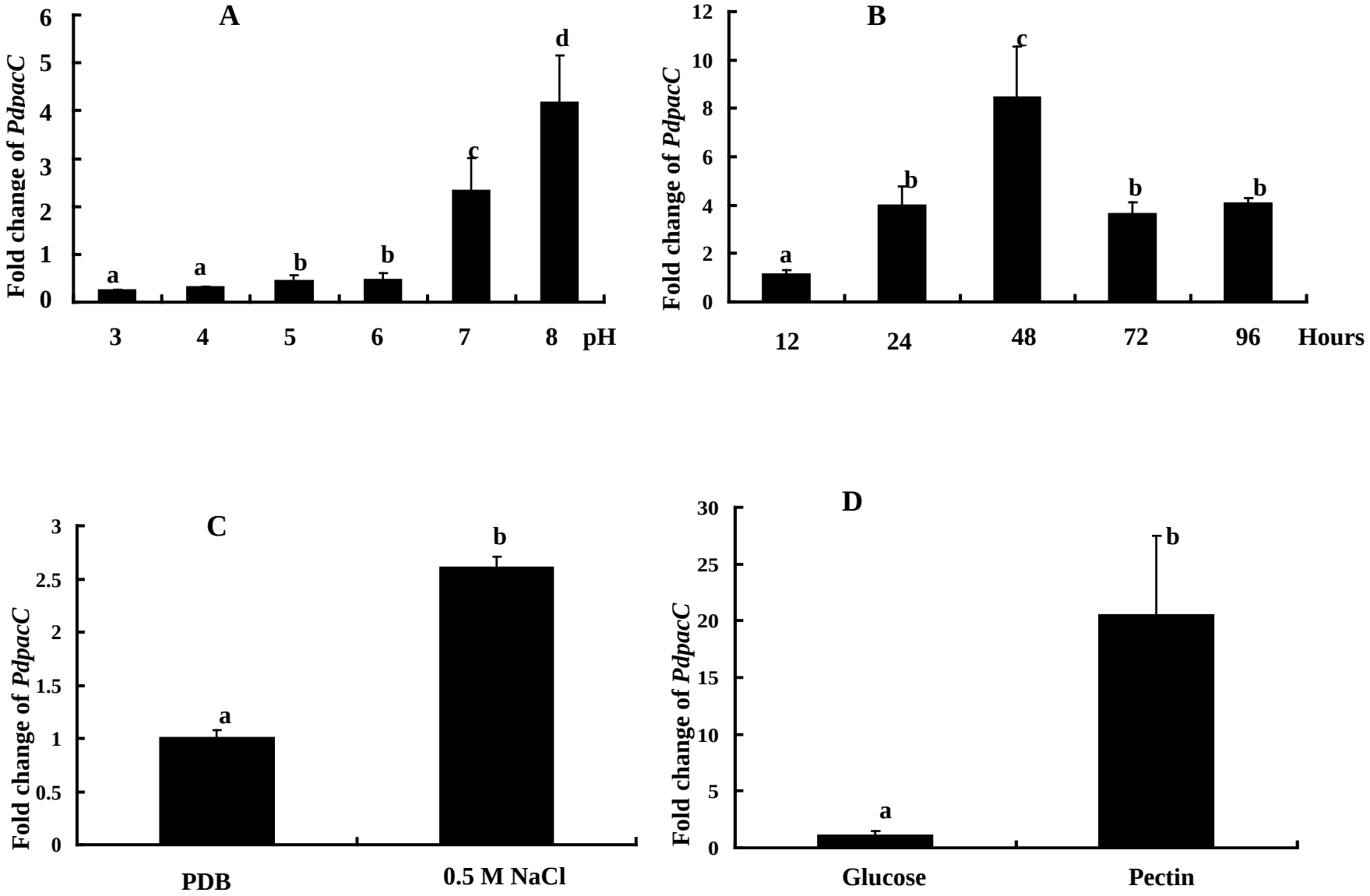

A
Fold change of PdpacC
6
5
4
3
2
1
0
a
a
b
b
c
d
3
4
5
6
7
8
pH
B
Fold change of PdpacC
12
10
8
6
4
2
0
a
b
c
b
b
12
24
48
72
96
Hours
C
Fold change of PdpacC
3
2.5
2
1.5
1
0.5
0
a
b
PDB
0.5 M NaCl
D
Fold change of PdpacC
30
25
20
15
10
5
0
a
b
Glucose
Pectin

Fig. 3

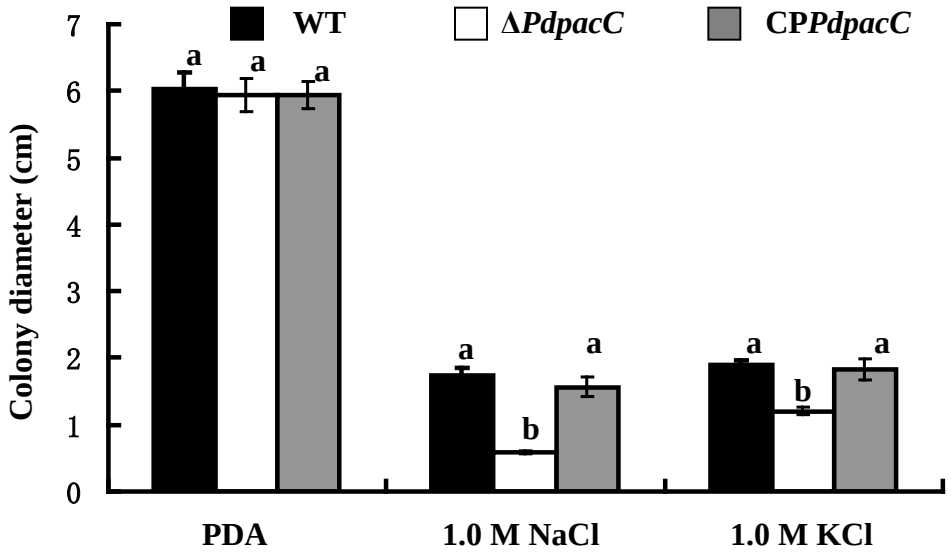

WT
ΔPdpacC
CPPdpacC
Colony diameter (cm)
0
1
2
3
4
5
6
7
a
a
a
a
b
a
a
b
a
PDA
1.0 M NaCl
1.0 M KCl

Fig. 4

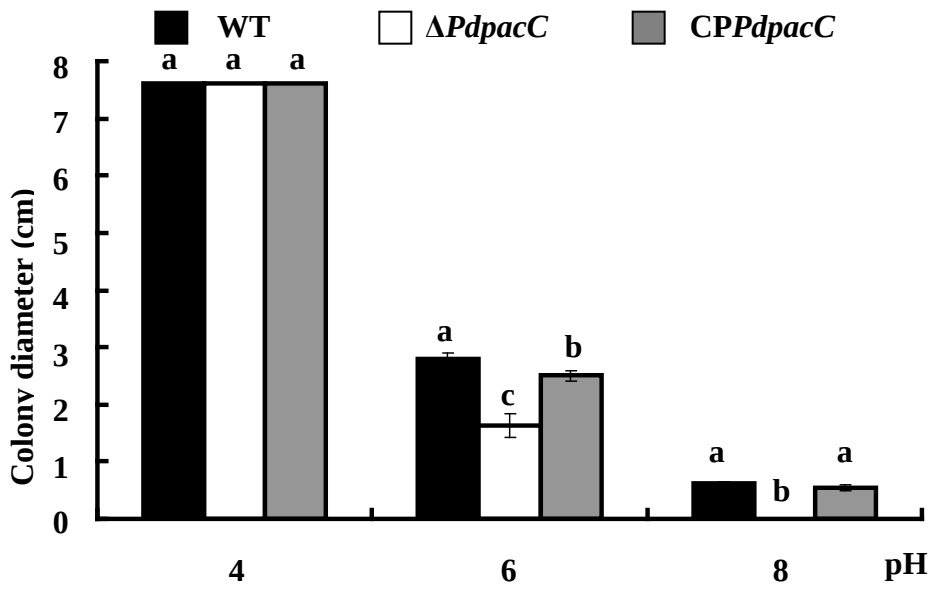

WT
ΔPdpacC
CPPdpacC
Colonv diameter (cm)
0
1
2
3
4
5
6
7
8
a
a
a
a
c
b
a
b
a
4
6
8
pH

Fig. 5

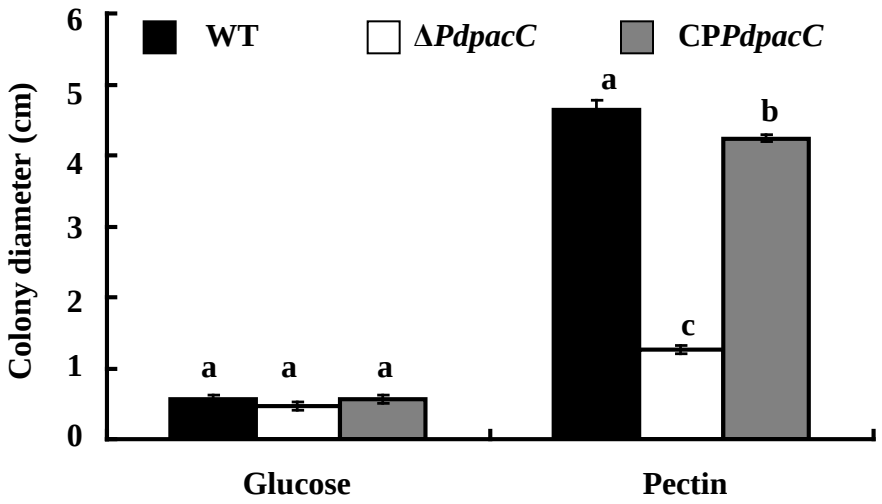

WT
ΔPdpacC
CPPdpacC
Colony diameter (cm)
6
5
4
3
2
1
0
a
a
a
a
c
b
Glucose
Pectin

Fig. 6

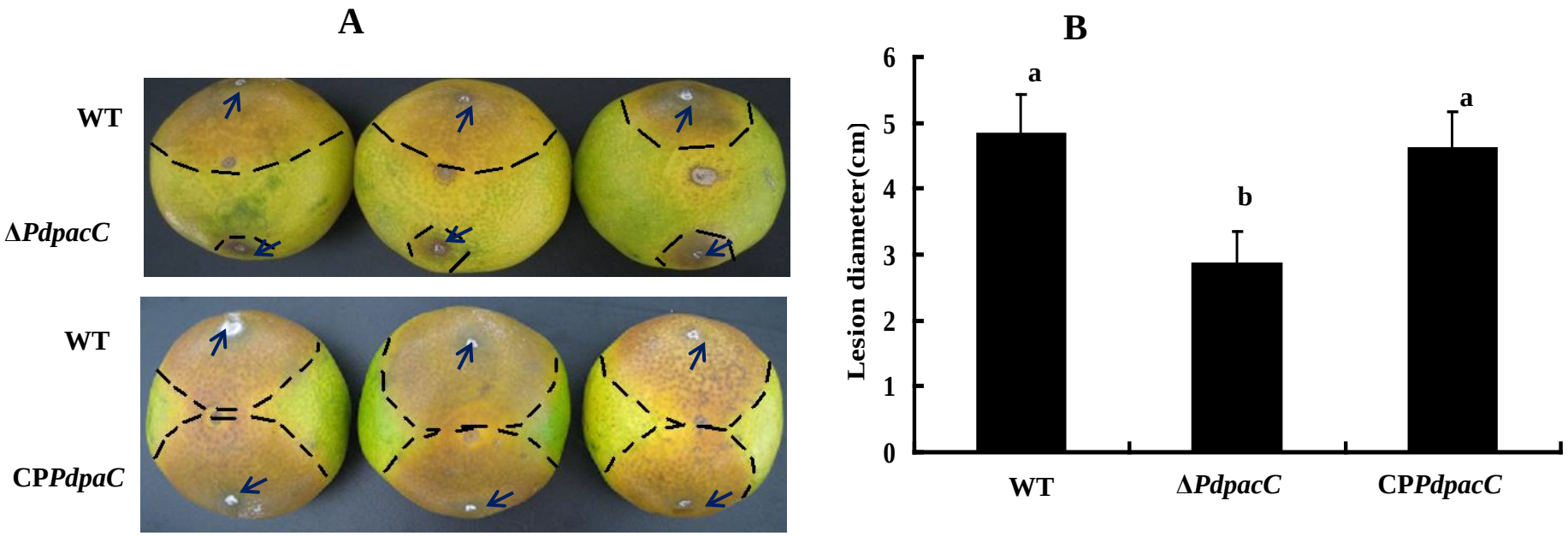

A
WT
ΔPdpacC
WT
CPPdpaC
B
Lesion diameter(cm)
6
5
4
3
2
1
0
a
b
a
WT
ΔPdpacC
CPPdpacC

Fig. 7

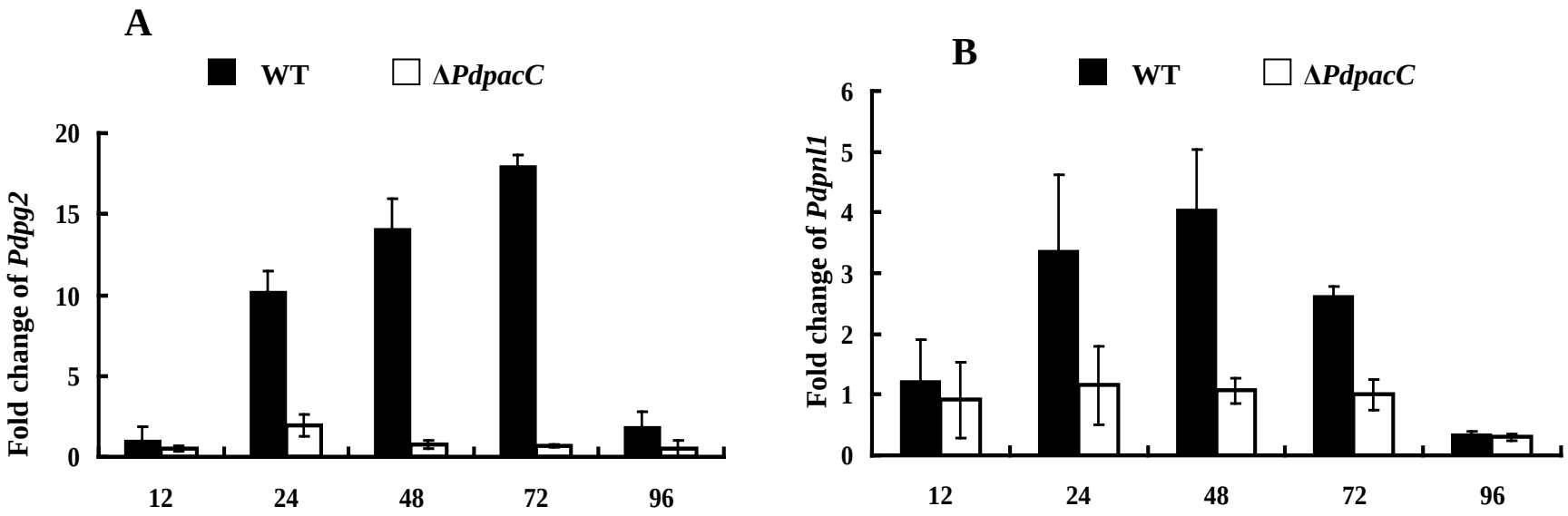
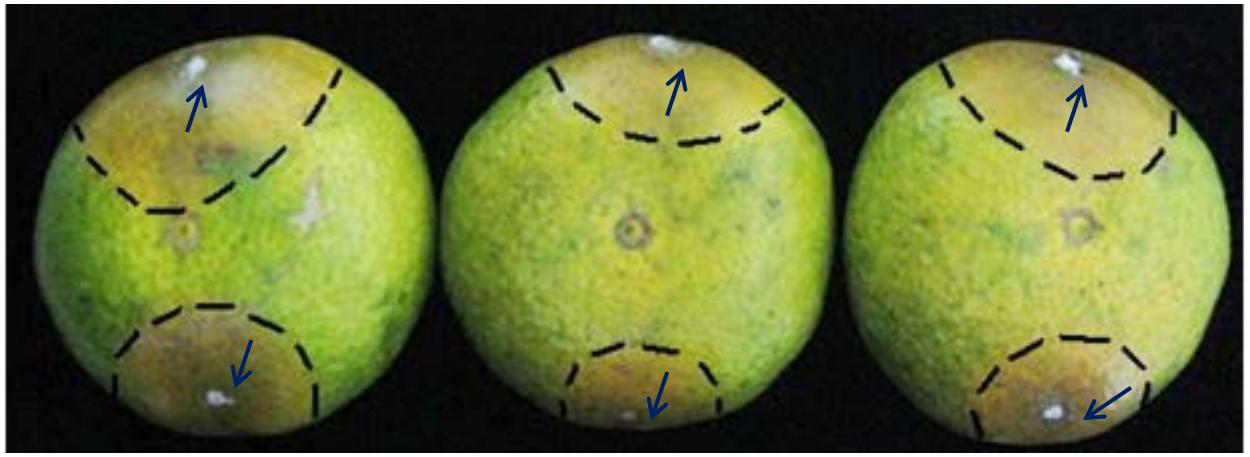
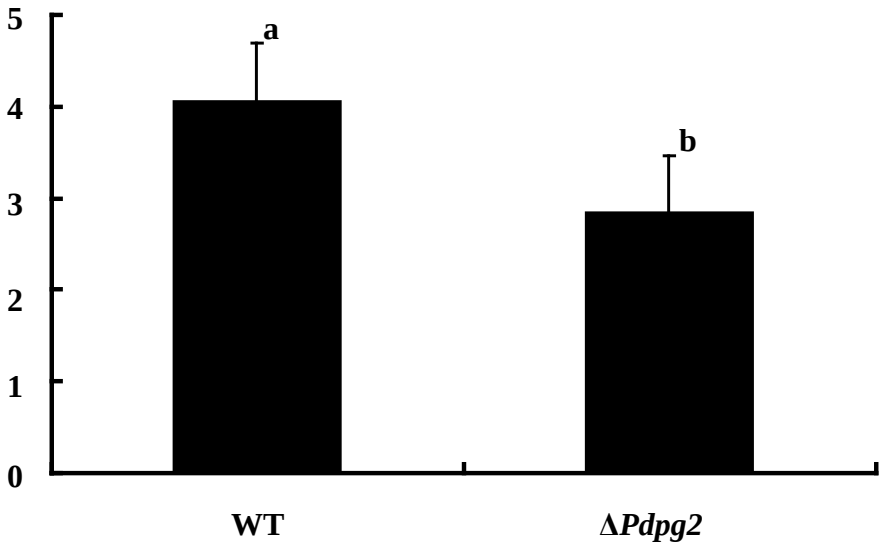

A
WT
ΔPdpacC
Fold change of Pdpg2
20
15
10
5
0
12
24
48
72
96
B
WT
ΔPdpacC
Fold change of Pdpnl1
6
5
4
3
2
1
0
12
24
48
72
96

Fig. 8

A



B