Multi-factor regulation of pectate lyase secretion by *Colletotrichum gloeosporioides* pathogenic on avocado fruits

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SUMMARY

Tissue alkalinization during *Colletotrichum gloeosporioides* attack enhances the expression of *PELB*, which encodes pectate lyase (PL), and PL secretion, which is considered essential for full virulence. We studied the regulation of PL secretion by manipulation of *C. gloeosporioides* *PELB*. *PELB* was down-regulated by knocking out *PAC1*, which encodes the PacC transcription factor that regulates gene products with pH-sensitive activities. We functionally characterized a *PACC* gene homologue, *PAC1*, from *C. gloeosporioides* wild-type (WT) Cg-14 and two independent deletion strains, $\Delta pac1_{372}$ and $\Delta pac1_{761}$. Loss-of-function *PAC1* mutants showed 85% reduction of *PELB* transcript expression, delayed PL secretion and dramatically reduced virulence, as detected in infection assays with avocado fruits. In contrast, *PELB* was up-regulated in the presence of carbon sources such as glucose. When glucose was used as a carbon source in the medium for the WT strain and the $\Delta pac1$ mutant at pH 6.0, *PELB* transcript expression and PL secretion were activated. Other sugars, such as sucrose and fructose (but not galactose), also activated *PELB* expression. These results suggest that the pH-regulated response is only part of a multi-factor regulation of *PELB*, and that sugars are also needed to promote the transition from quiescent to active necrotrophic development by the pathogen.

INTRODUCTION

Colletotrichum gloeosporioides is a broad-host-range plant-pathogenic fungus that secretes ammonia to alkalinize the host tissue (Drori *et al.*, 2003). This alkalinization process strongly activates the expression of *PELB*, which encodes pectate lyase (PL), and the secretion of PL, which has been implicated as a

virulence factor of *C. gloeosporioides* in avocado fruit (Drori *et al.*, 2003; Wattad *et al.*, 1997; Yakoby *et al.*, 2001). Drori *et al.* (2003) have suggested that PL secretion is also influenced by nutritional signals, such as the presence of nitrogen. The regulation of nitrogen assimilation is complex, and is important for disease development (Marzluf, 1997). In several *Colletotrichum* species, nitrogen starvation stimulates synchronous pre-infection development (Horowitz *et al.*, 2006) and the expression of genes that affect appressorium differentiation and formation (Donofrio and Dean, 2005; Soanes *et al.*, 2002). For *C. gloeosporioides*, the presence of a nitrogen source seems to be an important regulator of the expression of virulence factors. Nutritional deprivation of primary nitrogen sources has demonstrated that they are critical for PL secretion and fungal pathogenicity (Kramer-Haimovich *et al.*, 2006): PL secretion was observed only when there was a suitable environment (pH 6.0) as well as ammonia production. Increasing the amount of ammonia or glutamate added to the growth medium also increased *PELB* expression and PL secretion. Ammonia may therefore have two key functions: (1) tissue alkalinization, and (2) activation of *PELB* expression and PL secretion (Kramer-Haimovich *et al.*, 2006; Prusky *et al.*, 2001; Yakoby *et al.*, 2000).

Host alkalinization, which has been observed in avocado, apple, tomato and other infected fruit hosts (Prusky *et al.*, 2001), represents the basic signal for activation of the pH-dependent transcriptional regulator Pac1, which could modulate the activation of *PELB* and the secretion of PL. In the *Aspergillus nidulans* model system, the expression of genes encoding extracellular enzymes with differing pH optima has been shown to be governed by an ambient-pH-sensing signal-transduction pathway consisting of at least six *PAL* gene products (Negrete-Urtasun *et al.*, 1999). The target for regulation by this pathway is the zinc finger transcription factor PacC. This system has been thoroughly reviewed recently (Peñalva and Arst, 2004). Within this regulatory circuit, PacC is positively regulated under alkaline pH conditions, to promote transcription of alkaline-expressed genes and simultaneously repress the expression of acid-expressed genes (Tilburn *et al.*, 1995). The promoter regions of alkaline-expressed genes contain

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multiple copies of the 5'-GCCARG 3' consensus site that are recognized by PacC (Espeso *et al.*, 1997). Under acidic ambient pH, PacC is not activated. This system of gene regulation ensures that enzymes with pH-sensitive activity are produced under environmental pH conditions that favour their effective functioning.

Loss-of-function mutants of *PACC* in *A. nidulans* produce a constitutive acid-expressing phenotype that is characterized by increased acid phosphatase and decreased alkaline phosphatase activities, decreased sensitivity to aminoglycoside antibiotics, increased sensitivity to molybdate and lack of growth at alkaline pH (Arst *et al.*, 1994; Caddick *et al.*, 1986; Díez *et al.*, 2002; Espeso *et al.*, 2000; Orejas *et al.*, 1995). Dominant activating mutations of *PACC* reverse these effects (Caddick *et al.*, 1986; Espeso *et al.*, 1993; Shah *et al.*, 1991). Homologues of *PACC* have been isolated and characterized from ascomycete fungi closely related to *A. nidulans*, including *Aspergillus niger* and *Penicillium chrysogenum* (MacCabe *et al.*, 1996; Suárez and Peñalva, 1996) and from the cephalosporin-producing ascomycete, *Acremonium chrysogenum* (Schmitt *et al.*, 2001). A *PACC* homologue has been identified in *Sclerotinia sclerotiorum*, based on sequence homology and functional complementation of an *A. nidulans* *PACC*-null strain (Rollins and Dickman 2001).

As PL secretion is transcriptionally regulated by pH in *C. gloeosporioides*, it has been suggested that Pac1, which mediates the expression of the pH-regulated gene, is present in this fungus and that it modulates *PELB* (Denison, 2000; Drori *et al.*, 2003; Ramon *et al.*, 1999; Shah *et al.*, 1991). Drori *et al.* (2003) showed that five repeats of the sequence GCCAAG, the core binding site for *PACC*, are found in the *PELB* promoter

sequence. These repeats are situated at positions -547, -493, -410, -368 and -262, relative to the translation-initiation codon (ATG).

The goals of the present study were to elucidate the different factors modulating PL secretion and *C. gloeosporioides* pathogenicity. Our results suggest that the *C. gloeosporioides* loss-of-function *PAC1* mutant exhibits diminished PL secretion, limiting the colonization of avocado fruits. In addition to ammonia, the importance of which has been discussed previously (Kramer-Haimovich *et al.*, 2006), the sugars that become available during ripening of the attacked host fruit are key factors in promoting *C. gloeosporioides* pathogenicity. We hypothesize that pH, sugar and nitrogen are independent signals for the transcriptional regulation of genes required for the pathogenicity process of *C. gloeosporioides* and for the transition from the quiescent-biotrophic to necrotrophic stage of *Colletotrichum* colonization.

RESULTS

Characterization of $\Delta pac1$ mutants

Out of 140 transformants, only two independent hygromycin-resistant mutants ($\Delta pac1_{372}$ and $\Delta pac1_{761}$) showed correct integration into the *PAC1* gene (Shafran *et al.*, 2007b; Fig. 1). A series of mutants that included those with the ectopic integration construct (a control for *PAC1* integration) or the *PAC1* overexpression construct (that positively modulates the *PAC1*-affected genes) (for details see Experimental procedures) were used for further tests. When the fungus was grown on M₃S medium, no differences in mycelial growth were evident between the wild-type (WT;

Fig. 1 Schematic representation of homologous recombination events with replacement of the full-length (13 bases upstream of the ATG to the stop codon) *PAC1* gene (position 925–2778) of *C. gloeosporioides* with the hygromycin-resistance gene cassette HygR flanked by 5' (position 108–924) and 3' (position 2779–3485) *PAC1* genomic fragments. The HygR gene cassette includes the promoter and terminator of the *TRPC* gene of *Aspergillus nidulans* flanking the coding region of the *hphB* gene of *Escherichia coli*.

Fig. 2 Radial growth of wild-type and $\Delta pac1_{372}$ mutant strains on PDA-citrate phosphate buffer (0.1 M) at pH values ranging from 4 to 8. Colony diameters were measured 3 days after inoculation. Each point represents the mean diameter $\pm$ the standard deviation of three independent colonies.

isolate Cg-14) and the $\Delta pac1_{372}$ or $\Delta pac1_{761}$ gene-deletion mutants. Spore germination and appressorium formation did not differ between the WT progenitor and mutant strains. To determine whether the $\Delta pac1$ mutation affects the growth response to external pH, the radial growth rates of the WT and two $\Delta pac1$ mutants were examined on buffered potato–dextrose agar (PDA)

media. The radial growth rates of the WT and $\Delta pac1_{372}$ strain were similar at pH 4.0 (Fig. 2), but at pH 6.0 and 8.0, that of the mutant was inhibited by 32 and 44%, respectively, compared with the WT. Similar inhibition at pH 6.0 and 8.0 (31.5 and 45%, respectively) was observed with the $\Delta pac1_{761}$ mutant. Transformants with the ectopic integration or the *PAC1* overexpression construct exhibited growth similar to that of the WT throughout the pH range. In addition, the $\Delta pac1_{372}$ and $\Delta pac1_{761}$ transformants showed 63% (0.041 ± 0.01 U/mg protein) and 65% (0.038 ± 0.01 U/mg protein) inhibition in the secretion of alkaline phosphatase into the culture media at pH 6.0 relative to the WT (0.108 ± 0.03 U/mg protein). The ectopic-integration transformant showed alkaline phosphatase activity similar to that of the control (0.096 ± 0.04 U/mg protein) while the *PAC1*-overexpressing transformant showed an increase of 39% (0.151 ± 0.01 U/mg protein) in alkaline phosphatase secretion. The WT and the $\Delta pac1_{372}$ and $\Delta pac1_{761}$ transformants did not differ in the amounts of ammonia they secreted into the medium, or in their alkalization process.

The mesocarp and pericarp of avocado fruit were inoculated to determine the effect of *PAC1* loss-of-function on pathogenicity. By 3 days post-inoculation, the WT strain had induced decay on the avocado mesocarp, whereas almost none had been induced by the $\Delta pac1_{372}$ or $\Delta pac1_{761}$ mutants (Fig. 3A), and by

Fig. 3 Pathogenicity assays of *C. gloeosporioides* wild-type, ectopic and $\Delta pac1$ mutant strains. (A) Decay development of wild-type, ectopic-integrated construct and mutant strains after inoculation of peeled (on the mesocarp) or unpeeled (on the pericarp) freshly harvested avocado fruits. (B) Decay development on avocado fruit mesocarp tissue infected by wild-type, ectopic₃₆₄, $\Delta pac1_{372}$ and $\Delta pac1_{761}$ strains. (C) Relative expressions of *PELB* and *PAC1* in the decayed mesocarp of infected avocado fruit tissue. For decay development assessment, spores of the four strains were inoculated in holes (1 mm deep and 5 mm diameter) in the peeled fruits or placed directly on the pericarp, and the inoculated fruits were incubated at 25 °C under high humidity. Pictures were taken 3 and 7 days after inoculation of the mesocarp and pericarp, respectively. The average data for ten infected fruits are reported. For *PELB* and *PAC1* expression, the relative expression values obtained by quantitative RT-PCR normalized against 18S rRNA are the averages $\pm$ standard deviations, obtained from three replications of the treatment.

(C)

Relative expression	WT	$\Delta pac1_{372}$	Ectopic ₃₆₄	No fungi
<i>PELB</i>	470 $\pm$ 67	1 $\pm$ 0.21	421 $\pm$ 91	N.D.
<i>PAC1</i>	1 $\pm$ 0.2	0.003 $\pm$ 7 $\times 10^{-4}$	0.9 $\pm$ 0.15	N.D.

Fig. 4 Relative expression of *PAC1* and *PL* secretion by the wild-type, $\Delta pac1_{372}$ and $\Delta pac1_{761}$ mutants, an ectopic integrated-construct strain (Ectopic₃₆₄) and a *PAC1*-overexpressing strain overexp₇ of *C. gloeosporioides* at pH 6.0, 16 h after transfer of the growing hyphae from the primary medium to the secondary inducing medium. The relative expression values obtained by quantitative RT-PCR normalized against 18S rRNA are the averages $\pm$ standard deviations from three replications of the treatment. Western blot analyses were repeated three times and quantified by TINA 2.0 software (raytest Isotopenmeßgeräte GmbH); results of a representative experiment are presented (bottom).

7 days post-inoculation the diameter of the lesion induced by the $\Delta pac1_{372}$ and $\Delta pac1_{761}$ mutants had grown to 5 and 6 mm, respectively, compared with 21.5 mm for that induced by the WT strain (Fig. 3B) and the ectopic₃₆₄ integrated-construct strain. A similar pattern of decay inhibition was observed when the WT, ectopic₃₆₄, $\Delta pac1_{372}$ and $\Delta pac1_{761}$ mutants strains were applied to intact fruits and observed 7 days after inoculation (Fig. 3A). Strong transcript expression levels of *PELB* and *PAC1* were found in the decayed mesocarp tissue inoculated by the WT and ectopic₃₆₄ strains, compared with their minor expression with the $\Delta pac1_{372}$ mutant (Fig. 3C). No *PELB* or *PAC1* was detected in uninoculated fruits.

Previous studies have shown that accumulation of *PELB* transcript is promoted by an alkaline growth environment (Yakoby *et al.*, 2001; Drori *et al.*, 2003). When the levels of *PAC1* transcript were compared with *PL* accumulation by the WT, the $\Delta pac1$ strains, an ectopic strain and a *PAC1*-overexpressing strain, a

Fig. 5 Relative expression of *PAC1* and *PELB* and *PL* secretion by the wild-type and $\Delta pac1_{372}$ mutant strains of *C. gloeosporioides* at pH 6.0. (A) Expression of *PAC1*. (B) Expression of *PELB*. (C) *PL* secretion. Relative expression was detected by RT-PCR 7 and 15 h after transfer of the growing hyphae from the primary medium to the secondary inducing medium. The relative expression values normalized against 18S rRNA are the averages $\pm$ standard deviations of three replications of the treatment. Western blot analyses were repeated three times, and results from a representative experiment are presented.

direct relationship was found between the level of *PAC1* transcript in each strain and the molecular phenotypic fungal response of *PL* accumulation (Fig. 4). To determine more specifically the effect of the *PAC1* loss-of-function mutation on *PELB* transcription and *PL* accumulation, the transcript and *PL* accumulation levels were compared between WT and $\Delta pac1_{372}$ strains after 7 and 15 h of incubation at pH 6.0. Seven hours after transfer of the $\Delta pac1$ hyphae from the primary to the secondary inducing media, no *PELB* expression or *PL* secretion were detected, whereas *PL* was already being secreted by the WT strain (Fig. 5). Eight hours later, the $\Delta pac1_{372}$ mutant still showed a significantly lower level of *PELB* expression than the WT, but *PL* secretion by the mutant was detected (Fig. 5).

Fig. 6 Effect of carbon sources in the culture media on PL secretion by *C. gloeosporioides* wild-type and $\Delta pac1_{372}$ mutant strains. Avocado cell wall (1%, w/v) or 50 mM glucose was added to the inductive secondary medium as the carbon source. PL accumulation was detected 15 h after transfer of the growing hyphae from the primary medium to the secondary inducing medium. Western blot analyses were repeated three times, and results from a representative experiment are presented.

Fig. 7 Effect of glucose concentration on the induction of PL secretion and *PELB* expression by the *C. gloeosporioides* $\Delta pac1_{372}$ mutant strain. Relative gene expression was determined by RT-PCR, and PL secretion was detected 15 h after transfer of the growing hyphae from the primary medium to the secondary inducing medium at pH 6.0. The relative expression values normalized against 18S rRNA are the averages $\pm$ standard deviations of three replications of the treatment. Western blot analyses were repeated three times; results from a representative experiment are presented.

Effect of glucose on up-regulation of *PELB* gene expression at pH 6.0

To determine whether the effect of carbon source on *PELB* transcript levels was independent of *PAC1* regulation, PL secretion levels were compared in the presence of 1% avocado cell wall or 50 mM glucose, in the WT strain and in the $\Delta pac1_{372}$ mutant strain. The presence of glucose enhanced PL secretion in both the $\Delta pac1$ mutant and WT strains (Fig. 6). Growth of the $\Delta pac1_{372}$ mutant at glucose concentrations of 0, 25, 50 and 100 mM enhanced *PELB* transcript expression by 6-, 8- and 26-fold, respectively, and also increased PL secretion (Fig. 7).

Glucose enhanced PL secretion when added to several other less efficient carbon sources for *C. gloeosporioides* growth. Secretion of PL was always enhanced by the addition of 50 mM glucose when the WT strain was grown in the presence of

Fig. 8 Relative expression of *PELB* and PL secretion by *C. gloeosporioides* in the presence of different carbon sources added to the secondary inducing medium at pH 6.0. (A) Effect on PL secretion of galacturonic acid (GA, 1%), polygalacturonic acid (PGA, 1%) or pectin (1%), with or without glucose. (B) Effect of glucose, fructose or galactose on *PELB* expression and PL secretion. The relative expression values are the averages $\pm$ standard deviations of three replications of the treatment. Western blot analyses were repeated three times; results from a representative experiment are presented.

Fig. 9 Effect of the combination of pH, nitrogen and glucose on the secretion of PL by *C. gloeosporioides*. PL secretion was assayed 15 h after transfer of the growing hyphae from the primary medium to the secondary inducing medium in the presence of various combinations of pH, and nitrogen and carbon sources. Western blot analyses were repeated three times; results of a representative experiment are presented.

galacturonic acid (GA), polygalacturonic acid (PGA) or pectin (Fig. 8A). Levels of *PELB* transcripts and PL secretion were also enhanced in the presence of single sugars such as fructose, but not by galactose (Fig. 8B).

Multiple signals regulate PL secretion

When the WT strain was grown in the presence of glucose, a nitrogen source (NO_3) or both at pH 4.0, no secretion of PL was detected (Fig. 9). PL was not detected if the fungus was grown at

pH 6.0 with the addition of glucose or KNO₃; it was secreted only when the fungus was grown at pH 6.0 in the presence of both glucose and a nitrogen source (Fig. 9).

DISCUSSION

PAC1 deletion in *C. gloeosporioides* and its effect on pathogenicity

Previous studies (Drori *et al.*, 2003) have shown that the expression of *PELB*, which encodes PL, a key factor for pathogenicity of *C. gloeosporioides* in avocado, is up-regulated under alkaline conditions and during pathogenicity, in parallel to the expression of *PAC1*. As a first step in analysing the functional regulation of *PELB* by the transcription factor *PACC*, the encoding gene, *PAC1* from *C. gloeosporioides*, was cloned (Kramer-Haimovich *et al.*, 2006). Targeted gene deletion in WT *C. gloeosporioides* resulted in two independent *PAC1* loss-of-function mutants; these mutations showed no effect on germination or appressorium formation, but at pH 6.0 and 8.0, the growth of the mutants was reduced by 32 and 44%, respectively, compared with that of the WT. The $\Delta pac1_{372}$ and $\Delta pac1_{761}$ mutants also showed an average 64% inhibition of alkaline phosphatase activity compared with the WT. This differential physiological response of *PACC* mutants was similar to those found for *S. sclerotiorum* (Rollins, 2003), *Fusarium oxysporum* (Caracuel *et al.*, 2003) and *Colletotrichum acutatum* (You *et al.*, 2007).

The attenuated virulence of the two $\Delta pac1$ mutants clearly suggests that the pH-responsive gene expression acts as a significant molecular regulator of pathogenicity in this pathogen. A comparison of the decreases in virulence of the $\Delta pelB$ (Yakoby *et al.*, 2001) and the $\Delta pac1$ mutants relative to that of the WT (c. 40 and 72%, respectively) suggests that deletion of *PAC1* further reduces fungal colonization by about 32%. These results suggest that other genes, in addition to *PELB*, that are regulated by *PAC1* contribute to the development of *C. gloeosporioides* decay in avocado fruit. The full range of genes and processes regulated by *PAC1* in *C. gloeosporioides* is unknown, but the possibility of significant roles of other factors in *C. gloeosporioides* virulence, in addition to those revealed by $\Delta pac1$ deletion, cannot be discounted (Caddick *et al.*, 1986; Flaherty *et al.*, 2003; Lahey *et al.*, 2004; Li and Mitchell 1997; Li *et al.*, 2003; You *et al.*, 2007). Our results are consistent with a decrease in PL production accounting for virulence attenuation.

PELB gene regulation in *C. gloeosporioides*

The role of Pac1 as a regulator of the response of *C. gloeosporioides* gene expression to ambient pH is supported by the observation that PL accumulation at pH 6.0 is reduced in the $\Delta pac1$ loss-of-function mutants. However, two observations add to the perceived

complexity of *PELB* regulation, and indicate that *PAC1* is not the only regulator of *PELB* expression and PL production in *C. gloeosporioides* (Peñalva and Arst, 2002). The first is that although accumulation of *PELB* transcripts was greatly reduced in the $\Delta pac1_{372}$ loss-of-function mutant, PL secretion increased in the mutant and in the WT strain at pH 6.0, when grown in the presence of glucose as a carbon source. Second, glucose induced *PELB* expression and PL secretion in the $\Delta pac1_{372}$ mutant, whereas no *PAC1* expression was observed. These results suggest that although Pac1 is an important factor for pathogenicity under alkaline pH, a combination of environmental factors, including sugar sources, might be of great importance for the activation of pathogenicity genes.

The investigation of pathogenicity regulation by carbon nutrition has primarily focused on repression of cell-wall-degrading enzymes by carbon catabolites (Dean and Timberlake, 1989; Gómez-Gómez *et al.*, 2002; Reymond-Cotton *et al.*, 1996; Tonukari *et al.*, 2000; Wubben *et al.*, 2000). *CREA* is a major regulatory gene of carbon catabolites in filamentous fungi, and is functionally homologous to *MIG1* from yeast. Transcriptional regulators belonging to this class function in the presence of glucose, to repress genes required for the uptake and catabolism of more complex carbon sources. In the above studies, analysis of the *PELB* promoter could not detect the presence of a *CREA*-binding sequence. In the present study, glucose not only did not repress *PELB*, it significantly enhanced *PELB* expression and PL secretion, indicating that the ability to sense and respond properly to ambient carbon can modulate virulence in phytopathogenic fungi. Moreover, in *C. gloeosporioides* f. sp. *malvae*, the gene *PNL1*, encoding pectin lyase, was induced by pectin and glucose, whereas *PNL2* was induced mainly by mallow cell-wall extracts (Wei *et al.*, 2002). The basis for this differential gene activation by glucose in *C. gloeosporioides* has not been elucidated. Glucose behaves as a positive regulator of genes in general and of fungal pathogenicity genes in particular. In *Saccharomyces cerevisiae*, a glucose-activated transcription factor, *AZF1*, was found to be a positive regulator of *CLN3 G₁* cyclin; this activation involved a set of DNA elements with the sequence AAGAAAAA (A₂GA₃) (Newcomb *et al.*, 2002). In the present study, we found the specific antisense sequence T₂CT₅ in the *C. gloeosporioides* *PELB* promoter at -57 bp from the ATG. In addition, the yeast protein Azf1 functions as a glucose-dependent transcriptional activator in yeast (Newcomb *et al.*, 2002), which could account for the specific activation of *PELB* and *PNL1*.

Why is glucose activation important for *Colletotrichum* pathogenicity? Clear differences in gene expression levels of cell-wall-degrading enzymes have been observed among members of the *BCPG* gene family of *Botrytis cinerea* (Wubben *et al.*, 1999, 2000): expression of some members of the gene family (*BCPG3* and 5) was predominantly exhibited in the presence of glucose. Coordinated regulation of gene expression can be envisaged

to occur during infection of plants and fruits: endoPGs from constitutively expressed genes might release pectin-degradation products, which could induce the expression of other endoPG-encoding genes. This may be the case in several host fruits when attacked by pathogens such as *Colletotrichum*; during ripening of these fruits, the complex carbohydrates are transformed to simpler sugars such as sucrose, glucose and fructose, which induce PELB expression and PL secretion.

The regulation of pathogenicity in *Colletotrichum*, however, is even more complex, because it is also affected by nitrogen availability. Previous analyses of global, environment-responsive pathogenicity regulators in *Colletotrichum* have also focused on signalling pathways and regulatory factors involved in nitrogen uptake and utilization (Drori *et al.*, 2003; Pellier, *et al.*, 2003). The dynamic ambient pH environment induced by several *Colletotrichum* species, which, during host infection, secrete sufficient ammonia to raise the ambient pH, also induces PELB expression and PL secretion (Kramer-Haimovich *et al.*, 2006). In *Magnaporthe grisea*, *NPR1* and *NPR2*, which are involved in nitrogen utilization, are also required for expression of the pathogenicity-related hydrophobin gene *MPG1* (Lau and Hamer 1996). The loss-of-function mutation in *NPR1* or *NPR2* results in severely attenuated pathogenicity. These findings, together with those of Drori *et al.* (2003) and Kramer-Haimovitch *et al.* (2006), suggest that global nitrogen-regulating genes are also important for the regulation of virulence factors and pathogenicity (Pérez-García *et al.*, 2001).

The finding of joint or multi-factor regulation of a single pathogenicity gene by several different environmental factors is not new, but most reported examples involve complex combinations of positive and negative regulation. Expression and secretion of Acp1 protease by *S. sclerotiorum*, and secretion of PepA and PepB by *A. niger* are induced under glucose and nitrogen starvation, but are inhibited under alkaline conditions (Jarai and Buxton, 1994; Poussereau *et al.*, 2001). Espeso *et al.* (1993) has shown that although transcription of the *A. nidulans* gene encoding isopenicillin N synthase during the synthesis of penicillin is under the control of the pH regulatory system mediated by the PacC protein, external alkaline pH overrides carbon regulation. In contrast to penicillin synthesis, aflatoxin production by *A. nidulans* increases in glucose- and ammonium-based media and decreases in nitrate-based media, but in this case, too, an external alkaline pH overrides carbon regulation (Keller *et al.*, 1997). In contrast, PELB regulation, as observed in the present study, is unique in its joint involvement with alkaline pH, glucose and nitrogen in the activation of PELB transcription.

What is the significance of this joint regulation of PELB? The involvement of several different factors in PL regulation and secretion is indicative of the wide range of tools used by the fungus to exert its pathogenicity in the fruit. The active accumulation of ammonia at the leading edge of the infection court is the first factor contributing to the activation of fungal pathogenicity by

providing optimal pH. This early process, together with local enhancement of fruit ripening, could contribute to the availability of the sugar and nutritional conditions that lead to the enhanced activation of PELB expression. Alteration of ammonia secretion and pH-regulated processes could form the basis for a viable strategy to delay the transformation from the quiescent-biotrophic to necrotrophic stage of fungal colonization (Eshel *et al.*, 2002; Prusky *et al.*, 2001), and thereby reduce or prevent disease development by this plant-pathogenic fungus.

EXPERIMENTAL PROCEDURES

Construction of plasmids

The full sequence of *PAC1* was obtained from Dr Jeffrey Rollins (Gainesville, FL; accession no. EF585491). Full deletion of *PAC1* from 13 bases upstream of the ATG (position 925) to the stop codon (position 2879) was created by using *hphB* driven by the *TRPC* promoter and terminator from plasmid pGEM:hyg (Horowitz *et al.*, 2006) as a selectable marker. The hygromycin marker (HygR) was flanked by an upstream sequence (108–925) and a downstream sequence (2879–3486) of the *PAC1* gene (Fig. 1). The construct was assembled with the Multi Site Gateway Three-Fragment Vector Construction Kit (Invitrogen, Carlsbad, CA). Full details of the development of this method are described elsewhere (Shafran *et al.*, 2007a,b). The construct was transformed into *C. gloeosporioides* by electroporation (Yakoby *et al.*, 2001). The transformants were regrown as single colonies on M₃S agar with hygromycin B (70 mg/L), and DNA was extracted with the Master Pure Yeast Purification Kit (Epicenter Biotechnologies, Madison, WI). Two colonies out of 140 transformants were found to be positive by PCR and were identified as positive for *PAC1* deletion: $\Delta pac1_{372}$ and $\Delta pac1_{761}$. Single colonies of these deletion candidates were grown, and DNA was extracted from them. Southern analyses of these colonies revealed full deletion of *PAC1*, and this was confirmed by sequencing of the deletion area in the genome (Shafran *et al.*, 2007a,b).

To create an overexpression construct, we used the pSilent1 plasmid (Nakayashiki *et al.*, 2005), obtained from the Fungal Genetic Stock Center. In the first stage, a Gateway conversion cassette A (Invitrogen 11828-029, Carlsbad, CA) was inserted into the *XhoI* site of Silent1, which was blunted by Klenow reaction. In the next stage, we deleted the intron that exists in pSilent1 by cutting the plasmid with *SphI* and *BglII*, and blunting the ends by Klenow reaction and self-ligation. The new overexpression plasmid (FGOE1) can express fungal proteins under the *TRPC* promoter. A dominant truncated *PAC1* that mimics the *pac481* mutation (Caracuel *et al.*, 2003) was created by using oligos, *PAC1* attB1-5' GGGGACAAGTTTGTACAAAAAGCAGGCTATGTCCA-CCAAGCAAGAACAC and *pac1* stop2520 attB2 3' GGGGACCACT-TTGTACAAGAAAGCTGGGTTTACTCGTGTGTCGAAGCTGGGTCC.

The PCR product was cloned into FGOE1 using standard Gateway protocols (Invitrogen). When the fungus was grown on M₃S medium, no differences in mycelial growth were evident between the WT (isolate Cg-14) and two overexpressing mutants (overexpress₇, overexpress₂).

Fungal isolates, media and growth conditions

Single-spore cultures of a Cg-14 isolate of *C. gloeosporioides*, obtained from a decayed avocado fruit (*Persea americana* cv. Fuerte) in Israel, were routinely cultured on M₃S agar (Tu, 1985) containing 250 mg/L chloramphenicol. To determine sensitivity to pH, the fungus was grown on PDA medium (Difco, Detroit, MI) buffered to the desired pH level with 0.1 M citrate-phosphate buffer. The fungus was also grown in 40 mL M₃S liquid medium at pH 5.0, at an inoculation density of 1×10^6 spores per flask. The M₃S medium contains the following reagents (per litre): 2.5 g MgSO₄·7H₂O, 2.7 g KH₂PO₄, 1.5 g Bacto peptone, 1.5 g Bacto yeast extract (Difco), 15 g sucrose and 250 mg chloramphenicol (primary medium). The cultures were incubated at 22–24 °C in a shaking incubator at 150 r.p.m. for 4 days, and were harvested by filtration through a sterile Büchner funnel fitted with filter paper. The hyphal mat was washed twice with 40 mL of sterile distilled water. To determine the amount of PL protein accumulation, the washed mycelia were resuspended in 40 mL of fresh medium (secondary inducing medium) containing the following reagents (per litre): 4 g K₂HPO₄, 2 g MgSO₄·7H₂O, 5 g KNO₃, 0.3 g CaCl₂·2H₂O, 10 mg FeCl₃ and a carbon source [50 mM glucose, fructose or galactose, or 1% avocado cell wall, galacturonic acid (GA), PGA or pectin]. The medium was buffered with 50 mM phthalate-hydroxide buffer (Sigma, St Louis, MO) to obtain an initial pH between 4.0 and 7.0. The initial pH for each flask was determined after the medium had been autoclaved, but prior to inoculation. For alkaline phosphatase detection, the washed mycelia were resuspended in 40 mL limiting-phosphate medium (Dorn, 1965) containing (per litre): 6 g NaNO₃, 0.52 g MgSO₄·7H₂O, 0.52 g KCl, 10 g dextrose, 1 g trishydroxymethyl-aminomethane, 0.03% (w/v) Difco casamino acids, zinc and iron traces, adjusted to pH 7.4 with 4 N HCl. The experiments were repeated at least three times, and the results of a single representative experiment are shown here. Differences between experiments in the average values for each treatment did not exceed 2–3%.

pH measurement and ammonia detection

The pH was measured in 0.5-mL aliquots that were sampled at various times after fungal inoculation, with a Thermo-Orion Model 9810BN microcombination pH electrode (Thermo Fisher Scientific, Inc. Waltham, MA). Ammonia was detected with an ammonium test kit (Merck, Darmstadt, Germany), by means of a colorimetric reaction. Samples (0.5 mL) of the culture media were diluted with

4.5 mL double-distilled water, and the concentration of ammonia was determined according to the manufacturer's instructions. In brief, the sample containing ammonium was adjusted to pH 13 so that the ammonium was transformed to ammonia, which could be detected in the colorimetric reaction. Concentrations were reported as millimole ammonia.

Fruits, ripening parameters and fruit treatments

Freshly harvested avocado fruits, cv. 'Fuerte', from an orchard at Kibbutz Givat Brenner, Israel, were used for treatment and inoculations. When fruits were inoculated on the peel, 7 µL of a conidial suspension (10^6 conidia/mL) was placed on each of five longitudinally spaced inoculation spots on each side of each of ten different fruits per treatment, i.e. 100 inoculation replicates per treatment. When fruits were inoculated on the mesocarp, a 1- to 2-mm strip of peel tissue was removed from the fruit and an amount of suspension similar to that used for the peel inoculation was placed on each of three small holes (1 mm deep and 5 mm wide) in the underlying tissue, on each side of each of ten different fruits per treatment, i.e. 60 inoculation replicates per treatment. Following inoculation, the fruits were incubated at 22 °C and 95% relative humidity for 24 h in covered plastic containers containing wet paper towels. They were then transferred to ambient (70–80%) relative humidity at 22 °C until they ripened, and symptom development was monitored (Prusky *et al.*, 2001). The average decay diameter for ten fruits is reported. The inoculation experiments were repeated three times.

Detection of PL and alkaline phosphatases in liquid medium

Hyphae grown on secondary media were separated by filtration (see above), washed twice with sterile water, frozen with liquid N₂, lyophilized and stored at –80 °C pending extraction of RNA or protein. The culture medium filtrate was concentrated to 5 mL at 30 °C on a Rotavapor (BÜCHI Labortechnik AG, Switzerland), dialysed for 24 h against 5 L of 50 mM Tris-HCl, pH 8.5, with a SnakeSkin pleated dialysis tube (Pierce Biotechnology, Rockford, IL) with a cutoff at a molecular weight of 10 000, reconcentrated to 1 mL, lyophilized and resuspended in 150 µL of sterile water. Protein samples were quantified by the Bradford reagent (Bio-Rad, Munich, Germany) protein assay, with bovine serum albumin (Sigma) as the standard. A Mini-Protean II 12.5% sodium dodecyl sulphate-polyacrylamide gel (Bio-Rad) was loaded with 2.5-µg aliquots of secreted proteins and run for 1.5 h at a constant 100 V. Western blot analysis was performed with PL antiserum as described elsewhere (Bradford, 1976; Yakoby *et al.*, 2000, 2001). The blot analyses were repeated three times with similar results; the results from one representative experiment are presented here.

Alkaline phosphatase activity was detected by staining with nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) (Promega, Madison, WI). Concentrated culture medium was dialysed and concentrated as described above, and 10 µg of total protein, included in 1 mL of the reaction mixture containing 3.3 µL BCIP and 6.6 µL NBT in alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl and 5 mM MgCl₂) was used. The colour change caused by the alkaline phosphatase activity was measured at 660 nm, and the alkaline phosphate activity was determined by comparing the activity of the sample with that produced by the pure enzyme (Promega), as determined by means of a calibration curve. Units were expressed as alkaline phosphatase activity per milligram of total protein.

RNA extraction and real-time PCR analysis

RNA was extracted from 80 mg (dry weight) of mycelium or dry infected avocado mesocarp with an RNeasy Plant mini kit (QIAGEN Sciences, Hilden, Germany) and further purified by treatment with TURBO DNA free (Ambion, Austin, TX). The reverse-transcription reaction was performed on 10 µg of total RNA with the Reverse-It first-strand synthesis kit (ABgene, Surrey, UK). Samples of cDNA were diluted 1 : 10 (v/v) to the final template concentration for real-time PCR. Real-time quantitative PCR was performed with the RotorGene 3000 system (Corbett Research, Sydney, Australia). PCR amplification was performed with 3.5 µL of cDNA template in 10 µL of a reaction mixture containing 5 µL Syber-Green Amplification Kit (ABgene, Surrey, UK) and 300 nm of primers. PCR conditions were: initial denaturation for 15 min at 94 °C, 40 denaturation cycles of 10 s at 94 °C, annealing at 60 °C for 15 s, extension at 72 °C for 20 s, and melting at 72–95 °C. The samples were subjected to melting-curve analysis with the RotorGene program. All samples were normalized to 18S rRNA gene levels in the same real-time quantitative PCR, and the values were expressed as the increase/decrease of the levels relative to a calibrator sample. The forward and reverse primers for *PELB* were, respectively, 5'-CCGCTCTCTCCGACACAA-3' and 5'-CGAGGTCGACGTCATTGACA-3'; those for *PAC1* were, respectively, 5'-CACGTGCTCAGCTCCTAGAC-3' and 5'-AGCCGTCATCCCCCTGGT-3'; those for 18S rRNA were, respectively, 5'-AGCATTCTGGCGGGCAT-3' and 5'-AGCTGTAGGGCCCCAACAC-3'; each experiment was repeated three times with similar results; the results of one experiment are presented.

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