Global Aspects of *pacC* Regulation of Pathogenicity Genes in *Colletotrichum gloeosporioides* as Revealed by Transcriptome Analysis

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Colletotrichum gloeosporioides alkalinizes its surroundings during colonization of host tissue. The transcription factor *pacC* is a regulator of pH-controlled genes and is essential for successful colonization. We present here the sequence assembly of the *Colletotrichum* fruit pathogen and use it to explore the global regulation of pathogenicity by ambient pH. The assembled genome size was 54 Mb, encoding 18,456 genes. Transcriptomes of the wild type and $\Delta pacC$ mutant were established by RNA-seq and explored for their global pH-dependent gene regulation. The analysis showed that *pacC* upregulates 478 genes and downregulates 483 genes, comprising 5% of the fungal genome, including transporters, antioxidants, and cell-wall-degrading enzymes. Interestingly, gene families with similar functionality are both up- and downregulated by *pacC*. Global analysis of secreted genes showed significant *pacC* activation of degradative enzymes at alkaline pH and during fruit infection. Select genes from alkalinizing-type pathogen *C. gloeosporioides* and from acidifying-type pathogen *Sclerotinia sclerotiorum* were verified by quantitative reverse-transcription polymerase chain reaction analysis at different pH values. Knock out of several *pacC*-activated genes confirmed their involvement in pathogenic colonization of alkalinized surroundings. The results suggest a global regulation by *pacC* of key pathogenicity genes during pH change in alkalinizing and acidifying pathogens.

cence to necrotrophy is dependent on fruit maturation (Prusky et al. 2010). During its appressorial penetration and necrotrophic development, *C. gloeosporioides* is known to alkalinize the ambient pH by massive secretion of ammonia (Alkan et al. 2008; Miyara et al. 2010; Prusky et al. 2001; Shnaiderman et al. 2013), increasing the local pH by 4 pH units from pH 4.0 to 8.0 (Alkan et al. 2008; Prusky and Yakoby 2003). In a similar fashion, *C. higginsianum*, *C. graminicola*, *Alternaria alternata*, and *Fusarium oxysporum* alkalinize their surroundings by ammonia secretion (Eshel et al. 2002; Miyara et al. 2012; O'Connell et al. 2012). Ammonia secretion is known to activate host-programmed cell death (Alkan et al. 2008, 2009, 2012b), and ammonia-driven alkalization is thought to optimize pathogenicity for necrotrophic colonization (Alkan et al. 2008, 2009; Miyara et al. 2008). Environmental alkalization during infection is accompanied by the induction of pathogenicity factors, as was reported for pectate lyase (PelB) in *C. gloeosporioides* (Yakoby et al. 2000); polygalacturonase (PG1 and PG5) in *F. oxysporum* (Caracuel et al. 2003b), and endoglucanases in *A. alternata* (Eshel et al. 2002).

In filamentous fungi, the expression of genes in alkaline conditions is governed by a complex ambient-pH-sensing, signal-transduction pathway consisting of six phenylalanine ammonia lyase gene products that cleave the zinc finger transcription factor *pacC* at alkaline pH and was characterized in *Aspergillus nidulans* (Andersen et al. 2009; Galindo et al. 2012; Negrete-Urtasun et al. 1999; Penalva and Arst 2002, 2004; Penalva et al. 2008). Thus, under alkaline pH conditions, *pacC* serves as a positive regulator promoting transcription of alkaline-expressed genes while it is suggested to simultaneously repress acid-expressed genes (Arst and Penalva 2003; Tilburn et al. 1995). For example, PelB, a secreted cell-wall-degrading enzyme (CWDE), is controlled by the transcription factor *pacC* under alkalinizing conditions (Miyara et al. 2008).

The promoter regions of alkaline-expressed genes controlled by *pacC* contain multiple copies of the transcription factor binding site 5'-GCCARG 3' (Espeso et al. 1997; Miyara et al. 2010). Importantly, loss-of-function mutants of $\Delta pacC$ result in an acid-mimicking phenotype even in alkaline conditions (AbuQamar et al. 2006; Arst et al. 1994; Caddick et al. 1986; Diaz et al. 2002; Espeso and Arst 2000; Orejas et al. 1995; Tilburn et al. 1995). Thus, the mutant behaves as if it is in an environment of lower ambient pH with increased expression of acid-expressed genes and decreased expression of alkaline-

Genus *Colletotrichum* is distributed worldwide and species in this family are important agents in causing fruit rot both in the field and postharvest (Prusky 1996; Wharton and Diéguez-Uribeondo 2004). *Colletotrichum gloeosporioides* displays sequential quiescent and active necrotrophic-infection strategies to promote colonization. During quiescence, the fungus remains dormant for weeks until fruit ripening; the switch from quies-

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expressed genes. Indeed, *ApacC* mutants of *C. gloeosporioides*, *C. acutatum*, *F. oxysporum*, and *Sclerotinia sclerotiorum* show reduced virulence (Caracuel et al. 2003a; Rollins 2003; Miyara et al. 2008; You et al. 2007).

Our hypothesis is that the impact of *pacC* on virulence is due to its regulation of pathogenicity genes under alkalinizing pH conditions. Thus, in the present work, we analyzed global aspects of *pacC* regulation by transcriptome and functional analysis. Based on high-throughput RNA sequencing, we demonstrate that *pacC* upregulates 478 and downregulates 483 genes, some of which appear to be involved in the development of necrotrophic pathogenicity. The regulated genes include transporters and antioxidants that function to maintain cellular homeostasis and control the secretion of pathogenicity factors. Functional analysis by gene knock-out in *C. gloeosporioides* of a number of genes expressed at alkaline pH confirms their involvement in pathogenicity. Thus, *pacC* is a key factor in the regulation of many genes that contribute to *C. gloeosporioides* pathogenicity. We also provide evidence that, in acidifying-type fungi as well, *pacC*-regulated genes appear to play a similar role in the control of pathogenicity.

RESULTS

Comparison of *Colletotrichum* genomes.

The genome of the postharvest fruit pathogen *C. gloeosporioides* Cg-14 isolate was sequenced to a depth of approximately

10× using 454 GS FLX Titanium technology. The sequence reads were assembled using the GS De Novo Assembler software (454 Corp.) yielding 4,907 contigs larger than 500 bp, with an average size of 24.5 kb and 53% GC content (GenBank accession number SUB133583). These contigs have a total length of 54.5 Mb, which is similar to the recently published genomes of *C. graminicola* (57.4 Mb) and *C. higginsianum* (53.4 Mb) (Table 1) (O'Connell et al. 2012). Two other strains of *C. gloeosporioides* that were isolated from leaves and stem were recently released (Joint Genome Institute) (Gan et al. 2013). Those genome sizes are 58.8 and 55.6 Mb, respectively (Table 1).

The GeneMark-ES algorithm predicted 18,456 protein-coding gene models in the *C. gloeosporioides* genome. This number of genes is 14 to 53% more than the number of genes predicted in the *C. higginsianum* and *C. graminicola* genomes (16,172 and 12,006, respectively) (O'Connell et al. 2012). The RNA-Seq transcriptome analysis of *C. graminicola* and *C. higginsianum* yielded 14,972 and 10,812 transcripts, respectively, compared with 16,603 in the transcriptome of *C. gloeosporioides* (Table 1). Thus, the major variance detected is in the number of transcripts, with the highest number of transcripts in *C. gloeosporioides*. In all cases, the genomes displayed 8 to 11% more predicted genes than that revealed by transcriptome analysis. This discrepancy is likely due to the difficulty in detecting genes that are expressed at low levels or undergo stage-specific expression. Additionally, similar numbers of secreted

Table 1. Genome comparisons in the genus *Colletotrichum*

Species ^a	Genome size (Mb)	Predicted proteins	In vivo transcript	Secretome	GC (%)	Reference
<i>C. gloeosporioides</i> (Cg-14)	54	18,456	16,603	1,955	53	Present work
<i>C. gloeosporioides</i> (Musae 23)	58.8	18,975	...	...	...	...
<i>C. gloeosporioides</i> (Nara gc5)	55.6	15,469	...	2,042	53.6	Gan et al. 2012
<i>C. orbiculare</i>	88.3	13,479	...	1,557	37.5	...
<i>C. higginsianum</i>	53.4	16,172	14,972	2,142	49.1	O'Connell et al. 2012
<i>C. graminicola</i>	57.4	12,006	10,812	1,650	55.1	...

^a Six genomes were compared: *Colletotrichum gloeosporioides* (Cg-14); isolated from avocado fruit; *C. gloeosporioides* (Joint Genome Institute), an endophyte obtained from oak seedling; *C. gloeosporioides* (Nara gc5) (Gan et al. 2012), isolated from strawberry leaves; *C. orbiculare* (Gan et al. 2012); *C. higginsianum* (O'Connell et al. 2012); and *C. graminicola* (O'Connell et al. 2012). Genome size, number of predicted proteins, total in vivo transcripts number identified by Cufflink transcriptome analysis, secreted proteins, and average GC (%) are shown. Expressed transcripts of *C. higginsianum* and *C. graminicola* are the combined expression from the transcriptomes of three different stages of colonization stages, including germination, biotrophic, and necrotrophic stages.

Fig. 1. Proteases in *Colletotrichum* spp. and other fungi. Number of predicted proteases encoded by the *Colletotrichum gloeosporioides* genomes in comparison with other fungi, based on MCL clustering of predicted protein sequences of the different genomes.

proteins were detected by in silico analysis in most *Colletotrichum* spp., with lower numbers of secreted proteins in *C. graminicola* and *C. orbiculare* (Table 1). Comparison of the numbers of peptidases in fungal genomes revealed that most *Colletotrichum* spp. have more serine and cysteine peptidase than other fungal species tested. Furthermore, *C. gloeosporioides* genomes that have a wider host range possess more peptidase than other *Colletotrichum* spp. (Fig. 1).

To identify conserved regions showing co-localization of genes on chromosomes (synteny) within *Colletotrichum* spp., we aligned the *C. gloeosporioides* genome sequence to the genome of *C. graminicola* using symap 4.0 (Soderlund et al. 2006). Symap identified 9,482 alignments (anchors) between the two species, representing 30% of the *C. gloeosporioides* genome; this value is similar to the 34% synteny reported between *C. higginsianum* and *C. graminicola* (O'Connell et al. 2012). The anchored assembly enabled identification of 340 syntenic blocks between the two species (Supplementary Fig. S1). In all, 90% of *C. gloeosporioides* anchors fall within gene models in both species showing higher conservation in coding genes than in the noncoding sequence of the genome.

The large number of contigs in *C. gloeosporioides* NARA gc5 genome assembly as well as in the *C. gloeosporioides* Cg-14 genome prevented a detailed analysis of conserved synteny. Instead, we aligned the two *C. gloeosporioides* genomes of NARA gc5 and Cg-14 using Mauve analysis (Darling et al. 2010). The Mauve analysis resulted in 19,043 anchor alignments covering 89.4% of the NARA gc5 genome sequence. The remaining 10.4% of the NARA gc5 genome that was not aligned to Cg-14 could be missing from the Cg-14 genome or it could be too divergent to be aligned by the Mauve algorithm.

Transcriptome analysis of *C. gloeosporioides* reveals genome-wide pacC-dependent regulation.

In order to characterize global aspects of pH-regulated genes that are important for environmental fitness and pathogenicity, we characterized the pacC-regulated transcriptome. To this end, RNA-seq was applied to the *C. gloeosporioides* wild type (WT) and a reduced-pathogenicity $\Delta pacC$ mutant (Miyara et al. 2008) grown at pH 7.0 for different periods post-culture inoculation (discussed below). The $\Delta pacC$ strain behaved as an acid-mimic mutant, because it misregulated pH-controlled genes (AbuQamar et al. 2006; Arst et al. 1994; Caddick et al. 1986; Diaz et al. 2002; Espeso and Arst 2000; Orejas et al. 1995; Tilburn et al. 1995). Thus, genes that are upregulated in the WT compared with the $\Delta pacC$ mutant at alkaline condition (at pH 7.0) can be considered to be alkaline expressed. Alternatively, genes that are downregulated in the WT compared with $\Delta pacC$ mutant in those conditions can be considered to be acid expressed. To properly define transcriptional units, sequenced reads were compared with the GeneMark predicted transcripts. Regions with over 90% similarity between the predicted transcripts (from genome analysis) and the transcribed RNA established by RNA-seq analysis are presented in conversion Supplementary Table S1, showing the intron, exons, and transcript length. Functional annotation was carried out using Blast2GO software of the Cufflink-identified transcripts. The annotation includes enzyme code, InterPro annotation, and KEGG number maps (Supplementary Table S2). Transcripts that were differently regulated between the WT and $\Delta pacC$ mutants transcriptome were chosen if a significant two-fold change in expression was detected. In this manner, 478 upregulated and 483 downregulated genes were detected representing together more than 5% of the transcripts (Supplementary Tables S3 and S4). The up- and downregulation of genes is consistent with pacC acting as a gene activator and repressor during alkalization.

PacC up- and downregulated genes are alkaline- and acid-expressed and showed different induction kinetics.

To verify the transcriptome analysis and the hypothesis that comparison of WT and $\Delta pacC$ mutants grown at pH 7.0 will reveal alkaline- and acid-expressed genes, several up- and downregulated genes were selected from the transcriptome data and examined by quantitative reverse-transcription polymerase chain reaction (RT-qPCR) for their relative expression at pH 4.0 and 7.0. The genes shown in Figure 2 were further selected based on their importance in ammonia transport, ion transport for homeostasis maintenance, and CWDE for pathogenicity. These genes include ammonia exporter (AMET),

Fig. 2. Relative expression of *Colletotrichum gloeosporioides* wild-type (WT) genes determined by quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR). *C. gloeosporioides* up- and downregulated genes selected from different Gene Ontology terms were measured 1 and 24 h after introducing cultures to pH 4.0 or 7.0. Their relative expression was determined by RT-qPCR with an actin-related gene as reference gene. WT growth for 24 h at pH 7.0 was assigned the relative number 1. **A**, Relative expression of pacC-upregulated genes AMET (TCONS_00010049), peIB (TCONS_00001544), Ca transporting ATPase (TCONS_00015152), and noX4 (TCONS_00008736). **B**, Relative expression of pacC-downregulated genes amino permease, (TCONS_00007335), β -glucanase (TCONS_00000095), Ca proton exchanger (TCONS_00010215), and serine carboxypeptidase (TCONS_00008162).

Fig. 3. Relative expression of *Sclerotinia sclerotium* wild-type (WT) genes. *S. sclerotium* up- and downregulated genes, representatives of different Gene Ontology terms were selected and their relative expression was measured 8 h after subculture at pH 4.0 or 7.0. The relative expression was determined by quantitative reverse-transcriptase polymerase chain reaction with actin as reference gene. The WT grown for 8 h in pH 4.0 was assigned the relative number 1. **A**, Relative expressions of the pacC-upregulated genes pelB (SS1G_00238), Ca transporter ATPase3 (SS1G_06668), and AMET (SS1G_06601). **B**, Relative expressions of the pacC-downregulated genes calcium proton exchange (SS1G_14108), serine carboxypeptidase (SS1G_13633), and amino acid permease (SS1G_10461).

amino permease, $\text{Ca}^{2+}/\text{H}^{+}$ exchanger, Ca^{2+} transporter, nox4, β -glucanase, PelB, and serine carboxypeptidase. Expression of the up- and downregulated transcripts, as established by RNA-seq analysis of the ΔpacC strain, were elevated or decreased in alkaline environments, respectively (Fig. 2). Thus the upregulated genes were alkaline-expressed genes and the downregulated genes were acid-expressed genes. Interestingly, RT-qPCR analysis showed that the alkaline-upregulated genes were elevated within 1 h after pH shift to pH 7.0. However, the expression of the acid-induced genes was relatively delayed and increased approximately 24 h later (Fig. 2). In addition, the RNA-seq results were examined for known pH regulation of alkaline and acid phosphatase-expressed genes (Dorn 1965). Indeed, alkaline phosphatase TCONS_00015964 was found to be upregulated whereas the acid phosphatase TCONS_00006263 and TCONS_00004441 is downregulated. These results validate the RNA-seq analysis and show that pacC upregulates alkaline-expressed genes and downregulates acid-expressed genes at alkaline condition.

Conserved pacC regulation between *C. gloeosporioides* and *S. sclerotiorum*.

We examined whether pacC-regulated genes, identified in a pathogen that alkalinizes its ambient pH (e.g., *C. gloeosporioides*), are similar in fungi that acidify their surroundings. To this end, the set of best homologs to the *C. gloeosporioides* transcripts shown in Figure 2 were identified in *S. sclerotiorum* and examined under alkaline and acidified condition (pH 7.0 and 4.0). Indeed, 8 h after introducing the fungus to pH 7.0 or 4.0, the expression of the predicted pacC-upregulated genes were elevated in pH 7.0, whereas pacC-downregulated genes were highly expressed at pH 4.0 (Fig. 3). This suggests that both alkalinizing and acidifying fungi possess homologous genes of alkaline- and acid-expressed transcripts to cope with pH change.

Gene family sizes of pacC-regulated genes were examined in *C. gloeosporioides* in comparison with related families in other diverse fungi. To this end, upregulated transcripts of *C. gloeosporioides* were clustered together with those of *C. graminicola*, *C. higginsianum*, *Neurospora crassa*, *A. nidulans*, *S. sclerotiorum*, and *Botrytis cinerea* using the clustering software MCL. Selected representative clusters are presented in

Table 2. Fungal gene clusters^a

Function ^b	Cluster ^c	Alkalinizing			Neutral		Acidifying	
		C.gl	C.h	C.gr	N.c	A.n	S.s	B.c
MFS transporter	2	179	158	90	27	90	31	40
Sugar transporter	4	127	111	70	32	96	47	63
Integral membrane protein	5	126	110	81	28	62	42	55
Amino acid permease	23	39	26	19	10	27	17	19
Calcium-translocating ATPase	48	17	19	13	7	13	6	8
C6 zinc finger	74	18	17	8	4	13	4	9
Isoflavone reductase	92	22	17	11	3	7	3	5
Pectate lyase	139	14	15	4	1	5	0	2
Glycosyl hydrolase	172	9	8	7	6	5	11	11
Epimerase	211	14	6	6	1	4	2	2
Endoglucanase	214	5	4	3	4	2	3	3
Galactoside O-acetyltransferase	231	9	5	4	3	5	3	2
GDSL-like lipase/acylhydrolase	287	9	5	5	1	5	2	1
Serine carboxypeptidase	384	6	3	3	1	1	3	5
Endoribonuclease L-PSP	582	10	5	3	0	5	1	0
Amidase	807	6	2	2	1	1	2	2
Amidohydrolase	1,044	4	5	2	1	2	0	0
Chromo domain-containing	5,383	7	2	1	0	0	0	0

^a *Colletotrichum gloeosporioides* pacC upregulated gene clusters (C.gl), compared with *C. higginsianum* (C.h), *C. graminicola* (C.gr), *Neurospora crassa* (N.c), *Aspergillus nidulans* (A.n), *Sclerotinia sclerotiorum* (S.s), and *Botrytis cinerea* (B.c).

^b Protein families were identified with the clustering program MCL. Functions of the proteins within each family were annotated using InterPro and BLAST searches. Selected clusters are presented.

^c MCL Cluster ID.

Table 2. The results show larger-sized gene families in *C. gloeosporioides* compared with other *Colletotrichum* spp., and larger gene families in fungi that alkalize their surroundings compared with fungi that acidify their ambient pH. This observation is consistent with the finding that *C. gloeosporioides* has more predicted genes in general than other *Colletotrichum* spp. The complete comparative gene cluster analysis between fungal species for up- and downregulated sets of genes is presented in Supplementary Table S5.

Members of the same functional family are differentially regulated by pacC.

The Ontologizer program (Bauer et al. 2010) was used in order to identify groups of genes that are regulated in *C. gloeosporioides* by pacC. Many groups with similar functional activities appeared to be both up- and downregulated by pacC (Fig. 4). For example, CWDE that could contribute to fungal pathogenicity, transport activity and antioxidants were both up- and downregulated and could balance fungal redox and homeostasis in coping with extremes of pH (Fig. 4). When the “cellulase family” that belongs to the CWDE is examined in detail (Table 3), it was found to include 23 transcripts with putative cellulase activity (GO:0008810). Of these, seven were significantly upregulated while six transcripts were significantly downregulated. Similarly, of seven sulfate transport members (GO:0008272), three were upregulated genes and another three were downregulated by pacC (Table 3).

PacC controls secreted proteins with catabolic activity.

PacC regulates many CWDE and a pacC null mutant showed a major reduction in pathogenicity (Miyara et al. 2008). Because

CWDE are secreted enzymes, we further analyzed the overlap between pacC regulation and secreted proteins. To this end, we identified 1,955 putative secreted proteins in the *C. gloeosporioides* genome using WoLF PSORT applied to the GeneMark predicted transcripts (Horton et al. 2007). Of these, 870 transcripts had over 90% homology to transcripts identified by the Cufflink algorithm and, of them, 88 and 39 genes were found to overlap with pacC up- and downregulated genes, respectively (Fig. 5A). The degree of overlap between the secretome and the pacC-upregulated set was significantly more than expected (3.3-fold overrepresentation). This indicates that the secretome is enriched for pacC-regulated genes. Annotation of the pacC-regulated and secreted genes classified them into three main groups: cell wall catabolic, macromolecule catabolic, and proteolysis (Fig. 5B). Selected pacC-upregulated secreted genes were analyzed on media at pH. 7.0 and 4.0. The results confirmed elevated expression under alkaline condition (Fig. 6A). Because the natural fruit infection court is alkaline, RT-qPCR analysis of the same genes was carried out using infected avocado fruit tissue and compared with fungal growth in M₃S media (Tu 1985) at pH 5.5. The results showed a significant enhancement in transcript expression. These results confirm the in vitro results and suggest that secreted pacC-regulated genes contribute to fungal pathogenicity at the necrotrophic stage (Fig. 6B).

The impact of pacC on fungal pathogenicity.

It has been reported that $\Delta pacC$ mutants of *C. gloeosporioides*, *C. acutatum*, *F. oxysporum*, *A. nidulans*, and *S. sclerotiorum* are less virulent compared with the WT (Bignell et al. 2005; Caracul et al. 2003a; Miyara et al. 2008; Rollins 2003;

Fig. 4. Gene Ontology terms overrepresentation in *Colletotrichum gloeosporioides* pacC up- and downregulated genes. *C. gloeosporioides* pacC up- and downregulated genes that changed by twofold and had a significance q value of less than 0.05 were associated to GO terms. The GO overrepresentation analysis used Ontologizer software limited by Benjamini-Hochberg settings (Bauer et al. 2010). The topology-weighted analysis was with P value lower than 0.05. **A**, In total, 11,603 genes were in the population set, 478 of which were in the upregulated set, the significance presented as $-\log_{10}(P \text{ value})$. **B**, In total, 11,603 genes were in the population set, 483 of which were in the downregulated set, the significance presented as $-\log_{10}(P \text{ value})$.

You et al. 2007). Because the elimination of *pacC* induces the misregulation of many genes, it raises the question of the importance, if any, of individual genes on pathogenicity. To assess this, we examined genes that impact *C. gloeosporioides* pathogenicity (Table 4). For example, $\Delta pelB$, the secreted PelB (TCONS_00014224); $\Delta mepB$, the ammonium permease (MEPB) (TCONS_00015558); $\Delta amet$, the AMET (TCONS_00010049); and $\Delta chip6$, the sterol glycosyl transferase (TCONS_00013643) all show significant reduced pathogenicity during necrotrophic colonization on avocado fruit (Kim et al. 2002; Miyara et al. 2012; Shnaiderman et al. 2013; Yakoby et al. 2001). They are also all significantly upregulated by *pacC* (Table 4). Interestingly, glutamate dehydrogenase (GDH) ($\Delta gdh2$) (TCONS_00001112), a gene responsible for producing ammonia at acidic pH, was found to be repressed by *pacC* and its deletion mutant has been shown to display reduced pathogenicity (Miyara et al. 2012).

Two other secreted *pacC*-controlled products were mutated by gene knock out through homologous recombination of chitin deacetylase (*CDA*) and aspartic protease (*ASP*) (Table 4). The Δcda homologous recombination of 16 bases upstream of the ATG to 340 bases upstream of the stop codon with hygromycin-resistance gene was validated by PCR analysis of the 5'- and 3'-end PCR fragment with appropriate primer pairs (Fig. 7). Testing in vitro by RT-qPCR for *CDA* gene expression in three Δcda mutant strains revealed elimination of *CDA* ex-

pression (Fig. 7). However, elimination of this gene expression did not play a significant role in pathogenicity on fruit (Supplementary Fig. S2). Because paralogs of *CDA* exist in the genome, its functionality may be redundant. This possibility was confirmed by the observation of two other *CDA* genes that had increased expression in the background of the Δcda mutant when compared with the WT and ectopic strains (Supplementary Fig. S3). The results suggest functional compensation in the Δcda mutant and only multiple knock down of the whole gene family may alter pathogenicity.

ASP (TCONS_00013084), a secreted putative ASP, showed significant activation by *pacC* and during necrotrophic colonization on avocado fruit. Knockout of *ASP* was achieved by homologous recombination with hygromycin-resistance gene insertion 384 bases downstream of the ATG to 442 bases upstream of the stop codon. The insertion was validated by PCR analysis of 5'- and 3'-end PCR fragments with the appropriate primer pairs (Fig. 8). In addition, testing in vitro *ASP* gene expression in three Δasp mutant strains by RT-qPCR revealed no expression of transcripts (Fig. 8). Importantly, Δasp showed a significant reduction in pathogenicity during necrotrophic colonization on avocado fruit (Fig. 9; Table 4).

Pathogenicity factors in acidifying fungi.

PacC mutants of neutral and acidifying fungi such as *A. nidulans* and *S. sclerotiorum* are also less virulent compared with

Table 3. *PacC* regulation of cellulase activity and sulfate transport gene families

Transcript	Seq. description	Fold	q Value
Cellulase activity ^a			
Upregulated			
TCONS_00011897	Glycosyl transferase	7.76	0.00
TCONS_00011898	Cellulose synthase catalytic subunit	7.06	0.00
TCONS_00008710	Glucan- β -glucosidase	2.08	0.00
TCONS_00000830	Endo- β -glycosyl hydrolase	1.33	0.00
TCONS_00016243	Endoglucanase a precursor	7.10	0.00
TCONS_00005776	Glycosyl hydrolase family 12	1.49	0.00
TCONS_00006290	Endoglucanase a precursor	1.59	0.00
Downregulated			
TCONS_00009010	Blycosyl hydrolase family 61	-4.13	0.00
TCONS_00014558	Endoglucanase b	-15.81	0.00
TCONS_00014559	Endoglucanase b	-12.28	0.00
TCONS_00012618	Glycoside hydrolase family 12	-10.87	0.00
TCONS_00008371	Family 45 cellulase homologue	-10.00	0.00
TCONS_00013473	Extracellular endoglucanase	-8.82	0.01
No significant regulation			
TCONS_00005237	Xyloglucan-specific endoglucanase	1.10	0.21
TCONS_00010110	Endoglucanase b	-1.37	0.30
TCONS_00002734	Hydrolyzing O-glycosyl	-2.00	0.35
TCONS_00014692	β -Glucanase	1	0.46
TCONS_00007104	Phenazine biosynthesis	-1.53	0.47
TCONS_00014691	β -Glucanase	1.50	0.53
TCONS_00006249	β -Glucanase	3.40	0.53
TCONS_00013650	Glycosyl hydrolase family 7	1	0.56
TCONS_00009903	Endoglucanase	1.70	0.64
TCONS_00016450	Endoglucanase ii	1.19	0.76
Sulfate transport ^b			
Upregulated			
TCONS_00002171	Sulfate permease ii	5.79	0.00
TCONS_00004756	Sulfate permease 2	2.76	0.00
TCONS_00002173	Sulfate permease ii	2.52	0.00
Downregulated			
TCONS_00014828	Cysteine synthase b	-1.67	0.00
TCONS_00017544	Sulfate permease ii	-2.03	0.00
TCONS_00014829	Cysteine synthase b	-2.36	0.00
No regulation			
TCONS_00013384	Rhodanese-containing protein	1.22	0.18

^a The 23 genes with putative cellulase activity were divided according to their expression in the $\Delta pacC$ mutant. Upregulated group and downregulated groups are shown.

^b Seven sulfate transport genes were similarly divided. For each gene the transcript number, the sequence description, fold change of *Colletotrichum gloeosporioides* wild type and $\Delta pacC$ mutant, and *q* value are presented.

their WT (Bignell et al. 2005; Rollins 2003). In order to estimate pacC regulation of acidifying fungi pathogenicity, we analyzed all known pathogenicity mutants of *B. cinerea* for their best homologs in *C. gloeosporioides* and found that 7 of 46 mutants are homologs to genes that are regulated by pacC in *C. gloeosporioides*. Interestingly, all the *C. gloeosporioides* homologs are downregulated by pacC; therefore, they are acid-expressed (Table 4).

DISCUSSION

C. gloeosporioides genome.

C. gloeosporioides (as well as other fungal pathogens) alkalizes the ambient surroundings during pathogenic colonization of fruit (Alkan et al. 2012a). In this work, the genome of *C. gloeosporioides*, a successful post-harvest pathogen (Prusky 1996), was sequenced in order to characterize the genes which are controlled by pH during pathogenicity. Comparison of the *C. gloeosporioides* genome with other *Colletotrichum* spp. indicates a similar genome size of 54 Mb and an average GC of 53%, with the exception of *C. orbiculare*. The latter has a significantly expanded genome with many AT blocks potentially arising as the result of transposable element inactivation (Gan et al. 2013). Strikingly, with similar genome size, the *C.*

gloeosporioides genome contains 14 to 53% more predicted proteins, suggesting a significantly higher genome complexity.

Moreover, gene cluster analysis showed that *C. gloeosporioides* has larger gene families than found in *C. higginsianum* and *C. graminicola*. *C. gloeosporioides* has a wider host range than *C. higginsianum* and *C. graminicola*. Thus, these results may suggest that it needs more genes with various optimum activities to maintain colonization and pathogenicity under the large variety of hosts' environments. Furthermore, the secretome of *C. gloeosporioides* shows overrepresentation of pathogenicity factors such as CWDE and proteases. When inspecting the numbers of secreted proteins in other *Colletotrichum* spp., there were lower numbers of secreted proteins in both *C. graminicola* and *C. orbiculare*, which have a narrower host range compared with *C. higginsianum* and *C. gloeosporioides* (Table 1). A possible reason for the higher number of predicted proteins and gene families could be the reduced host specialization of *C. gloeosporioides*. *C. graminicola* attacks mainly corn, *C. orbiculare* colonizes cucurbits, and *Nicotiana benthamiana* and *C. higginsianum* colonize a number of *Brassicaceae* spp. *C. gloeosporioides* colonizes over 470 different plant and fruit

Fig. 5. Characterization of *Colletotrichum gloeosporioides* pacC-regulated secretome. **A**, *C. gloeosporioides* Venn diagram intersection describing the overlap between transcriptome, secretome, and pacC-regulated genes. The *C. gloeosporioides* transcriptome of 9,592 transcripts that have over 90% homology to the genome-based predicted proteins overlapped with 860 secretome genes and 574 pacC-regulated genes. **B**, Overrepresentation of Gene Ontology term annotation by Ontologizer program of 88 transcripts, which overlapped between the *C. gloeosporioides* secretome and the pacC-upregulated genes, presented as $-\log_{10}$ of the *P* value.

Fig. 6. Relative transcript expression of *Colletotrichum gloeosporioides* secreted proteins. **A**, Quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) average and standard error (SE) relative expression of five selected *C. gloeosporioides* wild-type (WT) secreted and pacC-dependent genes during 2 days of growth on peeled avocado mesocarp fruit compared with expression during in vitro growth conditions in an M₃S plate of 1-week-old cultures. **B**, RT-qPCR average and SE relative expression of five selected *C. gloeosporioides* WT secreted and pacC-dependent genes (TCONS_00014945, TCONS_00000137, TCONS_00014224, TCONS_00015963, and TCONS_00011812) at pH 4.0 and 7.0 after 16 h.

Table 4. Summary of *Colletotrichum gloeosporioides* and *Botrytis cinerea* reduced pathogenicity mutants which are pacC-regulated^a

Candidate gene name	Reference	Pathogenicity ^b	TCONS	pacC fold change ^c
<i>C. gloeosporioides</i>				
Pectate lyase (PELB)	Yakoby et al. 2001	+	TCONS_00014224	46.8***
Ammonia exporter (AMET)	Shnaiderman et al. 2013	+	TCONS_00010049	6.9***
Chitin deacetylase (CDA)	This work	–	TCONS_00011812	7.01***
Aspartic protease (ASP)	This work	+	TCONS_00013084	2.04*** ^d
Sterol glycosyl transferase (CHIP6)	Kim et al. 2002	+	TCONS_00013643	1.5***
Ammonium permease (MEPB)	Miyara et al. 2012	+	TCONS_00015558	1.2***
Glutamate dehydrogenase (GDH)	Miyara et al. 2012	+	TCONS_00001112	–2.5**
<i>B. cinerea</i>				
Pectin methylesterase (PME)	Valette-Collet et al. 2003	+	TCONS_00004801 ^e	–87.23***
Polygalacturonase1 (PG1)	ten Have et al. 1998	+	TCONS_00010609	–24.5***
Cytochrome p450 monooxygenase (BOT1)	Siewers et al. 2005	+	TCONS_00016890	–3.5***
Endopolygalacturonase2 (PG2)	Kars et al. 2005	+	TCONS_00005487	–3.24**
Endo-β-1,4 xylanase	Brito et al. 2006	+	TCONS_00011297	–1.61*
Tetraspanin1 (PLS1)	Gourgues et al. 2004	++	TCONS_00017362	–1.4***
Chitin synthase1 (CHS1)	Soulie et al. 2003	+	TCONS_00006342	–1.4*

^a Summary of *C. gloeosporioides* reduced pathogenicity gene knockouts which are pacC regulated and *B. cinerea* pathogenicity reduced mutants whose best homologs are pacC regulated. Presented are the gene names, reference, pathogenicity effect, *C. gloeosporioides* TCONS number for each mutant, and pacC fold change in *C. gloeosporioides*.

^b Pathogenicity reductions: – represents nonsignificant pathogenicity reduction, + represents significant pathogenicity reduction, and ++ represents a complete loss of pathogenicity.

^c Statistical significance (*q* value of RNA-seq after false discovery rate correction) is represented by asterisks, where *, **, and *** indicate lower than 0.05, 0.01, and 0.001, respectively.

^d Based on quantitative reverse-transcriptase polymerase chain reaction analysis.

^e Lowest e-value blast scores were chosen (5e-56 or below).

Fig. 7. Construction and analysis of chitin deacetylase (*CDA*) gene deletion transformants. **A**, Schematic representation of a double crossing-over homologous recombination event resulting in replacement of part of the *CDA* open reading frame sequence with the hygromycin-resistance gene cassette *HygR* flanked by 5' and 3' *CDA* genomic fragments. **B**, Polymerase chain reaction (PCR) analysis of the wild type (WT) and three independent *CDA*-disruption strains. Primer5 (*cda5Fctrl*) positioned upstream of the *CDA*:*HYG* region and reverse primer 7 located on the hygromycin resistance gene were used to identify positive gene replacement at the 5' end. Primer 8 from the hygromycin cassette and primer 6 downstream of the *CDA*:*HYG* region (*cda5Rctrl*) were used to identify gene replacement at the 3' end. Primer 1 and 7 set was used as a positive control for the ectopic strains, to confirm random integration of the *CDA*:*HYG* cassette. **C**, Expression of *CDA* gene in the *Δcda* strains compared with the ectopic-integration control and WT strains, as detected by real-time PCR. Cultures were initially grown in primary medium for 3 days and subsequently transferred to secondary media at pH 4.0 and supplemented with 50 mM glutamate for 16 h, prior to RNA extraction.

species and may employ a larger genome to maintain a more diverse array of genes to support colonization under varied conditions (Staats et al. 2005). Genome complexity is inversely related to specialization in other organisms, where evolution toward specialization was found to be associated with extensive genome reduction (Nilsson et al. 2005). Thus, in a similar manner, the root pathogen *Meloidogyne hapla* has fewer genes than the related free-living *Caenorhabditis elegans* (Opperman et al. 2008).

In addition to differences in genome complexity, the chromosome conservation between *Colletotrichum gloeosporioides* and *C. graminicola* shows only 30% synteny, with multiple gene rearrangements. The blocks of synteny were limited mainly to translated areas in the genome. This level of synteny is similar to that found between *C. higginsianum* and *C. graminicola* (O'Connell et al. 2012). Synteny analysis of transcripts between *C. orbiculare* and *C. gloeosporioides* showed 53 to 60% conservation of expressed genes (Gan et al. 2013). This observation is consistent with conservation among *Colletotrichum* spp. being higher at the gene level than at the chromosome level. Synteny comparisons between different strains of *C. gloeosporioides* (i.e., within the same species) resulted in 89% similarity, expectedly higher than the synteny between species of the *Colletotrichum* genus. However, the genome di-

vergence is still significant and is consistent with the relatively high dissimilarity found using select gene sequence comparisons of *C. gloeosporioides* isolates, as observed by Weir and associates (2012).

PacC shows dual regulation of genes under pH change.

Yeast and fungi have utilized *RIM* or *pacC*, respectively, as mechanisms to monitor and respond to external pH in order to survive under varied pH conditions (Penalva et al. 2008; Selvig and Alspaugh 2011). Because *pacC* is a transcription factor that regulates gene expression under alkaline conditions, we characterized the transcription of upregulated genes as alkaline-expressed genes and downregulated genes as acid-expressed genes. In the present work, we characterized the global control of *pacC* with RNA-seq by taking advantage of the fact that $\Delta pacC$ behaves as an acid-mimicking mutant even under alkaline conditions (Tilburn et al. 1995). We confirmed this rationale by measuring *pacC*-regulated genes with RT-qPCR in actual pH shifts. Furthermore, the expression of homologs of *pacC*-regulated genes from the acidifying type of pathogen, *S. sclerotiorum*, showed that their pH-dependent expression pattern was similar to a pathogen which alkalizes environment, *C. gloeosporioides*. This indicates similar control for homologous genes despite the different optimum of pH

Fig. 8. Diagram construction and analysis of aspartic protease (*ASP*) gene deletion transformants. **A**, Schematic representation of a double crossing-over homologous recombination event resulting in replacement of part *ASP* open reading frame sequence with the hygromycin-resistance gene cassette *HygR* flanked by 5' and 3' *ASP* genomic fragments. **B**, Polymerase chain reaction (PCR) analysis of the wild type (WT) and three potential independent *ASP*-disruption strains. Primer5 (*asp5Fctrl*) positioned upstream of the *ASP:HYG* region and reverse primer 7 located on the hygromycin resistance gene were used to identify positive gene replacement at the 5' end. Primer 8 from the hygromycin cassette and primer 6 downstream of the *ASP:HYG* region (*asp5Rctrl*) were used to identify gene replacement at the 3' end. Primer 1 and 7 set was used as a positive control for the ectopic strains, to confirm random integration of the *ASP:HYG* cassette. **C**, Expression of *ASP* gene in the Δasp strains compared with the ectopic-integration control and WT strains, as detected by real-time PCR. Cultures were initially grown in primary medium for 3 days and subsequently transferred to secondary media at pH 4.0 and supplemented with 50 mM glutamate for 16 h, prior to RNA extraction.

growth requirements for each fungus. Thus, it is likely that fungi with different pH preferences contain an arsenal of both alkaline- and acid-regulated genes in order to exploit changing pH conditions. Hence, at each pH, the fungus is likely to express an optimal gene combination.

Assessment of the functions of genes regulated by *pacC* revealed those that function in various cellular activities. These include transporters, antioxidants, and CWDE. Transporters, including those involved in sulfate, potassium, carboxylic acid, and ammonium transport, are likely candidates controlled by pH due to the direct pH effect on charge of inorganic or organic acid ions. The upregulation of transporters may compensate for changes in ion difference between intracellular and the extracellular regions in order to restore fungal homeostasis under changing pH, as observed in yeast (Bensen et al. 2004). Apparently, the pH shifts also affect cellular redox states, as exemplified by changes in antioxidants that include catalase activity and hydrogen peroxide catabolic process.

Major components of *pacC* regulation in *C. gloeosporioides* are CWDE pathogenicity factors. Groups of genes that are shown to be affected here by *pacC* include PelB, cellulase, α -mannosidase, and 1,4- β -xylanase activity. These findings extend the repertoire of pH-modulated CWDE from the previously identified PelB in *C. gloeosporioides* and endoglucanases in *Alternaria alternata* (Eshel et al. 2002; Prusky et al. 2001, 2004; Yakoby et al. 2000). The results are consistent with the observation that $\Delta pacC$ mutants of *C. gloeosporioides*, *C. acutatum*, *F. oxysporum*, and *S. sclerotiorum* were found to be less virulent than the WT (Caracuel et al. 2003a,b; Miyara et al. 2008; Rollins 2003; You et al. 2007).

Interestingly, gene families with members of similar functionality were both up- and downregulated by *pacC*. These included CWDE such as rhamnogalacturonan lyase activity, pectin catabolic, polygalacturonase activity, carbon-sulfurylase, β -1,4-glucanase, xyloglucan catabolic activity, aspartic-type peptidase, β -mannosidase activity, and cellulase activity. In an analogous manner, in *S. sclerotiorum*, it was demonstrated that the expression of polygalacturonase A and B was pH dependent and correlated with the optimal pKa of each protein (Favaron et al. 2004). The differential pH regulation of genes with similar activities suggests that genes are being selectively activated on the basis of their optimal enzymatic pH activity, allowing the

fungus to cope with variable pH conditions and make optimal use of the inventory of available enzymes. Differential regulation of family members with similar function extends to other functions, including transporters for organic alcohol, hydrogen ions, and sulfate. Thus, the function of *pacC* is not to switch on or off a new life stratagem but to assist in the fine-tuning of the expression of transporters and antioxidants to maintain homeostasis and expression of pathogenicity factors for orchestration of the genomic arsenal under changing pH. Conceptualization of fungal *pacC* pH-dependent regulation of gene expression is shown schematically in Figure 10.

It is of interest that, when known pathogenicity genes of the acidifying fungus *B. cinerea* are examined, the best homologs of those genes in *C. gloeosporioides* are actually under *pacC* control and were acid expressed. Furthermore, several polygalacturonases were acid expressed and *pacC*-dependent in *B. cinerea* and in *S. sclerotiorum* and affected fungal pathogenicity (Favaron et al. 2004; Kars et al. 2005; Rollins and Dickman 2001; Wubben et al. 2000). Those findings are consistent with a scenario in which *pacC* controls the fine-tuning of enzymes so that the optimum repertoire will be expressed at any given pH. Those acid-expressed-genes are crucial for *B. cinerea* pathogenicity because it thrives at low pH. Therefore, in a fungus that acidifies its surroundings, *pacC* will probably activate alkaline-expressed genes and repress acid-expressed genes transiently during initial colonization until the secretion of acids lowers the environmental pH. Reciprocally, in fungi that alkalinize the environment, *pacC* will be activated only after the fungi raise the surrounding pH. Because fungi are likely to encounter a broad spectrum of initial environmental pH, broad conservation of pH response has justification independent of the preferred pH for pathogenicity.

PacC regulation of alkalinizing and acidifying genes.

C. gloeosporioides secretes ammonia in order to alkalinize its surroundings (Prusky et al. 2001). Analysis of the $\Delta pacC$ transcriptome identified a number of genes involved in nitrogen and ammonia metabolism. Genes encoding the production of ammonia such as GDH and urease were downregulated and are acid expressed, whereas genes coding for ammonia transport such as AMET, urea transporter, and MEPB were upregulated (Supplementary Table S6). These observations are in agreement with the fact that GDH is acid expressed and MEPB is alkaline expressed (Miyara et al. 2010). Hence, *pacC* controls the ammonia production and secretion. When faced with an acidified environment, *Colletotrichum* spp. induced ammonia secretion in order to alkaline the environment to achieve

Fig. 9. Pathogenicity assay for Δasp mutants. Changes of decay size inoculated by the wild type and three independent Δasp mutants on avocado fruit 1 and 2 days postinoculation (dpi).

Fig. 10. Scheme illustrating *pacC* regulation in *Colletotrichum gloeosporioides* and in other fungi. Alkaline environments activate *pacC* that positively or negatively regulates alkaline- or acid-expressed genes, including pathogenicity factors (cell-wall-degrading enzymes [CWDE] and proteases), transporters, and antioxidants.

their optimum growth and pathogenicity (Alkan et al. 2008; Kramer-Haimovich et al. 2006).

Secretome and pathogenicity factors in fungi that alkalize or acidify.

The predicted secretome showed enrichment for *pacC*-upregulated genes containing 3.3-fold more genes than expected. Assessment of gene functions showed a significant overrepresentation of catabolic activity such as CWDE and proteolysis which we termed “pathogenicity factors”. Expression profiling of selected genes from the *pacC*-upregulated and -secreted genes confirmed transcript activation at alkaline pH and during necrotrophic infection, which indicates that they may contribute to fungal pathogenicity. Indeed, functional analysis of six *C. gloeosporioides* KO mutants that are regulated by *pacC* showed reduced pathogenicity (Table 4). Thus, a secreted CWDE, *PeiB*, was shown to be *pacC* regulated (46-fold) and the *peIB* null mutants showed reduced pathogenicity (30%) (Table 4) (Miyara et al. 2008; Yakoby et al. 2001). In addition, the *pacC*-controlled secreted *ASP* was shown to be a pathogenicity factor, as the *ASP* KO mutant showed 50% reduction of pathogenicity on avocado fruit (Fig. 9). Another *pacC*-controlled and secreted gene is the polysaccharide *CDA* that was found to be part of a gene family. However, its deletion did not show a reduction in pathogenicity. In that case, two additional putative *CDA* genes were shown to be strongly upregulated in the mutant background. Thus, the inability to detect a reduction in pathogenicity may be due to functional redundancy.

In addition to CWDE and proteases, ammonia was also shown to regulate *Colletotrichum* pathogenicity (Alkan et al. 2008, 2009). Here, we show that *pacC* controls the *AMET* and *MEPB* transcripts, and both of their *C. gloeosporioides* null mutants showed reduced pathogenicity (Table 4) (Miyara et al. 2012; Shnaiderman et al. 2013). Interestingly, *GDH2* is a main factor in the production of ammonia and is active at acidic pH. Thus, its activity contributes to ambient alkalization. *GDH2* is negatively regulated by *pacC* and its null mutant displays reduced pathogenicity (Table 4) (Miyara et al. 2012). This indicates that *pacC* control of *C. gloeosporioides* pathogenicity is not limited to regulation of CWDE, proteases, and ammonia transporters in alkaline surroundings. It also plays a direct role in the negative regulation of ammonia production under alkaline conditions, thus promoting ammonia accumulation under acidic conditions in order to alkalize the surroundings.

Interestingly, analysis of the results here reveals that deletion mutants of homologs of seven acid-expressed genes are crucial for *B. cinerea* pathogenicity. This indicates *pacC* control of alkaline- and acid-expressed genes that have a major role in fungal pathogenicity in both fungi that acidify or alkalize their surroundings. In fungi that thrive in alkaline environments such as *Colletotrichum* spp., the alkaline-induced genes display pathogenicity roles whereas, with fungi that thrive in acidic environment such as *Botrytis* spp., the acid-expressed genes display pathogenicity roles.

Summary.

The postharvest multihost pathogen *C. gloeosporioides* genome sequence is similar in size to the other *Colletotrichum* spp. but displays more complexity because it possesses more genes. Transcriptome analysis of pH control by *pacC* revealed an arsenal of pathogenicity factors, transporters, and antioxidants to control virulence and homeostasis under changing ambient pH conditions (Fig. 10). Comparison of diverse fungal genomes showed a similar strategy of control by *pacC*, indicating a conserved role in regulating fungal genes. The observations were supported by functional analyses of both fungi that

alkalize or acidify their surroundings. The results also indicated that several *pacC*-regulated genes have a major impact on pathogenicity.

MATERIALS AND METHODS

Fungal isolate, media, and growth conditions.

Single-spore cultures of the WT Cg-14 isolate of *C. gloeosporioides* were obtained from a decayed avocado fruit (*Persea americana* ‘Fuerte’) in Israel and were routinely cultured on M₃S agar. *C. gloeosporioides* Δ *PAC1* loss-of-function mutants that encode the *pacC* transcription factor (Miyara et al. 2008) were routinely cultured on M₃S agar (Tu 1985). M₃S medium without agar (primary medium) was also used for the initial growth of the fungus, prior to exposing it to secondary media (SM). The cultures were incubated at 22 to 24°C in a shaking incubator at 150 rpm for 3 days and were harvested by filtration through a sterile Buchner funnel fitted with Whitman filter paper. The hyphal mat was washed twice with 40 ml of sterile distilled water. The washed mycelia were resuspended in 40 ml of fresh SM containing the following reagents (per liter): 4 g of K₂HPO₄, 2 g of MgSO₄ • 7H₂O, 0.3 g of CaCl₂ • 2H₂O, 10 mg of FeCl₃, 50 mM KNO₃ and 50 mM glucose. The SM was buffered with 50 mM phthalate-hydroxide buffer (Sigma, Rehovot, Israel) to obtain pH 4.0 or 7.0. The efficacy of the pH buffering was confirmed at the end of the experiment. For transcriptome analysis (RNA-seq), a pool of mycelium was harvest after growth of the *C. gloeosporioides* WT and *pacC* mutant at pH 7.0 for 1, 3, 6, 12, and 19 h. Each time point was composed of five biological RNA extractions, and the pooled time-point and repeats were served for transcriptome analysis.

S. sclerotium isolate 1980 was routinely cultured on potato dextrose agar. Five 10-mm² plugs of 5-day-old *S. sclerotium* culture was grown initially for 48 h in 40 ml of potato dextrose broth (PDB) liquid media and transferred to PDB buffered with 50 mM phthalate-hydroxide (Sigma) to obtain pH 4.0 or 7.0 for 8 h.

Fruit inoculation.

Freshly harvested, ripe Fuerte avocado fruit were used for inoculations. Spores of *C. gloeosporioides* strains were inoculated on the avocado pericarp by placing 10 μ l of a conidial suspension (10⁶ conidia ml⁻¹) into 1-mm-deep, 1-mm-diameter inoculation spots. When fruit were inoculated on the meso-carp, a 1- to 2-mm strip of peel tissue was removed from the surface of fruit and 7 μ l of a conidial suspension was used. Five inoculation spots, longitudinally spaced on each side of each of 10 different fruit, were inoculated per treatment.

Genome sequencing, assembly, and annotation.

C. gloeosporioides was grown for 3 days on liquid M₃S media and DNA was extracted from 100 mg of mycelia using the Puregene DNA purification system (Gentra, Minneapolis, MN, U.S.A.). The purified genomic DNA was sequenced to a depth of approximately 10 $\times$ using 454 GS FLX Titanium technology. The sequence reads was assembled using the GS De Novo Assembler software (454 Corp) yielding 4,907 contigs larger than 500 bp (SUB133583). Sequencing and assembly were performed by the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Gene models were annotated using GeneMark-ES (Ter-Hovhannissyan et al. 2008).

Protein sequence clustering.

The protein sequences encoded by the gene models predicted for *C. gloeosporioides* were clustered together with the predicted proteins from *C. graminicola*, *C. higginsianum*, *Neurospora crassa*, *Aspergillus nidulans*, *S. sclerotium*, and *B.*

cinerea. The protein sequences for each species were downloaded from the respective genome portals on the websites of the Broad Institute and the Joint Genome Institute. The clustering was performed using the software MCL (van Dongen 2000) using an inflation parameter of 2.0.

Analysis of conserved synteny.

Conserved syntenic blocks between the *C. gloeosporioides* and *C. graminicola* genomes were identified using SyMAP 4.0 (Soderlund et al. 2006) using the default parameters. Pseudochromosome sequences for *C. graminicola* were reconstructed using the information available on the Broad Institute website. All *C. gloeosporioides* contigs larger than 10,000 bp were used for the synteny analysis. Due to the large number of contigs in the *C. gloeosporioides* Cg-14 and NARA cg-5 genome assembly, a detailed analysis of conserved synteny was prevented. Instead, we aligned the genomes of *C. gloeosporioides* NARA gc-5 and Cg-14 using Mauve (Darling et al. 2010). The progressive Mauve algorithm first constructs anchor alignments and then joins the anchors into locally collinear blocks.

RNA extraction and real-time PCR analysis.

RNA was extracted from 80 to 100 mg (dry weight) of *C. gloeosporioides* mycelium or inoculated fruit mesocarp. Samples were ground into fine powder and were extracted using Tri-reagent at 100 mg/ml (38% water saturated phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate [pH 5], and 5% glycerol). Aqueous phase was subsequently extracted twice with chloroform. Following isopropanol precipitation and two 70% ethanol washes, the RNA pellet was resuspended in sterile distilled water. The RNA was treated with 1 unit of DNase, amplification grade (Sigma), and stored at -80°C .

The RT reaction was performed on 1 μg of total RNA with the high-capacity cDNA Reverse-Transcription Kit (AB, Applied Biosystems, Foster, CA, U.S.A.). Samples of cDNA were diluted 1:10 to the final template concentration for real-time PCR. Real-time RT-qPCR was performed using StepOnePlus System (AB, Applied Biosystems, Singapore). PCR amplification was performed with 3.4 μl of diluted cDNA template in 10 μl of reaction mixture containing 5 μl of Syber-Green amplification kit (AB Gene) and 300 nM primers. PCR was carried out by using the following cycling program: 10 min at 94°C , followed by 40 cycles of 94°C for 10 s, 60°C for 15 s, and 72°C for 20 s. The samples were subjected to melting curve analysis, with efficiencies close to 100% for all primers pairs, and all products showed the expected size of 70 to 100 bp. All the samples were normalized to actin-related expression levels in *C. gloeosporioides* and actin in *S. sclerotium* (Miyara et al. 2012) and the values were expressed as the change in the increase or decrease of the relative levels of a calibrator sample. The actin-related gene was chosen as a suitable reference gene after its expression showed no change during transcriptome evaluation. All primers are listed in Supplementary Table S7. Each treatment consisted of three biological repeats and three technical replicates.

High-throughput sequencing RNA-seq of *C. gloeosporioides* ΔpacC .

RNA high-throughput sequencing (HTS) of *C. gloeosporioides* WT and ΔpacC grown at pH 7.0 was conducted (as described above). RNA of five biological repeats from five time points (1, 3, 6, 12 and 19 h) was extracted (as described above) and mixed to obtain a pool of 10 μg of total RNA. cDNA libraries were prepared for sequencing according to the manufacturer's instructions (True Seq; Illumina Inc., San Diego, CA, U.S.A.). Single-end reads of 101 bases were obtained using

Illumina HiSeq 2000. CASAVA version 1.8.1 was used to generate the raw fastq reads. Genome assemblies of *C. gloeosporioides* isolate Cg-14 using 454 pyro-sequencing technologies platforms of HTS were used as a reference. The hybrid assembly RNA HTS National Center for Biotechnology Information accession number is GSE41844. The *C. gloeosporioides* WT and ΔpacC pooled RNA-seq resulted in 6,974,185 and 4,242,258 reads, of which approximately 70% of the reads could be mapped perfectly to the *C. gloeosporioides* genome, resulting in 2,938,268 and 4,998,444 aligned reads, respectively.

The reads were aligned to the contigs of *C. gloeosporioides* genome using TopHat v1.3.0 (Trapnell et al. 2009). The resulting alignment data from TopHat were united and fed to Cufflinks v1.1.0 to assemble aligned RNA-Seq reads into transcripts. Transcript abundances were estimated for each sample in Partek Genomics Suite V6.6 beta. Reads per kilobase per million, as well as *P* value for differential expression using the log-likelihood ratio statistic, were calculated in Partek. The *P* values were corrected in Partek for multiple comparisons using the step-up method for false discovery rate (Benjamini and Hochberg 1995). Step-up-adjusted *P* values smaller than 0.05 were referred as significant.

Gene annotation and ontologies.

Functional annotation was performed using Blast2GO software (Conesa and Gotz 2008) on the Cufflink-identified transcripts searches of the nonredundant database to estimate potential function of the Cufflink-predicted transcript and includes enzyme code, InterPro annotation, and KEGG number maps. Predicted proteins were classified as proteases by querying the MEROPS database (Rawlings et al. 2012) using a BLASTp with cut-off at E-value of $1\text{E}-10$.

The up- and downregulated genes that were significantly changed by twofold or more were classified to groups according to their Gene Ontology annotation using the Ontologizer software (Bauer et al. 2010). Due to the presence of some genes in multiple GO annotation terms, a representative term from each cluster of was manually selected.

C. gloeosporioides CDA and ASP gene disruption and mutant characterization.

Gene disruption of CDA (TCONS_00011812) was done by integrating the hygromycin marker gene and CDA flanking sequences. This was carried out by amplifying a 668-bp 5' flanking sequence of the CDA gene on *C. gloeosporioides* genomic DNA. The CDA 3' region was isolated on genomic DNA by using the modified primer pair located in the CDA 3' flanking sequence. The two PCR products were assembled together with the Hyg entry clone plasmid, using the Multisite Gateway three-fragment vector construction system. Full details of this methodology are described by Shafran and associates (2008). The *NotI* fragment was used to create the CDA knockout. Fungal transformation was done by electroporation of germinated conidia, using an established protocol (Yakoby et al. 2001). A PCR screen of the putative transformants was employed with two sets of primers and primer set HYG. A similar procedure was used to create a Gene-blast construct for ASP (13155_t), with corresponding primers: primer asp1-asp2 and primer asp3-asp4 for the PCR ASP 5' and 3' flanking sequences (658 and 710 bp, respectively), and primer asp5-HygR(7), and primer asp6-HygF(8) for PCR screening of the putative transformants from the 5' and 3' ends, respectively.

Statistical analysis.

Statistical overrepresentation of GO annotation was done using Ontologizer software (Bauer et al. 2010), with the settings

of Benjamini-Hochberg, Topology-weighted analysis, and *P* value lower than 0.05. The statistical significance between overlapping groups was calculated using the representation fold factor that represents the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups. The possibility that these factors are a random event was calculated by hyper-geometric probability. The statistical differences between two average results from RT-qPCR were checked for significance by *t* test.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Blast2GO software website: www.blast2go.com/b2ghome
 Joint Genome Institute, Glomerata cingulata 23 webpage: genome.jgi.doe.gov/Gloci1/Gloci1.home.html
 KEGG PATHWAY database: www.genome.jp/kegg/pathway.html