A Role of AREB in the Regulation of PACC-Dependent Acid-Expressed-Genes and Pathogenicity of *Colletotrichum gloeosporioides*

Dana Ment,¹ Noam Alkan,¹ Neta Luria,¹ Fang-Cheng Bi,¹ Eli Reuveni,² Robert Fluhr,² and Dov Prusky¹

¹Department of Postharvest Science of Fresh Produce, Agricultural Research Organization, the Volcani Center, Bet Dagan 50250, Israel; ²Department of Plant Sciences, Weizmann Institute of Science, Rehovot 76100, Israel

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Gene expression regulation by pH in filamentous fungi and yeasts is controlled by the PACC/RIM101 transcription factor. In *Colletotrichum gloeosporioides*, PACC is known to act as positive regulator of alkaline-expressed genes, and this regulation was shown to contribute to fungal pathogenicity. PACC is also a negative regulator of acid-expressed genes, however; the mechanism of downregulation of acid-expressed genes by PACC and their contribution to *C. gloeosporioides* pathogenicity is not well understood. RNA sequencing data analysis was employed to demonstrate that PACC transcription factor binding sites (TFBS) are significantly overrepresented in the promoter of PACC-upregulated, alkaline-expressed genes. In contrast, they are not overrepresented in the PACC-downregulated, acid-expressed genes. Instead, acid-expressed genes showed overrepresentation of AREB GATA TFBS in *C. gloeosporioides* and in homologs of five other ascomycetes genomes. The *areB* promoter contains PACC TFBS; its transcript was upregulated at pH 7 and repressed in $\Delta pacC$. Furthermore, acid-expressed genes were found to be constitutively upregulated in $\Delta areB$ during alkalizing conditions. The *areB* mutants showed significantly reduced ammonia secretion and pathogenicity on tomato fruit. Present results indicate that PACC activates *areB* expression, thereby conditionally repressing acid-expressed genes and contributing critically to *C. gloeosporioides* pathogenicity.

Colletotrichum spp. cause extensive fruit rot, both in the field and after harvest (Prusky 1996; Wharton and Diéguez-Uribeondo 2004). During appressoria penetration and necrotrophic development, *Colletotrichum gloeosporioides* alkalizes the ambient pH by massive secretion of ammonia (Alkan et al. 2008; Miyara et al. 2010; Prusky et al. 2001; Shnaiderman et al. 2013), thereby increasing the local pH by up to 4 pH units from pH 4.0 to pH 8.0 (Alkan et al. 2008; Prusky and Yakoby 2003). The accumulation of ammonia is thought to optimize pathogenicity for necrotrophic colonization (Alkan et al. 2008, 2009; Miyara et al. 2008) and by activation of host *SIRBOH* to

generate reactive oxygen species and thereby accelerate local host-cell death (Alkan et al. 2009, 2012). In addition, ambient alkalization contributes to optimization of fungal pathogenicity by activation of alkaline-expressed fungal genes through the PACC/PAL signal transduction system (Alkan et al. 2013b; Miyara et al. 2008). Indeed, many fungi alkaline-induced genes were shown to be controlled by PACC and to be involved in key pathogenicity factors, including pectate lyase (*pelB*) in *C. gloeosporioides* (Yakoby et al. 2000), polygalacturonase (*pg1* and *pg5*) in *Fusarium oxysporum* (Caracuel et al. 2003), and endoglucanases in *Alternaria alternata* (Eshel et al. 2002a and b).

A large number of fungal plant pathogens modify host pH in several ways. These include host alkalization by, for example, *C. gloeosporioides*, *C. coccodes*, and *C. higginsianum* (Alkan et al. 2008; Miyara et al. 2010; O'Connell et al. 2012; Prusky et al. 2001); *A. alternata* and *F. oxysporum* (Eshel et al. 2002a; Eshel et al. 2002b), as well as host acidification by *Penicillium expansum* and *P. digitatum* (Lee and Dean 1993; Prusky et al. 2004); *Botrytis cinerea* (Manteau et al. 2003; ten Have et al. 2001; Wubben et al. 2000); *Sclerotinia sclerotium* (Rollins and Dickman 2001); *Phomopsis mangifera* (Davidzon et al. 2010); *Monilinia fructicola* (Perpetua et al. 1996); and *Aspergillus nidulans* (Asakura et al. 2012). Regardless of their optimal pH preference, fungi detect pH and react to it with the PACC/PAL signal transduction system. In *C. gloeosporioides*, under alkaline pH conditions, PACC serves as a positive regulator, promoting transcription of PACC-alkaline-expressed genes, and it was hypothesized to simultaneously repress acid-expressed genes by an unknown mechanism (Arst and Peñalva 2003; Tilburn et al. 1995). This mechanism of pH modulation of pathogenicity factors is thought to orchestrate optimal combinations of gene expression (Alkan et al. 2013a; Hamer and Talbot 1998). Indeed, $\Delta pacC$ mutants of *C. gloeosporioides*, *C. acutatum*, *F. oxysporum*, *Penicillium expansum*, and *S. sclerotium* showed reduced virulence (Caracuel et al. 2003; Miyara et al. 2008; Rollins 2003; You et al. 2007), which demonstrated the importance of PACC gene regulation during environmental pH changes. Because of its prominence, the PACC/PAL signal transduction system is the target for new drugs being developed against opportunistic fungal infections in human and plant diseases (Cornet and Gaillardin 2014).

The homologous pH sensing systems of RIM and PACC operate in yeasts and fungi, respectively (Peñalva et al. 2008; Selvig and Alspaugh 2011). The expression of genes in alkaline conditions is governed by a complex, conserved, ambient-pH-sensing, signal-transduction pathway consisting of *pal* gene products that cleave and thus activate the PACC product

D. Ment, N. Alkan, N. Luria, and F.-C. Bi contributed equally to this article.

Corresponding author: D. Prusky; Telephone: +972-3-9683880; Fax: +972-3-9683622; E-mail: dovprusk@agri.gov.il

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at neutral and alkaline pH (Andersen et al. 2009; Galindo et al. 2012; Negrete-Urtasun et al. 1999; Peñalva and Arst 2002, 2004; Peñalva et al. 2008). Thus, in *A. nidulans*, in response to a shift to neutral or alkaline conditions, the full-length 72-kDa PACC⁷² precursor is cleaved, generating an intermediate N-terminal 53-kDa fragment, PACC⁵³ (Diez et al. 2002; Peñalva et al. 2008), which is the substrate for another, possibly proteasomal, proteolytic event that yields the 27-kDa final product PACC²⁷ that is translocated to the nucleus (Hervas-Aguilar et al. 2007). Nevertheless, the phenotypes of *pacC* deletions do not fully overlap deletions of the pH-sensing PAL apparatus, which indicates unsolved complexities in PACC regulation (Peñalva and Arst 2004; Peñalva et al. 2008).

In *Saccharomyces cerevisiae*, the activated RIM101p transcription factor serves as a direct repressor, binding to transcription factor binding sites (TFBS). Indeed, activation of alkaline-expressed genes is indirect and carried out by RIM101p repression of the negative regulatory genes *nrg1* or *simp1* (Lamb and Mitchell 2003). In contrast to this indirect method of regulation, RIM101p in *Candida albicans* and PACC in *A. nidulans* act as direct transcriptional activator by binding to TFBS present in the promoters of PACC-alkaline-expressed genes (Espeso and Arst 2000). The concomitant repression of acid-expressed genes by PACC appears to be more complex: in *A. nidulans*, the *gabA* gene acid-expressed transcript is repressed through competition for binding of the INTA/AMDR transcriptional activator (Arst and Peñalva 2003; Espeso and Arst 2000; Tilburn et al. 1995); and, similarly, in *C. albicans*, RIM101 directly represses the *phr2* gene by competition (Baek et al. 2006). However, apart from these specific examples, it is unknown whether competitive binding of transcription factors (TF) is the general mode of action by which repression of acid-induced genes is achieved in fungi.

In order to assess the regulation of genes by PACC, the *ΔpacC* loss-of-function mutant (Miyara et al. 2008) was used as a means to mimic the phenotype of acid-expressed genes under alkaline conditions (AbuQamar et al. 2006; Arst et al. 1994; Caddick et al. 1986; Diez et al. 2002; Espeso and Arst 2000; Tilburn et al. 1995). PACC-expressed genes were recently

described by comparing the transcript expression of *Colletotrichum gloeosporioides* wild-type (WT) and *ΔpacC* (Miyara et al. 2008) strains that were grown at a fixed alkaline pH (Alkan et al. 2013b). In this manner, the transcripts of 478 up-regulated genes were characterized as belonging to the calls of alkaline-expressed genes, whereas those of 483 downregulated genes (5% of the transcripts) were characterized as acid-expressed genes (Alkan et al. 2013b). Both upregulated (alkaline-expressed) and downregulated (acid-expressed) genes of the same gene families described overlapping functions, including transporters, antioxidants, and cell-wall-degrading enzymes (CWDE) (Table 1). The differential, pH-dependent expression of genes with similar activities suggested that genes were being selectively activated on the basis of their optimal enzymatic pH activity, thus allowing the fungus to cope with variable pH conditions and to make optimal use of its inventory of available enzymes. This might indicate that the function of PACC is not to switch a new gene on or off but, rather, to assist in the continuous fine tuning of the expression of transporters, antioxidants, and CWDE so as to maintain homeostasis and expression of pathogenicity factors for orchestration of the genomic arsenal under changing pH (Alkan et al. 2013b). In addition to the question of how PACC contributed to the regulation of the acid-regulated genes, these findings raised the following question: Do acid-expressed genes contribute to pathogenicity of alkalizing pathogens as *C. gloeosporioides* (Miyara et al. 2010) as they contribute to the pathogenicity of acidifying *P. expansum* (Barad et al. 2014).

In the present work, we describe a possible mechanism for activation or repression of acid-expressed genes that are regulated by PACC during *C. gloeosporioides* alkalization. By integrating data from the genome of *C. gloeosporioides* and high-throughput RNA-sequencing of WT and *pacC*-deletion strains, we detected overrepresentation of PACC TFBS in the promoter region of the PACC-upregulated alkaline-expressed genes and showed that they were positively correlated with transcript expression. In contrast, no overrepresentation of PACC TFBS was detected in the downregulated, acid-expressed gene promoters; however, in those promoter regions, AREB-like TFBS

Table 1. Representative up- and downregulated genes of *Colletotrichum gloeosporioides* wild type (WT) compared with the *ΔpacC* mutant^a

PACC-upregulated genes			PACC-downregulated genes		
Sequence name	Gene description	Fold	Sequence name	Gene name	Fold
Degrading enzymes					
TCONS_00001544	Pectate lyase b	20.76	TCONS_00010609	Endopolygalacturonase	-24.47
TCONS_00010698	Pectate lyase	16.50	TCONS_00009538	Flavin-binding monooxygenase	-10.85
TCONS_00015967	Glycoside hydrolase 76	8.33	TCONS_00000095	β-endoglucanase	-8.07
TCONS_00011898	Cellulose synthase	7.06	TCONS_00004801	Pectinesterase precursor	-7.75
TCONS_00011204	Glycoside hydrolase 76	5.79	TCONS_00009497	Extracellular cellulase asp f7-	-5.00
TCONS_00014224	Pectate lyase	4.47	TCONS_00009010	Glycosyl hydrolase 61	-4.13
TCONS_00012730	Extracellular α-glucanase	3.83	TCONS_00000111	Mannan endo-β-mannosidase	-4.12
TCONS_00007011	Glycoside hydrolase 16	3.75	TCONS_00000114	Mannan endo-β-mannosidase	-4.10
TCONS_00008701	Benzoate 4-monooxygenase	3.69	TCONS_00012618	Glycoside hydrolase 12	-3.83
TCONS_00012326	Pectate lyase I	3.03	TCONS_00007001	Mannan endo-α-mannosidase	-3.80
TCONS_00004085	Glycoside hydrolase 76	2.82	TCONS_00006855	Glycosyl family	-3.40
TCONS_00009889	Endo-α-xylanase	2.79	TCONS_00008831	Aspartic-type	-8.41
TCONS_00004451	GdsI-lipase acylhydrolase	4.54	TCONS_00008162	Serine carboxypeptidase	-5.16
Transporters					
TCONS_00008736	NADPH oxidase 4	2.98	TCONS_00017386	Plasma membrane H ⁺ -atpase pma1	-3.06
TCONS_00015152	Calcium-transporting atpase	3.74	TCONS_00003818	Sugar transporter family protein	-12.10
TCONS_00008132	Calcium-transporting atpase	6.14	TCONS_00010215	Calcium proton exchanger	-7.11
TCONS_00014465	Calcium-transporting atpase	5.16	TCONS_00002452	Ca ²⁺ H ⁺ -exchanging protein	-3.94
TCONS_00017154	Sugar transporter stt1	5.74	TCONS_00003818	Sugar transporter	-12.10
TCONS_00010531	Amino-acid permease inda1	4.18	TCONS_00000475	Oligopeptide transporter opt	-29.69
TCONS_00014064	Amino-acid permease inda1	15.37	TCONS_00007335	Amino acid permease	-20.09
TCONS_00014256	Amino acid permease	5.64	TCONS_00009021	Purine-cytosine permease	-5.55
TCONS_00010049	Ammonium transporter	4.62	TCONS_00006367	Amino acid permease	-3.14

^a Transcripts TCONS number, gene description (Gene Ontology annotation), and fold induction compared with the WT are presented.

was observed to be overrepresented. This phenomenon of differential TFBS overrepresentation was shown to be conserved in other fungi. Furthermore, *areB* transcripts accumulated in a PACC-dependent manner. Indeed, the altered transcript accumulation of acid-expressed genes in the *areB* deletion strain and analysis of infection with the deletion strain suggested that AREB acts as a repressor during alkalization and is crucial for virulence. The results revealed novel, highly conserved regulation by PACC of acid-expressed genes, which regulate fungal pathogenicity.

RESULTS

PACC up- and downregulates genes with similar functions in *C. gloeosporioides*.

In order to create pH expression-specific probes and verify transcriptome results, genes that were up- or downregulated in the transcriptomic evaluation were selected for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). β -Endoglucanase (TCONS_00000095), amino acid permease (TCONS_00007335), and Ca^{2+} proton exchange (TCONS_00010215) are examples of *pacC*-downregulated genes that were upregulated at pH 4 compared with pH 7 (Fig. 1A), whereas chitin deacetylase (CDA) (TCONS_00011812), NADPH oxidase (NOX4) (TCONS_00008736), and Ca^{2+} -transporting ATPase (TCONS_00015152) are examples of genes that were upregulated by *pacC* at pH 7 (Fig. 1B). The comparison of normalized gene expression of the PACC up- and downregulated genes of the WT and $\Delta pacC$ strains (Fig. 1A and B) previously obtained by Alkan and associates (2013a) under pH 7 conditions showed proportional upregulation of the acid-expressed (Fig. 1C) and downregulation of the *pacC* alkaline-expressed genes (Fig. 1D). These results demonstrate the differential regulation of acid- and alkaline-expressed genes controlled by PACC TF.

Promoter analysis of PACC TFBS reveals potential modes of action.

Availability of large expression databases facilitates the definition of potential TFBS in the promoter regions. To prepare a “high-quality” data set, transcripts of the up- and downregulated genes were limited to those showing $\geq 90\%$ overlap be-

tween predicted GeneMark transcripts and the transcriptome data. Additionally, those genes had an identifiable ATG (based on GeneMark-ES), a translational start site (TSS), and a promoter of at least 1,000 bp upstream from the TSS. In all, the selected database included a total of 10,000 genes in the genome, in which the average length of the 5′ untranslated region (UTR) was 200 bp, which is similar to what has been reported for the *Neurospora crassa* 5′ UTR (Supplementary Fig. S1) and for 5′ UTR from other fungal genomes (Cvijovic et al. 2007). In that selected database of high-quality transcripts, 289

Table 2. Promoter analysis of *Colletotrichum gloeosporioides* PACC-regulated genes and their homologs in several fungal genomes with different optimal pH preference during pathogenicity^a

Species	Genes set	Transcripts	Z score ^b
Alkalizing			
<i>C. gloeosporioides</i>	Up	289	10.6*
	Down	303	1
	Control	2,214	1
<i>C. higginsianum</i>	Up	215	4.4*
	Down	250	−0.5
	Control	218	−1
Acidifying			
<i>Sclerotinia sclerotiorum</i>	Up	195	6.5*
	Down	220	0.9
	Control	162	1.8
<i>Botrytis cinerea</i>	Up	215	8.6*
	Down	265	4.6
	Control	224	2.9
Neutral			
<i>Neurospora crassa</i>	Up	214	3.5*
	Down	215	1.7
	Control	170	0.9
<i>Aspergillus nidulans</i>	Up	187	4.0*
	Down	221	−0.2
	Control	195	0.5

^a Fungal homologs of *C. gloeosporioides* up- and downregulated genes were identified by BLAST of *C. gloeosporioides* PACC-regulated transcripts to *C. higginsianum*, *S. sclerotiorum*, *B. cinerea*, *N. crassa*, and *A. nidulans* genomes. The set of best homolog promoters were analyzed by Genomatix software. The PACC transcription factor binding site overrepresentation of PACC upregulated, downregulated, and nonregulated (control) genes is represented by the Z score.

^b Asterisks (*) indicate significant overrepresentation Z score ≥ 2 .

Fig. 1. Genes of different functional classes are regulated by *pacC*. Effect of pH on relative expression of **A**, PACC-downregulated genes and **B**, PACC-upregulated genes. Total RNA was isolated from wild-type mycelia which were initially grown in M3S-rich medium (Miyara et al. 2008), transferred to secondary media at pH 4 or 7 for 24 h, and analyzed by quantitative reverse-transcriptase polymerase chain reaction. The reference 18S gene was used to normalize the expression data. Values represent means of duplicates. Genes shown are β -endoglucanase (TCONS_00000095), amino acid permease (TCONS_00007335), Ca^{2+} proton exchanger (TCONS_00010215), CDA (TCONS_00011812), NOX4 (TCONS_00008736), and Ca^{2+} -transporting ATPase (TCONS_00015152); * indicates $P < 0.05$ (Student's *t* test).

PACC-upregulated and 303 PACC-downregulated genes were found (Table 2). Next, the promoter sequences located between 1,000 bp upstream and 100 bp downstream from the translational start site were queried with Genomatix analysis software for the presence of TFBS (discussed below). The results were compared with those obtained with a large control group database of 2,214 promoters selected from a random list of similar high-quality genes which were changed by less than twofold by PACC. The comparison showed that alkaline-expressed promoters resulted in significant overrepresentation of the PACC TFBS (GCCARG motif) (Espeso et al. 1997), with a Z score of 10.6 (Table 2; Supplementary Table S1). To examine whether the number of TFBS present in a promoter could influence its expression, the transcript fold change was compared with the number of PACC TFBS detected. A positive correlation was obtained between the number of TFBS and the level of transcript induction (Fig. 2). However, no correlation was observed in the datasets of the downregulated or the unregulated control genes from *C. gloeosporioides* (Fig. 2;

Fig. 2. Correlation between the number of PACC transcription factor binding sites (TFBS) and fold change of gene expression in *ΔpacC* and wild-type strains. Numbers of TFBS in the promoter region of *Colletotrichum gloeosporioides* were analyzed for sets of 287 upregulated, 285 downregulated, and 2,214 nonregulated genes obtained in the RNAseq data of wild-type and *ΔpacC* strains cultured in secondary media at pH 7. *P* value of the linear regression of the upregulated set was lower than 0.001. Analysis of variance *P* value between the upregulated and the downregulated set was lower than 0.05.

Table 2; Supplementary Table S2). The results are consistent with a scenario in which PACC plays a positive role in the up-regulation of genes at high pH, as was shown for RIM101 in yeasts (Lamb et al. 2001). However, in the present study, the absence of PACC-related TFBS in acid-expressed genes suggests that a different TF is present and that PACC imposes only indirect control on those genes.

To examine the possibility of conservation of positive control by PACC in other fungi, we analyzed their genomes for the presence of PACC TFBS in their gene homologs. Fungal species have been shown to modify the pH of the host and exhibit acidifying, alkalizing, or neutral growth styles, assuming in accord with the optimal pH for species-specific necrotrophic growth (Prusky and Yakoby 2003). Because PACC has been shown to play roles in diverse growth styles (Barad et al. 2014; Miyara et al. 2008, 2012), we expanded our analysis of TFBS to include species with an alkalizing form of life such as *C. higginsianum* (O'Connell et al. 2012), species with an acidifying form of life such as *B. cinerea* and *Sclerotinia sclerotiorum* (Rollins and Dickman 2001; Manteau et al. 2003), and other species (*A. nidulans* and *N. crassa*) which change their ambient pH by only a minor degree during colonization (Freitas et al. 2007).

Previous work has described that orthologs of both acid- and alkaline-expressed genes of the alkalizing *C. gloeosporioides* and acidifying *S. sclerotiorum* species (Alkan et al. 2013b) had similar pH expression patterns at pH 4 and 7, suggesting a common mechanism of PACC regulation between them. Analysis of the representation of the PACC TFBS motifs in potentially pH-controlled homologous genes revealed highly significant overrepresentation of PACC TFBS in the homologs of PACC-activated, alkaline-expressed genes (Table 3). However, no PACC TFBS overrepresentation could be detected in homologs of the PACC-downregulated, acid-expressed genes in these species or in the control group of neutral genes (Table 3). The data suggest evolutionary conservation in PACC TFBS promoter distribution and gene function of *pacC* in several species for alkaline-expressed genes but not acid-expressed genes.

Analysis of the 303 acid-expressed downregulated genes of *C. gloeosporioides* showed that, in 229 of them, more than one GATA TFBS was present, suggesting a strong common regulator of this set of genes (Table 3; Supplementary Table S3). Similarly, promoter analysis of the best homologs of the acid-expressed genes from *C. higginsianum*, *F. oxysporum*, *B. cinerea*, *S. sclerotiorum*, *A. nidulans*, and *N. crassa* showed pre-dominance of GATA-like TFBS repressors (Fig. 3). The obser-

Table 3. Overrepresentation of transcription factor binding sites (TFBS) in the promoter region of PACC-regulated genes in *Colletotrichum gloeosporioides*^a

PACC upregulated genes			PACC downregulated genes		
TF	Cis-element motif	Z score	TF	Cis-element motif	Z score
PACC	GCCARG	10.69	ROX1	ycha TTGTt ctc	3.85
STB3	aaaw ttTTT Cact	3.41	GAT1*	cg ATAA g	3.38
MCM1	wnt CCYA awnnggtaa	3.15	YAP1	ttw GTMA	3.04
RPN4	nna CGCCaCC cka	3.08	SPT15	agnwa TATa tatns	2.98
LUE3	aCCGG cgwanncta	2.98	GAT4*	taGATC taywc	2.71
CUSE	yww tttnGCTG w	2.9	MATA1	t GATG ta	2.69
			GATA*	GATAa g	2.69
			MGA1	ata GAAC ay	2.59
			MATALPHA2	cta TTGT t	2.49
			GZF3*(AREB)	rct GATA agr	2.48
			HAP234	ycvd CCAAt nA	2.47
			DAL80*(AREB)	c GATA aag	2.21
			MIG2	aW tcGGG tar	2.11
			XCPE1	ggGCG gasc	2.1
			MET32	ast gTGGC gy	2.07

^a Overrepresentation of promoter TFBS found by Genomatix software analysis of 289 upregulated and 303 downregulated genes. Listed are the transcription factor (TF) name, the corresponding TFBS, and the representation Z score. Asterisks (*) indicate TF belonging to the GATA family; the core of the TFBS is in bold.

vation that GATA-like TFBS were conserved and statistically prominent in the PACC-downregulated, acid-expressed genes makes the GATA-like TF and, specifically, DAL80 and GZF3 (the homologs of *AREB*) repressors candidates for involvement in regulation of these genes. In all, the data suggest that PACC could regulate acid-expressed genes through AREB, which implies a dual mode of regulation by PACC: direct activation of alkaline-expressed genes through PACC TFBS and indirect downregulation of acid-expressed genes via a PACC-activated GATA-like repressor.

Inspection of the promoter analysis of acid-expressed genes suggests that there are multiple transcriptional regulators with Z score ≥ 2 (Table 3). Among them, reactive oxygen species responsive transcriptional regulators appear to be particularly prominent (i.e., ROX1 and YAP1). Searches for the best homologous genes in *C. gloeosporioides* genome indicate the presence of ROX1 (TCONS_00008475 and TCONS_00008476) and YAP1 (TCONS_00007085). YAP1 was hardly modified by PACC. In contrast, ROX1 had an interesting alternative splicing; whereas the full-length transcript was twofold upregulated by PACC, the shorter alternative splicing was fourfold downregulated by PACC. However given the results that i) five GATA-like TFBS such as GATA, GAT1, GAT4, GZF3, and DAL80 were significantly (Z score ≥ 2) over-represented in acid-expressed genes (Table 3); ii) the GATA/AREB TF was the only common TF between various Ascomycota fungi analyzed (Fig. 3); and iii) *areB* was upregulated by PACC (Alkan et al. 2013a and b), we concentrated on the functional characterization of the *areB*.

Identification of the GATA-like factor *areB* in *C. gloeosporioides*.

In one scenario, PACC upregulates GATA-like TF repressors and, thus, may inhibit the expression of acid-expressed genes. It was noted that a putative *areB* homolog, that belongs to GATA-like TF repressor family, from *C. gloeosporioides* was activated 2.7-fold by PACC (Alkan et al. 2013b). Indeed, this

Fig. 3. Overrepresentation of transcription factor binding sites (TFBS) in the promoters of acid-expressed genes in multiple fungal species. Venn diagram illustrating the overlap of TFBS in the PACC-downregulated acid-expressed set of genes from multiple fungal species. The best homologs for the acid-expressed genes from five ascomycete fungi were analyzed for TFBS with Genomatix software.

homolog (TCONS_00010332) was the best *areB* candidate gene in the *C. gloeosporioides* genome based on tBLASTx comparison with the known *areB* sequences from the genera *Neurospora* ($2e^{-22}$) (Feng et al. 2000), *Fusarium* ($2e^{-44}$) (Michielse et al. 2014), and *Aspergillus* ($6e^{-27}$) (Macios et al. 2012a). The relation of the *areB* candidate (TCONS_00010332) toward published GATA factors from several ascomycetes was carried out by Phylogeny.fr (Dereeper et al. 2008, 2010). The results further show highest phylogenetic comparison with AREB-type genes (Fig. 4A). In addition, direct tBLASTx of the *C. gloeosporioides areB* sequence showed high similarity to a predicted GATA zinc-finger EFQ27611.1 from *C. graminicola* (strain M1.001/M2/FGSC10212) with a 79% identity (e-value of 3×10^{-25}), with *Cyphellophora europea* DAL80 W2RNE6 (e-value $3e^{-22}$), *N. crassa* ASD4 Q9HEV5 (e-value $2e^{-22}$), *F. fujikori* AREB A1IU22 (e-value $1e^{-22}$), *Metarhizium acridum* AREB E9EB17 ($3e^{-25}$), and *A. nidulans* AREB α Q9HEV2, β Q9HEV3, and γ Q9HEV4 ($6e^{-21}$, $5e^{-20}$, and $1e^{-19}$, respectively). To summarize, the putative *Colletotrichum gloeosporioides* AREB protein showed high sequence conservation, specifically in the diagnostic zinc-finger DNA-binding domain, with many to fungal AREB homologs (Fig. 4B).

areB expression is controlled by PACC.

The role of AREB in regulation of acid-expressed genes was assessed by creating *areB*-deletion mutants. Correct homologous recombination was verified by genomic PCR and absence of *areB* expression was validated by qRT-PCR (Supplementary Fig. S2B and C). Complementation of the *areB* mutant was generated by integration of a cassette containing the WT *areB* gene with 768-bp upstream and 300-bp downstream noncoding sequences and the phleomycin-resistance gene. Two complementary transformants were obtained by insertion of the full-length *areB* transcript (approximately 4,000 bp) in the background of the *areB* deletion mutant, one of which was then used for comparison with the WT strain. Integration of the complementation constructs was verified by genomic PCR and transcript expression was verified by qRT-PCR. The complemented strain regained the original levels of *areB* expression. Spore germination and colony radial growth on synthetic M3S media (Miyara et al. 2008) of the $\Delta areB$ mutants, ectopic transformants, and complemented strains were not impaired in comparison with those of the WT (data not shown).

Deep sequencing analysis indicated that *areB* transcripts were 2.7-fold significantly higher in the $\Delta pacC$ strain compared with the WT (Alkan et al. 2013b). These preliminary results suggested that PACC may activate *areB*. To examine the nature of the regulation of *areB* by PACC, the relative expression of *areB* was subjected to qRT-PCR analysis against the background of active (WT) and inactive *pacC* ($\Delta pacC$) under nitrogen-sufficient conditions containing 70 mM glutamate at initial pH 4 and 7. Relative expression of *areB* at pH 7 was significantly higher than at pH 4 in the WT strain and its expression was not detected in the $\Delta areB$ strain (Fig. 5A). These results are consistent with the observation of the presence of three PACC TBFSs in the *areB* promoter region. Furthermore, relative expression of *areB* in the $\Delta pacC$ -deletion strain at pH 7 was actually repressed compared with that at pH 4 (Fig. 5A). The present results suggest that *areB* was induced in a PACC-dependent manner. The relative expression of *pacC* was not affected by the $\Delta areB$ mutant at pH 4 or 7 and was not detected in the $\Delta pacC$ mutant (Fig. 5B). Similarly, under nitrogen-sufficient condition at pH 4 or 7, *areA* relative expression of *C. gloeosporioides* (Alkan et al. 2013b) (TCONS_00002904) was not affected by $\Delta pacC$ or $\Delta areB$ (Fig. 5C). This is contrary to previous reports (Michielse et al. 2014) that implied that AREB may regulate *areA* and *areA*-dependent genes.

secondary media, the WT and mutant strains did not differ in their expression levels of the selected genes. However, at pH 7, accumulation of transcripts was significantly higher in the $\Delta areB1$ mutant than in the WT, indicating that AREB down-regulated acid-expressed genes under alkaline conditions (Fig. 7B and C) as well as under dynamic alkalization conditions (Fig. 7A). As a further control, two independent lines of $\Delta areB$ mutants and two independent lines with ectopic expression of *areB* were tested. In all cases, transcripts were elevated in the $\Delta areB$ mutant lines, and expression levels in the ectopic lines were similar to those of the WT (Supplementary Fig. S3). The results are consistent with the scenario in which PACC upregulates a GATA-like TF that, in turn, serves to downregulate acid-expressed genes.

***areB* is necessary for the pathogenicity of *C. gloeosporioides* on tomato fruit.**

As a tool for understanding the function of *areB* under PACC regulation, the importance of AREB for the repression of acid-expressed genes and *C. gloeosporioides* pathogenicity was analyzed in ripe tomato fruit inoculated with $\Delta areB1$ and $\Delta areB2$ -deletion mutants, WT, and control *areB*-ectopic strains. Colonization of tomato fruit by the $\Delta areB$ -deletion mutants was reduced by 70%; furthermore, both the ammonia secretion and alkalization were inhibited in the background of

$\Delta areB$ (Fig. 8A through C). The same set of acid-expressed genes analyzed in synthetic media was assessed in the $\Delta areB1$ -colonized fruit. Relative expression of the PACC-acid-expressed genes in the decay tissue 4 days after inoculation of the $\Delta areB$ mutant was observed to be distributed in three ways: i) 25% of the genes showed significant upregulation relative to WT (glucanase (TCONS_00005189), flavin binding protein (TCONS_00002095), and cytochrome P450 (TCONS_00012358); ii) 50% of the genes showed no or little upregulation, with no differentiation from the WT; and iii) 25% of the genes showed statistically significant downregulation compared with the WT; it included MFS allantoinase (TCONS_00008514), acetamidase formamidase (TCONS_00014082), and endopolygalacturonase-1 (TCONS_00010609) (Fig. 8D). The unexpected differential pattern of induction, which contrasts with the upregulation of all genes in the background of the *areB* mutant (Fig. 7A and

Fig. 5. Expression patterns of *areB*, *pacC*, and *areA* at pH 4 and 7 in wild-type and mutant *Colletotrichum gloeosporioides* strains. Expression levels of **A**, *areB*; **B**, *pacC*; and **C**, *areA* (TCONS_00002904) were determined in wild-type, $\Delta pacC$, and $\Delta areB$ strains under nitrogen-sufficient conditions containing 70 mM glutamate. Quantitative reverse-transcriptase polymerase chain reaction was applied to total RNA isolated from mycelium that was initially grown in M3S-rich medium (Miyara et al. 2008) and then transferred to secondary media at pH 4 and 7 for 24 h. The reference 18S gene was used to normalize the expression data. Values represent means of duplicates; $P < 0.05$ according to one-way analysis of variance with Tukey-Kramer honestly significant difference.

Fig. 6. Expression patterns of *pacC*, *areB*, and PACC acid- and alkaline-controlled genes at various pH levels. Expression levels of *pacC* and *areB* in buffered media at **A**, pH 4 and **B**, pH 7. Expression of **C**, *pelB* (TCONS_00001544), an alkaline-expressed gene, and **D**, endopolygalacturonase (TCONS_00010609), an acid-expressed gene under buffered conditions at pH 4 and 7. Total RNA was isolated from wild-type mycelia which were initially grown in M3S-rich medium (Miyara et al. 2008), then transferred to secondary media. Mycelium was sampled for up to 24 h for analysis by quantitative reverse-transcriptase polymerase chain reaction. The reference 18S gene was used to normalize the expression data. Values represent means $\pm$ standard error of duplicates. Means with asterisks represent significant differences at $P < 0.05$ according to t test. Different letters are significantly different at $P < 0.05$ according to one-way analysis of variance with Tukey-Kramer honestly significant difference.

C), is unlikely to be due to differential alkalization; the pH changes in vitro and in vivo were mostly similar in the two cases. Thus, in a synthetic medium, PACC can regulate gene expression via AREB over a broad range of pH values. In contrast, during fruit infection, the contribution of dynamic pH change, together with the complex signals and characteristics of decaying fruit, indicated that factors in addition to pH may come into play. Nonetheless, these findings reveal a possible critical role for *areB* in fungal pathogenicity (Fig. 8A).

DISCUSSION

Fungal pathogens shift the ambient pH by secreting metabolites, thereby tailoring the environmental pH in order to optimize their pathogenic capabilities (Alkan et al. 2013a; Barad et al. 2012; Miyara et al. 2012). PACC in fungi is associated with upregulation of alkaline-expressed genes and inhibition of acid-expressed genes during alkalization (Alkan et al. 2013a; Baek et al. 2006; Peñalva et al. 2008; Rollins and Dickman 2001), and this inhibition was shown to be driven by competition for binding to the promoter of an acid-expressed gene, *gabA* (Arst and Peñalva 2003; Espeso and Arst 2000; Peñalva et al. 2008; Tilburn et al. 1995). However, this paradigm was based on the analysis of a single gene. Recent transcriptome analysis of PACC-regulated genes in *C. gloeosporioides* indi-

cated that, globally, 956 genes (approximately 5% of the fungal genome) are regulated by PACC, and half of them were either up- or downregulated. This finding presented an opportunity to carry an analysis of the mechanisms of regulation supported by statistical analysis. The present data support the paradigm of direct positive activation of alkaline-expressed genes by PACC but suggest a new mechanism for downregulation of acid-expressed genes by PACC induction of the GATA-like repressor AREB. Repression of gene expression via induction of a repressor, as described in the present article, and competition for TFBS, as described previously (Arst and Peñalva 2003; Espeso and Arst 2000; Peñalva et al. 2008; Tilburn et al. 1995) are not mutually exclusive mechanisms but could act in parallel within the genome. However, the magnitude of the global analysis that was applied in the present study (i.e., with hundreds of promoters in several types of fungi) suggests that the concurrent induction of a GATA-like repressor mechanism is both pervasive and conserved among many fungal genomes.

Promoter analysis of the set of 289 alkaline-expressed, PACC-upregulated genes showed that the PACC TFBS, “GCCARG” was overrepresented in the promoter of the set of upregulated genes ($Z = 10.26$), which is consistent with direct activation of alkaline-expressed genes by PACC in *C. gloeosporioides*. Such interaction is supported by the observation of a positive correlation between the increase in TFBS and the

Fig. 7. Analysis of acid-expressed genes in acidic and alkaline media in $\Delta areB1$ and wild-type (WT) strains. Expression levels of acid-expressed genes under various conditions: **A**, during dynamic alkalization at pH 4 to 5.5; **B**, at buffered pH 4 and **C**, at buffered pH 7. Total RNA was isolated from WT mycelia which were initially grown in M3S-rich medium (Miyara et al. 2008) and then transferred to secondary media. Mycelium was sampled at 24 h and analyzed by quantitative reverse-transcriptase polymerase chain reaction. The reference 18S gene was used to normalize the expression data. Values represent means of duplicates: $P < 0.0001$ according to Student's *t* test and analysis of variance between WT and $\Delta areB1$ (B) and $P < 0.05$ according to Student's *t* test between WT and $\Delta areB1$ (C).

fold change in expression elevation (Fig. 2). However, the correlation value was not high, which indicates that the binding of PACC to local TFBS is likely to be context-specific and that not all TFBS were equally active. Such a nonlinear effect of the number of PACC TFBS on gene expression has been shown for the isopenicillin N synthase gene promoter (Espeso and Peñalva 1996). In light of the documented involvement of PACC in the regulation of a wide variety of genes and in many fungal systems, we analyzed promoters of genes homologous of the alkaline-expressed genes of *C. gloeosporioides* in several other genomes, including fungi with contrasting alkaline, acid, and neutral preferred pH-regulating life styles. These included *C. higginsianum*, *B. cinerea*, *S. sclerotiorum*, *A. nidulans*, and *N. crassa*. In all cases, the alkaline set of orthologous genes overrepresented PACC TFBS in their promoter regions and showed mutually similar overrepresentation of TFBS PACC density with significant Z scores, which suggests that the role of PACC is conserved across ascomycetes, regardless of their life strategy and their preferred pH condition for pathogenicity.

If PACC was involved directly in the downregulation of acid-expressed genes, one would expect to find their promoter regions enriched in PACC TFBS. However, analysis of 303 promoters of PACC-downregulated, acid-expressed-genes of *C. gloeosporioides* showed no significant overrepresentation of PACC TFBS in their promoters, and no direct correlation was observed between the number of PACC TFBS in those promoters and the fold change of transcript expression in the

WT compared that in the *pacC* mutant background. Indeed, most of the 303 acid-expressed genes contained in their promoter GATA-like TFBS, suggesting that AREB and related TFBS may play an important role in mediating downregulation of acid-expressed genes by PACC. Thus, although some acid-expressed genes have been described as being regulated directly by PACC, such as *gabA* in *A. nidulans* (Espeso and Arst 2000), the analysis carried out here suggests an alternative scenario. Moreover, when this search was expanded to include further global analysis of the genomes of six pathogenic fungi, it revealed statistically relevant conservation of other TFBS, of which the most pervasive were annotated as GATA-like repressors, including DAL80 (Macios et al. 2012b), GAT1 (Crespo et al. 2001), and GZF3 (Soussi-Boudekou et al. 1997).

GATA transcription factors are a universal class of DNA-binding proteins in eukaryotes, characterized by one or more highly conserved zinc-finger motifs that recognize consensus WGATAR (in which W = T or A and R = G or A) TFBS sequences. The *C. gloeosporioides areB* was identified by examination of an orthologous relation of TCONS_00010332 with published GATA factors from several ascomycetes, through phylogenetic analysis. In addition, the putative protein sequence of AREB was aligned with orthologs from five ascomycetes fungi. The putative protein sequence was shown to display the canonically conserved C-X(2)-C-X(17)-C-X(2)-C zinc-finger motif and the presence of a conserved diagnostic DNA-binding region (Espeso and Peñalva 1996). Therefore, *areB* (TCONS_00010332) is the ortholog of both DAL80 and GZF1 from yeast

Fig. 8. pH change and ammonia accumulation of *ΔareB*, wild-type (WT), and ectopic *ΔareB* strains of *Colletotrichum gloeosporioides* during colonization on tomato fruit and the relative expression of acid-expressed genes. The colonization of tomato fruit by *C. gloeosporioides* WT, *ΔareB1*, *ΔareB2*, and *areB*-ectopic strains was followed by analysis of **A**, decay area (cm²); **B**, pH changes; and **C**, ammonia secretion (mM/gram of tissue). Wound-inoculated tomato fruit were monitored 4 days postinoculation (dpi) and were used for **D**, relative expression of acid-expressed genes in *C. gloeosporioides* WT and *ΔareB1* mutant dpi. The reference 18S gene was used to normalize the expression data. Values represent means of duplicates. A through C: Values represent means of quadruplicates. Means not followed by the same letter are significantly different at $P < 0.05$ according to one-way analysis of variance with Dunnett's method for comparison with control. D: Values represent means of duplicates; * indicates $P < 0.05$ (Student's *t* test).

and, in light of its PACC-dependent upregulation, it was considered the best candidate to be the GATA-like repressor of acid-expressed genes. The *areB* promoter contains three PACC TFBS and its upregulation at alkaline pH was detected by transcriptome analysis of *pacC* mutant and direct qRT-PCR analysis. Comparison between the transcript dynamics of *pacC* and *areB* expression showed that *pacC* was induced earlier than *areB* during incubation at pH 7 (Fig. 6). Furthermore, the Δ *areB* mutant showed enhanced upregulation of acid-expressed genes at pH 7 compared with the WT strain, consistent with the involvement of AREB in mediating repression of acid-expressed genes. However, in comparison with the results in synthetic media, analysis of acid-expressed genes during tomato fruit colonization by *C. gloeosporioides* showed that some of those genes were positively regulated by AREB. This indicates another dimension to the functional complexity of these key TF players in cellular signaling under complex colonization conditions, in which pH and availability of nutrients induced by the macerating pathogen contribute to its pathogenicity.

Modulation of *areB* activity by pH was reported in the yeast *areB* ortholog *GZF3/DAL80*. In that case, overexpression of *areB* was found to be related to alkalinity tolerance (Garcia-Salcedo et al. 2006), supporting a potential role of *GZF3/DAL80* in gene suppression at alkaline pH. However, in general, GATA TF have been associated with nitrogen metabolism (Reyes et al. 2004), and gene regulation by AREB was shown to be dependent on the nutrient conditions tested. In both *P. chrysogenum* and *A. nidulans*, *areB* expression was found to be nitrogen dependent (Conlon et al. 2001; Haas et al. 1997); whereas AREB negatively regulated AREA-dependent gene transcription under nitrogen starvation in *Aspergillus* spp. (Wong et al. 2009), under nitrogen-repressing conditions, AREB negatively regulated the arginine catabolism genes *agaA* and *otaA* (Dzikowska et al. 2003; Macios et al. 2012b). In *P. chrysogenum*, overexpression of the *areB* homolog *nreB* led to repression of nitrate-assimilatory genes, further supporting a role of AREB/NREB as a repressor (Haas et al. 1997). In *C. gloeosporioides*, the Δ *areB* mutant did not affect *areA* relative expression in nitrogen-sufficient condition containing 70 mM glutamate (Fig. 5), a finding that is supported by work in *N. crassa*, in which the AREB homolog ASD4 appeared to have no direct role in nitrogen regulation (Feng et al. 2000). Some reports have connected GATA TF with pathogenicity through their effects on nitrogen utilization (Lee et al. 2012). Because nitrogen- and carbon-repressing conditions modulate *areB* (Haas et al. 1997; Macios et al. 2012b), it is possible to suggest that the complex nutritional effects that occur under pH changes induced by *C. gloeosporioides* in colonized fruit might also contribute to the regulatory activity of AREB during pathogenicity. This might affect the transcriptional activity of acid-expressed genes, and could explain their differential gene activation under conditions of varying pH, as observed under the dynamically changing conditions in the fruit-pathogen interactions.

Previous reports have shown the effect of Δ *pacC* in the reduction of pathogenicity (Miyara et al. 2010). In the present work, Δ *areB* mutants of *C. gloeosporioides* exhibited 70% reduced pathogenicity in tomato fruit, accompanied by 60% reduced ammonia accumulation and inhibition of host alkalization. Previous RNAseq analysis indicated the presence of several nitrogen metabolism candidates that are significantly repressed in alkaline conditions, including α -ketoglutarate-dependent xanthine dioxygenase, urease accessory protein, Δ -1-pyrroline-5-carboxylate dehydrogenase, and glutamate decarboxylase (Alkan et al. 2013b). Interestingly, all of those transcripts contain two to eight GATA TFBS, suggesting their possible regulation by AREB.

Our present results indicate that PACC activated *areB* at alkaline pH and during alkalization in nitrogen-sufficient conditions. Consequently, it may be suggested that AREB controls pathogenicity by repressing a large set of acid-expressed genes under alkalization conditions, which would indicate that both alkaline- and acid-expressed genes contribute to *C. gloeosporioides* pathogenicity. In this relation, previous knock-out mutants of *Botrytis* genes, homologous to the PACC-regulated acid-expressed genes in *C. gloeosporioides*, as pectin methylesterase (Valette-Collet et al. 2003) polygalacturonase1 (ten Have et al. 1998), cytochrome p450-mono-oxygenase (Siewers et al. 2005), endopolygalacturonase2 (Kars et al. 2005), and endo- β -1,4 xylanase (Brito et al. 2006), showed a significant reduction in pathogenicity, supporting the claim that acid-expressed genes are important players in fungal pathogenicity. This indicates that the regulation of genes involved in necrotrophic pathogenicity is complex and mediated by multiple environmental and regulatory inputs and that AREB regulation of PACC acid-expressed-genes is one of the components that contribute to the virulence of *C. gloeosporioides*.

MATERIALS AND METHODS

Promoter analysis of *C. gloeosporioides* and best-homolog identification.

C. gloeosporioides genome (GSE41844)-predicted proteins were annotated using GeneMark-ES (Ter-Hovhannisyan et al. 2008). The *C. gloeosporioides* genome and transcriptome is publicly available online (transcriptome accession numbers GSM1025551 and GSM1025552) (Alkan et al. 2013b). The selected database included a total of 10,000 genes. The Genomatrix Genome Analyzer software suite was used to identify TFBS within the chosen gene set, and the Genomatrix RegionMiner software was then employed to estimate the enrichment of known fungal TFBS and the PACC TFBS “GCCARG” in each of the groups (PACC up-, down-, and nonregulated) compared with a “background set” of genes, which were a constant random selection of promoters from the same organism, not regulated by PACC. RegionMiner calculates the number of each TFBS within a background set of sequences and compares it with the number observed in the sampled sequences. It then computes a Z score, which is equivalent to the distance of the number of TFBS in the sampled set from the population mean, in units of the population standard deviation. TFBS alignments in the promoter set were determined with Genomatrix MatInspector software, which attributes a significance of alignment score to each of the TFBS, together with its location and strand orientation.

Best homologs were found by conducting a BLASTP search against the transcriptome of each of the fungi. Scores with e-value below 10^{-5} and at least 50% similarity were accepted. The search was carried out by the use of accession numbers in the gene bank database, as described above.

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 *areB* loss-of-function mutants that encoded the *areB* transcription factor were routinely cultured on M3S agar (Tu 1985), and M3S medium without agar (primary medium) was also used for the initial growth of the fungus, prior to its exposure to SM. The cultures were incubated at 25°C in a shaking incubator at 150 rpm for 3 days and were harvested by filtration through a sterile Buchner funnel fitted with Whatman filter paper. The hyphal mat was washed

twice with 40 ml of sterile distilled water, and the washed mycelia were resuspended in 40 ml of fresh SM containing the following reagents (per liter): 4 g of K_2HPO_4 , 2 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of $CaCl_2 \cdot 2H_2O$, 10 mg of $FeCl_3$, 70 mM glutamate, and 50 mM glucose. The SM was adjusted to pH 4 or 7 by addition of 1 N HCl or 1 N NaOH. For buffered conditions, the SM was buffered with 50 mM phthalate-hydroxide buffer (Sigma, Rehovot, Israel) to obtain pH 4.0 or with phosphate buffer to obtain pH 7.0. The efficacy of the pH buffering was confirmed at the end of the experiment by measuring the pH of the culture media.

Fruit inoculation.

Freshly harvested, ripe tomato fruit were subjected to inoculations. Spores of the various *C. gloeosporioides* strains were inoculated on the tomato pericarp by placing 10 μ l of a conidial suspension (10^6 conidia ml^{-1}) into inoculation spots (1 mm deep by 1 mm in diameter). The fruit were incubated at 25°C and approximately 85% relative humidity in covered containers containing wet paper towels until symptoms were observed for 4 days. In all experiments, the average decay area $\pm$ standard error, pH changes, and ammonia accumulation in the healthy and the decayed tissues were reported. The inoculation experiments were repeated three times, with 10 to 12 fruit per experiment; the results of one representative experiment are presented.

pH measurement and ammonia detection.

The pH was measured in 0.5-ml aliquots of the culture media, collected at various times after fungal inoculation; a Thermo-Orion Model 9810BN microcombination pH electrode (Thermo Fisher Scientific, Waltham, MA, U.S.A.) was used. The pH in the fruit was measured by grinding a sample of fruit tissue and centrifuging for 5 min at 10,000 rpm. The supernatant obtained was used for pH measurement and ammonia detection. Ammonia was detected calorimetrically with an ammonium test kit (Merck, Darmstadt, Germany); its concentration was determined in 5-ml samples of 10-fold-diluted culture or fruit tissue supernatants, according to the manufacturer's instructions. Concentrations were reported in millimolar of ammonia per 1 ml of culture media or per gram of fruit tissue.

Total RNA extraction.

Samples (80 to 100 mg, dry weight) of WT or mutant *C. gloeosporioides* mycelium were ground into fine powder and RNA was extracted with the SV-total RNA isolation system (Promega, Madison, WI, U.S.A.). Total RNA was extracted from inoculated tomato fruit mesocarp according to the cetyltrimethylammonium bromide-based method (Yang et al. 2008), with minor changes. In addition, RNA was treated with DNase (Turbo DNase, Ambion, CA, U.S.A.).

qRT-PCR analysis.

The expression levels of the selected genes were estimated by real-time qRT-PCR for single-stranded cDNA synthesized from 1 μ g of total RNA, as described previously (Miyara et al. 2012). The forward and reverse primers for all of the genes are listed in Supplementary Table S4. Each treatment comprised three biological repeats and two technical replicates.

C. gloeosporioides areB gene disruption and complementation.

For generation of the knockout construct, the 5' and 3' flanking fragments of the coding region of *C. gloeosporioides areB* were amplified by PCR from the gene deposited in GenBank with accession number KJ869059. For *areB*, primer sets attB *areB* 5'F + attB *areB* 5'R and attB *areB* 3'F + attB *areB* 3'R

were used to amplify the 5' and 3' fragments, respectively, in a PCR reaction that included 30 ng of genomic DNA, 10 pmol of oligonucleotide, and PCR ready mix (Thermo Scientific). The construct was generated through GATEWAY technology, according to the manufacturer's instructions (Shafran et al. 2008). The plasmid was isolated, sequenced, and digested with *NorI* to release the deletion construct. Electroporation of germinating conidia was performed essentially as previously described (Yakoby et al. 2001). Briefly, Cg-14 isolates were cultured on solid M3S medium for 14 days. Conidia were collected in pea juice, adjusted to 10^6 conidia/ml, and incubated at 28°C for 2.5 h to initiate germination. The germinated conidia were collected, washed with cold electroporation buffer (1 mM n-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid and 50 mM mannitol, pH 7.5), and concentrated to 10^8 conidia/ml. Aliquots of 100 μ l were distributed in cold electroporation cuvettes (Bio-Rad, Hercules, CA, U.S.A.). Electroporation and transfer to regeneration medium were performed according to Shafran and associates (2008). Transgenic colonies appeared 3 to 4 days after transformation. The transformants were regrown as single colonies on M3S agar with hygromycin B at 100 mg/liter, and DNA was extracted with the Master Pure yeast purification kit (Epicentre Biotechnologies, Madison, WI, U.S.A.). *areB* gene-disruption events were first analyzed by PCR screening for integration of the *areB* 5'-Hyg-*areB* 3' cassette at the *areB* locus. For that purpose, primer pairs *areB* control 5' + hygromycin 5'R and *areB* control 3' + hygromycin 3'F were designed on the basis of the regions flanking the disruption cassette at the 5' and 3' ends of the Hyg cassette, respectively. Primers attB *areB* 5'F and attB *areB* 3'R were designed to analyze ectopic integration strains at the 5' and 3' ends of the Hyg cassette, respectively.

For generation of complementation constructs a representative *areB* gene-replacement strain, $\Delta areB1$, was utilized as a recipient for complementation, via transformation with the pDEST-Phleo vector, which contains the intact genomic *areB*, including 768 bp of native promoter and 285 bp of native terminator, and a marker conferring resistance to phleomycin. Resistant transformants were recovered and analyzed by PCR using primer pairs attB *areB* comp 5'F and attB *areB* comp 3'R, with primers *areB* promoter 5'R and *areB* terminator 3'F designed on the basis of the genome.

Spore germination and colony radial growth.

To assess conidial germination, 100 μ l of a suspension containing 10^6 conidia/ml was spread on M3S agar and incubated for 12 h at 28°C. Germination was observed microscopically and 100 spores from each replicate were counted. To assess colony radial growth of the WT, the $\Delta areB$, and the *areB*-ectopic isolates, the isolates were grown for 10 days on M3S without selection. A 0.5-cm-diameter mycelial plug was placed on M3S medium and incubated for 7 days at 28°C. Each experiment was repeated three times.

Data analysis.

For statistical overrepresentation of TFBS in the promoters, the Genomatix genome analysis software was used for searches of TFBS within the input regions. This procedure generates statistics on TFBS and compares overrepresentation values and Z scores against the genomic and promoter background; a representation factor > 1 indicates more overlap than expected. Statistical differences between results of qRT-PCR data, conidia germination, fruit decay, pH changes, and ammonia secretion were checked for significance by applying Student's *t* test or analysis of variance in JMP software (SAS, Cary, NC, U.S.A.).

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LITERATURE CITED

- AbuQamar, S., Chen, X., Dhawan, R., Bluhm, B., Salmeron, J., Lam, S., Dietrich, R. A., and Mengiste, T. 2006. Expression profiling and mutant analysis reveals complex regulatory networks involved in *Arabidopsis* response to *Botrytis infection*. *Plant J.* 48:28-44.
- Alkan, N., Fluhr, R., Sherman, A., and Prusky, D. 2008. Role of ammonia secretion and pH modulation on pathogenicity of *Colletotrichum coccodes* on tomato fruit. *Mol. Plant-Microbe Interact.* 21:1058-1066.
- Alkan, N., Davydov, O., Sagi, M., Fluhr, R., and Prusky, D. 2009. Ammonium secretion by *Colletotrichum coccodes* activates host NADPH oxidase activity enhancing host cell death and fungal virulence in tomato fruits. *Mol. Plant-Microbe Interact.* 22:1484-1491.
- Alkan, N., Fluhr, R., and Prusky, D. 2012. Ammonium secretion during *Colletotrichum coccodes* infection modulates salicylic and jasmonic acid pathways of ripe and unripe tomato fruit. *Mol. Plant-Microbe Interact.* 25:85-96.
- Alkan, N., Espeso, E. A., and Prusky, D. 2013a. Virulence regulation of phytopathogenic fungi by pH. *Antioxid. Redox Signal.* 19:1012-1025.
- Alkan, N., Meng, X. C., Friedlander, G., Reuveni, E., Sukno, S., Sherman, A., Thon, M., Fluhr, R., and Prusky, D. 2013b. Global aspects of pacC regulation of pathogenicity genes in *Colletotrichum gloeosporioides* as revealed by transcriptome analysis. *Mol. Plant-Microbe Interact.* 26:1345-1358.
- Andersen, M. R., Lehmann, L., and Nielsen, J. 2009. Systemic analysis of the response of *Aspergillus niger* to ambient pH. *Genome Biol.* 10:R47.
- Arst, H. N., and Peñalva, M. A. 2003. pH regulation in *Aspergillus* and parallels with higher eukaryotic regulatory systems. *Trends Genet.* 19:224-231.
- Arst, H. N., Bignell, E., and Tilburn, J. 1994. 2 New genes involved in signaling ambient pH in *Aspergillus-nidulans*. *Mol. Gen. Genet.* 245:787-790.
- Asakura, M., Yoshino, K., Hill, A. M., Kubo, Y., Sakai, Y., and Takano, Y. 2012. Primary and secondary metabolism regulates lipolysis in appressoria of *Colletotrichum orbiculare*. *Fungal Genet. Biol.* 49:967-975.
- Baek, Y. U., Martin, S. J., and Davis, D. A. 2006. Evidence for novel pH-dependent regulation of *Candida albicans* Rim101, a direct transcriptional repressor of the cell wall beta-glycosidase PHR2. *Eukaryot. Cell* 5:1550-1559.
- Barad, S., Horowitz, S. B., Moskovitch, O., Lichter, A., Sherman, A., and Prusky, D. 2012. A *Penicillium expansum* glucose oxidase-encoding gene, GOX2, is essential for gluconic acid production and acidification during colonization of deciduous fruit. *Mol. Plant-Microbe Interact.* 25:779-788.
- Barad, S., Horowitz, S. B., Kobiler, I., Sherman, A., and Prusky, D. 2014. Accumulation of the mycotoxin patulin in the presence of gluconic acid contributes to pathogenicity of *Penicillium expansum*. *Mol. Plant-Microbe Interact.* 27:66-77.
- Brito, N., Espino, J. J., and Gonzalez, C. 2006. The endo-beta-1,4-xylanase xyn11A is required for virulence in *Botrytis cinerea*. *Mol. Plant-Microbe Interact.* 19:25-32.
- Caddick, M. X., Brownlee, A. G., and Arst, H. N. 1986. Regulation of gene-expression by pH of the growth-medium in *Aspergillus-nidulans*. *Mol. Gen. Genet.* 203:346-353.
- Caracuel, Z., Roncero, M. I. G., Espeso, E. A., Gonzalez-Verdejo, C. I., Garcia-Maceira, F. I., and Di Pietro, A. 2003. The pH signalling transcription factor PacC controls virulence in the plant pathogen *Fusarium oxysporum*. *Mol. Microbiol.* 48:765-779.
- Conlon, H., Zadra, I., Haas, H., Arst, H. N., Jones, M. G., and Caddick, M. X. 2001. The *Aspergillus nidulans* GATA transcription factor gene areB encodes at least three proteins and features three classes of mutation. *Mol. Microbiol.* 40:361-375.
- Cornet, M., and Gaillardin, C. 2014. pH signaling in human fungal pathogens: A new target for antifungal strategies. *Eukaryot. Cell* 13:342-352.
- Crespo, J. L., Daicho, K., Ushimaru, T., and Hall, M. N. 2001. The GATA transcription factors GLN3 and GAT1 link TOR to salt stress in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 276:34441-34444.
- Cvijovic, M., Dalevi, D., Bilsland, E., Kemp, G. J. L., and Sunnerhagen, P. 2007. Identification of putative regulatory upstream ORFs in the yeast genome using heuristics and evolutionary conservation. *BMC Bioinf.* 8:295.
- Davidzon, M., Alkan, N., Kobiler, I., and Prusky, D. 2010. Acidification by gluconic acid of mango fruit tissue during colonization via stem end infection by *Phomopsis mangiferae*. *Postharvest Biol. Technol.* 55:71-77.
- Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J. F., Guindon, S., Lefort, V., Lescot, M., Claverie, J. M., and Gascuel, O. 2008. Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* 36:W465-W469.
- Dereeper, A., Audic, S., Claverie, J. M., and Blanc, G. 2010. BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC Evol. Biol.* 10:8.
- Diez, E., Alvaro, J., Espeso, E. A., Rainbow, L., Suarez, T., Tilburn, J., Arst, H. N., and Peñalva, M. A. 2002. Activation of the *Aspergillus* PacC zinc finger transcription factor requires two proteolytic steps. *EMBO (Eur. Mol. Biol. Organ.) J.* 21:1350-1359.
- Dzikowska, A., Kacprzak, M., Tomecki, R., Koper, M., Scazzocchio, C., and Weglenski, P. 2003. Specific induction and carbon/nitrogen repression of arginine catabolism gene of *Aspergillus nidulans*-functional in vivo analysis of the otaA promoter. *Fungal Genet. Biol.* 38:175-186.
- Eshel, D., Lichter, A., Dinor, A., and Prusky, D. 2002a. Characterization of *Alternaria alternata* glucanase genes expressed during infection of resistant and susceptible persimmon fruits. *Mol. Plant Pathol.* 3:347-358.
- Eshel, D., Miyara, I., Ailing, T., Dinor, A., and Prusky, D. 2002b. pH regulates endoglucanase expression and virulence of *Alternaria alternata* persimmon fruit. *Mol. Plant-Microbe Interact.* 15:774-779.
- Espeso, E. A., and Peñalva, M. A. 1996. Three binding sites for the *Aspergillus nidulans* PacC zinc-finger transcription factor are necessary and sufficient for regulation by ambient pH of the isopenicillin N synthase gene promoter. *J. Biol. Chem.* 271:28825-28830.
- Espeso, E. A., and Arst, H. N. 2000. On the mechanism by which alkaline pH prevents expression of an acid-expressed gene. *Mol. Cell Biol.* 20:3355-3363.
- Espeso, E. A., Tilburn, J., Sanchez-Pulido, L., Brown, C. V., Valencia, A., Arst, H. N., Jr., and Peñalva, M. A. 1997. Specific DNA recognition by the *Aspergillus nidulans* three zinc finger transcription factor PacC. *J. Mol. Biol.* 274:466-480.
- Feng, B., Haas, H., and Marzluf, G. A. 2000. ASD4, a new GATA factor of *Neurospora crassa*, displays sequence-specific DNA binding and functions in ascus and ascospore development. *Biochemistry* 39:11065-11073.
- Freitas, J. S., Silva, E. M., and Rossi, A. 2007. Identification of nutrient-dependent changes in extracellular pH and acid phosphatase secretion in *Aspergillus nidulans*. *Genet. Mol. Res.* 6:721-729.
- Galindo, A., Calcagno-Pizarelli, A. M., Arst, H. N., and Peñalva, M. A. 2012. An ordered pathway for the assembly of fungal ESCRT-containing ambient pH signalling complexes at the plasma membrane. *J. Cell Sci.* 125:1784-1795.
- Garcia-Salcedo, R., Casamayor, A., Ruiz, A., Gonzalez, A., Prista, C., Loureiro-Dias, M. C., Ramos, J., and Arino, J. 2006. Heterologous expression implicates a GATA factor in regulation of nitrogen metabolic genes and ion homeostasis in the halotolerant yeast *Debaryomyces hansenii*. *Eukaryot. Cell* 5:1388-1398.
- Haas, H., Angermayr, K., Zadra, I., and Stoffler, G. 1997. Overexpression of nreB, a new GATA factor-encoding gene of *Penicillium chrysogenum*, leads to repression of the nitrate assimilatory gene cluster. *J. Biol. Chem.* 272:22576-22582.
- Hammer, J. E., and Talbot, N. J. 1998. Infection-related development in the rice blast fungus *Magnaporthe grisea*. *Curr. Opin. Microbiol.* 1:693-697.
- Hervas-Aguilar, A., Rodriguez, J. M., Tilburn, J., Arst, H. N., and Peñalva, M. A. 2007. Evidence for the direct involvement of the proteasome in the proteolytic processing of the *Aspergillus nidulans* zinc finger transcription factor PacC. *J. Biol. Chem.* 282:34735-34747.
- Kars, I., Krooshof, G. H., Wagemakers, L., Joosten, R., Benen, J. A. E., and van Kan, J. A. L. 2005. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *Plant J.* 43:213-225.
- Lamb, T. M., and Mitchell, A. P. 2003. The transcription factor Rim101p governs ion tolerance and cell differentiation by direct repression of the regulatory genes NRG1 and SMP1 in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 23:677-686.
- Lamb, T. M., Xu, W., Diamond, A., and Mitchell, A. P. 2001. Alkaline response genes of *Saccharomyces cerevisiae* and their relationship to the RIM101 pathway. *J. Biol. Chem.* 276:1850-1856.
- Lee, I. R., Lim, J. W. C., Ormerod, K. L., Morrow, C. A., and Fraser, J. A. 2012. Characterization of an NMR homolog that modulates GATA factor-mediated nitrogen metabolite repression in *Cryptococcus neoformans*. *PLoS One* 7:e32585.
- Lee, Y. H., and Dean, R. A. 1993. cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. *Plant Cell* 5:693-700.
- Macios, M., Caddick, M. X., Weglenski, P., Scazzocchio, C., and

- Dzikowska, A. 2012a. The GATA factors AREA and AREB together with the co-repressor NMRA, negatively regulate arginine catabolism in *Aspergillus nidulans* in response to nitrogen and carbon source. *Fungal Genet. Biol.* 49:189-198.
- Manteau, S., Aboune, S., Lambert, B., and Legendre, L. 2003. Differential regulation by ambient pH of putative virulence factor secretion by the phytopathogenic fungus *Botrytis cinerea*. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol.* 43:359-366.
- Michielse, C. B., Pfannmüller, A., Macios, M., Rengers, P., Dzikowska, A., and Tudzynski, B. 2014. The interplay between the GATA transcription factors AreA, the global nitrogen regulator and AreB in *Fusarium fujikuroi*. *Mol. Microbiol.* 91:472-493.
- Miyara, I., Shafraan, H., Haimovich, H. K., Rollins, J., Sherman, A., and Prusky, D. 2008. Multi-factor regulation of pectate lyase secretion by *Colletotrichum gloeosporioides* pathogenic on avocado fruits. *Mol. Plant Pathol.* 9:281-291.
- Miyara, I., Shafraan, H., Davidzon, M., Sherman, A., and Prusky, D. 2010. pH regulation of ammonia secretion by *Colletotrichum gloeosporioides* and its effect on appressorium formation and pathogenicity. *Mol. Plant-Microbe Interact.* 23:304-316.
- Miyara, I., Shnaiderman, C., Meng, X., Vargas, W. A., Diaz-Minguez, J. M., Sherman, A., Thon, M., and Prusky, D. 2012. Role of nitrogen-metabolism genes expressed during pathogenicity of the alkalizing *Colletotrichum gloeosporioides* and their differential expression in acidifying pathogens. *Mol. Plant-Microbe Interact.* 25:1251-1263.
- Negrete-Urtasun, S., Reiter, W., Diez, E., Denison, S. H., Tilburn, J., Espeso, E. A., Peñalva, M. A., and Arst, H. N. 1999. Ambient pH signal transduction in *Aspergillus*: Completion of gene characterization. *Mol. Microbiol.* 33:994-1003.
- O'Connell, R. J., Thon, M. R., Hacquard, S., Amyotte, S. G., Kleemann, J., Torres, M. F., Damm, U., Buiate, E. A., Epstein, L., Alkan, N., Altmüller, J., Alvarado-Balderama, L., Bauser, C. A., Becker, C., Birren, B. W., Chen, Z. H., Choi, J., Crouch, J. A., Duvick, J. P., Farman, M. A., Gan, P., Heiman, D., Henrissat, B., Howard, R. J., Kabbage, M., Koch, C., Kracher, B., Kubo, Y., Law, A. D., Lebrun, M. H., Lee, Y. H., Miyara, I., Moore, N., Neumann, U., Nordstrom, K., Panaccione, D. G., Panstruga, R., Place, M., Proctor, R. H., Prusky, D., Rech, G., Reinhardt, R., Rollins, J. A., Rounsley, S., Schardl, C. L., Schwartz, D. C., Shenoy, N., Shirasu, K., Sikhakolli, U. R., Stuber, K., Sukno, S. A., Sweigard, J. A., Takano, Y., Takahara, H., Trail, F., van der Does, H. C., Voll, L. M., Will, I., Young, S., Zeng, Q. D., Zhang, J. Z., Zhou, S. G., Dickman, M. B., Schulze-Lefert, P., van Themaat, E. V. L., Ma, L. J., and Vaillancourt, L. J. 2012. Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. *Nat. Genet.* 44:1060-1065.
- Peñalva, M. A., and Arst, H. N. 2002. Regulation of gene expression by ambient pH in filamentous fungi and yeasts. *Microbiol. Mol. Biol. Res.* 66:426-446.
- Peñalva, M. A., and Arst, H. N. 2004. Recent advances in the characterization of ambient pH regulation of gene expression in filamentous fungi and yeasts. *Annu. Rev. Microbiol.* 58:425-451.
- Peñalva, M. A., Tilburn, J., Bignell, E., and Arst, H. N. 2008. Ambient pH gene regulation in fungi: Making connections. *Trends Microbiol.* 16:291-300.
- Perpetua, N. S., Kubo, Y., Yasuda, N., Takano, Y., and Furusawa, I. 1996. Cloning and characterization of a melanin biosynthetic THR1 reductase gene essential for appressorial penetration of *Colletotrichum lagenarium*. *Molecular plant-microbe interactions*. *Mol. Plant-Microbe Interact.* 9:323-329.
- Prusky, D. 1996. Pathogen quiescence in postharvest diseases. *Annu. Rev. Phytopathol.* 34:413-434.
- Prusky, D., and Yakoby, N. 2003. Pathogenic fungi: Leading or led by ambient pH? *Mol. Plant Pathol.* 4:509-516.
- Prusky, D., McEvoy, J. L., Leverentz, B., and Conway, W. S. 2001. Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence. *Mol. Plant-Microbe Interact.* 14:1105-1113.
- Prusky, D., McEvoy, J. L., Saftner, R., Conway, W. S., and Jones, R. 2004. Relationship between host acidification and virulence of *Penicillium* spp. on apple and citrus fruit. *Phytopathology* 94:44-51.
- Reyes, J. C., Muro-Pastor, M. I., and Florencio, F. J. 2004. The GATA family of transcription factors in *Arabidopsis* and rice. *Plant Physiol.* 134:1718-1732.
- Rollins, J. A. 2003. The *Sclerotinia sclerotiorum* *pac1* gene is required for sclerotial development and virulence. *Mol. Plant-Microbe Interact.* 16:785-795.
- Rollins, J. A., and Dickman, M. B. 2001. pH signaling in *Sclerotinia sclerotiorum*: Identification of a pacC/RIM1 homolog. *Appl. Environ. Microbiol.* 67:75-81.
- Selvig, K., and Alspaugh, J. A. 2011. pH response pathways in fungi: Adapting to host-derived and environmental signals. *Mycobiology* 39:249-256.
- Shafraan, H., Miyara, I., Eshed, R., Prusky, D., and Sherman, A. 2008. Development of new tools for studying gene function in fungi based on the Gateway system. *Fungal Genet. Biol.* 45:1147-1154.
- Shnaiderman, C., Miyara, I., Kobiler, I., Sherman, A., and Prusky, D. 2013. Differential activation of ammonium transporters during the accumulation of ammonia by *Colletotrichum gloeosporioides* and its effect on appressoria formation and pathogenicity. *Mol. Plant-Microbe Interact.* 26:345-355.
- Siewers, V., Viaud, M., Jimenez-Teja, D., Collado, I. G., Gronover, C. S., Pradier, J. M., Tudzynski, B., and Tudzynski, P. 2005. Functional analysis of the cytochrome P450 monooxygenase gene *bcob1* of *Botrytis cinerea* indicates that botrydial is a strain-specific virulence factor. *Mol. Plant-Microbe Interact.* 18:602-612.
- Soussi-Boudekou, S., Vissers, S., Urrestarazu, A., Jauniaux, J. C., and Andre, B. 1997. Gzf3p, a fourth GATA factor involved in nitrogen-regulated transcription in *Saccharomyces cerevisiae*. *Mol. Microbiol.* 23:1157-1168.
- ten Have, A., Mulder, W., Visser, J., and van Kan, J. A. L. 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. *Mol. Plant-Microbe Interact.* 11:1009-1016.
- ten Have, A., Breuil, W. O., Wubben, J. P., Visser, J., and van Kan, J. A. L. 2001. *Botrytis cinerea* endopolygalacturonase genes are differentially expressed in various plant tissues. *Fungal Genet. Biol.* 33:97-105.
- Ter-Hovhannisyann, V., Lomsadze, A., Chernoff, Y. O., and Borodovsky, M. 2008. Gene prediction in novel fungal genomes using an ab initio algorithm with unsupervised training. *Genome Res.* 18:1979-1990.
- Tilburn, J., Sarkar, S., Widdick, D. A., Espeso, E. A., Orejas, M., Mungroo, J., Peñalva, M. A., and Arst, H. N. 1995. The *Aspergillus* PACC zinc-finger transcription factor mediates regulation of both acid-expressed and alkaline-expressed genes by ambient pH. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:779-790.
- Tu, J. C. 1985. An improved Mathur medium for growth, sporulation, and germination of spores of *Colletotrichum lindemuthianum*. *Microbios* 44:87-93.
- Valette-Collet, O., Cimerman, A., Reignault, P., Levis, C., and Boccara, M. 2003. Disruption of *Botrytis cinerea* pectin methylesterase gene *Bcpme1* reduces virulence on several host plants. *Mol. Plant-Microbe Interact.* 16:360-367.
- Wharton, P. S., and Diéguez-Urbeondo, J. 2004. The biology of *Colletotrichum acutatum*. *An. Jard. Bot. Madrid* 61:3-22.
- Wong, K. H., Hynes, M. J., Todd, R. B., and Davis, M. A. 2009. Deletion and overexpression of the *Aspergillus nidulans* GATA factor AreB reveals unexpected pleiotropy. *Microbiology* 155:3868-3880.
- Wubben, J. P., ten Have, A., van Kan, J. A. L., and Visser, J. 2000. Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. *Curr. Genet.* 37:152-157.
- Yakoby, N., Kobiler, I., Dinoor, A., and Prusky, D. 2000. pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits. *Appl. Environ. Microbiol.* 66:1026-1030.
- Yakoby, N., Beno-Moualem, D., Keen, N. T., Dinoor, A., Pines, O., and Prusky, D. 2001. *Colletotrichum gloeosporioides pelB* is an important virulence factor in avocado fruit-fungus interaction. *Mol. Plant-Microbe Interact.* 14:988-995.
- Yang, G., Zhou, R., Tang, T., and Shi, S. 2008. Simple and efficient isolation of high-quality total RNA from *Hibiscus tiliaceus*, a mangrove associate and its relatives. *Prep. Biochem. Biotechnol.* 38:257-264.
- You, B. J., Choquer, M., and Chung, K. R. 2007. The *Colletotrichum acutatum* gene encoding a putative pH-responsive transcription regulator is a key virulence determinant during fungal pathogenesis on citrus. *Mol. Plant-Microbe Interact.* 20:1149-1160.

AUTHOR-RECOMMENDED INTERNET RESOURCES

- Gene Expression Omnibus accession *C. gloeosporioides* genome:
www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41844
 Genomatix Genome Analyzer database:
www.genomatix.de/solutions/genomatix-genome-analyzer.html