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Carbon regulation of environmental pH by secreted small molecules that modulate pathogenicity in phytopathogenic fungi

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SUMMARY

Fruit pathogens can contribute to the acidification or alkalinization of the host environment. This capability has been used to divide fungal pathogens into acidifying and/or alkalinizing classes. Here, we show that diverse classes of fungal pathogens—*Colletotrichum gloeosporioides*, *Penicillium expansum*, *Aspergillus nidulans* and *Fusarium oxysporum*—secrete small pH-affecting molecules. These molecules modify the environmental pH, which dictates acidic or alkaline colonizing strategies, and induce the expression of PACC-dependent genes. We show that, in many organisms, acidification is induced under carbon excess, i.e. 175 mM sucrose (the most abundant sugar in fruits). In contrast, alkalinization occurs under conditions of carbon deprivation, i.e. less than 15 mM sucrose. The carbon source is metabolized by glucose oxidase (*gox2*) to gluconic acid, contributing to medium acidification, whereas catalysed deamination of non-preferred carbon sources, such as the amino acid glutamate, by glutamate dehydrogenase 2 (*gdh2*), results in the secretion of ammonia. Functional analyses of Δ*gdh2* mutants showed reduced alkalinization and pathogenicity during growth under carbon deprivation, but not in high-carbon medium or on fruit rich in sugar, whereas analysis of Δ*gox2* mutants showed reduced acidification and pathogencity under conditions of excess carbon. The induction pattern of *gdh2* was negatively correlated with the expression of the zinc finger global carbon catabolite repressor *creA*. The present results indicate that differential pH modulation by fruit fungal pathogens is a host-dependent mechanism, affected by host sugar content, that modulates environmental pH to enhance fruit colonization.

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Keywords: carbon regulation of pathogenicity, pathogenicity, pH regulation.

INTRODUCTION

The ability of microorganisms to sense, modulate and adapt to environmental changes is essential to their survival and reproduction (Biswas *et al.*, 2007). This is particularly important for species such as pathogens, symbionts or commensals that exhibit an intimate association with host organisms. It is also relevant to pathogenic fungi that attack fruit, as they must respond to the extracellular pH, which can vary from host to host (Prusky and Yakoby, 2003). In fruit, the initial host pH varies from <4 to >6, yet pathogens may increase it to as high as pH 8.0 or reduce it to as low as pH 3.2 (Prusky and Yakoby, 2003). The adjustment of local pH is critical for colonization (Prusky and Yakoby, 2003; Yakoby *et al.*, 2000).

Colletotrichum gloeosporioides is a widely distributed pathogen of fruits and vegetables. It secretes massive amounts of ammonia during the initial stages of penetration and necrotrophic colonization in ripe fruit (Alkan *et al.*, 2008; Miyara *et al.*, 2010; Prusky, 1996; Prusky and Yakoby, 2003; Prusky *et al.*, 2010; Wharton and Diéguez-Uribeondo, 2004); the secretion increases the local pH by up to 4 pH units, from pH 4.0 to pH 8.0 (Prusky *et al.*, 2001, 2013). Alkaline adaptation has been well studied in the model systems *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Candida albicans* (Baek *et al.*, 2006; Davis *et al.*, 2000; Su and Mitchell, 1993), as has the contribution of secreted ammonia to the alkalinization process and *Colletotrichum* pathogenicity via the optimization and activation of fungal pathogenicity factors (Prusky *et al.*, 2013). Indeed, ammonia accumulation has been shown to depend on the initial environmental pH and, at pH 4, *Colletotrichum coccodes* showed a 3.5-fold higher ammonia

production than at pH 7 (Alkan *et al.*, 2008). The accumulation of ammonia has been shown to further contribute to necrotrophic colonization (Prusky and Yakoby, 2003; Prusky *et al.*, 2013; Shnaiderman *et al.*, 2013) through the activation of host *RBOH* (NADPH oxidase) to generate reactive oxygen species, thereby accelerating localized host cell death (Alkan *et al.*, 2009). Hence, the modulation of host pH by fungi enhances their virulence in ripening fruit (Prusky, 1996). Other fungal pathogens, such as *Penicillium*, also secrete pH-modulating molecules, identified as organic acids, which acidify the environment and similarly contribute to host cell necrotization and the optimization of fungal colonization.

The induction of alkaline-expressed fungal genes requires the highly conserved pH-sensor system mediated by PACC (Alkan *et al.*, 2013b; Espeso & Arst, 2000; Miyara *et al.*, 2008). PACC is a zinc finger transcription factor that regulates pH-controlled genes expressed under alkaline conditions. About 480 genes are up-regulated and an equal number down-regulated by PACC amounting to 5% of the genome (Alkan *et al.*, 2013b). Indeed, PACC-regulated genes, such as pectate lyase (*pelB*) in *C. gloeosporioides* (Yakoby *et al.*, 2000), polygalacturonase (*pg1* and *pg5*) in *Fusarium oxysporum* (Caracuel *et al.*, 2003), endoglucanases in *Alternaria alternata* (Eshel *et al.*, 2002) and polygalacturonase in *Penicillium expansum* (Prusky *et al.*, 2004), have been shown to be central virulence factors. In *C. gloeosporioides* under alkaline pH conditions, PACC serves as a positive regulator, promoting the transcription of alkaline-expressed genes (Alkan *et al.*, 2013b; Caracuel *et al.*, 2003). It simultaneously represses acid-expressed genes by a mechanism involving AREB repressor activation (Ment *et al.*, 2015).

The evaluation of a *C. gloeosporioides pacC* mutant revealed the differential pH regulation of hundreds of acid- or alkaline-expressed genes (Alkan *et al.*, 2013b). Importantly, different members of the same gene families (transporters, antioxidants and cell wall-degrading enzymes) were found to belong to either PACC-up-regulated (alkaline-expressed) or PACC-down-regulated (acid-expressed) groups, suggesting that similar types of gene activity are expressed under both pH conditions (Alkan *et al.*, 2013b; Ment *et al.*, 2015). Differential pH-dependent expression of genes enables the fungus to adapt to variable pH conditions and to make optimal use of its inventory of available enzymes. Hence, PACC acts to orchestrate the genomic arsenal under dynamic pH conditions (Alkan *et al.*, 2013b). Although PACC is known to control optimal expression under specific pH conditions, how pathogens alter the specific extracellular pH conditions is less clearly understood. This is highly important because fungal pathogens, such as *Colletotrichum* and *Penicillium*, may alkalinize or acidify, respectively, the host environment, thereby creating conditions that differentially modulate the pathogenicity of the species (Prusky and Yakoby, 2003; Prusky *et al.*, 2013; Vylkova *et al.*, 2011; Yakoby *et al.*, 2000).

Fruits, the target of diverse pathogens, differ widely in their levels of soluble sugars—of which sucrose, glucose and fructose represent more than 75%–80% of the total soluble solids (TSSs) (Hulme, 1971; Prusky, 1996). Some fruits are nearly devoid of TSSs, whereas others, on ripening, metabolize polysaccharides to sugars to achieve TSS levels as high as 25%; the concentration of sucrose may reach up to 10%–12% (Prusky, 1996; Hulme, 1971). Little is known about the impact of the varying host carbon levels in maturing and ripening fruit on the pH-modulating lifestyle induced by fungal pH-affecting molecules. Carbon catabolite repression (CCR) by *creA* is a regulatory mechanism that ensures the utilization of a preferred carbon source, such as glucose (Fernandez *et al.*, 2012, 2014). This mechanism operates via the negatively acting zinc finger repressor CREA, which ensures preferential glucose utilization by preventing the expression of genes required for the metabolism of other carbon sources. Their role in providing cues for the secretion of specific small molecules is not known. In the present work, carbon availability is shown to be the major controlling factor for the activation of acidification or alkalinization processes *in vitro* and *in vivo*, and this modulation is shown to be correlated with *creA*. Acidification has been shown to be induced under conditions of excess sugar, via the oxidation of glucose to gluconic acid (GLA) by glucose oxidase (GOX2), even in pathogenic organisms that are classified as leading an alkalinizing lifestyle (Alkan *et al.*, 2013b; Ment *et al.*, 2015). In contrast, alkalinization is dominant under conditions of carbon deprivation and is catalysed by the deamination of non-preferred carbon sources, such as the amino acid glutamate, by glutamate dehydrogenase 2 (GDH2). The present results suggest a new mechanism by which fruit pathogens control ambient pH and pathogenicity in fruits.

RESULTS

Carbon concentration modulates extracellular acidification or alkalinization in *Colletotrichum*

To analyse the effect of carbon availability on pH modulation by *C. gloeosporioides*, the fungus was grown in the presence of limiting (15 mM, 0.5%) or excess (175 mM, 6%) sucrose levels in liquid medium with 0.3% (w/v) tryptone and 0.7% (w/v) $NaNO_3$ as nitrogen sources and an initial pH 5, previously used for the detection of *gox2* expression (Barad *et al.*, 2012, 2014). Analysis of the pH shift induced by *C. gloeosporioides* in the presence of 15 mM sucrose showed the accumulation of 0.22 mM ammonia, but no detectable accumulation of GLA (Fig. 1B,C). The pH of the medium increased from pH 5 to pH 7.65 after 72 h (Fig. 1A). In contrast, the growth of *C. gloeosporioides* in sugar-enriched medium (175 mM sucrose) elicited GLA accumulation to 0.3 mM as the main organic acid produced, no ammonia accumulation and a

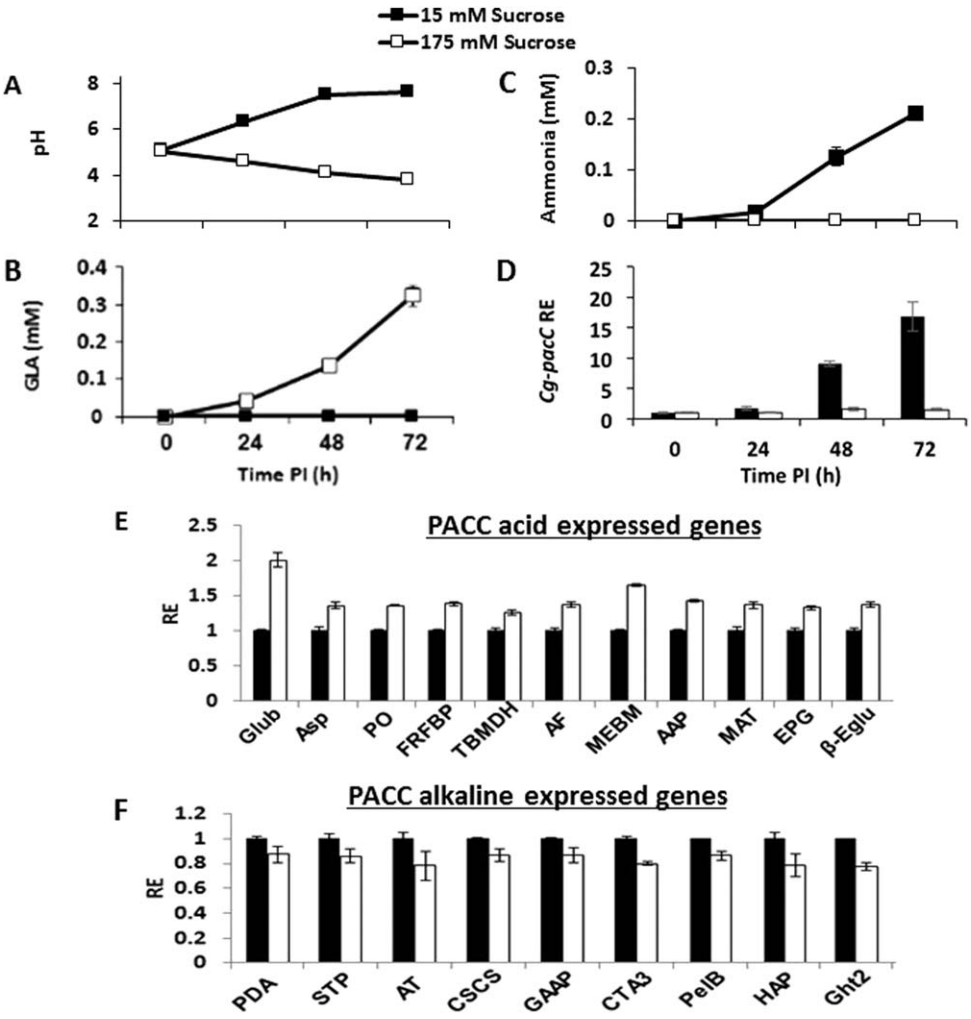

Fig. 1 Effects of carbon concentration on the induction of medium alkalinization or acidification by *Colletotrichum gloeosporioides* and the induction of PACC-regulated acid- and alkaline-expressed genes. Fungal mycelia of *C. gloeosporioides* (*Cg*) were grown in primary rich medium for 3 days and then transferred to secondary medium containing sucrose at 15 mM (■) or 175 mM (□), adjusted to pH 5, for 72 h. The effect of sucrose concentration was evaluated on the modulation of (A) pH, (B) gluconic acid (GLA) accumulation, (C) ammonia accumulation and (D) *pacC* relative expression (RE). RNA for *pacC* RE was extracted from mycelia at the indicated time points after transfer to inducing medium, and cDNA was used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR). RNA for the evaluation of *pacC*-regulated genes was extracted from mycelia 24 h after transfer to inducing medium. (E) The acid-expressed genes encoded glucanase b (*Glub*), aspartic type (*Asp*), proline oxidase (*PO*), flavin reductase fmn-binding protein (*FRFBP*), Tim barrel metal-dependent hydrolase (*TBMDH*), acetamidase formamidase (*AF*), mannan endo-β-mannosidase (*MEBM*), amino acid permease family (*AAP*), mfs allantotate (*MAT*), endopolygalacturonase (*EPG*) and β-endoglucanase (*β-EGlu*). (f) The PACC alkaline-induced genes encoded polysaccharide deacetylase family protein (*PDA*), sugar transport protein (*STP*), abc transporter (*AT*), cellulose synthase catalytic subunit (*CSCS*), general amino acid permease (*GAAP*), calcium-transporting ATPase 3 (*CTA3*), pectate lyase b (*PelB*), histidine acid phosphatase (*HAP*) and high-affinity glucose transporter (*Ght2*). The 18S gene was used to normalize the expression data for each fungus. Values represent means ± standard error (SE) of four biological replicates. The experiments were repeated three times. PI, post-inoculation.

c. 1 unit decrease in pH (Fig. 1A; Table S1, see Supporting Information).

Alkalinization of the medium at limiting sucrose levels elicited a 17-fold increase in the relative expression (RE) of *pacC*, 72 h after inoculation (Fig. 1D). No effect on *pacC* expression was observed when the fungus was grown under excess sucrose levels, i.e. when the pH decreased. PACC-regulated alkaline and acid genes have been identified previously in *C. gloeosporioides*; these genes are either activated or repressed, depending on the environmental pH (Alkan *et al.*, 2013b). A series of PACC-regulated gene expressions were analysed under conditions of high and low sugar content. PACC-regulated acid-induced genes were overexpressed by 25%–50% when the fungus was grown under high-sugar, acidifying conditions (Fig. 1E). In contrast, under the low sugar concentrations that generate alkalinizing conditions, a number of alkaline-induced, PACC-regulated genes were all up-regulated

when significant amounts of ammonia accumulated (Fig. 1F). These findings indicate bimodal pH modulation in *C. gloeosporioides* that can differentially activate PACC-regulated genes. Furthermore, reducing (1.5 g/L) or increasing (10 g/L) the nitrogen source (tryptone) in the growth medium (Fig. S1, see Supporting Information) under fixed sucrose concentrations of 15 or 175 mM did not change the accumulation pattern of ammonia or GLA, as reflected by the environmental pH, suggesting the importance of carbon concentration in the induction process.

A comparison of pH modulation by the growth of *C. gloeosporioides* in the presence of sucrose, glucose or fructose showed that limiting sugar concentrations (15 mM) induce similar ammonia accumulation, up to 0.4 mM, and a pH increase (Fig. S2A,C, see Supporting Information), suggesting that limited carbon levels of any sugar present in fruit can induce ammonia accumulation. In contrast, growth in the presence of excess glucose or sucrose (175 mM), but not fructose, induced GLA accumulation to 0.17 mM and a pH decrease (Fig. S2B,D), suggesting the importance of glucose oxidase (GOX) activation for GLA accumulation. Given that sucrose is one of the three most significant sugars in fruit and can be cleaved by the fungus to give both glucose and fructose, further experiments were carried out with sucrose.

Involvement of *gdh2* and *gox2* in the modulation of environmental pH under various carbon levels

To understand the mechanism regulating the secretion of pH-modulating molecules under conditions of limiting and excess carbon, the REs of the genes involved in ammonia or GLA synthesis were analysed. The gene *gdh2* (GenBank accession number EU182718) encodes glutamate dehydrogenase, which catalyses the oxidation of glutamate to α-ketoglutarate, resulting in ammonia release (Miyara *et al.*, 2010). GOX, encoded by *gox2*, catalyses the oxidation of glucose to GLA, as shown in *P. expansum* (Barad *et al.*, 2012; Hadas *et al.*, 2007). By homology to sequences of *Pe gox2* (Barad *et al.*, 2012; Hadas *et al.*, 2007) and several other GOX-containing species (Figs S3 and S4, see Supporting Information), we identified three *gox* orthologues in *C. gloeosporioides* (Table S2, see Supporting Information). One, *gox2* (TCONS_00011543), was induced 2.4-fold during the necrotrophic phase compared with the quiescent infection stage on tomato fruits (Alkan *et al.*, 2013b), and was further characterized in the present work to determine its relationship to GLA accumulation in *Colletotrichum*.

Transcripts of *gdh2* and *gox2* are not regulated by pH, as their expression was unchanged in the mutant *pacC* background (Alkan *et al.*, 2013b). Significantly, the analysis of *gdh2* RE during *Colletotrichum* growth in the presence of low sucrose (15 mM) showed 9–10-fold increases at 48 and 72 h after inoculation (Fig. 2A), concomitant with ammonia accumulation (Fig. 1C). Reciprocally, a 12- to 27-fold increase in *gox2* RE was observed during growth in high sucrose (175 mM) (Fig. 2B). The relative changes in pH induced by *gdh2* and *gox2* activity under both growth conditions were reflected in the activation, or lack thereof, of pH-sensitive *pacC* expression (Fig. 2C).

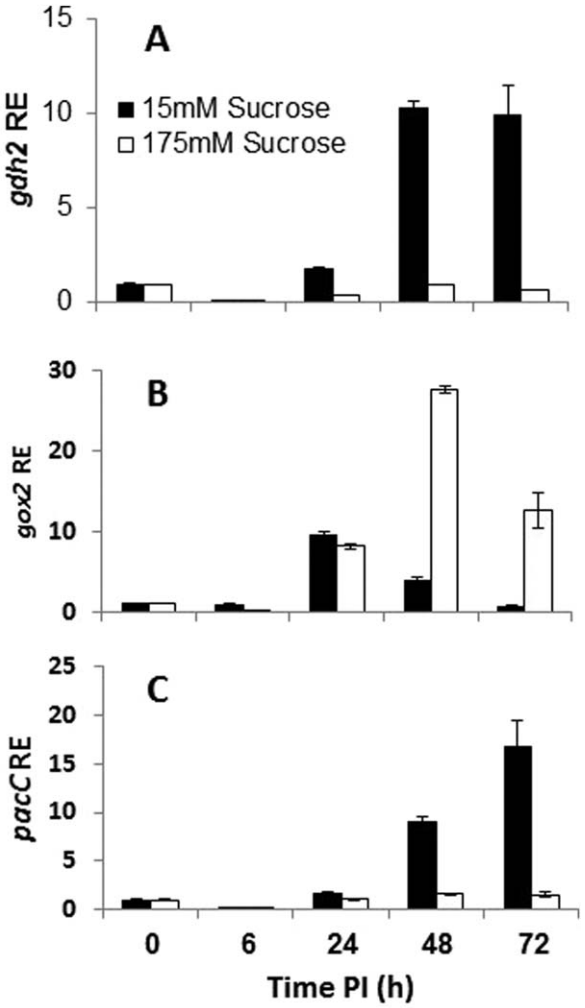

Fig. 2 Relative expression (RE) of *gdh2* (A), *gox2* (B) and *pacC* (C) in *Colletotrichum gloeosporioides* grown in different carbon levels. Fungal mycelia were grown in primary rich medium for 3 days and then transferred to secondary media (SM) medium containing sucrose at 15 mM (■) or 175 mM (□), adjusted to pH 5. RNA was extracted from mycelia at the indicated time points after transfer to secondary medium, and cDNA was used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The 18S gene was used to normalize the expression data. Values represent means ± standard error (SE) of duplicates. Experiments were repeated three times. PI, post-inoculation.

The contribution of *gdh2* to the pH change under limiting carbon was tested in the *C. gloeosporioides* Δ*gdh2* mutant (Miyara *et al.*, 2010). Compared with the wild-type (WT) strain under limited carbon, ammonia accumulation was reduced by 29% (Fig. 3A) and pH by 1.25 units (data not shown) in this mutant; 72 h after inoculation of the WT strain, when *pacC* expression was

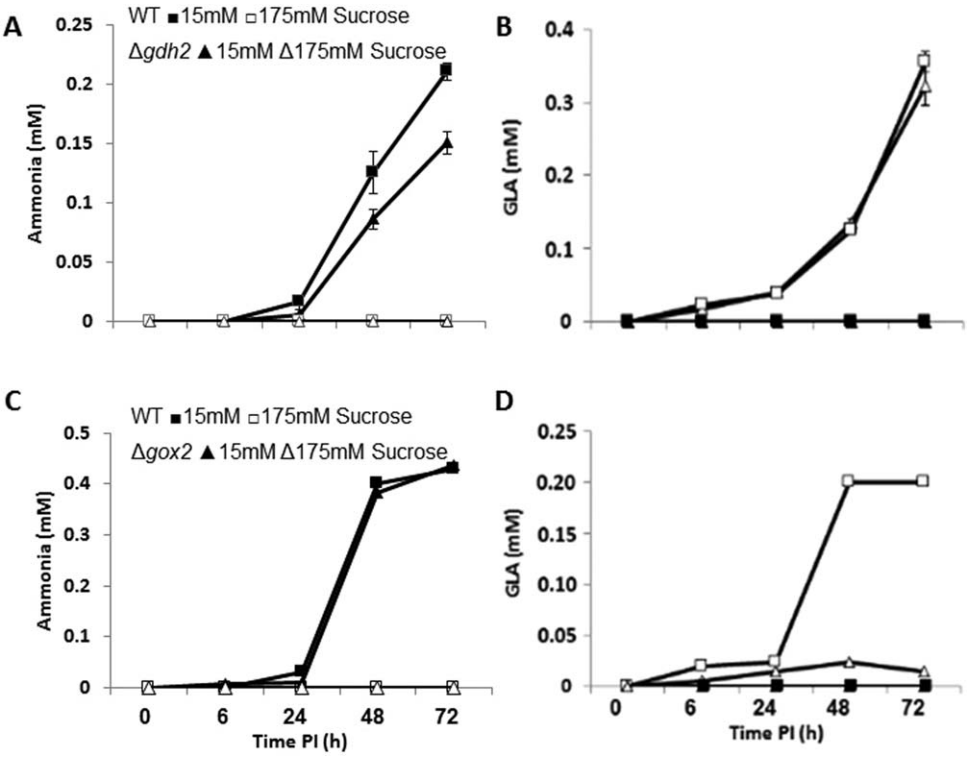

Fig. 3 Effects of carbon levels on alkalinization or acidification of the medium by wild-type (WT) and mutant strains of *Colletotrichum gloeosporioides*. (A, B) Δ*gdh2*. (C, D) Δ*gox2*. (A, C) Ammonia and (B, D) gluconic acid (GLA) accumulation. Fungal mycelia were grown in primary rich medium (M_3S) for 3 days and then transferred to secondary medium (SM) containing sucrose at 15 mM (■▲) or 175 mM (□Δ), adjusted to pH 5. Values represent means ± standard error (SE) of four biological replicates. Experiments were repeated three times. PI, post-inoculation.

highest (Fig. 2C), relative *pacC* expression in the Δ*gdh*2 mutant was reduced by 43%. However, as expected, GLA accumulation was not affected in the mutant compared with the WT strain when they were grown under excess sucrose, resulting in acid-inducing conditions (Fig. 3B). Thus, the deamination activity of *gdh2* induced under low-sucrose conditions is at least one potential source of small pH effector molecules.

The contribution of *gox2* to GLA accumulation under excess carbon was tested by developing a *C. gloeosporioides* Δ*gox*2 mutant (Fig. S5, see Supporting Information). Growth of the Δ*gox*2 mutant in excess sucrose decreased GLA accumulation almost 10-fold compared with the WT, 72 h after inoculation (Fig. 3D). No differences in ammonia accumulation were detected compared with the WT strain under limited carbon supply (Fig. 3C). Thus, acid production by *gox2* induced by high-sucrose conditions is at least one source of small pH-affecting molecules.

Interestingly, the growth of *C. gloeosporioides* in sucrose, glucose or fructose (main sugars in the TSSs of fruit) showed similar increases in *gdh2* RE (up to two-fold for fungal growth under excess sugar). However, when grown in the presence of fructose, which is not a substrate of GOX, *gox2* RE was significantly reduced (more than 2.5-fold) compared with that in *C. gloeosporioides* grown in the presence of sucrose or glucose (result not shown). Taken together, the results indicate that the balance between GLA and ammonia accumulation is carbon dependent, and probably a key factor in determining the modulation of host pH by *Colletotrichum*.

Carbon concentration modulates extracellular acidification or alkalinization by *Penicillium*, *Fusarium* and *Aspergillus*

To enhance the understanding of the induction of specific pH changes, pathogens with pH modulation of colonization strategies differing from that of *C. gloeosporioides* were analysed (Fig. 4): *P. expansum*, which has an acidification-inducing colonization strategy (Barad *et al.*, 2012); *F. oxysporum*, which has mixed alkalinizing and acidifying patterns (Caracuel *et al.*, 2003; Miyara *et al.*, 2010); and *A. nidulans*, with a less pronounced pH-modulating pattern (Espeso *et al.*, 1993). Qualitatively, all fungi showed a remarkably consistent pattern of ammonia production in low-sucrose medium. All fungi grown in the presence of 15 mM sucrose showed enhanced ammonia accumulation, similar to *C. gloeosporioides*, except for *A. nidulans* which exhibited smaller increases (Fig. 4A). The accumulated ammonia actually led to increases of ~2 pH units in *Penicillium* and *Fusarium* (Fig. 4A).

Similarly, when viewed qualitatively, all fungi showed a remarkably consistent pattern of GLA production in high-sucrose medium, but with widely varying levels (Fig. 4A). The copious amounts (20 mM GLA) produced by *P. expansum* were probably

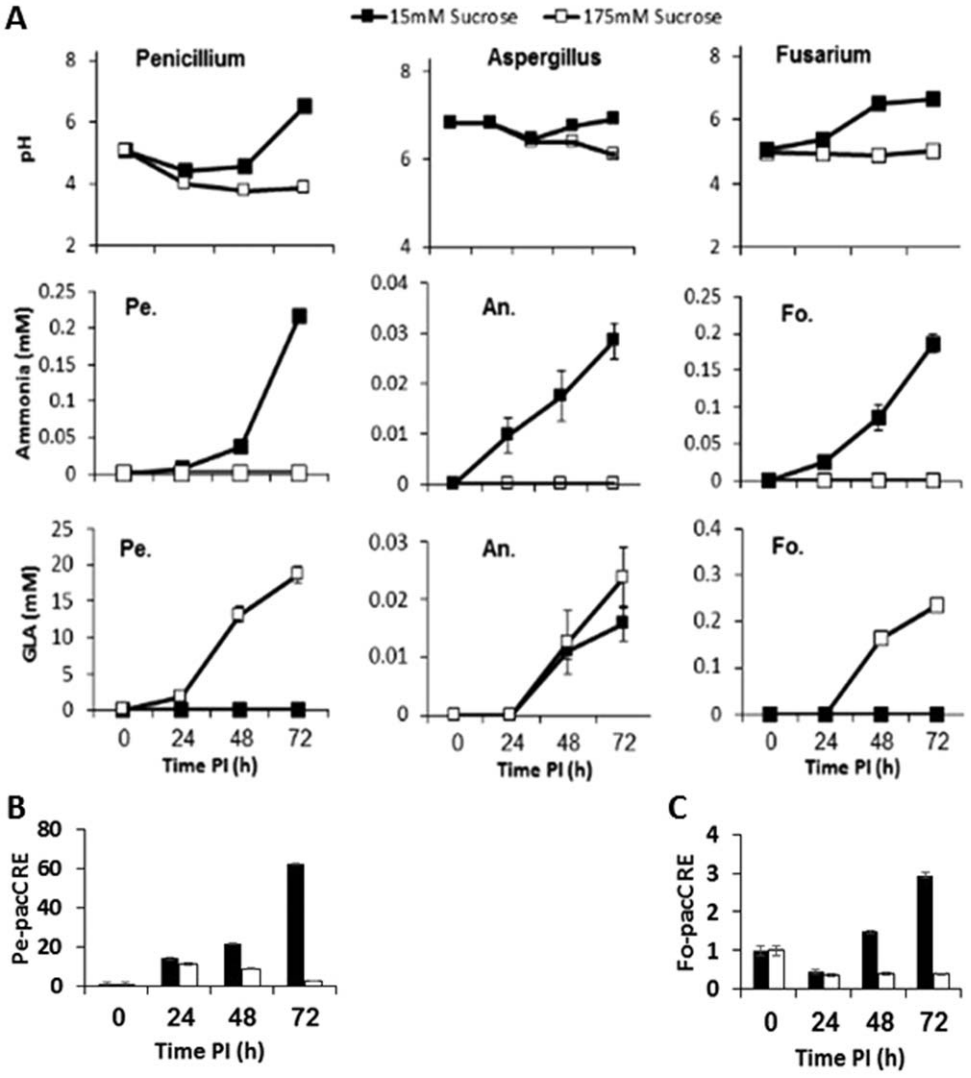

Fig. 4 Effect of carbon levels on the induction of medium alkalinization or acidification by *Penicillium expansum* (*Pe*), *Aspergillus nidulans* (*An*) and *Fusarium oxysporum* (*Fo*), and relative expression (RE) of *pacC*. (A) Fungal mycelia were grown in primary rich medium for 2–3 days and then transferred to secondary medium containing sucrose at 15 mM (■) or 175 mM (□), adjusted to pH 5, for 72 h. The effect of sucrose concentration was evaluated on pH modulation, and ammonia and gluconic acid (GLA) accumulation, for all three fungal species. Values represent means ± standard error (SE) of quadruplets. Experiments were repeated three times. (B, C) RE of *pacC* in *Pe* and *Fo*, respectively, during induction of alkalinization or acidification of the growth medium. RNA was extracted from mycelia at the indicated time points after transfer to inducing medium, and cDNA was used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Species-specific housekeeping genes (Table S3) were used to normalize the expression data for each fungus. Values represent means ± SE of duplicates. Experiments were repeated three times. PI, post-inoculation.

responsible for medium acidification, whereas the lower production in *F. oxysporum* and *A. nidulans* had only a modest effect on pH. These results indicate the importance of the balance between ammonia and organic acid accumulation for pH regulation of the environment during fungal growth (Figs 1 and 4). Thus, on a common background of carbon regulation and availability in all species, each showed similar qualitative, but very different quantitative, metabolic responses.

A pattern of increasing *pacC* expression, similar to that seen in *C. gloeosporioides*, was determined for *Fusarium* and *Penicillium*. During the alkalinization process in which ammonia accumulated, *pacC* was induced 2.94- and 62.1-fold in *Fusarium* and *Penicillium*, respectively (Fig. 4B,C). The results suggest that the ammonia/organic acid balance modulates the expression of *pacC* and PACC-induced genes.

The dynamic pH responses of *C. gloeosporioides* and *P. expansum*, known for their contrasting lifestyles, were compared under different carbon regimes (Fig. 5). *Penicillium* acidified the medium up to ambient concentrations of 50 mM sucrose (Fig. 5B), whereas, at the same concentration, *Colletotrichum* initiated

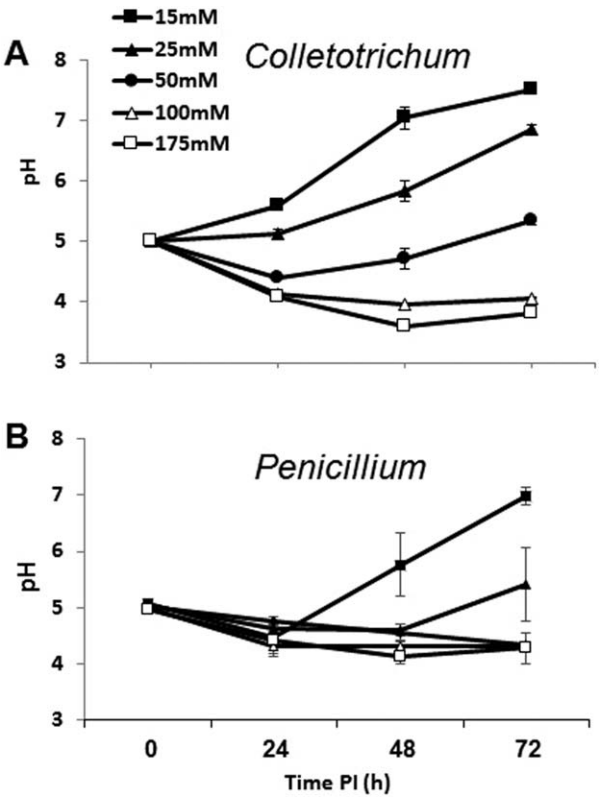

Fig. 5 Effects of carbon levels on the induction of medium alkalinization or acidification by *Colletotrichum gloeosporioides* (A) and *Penicillium expansum* (B). Fungal mycelia were grown in primary rich medium for 2-3 days and then transferred to secondary medium adjusted to pH 5 and containing 3 g/L tryptone amended with: 15 (■), 25 (▲), 50 (•), 100 (Δ) or 175 mM (□) sucrose. Values represent means ± standard error (SE) of quadruplets. Experiments were repeated three times. PI, post-inoculation.

moderate alkalinization (Fig. 5A). These results suggest that the balance between acidification and alkalinization patterns is the result of differential carbon utilization efficiencies in the respective pathogens.

Differential carbon regulation of gene expression and metabolites is effected by the negative carbon regulator *creA*

CCR, acting through the zinc finger repressor *creA*, is a global regulatory mechanism that ensures the utilization of preferred carbon sources. This mechanism operates by preventing the expression of the genes required for the metabolism of carbon sources other than glucose (Fernandez *et al.*, 2012, 2014). The mechanism of carbon regulation of *gox2* and *gdh2* under limiting and sufficient sucrose conditions was analysed by comparing their expression patterns with that of *creA* in *C. gloeosporioides* and *P. expansum*. *Colletotrichum gloeosporioides creA* shows 64% sequence identity and 73% sequence similarity to the *Magnaporthe oryzae* 70-15 *creA* (NCBI, XP_003714427.1), and 55% sequence identity and 67% sequence similarity to *creA* from *Aspergillus niger* (NCBI, XP_001399519.1) (Fig. S6, see Supporting Information). Similar to *creA* from other fungal species, the *C. gloeosporioides* strain Cg-14 *creA* has two zinc finger regions and a nuclear localization signal (Figs S6 and S7, see Supporting Information). Putative *creA* binding sites (SYGGRG; S, C/G; Y, C/T; R, A/G) were also identified in a 1500-bp upstream promoter region of *C. gloeosporioides* Cg-14 *gdh2* (one site) and *gox2* (four sites), suggesting a possible role for CREA in the regulatory process that controls the expression of these genes.

To determine the effect of carbon availability on gene regulation by *C. gloeosporioides*, the fungus was grown under increasing sucrose concentrations, from 15 to 175 mM. At the highest sucrose concentration, there was a 2.5-fold increase in *creA* expression paralleling a two-fold increase in *gox2* expression and a 10-fold decrease in *gdh2* expression (Fig. 6A–C). This indicated that glutamate metabolism is significantly restricted under excess carbon (Fig. 6C). A qualitatively similar trend emerged from the analysis of *creA* expression in *Penicillium* (Fig. 6D), although the relative changes were enhanced: 49-fold up-regulation of *gox2* expression and a reciprocal five-fold decline in *gdh2* expression (Fig. 6E,F). Thus, negative control of *gdh2* expression and positive control of *gox2* expression are correlated with *creA* expression. To functionally analyse the importance of *creA* during environmental acidification or alkalinization, *creA*-deletion mutants were sought in *C. gloeosporioides*. No *creA* knockout strains were identified in analyses of more than 300 transformants. The mutation is probably lethal, as has been reported for *F. oxysporum*, *Penicillium chrysogenum* and *M. oryzae* (Cepeda-Garcia *et al.*, 2014; Fernandez *et al.*, 2012; Jonkers and Rep, 2009).

The increase in *gox2* transcript in *Penicillium* paralleled the 400-fold increase in GLA accumulation and was much greater than that in *Colletotrichum* (compare Fig. 1 with Fig. 3). This highly specific response of *Penicillium* to *gox2* activation and GLA accumulation clearly suggests a different nutritional response by *Penicillium* vs. *Colletotrichum* (Fig. 6B,E). Although qualitatively similar, *Colletotrichum*'s predisposition for ammonia production appears to occur over a wider nutritional range than that of *Penicillium*, where mechanisms for acidification were more strongly expressed (compare Fig. 6B,C with 6E,F).

Carbon limitation during host colonization

To determine whether the fungal response to sugar concentrations when colonizing mature fruit follows a pattern similar to that described *in vitro*, a set of fruits with different sugar levels—avocado, tomato and plum—were inoculated with *C. gloeosporioides* (Table 1). Most of the imported sugars (glucose, fructose, sucrose and sorbitol) are known to accumulate in the vacuolar sink-tissue storage cells (Leigh *et al.*, 1979; Yamaki and Ino,

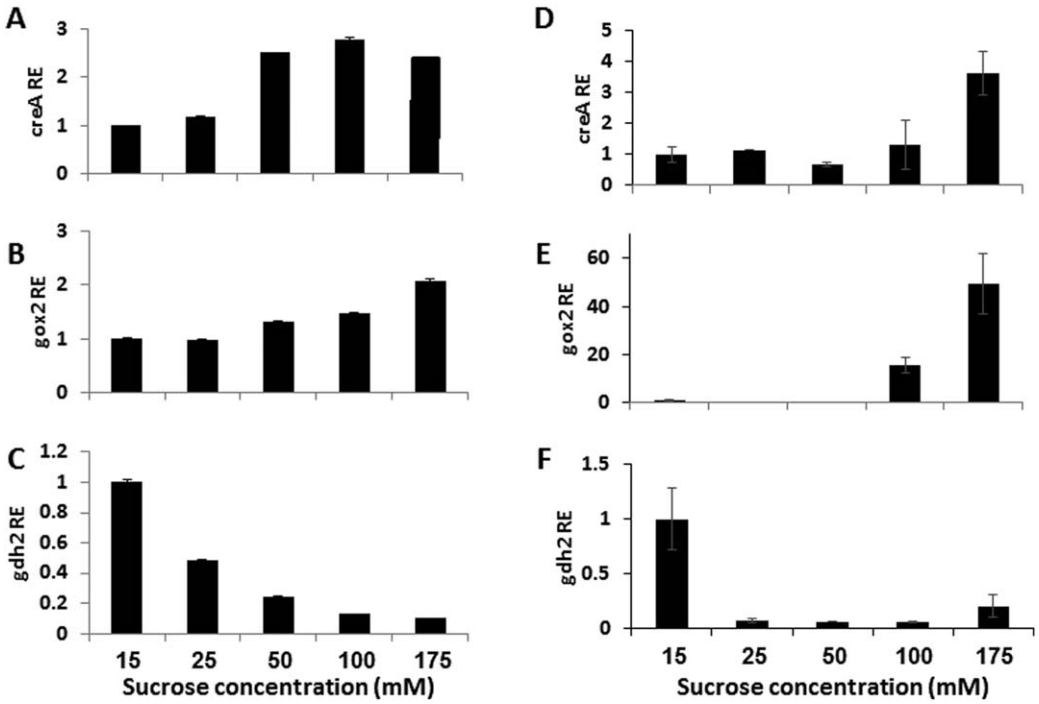

Fig. 6 Effects of carbon levels on relative expression (RE) of *creA*, *gox2* and *gdh2* in *Colletotrichum gloeosporioides* (A–C) and *Penicillium expansum* (D–F). Fungal mycelia were grown in primary rich medium for 3 days and then transferred to secondary medium containing increasing concentrations (15–175 mM) of sucrose and 3 g/L tryptone, adjusted to pH 5. RNA was extracted from mycelia at 48 h after transfer to inducing medium and cDNA was used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of c*reA*. Species-specific housekeeping genes (Table S3) were used to normalize the expression data for each fungus. Values represent means ± standard error (SE) of duplicates. Experiments were repeated three times.

1992). However, during fruit growth and maturation, the permeability of the tonoplast and plasma membrane to sucrose, glucose and fructose increases (John and Yamaki, 1994; Leigh *et al.*, 1979; Yamaki and Ino, 1992) to the point that, in older tomato fruit, the pericarp apoplasm is shown to be freely permeable to glucose movement (Ho, 1996). This significant release of sugar from the vacuole during fruit maturation enables fungal nutrient acquisition by the penetrating hyphae. Thus, avocado, tomato and plum were analysed to characterize the contribution of host carbon content to the modulation of host pH during colonization by *C. gloeosporioides* (Table 1). The fruit had relatively low concentrations of organic acids and different initial pH levels (Table 1). The differential modulation of GLA and/or ammonia accumulation was analysed by inoculating the fruit mesocarp with *C. gloeosporioides*. After inoculation of avocado and tomato fruit with relative low TSSs, pH increased (Fig. 7A,D) and ammonia accumulated (Fig. 7B,E). In contrast, inoculated plum fruit with high TSS showed a 2-unit decrease in pH, with no ammonia production, but the accumulation of a large amount of GLA (Fig. 7G–I). Importantly, less GLA was detected in the alkalinizing interactions in *C. gloeosporioides*-infected tomato or avocado (Fig. 7F and C, respectively), indicating that the balance of secreted ammonia and GLA is the major factor in host pH modulation.

To determine how carbon regulation modulates PACC-controlled gene expression, acid- and alkaline-expressed genes were analysed in plum fruit vs. tomato fruit (Fig. 7J). The PACC-regulated alkaline-expressed genes *pelB*, *nox4* and *cda*, and *pacC* itself, were markedly overexpressed when the fungus colonized tomato relative to plum fruit. In contrast, PACC-regulated acid-expressed genes encoding Ca^{2+} exchanger and endoglucanase b were overexpressed in plum (Fig. 7J). This suggests that fruit carbon content modulates *C. gloeosporioides* control of host environment pH in a manner that mimics growth in more defined synthetic medium (Fig. 1F).

To further determine the importance of host sugar content, we examined the colonization effects of *C. gloeosporioides* WT, Δ*gdh2* and Δ*gox* mutants on fruit. The Δ*gdh2* strain showed reduced alkalinization of the host (by 0.5 pH units), 72 h after inoculation, and reduced pathogenicity (by 70%) in tomato compared with the WT (Fig. 8A,B). The mutation did not affect the infection rate in plum, where the host was acidified from pH 5.0 to pH 3.8 during colonization (Fig. 8E,F). In contrast, the Δ*gox2* strain showed inhibited colonization and reduced acidification (by 0.8 pH units) in the high-sugar-content plums (Fig. 8G,H), but only a minor effect on pH and colonization in the low-sugar-content tomato fruit (Fig. 8C,D). This suggests that GDH2 and GOX2

Table 1 Physiological characterization of fruits prior to inoculation.

Parameter	Avocado cv. Fuerte	Tomato cv. Avigail	Plum cv. Santa Rosa
pH	6.03 ± 0.07	4.6 ± 0.04	5.4 ± 0.08
Total soluble solids (%)	7.2 ± 0.2	6.0 ± 0.25	14.3 ± 0.5
Total organic acids (%)	0.2 ± 0.03	0.85 ± 0.06	0.88 ± 0.3

activities play vital roles in the low- and high-sugar fruit, respectively, consistent with the differential gene activity noted in synthetic medium (Fig. 3A,B). The contribution of sucrose in the Δ*gdh2* background was obtained by adding 12% sucrose directly to the infected area the pathogencity of the Δ*gdh2* strain almost doubled (Fig. 8I), indicating that nitrogen and sugar make independent contributions to pathogenicity.

Next, host responses to infection with *Colletotrichum* and *Penicillium* were compared (Fig. 9). Although both pathogens acidified the host pH by 0.1–0.2 units in the high-sugar-content plum, their colonization of the lower-sugar-content tomato fruit induced increases in pH, suggesting that these pathogens are controlled by the limiting nutritional conditions present in these fruit. Therefore, fungal species increase or decrease host pH similarly.

DISCUSSION

Carbon regulation of pH modulation

The secretion of small organic molecules that modulate external pH serves to differentiate between organisms with acid- and alkaline-type colonization strategies in fruits and vegetables (Prusky *et al.*, 2013). Here, we show that the disparate physiological responses leading to environmental alkalinization or acidification of the medium or fruit are largely dictated by carbon availability. For example, the growth of *C. gloeosporioides* in the presence of 175 mM sucrose enhanced GLA accumulation and

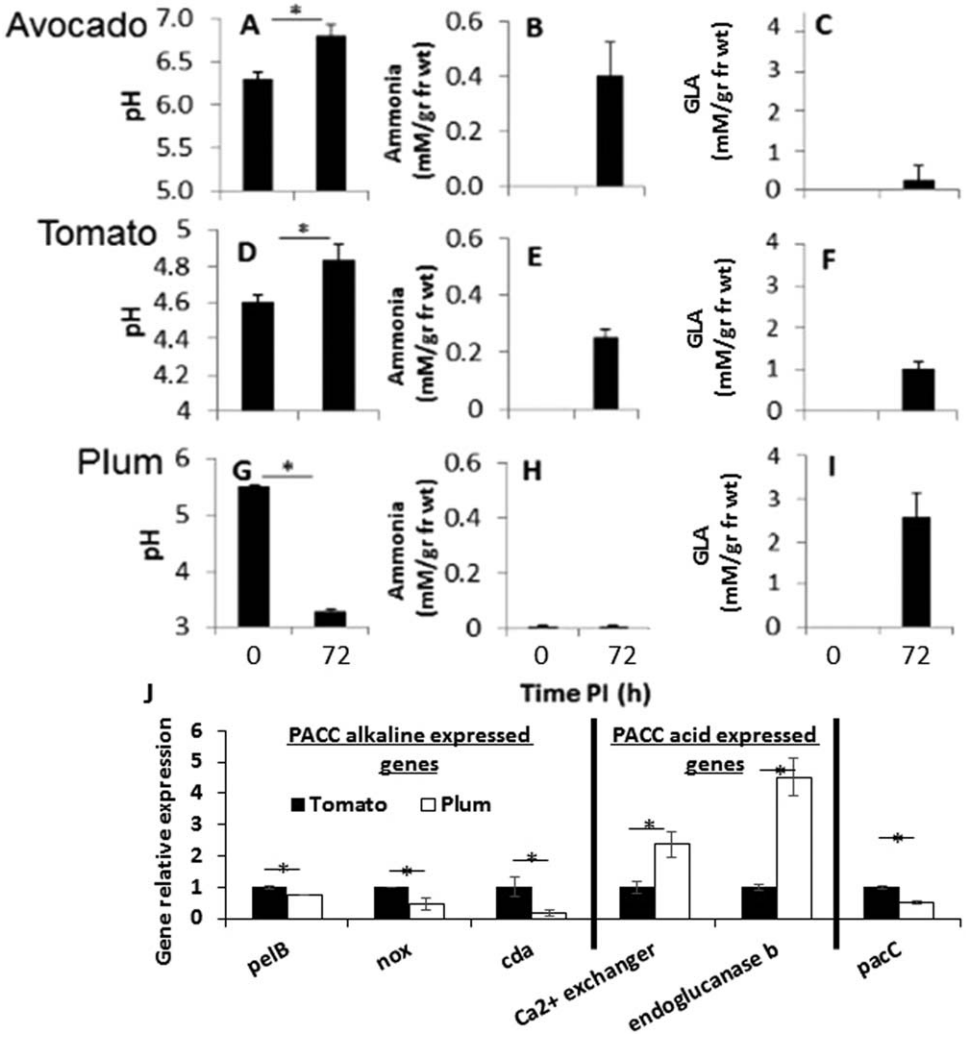

Fig. 7 Differential modulation of pH by *Colletotrichum gloeosporioides* on different fruits and effect on PACC-regulated genes. (A–C) avocado; (D–F) tomato; (G–I) plum; (J) PACC differentially regulated genes. Fruit pericarps were wound inoculated with a 10 μL suspension containing 10^6 spores/mL, and infected fruits were incubated at 25 °C, ~85% relative humidity (RH), for 3 days. Healthy and decayed tissues were sampled and used for the determination of pH, ammonia and gluconic acid (GLA). Values represent means ± standard error (SE) of four or five inoculations, each of eight fruits (*c.* 32 replications). Three days after fruit inoculation, RNA was extracted from the infected fruit tissues and the cDNA was used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The 18S gene was used to normalize the expression data. Experiments were repeated three times. $^*P < 0.05$ according to Student's *t*-test. PI, post-inoculation.

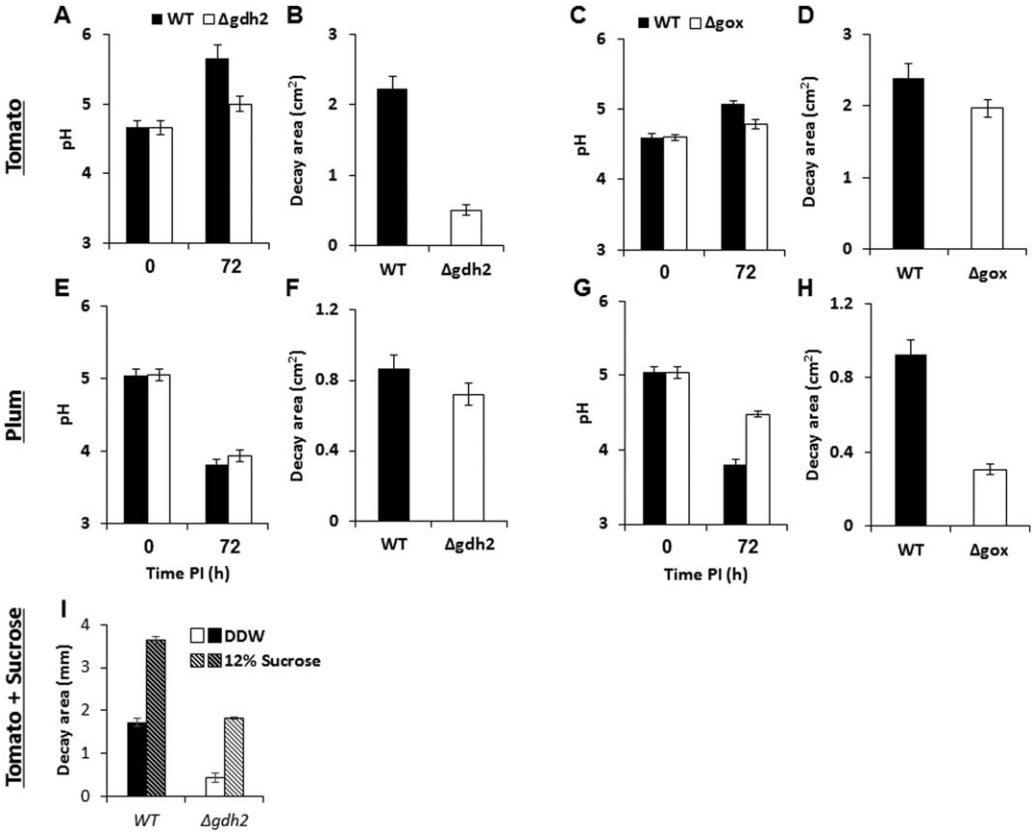

Fig. 8 Differential modulation of pathogencity and pH by *Colletotrichum gloeosporioides* wild-type (WT), Δ*gdh2* and Δ*gox2* strains on tomato (A–D, I) and plum (E–H). Fruits were wound inoculated with 10-μL suspensions of WT, Δ*gdh2* and Δ*gox2* strains containing 10^6 spores/mL. Infected fruits were incubated at 25 °C, ~85% relative humidity (RH), for 3 days. Inoculation of sucrose-treated tomato fruits with Δ*gox2* was carried out as described in Experimental procedures. The decay was examined after 3 days, and healthy and decayed tissues were sampled and subjected to pH analysis. Values represent means ± standard error (SE) of four or five inoculations, each of seven or eight fruits. The experiments were repeated three times. DDW, double distilled water; PI, post-inoculation.

decreased pH from 5 to 3.9, with no ammonia accumulation; under limited sucrose (15 mM), but similar nitrogen levels, ammonia accumulation was enhanced and pH was increased from 5 to 7.5. The differential response to carbon was retained in the presence of higher and lower (0.15%–1%) tryptone concentrations, and when the sucrose in the medium was substituted with glucose, but not fructose. These results demonstrate that key metabolizable carbon substrates present in fruit, and not nitrogen, are the regulatory molecules affecting growth medium pH.

Other phytopathogens, such as *P. expansum*, acidify the environment of their preferred host (Barad *et al.*, 2012). *Fusarium oxysporum* shows both acidifying and alkalinizing colonization strategies (Caracuel *et al.*, 2003), and *A. nidulans* produces no marked environmental change. Yet, all presented the same general pattern of regulation by carbon. Although similar in trend, there were striking differences in the levels of the secreted molecules and in the final pH, which are probably factors contributing to their overall pathogenicity on a particular host. Indeed, the accumulation of ammonia by *Colletotrichum* spp. during early biotrophic interactions under limited nitrogen conditions may lead to a quiescent infection (Alkan *et al.*, 2015; Prusky, 1996; Prusky *et al.*, 2013) in unripe fruit, whereas necrotrophic colonization by *Penicillium* occurs only in ripe fruit, in the presence of excess sucrose; in the latter case, the amount of GLA accumulated by *Penicillium* was 60 times higher than that by *Colletotrichum*, highlighting the different colonization patterns. Moreover, *Colletotrichum* and *Penicillium* accumulated similar amounts of ammonia, but exhibited different patterns of pH induction at increasing sugar content. These behavioural patterns suggest that the four fungal species described herein have similar sugar-controlled production of ammonia, but acid is modified by quantitative and qualitative differences, affecting the final pH.

How does the limiting balance between carbon and nitrogen nutrients affect fungal pathogenicity? Previous reports have indicated that nitrogen status serves as a regulatory switch for the activation of fungal infection development (Jurick *et al.*, 2012; Snoeijers *et al.*, 2000; Tavernier *et al.*, 2007). *In planta*, nitrogen availability seems to be limiting (Divon *et al.*, 2006), and several

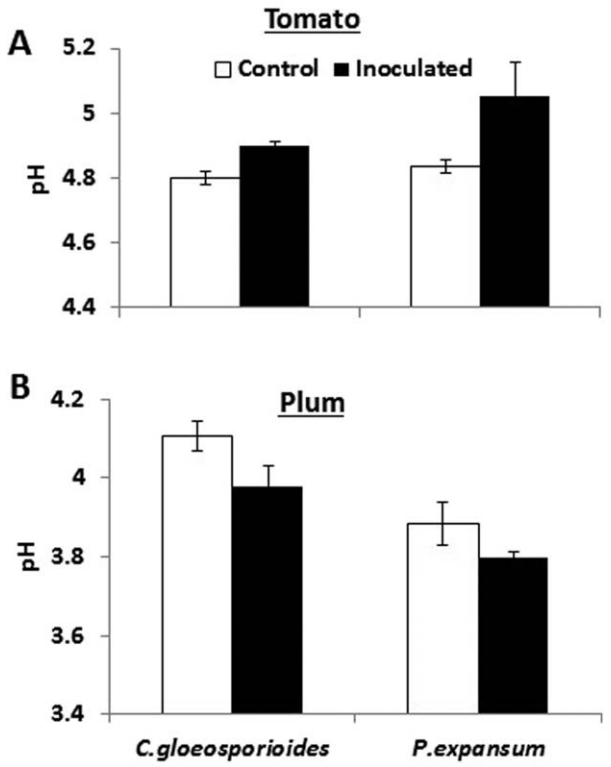

Fig. 9 pH modulation in plum and tomato by *Colletotrichum gloeosporioides* and *Penicillium expansum*. Fruits were wound inoculated with 10-μL suspensions containing 10^6 spores/mL, and infected fruits were incubated at 25 °C, ~85% relative humidity (RH), for 3 days. Healthy and decayed tissues were sampled and used for pH determination. Values represent means ± standard error (SE) of five inoculations, each of seven or eight fruits. The experiments were repeated three times.

fungal pathogenicity genes seem to be controlled by nitrogen starvation and may depend on nitrogen-responsive transcription factors (Talbot *et al.*, 1993; Thoma *et al.*, 2006). Nitrogen starvation has been widely used as a screen for the isolation of fungal genes that might be specifically induced *in planta* and/or involved in the pathogenicity of plant-pathogenic fungi. Thus, an avirulence gene (*avr9*), aldehyde dehydrogenase (*pSI-9*) and alcohol dehydrogenase (*pSI-10*) genes in the biotrophic fungal pathogen of tomato, *Cladosporium fulvum*, and the glutamine synthetase gene (*CgGS*) from *C. gloeosporioides*, all show induced expression under nitrogen—but not carbon—starvation (Coleman *et al.*, 1997; Stephenson *et al.*, 1997, 2000; Van den Ackerveken *et al.*, 1994). The *mpg1* gene of the rice blast hemibiotrophic fungus *Magnaporthe grisea*, encoding a hydrophobin-like protein involved in appressorium formation and necessary for pathogenicity, has also been shown to be expressed *in vitro* under carbon and nitrogen starvation (Talbot *et al.*, 1993). These results suggest that biotrophic and hemibiotrophic fungal pathogens encounter nitrogen-limiting conditions at the beginning of their infection process, and that nitrogen starvation constitutes one of the signals involved in the regulation of genes that are induced *in planta* (Snoeijers *et al.*, 2000).

Our study emphasizes the role of ambient carbon in determining the dynamic pH milieu of the pathogen in ripening fruit. Sugar availability in fruit (TSS) determines the optimal repertoire of pathogenicity factors at a particular pH, as determined in *C. gloeosporioides* (Fig. 1), where different pathogenicity genes for cell wall degradation were up-regulated at high pH, whereas other members of the same family were induced at low pH (Alkan *et al.*, 2013b).

Carbon regulation of *gdh2* and *gox2*

Carbon regulation modulates alkalinization or acidification of the host environment by regulating central metabolic pathways. *gdh2* and g*ox2* were shown here to be differentially regulated by carbon availability (Hadas *et al.*, 2007; Miyara *et al.*, 2008). In *Saccharomyces cerevisiae* (Coschigano *et al.*, 1991), carrot cell suspension (Robinson *et al.*, 1992) and glioblastoma cells (Yang *et al.*, 2009), carbon depletion restricted protein synthesis as a result of a lack of carbon skeletons. At the same time, catabolic oxidation of glutamate by *gdh2* was activated to provide carbon backbones for tricarboxylic acid cycle activity (Coschigano *et al.*, 1991; Yang *et al.*, 2009). Such metabolic changes have significant effects on fungal systems, in which nutritional factors may determine fungal development and colonization. The 2-oxoglutarate product of glutamate oxidation is preferentially metabolized through the tricarboxylic acid cycle, whereas the produced ammonium is either sequestered within the cell or excreted. Secretion of ammonia was shown here to occur in the presence of limited levels of key fruit sugars, i.e. sucrose, glucose and fructose (Fig. S2), and in a carbon-regulated manner among diverse fungi: *Colletotrichum*, *Penicillium*, *Aspergillus* and *Fusarium* (Figs (1 and 3) and S2).

Three GOXs with high homology to previously reported *gox* genes were noted in the *C. gloeosporioides* transcriptome (Alkan *et al.*, 2013b) (Figs S3 and S4). Whereas *gox1* and *gox3* showed mainly constitutive expression patterns, *gox2* expression was closely related to GLA accumulation. Furthermore, in the light of the signal peptide in GOX2 (cleavage site between positions 17 and 18, ALA-YP), GOX is considered to be extracellular and its catalytic product accumulates in culture (Barad *et al.*, 2012). The activation of *gox2* and GLA accumulation in the presence of high concentrations of sucrose has been reported in *P. expansum* (Barad *et al.*, 2012), and in *A. nidulans* (Whittington *et al.*, 1990) in the presence of glucose and sucrose, given the ability of both substrates to activate GOX activity. However, as shown here, the growth of *C. gloeosporioides* and other fungal species—namely *P. expansum*, *A. nidulans* and *F. oxysporum*—in culture medium at pH 5 containing 175 mM sucrose also enhanced GLA

accumulation. GLA, however, was not induced in the presence of fructose, which is not a substrate for GOX activities, suggesting that only metabolizable fruit sugars can induce GLA.

The modulation of *gdh2* and *gox2* activity was not dictated by pH, but by sugar concentration. This result indicates that the regulation of PACC-dependent genes, dictated by changes in environmental pH (Alkan *et al.*, 2013a; Barad *et al.*, 2012; Miyara *et al.*, 2010), occurs downstream of these nutritional responses. In the *C. gloeosporioides* Δ*gdh2* mutant growing under carbon-limiting conditions, ammonia accumulation and subsequent pH increase were reduced by 29%. The Δ*gox2* mutant growing under carbon excess showed a 90% reduction in GLA and subsequent decrease in pH modulation, indicating the critical importance of *gdh2* and *gox2* activities in initiating the PACC-regulated processes. The regulation of carbon and nitrogen metabolism has been extensively studied in the model filamentous saprobes *Neurospora crassa* (Marzluf, 1997) and *A. nidulans* (Arst and Cove, 1973; Bailey and Arst, 1975; Caddick *et al.*, 1994; Dowzer and Kelly, 1991; Mathieu and Felenbok, 1994; Wilson and Arst, 1998), but less so under the colonization conditions of fruit-pathogenic fungi. In *A. nidulans*, a GATA family transcription factor, AREA, functions in global nitrogen metabolite repression to enable the utilization of the most preferred nitrogen sources (Mathieu and Felenbok, 1994; Wilson and Arst, 1998). CCR, operating via the negatively acting zinc finger repressor CREA (Dowzer and Kelly, 1991; Mathieu and Felenbok, 1994), ensures the preferential utilization of glucose by preventing the expression of the genes required for the metabolism of carbon sources other than glucose (Fernandez *et al.*, 2014). In the work carried out here, nitrogen was maintained at a constant, non-limiting level, and changes in that level (0.15%–1%) did not affect the centrality of carbon regulation.

A number of global regulators of carbon metabolism have been characterized in fungi (Fernandez *et al.*, 2014). CCR has also been shown to be involved in the production of isocitrate lyase and cell wall-degrading enzymes in the tomato pathogen *F. oxysporum* (Jonkers and Rep, 2009). In that case, replacement of *CRE1* in *F. oxysporum* with a glutathione S-transferase (GST) fusion protein led to the de-repression of glucose-repressed transcript expression of endo-1,4-β-xylanase I (Jonkers and Rep, 2009; Katoh *et al.*, 2007). Overexpression of *creA* in the citrus fruit pathogen *Alternaria citri* resulted in its severe virulence under high-sugar conditions in citrus fruit (Katoh *et al.*, 2007). In *C. gloeosporioides* and *P. expansum*, the highest induction of *creA* transcript was observed at increasing sucrose concentrations. Under these conditions, increased pathogenicity was also observed for GLA-producing pathogens. If *creA* regulation in pathogenic fungi is similar to that found in *A. nidulans*, excess sugar will induce the oxidation of glucose to GLA whilst repressing the expression of *gdh2* to lower pH. In contrast, under the *creA*-limiting conditions observed in the presence of low sugar levels, CCR control will activate *gdh2* and increase pH. These findings suggest that environmental pH may be regulated in *Colletotrichum* via a dual CCR/nitrogen metabolite repression control that enables dynamic regulation in the background of changing sugar levels during fruit ripening.

The importance of carbon in pathogenicity

In the present work, observations of growth on defined media were extended to fruit. The effects of nitrogen and carbon on disease severity in fruit are varied because of the multiplicity of pathogen strategies, each involving different metabolic requirements and various ways of acquiring nutrients (Snoeijers *et al.*, 2000). Biotrophic and hemibiotrophic pathogens must interact with changing metabolite pools in the host cell (Hoffland *et al.*, 2000; Jensen and Munk, 1997). For example, during the quiescent biotrophic phase, when *C. gloeosporioides* has penetrated the unripe fruit, the availability of metabolic carbon pools is low (Prusky *et al.*, 2013). Alkan *et al.* (2015) recently reported an increase in the specific activities of enzymes directly related to ammonia production, including Cgl-GDH2 (*Cgl*_00001112). This was accompanied by significant transient alkalinization of the tissue surrounding the quiescent fungal structures, probably caused by secreted ammonia. In the light of the insights acquired here, these results are indicative of changes in carbon availability during germination and early stages of quiescence (Miyara *et al.*, 2008, 2012).

However, fungal colonization includes significant necrotrophic processes, in which sugars are released to the colonized tissue. Although most of the sugar is dumped during the assimilation process in the vacuole and may reach up to 30% of the fruit's nutritional content, cell membranes become significantly more permeable to sugars during maturation, until the necrotrophic stage, when the sugar may be released from the necrotic cells (Yamaki and Ino, 1992). It is clear that not all of the sugar detected by the TSS analysis (i.e. 9%–30%) is available for fungal growth. It is expected, however, that the concentrations of sugar released during fruit maturation and cell necrotization will be similar or higher than those tested *in vitro* in the present experiments. Our results showed ammonia accumulation and enhanced alkalinization during colonization and pathogenicity on tomato and avocado fruit, whose total sugar contents reached 6% and 7%, respectively. However, plum fruit, with a TSS of at least 14%, was devoid of ammonia accumulation, whereas GLA accumulation was two and five times higher, respectively, than in inoculated tomato and avocado fruit. This suggests that, during host colonization, the balance between ammonia and GLA accumulation also determines the final pH of the host environment. Importantly, the critical contribution of ammonia and GLA accumulation under low or high carbon was confirmed when fruit were inoculated with *Colletotrichum* Δ*gdh2* and Δ*gox2* mutant strains. The Δ*gdh2* mutant showed reduced colonization of low-sugar-containing

tomato fruit, but unaffected colonization of high-sugar-content plum fruit. The reduced growth was not caused by specific limitations of tomato fruit, as the addition of sucrose enhanced pathogenicity (Fig. 9l). Hence, sucrose treatments enhance colonization by strains with reduced nitrogen utilization. Thus, in analogy with growth on the high-sugar-containing plum, the need for *gdh2* activity becomes less relevant after the addition of sucrose.

The present results show that hosts with high or low sugar contents (plum relative to tomato) elicit fine-tuned changes in fungal species during colonization. The tuning does not necessarily mean that the nature of the activities will differ, but rather that the expressed activities will be well adapted to the prevailing pH following fungal host penetration. Hence, in nature, the predominant pathogens found on a particular fruit are adapted to their host niche. However, pathogens have retained at least residual general ability to grow on less favoured carbon sources, indicating an evolutionary pressure to be maintained as a generalist. Pathogens have developed efficient and rapid response systems to the fluctuating nutritional conditions occurring during fruit ripening in the preferred host and in serendipitous host encounters.

EXPERIMENTAL PROCEDURES

Fungal isolates, media and growth conditions

Colletotrichum gloeosporioides strain Cg-14 was obtained from decayed avocado fruit (*Persea americana* 'Fuerte') in Israel and was routinely cultured on primary rich agar medium (M_3S) agar (Tu, 1985). The *P. expansum* WT isolate Pe-21 was obtained from decayed apple fruit (*Malus domestica* 'Golden Delicious') purchased from a local market in Israel (Hadas *et al.*, 2007). Both strains originated from single-spore cultures (Alkan *et al.*, 2015; Hadas *et al.*, 2007). *Fusarium oxysporum* f. sp. *phaseoli* (*F. oxysporum* Schlechtend:Fr f. sp. *phaseoli* J.B. Kendrick and W.C. Kendrick) strain FOP-SP4 was obtained from the roots and stems of infected bean plants (*Phaseolus vulgaris*) collected in El Barco de Ávila (Spain) and originally described in previous studies (Alves-Santos *et al.*, 1999, 2002). *Aspergillus nidulans* was supplied by Eduardo Espeso, Spain, and grown under standard growth conditions for this organism (Espeso and Penalva, 1992).

Primary rich medium (M_3S) was used for the initial growth of *C. gloeosporioides* and *F. oxysporum* prior to their exposure to secondary medium containing (per litre): 2.5 g $MgSO_4.7H_2O$ (Merck), 2.7 g KH_2PO_4 (Merck, Germany), 1 g Bacto peptone (Becton Dickinson, MD, USA), 1 g Bacto yeast extract (Becton Dickinson, MD, USA) and 10 g sucrose (Duchefa Biochemie, Haarlem, Nederlands) (Miyara *et al.*, 2010). *Penicillium expansum* was grown in a different primary rich medium that contained (per litre): 10 g sucrose, 5 g yeast extract (Difco, MD, USA), 50 mL nitrate salts and 1 mL trace elements, at pH 4.5.

Penicillium expansum, *F. oxysporum* and *C. gloeosporioides* spores were inoculated at 10^6 spores/mL in 40 mL of primary rich medium in 125-mL flasks and grown for 2–3 days at 22–24 °C in a shaking incubator at 150 rpm; they were then harvested by filtration through a sterile Büchner funnel fitted with filter paper. The hyphal mat was washed twice with 40 mL of sterile distilled water, and the washed mycelia were resuspended in 40 mL of fresh secondary medium containing (per litre): 7 g $NaNO_3$, 3 g tryptone (Difco), 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$ and 0.5 g KCl. Sucrose was added to the secondary medium, as described for each experiment, at concentrations of 15–175 mM, without changing the other components of the medium unless otherwise stated. The secondary medium pH was adjusted to pH 5 with dilute HCl unless otherwise specified. The identical secondary medium amended with different fruit sugars—glucose, fructose or sucrose—was used to determine the growth responses of *C. gloeosporioides* under limited and excess sugar conditions.

Fruit sugar content evaluation and pathogenicity

The TSS index was used to evaluate sugar content, which included carbohydrates, organic acids, proteins, fats and minerals in the fruit. TSS represents 10%–20% of the fruit's fresh weight and increases as the fruit matures to produce a less acidic, sweeter product (Young *et al.*, 1993). TSSs were measured in fruit juice using a refractometer (Young *et al.*, 1993). Sugar concentration may range between 9% and 30% of the fresh weight, with sucrose, glucose, fructose and sorbitol being the predominant sugars in fruit (Coombe, 1976).

The fruit used for inoculation were ripe tomatoes (*Solanum lycopersicum* cv. Avigail), ripe plums (*Prunus domestica* cv. Santa Rosa) and ripe avocados (*Persea americana* cv. Fuerte). Spores of *C. gloeosporioides* strains were wound inoculated on the fruit pericarps by placing 10 μL of conidial suspension (10^6 conidia/mL) into inoculation spots (1 mm in diameter and 1 mm deep, using a 20-μL tip; the depth was determined by placing a rubber stopper at 1 mm from the end of the tip) on tomatoes and plums. Avocados were inoculated on the mesocarp after a 1–2-cm strip of peel tissue had been removed aseptically from the longitudinal axis of the fruit; 7 μL of conidial suspension (10^6 conidia/mL) were placed on top of 5-mm sterile filter paper discs, which were then placed on the fruit mesocarp, on three or four inoculation spots, longitudinally spaced on each side of the fruit. The fruits were incubated at 25 °C, 85%–95% relative humidity (RH), in a tightly covered plastic container containing wet paper towels for 4 days, at which point symptoms became visible. In all experiments, the average ± standard error (SE) of the decay area, pH changes and ammonia accumulation in healthy and decayed tissues are reported. The inoculation experiments were repeated three times with 10–12 fruits each time, and the results of one representative experiment are presented.

To determine the effect of excess sucrose on pathogencity, the inoculation points of the WT and Δ*gdh2* strains on fresh tomato fruits (*Lycopersicon esculentum* cv. Tory) were treated with a 12% sucrose solution, as described by Alkan *et al.* (2008). Fruit inoculation was carried out on the mesocarp. A 1-mm thick, 10-mm-diameter section of the peel pericarp tissue was removed from the fruit and 30 μL of conidial suspension (10^6 conidia/mL) were placed on the exposed tomato tissue. Following inoculation, the fruits were incubated at 22 °C and 95% RH in covered plastic containers containing wet paper towels until symptoms were evident. Sucrose and water treatments (30 μL) were initiated 15 h after inoculation and were applied twice a day. The diameter of decay development on the pericarp of the fruit, starting from the edge of the peeled mesocarp, was measured 48–72 h after inoculation and reported in millimetres over the 10-mm diameter of the initial removed tissue. Sixteen tomato fruit were inoculated with *C. gloeosporioides* WT and Δ*gdh2* strains (four

inoculations on each fruit: two WT and two Δ*gdh2*, alternately), and were supplemented with 30 μL of doubled distilled water (DDW) or 12% sucrose solution twice a day.

pH measurement, ammonia and GLA detection

Culture medium pH was measured with a Thermo-Orion Model 9810BN microcombination pH electrode (Thermo Fisher Scientific, Waltham, MA USA) in 0.5-mL aliquots collected at various times after fungal inoculation. The pH in the fruit was measured by sampling the tissue with a sterile scalpel and grinding in a sterile grinder. The ground tissue was centrifuged for 5 min at 10 621 ***g***. The supernatant was used for pH measurement, and ammonia and GLA determination. Ammonia was detected calorimetrically with an ammonium test kit (Merck), and its concentration was determined in 5 mL of 10-fold-diluted culture or fruit tissue supernatant, according to the manufacturer's instructions. The kit for the measurement of total GLA content (free GLA and D-glucono-δ-lactone) (Boehringer Mannheim, Germany) was used according to the manufacturer's instructions.

Sample preparation and analysis of organic acids by gas chromatography-mass spectrometry (GC–MS)

In the first stages of the evaluation of the culture filtrates, organic acids were identified using GC–MS (Table S1). Aliquots (1 mL) of acid-induced culture medium in which fungus was grown at pH 7 were taken at 48 h post-inoculation and evaporated to dryness in a vacuum concentrator (Test Tube Concentrator; Taitec, Saitama, Japan). The final dry residue was dissolved in 110 μL of a 10 : 1 (v/v) mixture of *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (Sigma, Rehovot, Israel). The solution was transferred to a 1-mL screw-cap vial which was tightly closed, and trimethylsilyl derivatization was performed at 25 °C for 30 min. Organic acid standards were prepared by the same derivatization methods.

Following the initial identification of GLA, by GC–MS, as the main organic acid in the filtrate samples, they were analysed in a model GC 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA. USA) coupled with a model 5975C mass spectrometer (Agilent Technologies) with a 30-m HP-5MS (5% phenyl-methylpolysiloxane) column (250 mM; Agilent Technologies); a film thickness of 0.25 μM was used to analyse the samples. An MSD Chemstation (Rev E02.00) (Agilent Technologies) was used for instrument control, data acquisition and processing. Metabolites were qualitatively identified by cross-validation against three standard GC–MS reference libraries—Wiley, NIST I and NIST EPA/NIH. Compounds were identified by comparison of retention times and mass spectra with WILEY 6 standard MS spectra databases and with the spectra of the standards. Data were reported as log (mg/mL) for each detected compound.

The standards were gluconic, citric, fumaric, malic and oxalic acids (Sigma). The absolute concentrations of the organic acids were determined by comparison with a standard calibration curve plotted from various concentrations of the standard solutions, which were derivatized concomitantly with the culture medium samples.

Total RNA extraction

Mycelia (80–100 mg dry weight) of *C. gloeosporioides* (WT or mutants), *P. expansum*, *F. oxysporum* and *A. nidulans* were ground to a fine powder and RNA was extracted with the SV-total RNA isolation system (Promega, Madison, WI, USA). Total RNA from inoculated tomato fruit mesocarp was extracted according to Yang *et al.* (2008), with minor changes. About 1–2 g of sample tissue was taken from pools obtained from four different fruits, ground to a fine powder in liquid nitrogen and transferred to 50-mL centrifuge tubes, together with 10 mL of cetyltrimethylammonium bromide (CTAB) RNA extraction buffer comprising 100 mM Tris–borate, pH 8, 2 M NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), pH 8, 2% (w/v) CTAB, 2% (w/v) polyvinylpolypyrrolidone (PVPP) and 2% (v/v) β-mercaptoethanol. The mixture was shaken for 3 min and then incubated at 65 °C for 15 min. Samples were extracted twice with an equal volume of 24 : 1 (v/v) chloroform–isoamyl alcohol, and the phases were separated by centrifugation at 10 000 ***g*** for 10 min. Lithium chloride was added to a final concentration of 2.5 M and RNA was precipitated overnight at 4 °C. RNA was pelleted at 4 °C for 30 min at 10 000 ***g***, washed with 70% ethanol and resuspended in SSTE buffer [10 mM Tris, pH 8, 1 M NaCl, 1 mM EDTA, pH 8, and 0.5% w/v sodium dodecylsulfate (SDS)] at 65 °C for 3 min. Samples were extracted with an equal volume of 24 : 1 (v/v) chloroform–isoamyl alcohol, and an equal volume of 24 : 1 : 25 chloroform–isoamyl alcohol–water-saturated phenol, and the phases were separated by centrifugation at 10 000 ***g***. The RNA was ethanol-precipitated overnight, suspended in diethyl pyrocarbonate-treated water and lastly treated with DNase (Turbo DNase, Ambion,Austin,TX, USA).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Sequences of *gdh2* (GenBank accession number EU182718) and *gox2* (TCONS_00011543) were obtained from GenBank. Single-stranded cDNA was synthesized from 1 μg of total RNA with the Verso cDNA synthesis kit (Thermo Fisher Scientific). The synthesized cDNA was used as a template for qRT-PCR to estimate the expression levels of the selected genes. The cDNA samples were diluted 1 : 10 (v/v) to the final template concentration for qRT-PCR. Real-time qRT-PCR was performed with a StepOne real-time PCR system (Applied Biosystems, Grand Island, NY, USA). PCR amplification was conducted with 3.4 μL of cDNA template in 10 μL of reaction mixture containing 5 μL of SYBR Green Amplification Kit (Abgene, Epsom, UK) and 300 nM primers. The PCR used 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 primer pairs, and all products showed the expected size of 80–120 bp. The results were analysed with StepOnePlus software v.2.2.2 (Applied Biosystems). Relative quantification was calculated by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). All samples were normalized to the 18S gene levels in the same qRT-PCR, and the values were expressed as increases or decreases in level relative to a calibration sample. The forward and reverse primers for all of the genes are listed in Table S3 (see Supporting Information). Each treatment comprised three biological samples and two technical replicates.

Colletotrichum gloeosporioides gox2 gene disruption

The knockout construct was generated by amplification of 556 bp of the 5′ and 494 bp of the 3′ flanking fragments using conventional PCR of the coding region of *C. gloeosporioides gox2* from the full gene deposited in GenBank (Submission ID: 1815308). For *gox2*, primer sets

attBgox2_5′F + attBgox2_5′R and attBgox2_3′F + attBgox2_3′R were used to amplify the 5′ and 3′ fragments, respectively (Table S3) in a PCR that included 30 ng of genomic DNA, 10 pmol of oligonucleotide and PCR Ready Mix (Thermo Scientific, USA). 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 *Not*I to release the deletion construct. Electroporation of germinating conidia was performed essentially as described previously (Yakoby *et al.*, 2001). Briefly, Cg-14 isolates were cultured on solid M_3S medium for 14 days. Conidia were collected in pea juice (Robinson and Sharon, 1999), 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), concentrated to 10^8 conidia/mL, and 100-μL aliquots were distributed in cold electroporation cuvettes (Bio-Rad, California, USA). Electroporation and transfer to regeneration medium were performed according to Shafran *et al.* (2008). Transformant colonies appeared 3–4 days after electroporation. The transformants were re-grown as single-spore colonies on M_3S agar with hygromycin B at 100 mg/L, and DNA was extracted with the Master Pure Yeast Purification Kit (Epicentre Biotechnologies, Madison, WI , USA). Two methods were used to confirm deletion of *gox2*. First, specific primers were designed outside the cassette region (gox2_5′ctrl _F and gox2_3′ctrl_R). The gox2_5′ctrl _F + Hyg_5′R primers were used to check the correct insertion of the 5′ (end), and hygromycin and the Hyg_3′F + gox2_3′ctrl_R primers were used for correct insertion of the hygromycin and the 3′ (end). The set of primers attbGOX2_5′F + Hyg_5′R and Hyg_3′F + attbGOX2_3′R were used to verify the ectopic transformants. In the case of homologous integration, the mutants showed the correct PCR fragments of 696 bp and 630 bp from the 5′ and 3′ ends, respectively (Fig. S5), which were sequenced for verification. Second, a single-spore colony was used to inoculate the liquid media as described earlier to verify the level of inhibition of *gox2* expression in the mutants compared with the WT and ectopic strains.

Statistical analysis

Statistically significant differences between qRT-PCR results, decay area, pH changes and ammonia and GLA secretion at a single time point were analysed by Student's *t*-test at the 0.95 confidence interval. At multiple time points, qRT-PCR results, pH changes and ammonia and GLA secretion were analysed by two-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$) and 0.95 confidence interval. Analysis was conducted by JMP software (SAS Institute, NC USA).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Effect of tryptone concentration on the differential alkalinization or acidification of the medium by *Colletotrichum gloeosporioides*, induced by different carbon levels. Fungal spores were inoculated in primary rich medium for 3 days and then transferred to secondary medium containing sucrose at 15 mM (■▲) or 175 mM (□Δ) in the presence of tryptone at 3 g/L (■□) or 10 g/L (▲Δ) in the medium, adjusted to pH 5. Values represent means ± standard error (SE) of quadruplets. The experiments were repeated three times. PI, post-inoculation.

Fig. S2 Effect of sucrose, glucose and fructose on differential pH modulation, and ammonia and gluconic acid (GLA) accumulation in the culture medium by *Colletotrichum gloeosporioides*. Fungal spores were inoculated in primary rich medium for 3 days and then transferred to secondary medium containing sucrose, glucose or fructose at 15 or 175 mM adjusted to pH 5 for 48 h. Values represent means ± standard error (SE) of quadruplets. The experiments were repeated twice.

Fig. S3 Phylogenetic analysis of glucose oxidase (GOX) proteins in various filamentous fungi. Based on sequence alignment and bootstrap analysis, the phylogenetic tree was generated using the neighbour-joining method of MEGA software, version 4.0. The examined fungal strains were as follows: *Colletotrichum gloeosporioides* Cg-14 GOX1 (*Cg*-14 GOX1; NCBI EQB52290.1; TCONS_00008235); *C. gloeosporioides* Cg-14 GOX2 (*Cg*-14 GOX2; TCONS_00011543); *C. gloeosporioides* Cg-23 (*Cg*-23 GOX; jgi|Gloci1|1716272|gm1.259_g); *C. gloeosporioides* Nara gc5 GOX (*Cg*-Nara gc5 GOX; XP_007275789); *Botrytis cinerea* (B05.10_vankan) GOX (*Bc* GOX; BROAD, B0510_9807); *Sclerotinia sclerotiorum* GOX (*Ss* GOX; SS1G_14293); *Magnaporthe oryzae* Mo-15 GOX (*Mo* GOX; XP_003711497.1); *Talaromyces variabilis* GOX (*Tv* GOX; CAE47418.1); *Penicillium expansum* GOX1 (*Pe* GOX1; AAT76526.2); *P. expansum* GOX2 (*Pe* GOX2; AAZ82018.1); *Aspergillus niger* GOX (*An* GOX; ACR56326.1).

Fig. S4 CLUSTAL omega alignment of glucose oxidase (GOX) proteins from various filamentous fungi. The shaded consensus amino acids showed a 60% identity threshold. The following fungal proteins were examined: *Colletotrichum gloeosporioides* Cg-14 GOX1 (*Cg*-14 GOX1; NCBI EQB52290.1; TCONS_00008235); *C. gloeosporioides* Cg-14 GOX2 (*Cg*-14 GOX2; TCONS_00011543); *C. gloeosporioides* Cg-14 GOX3 (*Cg*-14 GOX3; TCONS_00016375); *C. gloeosporioides* Cg-23 (*Cg*-23 GOX; jgi|Gloci1|1716272|gm1.259_g); *C. gloeosporioides* Nara gc5 GOX (*Cg*-Nara gc5 GOX; XP_007275789); *Botrytis cinerea* (B05.10_vankan) GOX (*Bc* GOX; BROAD, B0510_9807); *Sclerotinia sclerotiorum* GOX (*Ss* GOX; SS1G_14293); *Magnaporthe oryzae* Mo-15 GOX (*Mo* GOX; XP_003711497.1); *Talaromyces variabilis* GOX (*Tv* GOX; CAE47418.1); *Penicillium expansum* GOX1 (*Pe* GOX1; AAT76526.2); *P. expansum* GOX2 (*Pe* GOX2; AAZ82018.1); *Aspergillus niger* GOX (*An* GOX; ACR56326.1).

Fig. S5 Development of *gox2*-deletion mutants and relative expression (RE) of *gox2* and *gox1* in *Colletrotrichum gloeosporioides*. A double-cross-over homologous recombination event resulting in replacement of the original gox2 sequence with the gox2-5′-Hyg-gox2-3′ cassette was performed as described in Experimental procedures. (A) Scheme describing gene disruption by homologous recombination. The pairs of primers used to create the construct were attBgox2_5′F–attBgox2_5′R (located 356 bp before and 282 bp after ATG, respectively) for the 5′ end and attBgox2_3′F–attBgox2_3′R (located 230 bp before and 264 bp after TGA, respectively) for the 3′ end (Table S3). (B) Polymerase chain reaction (PCR) analysis of the wild-type (WT) strain, ectopic colony (Ectopic) and independent gox2-disrupted colony (Δgox2). gox2_5′ctrl_F (Table S3), flanking a position upstream of the gox2:HYG3 region, and reverse primer Hyg_5′R (Table S3), located on the hygromycin cassette, were used to identify positive *gox2* gene replacement at the 5′ locus. Hyg_3′F (Table S3), from the hygromycin cassette, and gox2_3′ctrl_R (Table S3), flanking the gox2:HYG3 region, were used to identify *gox2* gene replacement at the 3′ locus. gox2 attB primers for the 5′ and 3′ ends (Table S3) were used for WT DNA quality control. attBgox2_5′F–Hyg_5′R primers (Table S3) were used as a positive control for the ectopic strains, to confirm random integration of the 5′-gox2:HYG3 cassette. Hyg_3′F–attBgox2_3′R primers (Table S3) were used as a positive control for the ectopic strains, to confirm random integration of the 3′-gox2:HYG3 cassette. Expression of (C) *gox2* and (D) *gox1* in the WT strain compared with the Δ*gox2* and ectopic-integration strains, as detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The identity and similarity between sequences of *gox2* and *gox1* are

presented in Table S2. Cultures were initially grown in primary rich medium (M_3S) and then transferred to medium containing (per litre): 10 g sucrose, 5 g yeast extract, 50 mL nitrate salts, pH 7, for 24 h prior to RNA extraction. The RE values obtained by qRT-PCR were normalized against 18S rRNA. Values represent means ± standard error (SE) of duplicates.

Fig. S6 Phylogenetic analysis of carbon-regulating protein CREA in various filamentous fungi. Based on an analysis of sequence alignment and a bootstrap analysis, the phylogenetic tree was generated by the neighbour-joining method (MEGA software, version 4.0) using Mig1p as an outgroup. The following fungal strains were examined: *Colletotrichum gloeosporioides*-14 (*C.g*-14) CreA (NCBI, EQB49587.1; TCONS_00011180); *C. gloeosporioides*-Nara gc5 (*C.g* Nara gc5) Cre1 (XP_007279780.1); *C. higginsianum* (*C.h*) CreA (CCF41975.1); *C. orbiculare* (*C.o*) Cre1 (ENH80731.1); *Fusarium oxysporum* f. sp. *cubense* race 4 (*F.o*) CreA (EMT60442.1); *Metarhizium anisopliae* (*M.a*) CreA (KFG88228.1); *Trichoderma harzianum* (*T.h*) Cre1 (CAA64656.1); *Neurospora crassa* (*N.c*) Cre1 (AAC13555.1); *Botrytis cinerea* (B05.10_vankan) (*B.c*) CreA homologue (BROAD, B0510_4030); *Sclerotinia sclerotiorum* (*S.s*) CreA (XP_001589300.1); *Magnaporthe oryzae* 70-15 (*M.o*) CreA (XP_003714427.1); *Penicillium digitatum* PHI26 (*P.d*) CreA (EKV12335.1); *Aspergillus niger* (*A.n*) CreA (XP_001399519.1); *Saccharomyces cerevisiae* S288c (*S.c*) Mig1p (NP_011480.1).

Fig. S7 CLUSTAL omega alignment of CREA from various filamentous fungi. The consensus amino acids with a 60% identity threshold are shaded, amino acids with an 80% threshold are indicated with colons, and evolutionarily conserved amino acids are indicated with an asterisk. Two conserved zinc finger domains and one nuclear localization signal (NLS) in CreA are indicated by lines. The following fungi and proteins were examined: *Colletotrichum gloeosporioides*-14 (*C.g*-14) CreA (NCBI, EQB49587.1; TCONS_00011180); *C. gloeosporioides* Nara gc5 (*C.g* Nara gc5) Cre1 (XP_007279780.1); *C. higginsianum* (*C.h*) CreA (CCF41975.1); *C. orbiculare* (*C.o*) Cre1 (ENH80731.1); *Fusarium oxysporum* f. sp. *cubense* race 4 (*F.o*) CreA (EMT60442.1); *Metarhizium anisopliae* (*M.a*) CreA (KFG88228.1); *Trichoderma harzianum* (*T.h*) Cre1 (CAA64656.1); *Neurospora crassa* (*N.c*) Cre1 (AAC13555.1); *Botrytis cinerea* (B05.10_vankan) (*B.c*) CreA homologue (BROAD, B0510_4030); *Sclerotinia sclerotiorum* (*S.s*) CreA (XP_001589300.1); *Magnaporthe oryzae* 70-15 (*M.o*) CreA (XP_003714427.1); *Penicillium digitatum* PHI26 (*P.d*) CreA (EKV12335.1); *Aspergillus niger* (*A.n*) CreA (XP_001399519.1).

Table S1 Organic acids produced and secreted by *Colletotrichum gloeosporioides* during acidification of the environment. Gas chromatography-mass spectrometry (GC–MS) analysis of the organic acids secreted during a period of 24 h after transfer to inducing medium (175 mM sucrose, pH 7).

Table S2 Identities and similarities of glucose oxidases (GOXs) from *Colletotrichum gloeosporioides* strain Cg-14 and several other fungi.

Table S3 Primers used in this study.