Role of Nitrogen-Metabolism Genes Expressed During Pathogenicity of the Alkalinizing *Colletotrichum gloeosporioides* and Their Differential Expression in Acidifying Pathogens

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Submitted 25 January 2012. Accepted 28 April 2012.

Pathogens can actively alter fruit pH around the infection site, signaling modulation of pathogenicity-factor expression, as found for alkalinizing (*Colletotrichum* and *Alternaria* spp.) and acidifying (*Penicillium*, *Botrytis*, and *Sclerotinia* spp.) fungi. The nitrogen-metabolism genes *GDH2*, *GSI*, *GLT*, and *MEP* genes are differentially expressed during colonization by *Colletotrichum gloeosporioides*, and a *Δgdh2* strain reduces ammonia accumulation and pathogenicity. We analyzed the contribution of transporters *GLT* and *MEPB* to *C. gloeosporioides* pathogenicity. Germinating spores of *Δglt* strains showed reduced appressorium formation; those of *ΔnepB* mutants showed rapid ammonia uptake and accumulation inside the hyphae, indicating de-regulated uptake. Both mutants reduced pathogenicity, indicating that these transporters function during alkalinizing species pathogenicity. We compared the expressions of these genes in *C. gloeosporioides* and *Sclerotinia sclerotiorum*, and found five to 10-fold higher expression at the transcript level in the former. Interestingly, *GLT* and *MEPB* in the alkalinizing species showed no and very low sequence identity, respectively, with their counterparts in the acidifying species. Knockout analysis of *GLT* and *MEPB* and their differential transcript regulation in the alkalinizing and acidifying species suggest that the ammonia accumulation contributing to pathogenicity in the former is modulated by factors at the gene-regulation levels that are lacking in the acidifying species.

Colletotrichum gloeosporioides is a broad-host-range plant-pathogenic fungus that alkalinizes host tissue during disease

Nucleotide sequence data can be found in the EMBL and GenBank databases under the following accession numbers: for *GLT*, JN660151; *GDH2*, JN660152; *GSI*, JN660153; *MEPA*, JN660155; *MEPB*, JN660156; *MEPC*, JN660157; and *MEPD*, JN660154.

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*The e-Xtra logo stands for “electronic extra” and indicates that a supplementary table is published online.

development (Prusky et al. 2001). Host alkalinization results from active secretion of ammonia induced by relatively low pH values, as detected in several *Colletotrichum* spp.-infected fruit (Alkan et al. 2008; Diéguez-Urbeondo et al. 2008; Drori et al. 2003; Kramer-Haimovich et al. 2006; Prusky and Yakoby 2003). Whereas *Colletotrichum* spp. (Alkan et al. 2008; Prusky et al. 2001) and *Alternaria alternata* (Eshel et al. 2002) alkalinize the host environment, other species belonging to genera such as *Penicillium* (Hadas et al. 2007; Prusky et al. 2004), *Botrytis* (Manteau et al. 2003; ten Have et al. 2001; Wubben et al. 2000), *Sclerotinia* (Rollins and Dickman 2000; Tea Kim et al. 2007), *Phomopsis* (Davidzon et al. 2009), and *Aspergillus* (Karaffa and Kubicek 2003) tend to acidify it. Thus, some pathogens have been classified as alkalinizers or acidifiers according to the pH changes they induce during pathogenicity (Prusky and Yakoby 2003). This differential pH modulation is not a host-mediated effect, it is pathogen dependent. The same host attacked by acidifying or alkalinizing pathogens usually has opposite responses. In all cases, however, the induction of this differential pH shift represents an efficient tool for that particular pathogen for activating the production and secretion of pathogenicity factors, inducing host cell death and enhanced colonization processes (Alkan et al. 2009; Dickman 2007).

Miyara and associates (2008, 2010) monitored gene expression during ammonia secretion and colonization of avocado fruit, by using macroarrays carrying *C. gloeosporioides* cDNA. A set of genes involved in nitrogen metabolism and modulating the synthesis and catabolism of ammonia was differentially induced under different pH conditions. Among those genes induced at early stages of the alkalinization process (pH 4.0 to 4.5) were NAD⁺-specific glutamate dehydrogenase (*GDH2*, encoding for ammonia synthesis, JN660152) and the ammonia transporter *AMET* (modulated in yeast by the Ato protein). Among those induced at pH 6.0 to 7.0 during the alkalinization process were the ammonia permeases (*MEPB*, JN660156), glutamate transporter (*GLT*, JN660151), and glutamine synthetase (*GSI*, JN660153) (Table 1). The dynamics of ammonia accumulation in *C. gloeosporioides* showed that, at an early stage of the alkalinization process (pH 4.0 to 4.5), the production of ammonia by *GDH2* expression and its export by the ammonia exporter *AMET* contribute to the accumulation of ammonia in the medium (Miyara et al. 2010). At increasing pH values (pH 6 to 7), increased expression of *MEPB*

and *GLT*, together with *GS1* activity, regulates the internal level of ammonia in the hyphae (Miyara et al. 2010).

Activation of nitrogen metabolism is a highly complex process involving the activation of genes that contribute to the accumulation of ammonia in the host, induction of pH changes, and activation of pathogenicity factors that are expressed under the newly created environmental conditions. The present work was carried out to analyze the contribution of a set of nitrogen-metabolism genes, the transporters *GLT* and *MEPB*, to the modulation of pathogenicity by *C. gloeosporioides*. We also confirmed, by in-depth transcriptome comparison of the alkalizing and acidifying *C. gloeosporioides* and *Sclerotinia sclerotiorum*, respectively, the importance of differences at the sequence level in the regulation of the transporters *GLT* and *MEPB* in determining the differential ammonia accumulation between the two fungal species. In *C. gloeosporioides*, the alkalization process was the result of upregulation of ammonia synthesis and metabolism genes, the presence of an active *GLT* transporter that affects appressorium formation and pathogenicity, and a specific *MEPB* lineage involved in ammonia export, appressorium formation, and pathogenicity, none of which were found in *S. sclerotiorum*.

RESULTS

Sequence and functional analysis of the *GLT* transporter in *C. gloeosporioides*.

A BLAST search of the putative *GLT* sequence of *C. gloeosporioides* against other alkalizing species such as *Fusarium oxysporum* (Table 1; Fig. 1) revealed the presence of a homologous sequence with 51 to 59% similarity to the prokaryotic and eukaryotic high-affinity sodium-dependent glutamate transporters (Ren and Paulsen 2005; Ryan et al. 2009). Analysis of the putative *GLT* proteins displayed identical hallmark features described for high-affinity sodium-dependent glutamate transporters from eukaryotes and prokaryotes (Yernol et al. 2004):

Table 1. Pairwise sequence similarity between *Colletotrichum gloeosporioides* genes and homologs detected in four other species of filamentous fungi

Species	GDH2	GS1	GLT	MEPB
<i>Fusarium oxysporum</i>	76.86	76.52	59.45	71.31
<i>Aspergillus niger</i>	65.55	70.56	–	53.69
<i>Botrytis cinerea</i>	79.86	80.50	–	64.04
<i>Sclerotinia sclerotiorum</i>	71.82	81.97	–	63.71

sodium-binding, potassium-coupling, and glutamate γ -carboxylate-binding sites (Fig. 1).

To assess the function of *GLT* in *C. gloeosporioides*, gene knockout was performed to generate Δ *glt* strains (Fig. 2), by replacing the intact *GLT* with a *GLTB5'-Hyg-GLTB3'* cassette, as described below (Hadas et al. 2007). Deletion of the gene was confirmed by polymerase chain reaction (PCR) (Fig. 2). Gene expression of *GLT* was detected by quantitative reverse-transcriptase (qRT)-PCR (Fig. 2B) in the wild-type (WT) progenitor strain Cg-14 and ectopic₁₀ strains but not in Δ *glt*₅, Δ *glt*₆, or Δ *glt*₃₁ strains, confirming deletion of *GLT*.

The Δ *glt* strains grown for three generations on M₃S medium amended with reduced nitrogen sources levels (0.5 mM KNO₃) showed 37 to 56% inhibition of appressorium formation compared with the WT and ectopic strains (Fig. 3). Inoculation of avocado pericarp with strain Δ *glt*₃₁ inhibited anthracnose symptom development by 45 to 50% compared with the WT strain 7 days after symptom appearance, suggesting that inhibition of appressorium formation affects symptom development.

Functional analysis of *C. gloeosporioides* *MEPB*: Effect on ammonia import/export balance and pathogenicity.

To assess the function of *MEPB* of *C. gloeosporioides*, a gene-deletion experiment was carried out to generate Δ *mepB* mutant strains by homologous recombination (Fig. 4), replacing the intact *MEPB* with a *MEPB5'-Hyg-MEPB3'* deletion cassette, as described below (Shafran et al. 2008). Gene expression of *MEPB* was detected by qRT-PCR (Fig. 4B) in the WT progenitor Cg-14, ectopic₅, and the complemented strain Δ *mepB*_{12comp13}, but not in strains Δ *mepB*₃ or Δ *mepB*₁₂, confirming deletion of *MEPB*.

When the 3-day hyphal mats of the Δ *mepB* isolates were transferred from the initial M₃S medium to inductive secondary medium (SM) amended with 10 mM NH₄Cl, ammonia concentration in the growth culture declined fourfold from 23 to 6 mM ammonia per gram of dry weight of fungal mycelium (mM/g dw) 6 h postinoculation (hpi), suggesting rapid uptake, whereas minor changes were observed with the WT and ectopic strains which remained at a similar initial concentration (25 to 24 mM/g dw). After a further 6 h, ammonia level in the medium declined to similar levels in all strains (Fig. 5A). Analysis of ammonia levels inside the Δ *mepB* isolate, WT, and ectopic control strains showed a different pattern. Whereas the level of ammonia decreased drastically from 78 to 26 mM in the WT and ectopic strains during the first 6 hpi (Fig. 5B), it

Fig. 1. Sequence alignment of glutamate transporters. Alignment of amino acid sequences of *Pyrococcus horikoshii* GLT and rat GLT1 with putative GLT sequences identified in fungi. The sequence alignment highlights (dark gray) the conserved motifs shown to be involved in glutamate binding and transport. Light gray-shaded residues indicate the conserved arginine (R) suggested to be the glutamate γ -carboxylate-binding residue in *P. horikoshii* GLT (Yernol et al. 2004); amino acid G, sodium binding; amino acid YQ, potassium coupling. Amino acid sequences are: *P. horikoshii* (Ph) GLT (NP_143181); rat GLT1 (P31596); *F. oxysporum* (Fo) GLT (FOXG_03264.2); and *Colletotrichum gloeosporioides* (Cg) GLT (JN660151). The alignment was performed using ClustalW and adjusted manually.

remained constant (within 60 to 75 mM) in the $\Delta mepB$ strains. After an additional 6 h, the level of ammonia in the $\Delta mepB$ isolates was similar to that in the control strains. This rapid change in internal ammonia level suggests deregulation and a concurrent delay in ammonia export.

Germination of $\Delta mepB_3$ and $\Delta mepB_{12}$ strains was inhibited by 35% 14 h after germination initiation but, 2 h later, the $\Delta mepB$, WT, and ectopic control strains showed similar germination patterns (Fig. 6A). Appressoria formation showed a 55% inhibition in the $\Delta mepB$ isolate compared with WT and ectopic control strains (Fig. 6B). The colonization area of *C. gloeosporioides* $\Delta mepB_3$ and $\Delta mepB_{12}$ strains inoculated on peeled avocado fruit was significantly reduced compared with the WT strain (Fig. 7): 7 days after inoculation, the diameter of the mesocarp colonized by the WT and ectopic_{T5} strains reached 21.5 ± 0.5 mm, whereas average colonization by the $\Delta mepB_3$ and $\Delta mepB_{12}$ mutants was only 8.0 ± 0.1 mm. Complemented strains did not differ from the WT in any of the experiments (data not shown).

Comparison of the ammonia metabolism-modulating genes in alkalinizing and acidifying fungal species.

Five fungal species that either alkalinize their surroundings (*C. gloeosporioides* and *F. oxysporum* f. sp. *phaseoli*) or acidify them via the secretion of organic acids during colonization

Fig. 3. Appressorium formation by germinating spores of Δglt strains after growth on solid MS media containing KNO_3 as nitrogen source. Mycelia of the wild-type (WT) and Δglt strains were grown on M₃S transferred to a medium containing 0.5 M KNO_3 as nitrogen source for three generations and 2 weeks later collected for appressorium-formation assay. Spores were brought to a concentration of 10^6 spores/ml and a 10- μ l drop was placed on a glass slide for evaluation of appressorium formation 16 h later.

Fig. 2. Development of *GLT* mutant strains. A double crossing-over homologous recombination event resulting in replacement of the original *GLT* sequence with the *GLT5'-Hyg-GLT3'* cassette was carried. **A**, Scheme describing gene disruption by homologous recombination. The pairs of primers used in construct creation were 33-34 for the 5' end and 35-36 for the 3' end. **B**, Polymerase chain reaction (PCR) analysis of the wild-type (WT) strain, ectopic colony (Ectopic₁₀), and independent *GLT*-disruption colonies (Δglt). Primer 37 flanking a position upstream of the *GLT2:HYG3* region and reverse primer 1 located on the hygromycin cassette were used to identify positive *GLT* gene replacement at the 5' locus. Primer 38 from the hygromycin cassette and primer 2 flanking the *GLT:HYG3* region were used to identify *GLT* gene replacement at the 3' locus. *18S* gene primers 23 and 24 were used for WT DNA quality control. Primers 1 and 33 were used as a positive control for the ectopic strains, to confirm random integration of the *GLT:HYG3* cassette. **C**, Expression of *GLT* in the WT strain compared with the ectopic-integration control and the Δglt strains, as detected by quantitative reverse-transcriptase (qRT)-PCR. Cultures were initially grown in M₃S rich medium and were then transferred to broth minimal medium without nitrogen source for 5 h prior to RNA extraction. Relative expression values obtained by qRT-PCR are normalized against *18S* rRNA. Average $\pm$ standard deviation from three replications of one treatment is presented.

(*Botrytis cinerea*, *S. sclerotiorum*, and *Aspergillus niger*) were compared. Growth of *C. gloeosporioides* and *F. oxysporum* for 48 h on ammonia-inducing media containing 50 mM KNO₃ increased the pH from 4.0 to 5.82 and 5.40, respectively, and the ammonia concentration in culture filtrates to 110 and 70 μ mol/liter, respectively. In contrast, *B. cinerea* and *S. sclerotiorum* cultured in similar media decreased the pH values to 3.75 and 3.2 and the levels of secreted ammonia reached close to 52 and 47 μ mol/liter, respectively, indicating no significant secretion (Fig. 8A). Similar effects were observed when *C. gloeosporioides*, *F. oxysporum*, *B. cinerea*, and *S. sclerotiorum* were inoculated on tomato fruit, where pH in the infected tissue increased from 4.2 to 4.7 and 4.9 in *C. gloeosporioides*- and *F. oxysporum*-inoculated fruit, respectively; whereas, in fruit inoculated with *B. cinerea* and *S. sclerotiorum*, the pH decreased to 3.9 and 3.8, respectively (Fig. 8B).

Sequence analysis of *GDH2* and *GS1* in *C. gloeosporioides*, *F. oxysporum* f. sp. *phaseoli*, *B. cinerea*, *S. sclerotiorum*, and *A. niger* revealed a single copy of each gene with 82 and 72% sequence identity between all the alkalinizing and acidifying species for *GS1* and *GDH2*, respectively (Table 1). To compare the activities of glutamate and ammonium transporters in alkalinizing and acidifying strains, we searched for homologs of the GLT protein and of the highly hydrophobic AMTB ammo-

nium channel of *Escherichia coli* (Javelle et al. 2008) in the genome sequences. The BLAST search failed to find any sequence with homology to that of the *Colletotrichum* GLT protein in the acidifying species. Analysis of *GLT* promoters in the alkalinizing species revealed several PACC-binding motifs (three in *Colletotrichum* and four in *Fusarium* spp.), suggesting their expressions' high dependence on pH regulation and confirming the findings of Miyara and associates (2010).

A blast search of *MEPB* versus the proteomes of the acidifying species *B. cinerea*, *S. sclerotiorum*, and *A. niger* revealed distantly related BLAST hits with e values of 3×10^{-27} , 3×10^{-21} , and 2×10^{-31} , respectively, and subsequent analysis revealed that these hits are other members of the *MEP* family (Fig. 9). In contrast, the alkalinizing *F. oxysporum* species contained true orthologs of *MEPB* with an e value of 3×10^{-174} . The identified sequences presented 52 to 58% similarity to the AtmB from *E. coli* (not shown). Three putative MEP proteins were identified in *B. cinerea* and *S. sclerotiorum*, whereas four putative MEP sequences were identified in *C. gloeosporioides*, *F. oxysporum*, and *A. niger* (Fig. 9).

Phylogenetic analysis of the *MEP* gene family revealed at least four distinct lineages (Fig. 9). Only lineage C was conserved in all of the fungi included in this study. An ortholog of lineage A was missing from *F. oxysporum* but was otherwise

Fig. 4. Development of *MEPB* mutant strains. A gene-replacement construct for the *MEPB* sequence with the *MEPB*5'-*Hyg*-*MEPB*3' cassette and gene recovery with *MEPB* complementation cassette was followed. **A**, Scheme describing gene disruption by homologous recombination. Pairs of primers used in construct creation were 15-16 for the 5' end and 17-18 for the 3' end. **B**, Polymerase chain reaction (PCR) for identification of *MEPB* deletion events in the wild-type (WT) strain, ectopic colony (Δ mepB_{ectopic5}), independent *MEPB*-disruption colonies (Δ mepB⁻), and Δ mepB₁₂ complementation colonies (Δ mepB₁₂comp⁺). Primer 19 flanking the position upstream of the *MEPB2:HYG3* region and reverse primer 1 located on the hygromycin cassette were used to identify positive *MEPB* gene replacement at the 5' locus. Primer 2 from the hygromycin cassette and primer 20 flanking of the *MEPB2:HYG3* region were used to identify *MEPB* gene replacement at the 3' locus. *18S* gene primers 23 and 24 were used for WT DNA quality control. Primers 1 and 15 were used as a positive control for the ectopic₅ strains, to confirm random integration of the *MEPB2:HYG3* cassette. **C**, Expression of *MEPB* in the WT strain compared with the ectopic-integration control, the Δ mepB strains, and the Δ mepB₁₂ complementation strains, as detected by quantitative reverse-transcriptase (qRT)-PCR. Cultures were initially grown in M₃S rich medium and subsequently transferred to broth minimal medium without nitrogen source for 5 h, prior to RNA extraction. Relative expression values obtained by qRT-PCR were normalized against *18S* rRNA. Average $\pm$ standard deviation from three replications of one treatment is presented.

conserved in both the alkalinizing and acidifying fungi. Lineage B was only found in the alkalinizing species whereas lineage D was only found in the acidifying species. This confirms our early results on the B group's specific functions in the modulation of host-tissue pH by the alkalinizing species. Note that additional *MEP* homologs were discovered in *Aspergillus*, *Fusarium*, and *Colletotrichum* spp. that did not belong to any of the four main lineages. Although we have tentatively assigned these additional *MEP* genes as members of the *MEPD* lineage, this assignment is inconclusive and a more comprehensive phylogenetic analysis is needed for a more complete understanding of the evolution of this gene family.

MEPB promoter analysis in *Colletotrichum* and *Fusarium* spp. revealed several PacC-binding motifs (two in *Colletotrichum* and four in *Fusarium* spp.), suggesting their high dependence on pH regulation. Sequence analysis of *MEPA* and *MEPC* did not reveal PACC-binding sites, suggesting different or more complex regulation during the alkalinization process. Thus, *MEPB* is an ammonia transporter with no shared sequences in the acidifying species that is strongly expressed under active host colonization (Miyara et al. 2010) (Fig. 10) and represents an independent conserved group in the alkalinizing species (Fig. 9).

To analyze the transcriptional regulation of nitrogen-metabolism genes during the differential accumulation of ammonia and pH in culture, the transcript levels of the nitrogen-metabolism genes *GDH2*, *MEPA* (JN660155), *MEPB*, *GLT*, and *GSI* were compared in culture after transfer of one representative of the alkalinizing group (*C. gloeosporioides*) and one of the acidifying group (*S. sclerotiorum*) to an ammonia-inducing medium. Whereas a 10- to 90-fold increase in the relative expression of *GLT* and *GSI* and an up to 750-fold relative increase in *MEPB* were observed at 12 and 48 h after culture inoculation with *C. gloeosporioides* (Fig. 10A), relative expression during the same period in *S. sclerotiorum*-inoculated media was only 2- to 3-fold higher for *GDH2* and less than that for the *MEP* genes and *GSI* (Fig. 10B). These results indicated clear differential expression of the nitrogen-metabolism genes in the alkalinizing versus acidifying fungal strains under study. Under those conditions, *C. gloeosporioides* increased the pH of the medium from 4.2 to 5.7, and the final ammonia concentration in culture filtrates reached 10^5 $\mu\text{mol/liter}$ 48 hpi (Fig. 10C and D), whereas growth of *S. sclerotiorum* under the same conditions decreased the pH to 3.2 and final ammonia concentration reached only 40 $\mu\text{mol/liter}$ (Fig. 10C and D).

DISCUSSION

The ability to modulate the environment during pathogenicity, through alkalinization or acidification, has been described for several fungal pathogens (Alkan et al. 2008; Davidzon et al. 2009; Eshel et al. 2002; Hadas et al. 2007; Manteau et al. 2003; Prusky et al. 2001; Rollins and Dickman 2000). However, comparative analyses of genes contributing to pathogen colonization in the alkalinizing species or to different pathogenicity patterns are lacking. Whereas inoculation of culture and tomato fruit by *C. gloeosporioides* and *F. oxysporum* alkalinize the environment (by 1 to 2 pH units) via the secretion of significant amounts of ammonia, *S. sclerotiorum* and *B. cinerea* acidify culture and tomato fruit by 0.5 to 1.5 pH units. This response seems to be important for pathogenicity because it may determine the secretion of metabolites affecting the pH response and the consequent differential mechanism of pathogenicity for the acidifying and alkalinizing pathogens.

Previous work by Miyara and associates (2010) in *C. gloeosporioides* suggested the involvement of a set of nitrogen-metabolism genes in the synthesis and catabolism of ammonia.

The dynamics of ammonia accumulation suggested that acidic conditions induce *GDH2* production and accumulation, as well as the export of ammonia by *AMET*. Increasing pH outside the fungal cells increased the expression of *MEPB* and *GLT* which, together with *GSI* activity, regulate the internal level of ammonia (Miyara et al. 2010). The $\Delta gdh2$ mutants showed significant inhibition of ammonia accumulation and attenuation of fungal colonization (Miyara et al. 2010) but nothing was known about transporter genes regulating nitrogen metabolism that are upregulated during *Colletotrichum* colonization.

Contribution of nitrogen metabolism to fungal colonization.

The availability of recently published genome sequences of fungal pathogens that acidify or alkalinize the environment allowed us to carry out a genomic comparison of five fungi, three that acidify their environment (*B. cinerea*, *S. sclerotiorum*, and *A. niger*) and two that alkalinize it (*C. gloeosporioides* and *F. oxysporum*). *C. gloeosporioides* and *S. sclerotiorum* were chosen for transcriptome analysis of the genes determining the accumulation of ammonia in alkalinizing and acidifying species, respectively. Transcript analysis of the four genes modulating the alkalinization process (*GDH2*, *GSI*, *MEP* genes, and *GLT*) revealed their 45- to 500-fold upregulation in *C. gloeosporioides* compared with *S. sclerotiorum*, suggesting active regulation of ammonia accumulation in *Colletotrichum* spp.

What is the basis for the differential expression in these two groups? *GDH2* and *GSI*, coding for ammonia production and detoxification inside the fungal cells, were found as single-copy genes in all five genome sequences analyzed. Pairwise sequence similarity between *C. gloeosporioides* *GDH2* and *GSI* and their homologs in the alkalinizing and acidifying

Fig. 5. Ammonia accumulation induced in the wild-type (WT) and $\Delta mepB2$ *Colletotrichum gloeosporioides* strains. **A**, Ammonia concentration in the culture medium. **B**, Ammonia concentration inside the fungal cells. Spores of *C. gloeosporioides* were inoculated in primary medium and, 3 days later, transferred to secondary medium amended with 10 mM NH_4Cl , then analyzed at different hours postinoculation (hpi). Average of three replications $\pm$ standard deviation of one experiment out of three repeated experiments is presented.

groups ranged between 70 and 80%, suggesting that the differential transcript expression in the two groups is based mainly on differential regulation of those genes. Whereas *GDH2* has been reported to be mainly expressed at relatively low pH (4 to 5) and independently of the PacC response, *GS1* is pH dependent and mainly expressed at pH 6 to 7 (Miyara et al. 2010). Analysis of the upstream noncoding region of *GS1* revealed one GATA factor binding site and a PacC-binding motif in *Colletotrichum* spp. compared with none in the *GS1* sequence of *Sclerotinia* spp. However, similar analysis of the upstream promoter region of *GDH2* did not reveal any clear over-representation of any motif in either species and, thus, the basis for the differential expression of *GDH2* is still not fully understood (Schönig et al. 2008). Nevertheless, knockout of *GDH2* and *AREA*, a modulator of *GDH2*, in *C. coccodes* reduced its ammonia secretion and pathogenicity, highlighting the importance of this nitrogen-metabolism regulation during the alkalization process (Alkan et al. 2008; Miyara et al. 2010).

Contribution of GLT and MEP transporters to fungal colonization.

Early work, where GLT gene expression was monitored during colonization of avocado fruit by using macroarrays showed 180-fold induction (Miyara et al. 2010). In animal systems, uptake of extracellular glutamate is essential for regulating the activity of glutamate receptors, which play a role in neurological

diseases (Wipf et al. 2002). However, this is the first report of a glutamate transporter in fungal systems. In silico analysis of the GLT protein showed the classical hallmarks of glutamate transport, including sodium binding, potassium coupling, and glutamate γ -carboxylate-binding sites, suggesting an active glutamate transporter (Fig. 1). The *C. gloeosporioides* GLT sequence showed similarity to a hypothetical protein and to an unidentified amino acid transporter in *F. oxysporum*, with 70% identity, whereas no homologs were found in any of the acidifying fungal species. Functional analysis of Δ glt strains indicated that GLT contributes to appressorium formation and to pericarp colonization by *C. gloeosporioides* following growth under reduced nitrogen conditions. Miyara and associates (2010) reported that glutamate uptake led to the production of ammonia by activation of *GDH2* or by inducing *GS* and the production of glutamine and, from that, to amino acids that may be catabolized to ammonia (Fig. 11). In the present results, only after three transfers of the Δ glt strains on reduced KNO_3 media, when the internal glutamate pools have probably declined, was a different phenotypic response of the Δ glt strains from that of the WT observed. This suggests that the GLT transporter contributes to nitrogen metabolism via uptake of available glutamate during early biotrophic stages, when few nitrogen sources are available; whereas, during the necrotrophic stage, when amino acids are abundant, other types of transporters such as MEPB contribute to the import of nitrogen material.

Fig. 6. Comparisons of **A**, spore germination and **B**, appressoria formation induced in the wild-type (WT) and Δ mepB2 *Colletotrichum gloeosporioides* strains. Germination of the WT, Δ mepB2, and ectopic strains was evaluated at particular times (hours postinoculation [hpi]). Appressoria formation was evaluated 12 to 14 hpi and was compared to the complemented strains. The averages of three replications $\pm$ standard deviation are presented. Values within columns marked with different letters differ significantly according to Tukey-Kramer's highly significant differences test at $P \leq 0.05$.

An explanation for the absence of *GLT*-homologous sequences in acidifying species might be based on the analysis of the *GLT* transporters from *Streptococcus pneumoniae*, in which downregulation of gene expression and reduced activity of *GLT* occur under low acidic pH conditions (Krastel et al. 2010). *S. mutans* also shows decreased activity of amino acid transporters under acidic conditions, whereas their activities increase at pH 6.0 (Sato et al. 1989). A similar response has been reported for *C. gloeosporioides*, with *GLT* strongly expressed mainly under alkaline conditions, and was confirmed by analysis of the upstream regions of the coding sequences in the alkalinizing species which showed several *PACC* motifs (Miyara et al. 2010).

It might also be that some sequences in the acidifying species have diverged too extensively from the alkalinizing group to be recognized as homologs. For example, differences in the number of transporters between groups might suggest systematic gene loss and genome compaction as possible factors reducing the number of transport proteins in the acidifying species (Hong 1998; Linton and Higgins 1998; Nakanishi and Masu 1994; Tolner et al. 1955; Zientz et al. 2004). The observation of differential function in the Δ *glt* strains only following growth under low nitrogen conditions suggests that *GLT* function might be critical during the pathogen's long periods as ungerminated or quiescent germinated spores on host fruit (Prusky 1996).

The most significant contribution to alkaline fungal colonization, as well to the differences between the acidifiers and alkalinizers, was found to be the differential transcript expression of *MEP* genes and the specific sequence of *MEPB*, as determined by phylogenetic analysis of the *MEP* family. Miyara and associates (2010) suggested that *MEPB* is involved in nitrogen metabolism occurring during pathogenicity of *C. gloeosporioides* in fruit. Sequence analysis determined the presence of a well-conserved family of three ammonium transporters that has been characterized in most reported fungi (Teichert et al. 2008). However, the differential localization of *MEPB* within the alkalinizing species indicates a specific sequence not present in the acidifying fungal group.

Fig. 7. Pathogenicity assays of *Colletotrichum gloeosporioides* wild-type (WT) and Δ *mepB* mutant strains. Decay development of WT and mutant strains after inoculation of peeled freshly harvested avocado fruit. Average colonized area $\pm$ standard deviation for 10 infected fruit 3, 5, and 7 days postinoculation (dpi) is reported. Inoculation was carried out on a 1-mm-deep, 5-mm-diameter wound in the fruit mesocarp of peeled fruit followed by incubation at 25°C under high humidity.

The *MEP* proteins scavenge ammonia from the medium to be used as a nitrogen source (Rutherford et al. 2008; Teichert et al. 2008) and are abundantly expressed under the alkaline conditions induced by *C. gloeosporioides* (Miyara et al. 2010). The Δ *mepB* mutant strain showed rapid uptake of ammonia from the medium and accumulation of ammonia inside the mycelium compared with the WT and complemented mutants. This may suggest that *MEPB* regulates not only the import but also the export of ammonia. A possible bidirectional diffusion of ammonium was reported for *AMTB* transporters (homolog of *MEPB*) of *Saccharomyces cerevisiae* (Soupene et al. 2001). The reduced ammonia secretion by these Δ *mepB* mutants explains their reduced pathogenicity when inoculated on avocado fruit (Alkan et al. 2008, 2012). Considering its expression pattern in *Colletotrichum* spp., this transporter may represent a key component in the mechanisms that mediate and regulate ammonia production and secretion in alkalinizing species.

It is still not known whether *MEPB* is the main or only regulator of ammonia import and export. Protein analysis of *MEPB* showed the remarkable conservation of the histidine pore (at amino acid 168) (Javelle et al. 2006, 2008) and this suggests a role in allowing the substrate to cross the central part of the channel (Andrade et al. 2005; Khademi et al. 2004; Winkler 2006; Zheng et al. 2004). The transporter's precise function (i.e., serving as a proton acceptor for the entering ammonium

Fig. 8. Ammonia accumulation and pH changes induced by *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum*. **A**, In vitro: inducing medium amended with KNO_3 was inoculated with 10^6 spores per 40 ml in a 100-ml vial, and pH and ammonia concentrations were determined 48 h later. **B**, In planta: a wounded tomato fruit was inoculated with a 10- μ l drop (10^5 spores) and incubated at 20°C, and measurements were taken 4 to 5 days later. Values within columns marked with different letters (capitals and lowercase) differ significantly according to Tukey-Kramer's honestly significant difference test at $P \leq 0.05$.

ion or, more simply, shaping the pore and balancing its polarity) remains to be determined. Interestingly, in MEPC, His168 is changed to a glutamic acid (E). Javelle and associates (2006) demonstrated that the H168E point mutation does not affect the transporter's overall structure but reduces its activity by approximately 25% compared with WT proteins. This might imply that all fungal MEP C transporters have reduced activity compared with the proteins with a His168 pore, suggesting that MEPB might actually present different biochemical properties compared with other, less active MEP proteins, such as MEPC and MEPD, and this may further contribute to the differential expression in acidic and alkaline species.

Alkalinization of host tissue by enhanced nitrogen metabolism during necrotrophic growth is a major factor in the development of *Colletotrichum* spp. in different hosts. The present work demonstrated that *Fusarium* spp. also use ammonium secretion as a novel strategy for fungal colonization of different hosts. Ammonia's mechanism of action includes optimization of the pH of the infection court for activation of fungal virulence genes, and simultaneous activation of the host respiratory burst oxidase homolog which appears to stimulate salicylic acid pathway responses and suppress jasmonic acid pathway responses (Alkan et al. 2012). These processes correlate with local cell death and enhance the aggressiveness of the pathogen in its necrotrophic stage. Taken together, the differences that activate pathogenicity in the alkalinizing species include i) differential transcript expression of genes modulating ammonia accumulation; ii) presence of the *GLT* transporter, which modulates appressorium formation and pathogenicity; and iii) presence of specific permease *MEPB* that modulates ammonia import or

accumulation and pathogenicity. However, it is still unclear how the genes that determine ammonia accumulation are regulated. Functional analysis of the loss-of-function *Δpac1*, which reduces pathogenicity of the alkalinizing *C. gloeosporioides*, revealed downregulation of *GSI*, *GLT*, and *MEPB* expression (Miyara et al. 2010). This behavior was confirmed by the detection of consensus sequences of *PACC* in the promoter regions of *GLT* and *MEPB* in all of the alkalinizing species. However, analysis of the upstream untranslated regions of *GDH2* and *GSI* in the alkalinizing species showed few *AREA* and *PACC* motifs, suggesting a different modulating mechanism there. This differential expression pattern within the alkalinizing fungal species suggests that the classical transcription factors are not the only ones modulating the differential expression of genes in acidifying and alkalinizing species.

MATERIALS AND METHODS

Fungal isolates, media, and growth conditions.

Single-spore cultures of the WT Cg-14 isolate of *C. gloeosporioides*, obtained from a decayed avocado fruit (*Persea americana* 'Fuerte') in Israel, and all of the *C. gloeosporioides* *MEPB* mutant strains used in this work were routinely cultured on M₃S agar as described by Miyara and associates (2010). Cultures were supplied as follows: *F. oxysporum* (FOX Sp4) by J. M. Minguez (University of Salamanca, Spain), *B. cinerea* by L. Benito (University of Salamanca), and *Sclerotinia sclerotiorum* by M. Dickman (Texas A&M, College Station, TX, U.S.A.), and were grown on potato dextrose agar. M₃S medium (primary medium), used for the initial growth of the fun-

Fig. 9. Phylogenetic analysis of the *MEP* gene family. Phylogenetic reconstruction was performed using PHYML and evaluated using a bootstrap test with 100 replications. Numbers on branches are bootstrap support values. The *MEPB* clade, found only in alkalinizing species, is highlighted in light gray. The *MEPD* clade, found only in acidifying species, is highlighted in dark gray. Species codes: *Colletotrichum gloeosporioides* (Cg), *Fusarium oxysporum* f. sp. *phaseoli* (FOX), *Aspergillus nidulans* (An), *Sclerotinia sclerotiorum* (Ss), and *Botrytis cinerea* (Bc).

gus prior to exposing it to different nutritional induction factors (Miyara et al. 2010), contained (per liter) 2.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.7 g of KH_2PO_4 , 1.0 g of Bacto peptone, 1.0 g of Bacto yeast extract (Difco Laboratories, Detroit), 10 g of sucrose, and 250 mg of chloramphenicol. The cultures were incubated at 22 to 24°C in a shaking incubator at 150 rpm for 3 days and were harvested by filtration through a sterile Büchner funnel fitted with filter paper. The hyphal mat was washed twice with 40 ml of sterile distilled water. The washed mycelia were resuspended in 40 ml of fresh SM containing (per liter): 4 g of KH_2PO_4 , 2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mg of FeCl_3 , 50 mM glucose, and 1.0 g of KNO_3 . When *Fusarium*, *Botrytis*, or *Colletotrichum* spp. were compared for ammonia secretion, spore suspensions (10^6 spores per 40 ml in a 100-ml vial) were inoculated into inducing media. Inoculation of *S. sclerotiorum* for similar evaluations was performed with 10 plugs of 5 mm in diameter, sampled from the leading edge of a developing colony. The initial pH for each flask was determined after the medium had been autoclaved but prior to inoculation. The experiments were repeated at least three times, and the results of a single representative experiment are shown. Differences between experiments in the average values for each treatment did not exceed 2 to 3%.

Fruit inoculation and appressorium formation.

Freshly harvested tomato fruit from a local supermarket were used for inoculation. Spores of *C. gloeosporioides*, *F. oxy-*

sporum, and *B. cinerea* strains were inoculated on the mesocarp of tomato fruit by peel-wounding the fruit with a nail and placing 10 μl of a conidial suspension (10^6 conidia/ml) on each of three equally spaced inoculation spots. For inoculation of *S. sclerotiorum*, three 5-mm-diameter agar plugs from the leading edge of a developing colony were used. Each fungal inoculum was placed on the equatorial circle of five different fruit per treatment (i.e., 15 inoculation replicates per fungal strain per experiment).

In some experiments, *C. gloeosporioides* was inoculated on Fuerte avocado fruit freshly harvested from an orchard in Israel. Spores of the various *C. gloeosporioides* strains were inoculated on the avocado fruit peel by placing 7 μl of a conidial suspension (10^6 conidia/ml) on each of three longitudinally spaced inoculation spots on each side of 10 different fruit per treatment (i.e., 60 inoculation replicates per treatment). When fruit were inoculated on the mesocarp, a 1- to 2-mm strip of peel tissue was removed from the fruit and 7 μl of a conidial suspension (10^6 conidia/ml) was placed into 1-mm-deep, 1-mm-diameter inoculation spots in the fruit mesocarp. Five inoculation spots, longitudinally spaced, on each side of 10 different fruit were inoculated per treatment. The inoculation experiments were repeated three times and one representative experiment is presented.

For appressorium formation and maturation-detection experiments, *Colletotrichum* WT and mutant isolates were observed on glass slides. Briefly, 5 to 25 μl of a conidial suspension (10^6

Fig. 10. Comparison of pH change, ammonia secretion, and relative expression of *GDH2*, *MEP* genes, *GS1*, and *GLT* in alkalinizing *Colletotrichum gloeosporioides* and acidifying *Sclerotinia sclerotiorum* during growth on KNO_3 ammonia-inducing medium. **A** and **B**, Relative expression of *GDH2*, *MEPA*, *MEPB*, *MEPC*, *GLT*, and *GS1* in *C. gloeosporioides* and *S. sclerotiorum*, respectively. **C**, Induced pH changes. **D**, Ammonia ΔNH_3 (final-initial concentration). Samples were collected at various hours postinoculation (hpi) to perform the different assays. Initial ammonia concentration in the media was 35 $\mu\text{mol/liter}$. Relative expression of the genes was compared with that of the constitutive *18S* ribosomal gene. Average of three replications $\pm$ standard deviation is presented.

conidia/ml) of each isolate was placed on five glass slides and kept on moistened filter paper in a moist chamber. After 12 to 14 h of incubation, conidial germination and appressorium development were observed in three microscopic fields in each of five cells per slide. Maturation of appressoria was defined according to melanization level: mature appressoria were considered to be only those that were fully melanized compared with hyaline-less pigmented ones, which were considered immature.

pH measurement and ammonia detection.

pH was measured in 0.5-ml aliquots of the culture media collected at various times after fungal inoculation using a Thermo-Orion Model 9810BN microcombination pH electrode (Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.). Ammonia was detected colorimetrically with an ammonium test kit (Merck, Darmstadt, Germany). The concentration of ammonia was determined in 5 ml of 10-fold-diluted culture supernatants according to the manufacturer's instructions. Briefly, the sample containing ammonium was adjusted to pH 13 so that the ammonium was transformed to ammonia, which could then be detected colorimetrically at 690 nm. Concentrations were reported in millimolar ammonia. For determination of ammonia concentration inside the fungal cells, 100 mg of freshly collected mycelium was homogenized and then centrifuged at $10,500 \times g$ for 5 min; 50 μ l of the supernatant was diluted to

5 ml and ammonium was detected colorimetrically with the ammonium test kit.

RNA extraction and qRT-PCR analysis.

RNA for real-time qRT-PCR analysis was extracted from 80 mg (dry weight) of mycelium with RNeasy plant mini kit (Qiagen Sciences, Hilden, Germany) and further purified by treatment with a TURBO DNA Free kit (Ambion, Austin, TX, U.S.A.). The reverse-transcription reaction was performed on 1 μ g of total RNA with the Reverse-It first-strand synthesis kit (ABgene, Surrey, U.K.). Samples of cDNA were diluted 1:10 (vol/vol) to the final template concentration for qRT-PCR. Real-time qRT-PCR was performed with a RotorGene 3000 system (Corbett Research, Sydney, Australia). PCR amplification was run using 3.5 μ l of cDNA template in 10 μ l of a reaction mixture containing 5 μ l Syber-Green amplification kit (ABgene) and 300 nM primers. PCR conditions were initial denaturation for 15 min at 94°C; 40 denaturation cycles of 10 s at 94°C; annealing at 60°C for 15 s; extension at 72°C for 20 s (cycling A), 77°C for 6 s (cycling B), and 80°C for 6 s (cycling C); and melting at 72 to 99°C. The samples were subjected to melting-curve analysis with the RotorGene program. All samples were normalized to 18S rRNA gene levels in the same qRT-PCR, and the values were expressed as increase or decrease in levels relative to a calibration sample. The forward and reverse primers for all of the genes are given in Supple-

Fig. 11. Central pathways for nitrogen metabolism. Nitrogenous compounds in the cell are synthesized from either glutamate or glutamine. The major pathway for glutamate synthesis is through combination of ammonia with α -ketoglutarate, which is synthesized from acetyl CoA and oxaloacetate through the early steps of the citric acid cycle. Glutamine is synthesized by the combination of ammonia with glutamate. Pathways for utilization of a variety of nitrogen sources, including urea, proline, and arginine, are shown. The production of ammonia by *Colletotrichum gloeosporioides* is by the activation of GDH2. The presence of the MEPB and GLT transporters are shown (adapted from Magasanik and Kaiser 2002).

mentary Table S1. Each experiment was repeated at least three times with similar results, and the results of one experiment are presented.

Creation of *C. gloeosporioides* GLT and MEPB mutants.

Generation of knockout construct. The 5' and 3' flanking fragments of the coding region of *C. gloeosporioides* *MEPB/* *GLT* were amplified by PCR from the full gene deposited in GenBank. For *MEPB*, primer sets 15-16 and 17-18 were used to amplify the 5' and 3' fragments, respectively. For *GLT*, the primer sets 33-34 and 35-36 were used to amplify the 5' and 3' fragments, respectively. PCR tests included (in 25 µl of reaction volume) 30 ng of DNA, 10 pmol oligonucleotide primers, 12.5 mM dNTPs, 1 µl of *Taq* polymerase in 10× PCR buffer, and double-distilled water. 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 *NotI* to release the deletion construct.

Electroporation of germinating conidia was performed essentially as previously described (Horowitz et al. 2002; Yakoby et al. 2001). Briefly, Cg-14 isolates were cultured on solid M₃S medium for 14 days. Conidia were collected in pea juice (Robinson and Sharon 1999), adjusted to 10⁶ conidia/ml, and incubated at 28°C for 2 to 3 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 10⁸ conidia/ml, and 100-µl aliquots 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 80 mg/liter, and DNA was extracted with the Master Pure yeast purification kit (Epicentre Biotechnologies, Madison, WI, U.S.A.).

Gene deletion analysis. *GLT* gene-disruption events were first analyzed by PCR screening for integration of the *GLT5'-Hyg-GLT3'* cassette at the *GLT* locus (Fig. 5A and C). For that purpose, primer pairs 1-35 and 2-36 were designed on the basis of the region flanking the disruption cassette at the 5' and 3' ends of the Hyg cassette (Fig. 5A and C), respectively. Primer set 1-33 was designed to analyze ectopic integration strains (Fig. 6A). In the case of homologous integration, 1,995- and 900-bp amplicons from the 5' and 3' region, respectively, were observed in transformants *Δgl₅*, *Δgl₆*, and *Δgl₃₁*, indicating integration of the disruption cassette into the *GLT* locus (Fig. 6A). In the case of ectopic integration, an 830-bp amplicon was observed (Fig. 5A). The *18S* ribosomal gene was used for WT DNA quality control resulting in a 150-bp amplicon (Fig. 5A). All PCR fragments were sequenced for verification. Gene expression of *GLT* by qRT-PCR (Fig. 5B) was detected in WT progenitor Cg-14 and ectopic₁₀ strains but not in *Δgl₅*, *Δgl₆*, and *Δgl₃₁* strains, confirming the functionality of the disruption.

Gene-disruption events for *MEPB* were first analyzed by PCR screening for integration of the *MEPB5'-Hyg-MEPB3'* cassette at the *MEPB* locus (Fig. 8A and C). Primer pairs 1-19 and 2-20 were designed on the basis of the region flanking the disruption cassette at the 5' and 3' ends on the Hyg cassette (Fig. 8A and C), respectively. Primer set 1-15 was designed to analyze ectopic integration strains (Fig. 8A). In the case of homologous integration, 990- and 750-bp amplicons from the 5' and 3' regions, respectively, were observed in transformants *ΔmepB₃* and *ΔmepB₁₂*, indicating integration of the disruption cassette into the *MEPB* locus (Fig. 8A). In the case of ectopic

integration, an 890-bp amplicon was observed (Fig. 8A). An *18S* ribosomal gene was used for WT DNA quality control, resulting in a 150-bp amplicon (Fig. 8A). All PCR fragments were sequenced for verification. Gene expression of *MEPB* by qRT-PCR (Fig. 8B) was detected in WT progenitor Cg-14 and ectopic₅ strains but not in *ΔmepB₃* or *ΔmepB₁₂* strains, confirming the functionality of the disruption.

Generation of complementation construct. Transformant *ΔmepB₁₂*, a representative *MEPB* gene-replacement strain, was utilized as a recipient for complementation via transformation with the pDEST-Phleo vector, which contains the intact genomic *MEPB*, including 1,000 bp of native promoter and 300 bp of native terminator (accession number GH270299) and a marker conferring resistance to phleomycin. Resistant transformants were recovered, and three strains were analyzed by PCR using primer pairs 3-22 and 4-21, with primers 21 and 22 designed on the basis of the regions on the phleomycin cassette. In the case of positive integration, a 1,000-bp amplicon was observed in transformants *ΔmepB₁₂comp_{1,13}*, indicating integration of the complementation cassette into strain *ΔmepB₁₂* (Fig. 4A and D).

Genomic DNA extraction. Fungal cells were inoculated into liquid M₃S for 3 to 5 days at 28°C. For PCR analysis, 50 mg of wet tissue was used following the protocol of the Master Pure kit (Epicentre Biotechnologies). For Southern analysis, genomic DNA was prepared following the protocol of Möller and associates (1992) using 0.5 g of wet tissue.

Genome sequence and phylogenetic analysis.

A draft genome sequence of *C. gloeosporioides* was obtained by sequencing total genomic DNA to a depth of approximately 8× using the Roche 454 Titanium platform. Gene models were annotated using GeneMark (Ter-Hovhannisyan et al. 2008) and all of the genes used in this analysis were deposited in GenBank. Other sequenced genomes are publicly available from the National Center for Biotechnology Information.

The phylogenetic tree of the *MEP* gene family was constructed with PHYML software (Guindon and Gascuel 2003) using the JTT model and optimized for tree topology, branch length, and rate of evolution. Tree support was evaluated using a bootstrap test with 100 replications.

Accession numbers.

Sequence data from this study of each gene and its promoter from *C. gloeosporioides* can be found in the EMBL and GenBank data libraries under the following accession numbers: *GLT*, JN660151; *GDH2*, JN660152; *GSI*, JN660153; *MEPA*, JN660155; *MEPB*, JN660156; *MEPC*, JN660157; and *MEPD*, JN660154. The accession numbers for the *GDH2* genes are *Magnaporthe*, XP_399530; *Fusarium*, EUG76650.1; *Sclerotinia*, SS1G_00002; *Botrytis*, BC1G_15454.1; and *Aspergillus*, An02g14590. The accession numbers for other genes were obtained from the Broad Institute published database. The accession numbers for the *GSI* genes are *Magnaporthe*, XP_001402868; *Fusarium*, FOXG_05182; *Sclerotinia*, XP_001588876; *Botrytis*, BC1G_12011; and *Aspergillus*, An01g-08800. The accession numbers for the *GLT* genes are *Fusarium*, FOXG_03264.2 and *Magnaporthe*, XP_367728.1. The accession numbers for the *MEP* genes, other than of *Colletotrichum*, are as follows. *A. niger*: *AnMEPA* XP_001392437, *AnMEPC* XP_001400572, *AnMEPD* XP_001389574, and *AnMEPD2* XP_001400856; *B. cinerea*: *BcMEPA* CCD52297, *BcMEPC* CCD45586, *BcMEPD* CCD34682; *F. oxysporum*: *FOXMEPB* EGU79127, *FOXMEPC* EGU78832, *FOXMEPD* EGU75233, *FOXMEPD2* EGU81805; and *S. sclerotiorum*: *SsMEPA* XP_001592360, *SsMEPC* XP_001594694, and *SsMEPD* XP_001593457.

ACKNOWLEDGMENTS

This research was supported, in part, by research grant number IS-3945-06 from BARD, the United States-Israel Binational Agricultural Research and Development Fund. Part of the genome sequencing of *C. gloeosporioides* was funded by BARD projects. We thank L. Benito, University of Salamanca, Spain, for supplying *Botrytis* MEP sequences.

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