Differential Activation of Ammonium Transporters During the Accumulation of Ammonia by *Colletotrichum gloeosporioides* and Its Effect on Appressoria Formation and Pathogenicity

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Ammonium secreted by the post-harvest pathogen *Colletotrichum gloeosporioides* during host colonization accumulates in the host environment due to enhanced fungal nitrogen metabolism. Two types of ammonium transporter-encoding genes, *AMET* and *MEP*, are expressed during pathogenicity. Gene disruption of *AMET*, a gene modulating ammonia secretion, showed twofold reduced ammonia secretion and 45% less colonization on avocado fruit, suggesting a contribution to pathogenicity. *MEPB*, a gene modulating ammonium transport, is expressed by *C. gloeosporioides* during pathogenicity and starvation conditions in culture. Gene disruption of *MEPB*, the most highly expressed gene of the *MEP* family, resulted in twofold overexpression of *MEPA* and *MEPC* but reduced colonization, suggesting *MEPB* expression's contribution to pathogenicity. Analysis of internal and external ammonia accumulation by *ΔmepB* strains in mycelia and germinated spores showed rapid uptake and accumulation, and reduced secretion of ammonia in the mutant versus wild-type (WT) strains. Ammonia uptake by the WT germinating spores but not by the *ΔmepB* strain with compromised ammonium transport activated cAMP and transcription of *PKA* subunits *PKAR* and *PKA2*. *ΔmepB* mutants showed 75% less appressorium formation and colonization than the WT, which was partially restored by 10 mM exogenous ammonia. Thus, whereas both *AMET* and *MEPB* genes modulate ammonia secretion, only *MEPB* contributes to ammonia accumulation by mycelia and germinating spores that activate the cAMP pathways, inducing the morphogenetic processes contributing to *C. gloeosporioides* pathogenicity.

pH modulation by pathogenic fungi of the host environment has been shown to be a significant factor for modulation of pathogenicity in several fungi via either alkalization or acidification as a result of ammonia or organic acid secretion, respectively (Prusky and Yakoby 2003). The extent of the pH

change in the plant tissue depends on host buffer capacity and initial pH (Alkan et al. 2008; Kramer-Haimovich et al. 2006; Prusky et al. 2001). The pathogen's ability to alter pH locally was initially described for *Sclerotinia sclerotiorum* but similar findings were then extended to other pathogens, such as *Colletotrichum* spp., *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum*, *P. digitatum*, *P. italicum*, *Phomopsis mangiferae*, and *Fusarium oxysporum* (Barad et al. 2012; Davidzon et al. 2010; Eshel et al. 2002; Manteau et al. 2003; Miyara et al. 2010, 2012; Prusky et al. 2001, 2004; Rollins and Dickman 2001). The wide number of species modulating host pH stresses the importance of this mechanism during fungal colonization (Miyara et al. 2012).

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. is a necrotrophic ascomycete fungus that secretes ammonia under inductive environmental conditions during fruit colonization as a mechanism for the induction of alkalization during pathogenicity (Diéguez-Urbeondo et al. 2008; O'Connell et al. in press; Prusky and Yakoby 2003). Ammonium accumulation has been detected in association with host alkalization and pathogenicity of many *Colletotrichum* spp., including *C. gloeosporioides*, *C. acutatum*, *C. higginsianum*, *C. graminicola*, and *C. coccodes* (Alkan et al. 2008; Diéguez-Urbeondo et al. 2008; Miyara et al. 2010; O'Connell et al. 2012), *A. alternata* (Eshel et al. 2002) and *F. oxysporum* (Miyara et al. 2012). The roles suggested for the ammonia accumulated during *Colletotrichum* colonization include i) activation of fungal pathogenicity factors such as pectate lyase (Prusky and Yakoby 2003), ii) induction of host-cell death and host response during necrotrophic development (Alkan et al. 2008, 2009, 2012), and iii) induction of appressorium formation during host penetration (Miyara et al. 2010).

Macroarrays carrying *C. gloeosporioides* cDNA expressed during the alkalization process have been used to monitor the genes expressed during ammonia metabolism (Miyara et al. 2010). Genes expressed during early stages of alkalization at pH 4.0 include *GDH2* encoding NAD⁺-specific glutamate dehydrogenase and *AMET* encoding for a protein with homology to an ammonia transporter, whereas *MEPB*, *GLT*, and *GSI*, encoding for protein sequence homology to methyl ammonia permease, glutamate transporter, and glutamine synthetase, respectively, are induced at increasing pH close to 7.0 (Miyara et al. 2010). The importance of ammonia in pathogenicity has been studied via modulation of ammonia production. Reduction of ammonia production by *nit*⁻, *areA* or *Agdh*

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mutant strains elicits a reduction in host colonization (Alkan et al. 2009; Miyara et al. 2010). Some mutants, such as the *Agdh2* strains of *C. gloeosporioides* and *Magnaporthe oryzae* and the *AmepB* of *C. gloeosporioides*, show reduced appressorium formation as well (Miyara et al. 2010, 2012; Oh et al. 2008). Miyara and associates (2012) analyzed a *AmepB* mutant strain for the contribution of the *MEPB* gene to ammonia uptake and accumulation by *C. gloeosporioides* in comparison with the *GLT* gene modulating glutamate transport; both mutants inhibited appressoria formation and pathogenicity.

In the present study, we compared the ammonia uptake or secretion by two groups of genes encoding for ammonia transporters, *AMET* and *MEPA*, *MEPB*, and *MEPC*, and analyzed the effect of gene knockout on ammonia accumulation, induction of appressorium formation, and pathogenicity of *C. gloeosporioides*. Functional analysis of *AMET* mutants suggested that this gene encodes a protein with minimal effects on ammonia uptake and accumulation in the hyphae. *AMET* contributes to the pathogenicity of *C. gloeosporioides* by its effect on ammonia secretion. Functional analysis of the *MEPB* mutant of *C. gloeosporioides* indicated that, of the *MEP* genes encoding for a methylammonium permease, *MEPB* showed the main regulation of the influx, efflux, and accumulation of ammonia in the fungal hyphae. Because previous data had indicated the importance of cAMP-PKA pathways as signaling factors for the induction of appressorium formation (Gold et al. 1997; Kronstad 1997; Lee et al. 2003; Takano et al. 2000; Taylor et al. 1990), we used the *AmepB* mutant strain to show that processes involving ammonia uptake and cAMP activity occur concurrently and enhanced ammonia accumulation activated PKA subunits *PKA2* and *PKAR*. These results suggest that the balance between uptake and secretion of ammonia by germinating spores may induce signaling pathways that enhance appressorium formation, com-

pared with the developing mycelia where the secretion of ammonia and host alkalization contribute mainly to necrotrophic colonization by *C. gloeosporioides*.

RESULTS

Functional analysis of *C. gloeosporioides* *AMET*: Effect on ammonia export and pathogenicity.

The sequences of the *Amet* proteins in *C. gloeosporioides* were analyzed in the conserved domains database of the National Center for Biotechnology Information and found to belong to the GPR1/FUN34/yaaH family, with an *e* value of $9.25e^{-34}$. The single copy of *AMET* found in *C. gloeosporioides* preliminary genome sequence (D. Prusky, unpublished) showed 90% homology to FUN34 of *Glomerella graminicola* and FUN34 of *C. higginsianum*. Promoter analysis of the 1,000-bp region upstream of the 5' untranslated region (UTR) of the *AMET* sequence revealed 13 putative *CREA* binding motifs, one *AREA* binding motif that might modulate ammonia export (Monahan et al. 2006), and no putative *PACC* binding motifs.

To assess the function of *AMET* in *C. gloeosporioides*, gene knockout was performed to generate *Δamet* strains (Fig. 1A) in which the intact *AMET* was replaced with an *AMET5'-Hyg-AMET3'* cassette, as described by Shafran and associates (2008). Deletion of the gene was confirmed by polymerase chain reaction (PCR) (Fig. 1B). Expression of *AMET* was detected by quantitative reverse-transcriptase PCR (Fig. 1C) in the wild-type (WT) progenitor strain Cg-14 and ectopic₄ strains, whereas minimal levels of expression were detected in *Δamet*₃ or *Δamet*₁₁ strains, confirming deletion of *AMET*.

Characterization of the *AMET* mutants in solid media showed that none of them are affected in fungal growth in vitro or in spore germination. To characterize the mutants in

Fig. 1. Development of *AMET* mutant strains. A double crossing-over homologous recombination event resulting in replacement of the original *AMET* sequence with the *AMET5'-Hyg-AMET3'* cassette was carried out (Miyara et al. 2012). **A**, Scheme depicting gene disruption by homologous recombination. Primer pairs 13-14 were used for the 5' end and 15-16 for the 3' end. **B**, Polymerase chain reaction (PCR) analysis of the wild-type (WT) strain, ectopic colony (Ectopic₄ and Ectopic₁₇), and independent *AMET*-disruption colonies (*Δamet*). Primer 17 flanking a position upstream of the *AMET2:HYG3* region and reverse primer 11 located on the hygromycin cassette were used to identify positive *AMET* gene replacement at the 5' locus. Primer 12 from the hygromycin cassette and primer 18 flanking the *AMET:HYG3* region were used to identify *AMET* gene replacement at the 3' locus. *18S* gene primers 1 and 2 were used for WT DNA quality control. Primers 11 and 13 were used as a positive control for the ectopic strains, to confirm random integration of the *AMET:HYG3* cassette. **C**, Expression of *AMET* in the WT strain (relative expression to value 1) compared with the ectopic-integration control and the *Δamet* 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.

liquid media, 3-day hyphal mats of the *Δamet* isolates were transferred from the initial M₃S medium to ammonia-inducing secondary medium (SM) amended with 10 mM KNO₃. Under these conditions, the concentration of ammonia accumulated in the growth culture was reduced by almost 50% in all the mutant strains (from 2.8 to 1.35 mM ammonia per gram dry weight of fungal mycelium [hereafter mM g⁻¹]) 24 h postinoculation of SM. At the same time, the pH of the medium increased from 4.0 to 6.0 for the WT and ectopic strains, whereas it only increased to 5.5 for the *Δamet* strains (Fig. 2), suggesting that *AMET* knockout affects the secretion of ammonia from growing fungi. If the *Δamet* isolates were transferred from the initial M₃S medium to SM amended with 10 mM NH₄Cl, the average uptake of ammonia from the media increased 6 h after transfer by 20% by both *Δamet*₁₁ and *Δamet*₃ strains compared with the WT and ectopic *Δamet*₄ strains. However, 3 h later, no difference in the remaining ammonia in the media between the mutant and the controls was observed, suggesting only a minor capability for the *AMET* knockout to affect the uptake of ammonia from growing fungi. This was confirmed by the lack of differential accumulation of ammonia inside the hyphae 9 h after transfer of the *Δamet* and control strains to the SM amended with NH₄Cl. Pathogenicity assays of *Δamet*₃ and *Δamet*₁₁ strains compared with the WT showed a 42% reduction in avocado fruit colonization (Fig. 3), indicating the contribution of *AMET* to *C. gloeosporioides* colonization. No effect was observed also on the amount of appressoria formed by the *Δamet* strains when the germination occurred on avocado fruit pericarp, suggesting that attenuation of fungal colonization on fruit results from the reduced ammonia production.

Expression of *C. gloeosporioides* ammonium permeases *MEPA*, *MEPB*, and *MEPC* and their effect on pathogenicity.

The sequences of the three Mep proteins in *C. gloeosporioides* were analyzed by PSORT and TMHMM and were found to share 55 to 60% homology. Protein analysis predicted nine transmembrane-spanning domains (TMD) in *MEPB* and 11 TMD each in *MEPA* and *MEPC*. Promoter analysis of the 1,000-bp region upstream of the 5' UTR of the *MEPB* sequence revealed 17 putative *CREA*-binding motifs, seven *AREA*-binding motifs, and two putative *PACC*-binding motifs. In the *MEPA* promoter, 17 *CREA*-, three *AREA*-, and no *PACC*-binding motifs were found whereas, in the *MEPC* sequence, only a single *CREA*-binding site was found in the promoter region (only 184 bp upstream of the 5' UTR of *MEPC* were analyzed).

Growth of *C. gloeosporioides* in culture under nitrogen starvation (no N) at pH 4 or 7 showed that, at both pH values, the relative expression of *MEPB* is approximately three to 50 times greater than that of *MEPA* or *MEPC* (Fig. 4). Growth of the WT strain in the presence of 10 mM NH₄Cl severely repressed expression of all *MEP* genes, regardless of pH (Fig. 4).

To functionally analyze the effects of *MEPA*, *MEPB*, and *MEPC* on the balance between ammonia uptake and secretion by the fungus, the levels of ammonia were quantified in the mycelium and freshly produced spores of *C. gloeosporioides*. A 3-day hyphal mat of the *Colletotrichum* isolates was transferred from the initial M₃S medium to SM amended with NH₄Cl (Fig. 5A). At 6 h postinoculation, ammonia concentration in the growth media of the previously developed *ΔmepB* strains (Miyara et al. 2012) declined from 25 to 6 mM g⁻¹, whereas it remained steady in the WT, ectopic, and complemented strains. Then, 3 h later, the level of ammonia in the media had declined for the WT, ectopic, and complemented strains as well—to 6 mM g⁻¹—and the ammonia concentration remaining in the growth media of the *ΔmepB* strains had further decreased to the almost undetectable level of 0.01 mM g⁻¹ (Fig. 5A).

Analysis of ammonia level inside the hyphae showed the opposite behavior (Fig. 6B). Whereas, in the WT, ectopic, and

Fig. 3. Pathogenicity assays of *Colletotrichum gloeosporioides* wild-type (WT), ectopic, and *Δamet* mutant strains. Decay development of WT and mutant strains after inoculation of the pericarp of freshly harvested 'Fuerte' avocado fruit. Inoculation was carried out by placing spore suspension on the pericarp of unwounded fruit followed by incubation at 25°C under high humidity. Average colonized area for 10 infected fruit 10 to 15 days after inoculation is reported. Values within averages marked with different letters at each sampling time differ significantly according to Tukey-Kramer honestly significant difference test at $P \leq 0.05$.

24 hours in secondary media

Fig. 2. Ammonia accumulation and pH changes induced in the wild-type (WT) and *Δamet* *Colletotrichum gloeosporioides* strains in culture medium. Spores of *C. gloeosporioides* were inoculated in primary medium and 3 days later, transferred to secondary medium amended with 10 mM KNO₃, and then analyzed 24 h postinoculation (hpi). Average $\pm$ standard deviation of three replications of one experiment out of three repeated experiments is presented. Values within averages marked with different letters at each sampling time differ significantly according to Tukey-Kramer honestly significant difference test at $P \leq 0.05$. OD, optical density; DW, dry weight.

complemented strains, the concentration of ammonia in the mycelium decreased dramatically from 78 to 26 mM g⁻¹ during the first 6 h postinoculation, in the $\Delta mepB$ strains, it remained steady (within the range of 60 to 75 mM g⁻¹), indicating accumulation of ammonia in these mutants (Fig. 5B). Then, 3 h later, the level of ammonia in the $\Delta mepB$ strain had declined to 45 mM g⁻¹ and that in the WT and ectopic strains to approximately 40 mM g⁻¹ (Fig. 5B).

Analysis of the relative expressions of *MEPA*, *-B* and *-C* (Fig. 5D to F) in the WT in the inducing media showed that the initial expression of *MEPB* is 4.5- and 9.0-fold greater than that of *MEPA* and *MEPC*, respectively (Fig. 5D to F). At 6 h after culture inoculation of $\Delta mepB$, relative expressions of its *MEPA* and *MEPC* were upregulated by three- and 15-fold, respectively, compared with their expression in the WT strain. These results suggest that the increased expression of *MEPC* does not contribute to the secretion of ammonia and the produced ammonia remains restricted to the fungal mycelia.

Similar results were observed when the expressions of *MEPA*, *MEPB*, and *MEPC* were analyzed in inoculated avocado fruit. Highest expression of *MEPB* was also observed when the mycelia of the WT *C. gloeosporioides* colonized avocado pericarp (Fig. 6A). The relative expression of *MEPB* in the WT strain in the colonized avocado tissue was 2 and 1.25 times greater than that of *MEPA* and *MEPC*, respectively (Fig. 6A). When the $\Delta mepB$ strain was used to inoculate avocado pericarp, the relative expression of *MEPA* and *MEPC* by

Fig. 4. Expression analysis of *MEPA*, *MEPB*, and *MEPC* under NH_4Cl and pH growth conditions. Three-day-old mycelium grown in M_3S was transferred to secondary medium at pH 4.0 or 7.0, amended with 10 mM NH_4Cl , or not amended ("No nitrogen" treatment). Samples were taken 9 h postinoculation for relative expression determination by quantitative reverse-transcriptase polymerase chain reaction. Relative expressions of the different treatments were compared with that of the *MEPA* in the wild-type (WT) strain, which was given a value of 1. Values were normalized against 18S rRNA. Average $\pm$ standard deviation of relative expression from three technical replications of one treatment is presented.

the $\Delta mepB$ strain was 50 and 40%, respectively, greater than their expression in the WT, paralleling an 87% reduction in fruit colonization (Fig. 6B) and indicating the importance of *MEPB* in pathogenesis.

The increased internal ammonia accumulation by the $\Delta mepB$ strains raised the question of a possible mechanism for detoxification by the pathogen. Stephenson and associates (1997) described a mechanism for ammonia detoxification via the activation of glutamate synthetase (GS). Analysis of *GS* during fungal growth in inducing media showed two- to four-fold higher relative expression levels during the first 6 h in the hyphae of the $\Delta mepB_{12}$ isolate (Fig. 5C), suggesting that the process of glutamate enhancement is induced in the $\Delta mepB_{12}$ strain.

Reduction of ammonia secretion by germinating spores inhibited appressorium formation in the $\Delta mepB$ strains.

Activity of the nitrogen metabolism that modulates ammonia accumulation in germinating spores differed from that in the mycelium (Fig. 5). In germinating spores of the WT strain, continuous secretion of ammonia was observed during the first 9 h of germination, in contrast to lack of secretion in the $\Delta mepB$ strains, suggesting that the spores of the mutant strains were impaired in ammonia-secretion capability (Fig. 7A). Analysis of the internal ammonia concentrations showed a buildup from 9 to 42 mM per 10^6 spores in the WT strains in the first 9 h after initiation of germination (Fig. 7B). In the first 6 h of germination, the internal ammonia level in the $\Delta mepB$ strains remained higher, 42 to 43 mM per 10^6 spores (Fig. 7B). The increased accumulation of ammonia led us to search for a possible mechanism of detoxification of ammonia accumulated inside the spore. GS relative expression in the germinating spores of the $\Delta mepB_{12}$ isolate was fivefold higher than in the WT strain during the first hour of germination, suggesting that accumulation of ammonia (Fig. 7C) activates the enhancement of GS transcript expression levels. Accumulation of high internal levels of ammonia in pre- and post-germinated $\Delta mepB_{12}$ spores, in contrast to the WT, was easily confirmed by detection of strong fluorescence following treatment with the alkaline fluorescence dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (Fig. 8).

The effect of the external accumulation of ammonia by *C. gloeosporioides* germinating spores on mycelial growth, conidial germination, and appressorium formation was determined by comparing ammonia-secreting strains—the WT and complemented strain—to the nonsecreting strains $\Delta mepB_3$ and $\Delta mepB_{12}$. Mycelial growth of $\Delta mepB_3$ and $\Delta mepB_{12}$ strains on water or SM amended with 5 mM NH_4Cl did not affect fungal growth (results not shown) or spore germination compared with the WT, ectopic5, and complemented strains (Table 1). However, germinated $\Delta mepB$ strain spores showed 75% inhibition of appressorium formation compared with the WT and ectopic strains (Table 1). Pharmacological complementation of the $\Delta mepB$ strains by addition of 5 mM volatile NH_4Cl to germinated spores enhanced the percentage of appressoria formed by 20% (Table 1). This differential appressorium formation affected overall fungal colonization, as reflected by the diameters of the $\Delta mepB_3$ and $\Delta mepB_{12}$ strains' colonies on the pericarp of avocado fruit, which were 78% smaller than those of the WT 7 days after inoculation: 4 ± 0.1 mm² compared with 18 ± 0.2 mm² (Fig. 6B).

Based on early reports describing the impact of the cAMP-PKA pathways on appressorium formation (Kojima et al. 2002; Lee and Dean 1993, 1994; Takano et al. 2000; Taylor et al. 1990), we used the non-ammonium-secreting spores of the $\Delta mepB$ strains with exogenous ammonia treatment as a

tool to analyze the mechanism of induction of appressorium formation. Analysis of ammonia secretion of germinating spores of WT and complemented strains treated with exogenous NH_4Cl showed rapid uptake and time-dependent resecretion of ammonia to the medium (Fig. 9A and C) accompanied by enhanced cAMP activity (Fig. 9D and F), in contrast to the slow release of ammonia and low cAMP activity (Fig. 9B and E) in untreated WT spores. Exogenous ammonia treatment of ΔmepB spores did not enhance ammonia uptake or induce cAMP activity (Fig. 9B and E), suggesting the importance of ammonia uptake for cAMP activation during spore germination.

To further evaluate the role of ammonia on the PKA pathway of germinated spores, transcript activation of the genes *PKAR* (encoding the PKA regulatory subunit) (Kamaruddin et al. 2008) and *PKA2* (encoding a PKA catalytic subunit) (Liebmann et al. 2004) were compared in the ammonia-secreting WT strain and a nonsecreting strain, ΔmepB_{12} . Transcript activation in both strains was compared 8 h after initiation of germination when no ammonia was produced by the ΔmepB_{12} strain (Fig. 10). Analysis of the relative expressions of *PKA2* and *PKAR* showed 20- and 30-fold upregulation, respectively, in contrast to inhibited expression in the ΔmepB_{12} strain (Fig. 10), suggesting that ammonia uptake may activate the PKA pathway as well.

DISCUSSION

C. gloeosporioides ammonium transporters.

The *Colletotrichum* sp. alkalinizes its immediate environment during colonization by secreting large amounts of ammonium ions (Drori et al. 2003; Kramer-Haimovich et al. 2006; O'Connell et al. 2012). The dynamic nature of the system, which involves modulating ammonia export and import, indicates that the secreted ammonia is reassimilated by the ammonium transporters of *C. gloeosporioides* (Miyara et al. 2012). The *C. gloeosporioides* *AMET* gene encoding for an ammonium transporter showed 90% homology to FUN34 of *C. graminicola* and *C. higginsianum* (O'Connell et al. 2012). Three *C. gloeosporioides* genes of the *MEP* family—*MEPA*, *MEPB*, and *MEPC*—also shared 55 to 60% homology with published sequences of methyl ammonium transporters (Miyara et al. 2012). Protein analysis predicted the presence of 11 TMD in *MEPA* and *MEPC*, similar to the predictions in *Saccharomyces cerevisiae* (Lengeler et al. 2000; Rohde and Cardenas 2004), whereas only nine TMD were predicted for *MEPB*.

MEP genes are strictly regulated by the nitrogen regulator *AREA* (Teichert et al. 2008). Similarly, the four *AMT/MEP* genes of *Aspergillus nidulans* are differentially regulated in response to nitrogen status of the cell but their full expression requires the function of the GATA transcription factor *AREA*

Fig. 5. Ammonia uptake and secretion and *MEPA*, *MEPB*, and *MEPC* expression analysis for mycelia of *Colletotrichum gloeosporioides* wild-type (WT), ectopic, complemented, and ΔmepB strains. **A**, Ammonia concentration in the growth medium (mM ammonia per gram dry weight of mycelium). **B**, Ammonia concentration inside the fungal mycelium (mM ammonia per gram dry weight of mycelium). **C**, Relative expression of glutamine synthetase (*GS*). **D**, **E**, and **F**, Relative expressions of *MEPA*, *MEPB*, and *MEPC*, respectively. Relative expressions for the different treatments were compared with the lowest expression in the different treatments, which was given a value of 1. Spores of *C. gloeosporioides* were inoculated into primary medium and, 3 days later, mycelium was transferred to secondary medium amended with 10 mM NH_4Cl and analyzed at various intervals postinoculation. Average $\pm$ standard deviation of three technical replications of one single biological experiment out of three repeated experiments is presented.

(Monahan et al. 2006). Promoter analysis of *AMET* and *MEPB* in *C. gloeosporioides* indicated the presence of one and seven *AREA*-binding motifs, respectively, suggesting their possible regulation in *C. gloeosporioides* as well (Monahan et al. 2006). To further demonstrate the importance of *AREA* in ammonia export, Alkan and associates (2009) developed *Colletotrichum ΔareA* mutant strains that were impaired in ammonia secretion and attenuated in decay development. This suggested that modulation of ammonia accumulation by either *AMET* and *MEPB* or the transcription factor *AREA* is a critical factor contributing to *Colletotrichum* pathogenicity on fruit. However, *MEPB* is also regulated by *PACC* during the alkalization process induced by ammonia and is downregulated in *ΔpacC* mutants, indicating the presence of more than one mechanism of nitrogen-metabolism regulation during the colonization process of *Colletotrichum* spp. (Miyara et al. 2010).

Functional analysis of *C. gloeosporioides* *AMET* and *MEPB* genes encoding for ammonium transporters by gene knock-out showed mutant strains with reduced pathogenicity on avocado fruit and confirmed early results suggesting that attenuation of ammonia secretion reduces pathogenicity (Alkan et al. 2008; Miyara et al. 2010). Comparison of the attenuation of pathogenicity by the *AMET* and *MEPB* knockout strains indicated that, whereas the differences in colonization between the WT and *ΔmepB* strains were observed within 5 to 7 days postinoculation with up to 80% inhibition of pathogenicity, differences between the WT and *Δamet* strains were observed

Fig. 6. Expression analysis of *MEPA*, *MEPB*, and *MEPC* in the wild-type (WT) and *ΔmepB3* strains on inoculated pericarp of 'Fuerte' avocado fruit. **A**, Six days after inoculation of the WT, ectopic, and *ΔmepB* strains on avocado pericarp, samples for relative expression determination by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) were taken from inoculated fruit and **B**, the colonized area was evaluated. Values of RT-PCR were normalized against *18S* rRNA. Relative expressions in the different treatments were compared with that of *MEPA* in the WT strain, which was given a value of 1. Average $\pm$ standard deviation of relative expression from three replications of one treatment is presented. The figure presents decay development of WT, mutant *ΔmepB3* and *ΔmepB12*, and *Ectopic6* strains after inoculation (on the pericarp) of unpeeled freshly harvested avocado fruit. Average $\pm$ standard deviation of colonized areas on 10 infected fruit after inoculation is presented.

13 to 15 dpi with only 50% inhibition of pathogenicity, suggesting a stronger contribution of ammonia accumulation by the *MEPB* versus *AMET* genes.

In *C. gloeosporioides*, the relative expression of *MEP* genes during growth in vitro under conditions of pathogenicity clearly showed overexpression of *MEPB* in all cases; the highest tran-

Fig. 7. Ammonia accumulation by spores of *Colletotrichum gloeosporioides* wild-type (WT), ectopic, complemented, and *ΔmepB* strains. **A**, Outside the germinating spores. **B**, Inside the germinating spores. **C**, Glutamate synthase (*GS*) transcript expression. Spores were subjected to germination for 1 to 9 h in water on a stable hard surface and the amounts of ammonia secreted and accumulated in the spores were analyzed after various intervals postgermination. Average $\pm$ standard deviation of three technical replications of one single biological experiment out of three repeated experiments is presented.

Fig. 8. Alkalization determined by fluorescence of wild-type (WT) and *ΔmepB12* *Colletotrichum gloeosporioides* spores containing 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein dye 5 h after germination induction on a hard surface. Spores and germinated *ΔmepB12* spores showed enhanced alkalization as determined by the strong fluorescence compared with the WT strain.

script expression was found under starvation, as well as during fungal colonization. However, *MEPB*-deleted strains revealed fourfold enhanced ammonia uptake into the fungal hyphae and reduced accumulation of ammonia in the medium. This might result from either inactivation of the *MEPB* transporter protein or upregulation of *MEPA* and *MEPC* transcripts. Upregulation of *MEPA* and, especially, *MEPC* was observed when the *ΔmepB* strains were inoculated in cultures (Fig. 5 D to F) and on avocado fruit (Fig. 6), suggesting that they contribute to the internal accumulation of ammonia by the *ΔmepB* strain but they slightly differ, probably because of the wide number of nitrogen sources

Table 1. Percentage germination and appressorium formation by wild-type (WT) and *ΔmepB* strains in the presence of water and volatile ammonia^a

Strains	Germination (% ± SD)		Appressoria (% ± SD)	
	H ₂ O	NH ₄ Cl	H ₂ O	NH ₄ Cl
WT	93 ± 2	97 ± 1.2	89 ± 3.5	96 ± 1.5
<i>ΔmepB₁</i>	90 ± 1	88 ± 5.1	20 ± 1.5	44 ± 0.5
<i>ΔmepB₁₂</i>	90 ± 2	83 ± 5.5	24 ± 1.5	48 ± 1
Ectopic ₆	92 ± 3	95 ± 5.0	90 ± 3.5	95 ± 1.5

^a Spores were subjected to germination in a 10-μl water drop containing 10⁶ spores ml⁻¹ on a glass slide. In the ammonia treatment (NH₄Cl), 5 mM exogenous volatile ammonia at pH 8.5 was supplied and effects were compared with water control. At 14 h postinduction, the spores were stained with bromophenol blue and the percentages of germination and appressorium formation were determined. Average data ± standard deviation (SD) are presented.

present in the fruit. However, the 80% reduction in avocado pericarp colonization by the *ΔmepB* strains indicated that the pathogenicity of *C. gloeosporioides* is mainly affected by down-regulation of *MEPB* and not by upregulation of *MEPA* and *MEPC*. These results contrast with the effects of a single deletion of three *MEP* genes in *Saccharomyces* spp. or two *MEP*

Fig. 10. Relative expressions of *PKA2* and *PKAR* and ammonia accumulation by germinating *Colletotrichum gloeosporioides* spores. Quantitative relative expression of *PKA2* and *PKAR* 8 h after initiation of germination (i.e., at full germination). Values of reverse-transcriptase polymerase chain reaction were normalized against *18S* rRNA. Relative expression in the different treatments was compared with the lowest value of expression in the respective treatments, which was given a value of 1. Average ± standard deviation of three technical replications of one single biological experiment out of three repeated experiments is presented.

Fig. 9. A to C, Ammonia uptake and efflux and D to F, cAMP activity by germinating *Colletotrichum gloeosporioides* spores of the wild-type (WT), *ΔmepB₁₂*, and complemented strains. Spores of WT, *ΔmepB₁₂*, and complemented strains were subjected to germination for 1 to 14 h in water with added NH₄Cl (5 mM) on a stable hard (glass) surface and, after various intervals, the concentration (pmol) of accumulated cAMP in the spores was determined. Values were normalized against weight (milligrams) of total cell protein. Average ± standard deviation of three technical replications of one single biological experiment out of three repeated experiments is presented.

genes in *Aspergillus* spp., which did not elicit any effect or rendered the strains unable to grow under ammonium concentrations of less than 10 mM (Brendon et al. 2002; Magasanik 2005; Marini et al. 1997).

Do AMET and MEPB participate in nitrogen import and export?

The molecular mechanisms activating transporter-mediated ammonium regulation are not fully known. The fungal ammonium permeases have evolved regulatory functions of activation under starvation conditions, resulting in physiological and morphogenetic processes that affect fungal development. In *S. cerevisiae*, the plasma-membrane-localized permease MEP2 is able to sense environmental nutritional limitations that might modulate pathogenicity (Forsberg and Ljungdahl 2001; van Nuland et al. 2006) or pseudohyphal growth (Lorenz and Heitman 1998). CaMEP2 in *Candida albicans* induces filamentous growth under nitrogen-limited conditions, via activation of both mitogen-activated protein kinase and the Ras1-cAMP-dependent pathways (Biswas and Morschhäuser 2005). Similarly, in *Ustilago maydis*, the expression of two ammonium permease-encoding genes is required for the induction of filamentous growth (Smith et al. 2003).

In most cases, MEP transporters have been assayed by direct ammonia uptake (Javelle et al. 2003; Teichert et al. 2008). This is a simple and efficient assay but is not always representative of the complex processes occurring during uptake and secretion of ammonia (Miyara et al. 2010, 2012) in developing fungal cells. To investigate possible ammonia transport in *C. gloeosporioides*, a real-time assessment of uptake and secretion of ammonia was carried out on developing hyphae and the germinated spores. Functional analysis of *Δamet* and *ΔmepB* strains showed no effect on fungal growth but 50% inhibition of ammonia accumulation in the culture media (Table 2). Knockout of *MEPB* showed immediate, 2.7-fold enhanced ammonia accumulation in the hyphae compared with the WT strains whereas no differential accumulation was observed between the *Δamet* and control strains. This might indicate that, in colonizing mycelia of *C. gloeosporioides*, *MEPB* is an important regulator of efflux or uptake of ammonium and that, in the *ΔmepB* strains, *MEPA* and *MEPC* can contribute to the uptake but not the efflux. The functional reduction of ammonia secretion by *Δamet* and *ΔmepB* strains affects fungal pathogenicity via modulation of ammonia secretion to the colonized tissue, reduction of the alkalization process, and a reduction in fungal gene expression of *Colletotrichum* pathogenicity factors (Table 2) (Alkan et al. 2009, 2012; Miyara et al. 2010).

The balance of ammonia influx and efflux in germinating spores was different from that in the mycelium. Whereas the WT spores exhibited a basic level of ammonia secretion (0.1 mM 10⁶ spores⁻¹) during the 9 h of germination, significant uptake—from 8 to 45 mM 10⁶ spores⁻¹—was observed during the same period. *ΔmepB* mutants did not secrete ammonia; on

the contrary, they showed an immediate accumulation of ammonia inside the spore, as confirmed by fluorescence microscopy, to the levels found in the WT 9 h postinitiation of spore germination. This bidirectional activity of ammonia transporters has been reported in *Salmonella typhimurium* (AmtB), where the capability to take up and release ammonium into the medium by outward diffusion is dependent on growth on an alternative nitrogen source, such as arginine (Javelle et al. 2005; Soupene et al. 1998, 2002). Soupene and associates (2002) claimed that this type of outward diffusion requires a pH of 7.0 and a low concentration of ammonia (≤50 nM) in the culture media. However, the high level of ammonia inside the germinating spores of the *ΔmepB* strain and the lack of ammonia secretion suggest deregulation of ammonia transport. Interestingly, the fungus developed a simple mechanism for ammonia detoxification in both the hyphae and germinating spores, via increased relative expression of *GS*, which also probably contributed to the reduction in outward diffusion of the fungal-produced ammonia by the *ΔmepB* strain.

All of these findings are consistent with the view that ammonium permease proteins increase the rate of ammonium uptake across the cytoplasmic membrane (von Wiren and Merrick 2004) during spore germination, as a mechanism for the induction of appressorium formation (Miyara et al. 2010, 2012). Only those permeases that modulate uptake of ammonia modulate that mechanism of appressoria formation (Table 2). However, the critical role of the cAMP and PKA signaling pathway in appressorium formation and pathogenesis of several fungal pathogens (Adachi and Hamer 1998; Choi and Dean 1997; Gold et al. 1997; Kronstad 1997; Lee et al. 2003; Lee and Dean 1994; Mitchell and Dean 1995; Taylor et al. 1990; Xu 2000) led us to check whether the inhibition of appressorium formation in the mutants with compromised ammonium transport capacity is dependent on lack of activation of the cAMP and PKA signaling process. Our results indicated that the strain with compromised ammonium transport is not able to import ammonia to enhance cAMP activity, exhibits reduced expression of *PKA2* and *PKAR*, and shows reduced appressorium formation compared with the WT strain that exhibited rapid ammonia uptake, increased cAMP activity, enhanced expression of *PKA2* and *PKAR*, and exhibited a high percentage of appressorium formation. These results suggest that ammonia secretion by developing hyphae contributes to necrotrophic colonization by *Colletotrichum* spp. (Miyara et al. 2012; O’Connell et al. 2012) but, at early stages of spore germination, ammonia modulates the induction of appressorium formation as a signal for appressorium formation and pathogenicity (Miyara et al. 2010).

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 (*Persea ameri-*

Table 2. Comparison of the functional activity of MEPB and AMET1

Phenotypic response	Strains	
	<i>ΔmepB</i>	<i>Δamet1</i>
Spore germination	No effect	No effect
Appressoria formation	Inhibited	No effect
Fungal growth	No effect	No effect
Export of ammonia	Inhibited	Inhibited
Import of ammonia	Inhibited	Minor effect
Accumulation and pH increase out of the fungus in the inducing media	Inhibited	Inhibition
Pathogenicity on avocado fruit	Inhibition	Inhibition
Mode of action	Inhibition of appressoria formation and inhibition of alkalization that leads to reduced colonization	Inhibition of alkalization that leads to reduced colonization

cana 'Fuerte') fruit in Israel, and all strains used in the present study (Table 1) were routinely cultured on M₃S agar (Tu 1985). M₃S medium without agar (primary medium) was also used for initial growth of the fungus, prior to its exposure to various nutritional induction factors. The medium contained 2.5 g of MgSO₄ · 7H₂O (Merck, Darmstadt, Germany), 2.7 g of KH₂PO₄ (Merck), 1 g of Bacto peptone (Becton, Dickinson, Sparks, MD, U.S.A.), 1 g of Bacto yeast extract (Becton, Dickinson), 10 g of sucrose (Duchefa Biochemie, Haarlem, the Netherlands), and 25 mg of chloramphenicol (Sigma-Aldrich, St. Louis) per liter. The cultures were incubated for 3 days at 22 to 24°C in a shaking incubator at 150 rpm 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 4 g of KH₂PO₄, 2 g of MgSO₄ · 7H₂O, 0.3 g of CaCl₂ · 2H₂O (Sigma-Aldrich), 10 mg of FeCl₃ (Sigma-Aldrich), and 50 mM glucose (Duchefa Biochemie) per liter. To monitor ammonia consumption, SM was amended with 5 to 10 mM NH₄Cl (Sigma-Aldrich) as a nitrogen source (ammonia-consuming medium). The medium was buffered with 50 mM phthalate-hydroxide buffer (Sigma-Aldrich) to obtain an initial pH between 4.0 and 7.0. The initial pH for each flask was determined after the medium had been autoclaved but prior to inoculation. At least three biological repetitions for each experiment with three technical replicates were carried out. Low differences between the biological repetitions (with 2 to 3% between them) were usually found. A single biological experiment with three technical repetitions showing the differences between treatments are presented for all the experiments.

Analysis of *C. gloeosporioides* MEPB mutants.

Generation of MEPB knockout strains is described in detail elsewhere (Miyara et al. 2012). The transformants were re-grown as single colonies on M₃S agar. For genomic DNA extraction, a plug of grown mycelium was taken for extraction with Pure Yeast DNA Purification Kit Master (Epicenter, New London, CT, U.S.A.).

Fruit inoculation.

Freshly harvested Fuerte avocado fruit from an orchard in Kibbutz Givat Brenner, Israel, were used for inoculation. Spores of the various *C. gloeosporioides* strains were inoculated onto the avocado fruit peel by placing 7 µl of a conidial suspension containing 10⁶ conidia ml⁻¹ on each of three longi-

tudinally spaced inoculation spots on each side of 10 different fruit per treatment (i.e., 60 inoculation replicates per treatment). To inoculate fruit on the mesocarp, a 1- to 2-mm strip of peel tissue was removed and 7-µl aliquots of a conidial suspension containing 10⁶ conidia ml⁻¹ were placed into 1-mm-deep, 1-mm-diameter inoculation spots in the fruit mesocarp. In each treatment, five inoculation spots, longitudinally spaced on each side of 10 different fruit, were inoculated. For detection of gene expression during assessment of avocado decay development, a bulk-inoculation method similar to that described by Alkan and associates (2008) was used. Briefly, 5-day-old mycelium of *C. gloeosporioides* strains was cultured for 3 days in M₃S medium and was then washed twice with sterile water. The washed mycelium was inoculated into holes, 2 mm deep and 5 mm in diameter, pierced into the mesocarp. After inoculation, the fruit were incubated for 24 h at 22°C and 95% relative humidity in covered plastic containers containing wet paper towels, then were further incubated until symptoms were observed. For all experiments, the average decay diameters ± standard deviation are reported. The inoculation experiments were repeated three times, and one representative experiment is presented. The decayed tissue was sliced from the fruit, frozen in liquid nitrogen, and saved at -80°C pending RNA extraction.

Spore-germination and appressorium-induction assay.

For RNA extraction and ammonia detection in germinating spores producing or not producing appressoria (i.e., undergoing "pathogenic" or "saprophytic" germination, respectively, according to Barhoom and Sharon [2004]), 10-ml samples of suspensions of *C. gloeosporioides* conidia containing 10⁶ conidia ml⁻¹ of double-distilled water (DDW) were spread onto 20-cm-diameter petri dishes and were incubated for 12 to 14 h. The germinated conidia were then harvested by scraping them off the petri dishes and centrifuging at 3,200 × g for 20 min. The pellet containing conidia and appressoria was lyophilized and subjected to RNA extraction. The supernatant was used for ammonia detection. For appressorium-formation and maturation-detection experiments, *C. gloeosporioides* WT and MEPB-disruption isolates were observed on glass slides. Briefly, 5 to 25 µl of a conidial suspension of each isolate in DDW, containing 10⁶ conidia ml⁻¹, was placed on each of five glass slides that were kept on filter paper moistened with DDW or volatile ammonia in a humid chamber. After 12 to 16 h of incubation, conidial germination and appressorium development were observed in three microscope fields in each of five cells per slide. The spores were stained with bromophenol blue

Table 3. Primers used in this study

Number	Primer name ^a	Sequence 5'-3'
1	Cg 18S F	GTG AGG CCC TCA AAG GTA GTG G
2	Cg 18S R	GGA TCC CAG TGC GAG ACG T
3	Cg qRT-PCR MEPA F	AGC CTC GTT GTC CAT GTC ATT C
4	Cg qRT-PCR MEPA R	AGG CCG AGA TTC TAG GTA TCG AC
5	Cg qRT-PCR MEPB F	ATG GTC TTC GCC TTC ATC TGG
6	Cg qRT-PCR MEPB R	AGG AAA TGT GGA CGG GAG TA
7	Cg qRT-PCR MEPC F	ACA CCG TCG AGA CCG ATA AT
8	Cg qRT-PCR MEPC R	CGA TGA CTC CCT CGA TGT CT
9	Cg qRT-PCR MAPCK1 F	GTT GCA TGT GCG CCT GG
10	Cg qRT-PCR MAPCK1 R	TGG TAC CGT GCT CCC GAG
11	HYG 5'R ctrl	GCC TGG ACG ACT AAA CCA AA
12	HYG 3'F ctrl	CTG GAT CCA TCT CCT TGC AT
13	AMET 5'F	GGG GAC AAC TTT GTA TAG AAA AGT TGG CGG CCG CGG ACG TGC ATA CAT CTG GTG
14	AMET 5'R	GGG GACTGC TTT TTT GTA CAA ACT TGC GGT TCG TAT TGC GTT TCT T
15	AMET 3'F	GGG GAC AGC TTT CTT GTA CAA AGT GGA CCA TTG GAT TAC AGG GAG AT
16	AMET 3'R	GGG GAC AAC TTT GTA TAA TAA AGT TGG CGG CCG CAG GAT GAT TAG CAA ATG GAA TC
17	AMET 5'F ctrl	AAT GGC CAC GCA CGA G
18	AMET 3'F ctrl	CCC TAT TGC CAA AGC ACA TT

^a qRT-PCR, primer used in real-time quantitative reverse-transcriptase polymerase chain reaction experiments.

and the percentages of germination and appressorium formation of 300 spores in 10 repeats were determined. Maturation of appressoria was defined according to melanization level; only fully melanized appressoria were considered mature, hyalineless pigmented appressoria were considered immature. External ammonia was supplied to the humid chamber as 5 mM NH_4Cl at pH 8.5 without contacting the spore suspension.

Analysis of *C. gloeosporioides* MEPA, MEPB, MEPC, PKA2, and PKAR.

The genes encoding *C. gloeosporioides* MEPA, MEPB, MEPC, PKA2, and PKAR were analyzed by D. Prusky from *C. gloeosporioides* genome sequences (unpublished) and were deposited in GenBank under the following accession numbers: MEPA, JN660155; MEPB, JN660156; MEPC, JN660157; CPK2, JQ345691; and PKAR, JQ345692.

Quantitative PCR analysis.

The reverse-transcription reaction was performed on 1 µg 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 quantitative (q)PCR. Real-time qPCR was performed with the RotorGene 3000 system (Corbett Research, Sydney, Australia). PCR amplification was applied to 3.4 µl of cDNA template in 10 µl of a reaction mixture containing 5 µl of 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 99°C and annealing for 15 s at 60°C and for 6 s at 80°C, extension at for 20 s 72°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 qPCR, and the values were expressed relative to a calibration sample. The forward and reverse primers for all of the genes are provided in Table 3. The experiment was repeated at least three times with similar results; the results of one biological experiment with three technical repetitions are presented.

pH measurement and ammonia detection.

The pH in 0.5-ml aliquots taken at various times after fungal inoculation was measured with a Thermo-Orion Model 9810BN microcombination pH electrode (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Ammonia was detected colorimetrically with an ammonium test kit (Merck). Samples (0.1 ml) of the culture media were diluted with 4.9 ml of DDW, and the concentration of ammonia was determined 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 as millimoles of ammonia per gram (dry weight) of mycelium. For determination of ammonia accumulation and alkalization inside the germinated spores, spores were stained with the fluorescent dye BCECF (Molecular Probes, Invitrogen, Leiden, The Netherlands), according to Alkan and associates (2008).

Assay for spore cAMP activity.

To measure cAMP activity in the spores, the WT strain was grown on 20-cm petri dishes as described above. Germinating spores were harvested, frozen in liquid nitrogen, ground, homogenized in 10 volumes of 0.1 M HCl, and centrifuged at 600 × g. cAMP level was measured with the Direct cAMP enzyme immunoassay kit (Sigma-Aldrich) according to the manufacturer's instructions. Protein concentration was determined with the Bio-Edit protein assay kit (Bio-Rad, Foster

City, CA, U.S.A.). The intraspore cAMP concentration in total protein was expressed as picomoles per milligram.

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