Expression of *Magnaporthe grisea* Avirulence Gene ACE1 Is Connected to the Initiation of Appressorium-Mediated Penetration

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*Magnaporthe grisea* is responsible for a devastating fungal disease of rice called blast. Current control of this disease relies on resistant rice cultivars that recognize *M. grisea* signals corresponding to specific secreted proteins encoded by avirulence genes. The *M. grisea* ACE1 avirulence gene differs from others, since it controls the biosynthesis of a secondary metabolite likely recognized by rice cultivars carrying the Pi33 resistance gene. Using a transcriptional fusion between ACE1 promoter and eGFP, we showed that ACE1 is only expressed in appressoria during fungal penetration into rice and barley leaves, onion skin, and cellophane membranes. ACE1 expression is not induced by cellophane and plant cell wall components, demonstrating that it does not require typical host plant compounds. Cyclic AMP (cAMP) signaling mutants ΔcplA and Δmacl sum1-99 and tetraspanin mutant Δpsl1::hph differentiate melanized appressoria with normal turgor but are unable to penetrate host plant leaves. ACE1 is normally expressed in these mutants, suggesting that it does not require cAMP signaling or a successful penetration event. ACE1 is not expressed in appressoria of the buf1::hph mutant defective for melanin biosynthesis and appressorial turgor. The addition of hyperosmotic solutes to buf1::hph appressoria restores appressorial development and ACE1 expression. Treatments of young wild-type appressoria with actin and tubulin inhibitors reduce both fungal penetration and ACE1 expression. These experiments suggest that ACE1 appressorium-specific expression does not depend on host plant signals but is connected to the onset of appressorium-mediated penetration.

**Magnaporthe grisea** species complex attacks a wide range of grasses, including wheat, barley, and rice (10, 26), and is a model organism for the study of plant fungal interactions (11, 42). The *M. grisea* infection cycle is characteristic of grass leaf spot diseases. After spore attachment and germination, the fungus differentiates an appressorium through the perception of physical and chemical surface parameters (hydrophobicity, hardness, and cuticle monomers) (21, 42). This differentiation is the result of a complex morphogenetic process that involves cyclic AMP (cAMP), mitogen-activated protein kinases, and calcium signaling pathways (7, 45, 50). Early stages of appressorium development are associated with the deposition of a melanin layer between the cell wall and plasma membrane (21), migration of lipid bodies from spore to appressorium, mobilization of glycolgen, and the formation of a septum sealing the appressorium (5, 43). Maturation of the appressorium is characterized by the degradation of lipid bodies and glyco- gen (43) and the generation of a high turgor (22). Finally, a reorganization of the cytoskeleton is induced at the point of emergence of the penetration peg that penetrates the host cuticle and cell wall (5, 35). Inside the plant, *M. grisea* differentiates bulbous infectious hyphae (44) that colonize host tissues without visible damage for 4 to 5 days after penetration. Then the fungus rapidly expands and destroys colonized tissues, leading to small necrotic lesions producing spores spreading the disease.

Most *M. grisea* genes identified as essential for infection encode proteins involved in appressorium differentiation and appressorium-mediated penetration. They are involved in surface sensing, signaling, melanin/sugar/lipids metabolism, secretion, and membrane remodeling (42). *M. grisea* genes expressed in infected tissues (28) and appressoria (3, 11, 16, 23, 30, 41) were also identified using genomic tools (expressed sequence tags, arrays). Up to now, only a few of these genes are specifically expressed in appressoria or during infection. *GAS1* and *GAS2* encode related proteins of unknown function involved in penetration and specifically expressed in appressoria (48). *PLS1* encodes a membrane protein from the tetraspanin superfamily required for penetration and specifically expressed in appressoria (9). *CBP1* encodes a secreted chitin-binding protein that is not required for penetration and is specifically expressed in appressoria (25, 41). Two other genes identified as specifically expressed in appressoria encode a putative secreted protein (AI068463) (3) and a glucose dehydratase (AP3C19) (41). Yet their role in penetration is unknown. *M. grisea* avirulence (*AVR*) genes *PWL2*, *AVR-PITA*, and *AVR1-CO39* (18, 34, 39) encode small cysteine-rich proteins with putative secretion signal peptides that are likely recognized by plants carrying the corresponding resistance gene (19, 24). *AVR-PITA* and *PWL2* are specifically expressed during penetration, fungal colonization, and late infection (34, 29). ACE1 differs from previous *AVR* genes, as it encodes a cytoplasmic enzyme involved in secondary metabolism exclusively expressed in appressoria (4). Since Ace1 biosynthetic
activity is required for avirulence, the signal recognized by rice plants carrying Pi33 resistance gene is supposed to be a secondary metabolite whose biosynthesis requires Ace1 (4).

In this report, we have studied the factors involved in Ace1 appressorium-specific expression. Ace1 expression was monitored during appressorial differentiation and penetration into plant tissues or artificial membranes using a transcriptional fusion between Ace1 promoter and eGFP, and quantitative reverse transcriptase PCR (RT-PCR). We showed that Ace1 is only expressed in appressoria during penetration of either leaves or cellophane-based membranes, but not on Mylar or Teflon artificial membranes. Ace1 expression was not induced by cellophane or plant cell wall components. Using M. grisea penetration-deficient mutants, we showed that Ace1 is expressed in eAMP or PL5I-deficient mutants but not in melanin-deficient mutant buf1:bgph. Addition of actin or tubulin inhibitors reduces both Ace1 expression and fungal penetration into the host plant. Based on these results, we propose that the induction of Ace1 expression is connected to the initiation of appressorium-mediated penetration.

MATERIALS AND METHODS

Fungal strains, growth conditions, and transformation. Guy11 is a fertile M. grisea field isolate pathogenic on rice (33). Phenotypes of Guy11 Δpkrk mutant 127 (47) and Guy11 Anu1a1 mut1-99 mutant DA99 (1) were recently redescribed by Thines et al. (43). buf1:bgph (unpublished) and pl51:bgph (9) were obtained by REMI mutagenesis using the P1.2 M. grisea strain pathogenic on rice. Fungal strains were grown and stored as described by Doh et al. (14). Strains were grown on osmotic stress conditions on complete liquid medium (Tanaka minimal medium with yeast extract described in reference 14) containing either 0.4 M NaCl or 1 M sorbitol. M. grisea strains were transformed as described by Segard et al. (38) and modified as described by Bohnert et al. (4). For hygro-mycin selection, transformants were selected on complete medium containing 120 ng/ml hygromycin (Sigma-Aldrich, St. Louis, MO). For Basta and sulfo-nylurea selection, transformants were selected on the complex medium defined by Segard et al. (38) containing 35 mg/liter glufosinate or 100 mg/liter chloro- rinuron-ethyl (Chenuzco Info Labo, Ste, Foy la Grande, France), respectively. Transformants were purified by isolation of single spores.

Cloning procedures and plasmid constructions. Exospheric coli strain DH5α (Bethesda Research Laboratories) was used for cloning. Molecular methods followed protocols described by Sambrook et al. (37). eGFP was fused to the promoter and the terminator of Ace1 (promAce1::eGFP) and introduced into a plasmid conferring resistance to hygromycin as already described by Bohnert et al. (4). promACE1::eGFP was digested by EcoRI, and the 3.75 kb fragment containing the Ace1 promoter, eGFP open reading frame, and Ace1 terminator was introduced into pCB1635 (40), a vector conferring resistance to glufosinate. The resulting vector was called pCB1635-promACE1::eGFP. A genomic fragment containing the BUF1 gene was obtained from M. Farman (17) and cloned into pCB1004 vector (40). The sulfonyleurea resistance cassette from pCB1637 was introduced in this plasmid using Sall restriction sites, and the resulting vector, pCB1004-BUF1-SULF, was used to complement our buf1:bgph mutant.

Nucleic acid extraction and analysis. Genomic DNA was isolated from M. grisea by following the miniprep procedure (39) with modifications described by Bohnert et al. (4). Total RNA was extracted from M. grisea liquid cultures following the miniprep procedure (39) with modifications described by Sweigard et al. (38) and modified as described by Bohnhert et al. (4). For hygro-mycin selection, transformants were selected on the complex medium defined by Sweigard et al. (38) containing 35 mg/liter glufosinate or 100 mg/liter chloro-rinuron-ethyl (Chenuzco Info Labo, Ste, Foy la Grande, France), respectively. Transformants were purified by isolation of single spores.

RESULTS

Expression of Magnaportha grisea avirulence gene Ace1 during appressorium-mediated penetration. Ace1 was previously shown to be exclusively transcribed in appressoria during early stages of plant infection (4). We monitored Ace1 transcription during barley leaf infection by quantitative RT-PCR relative to transcripts from the constitutively expressed gene ILV5 (MGG_01808, aceto-hydroxy-isomero-reductase, unpublished data) (Fig. 1). Ace1 transcripts were detected in trace amounts (2% of maximum expression) as soon as 8 hai, rapidly reaching a peak at 17 hai, followed by a decreased at 24 hai (20% of maximum) to 30 hai (5% of maximum). Ace1 transcripts were not detected in RNA from mycelium. To easily monitor Ace1 transcription, we constructed an expression vector corresponding to a transcriptional fusion between the eGFP reporter gene and Ace1 promoter and terminator sequences (promACE1::eGFP). This vector was introduced by transformation into M. grisea avirulent strain Guy11. Most transfor-
mants (18/25, 72%) displayed a strong appressorium-specific eGFP fluorescence. Two of these transformants carrying a single copy of promACE1::eGFP (data not shown) were used to monitor ACE1 transcription. These transformants did not display eGFP fluorescence in young (1 to 6 days) and old (7 to 15 days) mycelia grown in liquid (still/shake) or agar media (complete, minimal, or rice) nor in conidia produced from these cultures. A weak eGFP fluorescence was first observed in appressoria differentiated on barley leaves at 16 hai (Fig. 2A). This observation suggests that ACE1 transcripts that are already abundant at 17 hai (Fig. 1) are not yet efficiently translated into eGFP. ACE1 expression peaked at 24 hai, leading to a strong eGFP fluorescence of both appressoria and primary infectious hyphae (Fig. 2B). After 24 hai, ACE1 expression gradually decreased, and only 50% of appressoria were still fluorescent at 48 hai (Fig. 2C). As the ACE1 transcript is already at a very low level at 48 hai (Fig. 1), the eGFP fluorescence observed at that time likely results from the long half-life of this protein. eGFP fluorescence was never observed in secondary infectious hyphae formed inside infected epidermal cells at 30 to 48 hai nor later during infection, including sporulating lesions. We have previously shown that the Ace1-eGFP fusion protein was exclusively localized in appressoria and was not detected in primary infectious hyphae (4). This observation suggests that the eGFP fluorescence we detected in the primary infectious hyphae of transformants expressing the promACE1::eGFP transcriptional fusion results from the diffusion of soluble eGFP from appressoria to primary infectious hyphae, as these two structures are linked by the penetration peg. ACE1 displayed a similar expression pattern on onion epidermis (Table 1). These results show that ACE1 appressorium-specific expression is controlled at the transcriptional level and is restricted to a specific stage of infection (16 to 48 hai) corresponding to the penetration into host plant tissues (16 to 30 hai).

**Effect of host plant on ACE1 expression.** We monitored ACE1 expression in appressoria differentiated on Teflon, Mylar artificial membranes, and PUDO-193 cellophane membranes (5) using Guy11 transformants expressing the promACE1::eGFP vector. Appressoria differentiated on these membranes are similar to those observed on leaves and develop a similar turgor (Table 1). The frequency of appressoria expressing ACE1 was strongly reduced on Teflon or Mylar membranes compared to those formed on barley leaves, as only 1 to 5% of appressoria were fluorescent at their peak of expression (24 hai). On the contrary, ACE1 expression was...
TABLE 1. *ACE1* expression in appressoria differentiated on host plants or artificial membranes

| Membrane or host plant | Appressorium differentiation (%) | Appressorial turgor (MPa)<sup>a</sup> | Penetration | Appressoria expressing *ACE1* (%)<sup>b</sup> | Time (h) of maximum *ACE1* expression |
|------------------------|-------------------------------|-------------------------------|-------------|----------------------------------|-----------------------------------|
| Teflon                 | 95                            | 4                             | –           | 1–5                              | 24                                |
| Mylar                  | 95                            | 5.5                           | –           | 1–5                              | 24                                |
| Cellophane             | 90                            | ND                            | +           | 50–75                            | 72                                |
| Barley                 | 98                            | 5.5                           | +           | 50–75                            | 24                                |
| Onion                  | 98                            | ND                            | +           | 50–75                            | 24                                |

<sup>a</sup> Turgor was determined using a cytorrhysis assay. ND, not determined.

<sup>b</sup> eGFP fluorescence of appressoria from *M. grisea* Guy11 transformants carrying the prom*ACE1::eGFP* vector was monitored after inoculation of spores on barley leaves, onion epidermis, or artificial membranes.

detected in up to 75% of appressoria formed on cellophane membranes at 48 hai with a peak of fluorescence at 3 days postinoculation when the fungus penetrates this membrane (Fig. 2D). *ACE1* was not expressed in pseudoinfectious hyphae produced within the cellophane membrane. Since such membranes contain cellulose and related oligosaccharides, we tested the effect of ground PUDO-193 cellophane membranes (0.5%) and several plant cell wall components (0.5% cellulose, 25 mM cellobiose, 0.5% xylan, and 0.5% pectin) on *ACE1* expression. We have not observed the induction of eGFP fluorescence when these compounds were added to 8-h-old appressoria differentiated on Teflon. These results demonstrate that *ACE1* expression is induced only in appressoria formed on host plants or cellophane membranes, but this induction does not involve the cellophane itself or host cell wall components.

**Relationship between cAMP signaling and *ACE1* expression.** *ACE1* encodes a multifunctional enzyme involved in *M. grisea* secondary metabolism. Since the cAMP signaling pathway negatively regulates the expression of genes involved in secondary metabolism in *Aspergillus nidulans* (49), we investigated whether this pathway is involved in the control of *ACE1* expression. In *M. grisea*, cAMP signaling is required for both the differentiation of appressoria on hydrophobic surfaces and for appressorium-mediated penetration (1, 32, 43, 47). Some cAMP pathway mutants are able to form appressoria but are impaired in penetration. Deletion of the *CPKA* gene that encodes the catalytic subunit of cAMP-dependent protein kinase A affects appressorium morphogenesis, leading to a delayed formation of smaller, nonfunctional appressoria (32, 47). Δ*cpkA* mutants are retarded for glycolysis and lipid mobilization during appressorium formation (43). These mutants are highly reduced in pathogenicity, inducing rare lesions and producing defective penetration pegs on onion epidermis (35). Δ*mac1 sum1-99* is a suppressor of Δ*mac1* mutation corresponding to a deletion of *M. grisea* adenylate cyclase gene MAC1 (1). sum1-99 corresponds to a mutation of the cAMP-binding pocket from the protein kinase A regulatory subunit, which leads to a constitutive activation of the cAMP pathway (1). Although it displays an accelerated conidial germination and appressorium development, this mutant is impaired in penetration, as its glycogen and lipid degradation is accelerated and completed before the onset of penetration (43). The prom*ACE1::eGFP* vector was introduced into Δ*cpkA* and Δ*mac1 sum1-99* mutants and *ACE1* expression was monitored as eGFP fluorescence. *ACE1* was normally expressed in appressoria of Δ*cpkA* and Δ*mac1 sum1-99* mutants formed on barley leaves (Table 2). These results demonstrate that *ACE1* appressorium-specific expression is independent of the cAMP signaling pathway.

**ACE1 expression in appressoria of *M. grisea* penetration-deficient mutants.** *ACE1* expression is restricted to appressoria penetrating leaves or cellophane membranes. To assay if *ACE1* expression requires a successful penetration of host tissues, we expressed prom*ACE1::eGFP* in penetration-defective mutants Δ*pls1::hph* and Δ*buf1::hph* (Table 2). The Δ*pls1::hph* mutant, defective for Pls1 tetrasin, differentiates melanized appressoria with normal turgor (Table 2) that are unable to penetrate host leaves and cellophane membranes (9). This mutant is likely blocked at a late stage of appressorial development, as it is unable to degrade its glycogen (9). The naphthalene reductase mutant Δ*buf1::hph* differentiates nonmelanized appressoria that cannot build up turgor and are unable to penetrate intact leaves or cellophane membranes (8, 12, 20, 22). *ACE1* was normally expressed in appressoria of the Δ*pls1::hph* mutant (Table 2). This result demonstrates that *ACE1* appressorium-specific expression is independent of the PLS1 pathway required for appressorium-mediated penetration and does not require a successful penetration event. In contrast, *ACE1* was not expressed in unmelanized appressoria from the Δ*buf1::hph* mutant differentiated on barley leaves or cellophane membranes (Table 2). The Δ*buf1::hph* melanin-deficient mutant tested was obtained by REMI mutagenesis during the screening of nonpathogenic mutants (9). Since secondary mutations are frequently observed during REMI mutagenesis (2, 38), we tested whether this Δ*buf1::hph* mutation was responsible for the lack of *ACE1* expression or not. To obtain melanin-deficient appressoria independently of the *BUF1* null mutation, we inhibited melanin biosynthesis using tricyclazole, a specific inhibitor of naphthalene reductase encoded by *BUF1* (8, 46).

**TABLE 2. *ACE1* expression in appressoria from *M. grisea* penetration-deficient mutants**

| *M. grisea* mutant | Pathway altered | Appressorium formation | Appressorial turgor<sup>b</sup> | Penetration | *ACE1* expression<sup>b</sup> |
|-------------------|----------------|------------------------|-------------------------------|-------------|--------------------------|
| Guy11             | Wild type      | +                      | +                             | +           | +                        |
| Δ*cpkA*           | cAMP signaling| +                      | +                             | +           | +                        |
| Δ*mac1 sum1-99*   | cAMP signaling| +                      | ND                            | +/−         | +                        |
| Δ*pls1::hph*      | Tetrasin       | +                      | +                             | −           | −                        |
| Δ*buf1::hph*      | Melanin biosynthesis | +                  | +                             | +           | +                        |

<sup>a</sup> Turgor was determined using a cytorrhysis assay for *pls1::hph* (9), Δ*cpkA* (47), and Δ*buf1::hph* (20, 47). ND, not determined.

<sup>b</sup> eGFP fluorescence of appressoria from different *M. grisea* transformants carrying prom*ACE1::eGFP* vector was monitored 24 h after inoculation of spores on barley leaves.
Tricyclazole was added to spore suspensions of Guy11 transformants expressing promACE1::eGFP inoculated on barley leaves or cellophane membranes. Tricyclazole-treated appressoria were not melanized (Fig. 3A) and unable to penetrate plant tissues or cellophane (Fig. 3C). ACE1 was not expressed in tricyclazole-treated appressoria differentiated on barley leaves (Fig. 3B) or cellophane (data not shown). We also complemented our buf1::hph promACE1::eGFP transformants with a vector carrying a wild-type BUF1 allele (17). The resulting buf1::hph/BUF1 transformants differentiated melanized appressoria identical to the wild type, and their pathogenicity on barley and rice was restored (Fig. 4). These buf1::hph/BUF1 transformants displayed a normal ACE1 appressorium-specific expression (Fig. 4). These results demonstrate that the inhibition of melanin biosynthesis either genetically (buf1::hph mutant) or chemically (tricyclazole) abolishes ACE1 expression in appressoria.

Effect of hyperosmotic solutes on ACE1 expression in buf1::hph melanin-deficient appressoria. During appressorium maturation, a high internal turgor pressure (4 to 8 Mpa) (22) is built up as a result of the accumulation of an osmolyte thought to be glycerol (12). Melanin-deficient mutants are unable to retain osmolytes accumulated in appressoria (8, 12, 20) and are therefore unable to build up appressorial turgor. We hypothesized that addition of hyperosmotic solutes to buf1::hph appressoria could mimic the high solute concentration reached in wild-type appressoria and induce ACE1 expression. We applied different hyperosmotic solutes to appressoria of buf1::hph transformants carrying promACE1::eGFP. We performed the same experiments with appressoria of Guy11 transformants expressing eGFP under the control of either ACE1 or MPG1 promoters (27) as controls. Six hours after inoculation of spores from Guy11 wild-type or buf1::hph promACE1::eGFP transformants on barley leaves or Teflon, young appressoria were fully differentiated. Residual water drops covering appressoria were replaced by hyperosmotic solutes, and eGFP fluorescence was monitored for 48 h. The concentrations of these solutes were chosen to generate an osmotic potential equivalent to those of wild-type appressoria (0.15 M PEG, 0.6 M sucrose, 1 M glycerol, 1 M sorbitol) (22) or to induce a hyperosmotic stress, as already observed for M. grisea mycelia (0.4 M NaCl) (15). Glycerol slightly decreased the proportion of eGFP fluorescent appressoria (60%) of Guy11 transformants formed on barley leaves compared to water (95%), whereas PEG, NaCl, sucrose, and sorbitol had no obvious effect on ACE1 expression (80 to 85%). Addition of PEG or glycerol did not restore ACE1 expression in buf1::hph appressoria differentiated on barley, while addition of NaCl, sucrose, or sorbitol significantly restored ACE1 expression (Table 3). Fifty-three percent of buf1::hph appressoria differentiated on barley leaves strongly expressed ACE1 when treated with NaCl, 58% with sorbitol, and 67% with sucrose. Interestingly, buf1::hph appressoria differentiated secondary hyphae on the surface of barley leaves or Teflon following NaCl, sucrose, or sorbitol treatment (Fig. 5). Similar secondary hyphae were not observed with wild-type appressoria treated with hyperosmotic solutes nor with untreated buf1::hph appressoria.

Although ACE1 expression was restored in 6-h-old buf1::hph appressoria by a treatment with hyperosmotic solutes, this expression was observed only 18 h after this treatment (24 hai).
To analyze the effect of the application time on *ACE1* expression, we treated *buf1::hph* appressoria with 0.6 M sucrose at 15 hai. Forty percent of appressoria expressed *ACE1*, but eGFP fluorescence was only observed at 48 hai (no fluorescence at 24 hai). We conclude that treatments with hyperosmotic solutes restore *ACE1* expression in 6- to 15-h-old *buf1::hph* appressoria after a delay of 18 to 24 h. We also tested if *ACE1* was expressed in mycelia following treatment with hyperosmotic solutes. We could not detect eGFP fluorescence in mycelia of Guy11 transformants expressing prom*ACE1::eGFP* grown in liquid medium containing either 0.4 M NaCl or 1 M sorbitol. *ACE1*-specific RT-PCR was performed on total RNA extracted from Guy11 mycelia grown under these hyperosmotic conditions for 15, 30, 60, or 120 min. An *ACE1* RT-PCR product was detected 60 min after treatment with NaCl and 120 min after treatment with sorbitol (data not shown). Quantification of these transcripts by quantitative RT-PCR showed that only a very low level of transcript was produced (around 1/1,000 of the maximum transcript level in appressoria). These experiments suggest that *ACE1* expression can be induced in mycelia grown under hyperosmotic conditions, but this expression level is very low compared to *ACE1* appressorium expression.

**Effect of cytoskeleton inhibitors on *ACE1* expression.**

The onset of appressorium-mediated penetration is associated with important reorganizations of actin and tubulin cytoskeleton associated with the formation of the penetration peg (5, 35). We speculated that the inhibition of these cytoskeleton modifications by inhibitors of actin and tubulin could inhibit appressorium-mediated penetration and consequently *ACE1* expression. We used carbendazim, which induces the depolymerization of microtubules, and cytochalasin A, which represses actin polymerization (36). Eight-hours-old appressoria differentiated on onion epidermis were treated with carbendazim (30, 100, or 300 ppm) and cytochalasin A (1, 3, or 10 μM) to avoid any interference with appressorium differentiation and to specifically inhibit penetration peg formation. Carbendazim treatment reduced the penetration of the fungus into onion epidermis in a dose-dependent relationship starting from 21% inhibition at 30 ppm to a complete inhibition at 300 ppm (Table 4). This high concentration also completely inhibited *ACE1* expression. At lower carbendazim concentrations, the expression of *ACE1* was reduced to the same extent (30 ppm) or more (100 ppm) than penetration. Cytochalasin A treatments reduced the penetration of the fungus into onion epidermis in a dose-dependent relationship, as observed for carbendazim. For example, 3 μM cytochalasin A reduced penetration by 29% while 10 μM cytochalasin A strongly reduced penetration (67%). At these two concentrations, the expression of *ACE1* was reduced to the same extent as penetration. In these experiments, we have observed that *ACE1* is only expressed in appressoria penetrating into host tissues. Since penetration and *ACE1* expression were coupled, we conclude that *ACE1* expression depends on the initiation of penetration.
**DISCUSSION**

Induction of *ACE1* appressorium-specific expression is independent of host plant. We monitored *ACE1* expression during fungal development and host plant infection using quantitative RT-PCR and a transcriptional fusion between the *ACE1* promoter and eGFP reporter gene. We showed that *ACE1* was only transcribed in mature appressoria, reaching a maximum at 17 hai and decreasing after 24 hai (real-time RT-PCR). *ACE1* expression monitored by eGFP fluorescence followed the same kinetics, although with a 6-h delay. *ACE1* appressorium-specific expression was also observed on artificial membranes, although at different degrees, ranging from 1 to 5% of appressoria on Mylar and Teflon to 75% on cellophane. A major difference between cellophane and Teflon/Mylar membranes is their chemical nature, with cellophane containing cellulose also found in plant cell walls, while Teflon and Mylar are inert chemical polymers. We tested different plant cell wall components (xylan, cellulose, pectin) and ground PUDO-193 cellophane on *ACE1* expression. These compounds are unable to induce *ACE1* expression in spores, germ tubes, and appressoria formed on a Teflon membrane, demonstrating that *ACE1* is not induced by cellophane components. Appressoria formed on Teflon or Mylar could differ in their physiology from those produced on the leaf surface, although they reach turgor levels similar to appressoria on host leaves (Table 1). Indeed, appressoria formed on Mylar or Teflon are unable to penetrate these membranes, and only a few of these appressoria are able to differentiate penetration pegs (22). The small number of appressoria expressing *ACE1* on Teflon and Mylar may correspond to the few appressoria initiating a penetration peg. Overall, these observations show that *ACE1* is exclusively expressed in mature appressoria during penetration into a leaf or an artificial cellophane membrane. This induction is independent of compounds from the cellophane membrane or plant cell wall.

*ACE1* is not expressed in melanin-deficient mutant appressoria. Early stages of appressorial development are characterized by the deposition of a melanin layer between the fungal membrane and cell wall that is required for turgor buildup (20, 21). This melanin layer acts as a semipermeable membrane, retaining solutes such as glycerol (12) accumulated in appressoria and allowing the buildup of a high internal hydrostatic pressure as water flows into this cell (20, 21). This turgor is required for appressorium-mediated penetration (8, 22). Melanin-deficient mutants do not retain appressorial solutes and are unable to penetrate leaves or cellophane (8, 12, 20). We clearly showed that *ACE1* was not expressed in the melanin-deficient mutant *buf1::hph*. Since the major defect of this mutant is the lack of turgor, we first hypothesized that appressorial turgor is needed for the induction of *ACE1* appressorium-specific expression. This is obviously not the case, as *ACE1* was not expressed in appressoria formed on Teflon and Mylar that generate a normal turgor. Treatments of wild-type appressoria with external hyperosmotic solutes reduce internal turgor dramatically (22). These treatments did not reduce *ACE1* expression, confirming that turgor is not required for *ACE1* expression. The other major defect of melanin-deficient mutant *buf1::hph* is the absence of accumulation of solutes. Therefore, we hypothesized that the addition of hyperosmotic solutes to *buf1::hph* appressoria could mimic the high solute concentration reached in wild-type appressoria and induce *ACE1* expression. Indeed, addition of hyperosmotic NaCl, sorbitol, or sucrose solutions to *buf1::hph* appressoria restored *ACE1* expression in mature mutant appressoria, while it did not induce its expression in mycelium, spores, germ tubes, and young appressoria. The wide range of compounds able to restore *ACE1* expression in *buf1::hph* appressoria suggests that this induction is not a consequence of the presence of a particular solute at a high concentration. NaCl, sorbitol, and sucrose that induce *ACE1* expression are not accumulated in wild-type appressoria (12). On the contrary, glycerol that is normally accumulated in appressoria during turgor buildup (12) did not induce *ACE1* expression. These results suggest that the restoration of *ACE1* expression in *buf1::hph* appressoria is a direct or indirect consequence of the hyperosmotic stress induced by these solutes.

When *buf1::hph* appressoria were treated with hyperosmotic solutes, we always observed the restoration of *ACE1* expression after a delay of at least 12 h. Treatment of *M. grisea* mycelia with hyperosmotic solutes induces the transcription of target genes after a short delay of 1 to 2 h (13, 15). This short delay in the direct transcriptional response of fungal cells to hyperosmotic stress suggests that the induction of *ACE1* expression in *buf1::hph* appressoria is not the result of a direct response to osmotic stress. The same osmotic stress did not induce *ACE1* expression in spores, young appressoria, or mycelia, even though a very small induction was observed at the mRNA level in stressed mycelia. Alternatively, *buf1::hph* appressoria could be blocked at an early stage of appressorial development. Hyperosmotic solutes could reinitiate appressorial development in *buf1::hph* appressoria, allowing them to reach the developmental stage required to induce *ACE1* expression. This hypothesis is strengthened by the fact that *buf1::hph* appressoria treated by hyperosmotic solutes reach a novel developmental stage associated with the differentiation of secondary hyphae formed at the base of the appressorium. These secondary hyphae likely arise from penetration pegs, suggesting that treated *buf1::hph* appressoria reach the penetration stage, although they are unable to pierce host cell wall.

*ACE1* expression is connected to the onset of appressorium-mediated penetration. *M. grisea* penetration-deficient mutants were used to assess whether a successful penetration was required for *ACE1* appressorium-specific expression or not. *ACE1* was normally expressed in the Δ*pkA* and Δmac1 *sum1-99* mutants deficient for or with a constitutively active cAMP signaling pathway, respectively (1, 32, 43, 47). These observations demonstrate that the control of *ACE1* expression is independent of the cAMP signaling pathway. Additionally, *ACE1* was also normally expressed in appressoria of the mutant Δ*pks1::hph* (9) unable to penetrate host tissues, demonstrating that its expression does not require a successful penetration event. We have previously shown that *ACE1* is not fully expressed in wild-type appressoria formed on Teflon and Mylar membranes that do not allow penetration peg formation (22). These apparently contradictory observations suggest that Δ*pkA* and Δ*pks1::hph* appressoria reach a developmental stage connected to *ACE1* expression, while appressoria formed on Teflon and Mylar do not. This developmental stage corresponds to the onset of appressorium-mediated penetration, as...
\( \Delta pkA \) mutant appressoria are still able to differentiate penetration pegs and attempt to penetrate the host cell wall (35).

To test this hypothesis, we used actin and tubulin inhibitors that should disturb the reorganization of cytoskeleton observed at an early stage of penetration peg formation (5, 35) and consequently inhibit penetration. Both carbenazim and cytochalasin A inhibited the penetration of \( M. grisea \) into onion epidermal cells in a dose-dependent manner. These treatments also inhibited \( ACE1 \) expression quantitatively, and the only appressoria expressing \( ACE1 \) were those which penetrated successfully into onion epidermis. These results show that the inhibition of cytoskeleton reorganization in the appressorium and, consequently, penetration peg formation also abolish \( ACE1 \) expression.

Overall, these experiments demonstrate that the induction of \( ACE1 \) expression in appressoria is connected to a specific appressorial developmental stage associated with penetration peg formation. \( ACE1 \) expression is therefore a landmark of this early stage of appressorium-mediated penetration.

\( ACE1 \) is a secondary metabolism gene with a novel expression pattern. \( ACE1 \) encodes a putative hybrid polyketide synthase (PKS)-nonribosomal peptide synthetase (NRPS) (4). Expression of fungal PKS- and NRPS-encoding genes is frequently induced during stationary phase (49) and is affected by environmental and nutritional factors such as temperature, pH, carbon and nitrogen sources, and lipids (6, 31). These genes are also frequently repressed during mycelial growth and induced during sporulation (49). We have shown that \( ACE1 \) expression is specifically connected to the onset of appressorium-mediated penetration. The \( ACE1 \) expression pattern therefore confirms the general assumption that genes from secondary metabolism are expressed at particular developmental stages. The regulatory networks involved in the tight appressorial expression of \( ACE1 \) remain to be discovered, since it is independent of appressorial signaling pathways identified so far. The identification of these appressorium-specific regulatory networks will be very helpful to understand the early stages of appressorium-mediated penetration.

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