EUKARYOTIC CELL, Mar. 2007, p. 546–554 Vol. 6, No. 3
1535-9778/07/$08.00+0 doi:10.1128/EC.00330-05

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

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Received 25 October 2005/Accepted 10 November 2006

***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* is almost not expressed in appressoria differentiated on Teflon and Mylar artificial 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 Δ*cpkA* and Δ*mac1 sum1-99* and tetraspanin mutant Δ*pls1*::*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 glycogen, and the formation of a septum sealing the appressorium (5, 43). Maturation of the appressorium is characterized by the degradation of lipid bodies and glycogen (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

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▽ Published ahead of print on 1 December 2006.

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 cAMP or *PLS1*-deficient mutants but not in melanin-deficient mutant *buf1*::*hph*. 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 Δ*cpkA* mutant I27 (47) and Guy11 Δ*mac1 sum1-99* mutant DA99 (1) were recently redescribed by Thines et al. (43). *buf1*::*hph* (unpublished) and *pls1*::*hph* (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 Dioh et al. (14). Strains were grown under 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 Sweigard et al. (38) and modified as described by Böhnert et al. (4). For hygromycin selection, transformants were selected on complete medium containing 120 mg/liter hygromycin (Sigma-Aldrich, St. Louis, MO). For Basta and sulfonylurea selection, transformants were selected on the complex medium defined by Sweigard et al. (38) containing 35 mg/liter glufosinate or 100 mg/liter chlorimuron-ethyl (Cluzeau Info Labo, Ste. Foy la Grande, France), respectively. Transformants were purified by isolation of single spores.

Cloning procedures and plasmid constructions. *Escherichia 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* (prom*ACE1*::*eGFP*) and introduced into a plasmid conferring resistance to hygromycin as already described by Böhnert et al. (4). prom*ACE1*::*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-prom*ACE1*::*eGFP*. A genomic fragment containing the *BUF1* gene was obtained from M. Farman (17) and cloned into pCB1004 vector (40). The sulfonylurea resistance cassette from pCB1637 was introduced in this plasmid using SalI restriction sites, and the resulting vector, pCB1004-BUF1-SULFR, was used to complement our *buf1*::*hph* mutant.

Nucleic acid extraction and analysis. Genomic DNA was isolated from *M. grisea* by following the miniprep procedure (39) with modifications described by Böhnert et al. (4). Total RNA was extracted from *M. grisea* liquid cultures using the hot acid-phenol protocol (9) or using Trizol reagent (Invitrogen, Carlsbad, CA). RT-PCR was carried out with 6 μg of total RNA as starting material using ReadyToGo You-Prime first-strand beads (Amersham Biosciences, Little Chalfont, United Kingdom) according to the manufacturer's protocol. The following *ACE1*-specific primers used in this study hybridize on both sides of the *ACE1* second intron: I3O+, 5′-GCGACACACTGACGGCGACC-3′ (6,208 bp from ATG); I3O−, 5′-GGAGCCGTTGCCCATGATGC-3′ (7,124 bp from ATG); I3i+, 5′-CCGCCGTCGTCACTCCCACC-3′ (6,346 bp from ATG); I3i−, 5′-TGACAGAGGACAGGAAGACG-3′ (6,987 bp from ATG).

Real-time RT-PCR. Reverse transcription was carried out using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, CA) with 5 μg of total RNA extracted from infected barley leaves at 0 h, 8 h, 17 h, 24 h, 30 h, 48 h, 52 h, and 72 h and from mycelium grown in complete liquid agitated medium for 24 h (three biological replicates for each time point). Real-time PCR was carried out with a LightCycler 1.0 (Roche Diagnostics, Indianapolis, IN) using Fast-Start DNA master SYBR green I kit (Roche Diagnostics, Indianapolis, IN). The following primers were designed using Primer Express (Applied Biosystem, Foster City, CA): qACE1-F, 5′-AGACGATGCCATTGGCAAA-3′; qACE1-R, 5′-AGCCAGCATGGAGTCCAATC-3′; qILV5-F, 5′-CCAGCTCTACGACTCGGTCAA-3′; qILV5-R, 5′-AGTCGGGCTGGCTGTTGTAGT-3′. *ACE1* expression is calculated relative to the transcript levels of the constitutively expressed gene *ILV5* (MGG_01808) using the formula $2^{-\Delta Ct} = 2^{-(Ct_{ACE1} - Ct_{ILV5})}$. StatBox V6.5 (Grimmersoft, Paris, France) was used for statistical analyses (critical threshold $\alpha = 0.05$).

Phenotypic analysis and cytology. Seedlings from barley cultivar Express were cultivated for 10 days (20°C during the day, 15°C at night). The detached barley leaf assay was carried out as described previously (4, 9). The onion skin assay was performed using the same protocol as the barley leaf assay. Appressoria were differentiated on artificial membranes composed of Teflon (Goodfellow, Cambridge, United Kingdom), Mylar (polyethylene terephtalate, Rhodia, Lyon, France), and PUDO-193 cellophane (gift from T. Bourett, DuPont de Nemours, Wilmington, DE) (5). Enhanced green fluorescent protein (eGFP) fluorescence was observed with a Nikon Optiphot fluorescence microscope equipped with a 488/DM510-550 eGFP-specific filter. Conidia and appressoria were observed on the leaf surface after a treatment for 1 min with a highly diluted calcofluor solution (fluorescent brightener 26 [Sigma-Aldrich], 0.01 mg/ml in water, pH 8), followed by water rinse. Cell wall calcofluor fluorescence was observed under UV light with a Nikon Optiphot fluorescence microscope. For melanin inhibition, 10 ppm tricyclazole (gift from E. Lilly Research Center Ltd., United Kingdom) (46) was added to spore suspensions before inoculation on membranes or leaves. Appressoria were treated with hyperosmotic solutions by replacing water droplets at 6 h after inoculation with solutes at the following concentrations: 0.6 M sucrose, 0.4 M NaCl, 1 M sorbitol, 0.15% polyethylene glycol (PEG), and 1 M glycerol. Similarly, 0.5% cellophane powder or plant cell wall components were added to appressoria by replacing water droplets at 8 h after inoculation (hai) with the following solutions: 0.5% cellulose, 25 mM cellobiose, 0.5% xylan (from beechwood or oat spelts), and 0.5% citrus pectin (Sigma-Aldrich, St. Louis, MO). Thirty, 100, and 300 ppm carbendazim (Ehrenstorfer GmbH, Augsburg, Germany) or 1, 3, and 10 μM cytochalasin A (Calbiochem, La Jolla, CA) were added at 8 hai on onion epidermis assays by replacing water droplets with inhibitor solutions.

Turgor assay using cytorrhysis. *M. grisea* spore suspensions were deposited on artificial membranes or detached barley leaves. At selected times (8 to 24 hai), water droplets were replaced with increasing concentrations of KCl or PEG4000 (Sigma-Aldrich, St. Louis, MO) solutions. Following 15 min of incubation in the solute, appressoria collapses were observed under light microscope (magnification, ×100; Nikon Optiphot), and the percentage of cytorrhysis was determined for 100 appressoria in three independent droplets. This experiment was repeated at least twice. The 50% cytorrhysis was calculated from the solute dose-percent collapse curve and used as an estimate of appressorium turgor pressure using the relationships defined by Howard et al. (22), allowing a correspondence between the molarity of variable solutes and turgor pressure.

RESULTS

Expression of *Magnaporthe 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 (prom*ACE1*::*eGFP*). This vector was introduced by transformation into *M. grisea* avirulent strain Guy11. Most transfor-

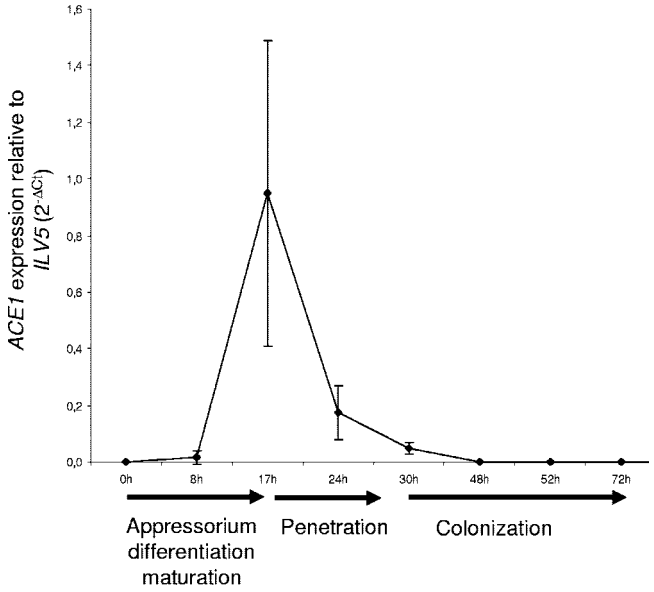

FIG. 1. Quantification of *ACE1* mRNA by real-time RT-PCR during infection of barley leaves. *ACE1* expression was quantified by real-time RT-PCR using RNA extracted from infected barley leaves collected at different times after droplet inoculation of Guy11 spore suspensions. *ACE1* expression is calculated relative to the transcript levels of the constitutively expressed gene *ILV5* using the formula $2^{-\Delta Ct} = 2^{-(Ct_{ACE1} - Ct_{ILV5})}$. Each data point is the average of results from three biological replicates. Standard deviations are indicated by error bars.

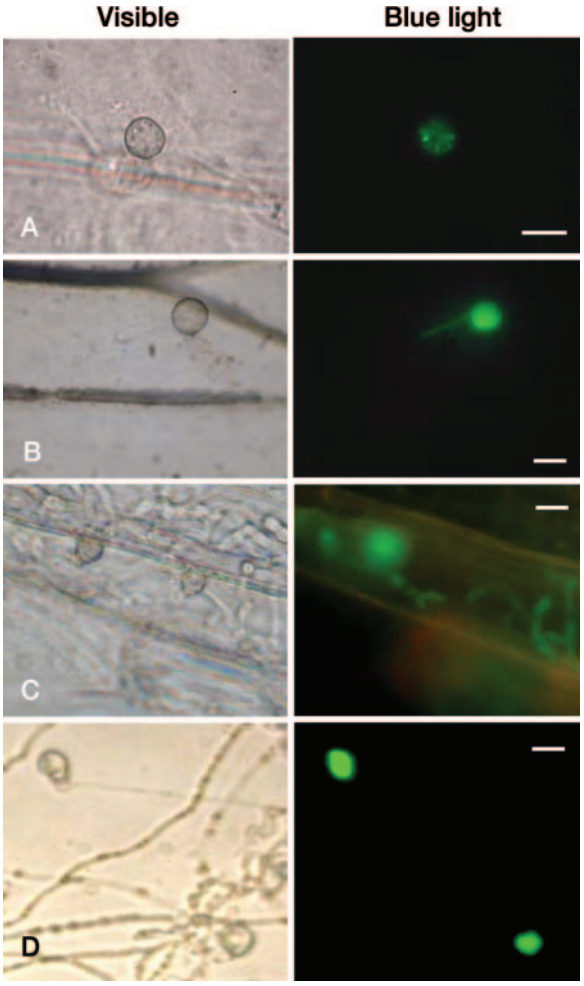

FIG. 2. *ACE1* appressorium-specific expression on leaves and cellophane membranes. *M. grisea* Guy11 transformants carrying the prom*ACE1*::*eGFP* vector were used to monitor *ACE1* expression as eGFP fluorescence at 16 (A), 24 (B), and 48 (C) h after inoculation of spores on barley leaves and at 24 h after inoculation of spores on a cellophane membrane (D). Bar, 10 μm. Bright field (visible): observation with a microscope at ×40 magnification under bright light. Fluorescence (blue light): observation with a microscope at ×40 magnification under UV light with an eGFP-specific filter.

mants (18/25, 72%) displayed a strong appressorium-specific eGFP fluorescence. Two of these transformants carrying a single copy of prom*ACE1*::*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 prom*ACE1*::*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 prom*ACE1*::*eGFP* fusion. 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)[a]	Penetration	Appressoria expressing *ACE1* (%)[b]	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

[a] Turgor was determined using a cytorrhysis assay. ND, not determined.
[b] 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 glycogen 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-sum99* 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 *buf1*::*hph* (Table 2). The Δ*pls1*::*hph* mutant, defective for Pls1 tetraspanin, 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 *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 *buf1*::*hph* mutant differentiated on barley leaves or cellophane membranes (Table 2). The *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 *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[a]	Penetration	*ACE1* expression[b]
Guy11	Wild type	+	+	+	+
Δ*cpkA*	cAMP signaling	+	+	−	+
Δ*mac1 sum1-99*	cAMP signaling	+	ND	+/−	+
pls1::*hph*	Tetraspanin	+	+	−	+
buf1::*hph*	Melanin biosynthesis	+	−	−	−

[a] Turgor was determined using cytorrhysis assays for *pls1*::*hph* (9), Δ*cpkA* (47), and *buf1*::*hph* (20, 47). ND, not determined.
[b] 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.

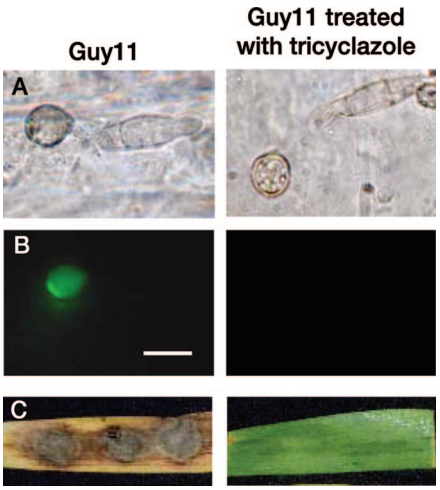

FIG. 3. Effect of tricyclazole on *ACE1* appressorium-specific expression. (A) Inhibition of appressorium melanization by tricyclazole. Microscopic observations were performed at ×100 magnification under bright light. (B) Inhibition of *ACE1* expression in appressoria differentiated on barley leaves treated with tricyclazole 24 h after inoculation. *M. grisea* Guy11 transformants carrying the prom*ACE1*::*eGFP* vector were used to monitor *ACE1* expression as eGFP fluorescence. Microscopic observations were performed at ×100 magnification under UV light with an eGFP-specific filter. (C) Lack of pathogenicity on barley leaves of Guy11 transformants carrying prom*ACE1*::*eGFP* vector treated with tricyclazole 5 days after inoculation. Bar, 10 μm.

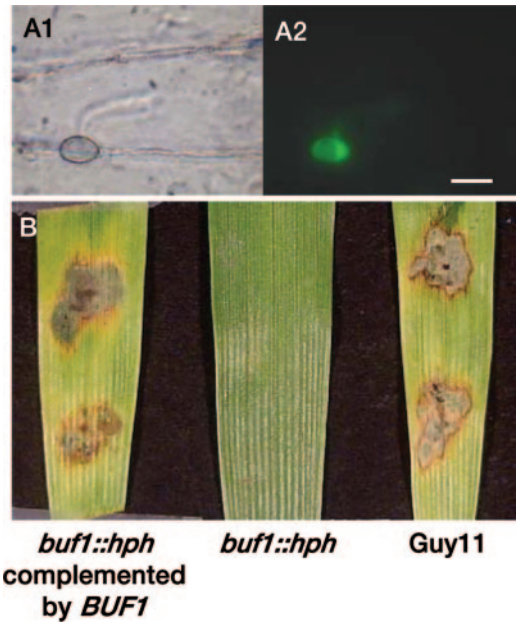

FIG. 4. Complementation of *buf1*::*hph* mutant with *BUF1* restores *ACE1* expression. (A) *M. grisea* P1.2 *buf1*::*hph*/*BUF1* transformants carrying prom*ACE1*::*eGFP* vector were used to monitor *ACE1* expression as eGFP fluorescence. Microscopic observations were performed at ×100 magnification under bright light (A1) or UV light with an eGFP-specific filter (A2). Bar, 10 μm. (B) Pathogenicity on barley leaves of the P1.2 *buf1*::*hph* mutant (*buf1*::*hph*) and P1.2 *buf1*::*hph* mutant complemented with the *BUF1* wild-type allele (*buf1*::*hph*/*BUF1*) and wild-type Guy11 5 days after inoculation.

Tricyclazole was added to spore suspensions of Guy11 transformants expressing prom*ACE1*::*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* prom*ACE1*::*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 prom*ACE1*::*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* prom*ACE1*::*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).

TABLE 3. Effect of hyperosmotic solutes on *ACE1* expression in appressoria from *buf1*::*hph* melanin-deficient mutants

Molarity	Osmotic solute[a]	Appressoria expressing *ACE1* (%) from[b]:	
		Guy11	*buf1*::*hph*
	Water	95 ± 7	0
0.15	PEG	83 ± 2	0
1.00	Glycerol	63 ± 16	0
0.40	NaCl	84 ± 7	53 ± 14
0.60	Sucrose	81 ± 12	67 ± 7
1.00	Sorbitol	81 ± 2	58 ± 8

[a] Spores from Guy11 wild-type transformant and P1.2 *buf1*::*hph* transformant expressing prom*ACE1*::*eGFP* vector were inoculated on barley leaves. After 6 h, water droplets were replaced with the osmotic solute.
[b] Appressoria from *M. grisea* Guy11 or P1.2 *buf1*::*hph* transformants were monitored for eGFP fluorescence 24 h after inoculation of spores on barley leaves.

TABLE 4. Effect of cytoskeleton polymerization inhibitors on *ACE1* appressorium-specific expression

Treatment[a]	Appressorium differentiation (%)	Penetration (%)[b]	Appressoria expressing *ACE1* (%)[b]	*ACE1* expression inhibition rate (%)
Water	100	57 ± 14*	52 ± 8*	0
Carbendazim				
30 ppm	100	37 ± 7†	41 ± 9†	21
100 ppm	100	41 ± 16†	14 ± 7‡	73
300 ppm	100	1 ± 1§	0§	100
Cytochalasin A				
1 μM	100	54 ± 7*	40 ± 13†	23
3 μM	100	46 ± 15†	37 ± 13†	29
10 μM	84 ± 12	22 ± 12‡	17 ± 6‡	67

[a] Inhibitors were applied 8 h after inoculation. eGFP fluorescence of appressoria from *M. grisea* Guy11 transformants carrying the prom*ACE1*::*eGFP* vector was monitored 24 h after inoculation of spores on onion epidermis.
[b] Statistical groups indicated by symbols (*, †, ‡, and §) are based on mean comparisons using Student or Mann-Whitney tests (critical threshold $\alpha/2 = 0.025$).

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.

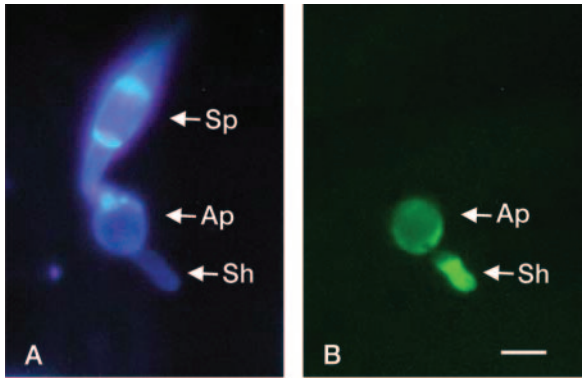

FIG. 5. Effect of hyperosmotic solute (0.4 M NaCl) on *ACE1* expression in a *buf1*::*hph* mutant. Spores from a P1.2 *buf1*::*hph* transformant expressing prom*ACE1*::*eGFP* vector were inoculated on barley leaves. After 6 h, water droplets were replaced by 0.4 M NaCl and leaves were observed under a microscope 24 h after inoculation. (A) Microscopic observation was performed at ×100 magnification under UV light after staining with calcofluor. A spore (Sp), an appressorium (Ap), and a secondary hypha (Sh) originating from the appressorium are visible through the bright blue fluorescence of their cell walls. (B) Microscopic observation was performed at ×100 magnification under UV light with an eGFP-specific filter. e*GFP* fluorescence was detected only in the appressorium (Ap) and in the secondary hypha (Sh). Bar, 10 μm.

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 pierce 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*cpkA* and Δ*mac1 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*pls1*::*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*cpkA* and Δ*pls1*::*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

Δ*cpkA* 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 carbendazim 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.

ACKNOWLEDGMENTS

This work was supported by EU grant ICA4-CT-2000-30021 (INCO-DEV project "RESIDIV"), including a Ph.D. fellowship to I.F., and by the French Ministère de la Recherche with the MENRT Ph.D. fellowship to J.C.

We thank Jin-Rong Xu (Purdue University) for Δ*cpkA-I27* and Δ*mac1 sum1-99* mutants and Mark Farman (University of Kentucky) for the *BUF1* wild-type gene. We also thank Tim Bourret (E. I. Du Pont de Nemours, Wilmington, DE) for PUDO-193 cellophane membrane, Ronald De Vries (Utrecht University, The Netherlands) for helpful advices on cell wall components, and Bernard Dumas (CNRS-UPS, Toulouse, France) for critical reading of the manuscript.

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