A Putative Polyketide Synthase/Peptide Synthetase from *Magnaporthe grisea* Signals Pathogen Attack to Resistant Rice [W]

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Isolates of the rice blast fungus *Magnaporthe grisea* that carry the gene encoding *Avirulence Conferring Enzyme1* (*ACE1*) are specifically recognized by rice (*Oryza sativa*) cultivars carrying the resistance gene *Pi33*. This recognition enables resistant plants to activate a defense response. *ACE1* was isolated by map-based cloning and encodes a putative hybrid between a polyketide synthase and a nonribosomal peptide synthetase, enzymes involved in microbial secondary metabolism. *ACE1* is expressed exclusively during fungal penetration of host leaves, the time point at which plant defense reactions are triggered. Ace1 appears to be localized in the cytoplasm of the appressorium. Mutation of the putative catalytic site of the β-ketoacyl synthase domain of Ace1 abolishes recognition of the fungus by resistant rice. This suggests that Ace1 biosynthetic activity is required for avirulence. Our results are consistent with the hypothesis that the fungal signal recognized by resistant rice plants is the secondary metabolite whose synthesis depends on Ace1.

INTRODUCTION

In the interaction between plants and their microbial pathogens, naturally occurring disease resistance often depends on specific interactions governed by pathogen avirulence (*AVR*) and plant disease resistance (*R*) genes (Flor, 1971). In these interactions, the pathogen produces a signal under the control of the *AVR* gene, whose recognition by the plant requires *R* gene activity. *R* genes encode related proteins that are classified based on the presence of leucine-rich repeats, nucleotide binding site, leucine zipper, Toll/Interleukin-1 receptor, and transmembrane domains (Martin et al., 2003). The predominant class of R proteins belongs to the nucleotide binding site–leucine-rich repeat type and appears to be directly involved in translating the presence of the pathogen-derived AVR signal into a plant response leading to resistance. It was originally proposed that the R protein acts as receptor for the AVR signal. However, after a series of unsuccessful attempts to demonstrate direct interaction between R protein and the corresponding AVR factor, this situation is now considered to occur rarely (Hammond-Kosack and Parker, 2003). Accumulating evidence supports a model termed the guard hypothesis that proposes that the R protein recognizes a complex containing the AVR signal and other plant proteins (van der Biezen and Jones, 1998).

Understanding the molecular basis of disease resistance requires not only knowledge about the mechanisms underlying perception of AVR signals by the host but also about the biological processes involved in avirulence signaling and their role in pathogenicity. Most of our current knowledge about *AVR* genes was gained from studies on plant pathogenic bacteria. More than 40 bacterial *AVR* genes were characterized to date, mostly in the genera Pseudomonas and Xanthomonas (Vivian and Arnold, 2000). Most bacterial *AVR* genes encode proteins without known functions that share a common active delivery system into the host cell (type III secretion). Bacterial *AVR* genes with known functions belong to the AvrBs3 family that displays typical features of eukaryotic transcription factors and modulate host gene expression (Marois et al., 2002) and to the AvrPtoB family that suppresses plant defense by inhibition of host programmed cell death (Abramovitch et al., 2003). The two unrelated Pseudomonas AVR factors AvrRpm1 and AvrB interact with RIN4, a protein that is involved in regulating basal defense responses in *Arabidopsis thaliana*. These interactions support the guard hypothesis because RIN4 appears to be guarded by the Arabidopsis R protein RPM1 (Mackey et al., 2002).

Despite the fact that fungi represent some of the most damaging crop pathogens, our knowledge about fungal *AVR* genes is still limited. Twelve fungal *AVR* genes have been characterized to date (Farman et al., 2002; Knogge, 2002; Luderer et al., 2002a, 2002b; Dodds et al., 2004) as well as one oomycete *AVR* gene (Shan et al., 2004). They encode small proteins or peptides without homology to each other that are secreted into host plants during infection. As for the bacterial

AVR factors, predicting function for the proteins encoded by fungal *AVR* genes has proven difficult. *NIP1* from the barley (*Hordeum vulgare*) pathogen *Rhynchosporium secalis* encodes a peptidic toxin that contributes to pathogenicity by inducing necrotic lesions (Rohe et al., 1995). *ECP2* from the tomato (*Lycopersicon esculentum*) pathogen *Cladosporium fulvum* encodes a protein without known biochemical function that acts as pathogenicity factor (Laugé et al., 1998), and *AVR4* from *C. fulvum* encodes a secreted protein that protects chitin from fungal cell walls (van den Burg et al., 2004). Finally, *AVR-Pita* from the rice (*Oryza sativa*) pathogen *Magnaporthe grisea* encodes a putative zinc-dependent metalloprotease that is dispensable for pathogenicity (Orbach et al., 2000). The interaction between AVR-Pita and the corresponding rice R protein Pi-ta is one of the few examples of direct interaction between R and AVR proteins (Jia et al., 2000). Clearly, more fungal *AVR* genes need to be characterized to investigate the spectrum of signals recognized by resistant host plants. In this context, the *M. grisea*–rice pathosystem is an attractive model for studying the interaction between an avirulent fungal plant pathogen and its resistant host. *M. grisea* is responsible for the most devastating disease of cultivated rice, called blast. Disease control relies mainly on the use of resistant rice cultivars, and several *AVR* genes have been identified in this fungus using classical genetics (Notteghem et al., 1994). Besides the above mentioned *AVR-Pita*, only one other *AVR* gene, *AVR-CO39*, and a set of host specificity genes, the *PWL1-4* genes, have been characterized so far (Kang et al., 1995; Sweigard et al., 1995; Farman et al., 2002).

The *AVR* gene *Avirulence Conferring Enzyme1* (*ACE1*) from *M. grisea*, previously named *AVR-Irat7*, was identified in a cross between Guy11, a hermaphroditic isolate from South America, and ML25, a male isolate from Africa (Silué et al., 1992). Guy11 is unable to infect resistant rice cultivars Irat7, DJ8-341, and Carreon, whereas the full-sib progeny 2/0/3 is virulent on these cultivars (Dioh et al., 2000). The rice resistance gene *Pi33* is responsible for the recognition of isolates carrying *ACE1* (Berruyer et al., 2003). *Pi33* was identified in different semi-dwarf indica rice cultivars, including the high yielding rice cultivar IR64 extensively used for rice breeding at the International Rice Reseach Institute. *Pi33* mapped on rice chromosome 8 and was distinguished from known neighboring resistance genes using allelism tests (Berruyer et al., 2003). *ACE1* segregates as a single Mendelian locus in the backcross between Guy11 and 2/0/3. Using progeny from this backcross, a genetic map was constructed based on restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers. *ACE1* was found to cosegregate with RAPD marker OPE-Y13, which maps on chromosome 1 at 20 centimorgans from *AVR1-CO39* (Farman and Leong, 1998; Dioh et al., 2000).

Here, we describe the cloning and characterization of the *AVR* gene *ACE1* from *M. grisea*. *ACE1* encodes a putative hybrid between a polyketide synthase (PKS) and a nonribosomal peptide synthetase (NRPS). PKS and NRPS are two distinct classes of enzymes involved in the production of secondary metabolites. *ACE1* is expressed specifically during penetration, and its translation product appears to be localized in the cytoplasm of appressoria. A single amino acid exchange in the β-ketoacyl synthase catalytic site of Ace1 abolishes recognition of the

fungus by resistant cultivars. This suggests that Ace1 biosynthetic activity is required for avirulence and that the detection of the invading fungal pathogen involves recognition of a secondary metabolite whose biosynthesis depends on Ace1.

RESULTS

Cloning of *M. grisea* Avirulence Gene *ACE1*

The single copy RAPD marker OPE-Y13 that cosegregates with the avirulence gene *ACE1* (Dioh et al., 2000) was used to screen a genomic cosmid library from avirulent progeny 96/0/76 (Dioh et al., 1997). The cosmid clone D31C12 was found to carry OPE-Y13. Using cosmid ends as probes, we isolated a single overlapping cosmid, D22F11. Together, both cosmids span a genomic region of 55 kb (Figure 1). To test for presence of *ACE1*, circular cosmids D31C12, D22F11, and the empty cosmid vector pMOcosX were introduced by transformation into virulent parent 2/0/3. Three pMOcosX transformants and nine D22F11 transformants were inoculated on susceptible rice cultivar Maratelli and resistant rice cultivar Irat7 carrying *Pi33*. These transformants were virulent on both cultivars (Figure 1), indicating that cosmid D22F11 does not carry *ACE1*. By contrast, six of ten transformants obtained with D31C12 were unable to infect
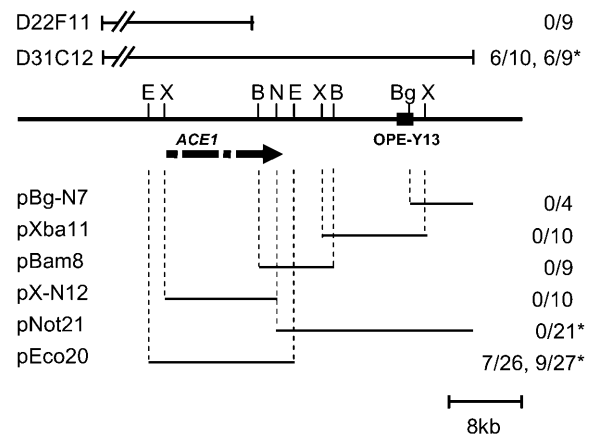

Figure 1. Isolation of *ACE1* by Complementation.

Schematic representation of the *ACE1* locus in Guy11, cosmids, and subclones tested for complementation to avirulence. The genomic locus is represented as a solid line, and the positions of RAPD marker OPE-Y13 and relevant restriction sites are indicated as follows: E, *Eco*RI; X, *Xba*I; B, *Bam*HI; N, *Not*I; Bg, *Bgl*II. The *ACE1* ORF is indicated as an arrow, and interruptions indicate positions of the three introns. Above the genomic locus map, the relative positions of the noncomplementing cosmid D22F11 and the complementing cosmid D31C12 are shown. Below the genomic locus map, the relative positions of D31C12 subclones used for complementation are shown. Fractions given to the right of each construct indicate the number of avirulent transformants identified over the number of transformants tested. Numbers without asterisks represent results obtained with virulent parent 2/0/3, numbers with asterisks represent results obtained with virulent field isolate PH14. Cosmid D31C12 and plasmid pEco20 complement both virulent strains to avirulence, demonstrating that they carry *ACE1*.

resistant cultivar Irat7 (Figure 1), although they retained full pathogenicity toward susceptible cultivar Maratelli (data not shown), suggesting that D31C12 carries *ACE1*.

To locate *ACE1* within D31C12, five overlapping subclones (pBg-N7, pXba11, pBam8, pX-N12, and pEco20; Figure 1) were constructed in the vector pCB1004 and introduced by transformation into virulent parent 2/0/3. The resulting transformants and two control 2/0/3 transformants obtained with cloning vector pCB1004 were inoculated on cultivars Irat7 and Maratelli. The 33 transformants obtained with the subclones pBg-N7, pXba11, pBam8, and pX-N12 and the two 2/0/3-pCB1004 transformants were virulent toward both resistant and susceptible rice cultivars (Table 1, Figure 1). By contrast, subclone pEco20 yielded seven avirulent transformants toward resistant cultivar Irat7 among 26 tested (Figure 1, transformant HBE20 in Figure 2B). These avirulent transformants were fully pathogenic toward susceptible cultivar Maratelli, suggesting that pEco20 carries *ACE1*. To confirm their specific interaction with *Pi33*, these avirulent transformants were inoculated on rice cultivars IR1529, C101lac, and Bala that carry *Pi33* but are of different genetic origin than Irat7 (Berruyer et al., 2003). Rice cultivar Sariceltik was included as additional susceptible control. As expected, transformants that were avirulent toward Irat7 were also avirulent toward IR1529, C101lac, and Bala (Table 1), confirming that pEco20 carries the dominant avirulence gene *ACE1* that interacts specifically with *Pi33*.

To corroborate the results of the complementation assays performed with virulent parent 2/0/3, we tested some selected D31C12 subclones for their ability to confer avirulence to the virulent field isolate PH14. Cosmid D31C12 and the subclones pEco20 and pNot21 that together span the entire genomic fragment carried by D31C12, were introduced by transformation into PH14. Six of nine transformants obtained with D31C12 and nine of 27 obtained with pEco20 were unable to infect the *Pi33* rice cultivars Irat7 and IR1529 but were fully virulent toward Maratelli (Figure 1). By contrast, the 21 PH14 transformants carrying pNot21 or the empty vector pCB1004 were as virulent on resistant rice cultivars as the recipient strain PH14. These results indicate that cosmid D31C12 and its subclone pEco20 have the ability to confer avirulence toward rice cultivars carrying *Pi33* independently of the virulent strain used as a recipient in transformation.

Subclone pEco20 carries a genomic *Eco*RI fragment of 15.4 kb. Sequence analysis of this fragment revealed a large open reading frame (ORF) of 12.5 kb interrupted by a few stop codons that correspond to three putative introns of 87, 91, and 70 bp, respectively (Figure 2A). The positions of these introns were confirmed experimentally by RT-PCR using total RNA extracted from barley leaves infected with avirulent isolate Guy11 17 h after inoculation and sequencing of the amplification products. Removal of the introns from the genomic sequence revealed an ORF of 12.1 kb. Transcription initiation and polyadenylation sites of *ACE1* were determined by rapid amplification of both cDNA ends (5′ and 3′ RACE). The 5′ and 3′ untranslated regions (UTRs) of *ACE1* were found to be 198 and 144 bp in length, respectively. The complete *ACE1* transcript is therefore expected to be 12,450-bp long. Besides the complete *ACE1* ORF, the complementing subclone pEco20 carries 1390 bp of upstream sequence (promoter region) and 1297 bp of downstream sequence (terminator region).

To prove that this ORF is the *ACE1* gene responsible for the avirulence of Guy11 toward rice cultivars carrying *Pi33*, we constructed mutants of Guy11 carrying a partial deletion of this ORF (*ace1Δ:hph*). To this end, we constructed a gene replacement vector pΔACE1 to replace an internal *ACE1* 3.6-kb *Age*I fragment by a hygromycin B resistance cassette (Figure 2A). After the introduction of pΔACE1 into Guy11 by transformation, we identified two transformants (HB26 and HB92) amongst 100 tested that had replaced their *ACE1* allele by the *ace1Δ:hph* allele. The two *ace1* deletion mutants HB26 and HB92 were inoculated on *Pi33* rice cultivars Irat7, IR1529, C101lac, and Bala and on the susceptible cultivars Maratelli and Sariceltik. The avirulent parental strain Guy11, the virulent strain 2/0/3, and three transformants (HB16, HB24, and HB33) that resulted from the ectopic integration of pΔACE1 served as controls. As expected, the three ectopic transformants HB16, HB24, and HB33 and the avirulent isolate Guy11 were unable to infect resistant cultivars but were fully pathogenic on Maratelli and Sariceltik (Table 1). By contrast, both *ace1* deletion mutants HB26 and HB92 were as pathogenic as the virulent parent 2/0/3 on the susceptible rice cultivars and on rice cultivars Irat7, IR1529, C101lac, and Bala, which contain *Pi33* (Table 1, HB26 in Figure 2B). These results demonstrate that the 12.5-kb ORF

Table 1. Pathogenicity Assays of 2/0/3-*ACE1* Transformants and Guy11-*ace1* Deletion Mutants

Cultivar	Guy11	2/0/3	2/0/3-31C12 #5	2/0/3-pMOcos X#1	2/0/3-pEco20 (HBE20)	2/0/3-pCB 1004#48	Guy11-HB26	Guy11-HB92	Guy11-HB24 (Ectopic)	Guy11-HB16 (Ectopic)	Guy11-HB33 (Ectopic)
Irat7 (R)	AVR	vir	AVR	vir	AVR	vir	vir	vir	AVR	AVR	AVR
IR1529 (R)	AVR	vir	AVR	vir	AVR	vir	vir	vir	AVR	AVR	AVR
C101lac (R)	AVR	vir	AVR	vir	AVR	vir	vir	vir	AVR	AVR	AVR
Bala (R)	AVR	vir	AVR	vir	AVR	vir	vir	vir	AVR	AVR	AVR
LS1 (s)	vir	vir	–	–	vir	vir	vir	vir	vir	vir	–
LTH (s)	vir	vir	–	–	vir	vir	vir	vir	vir	vir	–
Maratelli (s)	vir	vir	vir	vir	vir	vir	vir	vir	vir	vir	vir
Sariceltik (s)	vir	vir	vir	vir	vir	vir	vir	vir	vir	vir	vir

R, resistant (carrying *Pi33*); s, susceptible; AVR, avirulent; vir, virulent; –, not tested.
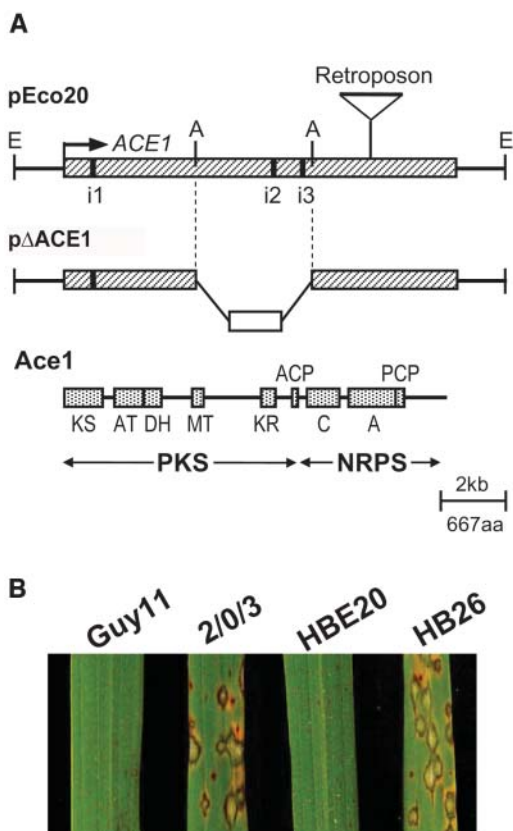
A

pEco20

B

Figure 2. Organization of Avirulence Gene *ACE1* and Analysis of Its Partial Deletion Mutants.

(A) Physical map of the complementing plasmid pEco20, insertion site of retroposon in 2/0/3 *ace1* virulent allele, the gene replacement vector pΔACE1, and Ace1 protein encoded by *ACE1*. The *ACE1* ORF is represented as a hatched box, the three introns (i1 to i3) as black bars, and restriction sites as E for *Eco*RI and A for *Age*I. In the *ace1* allele from virulent parent 2/0/3, a 2-kb sequence is inserted at 2.6 kb before the *ACE1* stop codon indicated by a retroposon sign on the pEco20 physical map. In the gene replacement vector pΔACE1, the *hph* resistance gene (open box) replaces an internal 3.6-kb *Age*I fragment. In the *ACE1* translation product (Ace1), stippled boxes represent enzymatic domains (drawn to scale): KS, β-ketoacyl synthase; AT, acyltransferase; DH, dehydratase; MT, methyl-transferase; KR, β-ketoreductase; ACP, acyl carrier protein; C, condensation domain; A, AMP binding domain; PCP, peptidyl carrier protein. PKS designates the polyketide synthase part of Ace1 and NRPS the nonribosomal peptide synthetase module of Ace1. aa, amino acids.

(B) Infection assays on resistant rice cultivar C101lac. Seedlings were spray inoculated with avirulent parent Guy11, virulent parent 2/0/3, 2/0/3 transformant HBE20 carrying pEco20, and Guy11 *ace1* deletion mutant HB26. Typical symptoms on rice leaves were obtained 7 d after inoculation. Guy11 is unable to infect the *Pi33*-carrying rice cultivar C101lac. Isolate 2/0/3 is virulent on C101lac and induces typical susceptible lesions. The 2/0/3 transformant HBE20 is avirulent on C101lac as a result of the introduction of *ACE1* carried by pEco20. HB26 is virulent on C101lac as a result of the partial deletion of *ACE1* by gene replacement using the pΔACE1 construct.

carried by pEco20 is the *ACE1* avirulence gene that interacts with the resistance gene *Pi33*.

Because the virulent *ace1* deletion mutants HB26 and HB92 are still pathogenic on rice, *ACE1* does not appear to be required for pathogenicity of *M. grisea* under our standard infection conditions. To test whether *ACE1* contributes to the aggressiveness of this fungus, we performed a quantitative pathogenicity assay with the virulent *ace1* deletion mutants HB26 and HB92, avirulent parent Guy11, and the two avirulent ectopic transformants HB16 and HB24. Additionally, we included avirulent transformant HBE20, control transformant 2/0/3-pCB1004, and virulent parent 2/0/3 (Table 1). Inoculations were performed on the following cultivars that do not carry *Pi33*: Sariceltik (highly susceptible japonica cultivar), Lijiangxintuanheigu (LTH; susceptible japonica cultivar), Maratelli (susceptible japonica cultivar), and Lung Sheng 1 (LS1; partially resistant japonica cultivar). These cultivars were inoculated with *M. grisea* conidial suspensions using two different methods, injection and spray inoculation. In these assays, virulent *ace1* deletion mutants HB26 and HB92 induce the same number of susceptible lesions on rice leaves (data not shown) as Guy11 and avirulent isogenic strains (HB16 and HB24). Furthermore, these lesions have the same size as those induced by avirulent strains (data not shown). These results suggest that *ACE1* does not contribute to lesion formation or invasive growth under our infection conditions. However, we cannot exclude that *ACE1* contributes to the aggressiveness of this fungus on other rice cultivars/host plants or under different infection conditions.

Loss of Avirulence in Virulent Parent 2/0/3

A survey of rice *M. grisea* isolates collected in rice fields at various worldwide locations revealed that virulence toward *Pi33* is less frequent in nature than avirulence (Berruyer et al., 2003). To understand the molecular events responsible for the loss of avirulence in the virulent parent 2/0/3, we compared the structure of the *ACE1* locus in 2/0/3 and avirulent parent Guy11. Genomic DNA gel blot hybridization revealed that *ACE1* is present in the genome of 2/0/3. RFLP analysis revealed a single difference in hybridization patterns between both strains. An internal 1.9-kb *Xho*I fragment of the *ACE1* allele from Guy11 appeared to be increased in size by 2 kb in 2/0/3 (see Supplemental Figure 1 online). The corresponding genomic region from 2/0/3 was amplified by PCR and sequenced. This revealed a 2-kb insertion in the last exon of *ACE1* (Figure 2A) that contains a fragment of the *MGL* Line1-like retroposon (GenBank accession number AF018033). Because no further RFLP was detected between Guy11 and 2/0/3, this insertion is likely to be responsible for the loss of avirulence.

ACE1 Encodes a Putative PKS/NRPS

The *ACE1* gene encodes a predicted 4035–amino acid polypeptide with a molecular mass of 438 kD. The deduced Ace1 polypeptide exhibits a high degree of similarity to type I fungal PKS at the N terminus and to bacterial NRPS at the C terminus. The PKS lovastatin nonaketide synthase (LNKS) from *Aspergillus terreus*, a key enzyme involved in the biosynthesis of the

antihypercholesterolemic lovastatin, shows a high degree of similarity to Ace1 (37% identity and 53% similarity). Fungal type I PKS are composed of a set of enzymatic domains that are used iteratively to catalyze the sequential condensation and modification of acyl-CoA precursors (Hutchinson et al., 2000). Ace1 is predicted to contain six enzymatic domains characteristic of fungal PKS (Figure 2A): β-ketoacyl synthase (KS), acyltransferase (AT), dehydratase (DH), methyltransferase (MT), β-ketoreductase (KR), and acyl carrier protein (ACP). The conserved active site motifs of KS, AT, DH, MT, and ACP domains (Hopwood and Sherman, 1990; Aparicio et al., 1996; Hopwood, 1997) were identified in the Ace1 protein sequence. The sequence of the putative NADPH binding site of the Ace1 KR domain (**GL**TG**GL**A) does not comply with the conserved consensus sequence described for PKS and fatty acid synthase (FAS) (GGxGxLG) and is therefore likely to be nonfunctional (Mathur and Kolattukudy, 1992). However, the KR domain is not essential for polyketide backbone biosynthesis but is involved in its modification by β-ketoreduction. Therefore, the PKS part of Ace1 is likely to be functional.

The C-terminal 1435–amino acid sequence of Ace1 is highly similar to MxaA from the myxobacterium *Stigmatella auranthiaca* (30% identity and 45% similarity). NRPS are composed of repeated, coordinated groups of enzymatic domains called modules. Each module consists of a condensation domain (C-domain), an adenylation domain (A-domain), and a peptidyl carrier protein (PCP) and is responsible for activation and covalent attachment of an amino acid (Marahiel et al., 1997). The C terminus of Ace1 represents a single complete NRPS module composed of a C-domain, an A-domain, and a PCP domain (Figure 2A). Because all core sequences described for bacterial NRPS (Marahiel et al., 1997) were identified in Ace1 protein sequence (shown for core motif C3 in Table 2), the NRPS module of Ace1 is likely to be functional. The structural basis of substrate recognition and activation by bacterial amino acid activating A-domains has been determined (Conti et al., 1997), allowing the deduction of the specificity-conferring code of bacterial adenylation domains. This code is contained in the signature sequence that is composed of the amino acid residues that line the amino acid binding pocket of the A-domain (Stachelhaus et al., 1999; Challis et al., 2000). Following the example of bacterial A-domains, we tried to predict which amino acid is activated by the Ace1 adenylation domain by comparing its signature sequence to the 31 bacterial NRPS signature sequences described by Stachelhaus and colleagues. Ace1 A-domain displayed closest similarity (70% identity) to Orn activating domains (**DM**AQ**LGG**INK for Ace1 and **DM**EN**LGL**INK for Orn1). How-

ever, because these signatures were defined for A-domains from bacterial NRPS, their specificity may be different from eukaryotic NRPS. In summary, our analyses suggest that Ace1 is a functional enzyme corresponding to a hybrid between a PKS and a NRPS. Previously, functional PKS/NRPS hybrids have been described exclusively in prokaryotes (Silakowski et al., 1999).

Ace1 Belongs to a Family of Fungal PKS/NRPS

Among fungal PKS with known biochemical activities, LNKS shows the highest degree of sequence identity to Ace1 (37%). LNKS carries the same PKS domains as Ace1 (KS, AT, DH, MT, KR, and ACP) and displays at its C terminus a sequence with similarity to NRPS condensation domains (C-domains; Hendrickson et al., 1999). In contrast with Ace1, the KR domain from LNKS is functional (Hutchinson et al., 2000), but the C-domain from the LNKS NRPS module lacks the second essential His of the core 3 motif HHxxxDG (Table 2; Marahiel et al., 1997) and is therefore unlikely to be functional. Additionally, LNKS does not carry the adenylation and PCP domains required for a functional NRPS module. The *MlcA* gene from *Penicillium citrinum* (Abe et al., 2002) encodes an enzyme with a biosynthetic activity similar to LNKS that carries a C-domain at its C terminus but has no adenylation and PCP domains.

The search for sequences homologous to Ace1 in fungal genome databases from the Broad Institute (http://www.broad. mit.edu/annotation/fungi/fgi/) and the Institute for Genomic Research (http://www.tigr.org) revealed 10 putative PKS/NRPS (Figure 3). In the *M. grisea* genome sequence, we identified apart from Ace1, five PKS/NRPS with a complete NRPS module (mg03810, mg03818, mg09589, Syn2, and Syn8) and two LNKS-like PKS/NRPS (Syn6 and Syn7) with a truncated NRPS module. Three of these PKS/NRPS are encoded by hypothetical proteins deduced from *M. grisea* ORFs (mg03810, mg03818, and mg09589) and four by novel genes we identified in the *M. grisea* genome (Syn2, Syn6, Syn7, and Syn8). Putative PKS/NRPS were also identified as hypothetical proteins deduced from ORFs of *Aspergillus nidulans* (AN8412), *A. fumigatus* (af04917), and *Fusarium graminearum* (FG10464) genomes. Independently, a phylogenomic analysis of fungal type I PKS reported the presence of seven other putative PKS/NRPS in the genomes of the ascomycetes *Botryotinia fuckeliana*, *Cochliobolus heterostrophus*, *Gibberella moniliformis*, and *G. zeae* (Kroken et al., 2003). A phylogenetic analysis of these putative PKS/NRPS was performed with the protein sequences of their KS and AT domains (see Supplemental Figure 2 online) using the neighbor-joining method. We included fungal PKS representatives of the monophyletic groups described by Kroken and coauthors, bacterial PKS (epoD, MxaC, and MxaD) related to fungal PKS, and the FAS from the silk moth *Bombyx mori* as outgroup. This phylogenetic analysis showed that PKS/NRPS clustered as a single group distinct from other fungal PKS because the node supporting this cluster has a bootstrap value of 100 (Figure 3). Interestingly, five PKS (BfPKS4, BfPKS6, ChPKS16, FG10464, and ncu08399) clustered with PKS/NRPS, suggesting they derive from PKS/NRPS by the complete loss of their NRPS module. Whereas all genomes from filamentous ascomycetes analyzed appeared to contain PKS/NRPS encoding genes, we could not

Table 2. Amino Acid Sequences of Core Motif C3 from the NRPS Condensation Domain[a]

Enzyme	Sequence
LNKS	**H**RLVG**DG**
Ace1	**HH**INM**DG**
Conserved motif	**HH**xxx**DG**

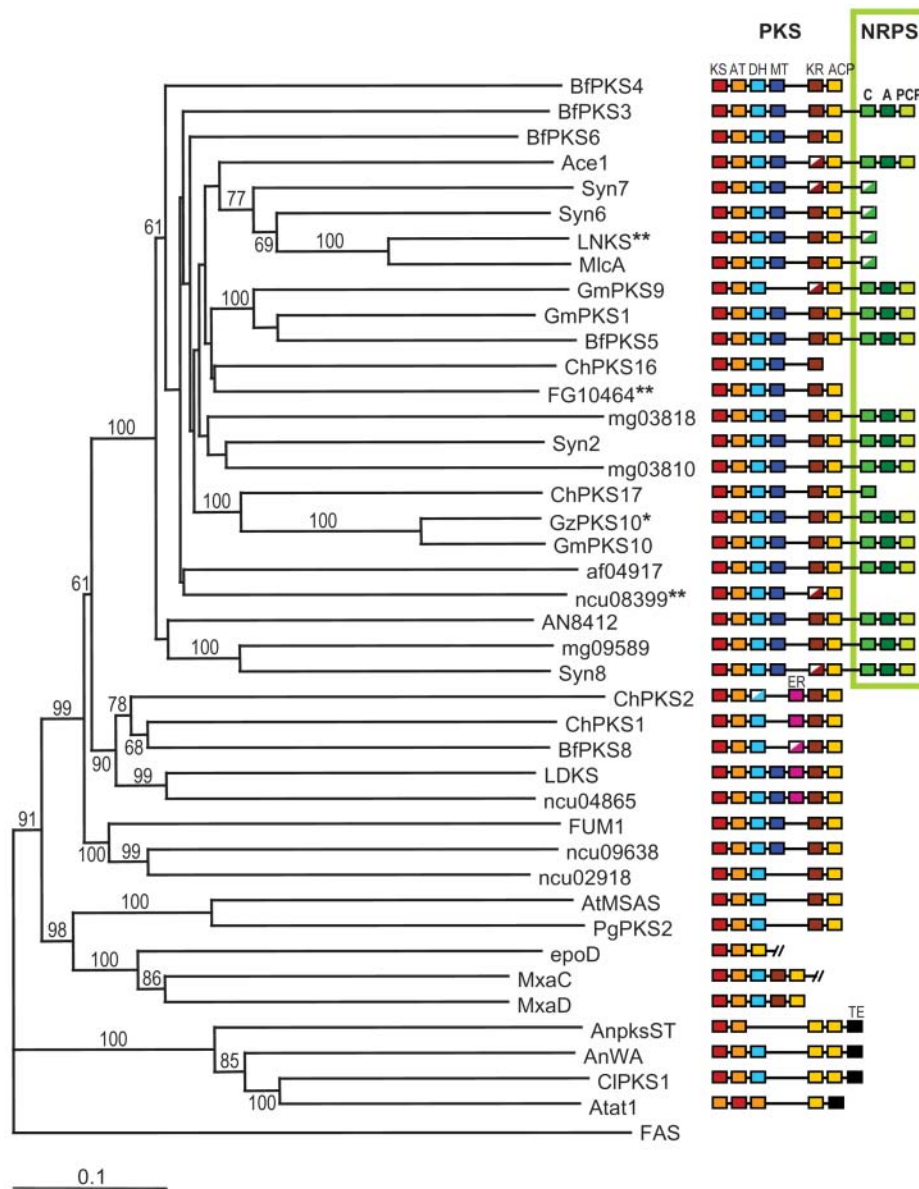[a] The core motif C3 is essential for NRPS catalytic activity (Marahiel et al., 1997).

Figure 3. Phylogeny of Fungal PKS Based on Protein Sequence of KS and AT Domains.

The consensus phylogenetic tree was obtained using the neighbor-joining method with distances. Bootstrap values are indicated above or below nodes of the tree. Colored boxes represent PKS and NRPS enzymatic domains (see Figure 2 for definitions). Sequences from the following organisms were included in this analysis (see Methods for accession numbers): *M. grisea* (Ace1, Syn2, Syn6,7,8, mg03810, mg03818, and mg09589); *A. fumigatus* (af04917); *A. nidulans* (AN8412, AnpksST, and AnWA); *F. graminearum* (FG10464); *N. crassa* (ncu08399, ncu04865, ncu09638, and ncu02918); *A. terreus* (LNKS, LDKS, AtMSAS, and Atat1); *P. citrinum* (MlcA); *P. griseofulvum* (PgPKS2); *C. heterostrophus* (ChPKS1); *G. moniliformis* (FUM1), and *C. lagenarium* (ClPKS1). We added fungal PKS and PKS/NRPS sequences described by Kroken et al. (2003) from *C. heterostrophus* (ChPKS2,16,17), *B. fuckeliana* (BfPKS3,4,5,6,8), *G. moniliformis* (GmPKS1,9,10), and *G. zeae* (GzPKS10) and bacterial PKS sequences (epoD, MxaC, and MxaD) as references. FAS from the silk moth *Bombyx mori* was included as an outgroup to root the tree and PKS/NRPS cluster as a monophyletic group. *, Although GzPKS10 is only described as a partial PKS sequence, its relatedness to GmPKS10 (90% identity) suggests that it has a full NRPS module as GmPKS10. **, FG10464 is almost identical (>91% identity) to GzPKS9 from Kroken et al. (2003). ncu08399 is identical to NcPKS4 from Kroken et al. (2003). LNKS is identical to lovB from Kroken et al. (2003).

identify such genes in the genomes of the hemi-ascomycetous yeast *Saccharomyces cerevisae*, the archeo-ascomycete *Schizosaccharomyces pombe*, and the basidiomycetes *Ustilago maydis*, *Coprinus cinereus*, and *Cryptococcus neoformans.* Overall, we did not identify PKS/NRPS orthologous to Ace1 in other fungal genomes, although we clearly showed that Ace1 belongs to a novel family of fungal PKS/NRPS.

ACE1 Is Expressed Exclusively during Penetration of *M. grisea* into the Leaf

The expression of *ACE1* in the avirulent *M. grisea* isolate Guy11 during mycelial growth and barley infection was analyzed by RT-PCR. RNA was isolated from mycelium grown in axenic culture and from infected barley leaves at the onset of penetration 17 h after inoculation. Primers were designed to amplify an *ACE1* genomic fragment of 453 bp carrying the first intron and corresponding to a 365-bp cDNA. The actin-encoding gene *ACT1* served as constitutive control. RT-PCR products were cloned and sequenced to confirm the specificity of amplification. An RT-PCR product corresponding to the *ACT1* transcript was obtained from both RNA samples (Figure 4A). By contrast, the *ACE1* transcript was detected only in RNA isolated from infected leaves but not in the mycelial RNA sample (Figure 4B). This suggested that *ACE1* expression is infection specific.

To follow the expression of *ACE1* during infection, we fused the green fluorescent protein (*GFP*) reporter gene to the *ACE1* promoter and terminator sequences. The resulting reporter construct, pProACE1:GFP, was introduced by transformation into avirulent isolate Guy11. Microscopic analysis of transformants carrying the *ProACE1:GFP* reporter construct confirmed that *ACE1* is not expressed at detectable levels in mycelium
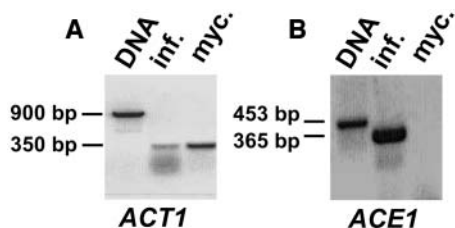

Figure 4. Expression Pattern of *ACE1* in Avirulent Isolate Guy11.

(A) PCR and RT-PCR amplification of the constitutively expressed *ACT1* gene as control of RNA integrity. The region spanning the first three introns of *ACT1* was amplified. Lane 1, *ACT1* PCR product obtained from genomic DNA (size, 900 bp). Lane 2, *ACT1* RT-PCR product obtained from RNA isolated from barley leaves infected with Guy11 17 h after infection (size, 350 bp). Lane 3, *ACT1* RT-PCR product obtained from RNA isolated from Guy11 mycelial culture. The amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide.
(B) PCR and RT-PCR amplification of *ACE1* from avirulent parent Guy11. The region spanning the first intron of *ACE1* was amplified. Lane 1, *ACE1* PCR product obtained from genomic DNA (size, 453 bp). Lane 2, *ACE1* RT-PCR product obtained from RNA isolated from barley leaves infected with Guy11 17 h after inoculation (size, 365 bp). Lane 3, No *ACE1* RT-PCR product was obtained with RNA isolated from Guy11 mycelial culture, suggesting that *ACE1* is not expressed in mycelium.

during axenic growth of the fungus in culture (data not shown). Transformants carrying the *ProACE1:GFP* reporter construct were inoculated on rice and barley leaves. They did not display GFP fluorescence in spores and germ tubes differentiated on the surface of rice and barley leaves. The characteristic green fluorescence of GFP was first detected in mature appressoria 15 h after inoculation of barley or rice leaves (data not shown). GFP fluorescence was strongest during penetration of the leaf surface ~24 h after inoculation (data not shown). After penetration into the epidermal cell (~36 h), GFP fluorescence was also detected in infectious hyphae (Figure 5A), illustrating cytoplasmic continuity between both structures (Bourett and Howard, 1990; Koga, 1994). The GFP fluorescence rapidly disappeared once secondary infectious hyphae began to spread within leaf tissues (~48 h; data not shown).

A similar appressorium-specific expression pattern was described for the *M. grisea* genes *GAS1* (*MAS3*), *GAS2* (*MAS1*), *CBP1*, and AI68463 (Banno et al., 2003; Takano et al., 2003). We searched for common transcription factor binding sites in the promoter sequences of *ACE1*, *GAS1* (AF363065), *GAS2* (AF264035), *CBP1* (BG809764), and AI068463. As control, we performed the same analysis on promoters from the constitutively expressed *M. grisea* genes encoding γ-actin (MG03982.4) and α-tubulin (MG06650.4). This revealed potential binding sites similar to the yeast stress response element (Transfac ID: F$STRE) in the promoters of *GAS1*, *GAS2*, *CBP1*, and AI068463, but not *ACE1*. This motif is located at similar distances from the transcription start site (TSS) of *GAS1*, *GAS2*, *CBP1*, and AI068463 (position −307 to −504, see Methods) and was not detected in the promoters of *ACT1* and *TUB1*. Thus, stress-related signaling may be involved in the regulation of expression of these four genes but is less likely to play a role in the regulation of *ACE1* expression. The promoters of all five appressorium-specific genes, including *ACE1*, carry potential GAL4 binding sites (Transfac ID: F$GAL4) that were not detected for the constitutive genes. However, the positions of these motifs vary considerably between promoters (position −136 to −685). It remains to be determined experimentally whether these putative binding sites play a role in the regulation of *ACE1* and other appressorium-specific genes.

Ace1-GFP Fusion Protein Is Localized in the Cytoplasm of the Appressorium

Fungal *AVR* genes characterized to date encode proteins that are either known or suspected to be secreted during infection (Knogge, 2002). By contrast, PKS and NRPS are typically cytoplasmic enzymes. Ace1 does not display a signal peptide sequence at its N terminus and was predicted to be cytoplasmic by PSORT II software (psort.nibb.ac.jp). To determine the cellular localization of Ace1, we constructed the vector pACE1:GFP, which encodes a C-terminal fusion between Ace1 and GFP, expressed under the control of the *ACE1* promoter and terminator sequences. We tested the effect of C terminus GFP fusion on Ace1 biological activity by introducing pACE1:GFP into the virulent parent 2/0/3. Nine of the eleven transformants obtained displayed a strong GFP fluorescence in their appressoria. These nine transformants were avirulent toward rice cultivars Bala and
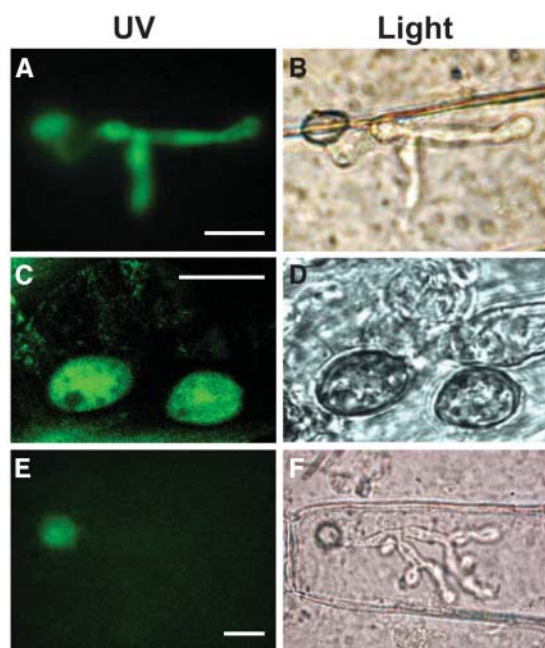
UV   Light

Figure 5. Localization of Ace1-GFP Fusion Protein in *M. grisea* Appressoria.

(A) GFP fluorescence of appressoria and infectious hypha after penetration of barley leaves. Conidia of transformant IF22 expressing GFP under the control of the *ACE1* promoter were inoculated on detached barley leaves and incubated at 26°C. At 36 h after infection, epidermal strips were removed from the infected leaf and observed under a microscope. Under blue light, GFP fluorescence is detected both in appressorium and in infectious hyphae. Bar = 10 μm.
(B) Same view as **(A)** under bright field. An appressorium and its infectious hypha located in the underlying epidermal cell are visible.
(C) Ace1-GFP fluorescence of appressoria on the surface of barley leaves. Conidia of transformant HB41 expressing the Ace1-GFP fusion protein under the control of *ACE1* promoter were inoculated on detached barley leaves incubated for 24 h at 26°C and observed with confocal laser scanning microscopy. Under blue light, fluorescence of the Ace1-GFP fusion protein is detected exclusively in the cytoplasm of the appressoria. Bar = 10 μm.
(D) Same view as **(C)** under bright field. Two germinated spores and appressoria are visible on the leaf surface.
(E) Ace1-GFP fluorescence of appressoria after penetration of barley leaves. Conidia of transformant HB44 expressing the Ace1-GFP fusion protein were inoculated on detached barley leaves and incubated at 26°C. Epidermal strips were removed from the infected leaf 40 h after inoculation and observed under a microscope. Under blue light, GFP fluorescence is detected in the appressorium but not in infectious hypha. Bar = 10 μm.
(F) Same view as **(E)** under bright field. An appressorium and its infectious hypha located in the underlying epidermal cell are visible.

C101Lac carrying *Pi33*, whereas they were fully virulent toward susceptible cultivar Maratelli. The two transformants that did not display GFP fluorescent appressoria remained virulent toward Bala and C101Lac. These results demonstrated that the Ace1-GFP fusion protein is functional as it conferred avirulence when introduced in a virulent strain. Microscopic observations of the

early infection process of these avirulent transformants (Figures 5C and 5E) showed that the kinetics of Ace1-GFP expression is similar to the kinetics observed with transformants expressing the *ProACE1*:*GFP* reporter construct (Figure 5A). Fluorescence of the Ace1-GFP protein was detected exclusively in the cytoplasm of appressoria (Figures 5C and 5E) during penetration of the fungus into barley or rice leaves, with the same expression pattern on susceptible and resistant rice cultivars (data not shown). Interestingly, the fluorescence of the fusion protein remained in the cytoplasm of the appressorium even after emergence of the infectious hyphae (Figure 5E). This is in contrast with the situation observed for transformants expressing GFP under the control of the *ACE1* promoter (Figure 5A) that displayed primary infectious hyphae with a strong GFP fluorescence. Fluorescence of the Ace1-GFP fusion protein disappeared gradually after completion of penetration and was not detected 48 h after inoculation (data not shown). Because we never detected Ace1-GFP fusion protein outside the appressorium, our observations suggest that Ace1 is not secreted into infected plant tissues. This raised the possibility that the host resistance response is not triggered by Ace1 protein but may depend on the biosynthesis of a metabolite by Ace1.

Site-Directed Mutagenesis of the Putative KS Domain of Ace1 Suggests That Its Enzymatic Activity Is Required for Avirulence

To test the hypothesis that Ace1 is involved in the biosynthesis of a secondary metabolite recognized by resistant rice plants, we decided to modify a domain of Ace1 that should be essential for its proposed enzymatic activity. We chose to modify the putative KS domain of Ace1 because it is known to be essential for the biosynthetic activity of PKS and mammalian FAS. Furthermore, the catalytic site motif of KS domains of bacterial PKS, fungal PKS, and mammalian FAS are highly similar in protein sequence and structure, and it has been well established that in these enzymes, a universally conserved Cys residue of the KS active site is essential for its catalytic activity (Keating and Walsh, 1999). Using site-directed mutagenesis, we replaced this Cys by an Ala (*ace1^{C183A}* allele) because this mutation is known to abolish the enzymatic activity of bacterial PKS and mammalian FAS without altering their folding and dimerization (Kao et al., 1996; Rangan et al., 1998). The *ace1^{C183A}* allele was fused to *GFP* to follow the expression of the mutant protein. The resulting construct, pace1^{C183A}:*GFP* was introduced by transformation into virulent parent 2/0/3. Among 44 transformants tested, 23 displayed a GFP fluorescence localized in the cytoplasm of appressoria, similar to those of transformants expressing a functional Ace1-GFP protein (Figure 6A). Whereas the 2/0/3 Ace1-GFP transformants were avirulent, transformants expressing the Ace1^{C183A}-GFP protein were as virulent on cultivars Bala and C101lac that carry *Pi33* as the virulent recipient strain 2/0/3 (Figure 6B). These results demonstrate that the *ace1^{C183A}*:*GFP* allele is unable to confer avirulence. Because the Ace1^{C183A} mutant is likely to have lost its ability to synthesize a polyketide, the observed loss of avirulence-conferring activity of the mutant protein is consistent with the hypothesis that the enzymatic activity of Ace1 synthase is required for avirulence.
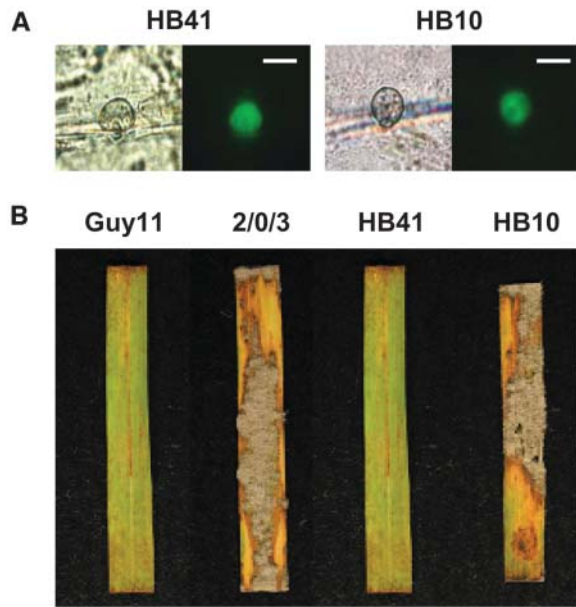
Figure 6. Lack of Avirulence Conferring Activity of the *ace1^{C183A}* Allele.

(A) Conidia of the transformants HB41 and HB10 were inoculated on detached barley leaves and incubated at 26°C. Epidermal strips were removed from the infected leaf 20 h after infection and observed under a microscope. Appressoria were observed under bright-field illumination (left) or UV light (right). The GFP fluorescence of Ace1-GFP and Ace1^{C183A}-GFP provides a control of the expression of the transgenes in appressoria and the correct localization of their respective translation product. Expression and localization of Ace1-GFP (HB41) and Ace1^{C183}-GFP (HB10) were similar, indicating that the Ace1^{C183}-GFP protein is expressed and localized as wild-type Ace1 protein. Bars = 8 μm.

(B) Avirulent isolate Guy11, virulent parent 2/0/3, and transformants of 2/0/3 expressing the *ACE1:GFP* fusion (HB41) or the mutant *ace1^{C183A}:GFP* allele (HB10) were inoculated on resistant rice cultivar C101lac. Guy11 and HB41 carrying *ACE1:GFP* are avirulent. Transformant HB10 and 2/0/3 carrying the mutant *ace1^{C183A}:GFP* allele are virulent.

DISCUSSION

Ace1-Mediated Avirulence Signaling

Fungal avirulence genes isolated to date encode small proteins that are known or supposed to be secreted during infection and are recognized by resistant plants (Knogge, 2002). The plant–pathogen interaction controlled by the avirulence gene *ACE1* from *M. grisea* and the rice resistance gene *Pi33* appears to be different because *ACE1* encodes a large cytoplasmic enzyme of 4035 amino acids that is unlikely to be secreted. Based on sequence homology, Ace1 protein appears to be a fungal PKS fused at its C terminus to a single complete NRPS module. Ace1 contains all sequence motifs known to be essential for the enzymatic activity of PKS and NRPS (Aparicio et al., 1996; Hopwood, 1997; Marahiel et al., 1997). These results suggest that Ace1 is an active enzyme that is likely to be involved in the biosynthesis of a secondary metabolite. Fungal PKS and NRPS are known to be involved in the production of mycotoxins and

host-specific toxins (HSTs) (Sweeney and Dobson, 1999; Wolpert et al., 2002). Wolpert and colleagues already pointed out that important parallels exist between the molecular and biochemical responses of host plants to HSTs and host plant defense reactions triggered by avirulence signals. The characterization of the PKS/NRPS Ace1 and its role in avirulence signaling may provide an example of the overlap between the concepts of HTS action and avirulence. Given the proposed biochemical function of Ace1, it is tempting to speculate that the metabolite produced by Ace1 could act as toxin that interferes with vital cellular functions or defense responses of the host plant, as illustrated by the *Cochliobolus carbonum*–maize (*Zea mays*) interaction (Walton, 1996). *C. carbonum* race 1 produces the NRPS-derived cyclic peptide HC-toxin that acts as HST. Specific maize cultivars are resistant to *C. carbonum* race 1, and this resistance depends on the presence of the dominant *HM1* gene in maize. The *HM1* resistance gene encodes a reductase that detoxifies HC-toxin. Lack of HC-toxin production results in complete loss of pathogenicity of *C. carbonum* on maize. By contrast, we did not observe a significant reduction in pathogenicity of *M. grisea ace1* deletion mutants. This suggests that Ace1 is not involved in the production of a toxin that acts as major pathogenicity determinant. Still, it remains possible that the proposed metabolite contributes to the aggressiveness of this fungus. Further experiments are needed to address this question.

To investigate the mechanisms involved in the recognition of the fungus triggered by *ACE1*, we addressed the following question. Is the Ace1 protein recognized by resistant rice cultivars? First, we determined the localization of Ace1 in the fungal cell to assess if this protein is secreted into infected host tissues where it could directly trigger resistance. For this study,

Table 3. *M. grisea* Strains Used in This Study

Strain	Description/Reference
Guy11	*ACE1*+ field isolate (Silué et al., 1992)
2/0/3	*ace1*− progeny of a cross between Guy11 and ML25 (Silué et al., 1992)
PH14	*ace1*− field isolate (Berruyer et al., 2003)
HBE20	*ACE1*+ transformant of 2/0/3 carrying p*ACE1* (this study)
HB26	*ace1*− deletion mutant of Guy11 obtained with pΔ*ACE1* (this study)
HB92	*ace1*− deletion mutant of Guy11 obtained with pΔ*ACE1* (this study)
HB16	*ACE1*+ ectopic transformant of Guy11 carrying pΔ*ACE1* (this study)
HB24	*ACE1*+ ectopic transformant of Guy11 carrying pΔ*ACE1* (this study)
HB33	*ACE1*+ ectopic transformant of Guy11 carrying pΔ*ACE1* (this study)
IF22	*ACE1*+ transformant of Guy11 carrying pProACE1:GFP (this study)
HB41	*ACE1*+ transformant of 2/0/3 carrying pACE1:GFP (this study)
HB44	*ACE1*+ transformant of 2/0/3 carrying pACE1:GFP (this study)
HB10	*ace1*− transformant of 2/0/3 carrying pace1^{C183A}:GFP (this study)

we used a vector expressing an *ACE1:GFP* translational fusion under the control of *ACE1* promoter that confers avirulence. Ace1-GFP fusion protein was detected exclusively in the cytoplasm of penetrating appressoria. We were unable to detect Ace1-GFP in the surrounding areas of appressoria. After penetration, the Ace1-GFP remained detectable in appressoria but not in infectious hyphae differentiated inside epidermal cells. This is in contrast with transformants that express GFP under the control of the *ACE1* promoter. In these strains, the cytoplasmic GFP is detectable both in appressoria and primary infection hyphae. Because there is a cytoplasmic continuity between appressoria and infectious hyphae, our observations suggest that Ace1-GFP fusion protein is retained in the appressorium. Similar observations have been described for the appressorium-specific proteins Gas1 and Gas2 from *M. grisea* (Xue et al., 2002), suggesting that retention of specific proteins in the appressorium during penetration is a common phenomenon. Localization of Ace1-GFP in the cytoplasm of the appressorium argues against its secretion into the underlying epidermal cell. However, it remains possible that small amounts of Ace1-GFP fusion protein below our detection limit are expressed in the infection hypha, secreted and delivered to plant cells. Additionally, Ace1 degradation products may be secreted and delivered to plant cells, triggering host resistance. Taking into consideration these possibilities, we decided to create a mutant protein that differs from the wild type only by the loss of its enzymatic function. The mutant Ace1^{C183A} protein fulfills these requirements as it carries a single amino acid modification in the KS domain catalytic site. The same amino acid exchange has been introduced in the KS domains of a bacterial PKS and mammalian FAS, where it has been shown to abolish enzymatic activity without interfering with the folding and dimerization of the mutant protein (Kao et al., 1996; Rangan et al., 1998). Thus, if direct recognition of Ace1 protein or its degradation products was involved in avirulence signaling, it should occur in the same way in transformants expressing Ace1^{C183A}. However, we found that Ace1^{C183A} was not able to confer avirulence to virulent strain 2/0/3, despite its correct expression and localization in appressoria. The most likely explanation for this lack of recognition is the loss of KS enzymatic activity of Ace1^{C183A} and the consequent loss of secondary metabolite production. To validate this hypothesis, we tried to identify the *ACE1* metabolite by liquid chromatography tandem mass spectrometry analysis of extracts from infected barley leaves 24 h after inoculation with either an avirulent or a virulent *M. grisea* isolate. These attempts were unsuccessful (data not shown), possibly because of the low amount of fungal material present in infected leaf tissue combined with the appressorium-specific expression of *ACE1*. Because appressoria formed on the artificial surfaces Teflon and Mylar do not appear to express *ACE1* (data not shown), we could not use these supports to generate large amounts of appressoria for the detection of the metabolite. Although we were not able to detect the *ACE1* metabolite, our results support the following hypothesis: Ace1 enzymatic activity is required for avirulence and is involved in the synthesis of a metabolite that triggers the recognition of the avirulent fungus by rice plants that carry the resistance gene *Pi33*.

To some extent, the requirement of a functional Ace1 enzyme for avirulence parallels results described for the bacterial type III–secreted Cys proteases AvrBsT and AvrPphB. For these three AVR factors, it was demonstrated that protease enzymatic activity is required for avirulence (Orth et al., 2000; Shao et al.,

Table 4. Primers Used in This Study

Primer	Sequence (5′/3′)	Purpose
H-OL1	TTGCCTCCATTGTGCTGAAG	RT-PCR (amplification of *ACE1* intron 1)
H-OL2	GCCTTCTGTGTGCCCAAT	RT-PCR (amplification of *ACE1* intron 1)
H-OL3	CCGCCGTCGTCACTCCCACC	RT-PCR (amplification of *ACE1* intron 2)
H-OL4	TGACAGAGGACAGGAAGACG	RT-PCR (amplification of *ACE1* intron 2)
H-OL5	CTCTTCTCGGTCGGCAACAC	RT-PCR (amplification of *ACE1* intron 3)
H-OL6	CGACGGCGACCAGTGAATCC	RT-PCR (amplification of *ACE1* intron 3)
H-OL7	ACATTGCTGGTTCCGTGGTGTTTATGA	5′ RACE of *ACE1*
H-OL8	CATCACGGGCGAGGGCACCAAT	3′ RACE of *ACE1*
H-OL9	TTGCGATTAGCGTCCATTGT	RT-PCR (amplification of *ACT1* introns 1 to 3)
H-OL10	TTCATCAGGTAGTCGGTCAA	RT-PCR (amplification of *ACT1* introns 1 to 3)
H-OL11	CGGAAATCCGCATCTCTAGCTTGGCGC	Construction of pProACE1:GFP
H-OL12	GTCACCCATGGTTGGTGATTTATTTGTTT	Construction of pProACE1:GFP
H-OL13	GGCCGGC	Construction of pProACE1:GFP
H-OL14	GCCACTCGGAATAAACAGAGGGTGGTC	Construction of pProACE1:GFP
H-OL15	GCTGAAGGGCGGCCGCTGATTCATGAACTTTGGAATACTGTAACTAG	Construction of pACE1:GFP
H-OL16	CAGTATTCCAAAGTTCATGACCATGGTC	Construction of pACE1:GFP
H-OL17	CCGTCCTTCAGC	Construction of pACE1:GFP
H-OL18	GCTGAAGGACGGGACCATGGTCATGAA	Construction of pACE1:GFP
H-OL19	CTTTGGAATACTG	Construction of pace1^{C183A}:GFP
H-OL20	GCTGAAGGGCGGCCGCTGATTCATGAACTTTGGAATACTGTAACTAG GCCACTCGGAATAAACAGAGGGTGGTC CGGTCACGGTCGATACCGCAGCTAGCTCCAGCTTGGTGGCCG CGGCCACCAAGCTGGAGCTAGCTGCGGTATCGACCGTGACCG	Construction of pace1^{C183A}:GFP

2003). Shao and coworkers postulated that these AVR proteases act on host plant target proteins and that the corresponding degradation products are the signals recognized by R proteins. The indirect signaling proposed for *ACE1* is different because this fungal protein is not expected to act through an enzymatic modification of host proteins, but through the biosynthesis of a secondary metabolite that may serve as avirulence signal. To our knowledge, there is currently only one precedent for metabolite-mediated avirulence signaling, described in the interaction between *Pseudomonas syringae* and soybean (*Glycine max*). In this case, the avirulence gene *AvrD* directs the production of a group of small glycolipid molecules called syringolides (Kobayashi et al., 1990). These amphipathic molecules induce the hypersensitive response only in soybean cultivars carrying the *Rpg4* resistance gene, possibly through interference with a photorespiration enzyme of the host (Atkinson et al., 1996; Okinaka et al., 2002). However, the role of syringolide production in the biology of Pseudomonas remains somewhat enigmatic. Apart from two sequences with similarity to *AvrD* that have been discovered in the genomes of *Ralstonia solanacearum* and *Streptomyces coelicolor* A3(2), *AvrD* genes were found exclusively in *P. syringae* isolates (Bentley et al., 2002; Salanoubat et al., 2002). Therefore, syringolides do not appear to be a major class of metabolites from plant pathogenic bacteria. By contrast, many microorganisms produce secondary metabolites using biosynthetic pathways that involve PKS or NRPS. In particular, plant-pathogenic microbes are notable producers of polyketides or nonribosomal peptides (Lugtenberg et al., 2002; Wolpert et al., 2002). Our analysis of *ACE1*-mediated avirulence signaling implies that the spectrum of molecular structures that can be recognized by resistant host plants is broad and that it could include PKS/NRPS-derived metabolites. Recognition of this class of metabolites by plants may provide disease resistance against a large variety of microbial pathogens through the perception of metabolites they produce during infection.

Ace1 Belongs to a Novel Family of Fungal PKS/NRPS

Ace1 belongs to a novel family of fungal PKS/NRPS that gathers at least 17 hypothetical proteins and two enzymes. Two fungal PKS/NRPS have known biochemical functions, the lovastatin biosynthetic enzyme LNKS from *A. terreus* (Kennedy et al., 1999) and the related enzyme MlcA from *P. citrinum* (Abe et al., 2002). LNKS and MlcA lack the adenylation and PCP domains required for a functional NRPS module. Therefore, their metabolic products may not be representative of the compounds produced by PKS/NRPS with a complete NRPS module. Two PKS/NRPS with a structure similar to LNKS were identified in the genome of *M. grisea* (Syn6 and Syn7). We identified eight PKS/NRPS with a complete NRPS module in the genomes of the ascomycetes *M. grisea*, *A. nidulans*, and *A. fumigatus* (see Supplemental Figure 3 online). Independently, a phylogenomic analysis of fungal type I PKS genes reported the presence of six novel putative PKS/NRPS with a full NRPS module in the genomes of the ascomycetes *B. fuckeliana*, *C. heterostrophus*, and *G. moniliformis* (Kroken et al., 2003). This study is complementary to ours in terms of the genomes analyzed and reaches similar conclusions.

Fungal PKS/NRPS belong to a monophyletic group (Figure 3) and are likely to derive from an ancestral enzyme corresponding to a type I fungal PKS fused to a complete NRPS module. This family may have evolved through duplications and independent truncations of the C-terminal NRPS module. Partial truncation may have led to PKS/NRPS related to LNKS (Syn6 and Syn7) or to ChPKS17. Independent loss of the entire NRPS module may have led to putative PKS, such as BfPKS4, BfPKS6, ChPKS16, FG10464, and ncu08399.

The genome of *M. grisea* appears to be relatively rich in PKS/NRPS with either a full or a truncated NRPS module (eight PKS/NRPS) compared with other fungi, such as *B. fuckeliana* (three PKS/NRPS), *C. heterostrophus* (one PKS/NRPS), and *G. moniliformis* (three PKS/NRPS) (Kroken et al., 2003). This may indicate a recent evolution of this gene family by gene duplication in *M. grisea*. By contrast, the closely related pyrenomycete *Neurospora crassa* has only one sequence related to fungal PKS/NRPS that may have lost its NRPS module. *M. griseae* and *N. crassa* are phylogenetically related species that differ most importantly in their respective life styles because the former is a pathogen of cereals, whereas the latter is a tropical soil/bark saprophyte. After the recent completion of the genome sequence of both organisms, one hope is that the direct comparison of their genomes may provide some insight into what it takes to make a pathogen. In this context, it is tempting to speculate that the observed richness of the Magnaporthe genome in PKS/NRPS may be part of the important differences between the pathogen and the saprophyte.

METHODS

Fungal Strains, Growth Conditions, and Transformation

The *M. grisea* strains used in this study are listed in Table 3. Guy11 is a fertile rice field isolate pathogenic on rice (*Oryza sativa*) and barley (*Hordeum vulgare*) (Notteghem et al., 1994), and 2/0/3 is a sib-progeny from the cross (Guy11 × ML25) (Silué et al., 1992). As all rice pathogenic *Magnaporthe grisea* isolates, Guy11 and 2/0/3 are also pathogenic on barley. Fungal strains were grown and stored as described (Dioh et al., 2000). *M. grisea* strains were transformed as described (Sweigard et al., 1995; Clergeot et al., 2001), with one modification for transformation of isolate Guy11 to hygromycin resistance: after incubation of the transformation plates at 30°C for 20 h, 5 mL of overlay agar (1% granulated agar [Difco, Becton Dickinson, Franklin Lakes, NY] and 300 mg/L of hygromycin) were added to each plate to reduce the occurrence of background growth from nontransformed colonies.

Phenotypic Analysis and Cytology

Rice cultivars Maratelli, Sariceltik, LTH, and LS1 were used as susceptible controls because they do not carry *Pi33*. Rice cultivars Irat7, IR1529, C101lac, and Bala were used as resistant controls because they carry the resistance gene *Pi33*. These rice cultivars were grown in the greenhouse as described (Dioh et al., 2000). Spray infections and detached leaf assays were performed as described (Silué et al., 1992; Clergeot et al., 2001). Fluorescence of the Ace1-GFP fusion protein was observed using a Nikon Optiphot-2 epi-fluorescence microscope equipped with a GFP-specific filter (excitation, 475/20; emission, 510/23; ref, XF100-2-NS4A; Nikon, Tokyo). Laser scanning electron confocal microscopy and image analyses were performed at the Centre de Quantimétrie (Lyon, France).

Nucleic Acid Extraction Analysis and Cloning Procedures

Genomic DNA was isolated from *M. grisea* following the miniprep procedure (Sweigard et al., 1997) with the following modifications: for protoplasting, Glucanex (Novo Nordisc Ferment, Dittingen, Switzerland) was used at 30 mg/mL in 0.7 M NaCl, pH adjusted to 6; protoplasts were lysed by vigorous vortexing with 0.3 g glass beads (425 to 600 μm, acid washed) for 45 s. Total RNA was extracted from *M. grisea* liquid cultures and infected barley leaves using the hot acid phenol protocol (Ausubel et al., 1994). For RFLP analysis, 5 μg of each genomic DNA were digested with *Xho*I, separated by gel electrophoresis on a 1% agarose gel, and transferred by vacuum blot to positively charged nylon membrane (Hybond-N+; Amersham Biosciences). Hybridization was performed with a probe composed of three individually labeled genomic restriction fragments spanning the *ACE1* ORF from 123 to 11,860 bp: a 2.8-kb *Eco*RV fragment, a 4.4-kb *Eco*RV fragment, and a 4.5-kb *Eco*RV-*Not*I fragment. Labeling was performed using the Megaprime DNA labeling system (Amersham Biosciences, Piscataway, NJ). Restriction enzyme digestion, gel electrophoresis, and hybridization were performed using standard procedures (Sambrook et al., 1989). For cloning purposes, the *Escherichia coli* strains DH5α (Bethesda Research Laboratories, Gaithersburg, MD) and JM110 (Stratagene, La Jolla, CA) were used. Molecular methods followed described protocols (Sambrook et al., 1989).

RT-PCR

RT-PCR was performed with 6 μg of total RNA using ReadyToGo You-Prime first-strand beads (Amersham Biosciences) according to the manufacturer's protocol. Positions of introns in the *ACE1* sequence were identified using the following strategy. Putative exon junction regions were amplified by RT-PCR using total RNA extracted from infected barley leaves 17 h after inoculation and the following primer pairs: intron 1, H-OL1 (for primer sequences, see Table 4) and H-OL2; intron 2, H-OL3 and H-OL4; intron 3, H-OL5 and H-OL6. PCR products were cloned in pGEM T-easy (Promega, Madison, WI) according to the manufacturer's protocol and sequenced. Transcription initiation and polyadenylation site of *ACE1* were determined by 5′ and 3′ RACE using GENE RACER kit (Invitrogen, Carlsbad, CA) and sequence analysis of amplification products. The 5′ UTR was amplified with gene-specific primer H-OL7, and the 3′ UTR was amplified using gene-specific primer H-OL8. The actin encoding gene *ACT1* was used as control in RT-PCR experiments. An EST corresponding to *ACT1* was isolated from a *M. grisea* cDNA library prepared from mycelial RNA (Clergeot et al., 2001) and used to identify the corresponding cosmid from a 96/0/76 genomic library (Dioh et al., 1997). Comparison of cDNA and genomic sequence revealed the presence of four introns. In the RT-PCR experiments, the region carrying the first three introns was amplified using primers H-OL9 and H-OL10.

Sequence Analysis

Sequencing was performed by Genome Express (Meylan, France). Sequence analysis was performed using DNA Strider 1.2 (Marck, 1988), BLAST (Altschul et al., 1997; http://www.ncbi.nlm.gov/BLAST/), PSORT (Nakai, 2000; http://psort.nibb.ac.jp/), and Pfam 6.5 (Bateman et al., 2002; http://pfam.wustl.edu/).

Promoter Analysis

For each gene, the TSS was determined by comparing EST sequences to the corresponding genomic sequence. The 1 kb of DNA sequence upstream from TSS was examined using the following software: TRES (http://bioportal.bic.nus.edu sg/tres/), MatInspector (http://www.genomatix.de), TF Scan (http://bioweb.pasteur.fr), and OTFBS (http://www.bioinfo.tsinghua.edu.cn/~zhengjsh/OTFBS/). Putative binding sites for the yeast stress response element (Transfac ID: F$STRE) were identified in the promoters of *GAS1* (positions −504 and −558), *GAS2* (position −416), *CBP1* (position −307), and AI068463 (position −331). Furthermore, putative GAL4 binding motifs were detected in the promoters of *ACE1* (positions −136 and −610), *GAS1* (position −387), *GAS2* (position −670), *CBP1* (overlapping motifs on the plus and minus strand, positions −158 and −160 and positions −668 and −685).

Identification of Ace1 Enzymatic Domains

The following domains were identified using the Pfam 6.5 database: KS, N-terminal and C-terminal domains (PF00109 and PF02801), AT (PF00698), condensation (C-) domain (PF00668), AMP binding enzyme (A-domain) (PF00501), and phosphopantheteine attachment site (ppt-domain) (PF00550). The remaining domains (DH, MT, KR, and ACP) were identified by direct comparison of the Ace1 sequence with the sequences of LNKS from *Aspergillus terreus* (AF151722), Pks1 from *Cochliobolus heterostrophus* (U68040), and Fum5 from *Gibberella moniliformis* (AF155773).

Phylogeny of Fungal PKS and PKS/NRPS

The Ace1 protein sequence was used as a query to search the non-redundant protein database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Additional fungal PKS/NRPS were identified using the TBLASTN program and Ace1 protein sequence as a query in the following databases. The fungal genomes database Web site (http://www.broad.mit.edu/annotation/fungi/fgi) was used for *M. grisea*, *Neurospora crassa*, *Aspergillus nidulans*, *Fusarium graminearum*, *Ustilago maydis*, *Coprinus cinereus*, and *Cryptococcus neoformans* genome sequences. The Institute for Genomic Research Web site (http://www.tigr.org) was used for the preliminary genomic sequences of *Aspergillus fumigatus*. Annotated ORFs with significant similarity to Ace1 were used directly for the analysis (mg03810, mg03818, mg09589, ncu08399, AN8412, af04917, and FG10464). Sequences that displayed high degrees of similarity but were not annotated (Syn2, Syn6, Syn7, and Syn8) were processed as follows. The nucleotide sequence spanning the region of similarity to *ACE1* was recovered and compared with *ACE1* at the protein level using BLASTX. Putative introns were detected as gaps in the collinearity of the aligned protein sequences, followed by manual search for motifs characteristic of fungal intron splice donor and acceptor sites (Edelmann and Staben, 1994). The protein sequences were obtained from the translation of ORF sequences resulting from the removal of the putative introns. Protein sequences from the KS and AT domains of each PKS and PKS/NRPS were aligned using the CLUSTALW program (http://www.ch.embnet.org/software/ClustalW.html). Ace1, fungal PKS, and PKS/NRPS sequences were analyzed with PAUP 4.0b10 (Swofford, 2002) using the neighbor-joining distance method. The following enzymes were included in the analysis: AnpksST (AAA81586) and AnWA (CAA46695) from *A. nidulans*; LNKS (AF151722), LDKS (AF141925), AtMSAS (AAC49814), and Atat1 (BAB88688) from *Aspergillus terreus*; ClPKS1 (BAA18956) from *Colletotrichum lagenarium*; ChPks1 (AAB08104) from *Cochliobolus heterostrophus*; Fum1 (AAD43562) from *Gibberella moniliformis*; mLcA (BAC20564) from *Penicillium citrinum*; PgPKS2 (AAB49684) from *Penicillium griseofulvum*. The following hypothetical proteins deduced from ORFs identified in genomic sequences were included in the analysis: mg03810 (EEA50051), mg03818 (EEA50059), mg09589 (EEA48052) from *M. grisea*, ncu02918.1 (EEA36364), ncu04865.1 (EEA29886), ncu08399.1 (EEA34002), and ncu09638.1 (EAA28933) from *N. crassa*, AN8412 (EAA67034) from *A. nidulans*, af04917 from *A. fumigatus*, and FG10464.1 (EAA68296) from *F. graminearum*. Syn2, Syn6, Syn7, and Syn8 were identified in the *M. grisea* genome during this study (*SYN2* as

AJ704623, *SYN6* as BN000505, *SYN7* as BN000506, and *SYN8* as BN000507). We added fungal PKS and PKS/NRPS sequences described by Kroken et al. (2003) from *C. heterostrophus* (ChPKS2,16,17), *B. fuckeliana* (BfPKS3,4,5,6,8), *G. moniliformis* (GmPKS1,9,10), and *G. zeae* (GzPKS10, partial sequence). GzPKS10 is highly related to GmPKS10 (90% identity) and is likely to have a full NRPS module as GmPKS10. The following three PKS/NRPS (GzPKS9, BfPKS7, and GmPKS8) identified by Kroken et al. were not added to this analysis because they lack some sequences of their KS or AT domains or have only KS domains (GzPKS9). GzPKS9 is almost identical (>91% identity) to FG10464. We also included in the analysis bacterial PKS epoD (AAF26922), MxaC (AAK57187), and MxaD (AAK57188) related to fungal PKS. FAS from *Bombyx mori* (U67866) served as outgroup to root the tree.

Plasmid Constructions

The cosmid library is described by Dioh et al. (1997). For cosmid D31C12 subclones, cosmid DNA was digested with each of the following enzymes: *Bam*HI, *Eco*RI, *Not*I, and *Xba*I and in double digests with *Bgl*II plus *Not*I and *Xba*I plus *Not*I, respectively. The following genomic fragments were recovered and cloned into pCB1004 (Sweigard et al., 1997): for pBam8, an 8.3-kb *Bam*HI fagment; for pEco20, a 15.4-kb *Eco*RI fragment; for pNot21, a 21.0-kb *Not*I fragment (one *Not*I site is derived from the cosmid polylinker); for Xba11, an 11.1-kb *Xba*I fragment; for pBg-N7, a 6.7-kb *Bgl*II-*Not*I fragment (*Not*I site derived from polylinker); for pX-N12, a 12.1-kb *Xba*I-*Not*I fragment.

For pΔACE1, the 15.4-kb *Eco*RI fragment carrying *ACE1* was cloned into pBluescript II KS−. The resulting vector was digested with *Age*I, and the internal 3.5-kb *Age*I fragment was replaced by the 1.4-kb hygromycin resistance cassette from pCB1004 (Sweigard et al., 1997).

For pProACE1:GFP, the 1.6-kb *ACE1* promoter region was amplified using primers H-OL11 and H-OL12, introducing an *Nco*I site at the start codon of *ACE1*; 1.5-kb *ACE1* terminator region were amplified using primers H-OL13 and H-OL14, introducing a *Not*I site at the stop codon of *ACE1*. The resulting PCR products were cloned in pGEMT-easy (Promega) and sequenced. Then, the *ACE1* promoter was excised as an *Eco*RI-*Nco*I fragment and the terminator as *Not*I-*Eco*RI fragment, and both were fused to the *Nco*I-*Not*I fragment from pEGFP (Clontech, Palo Alto, CA) carrying the EGFP reporter gene (Lorang et al., 2001). The *ProACE1:GFP* transcriptional fusion was introduced into a pBluescript II KS− (Stratagene) carrying at its *Eco*RV site the hygromycine resistance cassette from pCB1003 .

For pACE1:GFP, an *Nco*I site was introduced at the *ACE1* stop codon by PCR using primers H-OL15 and H-OL16. The *ACE1* terminator region was amplified with primers H-OL17 and H-OL18, introducing a *Not*I site immediately downstream of the stop codon of *ACE1*. The *Nco*I-*Not*I *GFP* cassette of pEGFP (Clontech) was cloned between the 3′ end of the *ACE1* ORF and the *ACE1* terminator. Expression of the tagged allele is under the control of the native promoter.

For pace1^C183A:GFP, the KS domain coding sequence of *ACE1* was subjected to site-directed mutagenesis using primers H-OL19 and H-OL20 and the QuikChange site-directed mutagenesis kit (Stratagene). This leads to replacement of catalytic residue Cys183 with Ala, introducing a diagnostic *Nhe*I restriction site. The resulting PCR product was sequenced, and the 470-bp *Bsp*EI-*Fse*I fragment carrying the introduced mutation was used to replace the native 470-bp *Bsp*EI-*Fse*I fragment in pACE1:GFP.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under the following accession numbers: *ACE1* as AJ704622, *SYN2* as AJ704623, *SYN6* as BN000505, *SYN7* as BN000506, and *SYN8* as BN000507.

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