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

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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*). 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* expression is exclusively during fungal penetration of host leaves, the time point at which plant defense reactions are triggered. Ace1 is 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 is 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; Knooge, 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 Confering 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 *P33* is responsible for the recognition of isolates carrying *ACE1* (Berruyer et al., 2003). *P33* was identified in different avirulent lines from tomato (*Lycopersicon esculentum*), which includes the high yielding rice cultivar IR64 extensively used for rice breeding at the International Rice Research Institute. *P33* 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 *ACE1* (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 peptidyl 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 parental 2/0/3. Three pMOcosX transformants and nine D22F11 transformants were inoculated on susceptible rice cultivar Maratelli and resistant rice cultivar *Irat7* carrying *P33*. 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*, EcoRI; *X*, XbaI; *B*, BamHI; *N*, Ncol; *BglII*. 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 tested (Figure 1, transformant HBE20 in Figure 2B). These 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 tested (Figure 1, transformant HBE20 in Figure 2B).

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

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>2/0/3-31C12 #5</th>
<th>2/0/3- pEco20 (HBE20) 1004#48</th>
<th>2/0/3- pPCB</th>
<th>Guy11-HB26</th>
<th>Guy11-HB92 (Ectopic)</th>
<th>Guy11-HB24</th>
<th>Guy11-HB16 (Ectopic)</th>
<th>Guy11-HB33 (Ectopic)</th>
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<tbody>
<tr>
<td>Irat7 (R)</td>
<td>AVR</td>
<td>AVR</td>
<td>vir</td>
<td>vir</td>
<td>vir</td>
<td>AVR</td>
<td>AVR</td>
<td>AVR</td>
</tr>
<tr>
<td>IR1529 (R)</td>
<td>AVR</td>
<td>AVR</td>
<td>vir</td>
<td>vir</td>
<td>vir</td>
<td>AVR</td>
<td>AVR</td>
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</tr>
<tr>
<td>C101lac (R)</td>
<td>AVR</td>
<td>AVR</td>
<td>vir</td>
<td>vir</td>
<td>vir</td>
<td>AVR</td>
<td>AVR</td>
<td>AVR</td>
</tr>
<tr>
<td>Bala (R)</td>
<td>AVR</td>
<td>AVR</td>
<td>vir</td>
<td>vir</td>
<td>vir</td>
<td>AVR</td>
<td>AVR</td>
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<tr>
<td>LS1 (s)</td>
<td>vir</td>
<td>–</td>
<td>–</td>
<td>vir</td>
<td>vir</td>
<td>–</td>
<td>–</td>
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<td>Sariceltik (s)</td>
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R, resistant (carrying Pi33); s, susceptible; AVR, avirulent; vir, virulent; –, not tested.

Subclone pEco20 carries a genomic EcoRI 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 P33, we constructed mutants of Guy11 carrying a partial deletion of this ORF (ace1313:hph). To this end, we constructed a gene replacement vector pACE1 to replace an internal ACE1 3.6-kb fragment by a hygromycin B resistance cassette (Figure 2A). After the introduction of pACE1 into Guy11 by transformation, we identified two transformants (HB26 and HB92) amongst 100 tested that had replaced their ACE1 allele by the ace1313:hph allele. The two ace1 deletion mutants HB26 and HB92 were inoculated on P33 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 P33 (Table 1, HB26 in Figure 2B). These results demonstrate that the 12.5-kb ORF...
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 *XhoI* 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
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), Ljiangxintuanheig (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.
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 (GLTGG LA) 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 aurantiaca (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 described exclusively in prokaryotes (Silakowski et al., 1999).

### Table 2. Amino Acid Sequences of Core Motif C3 from the NRPS Condensation Domain

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>LNKS</td>
<td>HRLVGDG</td>
</tr>
<tr>
<td>Ace1</td>
<td>HHINHDG</td>
</tr>
<tr>
<td>Conserved</td>
<td>HxkxkDG</td>
</tr>
</tbody>
</table>

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

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 HxkxkDG (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 McA 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 phylogenetic analysis of fungal type I PKS reported the presence of seven other putative PKS/NRPS in the genomes of the ascomycetes Botryotinia fuckeliana, Cochliobolus heterosporus, 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...
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, AnpkST, and AnWA); F. graminearum (FG10464); N. crassa (ncu08399, ncu04865, ncu09638, and ncu02918); A. terreus (LNKS, LDKS, AIMASAS, and Atat1); P. citrinum (MicA); P. griseofulvum (PgPKS2), C. heterostrophus (ChPKS1); G. moniliformis (FUM1), and C. lagenarium (CIPKS1). We added fungal PKS and PKS/NRPS sequences described by Kroken et al. (2003) from C. heterostrophus (ChPKS2,16,17), B. fuckeliana (BIPKS3,4,5,6,8), 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 \textit{Saccharomyces cerevisiae}, the archeo-ascomycete \textit{Schizosaccharomyces pombe}, and the basidiomycetes \textit{Ustilago maydis}, \textit{Coprinus cinereus}, and \textit{Cryptococcus neoformans}. Overall, we did not identify PKS/NRPS orthologs 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 \textit{M. grisea} into the Leaf**

The expression of \textit{ACE1} in the avirulent \textit{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 \textit{ACE1} genomic fragment of 453 bp carrying the first intron and corresponding to a 365-bp cDNA. The actin-encoding gene \textit{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 \textit{ACT1} transcript was obtained from both RNA samples (Figure 4A). By contrast, the \textit{ACE1} transcript was detected only in RNA isolated from infected leaves but not in the mycelial RNA sample (Figure 4B). This suggested that \textit{ACE1} expression is infection specific.

To follow the expression of \textit{ACE1} during infection, we fused the green fluorescent protein (GFP) reporter gene to the \textit{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 \textit{ACE1} is not expressed at detectable levels 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 \textit{M. grisea} genes \textit{GAS1} (\textit{MAS3}), \textit{GAS2} (\textit{MAS1}), \textit{CBP1}, and \textit{AI68463} (Banno et al., 2003; Takano et al., 2003). We searched for common transcription factor binding sites in the promoter sequences of \textit{ACE1}, \textit{GAS1} (AF363065), \textit{GAS2} (AF264035), \textit{CBP1} (BG009764), and \textit{AI68463}. As control, we performed the same analysis on promoters from the constitutively expressed \textit{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 \textit{GAS1}, \textit{GAS2}, \textit{CBP1}, and \textit{AI68463}, but not \textit{ACE1}. This motif is located at similar distances from the transcription start site (TSS) of \textit{GAS1}, \textit{GAS2}, \textit{CBP1}, and \textit{AI68463} (position ~307 to ~504, see Methods) and was not detected in the promoters of \textit{ACT1} and \textit{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 \textit{ACE1} expression. The promoters of all five appressorium-specific genes, including \textit{ACE1}, carry potential GAL4 binding sites (Transfac ID: F$S$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 \textit{ACE1} and other appressorium-specific genes.

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

Fungal \textit{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 \textit{pACE1:GFP}, which encodes a C-terminal fusion between Ace1 and GFP, expressed under the control of the \textit{ACE1} promoter and terminator sequences. We tested the effect of C terminus GFP fusion on Ace1 biological activity by introducing \textit{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...
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 appressorium. 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 hyphae. 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.

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 (ace1C183A 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 ace1C183A allele was fused to GFP to follow the expression of the mutant protein. The resulting construct, pace1C183A-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 Ace1C183A-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 ace1C183A-GFP allele is unable to confer avirulence. Because the Ace1C183A 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.
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 NRP-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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description/Reference</th>
</tr>
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<tbody>
<tr>
<td>Guy11</td>
<td>ACE1&lt;sup&gt;+&lt;/sup&gt; field isolate (Silué et al., 1992)</td>
</tr>
<tr>
<td>2/0/3</td>
<td>&lt;i&gt;ace1&lt;/i&gt;&lt;sup&gt;-&lt;/sup&gt; progeny of a cross between Guy11 and ML25 (Silué et al., 1992)</td>
</tr>
<tr>
<td>PH14</td>
<td>&lt;i&gt;ace1&lt;/i&gt;&lt;sup&gt;-&lt;/sup&gt; field isolate (Berruyer et al., 2003)</td>
</tr>
<tr>
<td>HBE20</td>
<td>ACE1&lt;sup&gt;+&lt;/sup&gt; transformant of 2/0/3 carrying pACE1 (this study)</td>
</tr>
<tr>
<td>HB26</td>
<td>&lt;i&gt;ace1&lt;/i&gt;&lt;sup&gt;-&lt;/sup&gt; deletion mutant of Guy11 obtained with pΔACE1 (this study)</td>
</tr>
<tr>
<td>HB92</td>
<td>&lt;i&gt;ace1&lt;/i&gt;&lt;sup&gt;-&lt;/sup&gt; deletion mutant of Guy11 obtained with pΔACE1 (this study)</td>
</tr>
<tr>
<td>HB16</td>
<td>ACE1&lt;sup&gt;+&lt;/sup&gt; ectopic transformant of Guy11 carrying pΔACE1 (this study)</td>
</tr>
<tr>
<td>HB24</td>
<td>ACE1&lt;sup&gt;+&lt;/sup&gt; ectopic transformant of Guy11 carrying pΔACE1 (this study)</td>
</tr>
<tr>
<td>HB33</td>
<td>ACE1&lt;sup&gt;+&lt;/sup&gt; ectopic transformant of Guy11 carrying pΔACE1 (this study)</td>
</tr>
<tr>
<td>IF22</td>
<td>ACE1&lt;sup&gt;+&lt;/sup&gt; transformant of Guy11 carrying pProACE1:GFP (this study)</td>
</tr>
<tr>
<td>HB41</td>
<td>ACE1&lt;sup&gt;+&lt;/sup&gt; transformant of 2/0/3 carrying pACE1:GFP (this study)</td>
</tr>
<tr>
<td>HB44</td>
<td>ACE1&lt;sup&gt;+&lt;/sup&gt; transformant of 2/0/3 carrying pACE1:GFP (this study)</td>
</tr>
<tr>
<td>HB10</td>
<td>&lt;i&gt;ace1&lt;/i&gt;&lt;sup&gt;-&lt;/sup&gt; transformant of 2/0/3 carrying pace1&lt;sup&gt;C183A&lt;/sup&gt;:GFP (this study)</td>
</tr>
</tbody>
</table>
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 appressorium 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>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'/3')</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-OL1</td>
<td>TTGCCCTCCATGGTCTGGAAG</td>
<td>RT-PCR (amplification of ACE1 intron 1)</td>
</tr>
<tr>
<td>H-OL2</td>
<td>GCCCTCTCTGTCGGCCCAAAT</td>
<td>RT-PCR (amplification of ACE1 intron 1)</td>
</tr>
<tr>
<td>H-OL3</td>
<td>CCGCGTCGGTCGATACCGCACCACCAAT</td>
<td>RT-PCR (amplification of ACE1 intron 2)</td>
</tr>
<tr>
<td>H-OL4</td>
<td>TGACAGAGGAGACGAGACG</td>
<td>RT-PCR (amplification of ACE1 intron 2)</td>
</tr>
<tr>
<td>H-OL5</td>
<td>CTCTTCTCGGTCGGCAACAC</td>
<td>RT-PCR (amplification of ACE1 intron 2)</td>
</tr>
<tr>
<td>H-OL6</td>
<td>CGACCGGAGGACGTAATCC</td>
<td>RT-PCR (amplification of ACE1 intron 2)</td>
</tr>
<tr>
<td>H-OL7</td>
<td>ACGATTCTGGTCCGGTGGTTATAGA</td>
<td>3' RACE of ACE1</td>
</tr>
<tr>
<td>H-OL8</td>
<td>CATCACGGGAGGAGCAACTACT</td>
<td>3' RACE of ACE1</td>
</tr>
<tr>
<td>H-OL9</td>
<td>TTGGCATGCTTCGTCATTGT</td>
<td>RT-PCR (amplification of ACT1 introns 1 to 3)</td>
</tr>
<tr>
<td>H-OL10</td>
<td>TTTCTGATTAGTCGGTCAA</td>
<td>RT-PCR (amplification of ACT1 introns 1 to 3)</td>
</tr>
<tr>
<td>H-OL11</td>
<td>CGGAAATCCGATCTCTAGCTTGGCGGC</td>
<td>Construction of pProACE1:GFP</td>
</tr>
<tr>
<td>H-OL12</td>
<td>GTCCACCATGTTGGATTATTATTGTGTTT</td>
<td>Construction of pProACE1:GFP</td>
</tr>
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<td>H-OL13</td>
<td>GGCAGGCACCGCGATTTG</td>
<td>Construction of pProACE1:GFP</td>
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<td>H-OL14</td>
<td>GCCAATCGGATTCACAGGAGGTGTGC</td>
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<td>H-OL15</td>
<td>GCTGAAGGACGGCGCCCTGATTTCCTAGAATCTTTGGAATACTGTAACTAG</td>
<td>Construction of pACE1:GFP</td>
</tr>
<tr>
<td>H-OL16</td>
<td>CAGTATTCGCAAGTACGTTAGACCATGTCGTC</td>
<td>Construction of pACE1:GFP</td>
</tr>
<tr>
<td>H-OL17</td>
<td>CCGTCCCTTCAGC</td>
<td>Construction of pace1 C183A:GFP</td>
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<tr>
<td>H-OL18</td>
<td>GCTGAAGGACGGCGCCCTGATTTCCTAGAATCTTTGGAATACTGTAACTAG</td>
<td>Construction of pace1 C183A:GFP</td>
</tr>
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<td>H-OL19</td>
<td>CTTGGGAAATACCT</td>
<td>Construction of pace1 C183A:GFP</td>
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<tr>
<td>H-OL20</td>
<td>GCTGAAGGACGGCGCCCTGATTTCCTAGAATCTTTGGAATACTGTAACTAG</td>
<td>Construction of pace1 C183A:GFP</td>
</tr>
</tbody>
</table>
terms of the genomes analyzed and reaches similar conclusions. (Kroken et al., 2003). This study is complementary to ours in B. fuckeliana cetes PKS genes reported the presence of six novel putative PKS/online). Independently, a phylogenomic analysis of fungal type I between olite-mediated avirulence signaling, described in the interaction 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 amphiphilic molecules induce the hypersensitive response only in soybean cultivars carrying the Rpd4 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 seems somewhat enigmatic. Apart from two sequences with similarity to AvrD that have been discovered in the genomes ofRalstonia 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 pathogenic fungal species. 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 MicA from P. citrinum (Abe et al., 2002). LNKS and MicA 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 lead 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 BiPKS4, BiPKS6, 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 × ML26) (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, Glucanase (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 XhoI, 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 EcoRV fragment, a 4.4-kb EcoR V fragment, and a 4.5-kb EcoRV-NotI 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* strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD) and JM110 (Strategene, 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 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 cosmicd 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, 1998), BLAST (Altschul et al., 1997; http://www.ncbi.nlm.nih.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://biportal.bic.nus.edu.sg/tres/), MatInspector (http://www.genomatix.de), TF Scan (http://bioweb.pasteur.fr), and OTFBS (http://www.biocinfo.tsinghua.edu.cn/~zhengsh/OTFBS/). Putative binding sites for the yeast stress response element (Transfac ID: FSTRE) were identified in the promoters of GAS1 (positions –504 and –558), GAS2 (position –416), CBP1 (position –307), and A0068463 (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

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/tf) was used for *M. grisea*, *Neurospora crassa*, *Aspergillus nidulans*, *Fusarium graminearum*, *Ustilago maydis*, *Coprinus cinereus*, and *Cryptococcus neoforms* genome sequences. The Institute for Genomic Research Web site (http://www.tigr.org) was used for the preliminary genomic sequences of *Aspergillus fumigatus*. Annoted 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*; LNSK (AF151722), LDKS (AF141925), AtMSAS (AAC49814), and Ata1 (BAB88688) from *Aspergillus terreus*; CIPKS1 (BA18856) from *Colletotrichum lagenarium*; ChPks1 (AAB08104) from *Cochliobolus heterostrophus*; Fum1 (AAO43562) from *Gibberella moniliformis*; mLCa (BAC20564) from *Penicillium citrinum*; PgPKS2 (A849684) from *Penicillium griseofulvum*. The following hypothetical proteins deduced from ORFs identified in genomic sequences were included in the analysis: mg03810 (EEA05051), mg03818 (EEA00569), mg09589 (EEA48052) from *M. grisea*, ncu02918.1 (EEA36364), ncu04865.1 (EEA29886), ncu08399.1 (EEA34002), and ncu09638.1 (EEA28933) from *N. crassa*, AN8412 (EA67003) from *A. nidulans*, atf04917 from *A. fumigatus*, and FG10464.1 (AA68296) from *F. graminearum*, Syn2, Syn6, Syn7, and Syn8 were identified in the *M. grisea* genome during this study (SYW2 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. fuscellana (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 PICS module and GmPKS10. The following three PKS/NRPS (GzPKS9, BfPKS7, and GmPKS8) identified by Kroken et al. were not added to the database because they lack some sequences of their KS or AT domains or have only KS domains (GzPKS9), GzPKS9 is almost identical (>91% identity) to FG01464. We also included in the analysis Bacterial EPS (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 cosm id library is described by D ih o et al. (1997). For cosm id D31C12 subclones, cosmid DNA was digested with each of the following enzymes: BamHI, EcoRI, NotI, and XbaI and in double digests with BglII plus NotI and XbaI plus NotI, respectively. The following genomic fragments were recovered and cloned into pCB1004 (Sewigard et al., 1997): for pBam8, an 8.3-kb BamHI fragment; for pEco20, a 15.4-kb EcoRI fragment; for pNot21, a 21.0-kb NotI fragment (one NotI site is derived from the codon polylinker); for Xba11, an 11.1-kb XbaI fragment; for pBglP7, a 6.7-kb BglII-NotI fragment (NotI site derived from polylinker); for pX-N12, a 12.1-kb XbaI-NotI fragment.

For pΔACE1, the 15.4-kb EcoRI fragment carrying ACE1 was cloned into pBluescript II KS− as BN000505, SYN7 as BN000506, and SYN8 as BN000507.

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

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