Introduction

The rice blast fungus *Magnaporthe oryzae*, which causes significant reductions in rice yields, is a well-known model for studying plant fungal interactions and pathogenicity. Rice resistance receptors (R) recognize *M. oryzae* secreted avirulence signals (AVR), allowing the rice plant to mount an effective defence response, killing or containing the fungus within the initial infected cell. Genetic and molecular studies have conclusively linked the avirulent phenotype of strain Guy11 on initial infected cell. Genetic and molecular studies have conclusively linked the avirulent phenotype of strain Guy11 to the resistance gene *R*.

The majority of known *AVR* genes encode small secreted proteins acting directly on the host plant, *ACE1* encodes a cytoplasmic biosynthetic protein responsible for the production of a low molecular weight compound.

*ACE1* belongs to an infection-specific secondary metabolite gene cluster (Scheme 1), expressed only during appressorium-mediated penetration, and not at any other stage of the *M. oryzae* life cycle. The 12.4 kb *ACE1* gene encodes an enzyme consisting of a fungal highly-reducing polyketide synthase (hrPKS) fused to a single module of a nonribosomal peptide synthetase (NRPS). Similar fungal synthetases have been investigated genetically and biochemically and are known to produce acyl tetramic acids or pyrrolidones including pretenillins A 1, 2, and 3 (refs. 10 and 12) as well as other potent bioactive compounds including cytochalasins K 4 and L 5 (refs. 13 and 14) and the important cholesterol biosynthesis inhibitor lovastatin 5 (ref. 15) (Scheme 1). It appears likely, therefore, that *ACE1* encodes a biosynthetic protein which makes a small molecule consisting of a polyketide fused to an amino acid. Because *ACE1* is under very tight temporal and cell type-specific control in *M. oryzae*, it has not yet been possible to identify or purify the avirulence compound.

Knowledge of the *ACE1* metabolite structure would be highly useful for investigating avirulence signalling and developing novel resistant cultivars. The research of our group and that of others has focused on the heterologous expression of other fungal PKS-NRPS genes in *Aspergillus oryzae* from gene clusters of unknown function. Here we set out to use heterologous expression of genes from a *cryptic pathway* to determine its
In initial experiments a construct known to express ACE1 in M. oryzae, containing ACE1 fused to egFP at its 3’ terminus (pPACE1-ACE1eGFP-hyg), was used to construct the vector pPamyB-ACE1eGFP-argB (Fig. 1A) in which the ACE1-eGFP gene fusion was located downstream of PamyB.

This plasmid was then used to transform A. oryzae M-2-3. Transformants were selected on minimal medium and 100 colonies were selected and grown further on both minimal medium and minimal medium plus maltose. Four transformants showed weak fluorescence and these were picked together with 10 non-fluorescent transformants and grown in liquid medium (see ESI†). The resultant organic extracts were examined by LCMS but no significant chemical differences were observed between extracts from any of the transformants and the untransformed A. oryzae M-2-3 strain (data not shown).

### Intron processing

Since A. oryzae is increasingly being used as a heterologous host for studying gene expression, we wanted to determine why our initial attempt at expressing ACE1 had failed. The ACE1 gene contains three introns located between exons corresponding to PKS domains (Fig. 1B). As there is a possibility that A. oryzae may not always splice heterologously expressed genes the same way as the host organism, we explored how the introns of ACE1 are processed by A. oryzae and M. oryzae.

Each ACE1 intron was amplified with ca. 20 bp of flanking 5’ and 3’ exon sequences and cloned as in-frame fusions with eGFP downstream of either the native ACE1 promoter (Pace1), the strong constitutive promoter Pglyc from Aspergillus nidulans or the inducible promoter Pamyb from A. oryzae. A total of nine plasmids were constructed (Fig. 1C). These constructs were transformed into either A. oryzae or M. oryzae, along with positive and negative controls, selected on the appropriate selection media and observed for fluorescence (Table 1 and Fig. 2). RT-PCR was performed for each intron construct using primers located either side of the introns and the products were sequenced (see ESI for details).

The results demonstrated that introns 1 and 3 are spliced identically in A. oryzae and M. oryzae whereas incorrect splicing of intron 2 by A. oryzae results in a frameshift and premature stop codon (Fig. S1†).

### Heterologous expression of ACE1

Based on results from the intron processing experiments, we created two modified expression plasmids for further attempts to produce the ACE1 compound (Fig. 1A). The first consisted of the entire ACE1 gene lacking all introns fused to eGFP (pPamyB-ACE1[Δintron1–3]eGFP-argB); the second consisted only of the PKS encoding portion of ACE1 – also fused to eGFP, and containing only intron-3 (pAmyB-ACE1ps[Δintron1–2] eGFP-argB, see ESI† for construction details). Controls included empty vector and eGFP alone (pPgpdA-eGFP-hyg, Fig. 1D). These plasmids were used to transform A. oryzae M-2-3 and selection was achieved on minimal medium supplemented with hygromycin where appropriate. On malt extract agar (MEA) the transformants containing the ACE1 constructs produced a bright yellow colour, while the controls were colourless. Twenty colonies were selected for each construct, grown in liquid medium and extracted as described above. All transformants tested clearly produced a new peak compared to the controls (Fig. 3A) in the neutral organic extract, and in lesser amounts, in the acidic and mycelial extracts.

The new compounds appeared to be identical for the two ACE1 expression constructs (RT = 33.2 min; uv max = 214, 272,
361 nm; m/z = 315 [M]Na⁺, 293 [M]H⁺, 275 [M – H₂O]H⁺). Both compounds were purified and shown to be identical by NMR and HRMS (m/z 291.1238 Da [M/C₈H₂O]₄H⁺), consistent with a molecular formula of C₁₆H₂₀O₅).

1H NMR analysis (see ESI†) showed that the sample consisted of a 1 : 1 mixture of diastereomers each of which contained three methyl groups, a CHX, five contiguous olefinic protons, and two sharp doublets (J = 2.0 Hz) in the olefinic region. COSY correlations were used to determine that the olefinic protons were part of a distinct spin-system with an olefinic methyl group, connected by a vicinal coupling, visible only as a slight broadening of the terminal olefin doublet in the 1D ¹H NMR. The COSY also showed that the CHX (which appeared as a complex multiplet) and one of the methyl groups (itself appearing as a pair of doublets) formed another distinct coupling system. An HMBC spectrum determined that the lone methyl group and olefins were all part of the same chain with a formula of C₁₁H₁₇O₂, leaving C₅H₃O₃ unaccounted for. The characteristic ¹³C NMR resonance of δ 163.6 ppm revealed that an ester group was present in the structure accounting for a further CO₂, and leaving C₄H₃O unassigned.

The remaining two unassigned protons showed HMBC correlations to the ester carbonyl and two other quaternary carbons, the chemical shifts of which (δ 162.4 ppm and δ 162.6 ppm) were consistent with enolic carbons. Although these protons were coupled, they did not appear to be on adjacent

| Construct name | Expression host | Conditions | Fluorescent transformants |
|----------------|----------------|------------|--------------------------|
| pPamyB-intron1-eGFP-argB | A. oryzae | M | 80% |
| pPamyB-intron2-eGFP-argB | A. oryzae | M | 0% |
| pPamyB-intron3-eGFP-argB | A. oryzae | M | 90% (Fig. 2A) |
| Empty vector | A. oryzae | M | 0% |
| eGFP | A. oryzae | M | 87% |
| pPgdA-intron1eGFP-hyg | M. oryzae | M, A | >70% |
| pPgdA-intron2eGFP-hyg | M. oryzae | M, A | >70% (Fig. 2B) |
| pPgdA-intron3eGFP-hyg | M. oryzae | M, A | >70% |
| Empty vector | M. oryzae | M, A | 0% |
| eGFP | M. oryzae | A | >70% |
| pPACE1-intron1eGFP-hyg | M. oryzae | A | >60% (Fig. 2C) |
| pPACE1-intron2eGFP-hyg | M. oryzae | A | >60% (Fig. 2D) |
| pPACE1-intron3eGFP-hyg | M. oryzae | A | >60% (Fig. 2E) |
| Empty vector | M. oryzae | M, A | 0% |
| eGFP | M. oryzae | M, A | >60% |
to rule out NRPS per-
gen expression system to remove the 3′-eGFP gene to create pPa-
methionine to

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Thus 6 consists entirely of a polyketide and does not contain the expected amino acid component. To rule out NRPS per-

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methyl]-13C]-methionine to A. oryzae

methyl] acetate and [methyl-13C]-methionine to A. oryzae

The polyketide origin of 6 was confirmed by feeding [1,2-13C2]-acetate and [methyl-13C]-methionine to A. oryzae

Pyrone 6 isolated after the labelled acetate feed showed the presence of distinctive doublets in the 13C NMR indicative of the incorporation of intact 13C units (Fig. 4 and ESI†). Matching of the 2JCC values showed intact acetate incorporations consistent with the heptaketide origin of 6. The origin of both methyls from methionine was confirmed by the observation of a 9.4% incorporation of label at C-15 and C-16.

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apdC and lovC respectively interact with the PKS to provide programmed enoyl reduction and have the effect of reinforcing PKS programming including methylation and chain-length fidelity. RAP1 also encodes a trans-acting ER (e.g. RAP1 has a 48% similarity to TENC and 58% similarity to LOVC) and thus it may play a similar role in concert with ACE1, possibly controlling fidelity as well as enoyl reduction. We therefore constructed plasmids to coexpress RAP1 with ACE1 in both A. oryzae and M. oryzae.

For A. oryzae we created a plasmid carrying both the ACE1 and RAP1 genes in which ACE1 lacking all introns was expressed under the control of PamyB, and RAP1 was expressed from the strong constitutive alcohol dehydrogenase promoter Padh from A. oryzae (pPamyB-ACE1[Δintrons 1–3]-argB·Padh·RAP1, Fig. 1A). A. oryzae M-2-3 protoplasts were transformed with pPamyB-ACE1[Δintrons 1–3]-argB·Padh·RAP1 and transformants were selected on minimal medium.

Twenty seven of the resulting A. oryzae transformants were selected and transferred to new agar plates. Ten of these transformants were grown in liquid medium containing starch selected and transferred to new agar plates. Ten of these transformants were selected on minimal medium.

A single A. oryzae transformant was grown at large scale (800 mL) to yield 65.5 mg of crude extract. The new compound was purified by HPLC and analysed by NMR and HRMS (m/z 482.3265 Da [M+H]+, consistent with a molecular formula of C30H43NO4). 1H NMR analysis showed that the compound contained four methyl groups, six olefinic protons and an aromatic group indicative of tyrosine. 13C NMR, COSY, HMBC and HSQC determined the structure as 8 (Fig. 6, ESIF†).

**Biological testing**

Purified magnaportheapyrones 6 and 7 were tested for biological activity on susceptible, (Maratelli, Sariceltik CO39) and resistant rice cultivars carrying Pi33 (IR64 and C101LAC). Compounds were deposited onto normal or wounded rice leaves. Both compounds induced localised brown necrosis on all cultivars, irrespective of host genotype (Fig. 7). These results suggest that magnaportheapyrones do not induce a phenotype specifically on rice cultivars carrying Pi33. Addition of these compounds to spore suspensions of an isolate virulent on Pi33 rice cultivars did not induce an AVR reaction, but slightly reduced pathogenicity on both susceptible and resistant cultivars. Compound 8 had no biological activity: no induction of leaf symptoms, no changes of a virulent interaction into AVR, no enhancement nor reduction in pathogenicity. Therefore compounds, 6, 7 and 8 do not appear to be the avirulence signal compound produced by ACE1/M. oryzae Guy11 during appressorium-mediated penetration.
Discussion

Initial attempts to express \textit{ACE1} in \textit{A. oryzae} produced no observable new metabolites. This was surprising as all of our previous efforts to express foreign fungal genes in this organism had been successful.

Further investigation using RT-PCR and subsequent sequence analysis clearly showed that \textit{ACE1} intron-2 is not spliced correctly in \textit{A. oryzae}, resulting in an mRNA with a frame-shift and premature stop codon. Intron-2 is located centrally in the PKS portion of \textit{ACE1}, upstream of the sequence encoding the key acyl carrier protein (ACP) domain. Any truncated \textit{ACE1} protein resulting from the translation of this incorrectly spliced mRNA would not be capable of producing a polyketide product.

Removal of the \textit{ACE1} introns resulted in transcripts which were translated correctly in \textit{A. oryzae}. Indeed more than 75% of transformants produced full-length \textit{ACE1}-eGFP protein fusions as evidenced by strong fluorescence of transformed hyphae, and the production of novel metabolites. Expression of both full-length and NRPS-deleted \textit{ACE1} intronless constructs resulted in the production of the same compound, 12,13-dihydroxymagnaporthepyrone which is exclusively of polyketide origin as shown by isotopic labelling. Expression of the same clones in \textit{M. oryzae} resulted in the production of the closely related 10,11-dihydroxymagnaporthepyrone. Similar compounds were observed by Vederas and coworkers when the lovasatin non-aketide synthase (LNKS) was expressed without its cognate ER encoded by \textit{lovC}.

These results can be interpreted if the PKS portion of \textit{ACE1} produces the polyunsaturated pyrone (which we name magnaporthepyrone, Scheme 2). This compound is then epoxidised by different monoxygenases in either \textit{A. oryzae} or \textit{M. oryzae} and spontaneous hydrolysis of the epoxides results in the observed diols and \textit{M. oryzae} and spontaneous hydrolysis of the epoxides results in the observed diols \textit{6} and \textit{7}. Support for this hypothesis comes from the expression of \textit{tenS} in \textit{A. oryzae} where we observed the presence of prototenellin \textit{C} \textit{10} (ref. 22) which displays exactly the same chemical motif as 12,13-dihydroxymagnaporthepyrone \textit{6} – thus \textit{A. oryzae} must possess a monooxidase selective for the terminal methylbutenyl motif of such precursors. Likewise, \textit{M. oryzae} is known to produce diol compounds such as pyriculol \textit{11} (ref. 23) which must also derive from epoxidation of a polyunsaturated polyketide followed by hydrolysis. Therefore, \textit{M. oryzae} must have a monooxygenase with selectivity for epoxidation of mid-chain polyenes.

The biosynthesis of \textit{6} and \textit{7} do not require an enoyl reductase step, consistent with the observation that the \textit{ACE1} ER domain is predicted to be inactive. Coexpression of \textit{ACE1} with \textit{RAP1}, which encodes a trans-acting ER, changed the biosynthetic pathway. In the heterologous host \textit{A. oryzae}, where we constructed a single vector to carry both genes, the amide \textit{8} was produced and compounds derived from \textit{9} were no longer observed.

Amide \textit{8} is closely related to pyrone \textit{9} and its biosynthesis can be rationalised by the intervention of the \textit{RAP1} ER at the pentaketide stage of biosynthesis (Scheme 2). Pentaketide intermediate \textit{12} is recognised by the \textit{ACE1} KS domain and extended to \textit{13}, but in the absence of prior enoyl reduction the next required CMeT step is prevented. Continued chain extension by an unselective KS then gives the tricarbonyl \textit{14} which can spontaneously offload itself from the synthase as the pyrone \textit{9}. Alternatively, in the presence of the \textit{RAP1} ER, pentaketide \textit{12} is reduced giving a substrate which can be extended by the KS and then correctly methylated to give \textit{15} before following three
more extension and processing cycles to give the fully extended nonaketide 16. Thus the ACE1 CMET domain appears to display an additional level of substrate selectivity in blocking correct chain processing in the absence of 10,11 reduction. Crucially, however, the KS is unselective and continues to extend the chain to the tricarbonyl 14 which can be off-loaded as the pyrone 9. Thus the lack of selectivity by the KS effectively removes incorrectly processed chains from the PKS, preventing its blockage.

In many other investigated PKS-NRPS systems the NRPS then attaches the amino acid to the fully extended polyketide and either reductively cleaves it, or performs a non-reductive Dieckmann cyclisation reaction to release an acyl tetratic acid. In this case it appears that tyrosine is used as the amino acid and attached to the polyketide in the same way as observed in the cases of pretenellin A 1 and preaspermydione 3. After amide formation, however, the fully elaborated product is probably reductively released. This could be via 2 electron reduction to form an aldehyde (as in the classic case of fungal lysine biosynthesis) which could be further reduced to a primary alcohol by an adventitious A. oryzae enzyme, or via double (i.e. 4 electron) reduction catalysed by the NRPS R-domain itself as observed during the biosynthesis of myochelin.

Consistent with this hypothesis is the observation that the ACE1 terminal-domain has an intact conserved NADPH binding motif (GXXXXG) and the catalytic triad Ser-Tyr-Lys more closely resembles ‘reducing’ R-domains than DKC cyclising domains (see ESI†). Oiao et al. predicted a reductive release for ccsA, the PKS-NRPS gene involved in cytochalasin K 4 biosynthesis in Aspergillus clavatus, based on the same rationale, and the co-expression of ccsA with ccsC by Oikawa and co-workers led to the production of the amide 17, also at the alcohol oxidation state.

The Oikawa compound 17 differs from 8 in being an octaketide rather than a nonaketide and being evidently constructed from phenylalanine rather than tyrosine (Scheme 3). A further difference is that 8 is unsaturated between carbons 8 and 9 of the polyketide, whereas 17 is fully saturated at the corresponding position. Genetic experiments have already shown that ccsA and ccsC are involved in cytochalasin K 4 biosynthesis, most likely via ketocytochalasin 18. Although 17 is unlikely to be a direct precursor of 4, it is probably a shunt metabolite from the corresponding aldehyde. Vederas, Tang and coworkers have also recently elucidated that ccsB from the cytochalasin K biosynthetic pathway is an FAD-dependent oxygenase which catalyses (among other reactions) the creation of the distinctive carbonate moiety of 4.

The structural similarities between the ccsA/C product 17 and the ACE1/RAP1 product 8 strongly suggest that 8 is also a shunt metabolite of a cytochalasan-like biosynthetic pathway. This is supported by the observation that 8 does not show the expected biological activity as an avirulence signalling compound.

To our knowledge, only two other gene clusters have been correlated with cytochalasin biosynthesis in fungi. These are the gene clusters for chaetoglobosin A 19 biosynthesis in Penicillium expansum and Chaetomium globosum. Chaetoglobosin A 19 is biosynthesised from tryptophan and a nonaketide likely to be almost identical to that of amide 8, except for the unsaturation between carbons 4 and 5. However, several chaetoglobosins are also known which are fully saturated at this position (e.g. chaetoglobosin C 20) indicating that amide 8 could include the correct polyketide precursor. Analysis of the tailoring genes in the ACE1 cluster show that they have more similarity to the chaetoglobosin biosynthetic genes than those in the ccs cytochalasin gene cluster (Table S2†).
The nonaketide nature of the amide 8 and the fact that tyrrosine is incorporated, distinguishes the RAP1/AE1 product from the ccsA/ccsC compound and mean that the likely ultimate product of the ACE1 pathway will be a member of the tyrosine-derived cytochalasans, an example of which is phompovichalin (also known as diaporthichalin) 21, 24. In addition, the differences between the ACE1 and ccs gene clusters, such as the lack of a ccsB homolog, plus additional oxidoreductases similar to those found in the chaetoglobosin A 19 gene cluster, indicate that the tailoring of the polyketide backbone is likely to be more similar to 19, which could arise from a polyketide identical or very similar to that of 8.

Thus we have shown that heterologous expression is an effective tool to probe cryptic gene clusters in fungi, which enables important metabolic relationships to be unravelled. It is now clear that avirulence signalling between the rice blast fungus *Magnaporthe oryzae* and rice is mediated by a natural compound responsible for avirulence signalling. A homolog, plus additional oxidoreductases derived cytochalasans, an example of which is phomopichalin, are now clear that avirulence signalling between the rice blast fungus *Magnaporthe oryzae* and rice is mediated by a natural compound responsible for avirulence signalling.

### Experimental

For construction of vectors, medium conditions, transformation protocols, LCMS methods, NMR data, isotope feeding experiments and details on biological testing see the ESI.†

### Characterisation of new natural products

12,13-Dihydroxy.magnaporthepyrone 6. Isolated as an amorphous yellow solid (15 mg) and a 1 : 1 mixture of two diastereoisomers (A and B) by preparative HPLC. 1H NMR (500 MHz acetone-\(d_6\)): \(\delta\) 1.09 ppm (1.5H, d, \(J_{HH} 6.7, H-14A\)), 1.11 ppm (1.5H, d, \(J_{HH} 6.3, H-14B\)), 1.255 (1.5H, s, H-15A), 1.260 (1.5H, s, H-15B), 2.02 (1.5H, s, H-16A), 2.03 (1.5H, s, H-16B), 3.60 (0.5H, q, \(J_{HH} 6.7, H-13A\)), 3.61 (0.5H, q, \(J_{HH} 6.3, H-13B\)), 5.39 (1H, d, \(J_{HH} 2.0, H-2A + H-2B\)), 6.07 (0.5H, d, \(J_{HH} 15.3, H-11A\)), 6.08 (0.5H, d, \(J_{HH} 15.3, H-11B\)), 6.20 (1H, d, \(J_{HH} 2.0, H-4A + H-4B\)), 6.55 (1H, m, H-10AB), 6.69 (2H, m, H-8AB + H-9AB), 7.09 (1H, d, \(J_{HH} 9.6, H-7AB\)); 13C NMR (151 MHz acetone-\(d_6\)): \(\delta\) 12.7 ppm (C-15), 18.1 & 18.3 (C-14), 24.5 & 23.7 (C-16), 74.2 (C-13), 126.3 (C-6), 126.3 (C-8), 132.2 (C-7), 136.8 (C-12), 142.0 (C-9), 162.0 (C-3), 163.6 (C-1), 170.7 (C-3); UV/vis \(\lambda_{\text{max}}\) (H\(_2\)O/MeOH) 248, 340 nm; MS (ESI) \(m/z\) (%): 293 (100) [M\(^+\)], 315 (50) [M\(^+\)]; MS (ESI) \(m/z\) (%): 291 (100) [M – H\(^+\)]; HRMS (ESI) calculated [M – H\(^+\)] \(275.1277\) found 275.0922; HRMS (ESI) calculated [M – H\(^+\)] \(275.1033\) found 291.1238.

Amide 8. 1H NMR (500 MHz, CDCl\(_3\)): \(\delta\) 0.92 ppm (d, 3H, \(J = 6.8\)), 1.35 (m, 2H), 1.51 (m, 2H), 1.56 (s, 3H), 1.72 (d, 3H, \(J = 9.1\)), 1.74 (s, 3H), 1.95 (t, 2H, \(J = 7.5\)), 2.03 (t, 2H, \(J = 7.1\)), 2.41 (m, 1H), 2.47 (m, 2H), 2.76 (dd, 1H, \(J = 7.4, 13.9\)), 2.81 (dd, 1H, \(J = 7.1, 13.9\)), 3.31 (d, 1H, \(J = 17.2\)), 3.34 (d, 1H, \(J = 17.2\)), 3.57 (m, 1H), 3.68 (m, 1H), 4.15 (m, 1H), 4.91 (d, 1H, \(J = 9.3\)), 5.52 (q, 1H, \(J = 6.8\)), 6.51 (ddd, 1H, \(J = 14.6, 7.3, 7.3\)), 6.05 (dd, 1H, \(J = 14.3, 9.3\)), 6.08 (dd, 1H, \(J = 14.6, 9.3\)), 6.13 (d, 1H, \(J = 14.6\)), 6.75 (brd, 2H, \(J = 8\)), 7.06 (brd, 2H, \(J = 8\)), 7.2 (brd, 1H, \(J = 7.8\)); 13C NMR (125 MHz, CDCl\(_3\)): \(\delta\) 14.6 ppm, 16.6, 18.8, 23.4, 25.4, 29.8, 35.4, 38.8, 41.9, 43.6, 46.4, 51.2, 56.1, 67, 118.1, 128.9, 129.2, 132.0, 133, 133.7, 134.6, 134.8, 135.3, 137.4, 138.1, 157.2, 169, 209.7. See ESI for full assignments. HRMS ESI [M\(^+\)]; found 482.32649; predicted for C\(_{30}\)H\(_{43}\)NO\(_4\) 482.32703.

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