RSC Advances

PAPER

Cite this: *RSC Adv.*, 2019, **9**, 35797

Function of pathway specific regulators in the *ACE1* and pyrichalasin H biosynthetic gene clusters†

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Received 3rd September 2019
Accepted 28th October 2019

DOI: 10.1039/c9ra07028a

rsc.li/rsc-advances

Ectopic expression of *BC1* which encodes a putative pathway specific transcription factor from the *ACE1* biosynthetic gene cluster of the rice pathogen *Pyricularia oryzae* Guy11 did not lead to the production of *ACE1*-related compounds. However the known compound hinnulin A was formed. A putative partial gene cluster potentially involved in the biosynthesis of hinnulin A and DHN melanin was validated by RT-PCR and a possible biosynthetic pathway is proposed. Ectopic expression of *pyiR* which encodes a pathway specific transcription factor from the pyrichalasin H biosynthetic gene cluster in *Magnaporthe grisea* NI980 led to the apparent up-regulation of the *pyi* cluster and a 3-fold increase in pyrichalasin production under standard fermentation conditions, but did not lead to the formation of new compounds.

Introduction

The fungus *Pyricularia oryzae* (=*Magnaporthe oryzae*) is a major pathogen of rice (*Oryza sativa*) causing significant annual reduction in yields.[1] The progress of rice blast disease caused by *P. oryzae* is well understood. Spores of the fungus are distributed by wind and germinate on rice leaf surfaces. A germ tube spreads across the cuticle, before forming an appressorium. The appressorium is a structure which mediates penetration of the fungus into the plant through the generation of turgor,[2] the generation of specific hydrolytic enzymes,[3] and the formation of 1,8-dihydroxynaphthalene (DHN) melanin.[4] After penetration, the fungal mycelium multiplies through the plant leaves and stems, killing cells and causing visible lesions. Eventually the mycelium breaks out of the cells and sporulates on the plant surface, repeating the disease cycle.

Rice resistance to *P. oryzae* infection is known, but resistant rice varieties only display this phenotype towards avirulent strains of *P. oryzae*. Work by the group of Lebrun has shown that avirulent strains of *P. oryzae* such as Guy11, contain the *ACE1* (Avirulence Conferring Enzyme 1) biosynthetic gene cluster (BGC) which is responsible for the avirulence phenotype.[5,6] It is hypothesised that the *ACE1* BGC encodes the biosynthesis of a signal compound which is detected by resistant rice[7] allowing the plant to mount an effective defence. The *ACE1* BGC was shown to be expressed only under very specific conditions during appressorium penetration into plant cells.[8] Under these conditions the *ACE1* metabolite is produced in very low quantities for a very short period and it has therefore been impossible to isolate and identify the avirulence signal compound.[9]

Comparisons to related BGC suggest that the *ACE1* metabolite is likely to be related to the cytochalasans[10] and heterologous expression experiments of *ACE1* and other genes from the BGC produce plausible cytochalasan intermediates or shunt metabolites.[11] The BGC consists of genes encoding a typical highly programmed fungal polyketide synthase nonribosomal peptide synthetase[12] hybrid (PKS-NRPS, *ACE1*) together with tailoring genes typical of cytochalasan biosynthesis (Fig. 1). The *ACE1* BGC also encodes various transport proteins and the transcription factor (TF) BC2. A closely related BGC is that involved with the biosynthesis of pyrichalasin H **1** in the related organism *Magnaporthe grisea* NI980.[13] The pyrichalasin (*pyi*) BGC also encodes a PKS-NRPS and includes a TF gene known as *pyiR*, but unlike the *ACE1* BGC the *pyi* cluster is active under a variety of physiological conditions.

Previous work reported by Hertweck and coworkers has shown that transcription factors encoded in PKS-NRPS BGC are pathway-specific positive regulators of BGC expression and can make useful tools for the activation of previously silent BGC.[14] For example, in the case of the aspyridone (*apd*) cluster the *apdR* TF was ectopically expressed in the native host *A. nidulans* under the control of the inducible alcohol dehydrogenase promoter P_{alcA}. This 'switched on' the pathway and resulted in the production of aspyridones A **2a** and B **2b** (Fig. 1).[15] Similarly, Tang and coworkers expressed *ccsR* which encodes a pathway-specific TF in the cytochalasin E **3** (*ccs*) BGC in *Aspergillus clavatus*. This resulted in an up-regulation of the cluster and a significant increase in the titre of **3**.[16]

We reasoned that a strategy involving the ectopic expression of *BC2* could be an effective way to activate the *ACE1* BGC with the aim of isolating and structurally characterising the *ACE1*

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9ra07028a

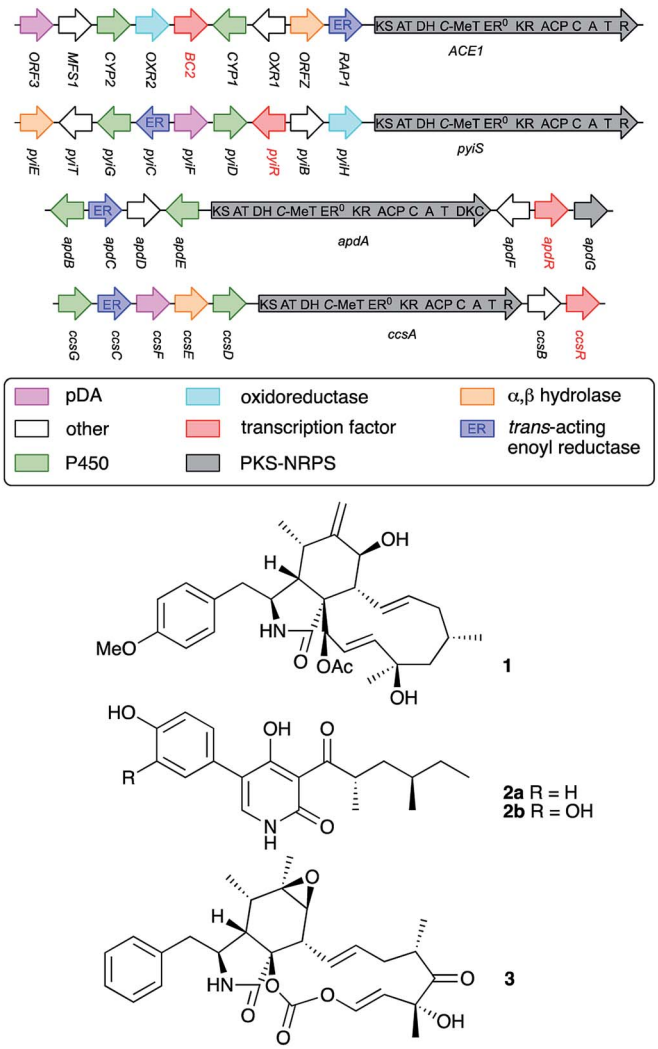


Fig. 1 Biosynthetic gene clusters involved in the biosynthesis of various PKS-NRPS-derived compounds. Open reading frames are represented by arrows which are not to scale. Abbreviations: ER, enoyl reductase; KS, ketosynthase; AT, acyl transferase; DH, dehydratase; *C*-MeT, *C*-methyl transferase; ER^0, non-functional ER; KR, ketoreductase; ACP, acyl carrier protein; C, condensation; A, adenylation; T, thiolation; R, reductive release; DKC, Dieckmann cyclase; pDA, putative Diels Alderase.

signal compound from *P. oryzae*. In parallel, we also experimented with ectopic over-expression of *pyiR* with the aim of increasing the titre of pyrichalasin H **1** production in *M. grisea* NI980.

Results

P. oryzae Guy11 is sensitive to the antibiotics hygromycin B and glufosinate (Basta).[17] In order to develop a reliable transformation method, genetic manipulation of *P. oryzae* Guy11 was established using the vector pTH-GS-*hygR-egfp*. The vector features a hygromycin B resistance cassette (*hyg*^{R}) as well as the *egfp* gene under the control of the strong inducible *amyB* promoter (P_{amyB}) from *Aspergillus oryzae*.[18,19]

Transformation of *P. oryzae* Guy11 was performed with vector pTH-GS-*hygR-egfp* using a PEG/$CaCl_2$-mediated protoplast protocol resulting in 12 hygromycin B resistant transformants. Six transformants and the wild type were cultivated for 5 days in DPY medium before the samples were analyzed by fluorescence microscopy. Successful production of GFP was ascertained in all transformants, but not in the wild type control (Fig. 2). This indicates the suitability of the *hyg*^{R}/P_{amyB} system for further manipulation of *P. oryzae* Guy11. *BC2* was then amplified from *P. oryzae* Guy11 genomic DNA (gDNA) by PCR and placed downstream of P_{amyB} and upstream of the terminator T_{amyB} to create the vector pTYGS-*hygR-BC2*. The gene *pyiR* was amplified from *M. grisea* gDNA and inserted downstream of the strong constitutive P_{gpdA} promoter in the vector pTYGS-*bar* which also contains a basta resistance gene (*bar*) under the control of the constitutive P_{trpC} promoter. This created the vector pTYGS-*bar-pyiR*. We previously showed that P_{gpdA} is effective in *M. grisea* NI980.[8]

P. oryzae PEG/$CaCl_2$ mediated protoplast transformation with pTYGS-*hyg*^{R}-*BC2* resulted in the isolation of 14 hygromycin resistant transformants. In the case of *M. grisea*, transformation with pTYGS-*bar-pyiR* produced 40 basta-resistant transformants.

Eleven *P. oryzae* pTYGS-*hyg*^{R}-*BC2* transformants were cultivated for 7 days in liquid DPY media (25 °C, 110 rpm) before cells and growth media were individually extracted and analysed by LCMS. In five transformants production of a new, dark brown compound **4** (MW = 348 g mol^{-1}) was observed in the media extracts (Fig. 3). The production of **4** was not observed in extracts of *P. oryzae* Guy11 wild type (WT) strain. To test whether the P_{amyB} *BC2* construct is present in these transformants, a PCR experiment was designed to amplify the *BC2* – T_{amyB} junction. This showed the expected integration of *BC2* into the genome of four genetically analysed transformants.

1.3 mg of **4** were purified by preparative LCMS and the structure was elucidated by NMR. HRMS confirmed a molecular formula of $C_{20}H_{12}NO_6$ ([M]H$^+$ calculated 347.0542, found 347.0529). Couplings in the ^{1}H–^{1}H COSY spectrum revealed characteristic signals for aromatic regions. **4** contains 8 aromatic protons (H-1, H-7, H-8, H-12, H-13, H-18, H-19 and H-20, Fig. 4). Notably, the signal for H-1 is a singlet (δ_H 6.24 ppm); it demonstrated no coupling signal in the COSY spectrum. Based on the HMBC experiment, H-1 correlates to two carbonyl

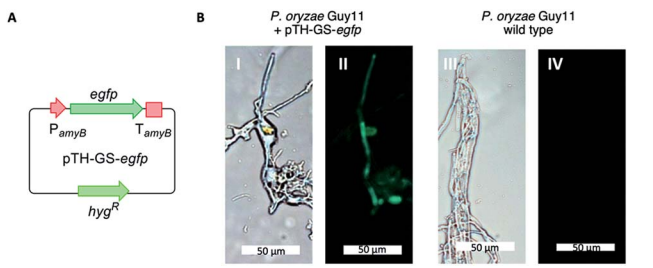


Fig. 2 Expression of *egfp* in *P. oryzae* Guy11. (A) Cartoon of expression system; (B) results of expression in *P. oryzae* Guy11. Left panels under white light, right panels under UV light.

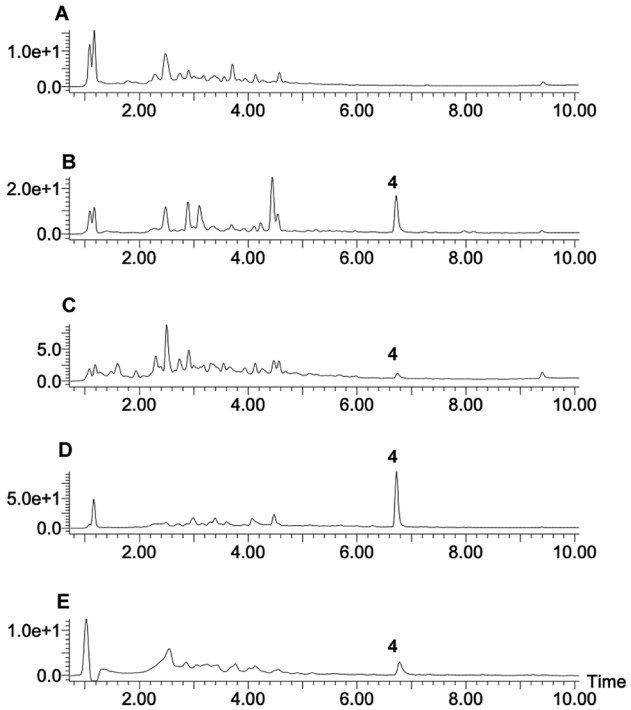


Fig. 3 LCMS traces of expression of *BC2* in *P. oryzae* Guy11. (A) Extract of WT *P. oryzae*; (B–E) extracts of four representative strains of *P. oryzae* pTYGS-*hyg*^{R}-*BC2*. Diode array UV traces shown.

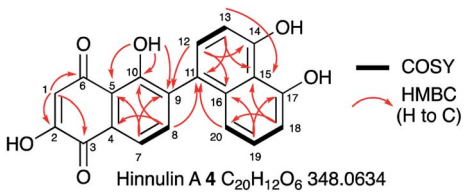


Fig. 4 Summary of key 2D NMR correlations observed for **4**. See ESI† for full data.

groups (C-6 at δ_C 193.5 ppm and C-3 at δ_C 182.6 ppm), one hydroxyl group (C-2 at δ_C 161.6 ppm) and one alkene (C-5 at δ_C 115.8 ppm) and is part of the first aromatic ring. Key HMBC signals for the adjacent aromatic ring originated from the hydroxyl group at C-10. Notably, only for 10-OH a signal (singlet at δ_H 12.86 ppm) was observed. Proton signals for the remaining three phenolic protons are missing (or too broad), presumably due to proton exchange in deuterated solvent. Starting from H-8, HMBC data revealed that the second and the third aromatic rings are connected by a C-9/C-11 carbon–carbon bond. Compound **4** was identified as the already known hinnulin A previously isolated from *Nodulisporium hinnuleum*.[20]

Transcription of *BC2* was determined by reverse transcriptase (RT) analysis of transformant VBI27-5. To this end, messenger RNA (mRNA) of VBI27-5 and WT *P. oryzae* Guy11 strains were extracted and converted into the corresponding complementary DNA (cDNA) before the samples were analyzed by PCR. RNA was used as negative control to ensure that no gDNA remained in the sample. As expected, expression of *BC2* was confirmed for VBI27-5, but was absent in the WT strain.

In addition, expression of genes from the *ACE1* BGC (*ACE1*, *RAP1*, *ORFZ* and *ORF3*) was tested. In accordance with the observed secondary metabolite profile, expression of all four genes was not observed. Apparently, expression of *BC2* did not activate the biosynthetic pathway of the ACE1 metabolite under the tested conditions.

In the case of the *M. grisea* pTYGS-*bar-pyiR* transformants, 20 strains were tested by PCR of gDNA for the correct integration of the P_{gpdA}-*pyiR* cassette. Five transformants were confirmed. One of these strains was grown in parallel to WT *M. grisea* NI980 in DPY liquid media for 7 days. After this time the liquid supernatant was extracted and the organic extract examined by LCMS. No differences in the chromatograms of the WT and pTYGS-*bar-pyiR* strains were observed (Fig. 5). However pyrichalasin H **1** was purified from each extract. The WT strain yielded 61.6 mg L^{-1} while the pTYGS-*bar-pyiR* strain yielded 190.4 mg L^{-1} (average over six flasks).

Remarkably, expression of *BC2* in *P. oryzae* appeared to have influenced expression of another BGC, leading to the production of **4**. Based on its structure, **4** seems to be related to fungal 1,8-dihydroxynaphthalene (DHN)-melanin. Therefore, the hinnulin BGC is likely to contain a gene encoding a PKS homologous to the *alb1* gene (AFUA_2G17600) known to be involved in the synthesis of 1,3,6,8-tetrahydroxynaphthalene (T_4HN) **5**, the precursor of DHN in fungi. The *Colletotrichum lagenarium PKS1* gene encodes a non-reducing-PKS (nrPKS) which is known to produce T_4HN **5**.[21,22] A BLAST search was performed with the *C. lagenarium PKS1* gene to identify a "melanin-like" BGC in *P. oryzae* Guy11. Two potential genes were identified: *MGG_07219*, (72% identity); and *MGG_00428* (35% identify). MGG_07219 also shows 43% identity to the *A. fumigatus* Alb1 PKS which is also known to be involved in fungal melanin biosynthesis.[23]

Genes adjacent to *MGG_07219* encode two transcription factors, a multicopper oxidase/laccase,[25] a T_4HN reductase[23,26] and several hypothetical proteins of unknown function (Table 1). Significantly, laccase-like multicopper oxidases (*MGG_07220*) and T_4HN reductases (MGG_07216) are known to be involved in melanin biosynthesis (*vide infra*).

The genes adjacent to the second nrPKS gene, *MGG_00428*, encode a short-chain dehydrogenase/reductase (SDR), an *O*-methyl transferase (*O*-MeT), a 1,2-dioxygenase and several other hypothetical proteins unlikely to be involved in the biosynthesis of DHN-melanin or **4**. Based on these findings, the *MGG_07219* BGC is the best bioinformatic hit and might be involved in the

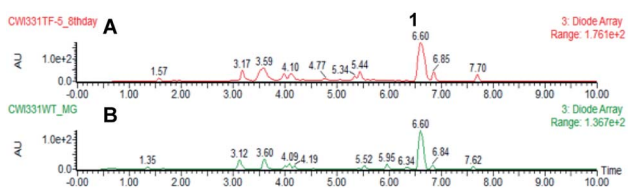
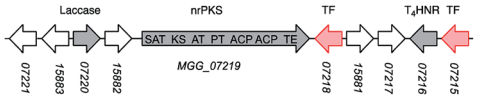


Fig. 5 Results of expression of *pyiR* in *M. grisea* NI980. (A) Extract of *M. grisea* pTYGS-*bar-pyiR*; (B) extract of *M. grisea* NI980 WT.

Table 1 Deduced functions (PBLAST) of the *P. oryzae* MGG_07219 genomic region



Gene	Putative function	Homolog	Ident. (%)	Ref.
MGG_07221	Hypothetical protein	TLD08818.1	94	—
MGG_15883	Hypothetical protein	N/A	—	—
MGG_07220	*Verticullium dahliae* laccase	XP_009649861.1	59	—
	Cu dep. DHN polymerase	ABR1	40	16
	Cu dep. DHN polymerase	ABR2	25	16
MGG_15882	Hypothetical protein	QBZ57338.1	94	—
MGG_07219	*C. lagenarium* PKS1	BAA18956.1	72	13 and 14
	A. fumigatus alb1 THN PKS	XP_756095.1	43	15
MGG_07218	Transcriptional regulator	KLU88469.1	65	—
MGG_15881	Hypothetical protein	ELQ35579.1	98	—
MGG_07217	Hypothetical protein	QBZ57332.1	81	—
MGG_07216	T_4HN reductase	1JA9_A	99	17 and 24
MGG_07215	Transcriptional regulator	AAF37291.1	99	—
		XP_009216426.1	72	

biosynthesis of **4**. However, further experiments were conducted to experimentally validate this hypothesis.

Expression of both PKS genes, *MGG_07219* and *MGG_00428*, was tested by RT-PCR. Expression of *MGG_07219* was observed in the transformant VBI27-5 but not in the WT *P. oryzae* Guy11 control. Expression of *MGG_00428* was not observed in either the transformant VBI27-5 or in the WT control. These findings support the hypothesis that *MGG_07219* might be involved in the biosynthesis of **4**. To gain more insight into the identified BGC containing *MGG_07219*, expression of the six adjacent genes was tested. These encode a T_4HN reductase, two transcription factors (TF1 and TF2), two hypothetical proteins and a laccase-like multicopper oxidase. Expression of the T_4HN reductase gene (*MGG_07216*) and the gene encoding TF1 (*MGG_07215*) was observed for the wild type and the transformant strain. However, expression of the other genes was not observed in either the wild type strain or in the transformant.

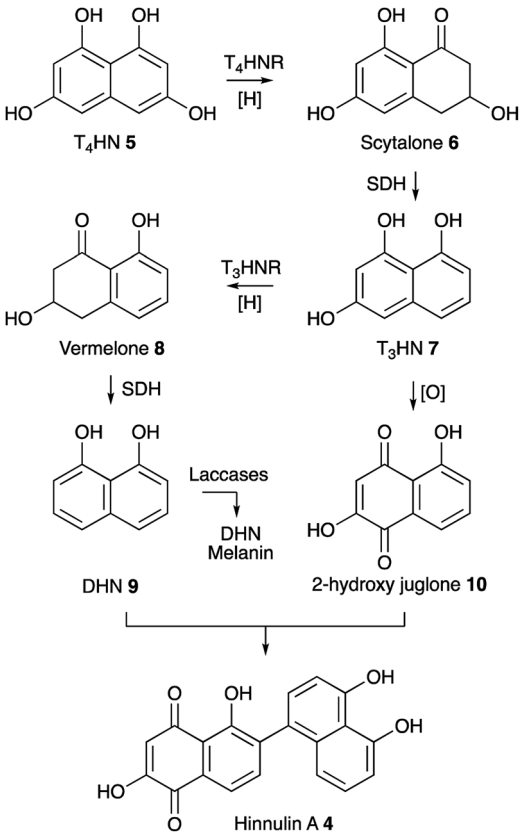
Discussion

Our results show that upon expression of *BC2* in *P. oryzae* Guy11, transcription of key genes from the *ACE1* BGC including *ACE1*, *RAP1*, *ORF3* and *ORFZ* was not observed. This corresponds with the absence of production of PKS-NRPS-related metabolites which are the known products of the *ACE1* BGC.[7] Apparently, this TF was not able to activate the *ACE1* BGC under the tested conditions. One reason for this might be that BC2 is insufficient alone to initiate transcription. Another explanation might be that BC2 was not able to activate the *ACE1* BGC if its expression is inhibited by the formation of heterochromatin in the region of the *ACE1* BGC.[27]

Notably, overexpression of *BC2* in *P. oryzae* Guy11 does lead to the expression of the nrPKS encoded by *MGG_07219* and to the production of hinnulin A **4** which was first identified in the fungus *Nodulisporium hinnuleum*. **4** belongs to the class of 1,2′-binaphthyl natural products,[15] and would appear to be structurally related to fungal DHN-melanin. Most fungal melanins are derived from T_4HN **5**.[28] The pathway involves reduction of T_4HN **5** to scytalone **6** by T_4HN reductase (T_4HNR) and then dehydration of scytalone **6** by scytalone dehydratase (SDH) to form 1,3,8-trihydroxynaphthalene (T_3HN) **7**. This is then reduced again to form vermelone **8**,[29] at which point SDH acts again to form 1,8-DHN **9**. 1,8-DHN **9** is then polymerised by various laccases to form melanin (Scheme 1). Oxidation of T_3HN **7** is also known to lead to 2-hydroxy juglone **10** (ref. 30) although the molecular basis for this step in fungi is not known.

The *MGG_07219* BGC also encodes a T_4HNR and a laccase related to ABR1 and ABR2 which are proteins known to be involved in melanin formation in *Aspergillus fumigatus*. However, genes encoding SDH, T_3HNR and other laccases are not present close to *MGG_07219*. Some fungi such as *Talaromyces marneffei* do contain a complete melanin BGC,[31] but in other organisms such as *Cladosporium fulvum* the melanin biosynthetic genes appear not to be fully clustered.[32] In the case of *P. oryzae* Guy11 suitable candidates for SDH-, T_3HNR- and laccase-encoding genes are present elsewhere on the genome (see ESI† for details). Under the conditions tested *MGG_07219* (nrPKS) and *MGG_07220* (T_4HNR) are expressed, likely giving scytalone **6** which is a known precursor of **9** and **10** and presumably **4**. However further work will be required to find the missing pathway steps.

Interestingly it is known that the *ACE1* signal molecule is produced in the appressoria of *P. oryzae*, which are also heavily melanized.[2] The linkage between the *ACE1* BGC and a putative melanin BGC in *A. oryzae* is therefore probably not coincidental. However further detailed experiments will be required to unpick these relationships. Our experiments also show that the *pyiR* encoded TF acts as expected as a pathway-specific activator



Scheme 1 Known pathway to DHN melanin in fungi and proposed biosynthesis of **4**. Abbreviations: T_4HNR, T_4HN reductase; SDH, scytalone dehydratase; T_3HNR, trihydroxynaphthalene reductase.

in *M. grisea* NI980, increasing the titre of **1** by 3-fold. The differences between the actions of *BC2* and *pyiR* emphasise the fact that the operation of regulatory genes in closely related BGC (*i.e.* the *pyi* and *ACE1* clusters) are surprisingly different and remain cryptic to bioinformatic analysis. Again, significant future work will be required to untangle the differing control pathways responsible for the activation of these pathways.

Experimental

Organisms

P. oryzae Guy11 and *M. grisea* NI980 were obtained from Professor Marc-Henri Lebrun.

Analytical and preparative methods

Analytical LCMS data was obtained using a Waters LCMS system comprising of a Waters 2767 autosampler, Waters 2545 pump, a Phenomenex Kinetex column (2.6 μm, C_{18}, 100 Å, 4.6 × 100 mm) equipped with a Phenomenex Security Guard precolumn (Luna, C_5, 300 Å) and a flow rate of 1 mL min^{-1}. Detection was carried out by a diode array detector (Waters 2998) in the range 210 to 600 nm and an ELSD detector (Waters 2424) together with a mass spectrometer, Waters SQD-2 mass detector, operating simultaneously in ES^+ and ES^- modes between 150 and 1000 *m*/*z*. A solvent gradient was run over 15 min starting at 10% acetonitrile/90% HPLC grade water (0.05% formic acid) and ramping to 90% acetonitrile.

Preparative LCMS was used to purify compounds from a raw extract or from a reaction mixture. This consisted of the same system described above with a Phenomenex Kinetex Axia column (5 μm, C_{18}, 100 Å, 21.2 × 250 mm) and Phenomenex Luna, C_5, 300 Å precolumn. A solvent gradient was run over 15 min starting at 10% acetonitrile/90% HPLC grade water (0.05% formic acid) and ramping to 90% acetonitrile. The flow was set to 20 mL min^{-1} and the post-column flow was split (100 : 1) and the minority flow was made up with HPLC grade MeOH + 0.045% formic acid to 1 mL min^{-1} for simultaneous analysis by diode array detector (Waters 2998) in the range 210 to 600 nm and an ELSD detector (Waters 2424) together with a mass spectrometry, Waters SQD-2 mass detector, operating simultaneously in ES^+ and ES^- modes between 150 and 1000 *m*/*z*. Detected peaks were collected into glass test tubes. Combined tubes were evaporated (vacuum centrifuge) and residues analysed directly.

NMR measurements were acquired on Bruker DRX 400, Bruker Avance 500 or Bruker Avance 600 MHz spectrometers (Institute for Organic Chemistry, Leibniz Universität Hannover). Chemical shifts are shown in parts per million (ppm) in comparison to the TMS (Tetramethylsilane) standard. Coupling constants *J* are quoted in Hz.

Hinnulin A.[19] Brown solid, 1.3 mg. δ_H, 12.86 (s, 1H, OH-10), 7.71 (d, $J = 7.6$, 1H, H-7), 7.58 (d, $J = 7.6$, 1H, H-8), 7.24 (d, $J = 7.7$, 1H, H-12), 7.23 (dd, $J = 8.1$, $J = 8.1$, 1H, H-19), 6.97, (d, $J = 8.5$, 1H, H-20), 6.87 (d, $J = 7.8$, 1H, H-13), 6.82 (d, $J = 7.6$, 1H, H-18), 6.24 (s, 1H, H-1). δ_C, 193.5 (C-6), 182.6 (C-3), 161.6 (C-2), 160.6 (C-10), 155.9 (C-14), 155.9 (C-17), 139.6 (C-9), 138.4 (C-8), 136.3 (C-16), 131.0 (C-4), 130.0 (C-12), 128.6 (C-19), 127.4 (C-11), 120.1 (C-7), 119.2 (C-20), 115.8 (C-5), 111.4 (C-1), 110.4 (C-18), 109.8 (C-13). HRMS $C_{20}H_{12}NO_6$ ($[M]H^+$ calculated 347.0542, found 347.0529).

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

DFG is thanked for the provision of NMR and LCMS equipment (INST 187/621-1, INST 187/686-1). VH was funded by DFG (CO 1328/2-1) and CW was funded by the China Scholarship Council (2016 08310143). Dr Colin Lazarus, University of Bristol, is thanked for the gifts of pTHGS-*eGFP* and pTYGS-*ade*. The publication of this article was funded by the Open Access Fund of the Leibniz Universität Hannover.

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