Function of Pathway Specific Regulators in the *ACE1* and Pyrichalasin H Biosynthetic Gene Clusters

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1.0 Construction of fungal expression vectors

1.1 pTYGS-hygR-BC2

The fungal expression vector pTYGS-hygR-BC2 was constructed by in vivo homologous recombination in *S. cerevisiae*. The BC2 gene was amplified by PCR using *P. oryzae* Guy11 cDNA as template, with oligonucleotides (P1 and P2) featuring 30 bp homologous overlaps. The hph gene was amplified from vector DNA (pTH-GS-egfp, P3 and P4); the “adh patch” from vector DNA pTYGS (P5 and P6). The vector backbone was digested with NotI and Ascl (New England Biolabs). PCR was performed using Q5 DNA Polymerase (New England Biolabs) and sequences were checked by DNA sequencing (Mix2Seq kit, Eurofins). The plasmids pTHGS-eGFP and pTYGS-ade were obtained, with thanks, from the Lazarus group at the University of Bristol.

![Figure S1: Cloning strategy of pTGS-hygR-BC2. HR = homologous recombination.](image1)

1.2 pTYGS-bar-pyiR

The fungal expression vector pTYGS-bar-pyiR was constructed by in vivo homologous recombination in *S. cerevisiae*. The pyiR gene was amplified by PCR using *M. grisea* NI980 genomic DNA as template, with oligonucleotides (P39 and P40) featuring 30 bp homologous overlaps. The “adh patch” (P5 and P6) and “eno patch” (P36 and P37) from vector DNA pTYGS-bar. The vector backbone was digested with Ascl (New England Biolabs). PCR was performed using Q5 DNA Polymerase (New England Biolabs) and sequences were checked by DNA sequencing (Mix2Seq kit, Eurofins). The plasmids pTYGS-bar were obtained, with thanks, from the Lazarus group at the University of Bristol.

![Figure S2: Cloning strategy of pTYGS-bar-pyiR. HR = homologous recombination.](image2)
## 1.3 Table of PCR primers

| Primer ID | Number | Sequence 5’-3’ |
|-----------|--------|----------------|
| BC2 PamyB F | P1 | CTGAACAAATACCCACGAAAGCTCCGAATGGATGGATTCAATTGAATAA |
| BC2 Tamyb B | P2 | CTCCACJCCTCACAGCTACTACAGATCTATGCGGCGGATGGTCTTC |
| hph Pgpda F | P3 | TAAACAGCTACCCCCGGCTTGAGCAGACATCAGATCTACCTGAACCTACCCGAC |
| hph Teno R | P4 | AGGGTGGCTGGTATAGCTATATAATATCATATACCTTTGGCTCAGGAC |
| Patch_PadH F | P5 | ATTCACCACTATTTTATCCTACATTAA |
| Patch_TadH R | P6 | GACACGCAAACAGACTTTCATCGTAAAA |
| BC2 F | P7 | ATGGTGATGGATTCAATTA |
| BC2 Tamyb B | P8 | CTCCACAGCTACTACGG |
| Tubulin F | P9 | TGGTTGCAAGATCAGGCATCAAT |
| Tubulin R | P10 | CATCACACAGGTCACAGCAGC |
| BC2 R | P11 | TATGCCCCGGGATGGGCTTC |
| ACE1 cDNA new F | P12 | CAGCACTACGGACCTTGGAC |
| cDNA ACE1 R | P13 | CTGCCCTGATAGGATTACAG |
| RAP1 cDNA F | P14 | GGCTATCACCACCTGTCGG |
| RAP1 R | P15 | CTACAGCCAGCAACAAATCT |
| cDNA ORF2 F | P16 | GATCTGGGATATCCCCGGTTT |
| cDNA ORF2 R | P17 | CAATTCCAAAGTCTCATCAG |
| cDNA ORF3 F | P18 | GCTACACTGGATCAGCCG |
| cDNA ORF3 R | P19 | CATAACACTGCTGTAAGGCT |
| melanin 1 F | P20 | CTGTAATGTGGTGATCACCTC |
| melanin 2 R | P21 | CAGGTCGGAGCTTGGTGG |
| melanin 2 F new | P22 | GCTGAGGAAGAAAGATCG |
| melanin 2 R new | P23 | GTGAACGTAGTCTAGGCTTTG |
| T4NR F | P24 | GGCCACATTTTCTCTGAGCAT |
| T4NR R | P25 | ACTAGCTCATGTCGCCACCAT |
| TF 1 F | P26 | TGGTGCCAGCCGGAATCTCAG |
| TF 2 R | P27 | CGGCCCTCTATCCTGCC |
| TF 2 F | P28 | CATGTACATGGTGCAAAGGTTG |
| TF 2 R | P29 | CGCCGATGGCCTGGAC |
| laccase F | P30 | GTCAAGGGGAAGAGCTAGG |
| laccase R | P31 | TCAACCATCGTACAGGTC |
| L2 F | P32 | CAGCTACAATGCGCGATGAG |
| L2 R | P33 | CTACGAGGGTGATAGGAT |
| L3 F | P34 | GTCTTGCCGGACGCTAGAG |
| L3 R | P35 | CATCAATTACGGCGAGC |
| Patch_Peno F | P36 | CTCTCAATAATCATGCTGATAACCTTCCTGTA |
| Patch_Peno R | P37 | CAAAGTATATTGGAGACATAGCTACTAG |
| Patch_Pgpda F | P38 | CTTTCTCTCTCTCTTTCTCTCCCCATCTTC |
| Pgpda TF FP | P39 | ACACGCTACCCGCCTTGGACAGACATCACCGACCGAGTCTTCAACCCAG |
| Pgpda TF RP | P40 | ACACGCAATTGTCATACCAATCATGACCTTAATTTCTCCATAACTCC |
2.0 Transformation, selection and analysis of Magnaporthe species

2.1 P. oryzae Guy11

Preparation and transformation of *P. oryzae* protoplasts followed reported methods described for *M. grisea*, the only modification was that glass beads were used for homogenization of the cells.

In brief, *P. oryzae* was grown on complete medium (CM) plates for 10-12 days and used to inoculate 100 ml CM liquid media, which was incubated at 25 °C for 3-4 days with shaking at 150 rpm. Mycelia were homogenised using glass beads and transferred to new 100 ml CM followed by incubation at 25 °C for 1-2 days. Mycelia was collected by filtration and protoplasts produced by an enzyme mixture (*Trichoderma harzianum* lysing enzymes (20 mg/ml) and Driselase enzyme (10 mg/ml) in 0.9 M NaCl). Protoplasts were filtered through miracloth, collected by centrifugation (2000 rpm for 10 min) and resuspended in STC buffer (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl2). Previously prepared protoplasts which had been frozen and stored at -80 °C were used directly for each transformation experiment. Protoplasts were diluted to 3 x 107/ml and approximately 5 μg (20 μl) of appropriate plasmid or DNA fragments were added to 200 μl protoplast solution. The mixture was incubated on ice for 30 min. After adding 1 ml of PTC buffer (60 % PEG 3350, 1 M Sorbito,50 mM CaCl2, 50 mM Tris-HCl, pH 7.5) the mixture was incubated at RT for 30 min. 4 ml of TB4 (200 g/l sucrose, 3 g/L yeast extract) recovery medium was added and the protoplast were allowed to regenerate overnight, gently shaking. The supernatant was reduced by centrifugation to 1 ml and added to 20 ml of molten TNK-SU-CP (10 g glucose, 2 g yeast extract, 15 g agar, 2 g NaNO3, 2 g KH2PO4, 0.5 g MgSO4.7H2O, 0.1 g CaCl2.2H2O, and 4 mg FeSO4.7H2O, 7.9 mg/l ZnSO4.7H2O, 0.6 mg/l CuSO4, 0.1 mg/l H3BO3, 0.2 mg/l MnSO4.7H2O, and 0.14 mg/l NaMoO4, 1 mg/l thiamine, 5 μg/l biotin) containing 250 μg/ml hygromycin B. Plates were incubated at 25 °C for 5-7 days until the appearance of colonies. Transformants were further selected to monoclonies on TNK-CP-Hyg plates.

Transformation of *P. oryzae* Guy11 with pTH-GS-egfp

In order to develop a reliable transformation method, genetic manipulation of *P. oryzae* Guy11 was re-established using the vector pTH-GS-egfp. The vector features a hygromycin B resistance cassette (hgyB) as well as the egfp gene (encoding an enhanced green fluorescent protein (eGFP)), as visible marker. The egfp gene is expressed under the control of the strong inducible (by starch or maltose) amyl promoter (PamyB) from *A. oryzae*. Transformation of *P. oryzae* Guy 11 was performed with vector pTH-GS-egfp and resulted in 12 hygromycin B resistant transformants. 6 transformants and the wild type were cultivated for 5 days in DPY medium before the samples were analyzed by fluorescence microscopy. Upon typical GFP excitation and emission conditions the successful production of GFP wasascertained in all transformants, which was not observed for the wild type control (Figure S3, B I-IV). Thus, the strain *P. oryzae* Guy11 was successfully transformed using hygromycin B as the selection marker. Furthermore, the amylB promoter efficiently initiates expression of egfp demonstrating that it can be used in further studies to express the gene of interest in *P. oryzae* strains.
Transformation of *P. oryzae* Guy11 with pTGS-hygR-BC2

*P. oryzae* PEG/CaCl2 mediated protoplast transformation with pTGS-hygR-BC2 resulted in the isolation of 14 hygromycin resistant transformants growing on tertiary selection plates. All transformants were cultivated for 7 days in DPY media (25°C, 110 rpm) before cells and growth media were individually extracted twice with ethyl acetate and analysed by LC-MS. In 5 transformants (VBI27-3, -4, -5, -12) production of hinnulin A was observed in extracts of the media. Hinnulin A was not observed in extracts of *P. oryzae* Guy11 wild type (WT) strain (Figure S4).

As part of the ACE1 BGC the BC2 gene is present in the *P. oryzae* Guy 11 WT strain and therefore also in all transformants. To test whether an extra copy of the BC2 gene (which is fused to the inducible amyB promoter (PamyB)) is present in the transformants, a PCR experiment was designed with the aim to amplify the PamyB - BC2 junction. The junction between BC2 and the terminator sequence (TamyB) was amplified using P7 and P8. As a positive control vector DNA (pTGY-hygR-BC2) was used. A copy of BC2 was integrated into the genome of the four genetically analysed transformants (VBI27-3, -4, -5, -12, Figure S5).
Figure S5: Genetic analysis of *P. oryzae* Guy 11 transformants.

**Reverse Transcriptase (RT) analysis of transformant VBI27-5**

Successful expression of the transcription factor BC2 was determined by reverse transcriptase (RT) analysis of transformant VBI27-5. To this end, messenger RNA (mRNA) of VBI27-5 and the *P. oryzae* Guy11 wild type strain (used as a control) was extracted using the ZR Fungal Bacterial RNA MiniPrep kit (Zymo Research) kit and converted into the corresponding complementary DNA (cDNA) using the RevertAid Premium Transcriptase kit (Thermo Scientific) before the samples were analyzed by PCR. RNA was used as negative control to ensure that no gDNA remained in the sample. In addition, a control PCR was performed to amplify the house-keeping gene MGG_06650, encoding tubulin alpha-B chain (P9 and P10). Since the gene MGG_06650 contains introns, the amplification product from gDNA is longer than the product from mRNA (Figure S6, A). As expected, expression of BC2 was confirmed for VBI27-5 (P7 and P11), but was absent in the WT (Figure S6, B).

In addition, expression of genes from the ACE1 BGC (*ACE1, RAP1, ORFZ and ORF3*) was tested (Figure S7). 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 tested conditions.
Expression of BC2 appeared to have influenced expression of another BGC, leading to the production of hinnulin A 4 which seems to be related to fungal 1,8-dihydroxynaphthalene (DHN)-melanin. The Colletotrichum lagenarium PKS1 gene encodes a non-reducing-PKS (nrPKS) which is known to produce T₄HN.²,³ A BLAST search was performed with the 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 % identity, Figure S8). MGG_07219 also shows 43% identity to the A. fumigatus alb1 PKS which is also known to be involved in fungal melanin biosynthesis.⁴ Genes adjacent to the second PKS encoded by MGG_00428 include a short-chain dehydrogenase/reductase (SDR), an O-methyl transferase, a 1,2-dioxygenase and several hypothetical proteins unlikely to be involved in the biosynthesis of melanin or hinnulin A 4. The genetic borders of the respective loci of both clusters are unknown. Based on these findings, the MGG_07219 BGC is the best bioinformatic hit and might be involved in the biosynthesis of hinnulin A 4. Further experiments were conducted to experimentally validate this hypothesis.

Figure S7: RT-PCR results of ACE1, RAP1, ORFZ and ORF3. WT = wild type, T = transformant VBI27-5, RNA isolated from VBI27 was used as negative control (-), M = marker.
Expression of both genes, *MGG_07219* and *MGG_00428*, was tested by PCR of the previously prepared cDNA template. Expression of *MGG_07219* was observed in the transformant VBI27-5 and not in the wild type control (Figure S9). Expression of *MGG_00428* was not observed; neither in the transformant VBI27-5 nor in the wild type control (Figure S9). These findings support the assumption that *MGG_07219* might be involved in the biosynthesis of hinnulin A4.

To gain more insight into the identified BGC containing *MGG_07219*, expression of the 6 adjacent genes was tested (Figure S10). These encode a T4HN reductase, two transcription factors (TF1 and 2), two hypothetical proteins (named in this work L2 and R3) and a laccase-like multicopper oxidase. The remaining genes (encoding hypothetical proteins named in this work L1, R1 and R2) were rather small (< 200 bp) and unlikely encode any functional enzymes. Therefore, these genes were excluded from further examinations. Expression of the T4HN 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; neither in the wild type strain nor in the transformant.
2.2 *M. grisea NI80*

Preparation and transformation of *M. grisea* protoplasts followed the same methods as *P. oryzae* described above.

**Transformation of *M. grisea NI980* with pTYGS-bar-pyiR**

Transformation of *M. grisea* NI980 was performed with vector pTYGS-bar-pyiR and resulted in 40 basta resistant transformants. Twenty transformants were tested by colony PCR of genomic DNA for the correct integration of the \( P_{gpd} \)-pyiR cassette. Five transformants were confirmed and transferred into 500 mL Erlenmeyer flasks containing 100 mL DPY medium for 7 days (25 °C, 110 rpm). A WT *M. grisea* NI980 strain were inoculated in DPY medium in the same time. The cells were separated and growth media were extracted with ethyl acetate twice and analysed by LC-MS (figure S11).
Figure S11: Analysis of M. grisea NI980. **Top**, Colony PCR result of 5 positive transformants: 1, *M grisea* WT; 2-6, five positive transformants (P38 & P40). **Bottom**, LC-MS chromatogram extracted on the 7th day from DPY medium: A, *M. grisea* NI980 wild type; B, *M. grisea* NI980 pTYGS-bar-pyIR.

3.0 **Fermentation and extraction procedures**

For extraction, *M. grisea* or *P. oryzae* spores were collected from DPY plates incubated for 7 days and inoculated into 500mL Erlenmeyer flasks containing 100 mL DPY. The spores were allowed to grow in the liquid culture for 7-8 days on shakers at 110 rpm at 25 °C.

Grown *P. oryzae* or *M. grisea* cultures were segregated into mycelia mass and aqueous phase by vacuum filtration. The aqueous phase was acidified with 2 M HCL to pH 3-4 and extracted twice with an equal volume of ethyl acetate. The mycelia were homogenised with a blender and stirred for 1-3 h in ethyl acetate. The extract was separated from mycelia by filtration. Combined organic layers were dried with anhydrous MgSO4 and the solvent removed with rotary evaporator. Extracts were dissolved in methanol to a concentration of 10 mg/ml, filtered over glass wool and analysed or purified by LCMS. All compounds produced were mainly present in the aqueous phase.
4.0 Analytical data

4.1 Hinnulin A 4

**Figure S12**: UV spectrum (left) and mass spectra (right) of hinnulin A 4.

**Figure S13**: HRMS of hinnulin A 4.
Table S1: NMR data of hinnulin A 4. Not all ^1H signals could be integrated due to overlap. Solvent: CD$_3$CN.

| Atom | $\delta_C$ ppm | $\delta_H$ ppm | Multiplicity (J, Hz) | COSY | HMBC | HSQC | Structure |
|------|----------------|----------------|----------------------|------|------|------|------------|
| 111.4 | 6.24 s | - | 2, 3, 5, 6 | CH |
| 161.6 | - | - | - | - |
| 182.6 | - | - | - | - |
| 131.0 | - | - | - | - |
| 115.8 | - | - | - | - |
| 193.5 | - | - | - | - |
| 120.1 | 7.71 d (7.6) | 8 | 3, 5, 8, 9 | CH |
| 138.4 | 7.58 d (7.6) | 7 | 4, 10, 11 | CH |
| 139.6 | - | - | - | - |
| 160.6 | - | - | - | - |
| 127.4 | - | - | - | - |
| 130.0 | 7.24 d (7.7) | 13 | 9, 14, 16 | CH |
| 109.8 | 6.87 d (7.8) | 12 | 11, 14, 15 | CH |
| 155.9 | - | - | - | - |
| 115.4 | - | - | - | - |
| 136.3 | - | - | - | - |
| 155.9 | - | - | - | - |
| 110.4 | 6.82 d (7.6) | 19 | 15, 17, 20 | CH |
| 128.6 | 7.23 t (8.1, 8.1) | 18, 20 | 16, 17 | CH |
| 119.2 | 6.97 d (8.5) | 19 | 11, 15, 16, 18 | CH |
| 10 - OH | - | 12.86 s | - | 5, 9, 10 | - |

Figure S14: $^1$H NMR of hinnulin A 4.
Figure S15: COSY spectrum of hinnulin A 4.
Figure S16: $^{13}$C NMR spectrum of hinnulin A 4.

Figure S17: HSQC spectrum of hinnulin A 4.
4.2 Pyricularin H 1

Table S2: NMR data of pyricularin H 1 recorded at 400 MHz in CDCl₃. Values are in agreement with published data.²
Figure S19: $^1$H NMR spectrum of pyrichalasin H 1.

Figure S20: $^{13}$C NMR spectrum of pyrichalasin H 1.
Figure S21: H-H COSY NMR spectrum of pyrichalasin H 1.

Figure S22: HMBC NMR spectrum of pyrichalasin H 1.
5.0 Bioinformatic analysis

PyiR and BC2 belong to different TF families and share only 18.1% identity. BC2 is predicted to be a GAL4-like Zn$_2$Cys$_6$ binuclear cluster DNA-binding domain; while PyiR is predicted to be a c2H2 zinc-finger protein. BlastP searches were performed to find the 5 closest known relatives of BC2 and PyiR. A phylogenetic tree was created by neighbour–joining method using the clustal omega server (Figure S24). Homologs of PyiR-like transcription factors (red in Figure S24) cluster more closely than the homologs of BC2-type transcription factors (black in Fig S24). ApdR involved in positive regulation of the aspyridone cluster and CcsR involved in positive regulation of the cytochalasin E cluster are also shown (blue).

Expression of the MGG_07220 gene (encoding a laccase) was not observed. However, several other genes encoding laccases are present in the genome of P. oryzae. A Protein Blast search was performed using the Abr1 protein from A. fumigatus as query. The 5 best bioinformatic hits are listed in Table S3.
The *Colletotrichum lagenarium* scytalone dehydratase (SD) gene (D86079.1) is known to be involved in the biosynthesis of DHN-melanin was used as query to identify homologous genes in *P. oryzae*. One gene was identified (Table S4).

To identify putative T₃HNR in *P. oryzae*, the *Curvularia lunata* 1,3,8-trihydroxynaphthalene reductase (T₄HNR) gene (AF419330) was used as query for a BlastP search (Table S5).\(^\text{12}\)

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### Table S3: Homologous Proteins to Abr from *A. fumigatus* in *P. oryzae*.

| Protein               | putative function                                      | identity [%] | references |
|-----------------------|--------------------------------------------------------|--------------|------------|
| XP_003708928.1        | iron transport multicopper oxidase FET3/5              | 43           | -          |
| XP_003720855.1        | laccase                                                | 30           | -          |
| XP_003718499.1        | hypothetical protein                                   | 28           | -          |
| XP_003718807.1        | laccase                                                | 28           | -          |
| XP_003712139.1        | laccase                                                | 28           | -          |

### Table S4: Homologous Proteins to Scytalone Dehydratase from *Colletotrichum lagenarium* in *P. oryzae*.

| Protein               | putative function                  | identity [%] | reference |
|-----------------------|------------------------------------|--------------|-----------|
| XP_003712572.1        | scytalone dehydratase              | 69           | 11        |

### Table S5: Homologous Proteins of T₃HNR from *Curvularia lunata* in *P. oryzae*.

| Protein               | putative function                                      | identity [%] | reference |
|-----------------------|--------------------------------------------------------|--------------|-----------|
| XP_003709023.1        | T₃HNR                                                  | 75           | 13, 14    |
| XP_003715430.1        | T₄HNR                                                  | 49           | 14        |
| XP_003718310.1        | rhamnolipids biosynthesis 3-oxoacyl-[acyl-carrier-protein] reductase | 29           | -         |
| XP_003720189.1        | 3-oxoacyl-[acyl-carrier-protein] reductase              | 33           | -         |
| XP_003711492.1        | acetoin(diacetyl) reductase                           | 29           | -         |
6.0 References

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