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

Verena Hantke,[a] Chongqing Wang,[a] Elizabeth J. Skellam[a] and Russell J. Cox*[a]

Institute for Organic Chemistry and BMWZ

Leibniz Universität Hannover

Schneiderberg 38

30167 Hannover, Germany

Electronic Supplementary Information

- **1.0 Construction of fungal expression vectors 2**
  - **1.1 pTYGS-*hygR-BC2* 2**
  - **1.2 pTYGS-*bar-pyiR* 2**
  - **1.3 Table of PCR primers 3**
- **2.0 Transformation and selection and analysis of *Magnaporthe* species 4**
  - **2.1 *P. oryzae Guy11* 4**
  - **2.2 *M. grisea* NI980 9**
- **3.0 Fermentation and extraction procedures 10**
- **4.0 Analytical NMR 11**
  - **4.1 Hinnulin A 4 11**
  - **4.2 Pyrichalasin H 1 15**
- **5.0 Bioinformatic analysis 18**
- **6.0 References 20**

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 *Not*I and *Asc*I (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.

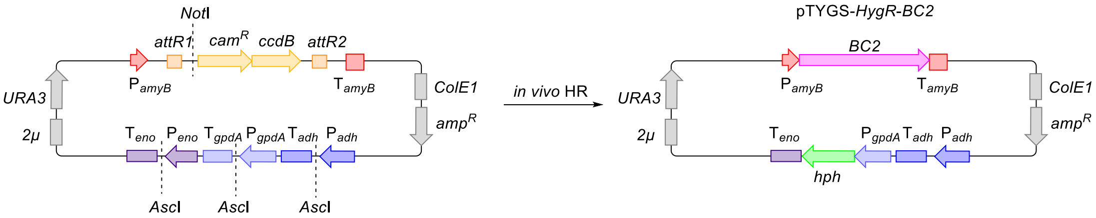


Figure S1: Cloning strategy of pTGS-*hygR-BC2*. HR = homolgous recombination.

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 *Asc*I (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.

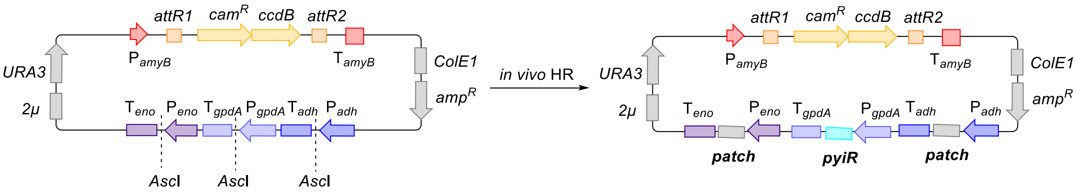


Figure S2: Cloning strategy of pTYGS-bar-pyiR. HR = homolgous recombination.

1.3 Table of PCR primers

Primer ID	**Number**	**Sequence 5’-3’**
BC2 PamyB F (1063)	P1	CTGAACAATAAACCCCACAGCAAGCTCCGAATGTTGATGGATTCGAATTA
BC2 TamyB R (1064)	P2	CTCTCCACCCTTCACGAGCTACTACAGATCCTATGGCCCGGGTGGTCTTC
hph Pgpd F (1065)	P3	TAACAGCTACCCCGCTTGAGCAGACATCACATGCCTGAACTCACCGCGAC
hph Teno R (1066)	P4	AGGTTGGCTGGTAGACGTCATATAATCATACTATTCCTTTGCCCTCGGAC
Patch_PadH F	P5	ATTCACCACTATTATTCCCACCCTATAATA
Patch_TadH R	P6	GAGACGAAACAGACTTTTTCATCGCTAAAA
BC2 F	P7	ATGTTGATGGATTCGAATTA
BC2 TamyB	P8	CTTCACGAGCTACTACAG
Tubulin F	P9	TGGTTGCCAGATCGCCAATTC
Tubulin R	P10	CATCACCACGGTACAGCAAGC
BC2 R	P11	CTATGGCCCGGGTGGTCTTC
ACE1 cDNA new F	P12	CAGCATCACGCAGCTGACG
cDNA ACE1 R	P13	CTGCCTTGATGAGGTATTCAG
RAP1 cDNA F	P14	GGCTATCACCACCTGCTCG
RAP1 R	P15	CTACAGCCGCACAACAATCT
cDNA ORFZ F	P16	GATCTGGGATATGCCGTTTTG
cDNA ORFZ R	P17	CAATTCCAAAGTGCTCATCGAG
cDNA ORF3 F	P18	CTCAACTCGGATTCGCTC
cDNA ORF3 R	P19	CATAATTGCTCTTGAACAGTGG
melanin 1 F	P20	CTCGTAATTTGCGGTCGCCTC
melanin 2 R	P21	CAGCTGCGTCAGCTTGTTTG
melanin 2 F new	P22	GCTCTGAGGAAAGAGATCG
melanin 2 R new	P23	GTGAACGTCTAGTCCCTTGG
T4NR F	P24	GGCACACTTTCTCTGAGCAT
T4NR R	P25	ACTAGCTCATGTCGACCCATC
TF 1 F	P26	TGGTGCCACCGCGAATCTCAG
TF 2R	P27	CGGCCTCTTCAATCTCCTTGA
TF 2 F	P28	CATGTACATGGTCCTGGTC
TF 2 R	P29	CGCCGATGTTGCTTGACCA
laccase F	P30	GTCAAGGGCAAGACCTACCG
laccase R	P31	TCACCCATGGTAGACAGTGTC
L2 F	P32	CAGCTACAATCGCGCATGCAC
L2 R	P33	CTATCGAGGCGTAATGGAT
L3 F	P34	GTCTTTGCCGACGCATCGAG
L3 R	P35	CATCAATTACGTTCGACCAG
Patch_Peno F	P36	CTTCTTAAATATCGTTGTAACTGTTCCTGA
Patch_Peno R	P37	CGAAGTATATTGGGAGACTATAGCTACTAG
Patch_Pgpda F	P38	CTTTTCTTTTCTCTTTCTTTTCCCATCTTC
Pgpda TF FP	P39	AACAGCTACCCCGCTTGAGCAGACATCACCATGCACAGGTTCAACCCACG
Pgpda TF RP	P40	ACGACAATGTCCATATCATCAATCATGACCTTAATTTCTTCCATAACTCC

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*,[1] 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 Tis-HCl, pH 7.5, 10 mM $CaCl_2$). 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 10^7/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 $CaCl_2$, 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 $NaNO_3$, 2 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, and 4 mg $FeSO_4.7H_2O$, 7.9 mg/l $ZnSO_4.7H_2O$, 0.6 mg/l $CuSO_4$, 0.1 mg/l H_3BO_3, 0.2 mg/l $MnSO_4.H_2O$, and 0.14 mg/l $NaMoO_4$, 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 monocolonies 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 (hyg^R) 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) *amyB* promoter (P_{amyB}) 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 was ascertained 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 *amyB* 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.

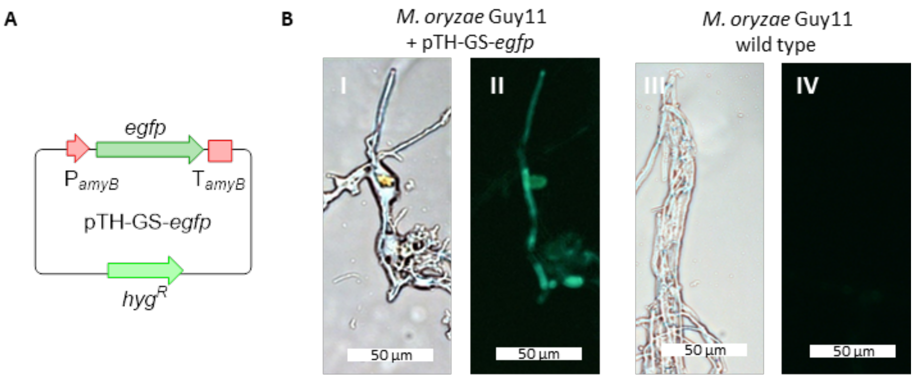


***Figure S3**: P. oryzae* Guy11 transformation: **A**, Vector map of pTH-GS-egfp; **B**, *P. oryzae* transformed with pTH-GS-*egfp* (I+II) and WT (III+IV), I+III) upon excitation (450-490 nm) and II+IV) bright-field.

Transformation of *P. oryzae* Guy11 with pTGS-*hygR-BC2*

P. oryzae PEG/$CaCl_2$ mediated protoplast transformation with pTGYS-*hyg*^{R}-*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 **4** was observed in extracts of the media. Hinnulin A **4** was not observed in extracts of *P. oryzae* Guy11 wild type (WT) strain (Figure S4).

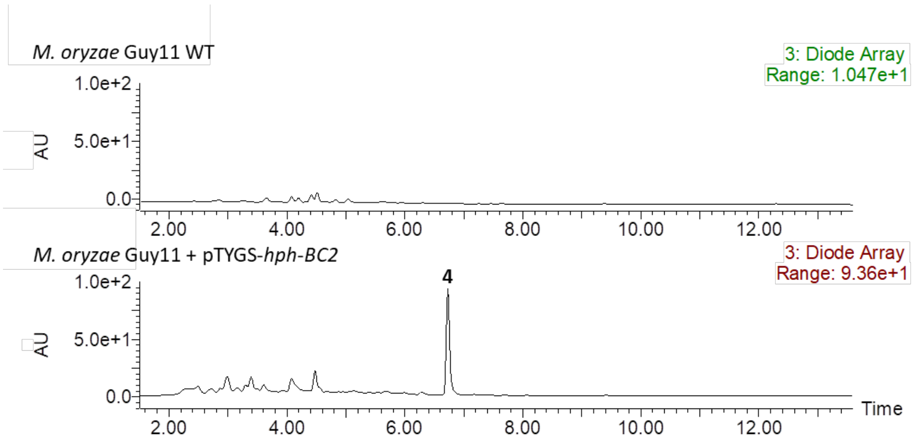


***Figure S4**:* Organic extracts of *P. oryzae* Guy11 wild type and transformant VBI27-5. Cultivation conditions: 100 mL DPY medium, 5 days, 25 °C, 110 rpm.

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 (P_{amyB}) is present in the transformants, a PCR experiment was designed with the aim to amplify the P_{amyB} - *BC2* junction. The junction between *BC2* and the terminator sequence (T_{amyB}) was amplified using P7 and P8. As a positive control vector DNA (pTGYS-*hyg*^{R}-*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**Error! Reference source not found.**).

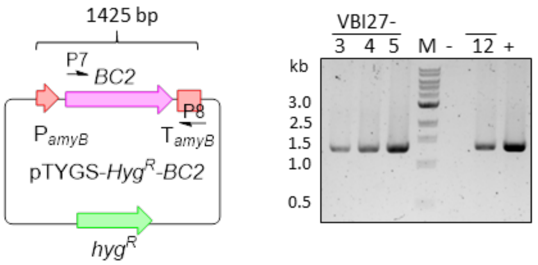


Figure S5: Genetic analysis of *P. oryzae* Guy 11 transformants.

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 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 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. 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).

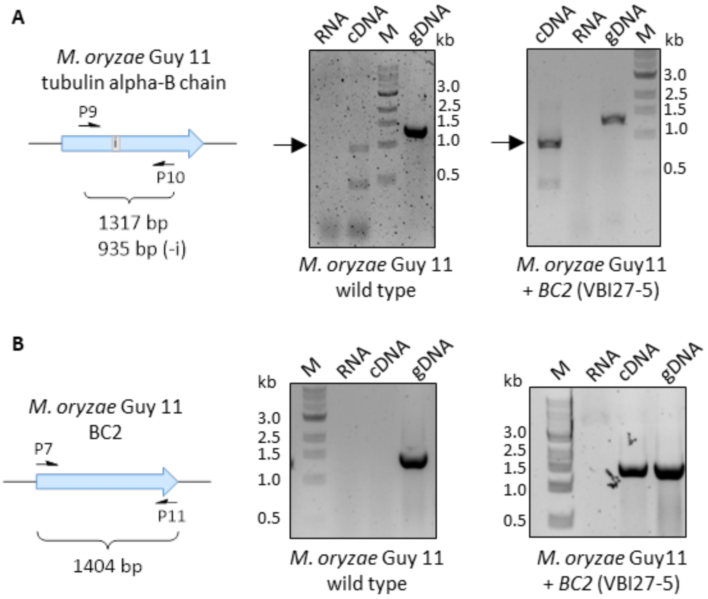


Figure S6: Reverse Transcriptase (RT) analysis of transformant VBI27-5: **A**, The gene encoding for tubulin alpha-B chain was used as control for gDNA contamination; **B**, Amplification of BC2 was observed in VBI27-5. M = marker.

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.

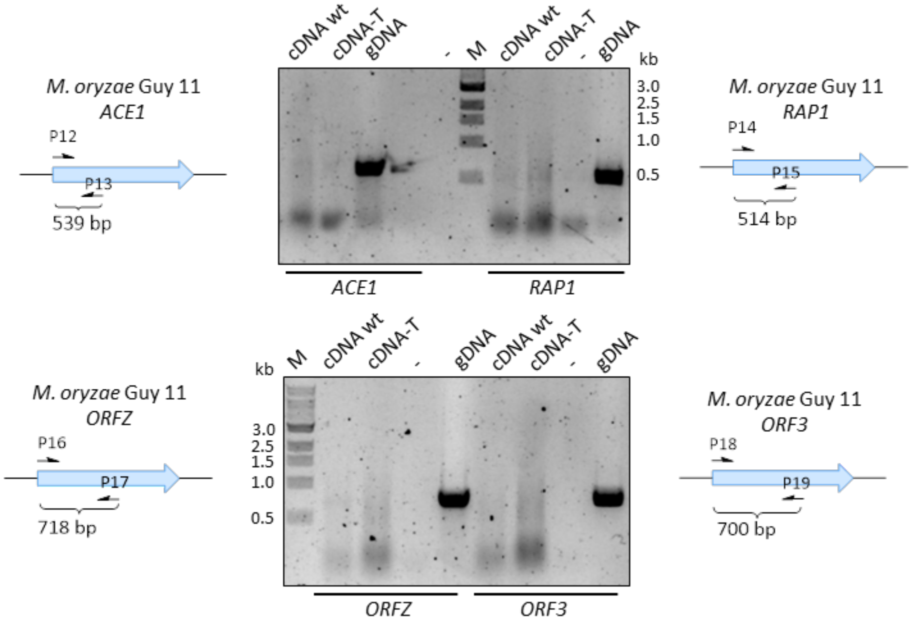


Figure S7: RT-PCR results of *ACE1, RAP1, ORFZ* and *ORF3*. WT = wild type, T = transformant VBI27-5, RNA isolated from VBI27 was used as negative control (-), M = marker.

Expression of *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 T$_4$HN.[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 % identify, 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 *MGG_07219* encode two transcription factors, an iron transport multicopper oxidase, a reductase and several hypothetical uncharacterised proteins. Notably, a BLAST search with the *MGG_07216* gene (mis-annotated as encoding versicolorin reductase) revealed its sequence similarity to a T$_4$HN reductase (98.4 % identity) in *M. grisea.* A BLAST search with the *MGG_07220* gene (annotated as an iron transport multicopper oxidase) exposed its sequence similarity to a laccase-like multicopper oxidase (79.1 % identity) in *Colletotrichum orbiculare.* Both enzymes, the T$_4$HN reductase and the laccase, are known to be involved in the biosynthesis of DHN-melanins.[5,6,7,8]

The 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 locus 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.

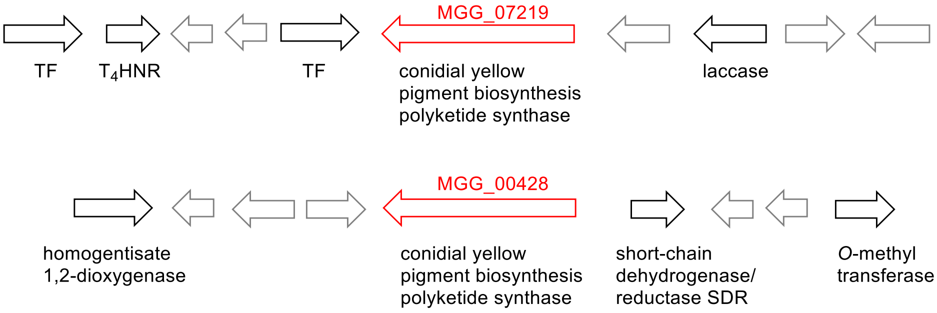


Figure S8: "Melanin-like" BGC in *P. oryzae* Guy11.

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 A **4**.

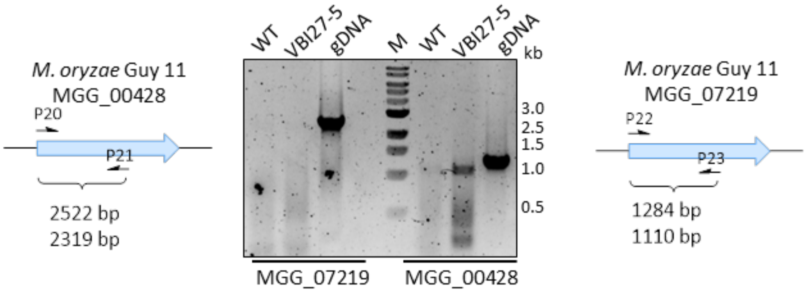


Figure S9:RT-PCR results of *MGG_07219* and *MGG_00428*. WT = wild type, M = marker.

To gain more insight into the identified BGC containing *MGG_07219*, expression of the 6 adjacent genes was tested (Figure S10). These encode a T_4HN 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 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; neither in the wild type strain nor in the transformant.

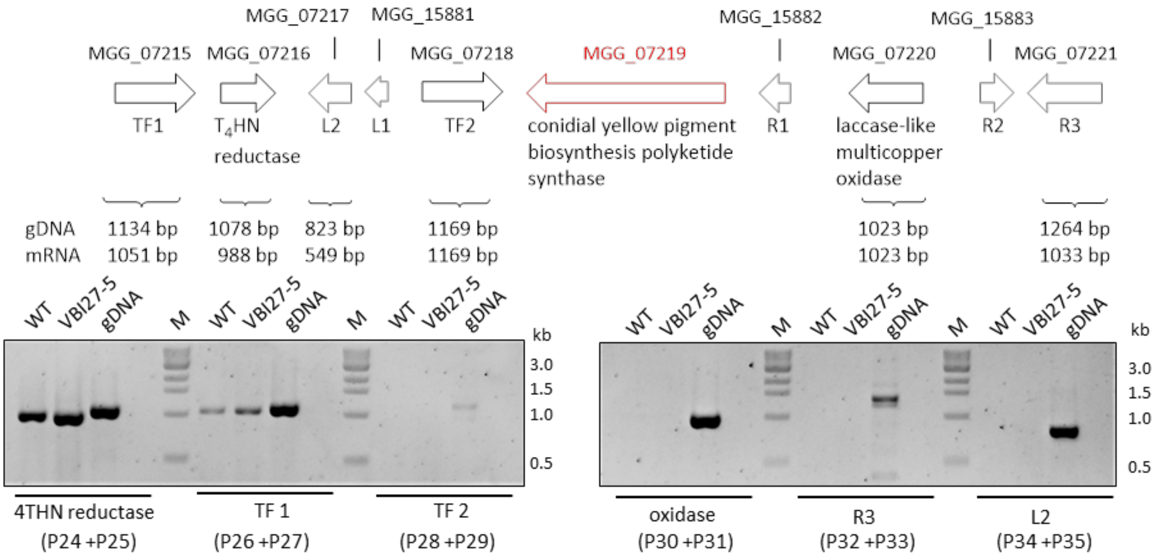


Figure S10: RT-PCR results of MGG_07219 and MGG_00428. WT = wild type, M = marker.

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_{gpdA}-*pyiR* cassette. Five transformants were confirmed and transferred into 500 mL Erlenmeyer flasks containing 100 mL DPY medium for 7days (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).

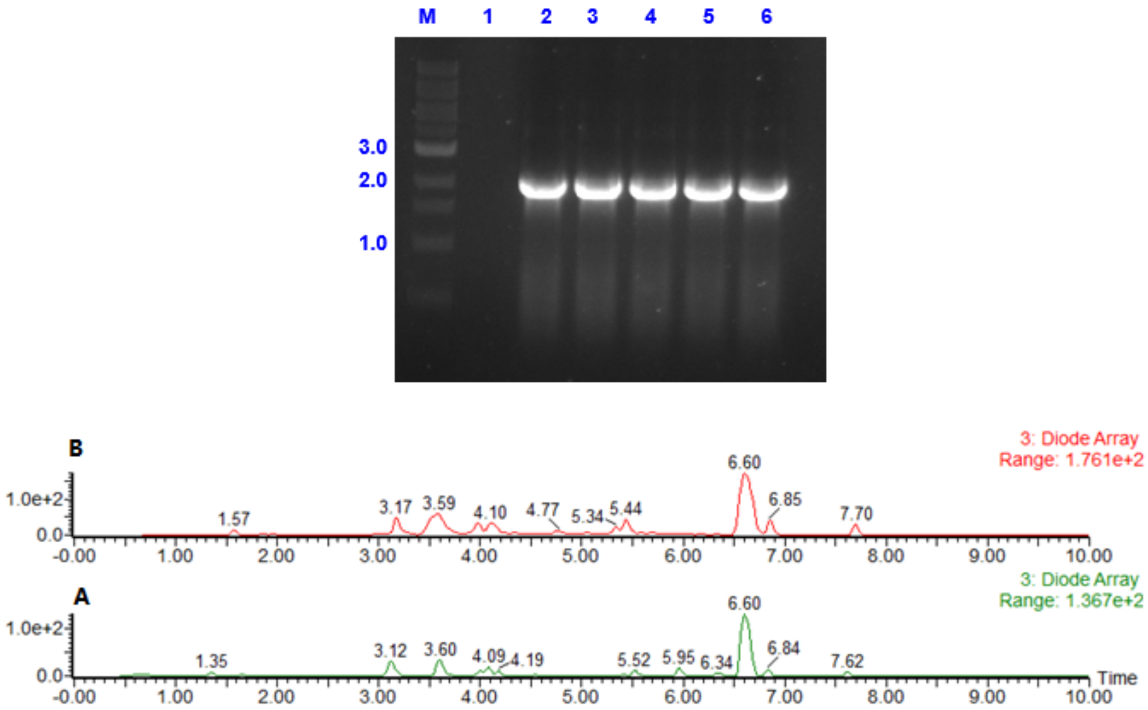


Figure S11: Analysis of M. grisea NI980. **Top**,Colony PCR result of 5 positive transformants: **1**, *M grisea* WT; **2-6**, five positive transformants (P38 & P40). **Bottom**, LC-MS chromatogram extracted on the 7th day from DPY medium: **A**, *M. grisea* NI980 wild type; **B**, *M. grisea* NI980 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 $MgSO_4$ 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

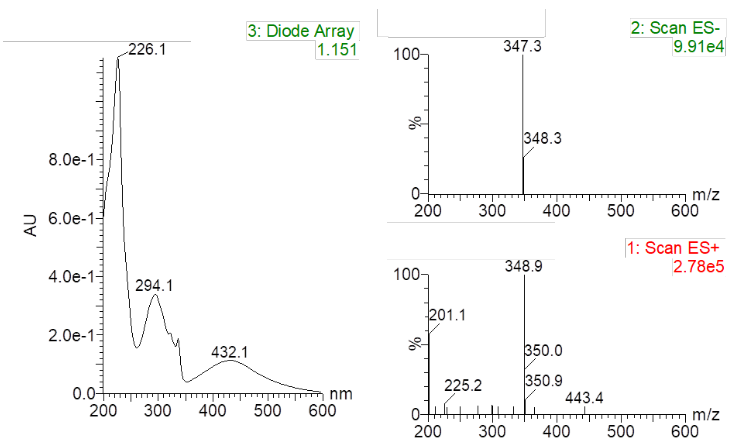
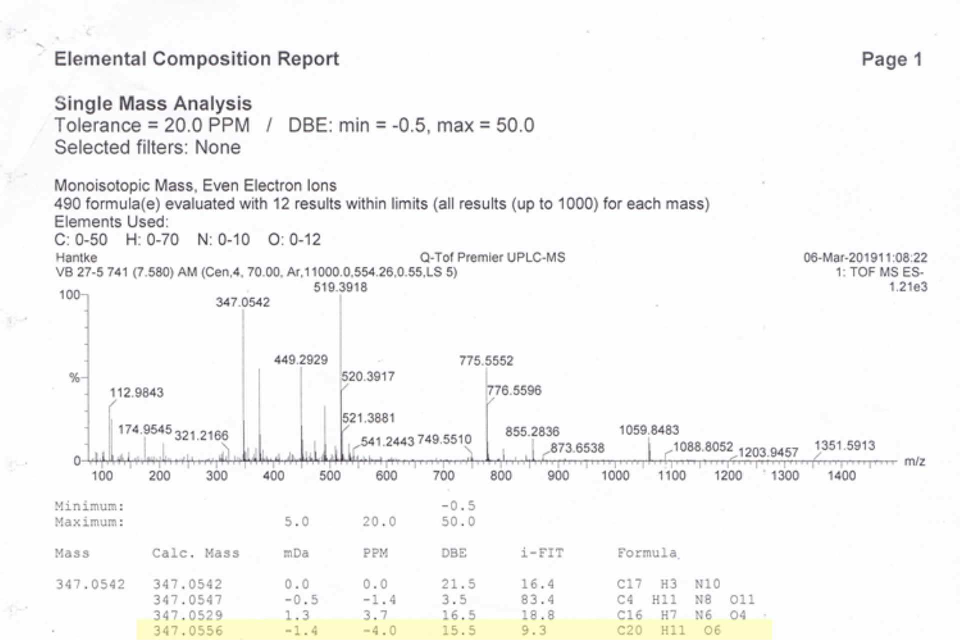


Figure S12: UV spectrum (left) and mass spectra (right) of hinnulin A **4**.



Elemental Composition Report

Page 1

Single Mass Analysis
Tolerance = 20.0 PPM / DBE: min = -0.5, max = 50.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
490 formula(e) evaluated with 12 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-50 H: 0-70 N: 0-10 O: 0-12

Hantke
VB 27-5 741 (7.580) AM (Cen,4, 70.00, Ar,11000.0,554.26,0.55,LS 5)

Q-Tof Premier UPLC-MS

06-Mar-201911:08:22
1: TOF MS ES-
1.21e3

```
Minimum:                                    -0.5
Maximum:                   5.0      20.0    50.0

Mass       Calc. Mass      mDa      PPM     DBE     i-FIT     Formula

347.0542   347.0542        0.0      0.0     21.5    16.4      C17  H3   N10
           347.0547        -0.5     -1.4    3.5     83.4      C4   H11  N8   O11
           347.0529        1.3      3.7     16.5    18.8      C16  H7   N6   O4
           347.0556        -1.4     -4.0    15.5    9.3       C20  H11  O6
```

Figure S13: HRMS of hinnulin A **4**.

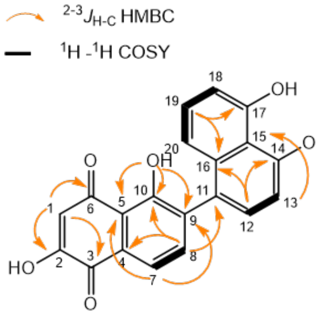
Atom	δ_C ppm	δ_H ppm	Multiplicity (*J*, Hz)	COSY	HMBC	HSQC	Structure
1	111.4	6.24	s	-	2, 3, 5, 6	CH	
2	161.6	-	-	-	-	-	
	182.6	-	-	-	-	-	
4	131.0	-	-	-	-	-	 **xxhin** $C_{20}H_{12}O_6$ MW: 348 g/mol
5	115.8	-	-	-	-	-	
6	193.5	-	-	-	-	-	
7	120.1	7.71	d (7.6)	8	3, 5, 8, 9	CH	
8	138.4	7.58	d (7.6)	7	4, 10, 11	CH	
9	139.6	-	-	-	-	-	
10	160.6	-	-	-	-	-	
11	127.4	-	-	-	-	-	
12	130.0	7.24	d (7.7)	13	9, 14, 16	CH	
13	109.8	6.87	d (7.8)	12	11, 14, 15	CH	
14	155.9	-	-	-	-	-	
15	115.4	-	-	-	-	-	
16	136.3	-	-	-	-	-	
17	155.9	-	-	-	-	-	
18	110.4	6.82	d (7.6)	19	15, 17, 20	CH	
19	128.6	7.23	t (8.1, 8.1)	18, 20	16. 17	CH	
20	119.2	6.97	d (8.5)	19	11, 15, 16, 18	CH	
10 - OH	-	12.86	s	-	5, 9, 10	-	

Table S1: NMR data of hinnulin A **4**. Not all ^{1}H signals could be integrated due to overlap. Solvent: CD_3CN.

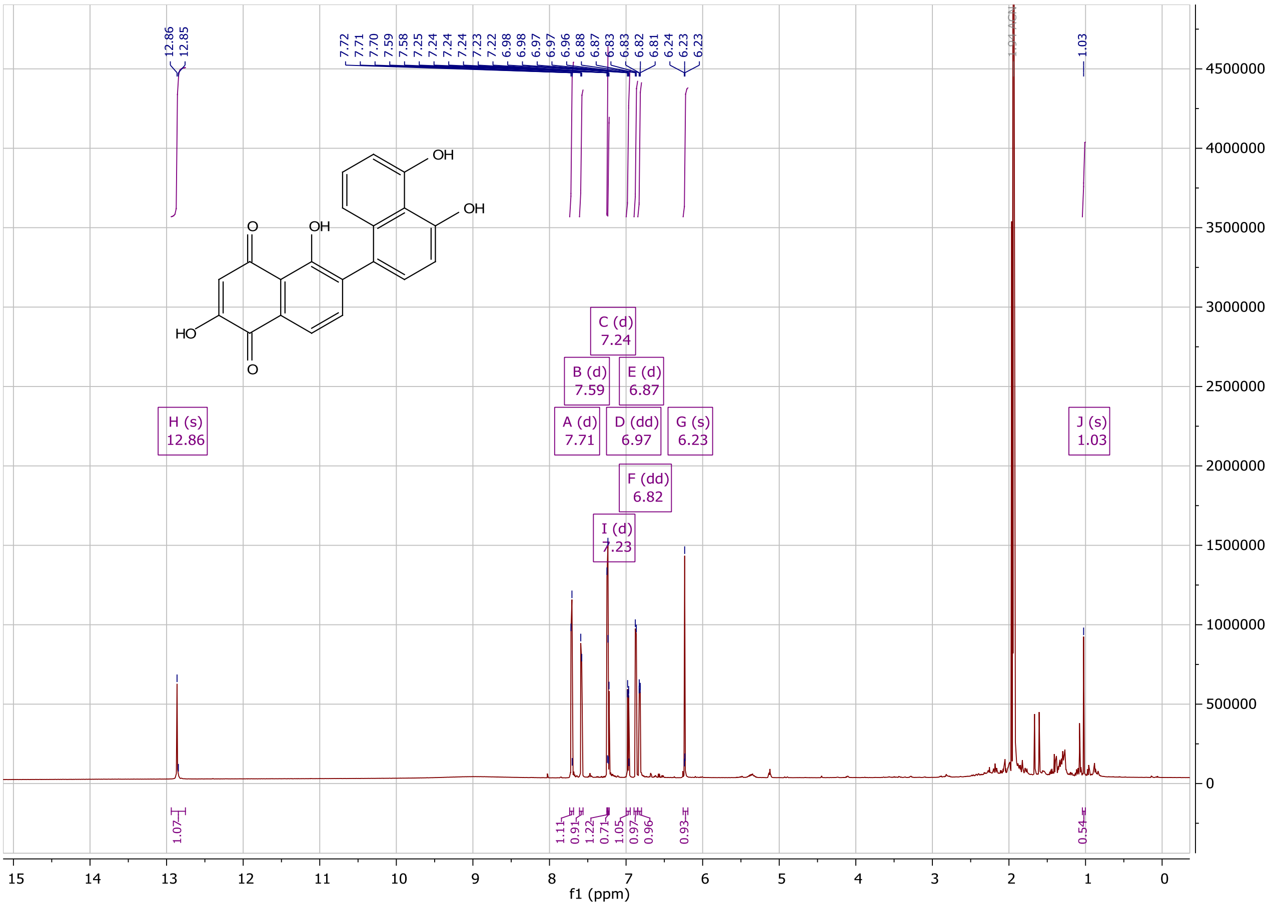


Figure S14: ^{1}H NMR of hinnulin A **4**.

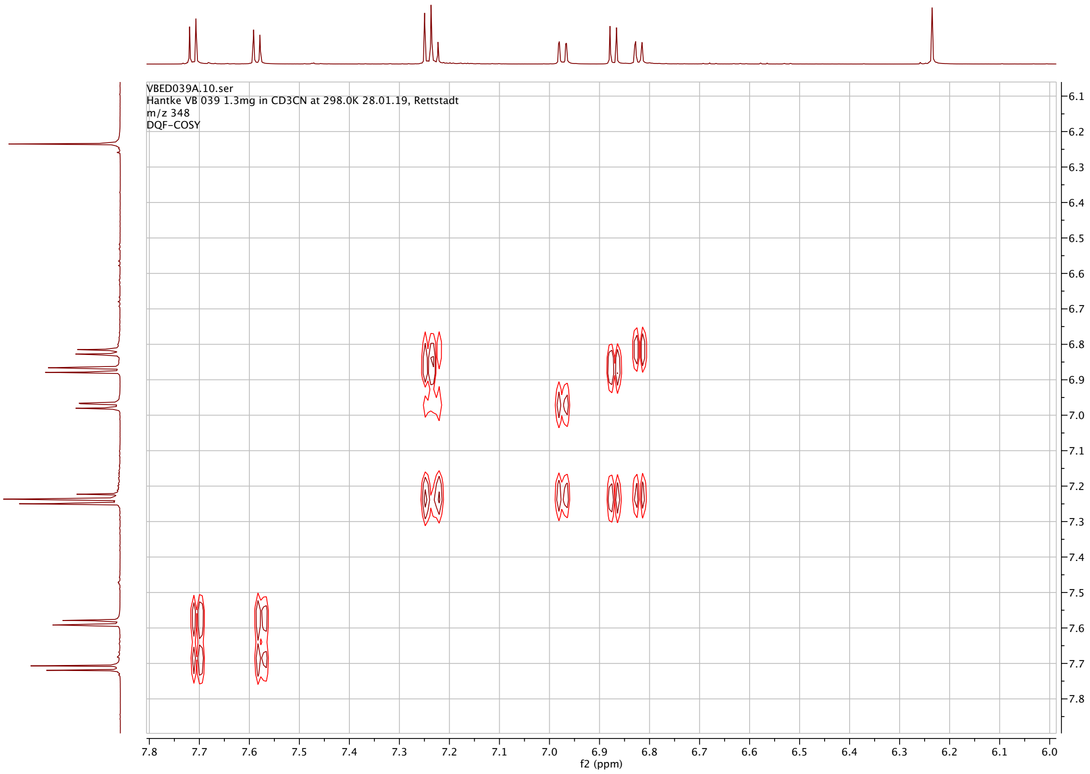


Figure S15: COSY spectrum of hinnulin A **4**.

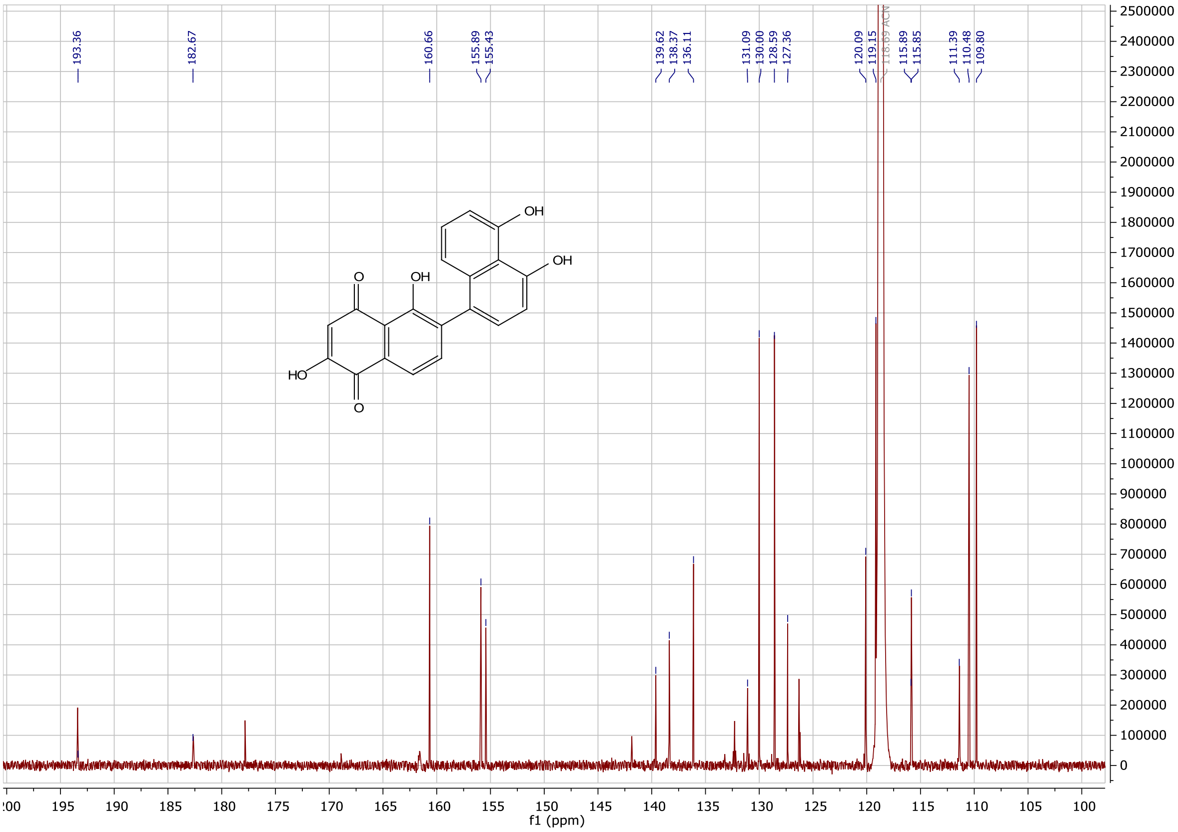


Figure S16: ^{13}C NMR spectrum of hinnulin A **4**.

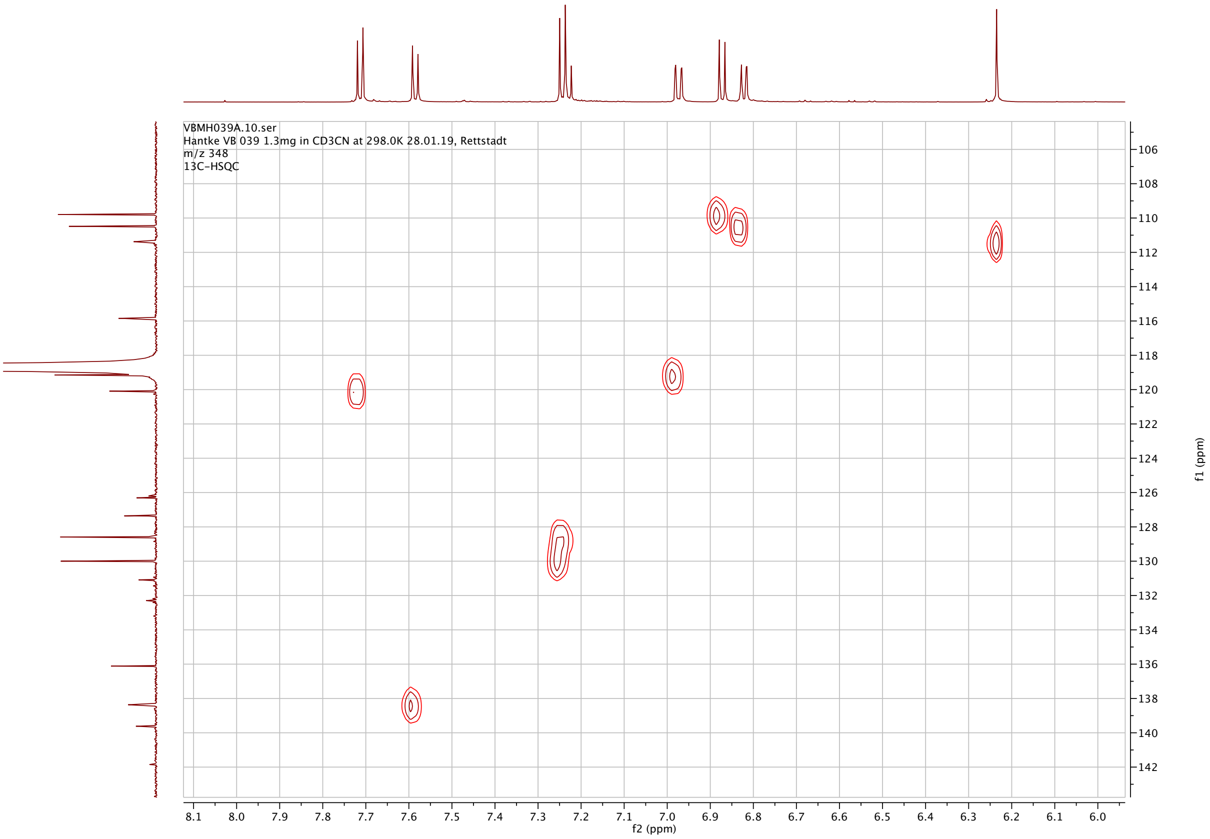


Figure S17: HSQC spectrum of hinnulin A **4**.

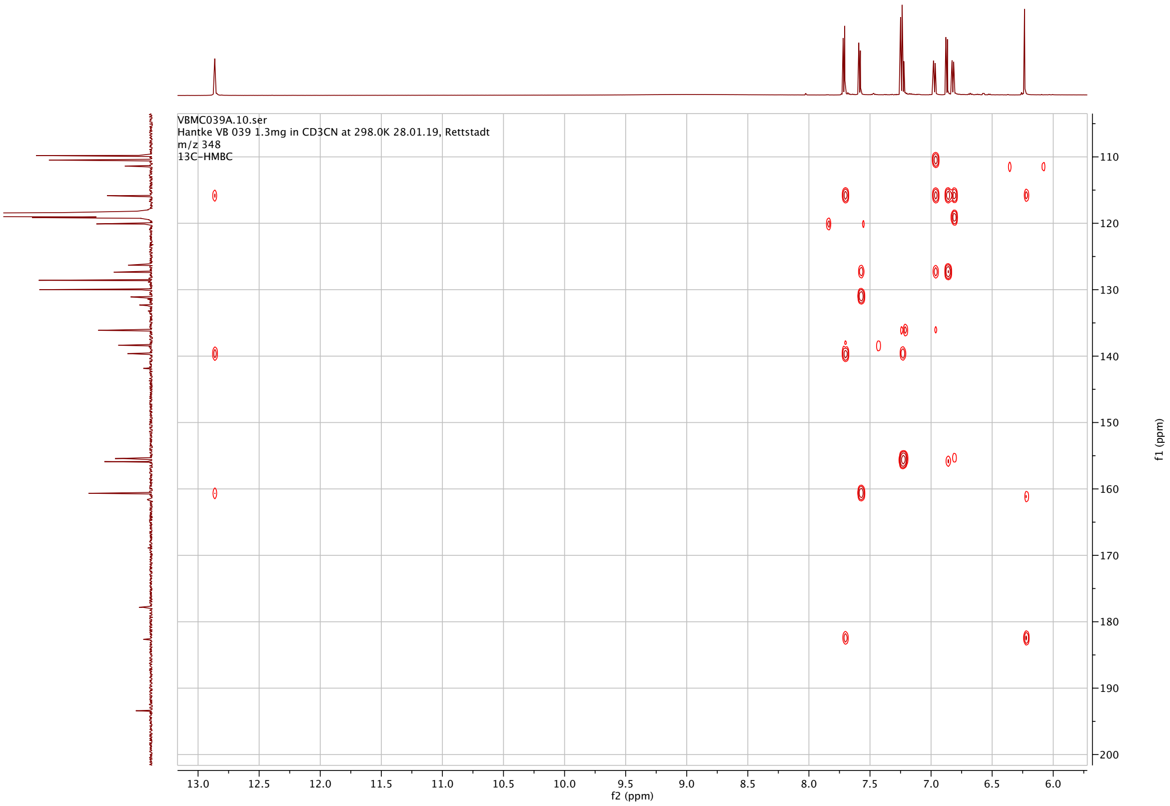
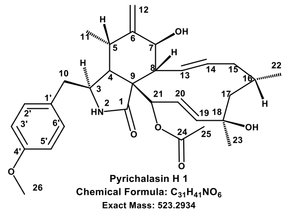


Figure S18: HMBC spectrum of hinnulin A **4**.

4.2 Pyrichalasin H 1



Pyrichalasin H 1
Chemical Formula: $C_{31}H_{41}NO_6$
Exact Mass: 523.2934

Position	δ_H	M	$J_{H\text{-}H}$/Hz	δ_C	HSQC	HMBC H to C	H-H COSY
1	-	-	-	174.4	-	-	-
2	5.57	s	-	-	-	-	-
3	3.23	ddd	4.6, 9.5, 4.5	54.0	CH	1‘, 1, 4, 9	4, 10
4	2.12	dd	4.5, 4.9	50.4	CH	5, 6, 10, 11, 21	3, 5
5	2.79	m	-	33.0	CH	1’, 7, 11	4, 12
6	-	-	-	148.1	-	-	-
7	3.84	dd	10.8, 1.3	69.9	CH	5, 6, 8, 9, 13	8, 12
8	2.95	dd	10.8, 9.8	47.3	CH	1, 3, 6, 7, 13, 14	7, 13
9	-	-	-	51.9	-	-	-
10a	2.61	dd	13.6, 9.5	44.8	CH_2	1’, 3, 4	3, 11
10b	2.80	dd	13.6, 4.6	-	-	-	-
11	0.99	d	6.7	14.2	CH_3	4, 5, 6	10
12a	5.12	brs	-	114.2	CH_2	5, 6, 7	5, 7, 15
12b	5.36	brs	-	-	-	-	-
13	5.75	ddd	9.8, 15.5, 1.3	127.3	CH	7, 8, 15, 16	8, 14
14	5.41	ddd	15.5, 5.2, 10.4	138.8	CH	8, 15, 16	13, 15
15a	1.83	m	-	42.9	CH_2	13, 14, 16, 17, 22	12, 14, 22
15b	2.05	m	-	-	-	-	-
16	1.83	m	-	28.4	CH	17	17
17a	1.56	dd	14.3, 2.7	53.8	CH_2	16	16
17b	1.88	m	-	-	-	-	-
18	-	-	-	74.5	-	-	-
19	5.55	dd	17.1, 2.3	138.2	CH	8, 9, 20, 21, 23	20
20	5.89	dd	17.1, 2.1	126.1	CH	8, 18, 19, 21	19, 21
21	5.56	dd	2.1, 2.3	77.6	CH	8, 19, 20, 23, 24	20
22	1.06	d	6.3	26.6	CH_3	15, 16, 17	15
23	1.37	s	-	31.3	CH_3	16, 17, 18, 19	-
1‘	-	-	-	129.5	-	-	-
2’ 6’	7.07	d	8.6	130.2	2 x CH	3’, 4’, 5’, 10	3’, 5’
3’ 5’	6.86	d	8.6	114.4	2 x CH	2’, 6’, 4’	2’, 6’
4’	-	-	-	158.8	-	-	-
24	-	-	-	170.3	-	-	-
25	2.26	s	-	21.0	CH_3	21, 24	-
26	3.81	s	-	55.4	CH_3	4’	-

Table S2: NMR data of pyrichalasin H **1** recorded at 400 MHz in $CDCl_3$. Values are in agreement with published data.[9]

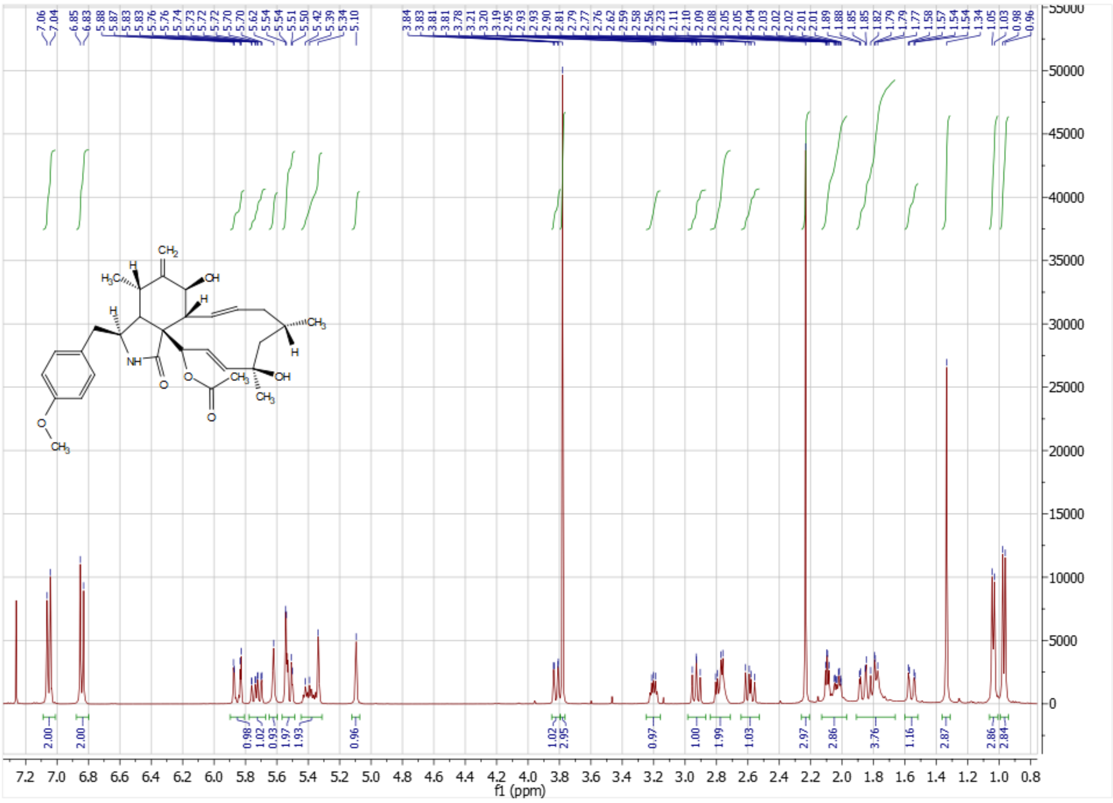


Figure S19: ^{1}H NMR spectrum of pyrichalasin H **1**.

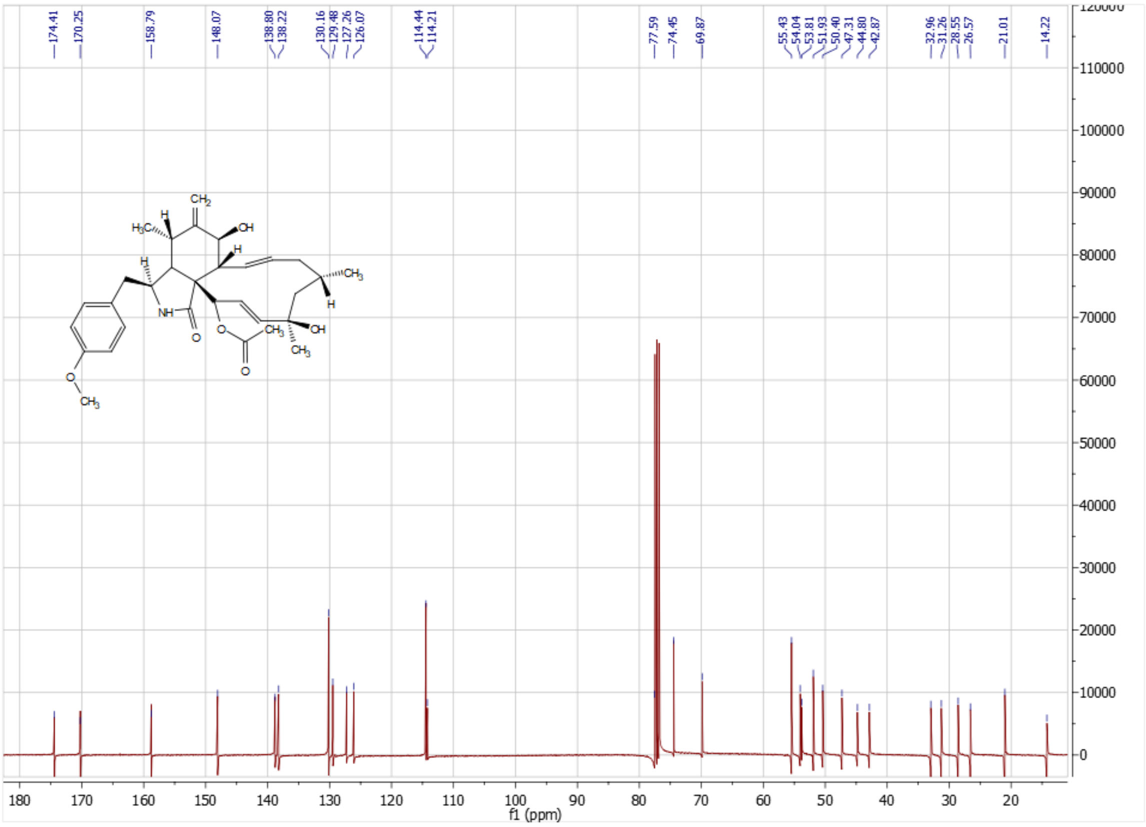


Figure S20: ^{13}C NMR spectrum of pyrichalasin H **1**.

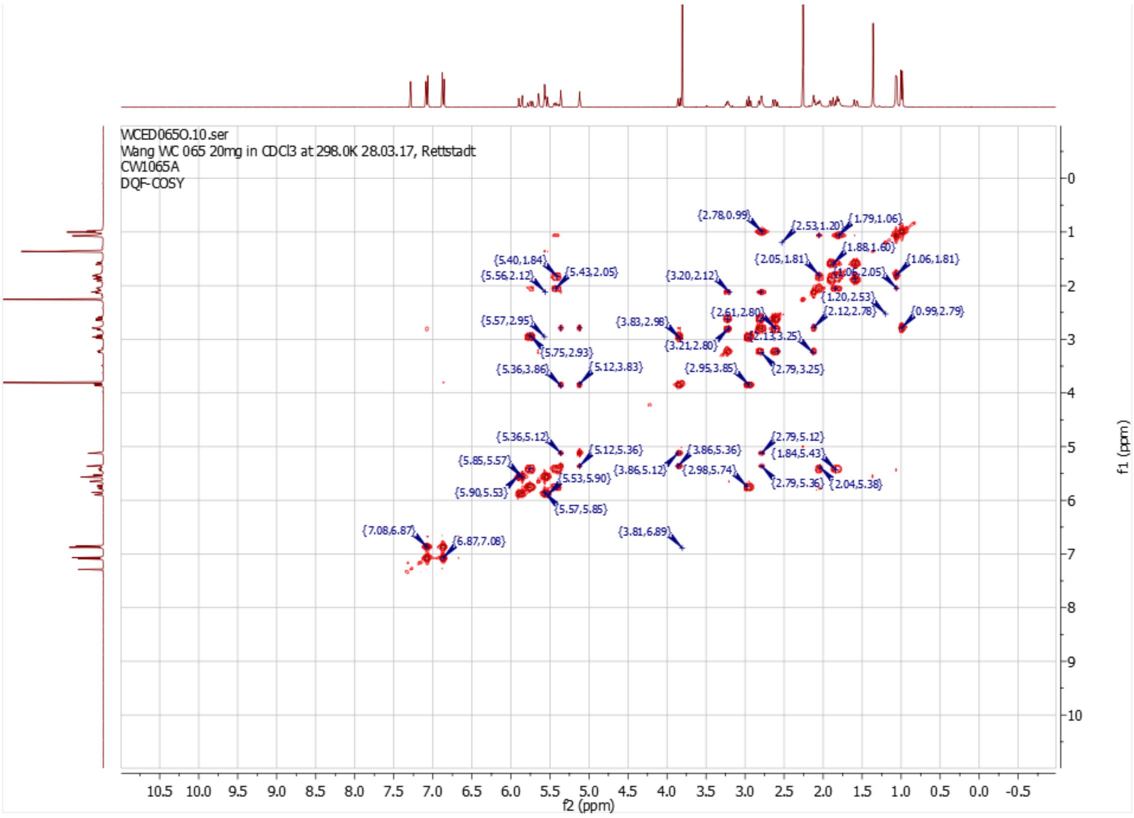


Figure S21: H-H COSY NMR spectrum of pyrichalasin H **1**.

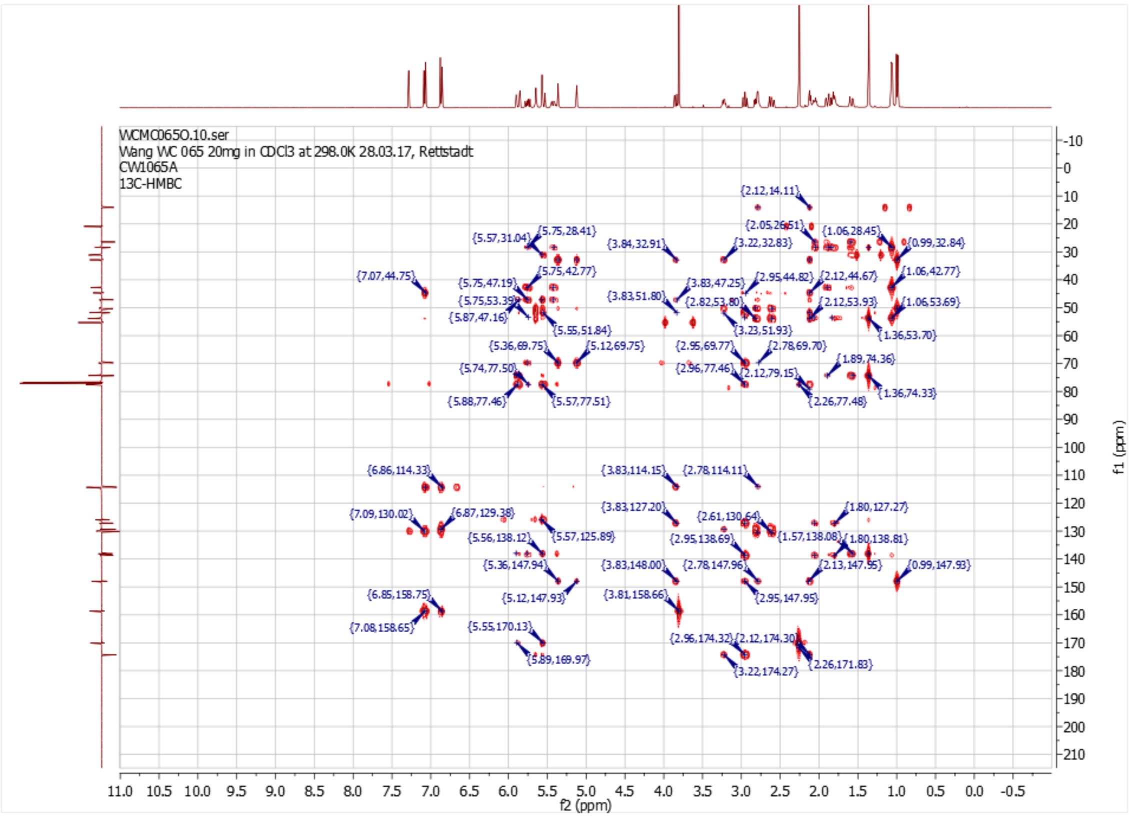


Figure S22: HMBC NMR spectrum of pyrichalasin H **1.**

Figure S23: HSQC 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_2Cys_6 binuclear cluster DNA-binding domain; while PyiR is predicted to be a c_2H_2 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 cytochalsin E cluster are also shown (blue).

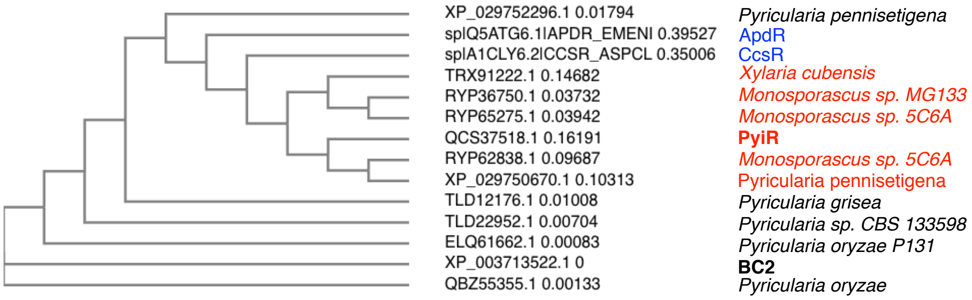


Figure S24: Phylogenetic relationship between *BC2*, *PyiR* and 10 homologous proteins identified by BLASTp. ApdR and CCsR are the pathway specific transcription factors from the aspyridone and cytochalasin E pathways respectively.

Expression of the *MGG_07220* gene (encoding a laccase) was not observed. However, several other genes encoding laccases are presnt in the genome of *P. oryzae*. A Protein Blast search was performed using the Abr1 protein from *A. fumigatus* as query.[10] The 5 best bioinformatic hits are listed in Table S3.

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

The *Colletotrichum lagenarium* scytalone dehydrate (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).

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

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

To identify putative T_3HNR in *P. oryzae*, the *Curvularia lunata* 1,3,8-trihydroxynaphthalene reductase (T_3HNR) gene (AF419330) was used as query for a BlastP search (Table S5).[12]

Protein	putative function	identity [%]	reference
XP_003709023.1	T_3HNR	75	13, 14
XP_003715430.1	T_4HNR	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	-

Table S5: Homologous Proteins of T_3HNR from *Curvularia lunata* in *P. oryzae*.

6.0 References

---

1. N. J. Talbot, D. J. Ebbole and J. E. Hamer, *Plant Cell*, 1993, **5**, 1575–1590.
2. Y. Takano, Y. Kubo, K. Shimizu, K. Mise, T. Okuno and I. Furusawa, *Mol. Gen. Genet.*, 1995, **249**, 162–167.
3. A. L. Vagstad, E. A. Hill, J. W. Labonte and C. A. Townsend, *Chem. Biol.*, 2012, **19**, 1525–1534.
4. A. Watanabe, I. Fujii, H. F. Tsai, Y. C. Chang, K. J. Kwon-Chung and Y. Ebizuka, *FEMS Microbiol. Lett.*, 2000, **192**, 39–44.
5. M. H. Wheeler, *Trans. Br. Mycol. Soc.*, 1983, **81**, 29–36.
6. A. A. Bell and M. H. Wheeler, *Annu. Rev. Phytopathol.*, 1986, **24**, 411–451.
7. M. J. Butler and A. W. Day, *Can. J. Microbiol.*, 1998, **44**, 1115–1136.
8. W. A. Edens, T. Q. Goins, D. Dooley and J. M. Henson, *Appl. Environ. Microbiol.*, 1999, **65**, 3071–3074.
9. M. Nukina, *Agric. Biol. Chem*., 1987, **51**, 2625-262.
10. H. Tsai, M. H. Wheeler and Y. U. N. C. Chang, *J. Bacteriol*., 1999, **181**, 6469–6477.
11. M. Nakasako, T. Motoyama, Y. Kurahashi and I. Yamaguchi, *Biochemistry*, 1998, **37**, 9931–9939.
12. T. Lanišnik Rižner and M. H. Wheeler, *Can. J. Microbiol.*, 2003, **49**, 110–119.
13. D. I. Liao, G. S. Basarab, G. S. Gatenby, B. Valent and D. B. Jordan, *Structure*, 2001, **9**, 19–27.
14. J. Thompson, S. Fahnestock, L. Farrall, D.-I. Liao, B. Valent, and D. Jordan, *J. Biol. Chem.,* 2000, **275**, 34867–34872.