Trichoderma reesei Contains a Biosynthetic Gene Cluster That Encodes the Antifungal Agent Ilicicolin H

Mary L. Shenouda 1,2, Maria Ambilika 1 and Russell J. Cox 1,*

1 Institute for Organic Chemistry and Biomolekulares Wirkstoffzentrum (BMWZ), Schneiderberg 38, 30167 Hannover, Germany; mary.shenouda@oci.uni-hannover.de (M.L.S.); m.ambilika@gmail.com (M.A.)
2 Pharmacognosy Department, Faculty of Pharmacy, Alexandria University, Alexandria 21521, Egypt
* Correspondence: russell.cox@oci.uni-hannover.de

Abstract: The trili biosynthetic gene cluster (BGC) from the well-studied organism Trichoderma reesei was studied by heterologous expression in the fungal host Aspergillus oryzae. Coexpression of trilIA and trilIB produces two new acyl tetramic acids. Addition of the ring-expanding cytochrome P450 encoded by triliC then yields a known pyridone intermediate to ilicicolin H and a new chain-truncated shunt metabolite. Finally, addition of the intramolecular Diels-Alderase encoded by triliD affords a mixture of 8-epi ilicicolin H and ilicicolin H itself, showing that the T. reesei trili BGC encodes biosynthesis of this potent antifungal agent. Unexpected A. oryzae shunt pathways are responsible for the production of the new compounds, emphasising the role of fungal hosts in catalysing diversification reactions.

Keywords: heterologous expression; PKS-NRPS; ilicicolin H; shunt metabolite; Trichoderma reesei

1. Introduction

Trichoderma reesei (Hypocrea jecorina) is a filamentous fungus widely used for the industrial production of cellulase [1]. The organism can grow to high cell density and can grow robustly on a variety of low-quality substrates. It has been subject to a wide variety of biotechnological improvements since the discovery of the wild-type QM6A strain in the 1940s, and many molecular tools are available for its genetic manipulation. Many Trichoderma species have been used in the remediation of fungal diseases. For example, Foster and coworkers have shown that some Trichoderma spp. can effectively treat Armillaria mellea (honey fungus) that causes root rot in many tree species [2]. T. reesei is also well-known for the production of a number of secondary metabolites such as sorbicillinol 1 [3,4], and numerous multimeric derivatives such as bisvertinolone 2 [5].

Other members of the Trichoderma genus are well-known producers of many secondary metabolites [6]. In particular, compounds such as acyl tetramic acids (acyl 2-pyrollones) and acyl 2-pyridones, synthesised by hybrid polyketide synthase-nonribosomal peptide synthetases (PKS-NRPS) are commonly produced by various Trichoderma species. These include metabolites such as harzianic acid 3 (T. harzianum) [7,8], and deacetyl-18-deoxy cytochalasin H 4 [9] among the pyrollones; and trichodin A 5 [10] and harzianopyridone 6 [11,12] among the 2-pyridones (Figure 1). The biosynthesis of related pyrollones such as fusarin C 7 in Fusarium spp [13] and acurin A 8 in Aspergillus aculeatus, [14] and 2-pyridones such as tenellin 19 in Beauveria bassiana [15] and ilicicolin H 10 in Talaromyces variabilis and Nectria spp [16,17] are well-understood.

However, despite decades of study, T. reesei itself has not been reported to produce any PKS-NRPS compounds. Since we have a broad interest in fungal PKS-NRPS compounds and we recently made detailed studies on the biosynthesis of sorbicillinoids in T. reesei [3,4], we set out to determine if this organism can also produce PKS-NRPS compounds.
2. Materials and Methods

All chemicals and media ingredients used in this work were purchased from Duchefa Biochemie, Roth, VWR, Fisher scientific, Sigma Aldrich, abcr and Formedium. All media, buffers, solutions and antibiotics were prepared with double-distilled water (ddH$_2$O), where deionized water was further purified by GenPure Pro UV/UF Milipore device from Thermo scientific. All growth media and sterile solutions were autoclaved at 121 °C for 15 min using Systec VX150 or a classic Prestige Medical 2100 autoclave. Antibiotic solutions were sterilized using a sterile syringe filter (0.45 µm pore size, Roth). See ESI for details of all growth media, buffers and solutions, enzymes and antibiotics.

### 2.1. Microbiological Methods

Bacterial and fungal strains and all microbiological methods used in this work are summarized in the Electronic Supplementary Information (ESI).

### 2.2. Construction of Heterologous Expression Vectors

The genomic DNA (gDNA) of *T. reesei* QM6a Δtmus53 Δpyr4 [18] was used as a template to amplify genes for yeast homologous recombination (without removal of introns). The vector pEYA was used to assemble the PKS-NRPS (TRIREDRAFT_58285, *triliA*) gene (~12 kb) in four different DNA fragments of around 3 kb each using yeast homologous recombination. This was followed by LR recombination between the entry plasmid pEYA-*triliA* (MSIII139) and the expression vector pTYGS-arg to yield vector pTYGS-argB-triliA (MSIII144). The enoyl reductase gene (TRIREDRAFT_58289, *triliB*) was also amplified from the gDNA of *T. reesei* using primers with overhang to *P_{gpdA}* and *T_{gpdA}*. Using yeast homologous recombination, the final vector pTYGS-argB-triliA-triliB (MSIII152) was constructed which contains the PKS-NRPS (*triliA*)- and ER (*triliB*)-encoding genes from the specified cluster. A vector for the expression of the first three genes of the cluster; PKS/NRPS (TRIREDRAFT_58285, *triliA*), ER (TRIREDRAFT_58289, *triliB*), P450$_{RE}$ (TRIREDRAFT_58953, *triliC*) was constructed using yeast homologous recombination using gDNA of *T. reesei* QM6a Δtmus53 Δpyr4 as a template for the amplification of the genes. A vector for the expression of the first four genes of the cluster; PKS/NRPS (TRIREDRAFT_58285, *triliA*), ER (TRIREDRAFT_58289, *triliB*), P450$_{RE}$ (TRIREDRAFT_58953, *triliC*) and Diels-Alderase (*triliD*) was constructed via yeast homologous recombination using gDNA of *T. reesei* QM6a Δtmus53 Δpyr4 as template for the amplification of the genes.
2.3. Chemical Analysis

All the chemicals and materials were purchased from one of the following companies: Bio-Rad (München, Germany), New England Biolabs (Beverly, MA, USA), Roth (Karlsruhe, Germany), Sigma Aldrich (Steinheim, Germany), and Thermo Fisher Scientific (Waltham, MA, USA).

2.3.1. Liquid Chromatography–Mass Spectrometry (LC–MS)

Analytical LC–MS data of the organic extracts were obtained by either a Waters mass-directed autopurification system (Waters, Wilmslow, UK) including a Waters 2767 autosampler, a Waters 2545 pump and Phenomenex Kinetex column (2.6 µm, C₁₈, 100 Å, 4.6 mm × 100 mm; Phenomenex) equipped with a Phenomenex Security Guard precolumn (Luna, C₅, 300 Å). Detection was performed by a diode array detector from 210–600 nm (DAD; Waters 2998 or Waters 996), an electron light-scattering detector (ELSD; Waters 2424) and an electrospray ionisation mass detector in the range of 100–1000 m/z (Waters SQD-2). Gradient was run over 15 min starting at 10% acetonitrile/ 90% HPLC grade water (+ 0.05% formic acid) and ramping to 90% acetonitrile/ 10% HPLC grade water (+ 0.05% formic acid). Flowrate was 1 mL/min and 20 µL of the sample was injected. Data were displayed using the software MassLynx. The LC–MS program is summarized in the ESI.

Single compounds were purified from the raw organic extracts by a Waters mass-directed autopurification system. It comprises a Waters 2767 autosampler, a Waters 2545 binary gradient module system and a Phenomenex Kinetex Axia column (5 µm, C₁₈, 100 Å, 21.2 mm × 250 mm) equipped with a Phenomenex Security Guard precolumn (Luna, C₅, 300 Å). A water/acetonitrile gradient was run over 15 min with a flowrate of 20 mL/min and a post-column flow split of 100:1. The minority flow was applied for simultaneous analysis by a diode array detector (Waters 2998) in the range 210 to 1600 nm, an evaporative light-scattering detector (ELSD; Waters 2424) and a Waters SQD-2 mass detector, operating in ES+ and ES− modes between 100 and 1000 m/z. Selected peaks were collected into test tubes and solvent was evaporated under reduced pressure. Compounds were dissolved in methanol (1 mg/mL). High-Resolution Mass Spectrometry was performed on a Q-Tof Premier mass spectrometer (Waters) coupled to an Acquity UPLC-domain (Waters). Electron spray Ionisation (Esi) mass spectroscopy was measured in positive or negative mode depending on the compound.

2.3.2. Nuclear Magnetic Resonance (NMR) Analysis

Bruker Ascend 400 MHz, Bruker DRX 500 MHz or a Bruker Ascend 600 MHz Spectrometer (Bruker) were used for NMR measurements of the samples. Raw data were then analyzed using the software Bruker TopSpin 3.5. Chemical shifts are expressed in parts per million (ppm) in comparison to the Tetramethylsilane (TMS) standard and are referenced to the deuterated solvent.

2.3.3. Extraction of Fungal Cultures

For A. oryzae transformants, a small cell sample was dried by Büchner filtration or gravity filtration and used for gDNA analysis. Mycelia were ground using a hand blender and removed from the culture supernatant by Büchner filtration. Supernatant was acidified with 2 M HCL to pH 3–4 and extracted twice with ethyl acetate. Combined organic layers were dried over MgSO₄ and solvents were removed under vacuum. Organic residue was dissolved in methanol or acetonitrile to a concentration of 5–10 mg/mL (analytical) or 50 mg/mL (preparative) and filtered over glass wool.

3. Results

The genome of T. reesei QM6A, the wild-type isolate, was sequenced in 2008 [19]. We examined this genome sequence using a combination of automated AntiSMASH [20] and manual screening, with a particular focus on PKS and PKS-NRPS encoding genes. Bioinformatic analysis of all PKS and PKS-NRPS genes in T. reesei QM6a revealed the
presence of 11 PKS and two hybrid PKS-NRPS encoding genes (see ESI for details). Of these, only the two PKS genes responsible for the sorbicillins and related compounds have been identified and confirmed (TRIREDRAFT_73618 and 73621) [4,21,22]. The HR-PKS TRIREDRAFT_65116 shows a very high similarity to the recently investigated PKS from *Trichoderma virens*, which is encoded in the biosynthetic gene cluster (BGC) that directs the production of virensols and trichoxide [23]. However, the other PKS and PKS-NRPS BGCs could not be linked to the production of any specific compound.

Although the *T. reesei* genome contains two hybrid PKS-NRPS encoding genes, no PKS-NRPS secondary metabolites have been reported from this fungus and these BGCs are likely to be silent under laboratory conditions. The two PKS-NRPS themselves show similarity (>60%) to proteins involved in fusarin C biosynthesis in *Fusarium moniliforme* [13] and illicicolin H biosynthesis in *Penicillium variabile* (= *Talaromyces variabilis*) [16]. Illicicolin H is an interesting antifungal agent that targets the cytochrome bc1 respiration complex in the 3–5 nM range [24], and it is conceivable that illicicolin H production may be responsible for the observed antifungal effects of *Trichoderma* species in general [2] and *T. reesei* in particular [25]. We therefore selected the putative *T. reesei* illicicolin H BGC (*trili*) for further investigation.

Based on the NCBI prediction, the *trili* BGC (Figure 2) consists of a hybrid PKS/NRPS (*triliA*), an ER (*triliB*), and a putative cytochrome P450 (*triliC*). Reannotation of the cluster by FungiSMASH and Clinker [26] and Artemis comparison to a closely related BGC in *T. citrinoviride* resulted in the identification of a putative S-adenosylmethionine (SAM)-dependent Diels-Alderase in the cluster (*triliD*), that shows high similarity to *iccD* from the illicicolin-H BGC in *Talaromyces variabilis* [16]. Finally, *triliE* encodes a putative TIM-like epimerase homologous to IccE, known to epimerise epi-illicicolin H in *T. variabilis*. Multigene analysis using cblaster [27] identified highly similar *ili* BGCs in other strains of *T. reesei* (RUT C-30) and other *Trichoderma* species, including *T. parareesei*, *T. virens* Gv29-8, *T. citrinoviride* TUCIM6016 and *T. longibrachiatum* ATCC 18648 (see ESI).

In an initial heterologous expression experiment, *triliA* and *triliB* were cloned into the vector pTYGS-argB [28] using rapid recombination of genome-derived PCR fragments in yeast and *in vitro* (Gateway) recombination. The PKS-NRPS-encoding gene *triliA* was placed downstream of the inducible *A. oryzae amyB* promoter (*PamyB*), while *triliB* was placed under the control of the *Aspergillus gpdA* promoter (*PgpDA*). The resulting vector pTYGS-argB-*triliA-triliB* was transformed into the fungal host *Aspergillus oryzae* NSAR1 [29]. Comparison of the organic extracts of transformants with those of untransformed *A. oryzae*.

![Figure 2](image-url)
NSAR1 revealed the production of new compounds by LC–MS analysis eluting at 6.7 min, 8.0 min and 11.0 min (Figure 3).

![LC-MS traces](image)

**Figure 3.** LC–MS traces (DAD 210–600 nm) of transformed *A. oryzae* with *illiA* and *illiB* (A), the extract of the wild-type *A. oryzae* NSAR1 (B–E), extracts of four transformants of *A. oryzae* NSAR1 with *triliA* and *triliB* showing the production of different PKS-NRPS-related compounds at RT 6.7, 8.0 and 11.0 min.

Compound 11 (RT 6.7 min, 30 mg) was isolated by preparative LC–MS, and the structure was elucidated using 1D and 2D NMR. HRMS analysis of 11 confirmed a molecular formula of C$_{23}$H$_{28}$NO$_6$ ([M+H]$^+$ calculated 414.1917, found 414.1917). Compound 12 (RT 8.0 min, 4 mg) was isolated by preparative LC–MS and the structure was elucidated using NMR. HRMS analysis of 12 confirmed a molecular formula of C$_{27}$H$_{32}$NO$_6$ ([M+H]$^+$ calculated 466.2230, found 466.2237).

NMR analysis of these compounds (see ESI for full details) showed that 11 is a linear acyl tetramic acid with a C$_{12}$ sidechain terminating in an unusual (at this position) carboxylic acid. Minor compound 12 was shown to be a related acyl tetramic acid featuring a decalin, with an unusual 3-propenoic acid substituent. Although the minor compound 13 at 11.0 min could not be fully identified by NMR, mass spectroscopic and UV analysis suggested that it is likely to be a third related tetramic acid with a C$_{16}$ sidechain, first reported by Tang and coworkers during biosynthetic studies of illicolin H (Scheme 1) [16].
In the next round of experiments, *triliC*, encoding a cytochrome P450 oxidase, was added to the *A. oryzae* strains, driven by *P_adh*. *TriliC* is related to ring-expanding cytochrome P450 oxidases first characterised from the tenellin pathway [15]. Coexpression of *triliA*, *triliB* and *triliC* resulted in production of two new compounds in comparison to the previous transformants and the untransformed *A. oryzae* (Figure 4), eluting at 6.7 (14), and 10.8 (15) min. Full NMR analysis of the RT 6.7 min compound showed it to be the pyridone homolog of 11. Similarly, the 10.8 min compound was shown to be the pyridone homolog of 13.

**Scheme 1.** Overall predicted pathway showing production of new compounds 11, 12 and 14 in *A. oryzae* NSAR1.
Similarly, the 10.8 min compound was shown to be the pyridone homolog of 11. Similarly, the 10.8 min compound was shown to be the pyridone homolog of 11.

Finally, a gene homologous to Diels-Alderase iccD (triliD) was added to the expression system downstream of $P_{eno}$ and transformed into $A. oryzae$. This experiment again produced 11 (RT 6.7 min). Additionally, two new peaks in comparison to the previous transformants and the untransformed $A. oryzae$ were also observed by LC–MS (Figure 5) at RT 10.76 and 11.0 min. The minor compound at 11.0 min was again assigned as 13 after analysis of partial data. NMR and HRMS analysis (see ESI for full details) showed the peak at 10.76 min consists of an almost coeleuting mixture of the known 8-epi ilicicolin H 16 and ilicicolin H 10 itself.
Figure 5. LC–MS traces (DAD 210–600 nm) of transformed *A. oryzae* with *illiA*, *illiB*, *illiC* and *illiD*. (A), the extract of the wild-type *A. oryzae* NSAR1; (B–C), Extracts of two representative transformants of *A. oryzae* with *illiA*, *illiB*, *illiC* and *illiD*.

4. Discussion

PKS-NRPS compounds have been reported before from the genus *Trichoderma*, but *T. reesei* QM6A itself has not been reported to produce these compounds. Genomic analysis, however, reveals two typical fungal PKS-NRPS BGCs. Heterologous expression experiments reveal that the *trili* BGC is fully functional, but apparently silent in *T. reesei* QM6A under laboratory conditions. Initial expression of the PKS-NRPS (*triliA*) and trans-ER (*triliB*) genes in *A. oryzae* led to production of two new acyl tetramic acids (11 and 12) and a third compound 13 that is likely to be a precursor of illicicolin H, recently described by Tang and coworkers [16] from *Nectria* sp. B13 and *T. variabilis* and Gao and coworkers [17] in *Neonectria* sp. DH2. Decalin 12 appears to have been oxidised to a carboxylic acid at its terminal polyketide methyl group. Addition of the ring-expanding P450 oxidase then produces the corresponding 2-pyridones 14 and 15. Compound 15 is identical to an illicicolin H precursor previously identified by Tang and coworkers [16], but 14 is new. Finally, addition of the Diels-Alderase encoded by *triliD* produces a mixture of 8-epi illicicolin H 16 and illicicolin H 10 itself.

The *T. variabilis icc* [16] and *Neonectra* DH2 *ili* [17] BGCs were previously investigated by expression in *A. nidulans*. Our results are highly similar to those obtained from these studies, except that *A. oryzae* as a host appears to catalyse some unexpected steps. For example, in *A. oryzae* the initial tetramic acid precursor 13 appears to be oxidatively degraded to remove the terminal four polyketide carbons and leaves a carboxylic acid in the structure of 11. Previous heterologous expression studies on *A. oryzae* also indicate that
this host has a propensity for the oxygenation of polyenes [30,31] and oxidative cleavage of olefins [32]. Likewise, oxygenation of the terminal methyl of 12 to a carboxylic acid is newly observed in A. oryzae. This is similar to the BueE-catalysed oxidation recently observed by Piggott and coworkers in the fungal polyketide decalin burnettiene A in Aspergillus burnetti [33]. It also appears that a spontaneous (or A. oryzae-catalysed) Diels–Alder reaction can occur to produce a decalin that is then oxidised to a carboxylic acid at the terminal methyl to generate new compound 12. In previous studies on ilicicolin H biosynthesis, using A. nidulans as the host, a specific Diels–Alder catalyst was required for this step. We also observed the chain-truncated pyridone 14—again a new natural product. In A. nidulans it appears that 8-epi ilicicolin H 16 is a stable compound, requiring expression of iccE for the conversion to ilicicolin H. However, in A. oryzae, observable amounts of ilicicolin H 10 are produced in the absence of this epimerase catalyst.

Our results therefore illustrate that T. reesei QM6A has a fully functional PKS-NRPS BGC that encodes the biosynthesis of ilicicolin H, a potent antifungal agent. The ability of T. reesei QM6A to make this compound has never before been observed [34], despite nearly 70 years of laboratory-based investigations. It is likely that this BGC can be activated under unknown environmental conditions in its native habitat and may contribute to its utility as an agricultural antifungal agent, but it appears to be silent under laboratory conditions. Finally, our results also show that different fungal heterologous hosts can introduce unexpected shunt pathways which can diversify known secondary metabolites and produce new-to-nature compounds.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7121034/s1. All experimental details including growth media, buffers, enzyme and antibiotic solutions, bacterial and fungal strains, vectors and oligonucleotides, bioinformatic procedures and results, structure elucidation details, tabulated NMR data, NMR spectra, and HRMS data.

Author Contributions: M.L.S. and R.J.C. designed the study. Experimental work was performed by M.L.S., assisted by M.A. The paper was written by R.J.C. and M.L.S., R.J.C. was responsible for providing physical resources. Funding was gained by M.L.S. (DAAD GERLS) and R.J.C. All authors have read and agreed to the published version of the manuscript.

Funding: M.L.S. thanks the German Academic Exchange Service (DAAD) and the Egyptian Ministry of Higher Education and Scientific Research (MHESR) for funding (GERLS programme, 2017, 57311832). This work was supported by DFG [CO 1328/2-1, INST 187/621-1, INST 187/686-1]]. The APC was funded by the Open Access Fund of the Leibniz Universität Hannover.

Acknowledgments: The authors would like to thank Eric Kuhnert and Kevin Becker for the helpful discussions and Astrid Mach-Aigner for donating T. reesei strain.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

References
1. Beier, S.; Hinterdobler, W.; Monroy, A.A.; Bazafkan, H.; Schmoll, M. The Kinase USK1 Regulates Cellulase Gene Expression and Secondary Metabolite Biosynthesis in Trichoderma reesei. Front. Microbiol. 2020, 11, 974. [CrossRef] [PubMed]
2. Rees, H.J.; Bashir, N.; Drakulic, J.; Cromey, M.G.; Bailey, A.M.; Foster, G.D. Identification of native endophytic Trichoderma spp. for investigation of in vitro antagonism towards Armillaria mellea using synthetic- and plant-based substrates. J. Appl. Microbiol. 2021, 131, 392–403. [CrossRef] [PubMed]
3. Al Fahad, A.; Abood, A.; Fisch, K.M.; Osipow, A.; Davison, J.; Avramović, M.; Butts, C.P.; Piel, J.; Simpson, T.J.; Cox, R.J. Oxidative dearomatisation: The key step of sorbicillinoid biosynthesis. Chem. Sci. 2014, 5, 523–527. [CrossRef] [PubMed]
4. Kahlert, L.; Bassiony, E.F.; Cox, R.; Skellam, E.J. Diels–Alder Reactions during the Biosynthesis of Sorbicillinoids. Angew. Chem. Int. Ed. 2020, 59, 5816–5822. [CrossRef]
5. Harned, A.M.; Volp, K.A. The sorbicillinoid family of natural products: Isolation, biosynthesis, and synthetic studies. Nat. Prod. Rep. 2011, 28, 1790–1810. [CrossRef]
6. Shenouda, M.L.; Cox, R.J. Molecular methods unravel the biosynthetic potential of Trichoderma species. RSC Adv. 2021, 11, 3622–3635. [CrossRef]
7. Sawa, R.; Mori, Y.; Iinuma, H.; Naganawa, H.; Hamada, M.; Yoshida, S.; Furutani, H.; Kajimura, Y.; Fuwa, T.; Takeuchi, T. Harzianic acid, a new antimicrobial antibiotic from a fungus. J. Antimi. 1994, 47, 731–732. [CrossRef]

8. Xie, L.; Zhang, X.; Cheng, W.; Zhang, Z.; Zhou, J.; Chen, M.; Tang, Y. Harzianic Acid from Trichoderma afroharzianum Is a Natural Product Inhibitor of Acetohydroxyacid Synthase. J. Am. Chem. Soc. 2021, 143, 9575–9584. [CrossRef]

9. Chen, H.; Daletos, G.; Okuye, F.; Lai, D.; Dai, H.; Proksch, P. A New Cytoxic Cytochalasin from the Endophytic Fungus Trichoderma harzianum. Natl. Commun. 2015, 10, 585–587. [CrossRef]

10. Wu, B.; Oesker, V.; Wiese, J.; Schmaljohann, R.; Imhoff, J.F. Two New Antibiotic Pyridones Produced by a Marine Fungus, Trichoderma sp. Strain MI106. Mar. Drugs 2014, 12, 1208–1219. [CrossRef]

11. Dickinson, J.M.; Hanson, J.R.; Hitchcock, P.B.; Claydon, N. Structure and biosynthesis of harzianopyridione, an antifungal metabolite of Trichoderma harzianum. J. Chem. Soc. Perkin Trans. 1 1989, 1, 1885–1887. [CrossRef]

12. Bat-Erdene, U.; Kanayama, D.; Tan, D.; Turner, W.C.; Houk, K.N.; Ohashi, M.; Tang, Y. Iterative Catalysis in the Biosynthesis of Mitochondrial Complex II Inhibitors Harzianopyridone and Atpenin B. J. Am. Chem. Soc. 2020, 142, 8550–8554. [CrossRef]

13. Song, Z.; Cox, R.J.; Lazarus, C.M.; Simpson, T.J. Fusarin C Biosynthesis in Fusarium moniliforme and Fusarium venenatum. ChemBioChem 2004, 5, 1196–1203. [CrossRef]

14. Wolff, P.B.; Nielsen, M.L.; Slot, J.C.; Andersen, L.N.; Petersen, L.M.; Isbrandt, T.; Holm, D.K.; Mortensen, U.H.; Nødvig, C.S.; Larsen, T.O.; et al. Acurin A, a novel hybrid compound, biosynthesized by individually translated PKS- and NRPS-encoding genes in Aspergillus aculeatus. Fungal Genet. Biol. 2020, 139, 103378. [CrossRef]

15. Halo, L.M.; Heneghan, M.N.; Yakasai, A.A.; Song, Z.; Williams, K.; Bailey, A.; Cox, R.; Lazarus, C.M.; Simpson, T.J. Late Stage Oxidations in the 2-Pyridone Terellin in the Entomopathogenic Fungus Beauveria bassiana. J. Am. Chem. Soc. 2008, 130, 17998–17996. [CrossRef]

16. Zhang, Z.; Jamieson, C.S.; Zhao, Y.-L.; Li, D.; Ohashi, M.; Houk, K.N.; Tang, Y. Enzyme-Catalyzed Inverse-Electron Demand Diels–Alder Reaction in the Biosynthesis of Antifungal Ilicicolin H. J. Am. Chem. Soc. 2019, 141, 5659–5663. [CrossRef]

17. Lin, X.; Yuan, S.; Chen, S.; Chen, B.; Xu, H.; Liu, L.; Li, H.; Gao, Z. Heterologous Expression of Ilicicolin H Biosynthetic Gene Cluster and Production of a New Potent Antifungal Reagent, Ilicicolin J. Molecules 2019, 24, 2267. [CrossRef]

18. Derntl, C.; Kiesenhofer, D.P.; Mach, A.; Mach-Aigner, A.R. Novel Strategies for Genomic Manipulation of Trichoderma reesi with the Purpose of Strain Engineering. Appl. Environ. Microbiol. 2015, 81, 6314–6323. [CrossRef]

19. Chia-Ling, C.; Huang, C.-H.; Chen, C.-L.; Tung, S.-Y.; Wang, T.-F. Trichoderma reesei complete genome sequence, repeat-induced point mutation, and partitioning of CAZyme gene clusters. Biotechnol. Biofuels 2012, 5, 239, 2473–2475. [CrossRef]

20. Blin, K.; Shaw, S.; Kloosterman, A.M.; Charlop-Powers, Z.; van Wezel, G.P.; Medema, M.H.; Weber, T. antiSMASH 6.0: Improving cluster detection and comparison capabilities. Nucleic Acids Res. 2021, 49, W29–W35. [CrossRef]

21. Derntl, C.; Rassinger, A.; Srebetonik, E.; Mach, R.L.; Mach-Aigner, A.R. Identification of the Main Regulator Responsible for Synthesis of the Typical Yellow Pigment Produced by Trichoderma reesi. Appl. Environ. Microbiol. 2016, 82, 6247–6257. [CrossRef]

22. Derntl, C.; Guzman-Chavez, F.; Mello-De-Sousa, T.M.; Busse, H.-J.; Driessen, A.J.M.; Mach, R.; Mach-Aigner, A.R. In Vivo Study of the Sorbicillinoid Gene Cluster in Trichoderma reesi. Front. Microbiol. 2017, 8, 2037. [CrossRef]

23. Liu, L.; Tang, M.-C.; Tang, Y. Fungal Highly Reducing Polyketide Synthases: Bioformsalicylaldehyde. The Chemical Registry of Acidic and Neutral Natural Products. J. Am. Chem. Soc. 2019, 141, 19538–19541. [CrossRef]

24. Singh, S.B.; Liu, W.; Li, X.; Chen, T.; Shafiee, A.; Card, D.; Abruzzo, G.; Flattery, A.; Gill, C.; Thompson, J.R.; et al. Antifungal Spectrum, In Vivo Efficacy, and Structure–Activity Relationship of Ilicicolin H. ACS Med. Chem. Lett. 2012, 3, 814–817. [CrossRef]

25. Damodaran, T.; Rajan, S.; Muthukumar, M.; Gopal, R.; Yadav, K.; Kumar, S.; Ahmad, I.; Kumari, N.; Mishra, V.K.; Jha, S.K. Biological Management of Banana Fusarium Wilt Caused by Fusarium Oxysporum f. sp. cubense Tropical Race 4 Using Antagonistic Fungal Isolate CSR-T-3 (Trichoderma Reesei). Front. Microbiol. 2020, 11, 595845. [CrossRef]

26. Gilchrist, C.L.M.; Chooi, Y.-H. Clinker & clustermap.js: Automatic generation of gene cluster comparison figures. Bioinformatics 2021, 37, 2473–2475. [CrossRef]

27. Gilchrist, C.L.M.; Booth, T.J.; van Wersch, B.; van Grieken, L.; Medema, M.H.; Chooi, Y.-H. clbaster: A remote search tool for rapid identification and visualization of homologous gene clusters. Bioinform. Adv. 2021, 1, vbab016. [CrossRef]

28. Pahirulzaman, K.A.K.; Williams, K.; Lazarus, C.M. A Toolkit for Heterologous Expression of Metabolic Pathways in Aspergillus oryzae. Methods Enzymol. 2012, 517, 241–260. [CrossRef]

29. Jin, F.J.; Maruyama, J.I.; Juvvadi, P.; Arioka, M.; Kitamoto, K. Development of a novel quadruple auxotrophic host transformation system by argBgene disruption usingiggA gene and exploiting adenosine auxotrophy in Aspergillus oryzae. FEMS Microbiol. Lett. 2004, 239, 79–85. [CrossRef]

30. Song, Z.; Baker, W.; Marshall, J.W.; Yakasai, A.A.; Khalid, R.M.; Collemare, J.; Skellam, E.; Tharreau, D.; Lebrun, M.-H.; Lazarus, C.M.; et al. Heterologous expression of the avirulence gene ACE1 from the fungal rice pathogen Magnaporthe oryzae. Chem. Sci. 2015, 6, 4837–4845. [CrossRef]

31. Heneghan, M.N.; Yakasai, A.A.; Williams, K.; Kadir, K.A.; Wasil, Z.; Baker, W.; Fisch, K.M.; Bailey, A.M.; Simpson, T.J.; Cox, R.J.; et al. The programming role of trans-acting enoyl reductases during the biosynthesis of highly reduced fungal polyketides. Chem. Sci. 2011, 2, 972–979. [CrossRef]
33. Roux, I.; Bowles, S.; Kalaitzis, J.A.; Vuong, D.; Lacey, E.; Chooi, Y.-H.; Piggott, A.M. Characterisation and Heterologous Biosynthesis of Burnettiene A, a New Polyene-Decalin Polyketide from Aspergillus Burnettii. *Org. Biomol. Chem.* **2021**, *19*, 9506–9513. [CrossRef] [PubMed]

34. Rush, T.A.; Shrestha, H.K.; Meena, M.G.; Spangler, M.K.; Ellis, J.C.; Labbé, J.L.; Abraham, P.E. Bioprospecting Trichoderma: A Systematic Roadmap to Screen Genomes and Natural Products for Biocontrol Applications. *Front. Fungal Biol.* **2021**, *2*, 716511. [CrossRef]