Combination Strategy of Genetic Dereplication and Manipulation of Epigenetic Regulators Reveals a Novel Compound from Plant Endophytic Fungus

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Article

1. Introduction

Filamentous fungi produce clinically important secondary metabolites (SMs), including many natural products developed into pharmaceutical drugs. The biosynthesis genes for these SMs are usually clustered in a chromosome, and are called biosynthetic gene clusters (BGCs) [1]. Under laboratory cultural conditions, most of BGCs in silence are unexpressed or little expressed. In recent years, many strategies have been developed to effectively activate the silent expressed BGCs for the discovery of new natural products in filamentous fungi, including heterologous expression, promoter engineering, genetic dereplication, modulations of transcription factor, global regulator, epigenetic regulator, and combinational strategies [2,3].

Genetic dereplication is a powerful approach to discover novel compounds from unknown biosynthesis pathways. Eliminating major SMs is particularly effective to increase the odds of detecting minor SMs as well as being useful for the heterologous expression of biosynthetic genes from other fungal species [4]. Deletion of eight of the most highly
expressed secondary metabolites gene clusters in *Aspergillus nidulans* resulted in the discovery of aspercryptin [4]. A genetic dereplication approach led to discovery of two novel polycyclic lactones, three new sesquiterpenes, and the known fusidilactone A from *Trichoderma hypoxylon* [5,6]. The biosynthesis gene cluster of atranorin in lichens was identified through genetic dereplication in *Cladonia* and heterologous expression in *Ascochyta rabiei* [7]. A metabolic shunting strategy by deleting the key gene for rubratoxins (with the high yield) biosynthesis combining with the optimization of culture conditions successfully activated multiple silent genes encoding for other polyketide synthases (PKSs), and led to the discovery of 23 new compounds in *Penicillium dangeardii* [8].

Epigenetic regulation also has been proved as an efficient activation approach to access chemical diversity and discover new natural products in filamentous fungi. Deletion or overexpression of histone-modifying enzyme-genes can trigger the expression of the BGCs located in the chromatin [2]. These modifying enzymes catalyze different modifications of histone, including acetylases, methyltransferases, demethylases, deacetylases, phosphatase, and ubiquitin enzyme, respectively [3]. Modulation of histone acetylation is typically associated with transcriptional activation in different fungal species. Deletion of the histone deacetylase-encoding gene *hdaA* could positively and negatively regulate the production of secondary metabolites in *Aspergillus fumigatus* [9], *Fusarium fujikuroi* [10] and *Pestalotiopsis microspora* [11]. Deletion of an *hdaA* homolog increased production of penicillin and sterigmatocystin in *A. nidulans* [12], melanin in *Magnaporthe oryzae* [13], trichothecenes in *Fusarium asiaticum* [13], pigment in *Aspergillus niger* [14]. Meleagrin/roquefortine alkaid production was upregulated by 84.8-fold in the *Penicillium chrysogenum ΔhdaA* strain [15]. Disruption of *hdaA* resulted in production of four novel natural products as well as deviant growth and physiologic function in *Calcarisporium arbuscular* [16], and production of unknown metabolites in *P. chrysogenum* [17]. In addition, histone methylation through CclA also takes part in the regulation of SMs in fungi. Deletion of *cclA* in *A. nidulans* activated the production of two polyketides F9775A and B as well as emodin analogues [18]. Deletion of a *cclA* homolog resulted in increase of several SMs in *A. fumigatus* [19] and *Aspergillus oryzae* [20], and production of new representatives in *Colletotrichum higginsianum* [21], respectively. Deletion of the *cclA* homolog that encodes for CCL1 led to the increase or loss of various SM production in *Fusarium graminearum* [22] and *F. fujikuroi* [23]. Furthermore, a combinational strategy based on epigenetic regulation provides ways to explore the influence on the secondary metabolism in filamentous fungi [3]. For instance, histone deacetylases HosA and HdaA affect the phenotype and transcriptomic and metabolic profiles in *A. niger*, and especially the yield of fumonisin was obviously reduced in the *A. niger ΔhosA ΔhdaA* mutant [14]. Deletion of both *cclA* and *sumO* (the ubiquitin-like modifier) led to obvious changes of the colony and a medium color, which reflects an impaired secondary metabolism in *A. nidulans* [24].

The plant endophytic fungi from *Pestalotiopsis* genus is well-studied for their SM production [25–27]. *Pestalotiopsis fici* has been reported to produce about 100 distinctive compounds by traditional isolation and genetic manipulation strategies [28–32]. Disruption of *cclA* and *hdaA* led to the identification of 15 new structures [29]. The diphenyl ether pestheic acid and its analogues are the major SMs produced in *Pestalotiopsis microspore*. Disruption of histone acetylation is typically associated with transcriptional activation in different fungal species. Deletion of the *cclA* homolog increased production of *P. fici* [28,33]. Here, we constructed the single knockout mutant of *PfptaA* (*PFICI_10824*), double knockout mutants of *PfptaA* and *PfclA* (*PFICI_05127*), and *PfptaA* and *PfhdaA*
Using the previously described transformation method, individually [29] (Figure 1 and Table S1). For deletion of PfptaA, we constructed a plasmid containing the upstream and downstream homologous arms of PfptaA with the resistance gene of G418 antibiotic. The fragments of deletion cassette of PfptaA were amplified by PCR, and the plasmid construct was transformed into the P. fici wild type (WT) strain. Then PfptaA deletion mutants were verified by diagnostic PCR analysis using designated primers (Figure 1a,b and Table S2). Subsequently, the PfptaA deletion plasmid construct was transformed into strains of TYXW7 (ΔPfcclA) and TYXW8 (ΔPfhdA). The genomic DNAs of transformants were extracted and correct mutants were verified by diagnostic PCR using designated primers (Figure 1c,d; Figure S1 and Table S2).

![Figure 1](image)

**Figure 1.** Generation of gene deletion strains. (a) Schematic illustration for disruption of PfptaA gene in P. fici; (b) PCR verification for PfptaA gene deletion in P. fici wild-type strain. The primer pairs of P1F/R, P2F/R, P3F/R, were designed for screening and the products should be 850, 1850, 1780 bp, respectively; (c) PCR verification for PfhdA gene deletion in ΔPfptaA host; (d) PCR verification for PfcclA gene deletion in ΔPfptaA host.

### 2.2. Assessment of Secondary Metabolites

To evaluate the modulation of secondary metabolites production via the deletion of the target genes, the strains were grown on a rice-based medium and the culture extracts were analyzed by HPLC and LC-MS. The results were changes of SM production profile in ΔPfpta AΔPfcclA and ΔPfpta AΔPfhdA mutants, compared with WT and ΔPfpta strains (Figure 2a). A new peak was obviously detected along with 11 known SMs. Subsequently, the new peak was further isolated and purified to obtain pure compound 1, produced in ΔPfpta AΔPfhdA mutant (Figure 2b). Compounds 7 and 12 produced in WT were disappeared in both ΔPfpta AΔPfcclA and ΔPfpta AΔPfhdA mutants. Compound 10 produced in WT was obviously decreased in both of the double-deletion strains. Compounds 2, 5, 8, and 9 were increased in the deletion strains, in comparison with WT. Compounds 3 and 4 were novel peaks in both of the double deletion strains compared with WT, and compound 4 also was produced in ΔPfpta mutant. The known SMs identified in P. fici wild type, ΔPfcsnE (the fifth subunit of COP9 signalosome, CsnE), and ΔPfhdA strains are pestaloficilic M (2), pestaloficin D (3), ficiolide J (4), asperpentyn (5), ficiolide C (6), isosulochrin (7), chloropupukeanalin (8), ficiolide K (9), pestaloficilic J (10), hydroxyisoseiridin (11), and pestheic acid (12) [29,33–38] (Figure 2c). All of these known structures were elucidated by comprehensive analysis with spectroscopy and HR-ESI-MS (Figures S8–S18).
The key HMBC correlation of H-1 of 13-enynes-based cyclohexanoid terpenoids. Further analysis of 1D NMR data provided the evidence that backbone of 1 is identical to siccayne which was first isolated as an antibiotic from the deuteromycete Helminthosporium siccans in 1981 [39]. The remaining NMR signals and unsaturation revealed that the hexose moiety is proposed. H-1/C-1′′ and H-5′′/C-1′′ HMBC correlations established that the hexose moiety underwent ring closure. The key HMBC correlation of H-1′′ with C-2 allowed us to assemble the intact planar structure by ether bond between the siccayne and hexose moiety (Figures 2b and S2–S6 and Table 1). Unfortunately, due to the lower content of 1, the configuration of hexose moiety remained unclear. Finally, compound 1 was determined to be a new structure and named pestaloficiol X. Glycosylation of small molecular plays a significant role in drug discovery and development [40]. As the precursor of 1, siccayne exhibited moderate antibiotic activity of Gram-positive bacteria and some fungal strains [39], and cytotoxic activity against multiple human
cancer cell lines [41]. The glycosylated siccayne such as 1 was speculated to improve the solubility in water and druggability.

Table 1. $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR spectroscopic data for 1 in DMSO-$d_6$.

| Position | $\delta_{C}$, Type | $\delta_{H}$, Multi., J in Hz | HMBC Correlation | $^1$H-$^1$H COSY |
|----------|------------------|---------------------------|-----------------|-----------------|
| 1        | 113.0 C          | -                         | -               | -               |
| 2        | 150.5 C          | -                         | -               | -               |
| 3        | 117.0 CH         | 6.99, d, 9.8              | C-1’, 1, 2, 5   | H-4             |
| 4        | 116.8 CH         | 6.71, m, overlap          | C-2, 6         | H-3             |
| 5        | 151.7 C          | -                         | -               | -               |
| 6        | 118.2 CH         | 6.72, m, overlap          | C-1’, 4, 5     | -               |
| 1’       | 85.3 C           | -                         | -               | -               |
| 2’       | 94.1 C           | -                         | -               | -               |
| 3’       | 126.7 C          | -                         | -               | -               |
| 4’       | 122.1 CH$_2$     | 5.38, s                   | C-2’’, 3’, 5’   | H-5’            |
| 5’       | 23.2 CH$_3$      | 1.93, s                   | C-2’, 3’, 4’   | H-4’            |
| 1’’      | 100.7 CH         | 4.81, d, 7.2              | -               | H-2’’           |
| 2’’      | 73.9 CH          | 3.26–3.22, overlap        | C-1’’, 4’’     | H-1’’           |
| 3’’      | 76.9 CH          | 3.26–3.12, overlap        | C-4’’          | -               |
| 4’’      | 70.1 CH          | 3.26–3.22, overlap        | C-5’’          | -               |
| 5’’      | 77.0 CH          | 3.26–3.12, overlap        | C-4’’          | -               |
| 6’’      | 60.7 CH$_2$      | 3.51–3.16, overlap        | C-5’’          | -               |
| -OH      |                 | 9.39, brd                 | -               | -               |
| -OH      |                 | 4.53, brs                 | -               | -               |
| -OH      |                 | 5.07, brs                 | -               | -               |
| -OH      |                 | 7.40, brs                 | -               | -               |
| -OH      |                 | 8.42, brs                 | -               | -               |

2.4. Proposed Biosynthesis Pathway of 1 in P. fici

Alkyne is a typical active group in many natural products with antitumor and anti-HIV activities. There have been several analogues of 1 with alkynyl pattern isolated in P. fici [29,33], Biscogniauxia sp. [42], Eutypa lata [43] and Aspergillus sp. [44] (Figure 3a). The biosynthesis gene cluster iac for iso-A82775C has been identified in P. fici. While the prenyltransferase IacE is responsible for the modification of isopentenyl, the mechanism of alkyne formation in the biosynthetic pathway was not elucidated in the work [45]. Inspired by this research, the bis gene cluster for biscognienyne B biosynthesis was identified in Biscogniauxia sp., in which cytochrome P450 enzyme (BisI) is confirmed to catalyze the alkynylation of the prenyl chain [46]. Surprisingly, there was no homologous gene of bisI in iac gene cluster in P. fici. In addition, the oxidoreductase gene iacJ in the iac cluster was unrelated to the formation of alkyne [45]. Simultaneously, another P450 monooxygenase (AtyI) was verified to catalyze dehydrogenation of the prenyl chain and to yield an alkene moiety in compound asperpentyn in Aspergillus sp. [47]. The cytochrome P450 gene Pfici_01577 located outside of iac gene cluster in P. fici was found by BlastP analysis to be the cytochrome P450 homologous protein of both BisI and Atyl with identity and similarity of both 89%/79% and 78%/87%, respectively (Figure 3b) [46,47]. So, there is an obvious difference in the biosynthetic gene clusters in different fungal species that produced siccayne and its analogues. In addition, glycosylation of natural products is catalyzed by glycosyltransferases (GTs). A phenolic GT MhGT1 identified in Mucor hiemalis exhibited...
broad substrate scope and regio- and stereospecificy [48]. The several O-GTs predicted in \textit{P. fici} were not within the gene clusters and distributed throughout the genome [40]. The biosynthesis of 1 was proposed according to the above-mentioned studies. Compound 1 was synthesized via the biosynthesis pathway of iac gene cluster along with a putative cytochrome P450 enzyme (Pfici_01577), a putative hydroxylase (Pfici_01576), and an unknown glycosyltransferase in \textit{P. fici}. The proposed biosynthetic pathways of pestaloficiol X (1) in \textit{P. fici} was shown in Figure 3c.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{The structure analogues with alkynyl of 1 and biosynthesis of 1. (a) The structure analogues of 1 produced in filamentous fungi; (b) the gene clusters related to biosynthesis of siccayne and 1 in filamentous fungi; (c) proposed biosynthetic pathway for 1 in \textit{P. fici}.}
\end{figure}

2.5. Assessment of Conidia Development in the Mutant Strains

Deletion of the cclA can observably decrease the amount of asexual spores and block the production of mature fruiting bodies and sexual development in \textit{A. nidulans} [24]. To examine the effect on the morphology and conidia development, the strains of \textit{P. fici} WT, \textit{ΔPfptaA}, \textit{ΔPfclA}, \textit{ΔPfhdaA}, \textit{ΔPfptaA ΔPfclA}, and \textit{ΔPfptaA ΔPfhdaA} were cultivated on Potato Dextrose Agar (PDA) plate. The differences in morphology between the mutants and WT strain were shown, respectively (Figure 4a), and the differences in conidia number among all of the strains were analyzed (Figure 4b). In the \textit{ΔPfptaA} mutant, the conidia number increased about 10-fold compared with \textit{P. fici} WT. Both deletion of PfclA and PfhdaA led to no obvious change in conidia number compared with \textit{P. fici} WT. This suggested that
PfptaA is involved in the conidia development and formation, and PfcclA and PfhdaA has little effect on conidia development and formation. Furthermore, deletion of PfcclA and PfhdaA in ΔPfptaA mutant had no differential effect on the conidia number compared with ΔPfcclA and ΔPfhdaA mutants, respectively, but both double mutant strains had decreased numbers of conidia in comparison with ΔPfptaA mutant. The results indicated that deletion of PfcclA or PfhdaA neutralises the enhancement of conidia formation in ΔPfptaA host, suggesting interconnected regulatory network among these genes and products in conidia development and formation.

![Figure 4](image)

**Figure 4.** Phenotypic effect on conidia development in *P. fici* strains. (a) Phenotype observations of strains of *P. fici* WT, ΔPfptaA, ΔPfcclA, ΔPfhdaA, ΔPfptaA ΔPfcclA, and ΔPfptaA ΔPfhdaA; (b) the difference of conidia production in strains of *P. fici* WT, ΔPfptaA, ΔPfcclA, ΔPfhdaA, ΔPfptaA ΔPfhdaA, and ΔPfptaA ΔPfhdaA. All of the strains were grown on PDA plates for number determination at 25 °C for 14 days. Three replicates were done for each culture of strain. Error bars represent the standard deviations. Asterisks indicated significant differences in mean values (*p* < 0.01(**); *p* > 0.05 (ns)).

### 2.6. Assessment of Oxidative Stress Response of the Mutant Strains

Epigenetic regulators also influence the fungal growth, development, infection, and their adaptation to environment. For instance, the loss of hdaA did not affect the growth rate of *A. nidulans* [49], but *A. fumigatus* ΔhdaA strain showed a statistically significant reduction of growth compared with the wild type [9]. HdaA was involved in sclerotia formation in *A. flavus* [50], and the deletion of hdaA reduced the oxidative stress tolerance of *A. nidulans* [49]. On the other hand, the deletion of cclA strongly reduced mycelial growth, asexual sporulation and spore germination, but did not impair the morphogenesis of specialized infection structures in *C. higginsianum* [21]. To assess any impact of target genes on oxidative stress response, *P. fici* WT strain and all of mutants were subjected to three oxidative reagents, including diamide, tert-butylhydroperoxide (tBOOH), and menadionensodium bisulfite (MSB). Separate deletion of PfptaA, PfcclA, and PfhdaA partly lowered the growth rate on PDA medium, and the inhibition was more obvious in the ΔPfcclA mutant than the ΔPfptaA and ΔPfhdaA mutants. There were obvious differences of sensitivity to tBOOH, diamide, and MSB in different mutants in the third day and fifth day as determined via the measurement of colony diameter. The smaller colony size might indicate the more sensitivity. Almost all of the mutants were more sensitive to tBOOH, diamide, and MSB than *P. fici* WT, and displayed slower growth in the presence of oxidative stressors besides the ΔPfptaA ΔPfhdaA mutants treated with MSB (Figure 5a–d). Moreover, the MSB effects on colony size on the 3rd day and the 5th day were opposite between the *P. fici* WT and ΔPfptaA mutant. For the double genes-deficient mutants, the ΔPfptaA ΔPfcclA mutant was more sensitive to all of the stressors than the ΔPfptaA mutant, and only more sensitive to tBOOH than ΔPfptaA. The ΔPfptaA ΔPfhdaA mutant was not sensitive to MSB as well as ΔPfptaA mutant, and it was apparently not as sensitive as the
\Delta PfhdaA\) mutant to MSB. The effects on strain sensitivity to the oxidative stress agents were generally different by deletion of epigenetic regulators in various fungal species. The \(A. \text{nidulans}\ \Delta hdaA\) mutant increased susceptibility to oxidative stress compared with the wild type [49], but the growth of the \(A. \text{fumigatus}\ \Delta hdaA\) mutant was not affected under oxidative stress conditions compared with the wild type [9]. Moreover, conidial production was indistinguishable between \(A. \text{fumigatus}\ \Delta hdaA\) and wild-type strains [9]. The \(A. \text{fumigatus}\ \Delta cclA\) mutant was more sensitive to chemical 6-azauracil (6AU) compared with the wild type [19]. The cause leading to these different effects may be involved in the changes of SMs, and the regulatory mechanism should be explored in the future.

\[\Delta PfhdaA\]

Figure 5. Comparison of oxidative stress tolerances of \(P. \text{fici}\) strains. (a) Mycelia growth of the mutants under oxidative stress on the 3rd day; (b) the colony diameters of the testing strains were measured on the 3rd day; (c) mycelia growth of the mutants under oxidative stress on the 5th day; (d) the colony diameters of the testing strains were measured on the 5th day. The spores of WT strain and mutants were inoculated on PDA media with or without tBOOH (1.8 mM), diamide (0.5 mM), or MSB (0.5 mM), and cultured at 25 °C for 5 days. Three replicates were done for each culture of strain. OR is the abbreviation of oxidative reagents. Error bars represented the standard deviations. Asterisks indicate significant differences in mean values (\(p < 0.0001\) (***)), \(p < 0.001\) (**), \(p < 0.01\) (*), \(p < 0.05\) (*)).
3. Materials and Methods

3.1. Strains, Media and Culture Conditions

*P. fici* CGMCC3.15140 and its correct transformants were grown at 25 °C on Potato Dextrose Agar (PDA) medium with appropriate antibiotics as required (Table S1). All of the strains were grown on the rice medium with water at 25 °C for 20 days for extraction and analysis of secondary metabolites. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* AGL-1 were propagated at 37 °C in LB medium with appropriate antibiotics for plasmid DNA amplification and transformation, respectively.

3.2. Plasmids for Deletion of *PfptaA*

The plasmids and primers are listed in Table S1 and Table S2, respectively. PCR amplifications were executed in the T100TM Thermal cycler (Bio-Rad, Hercules, CA, USA). TransStart-FastPFu DNA polymerase as a High-Fidelity DNA polymerase (TransGene Biotech, Beijing, China) was used to amplify the gene fragments. PCR screenings for transformants were performed by using 2× Taq Mix kit (Tiangen Biotech, Beijing, China). PCR reaction and thermal profiles were referred to the manufacturer’s instructions. The restriction enzymes used in this work were obtained in New England Biolabs (New England Biolabs Inc. (NEB), Ipswich, MA, USA). To generate the deletion cassette, we used Fusion PCR strategy as described previously [51]. Briefly, G418 was amplified from the pAG1-H3-G418, and around 1.1 kb of fragments upstream and downstream of the gene *PfptaA* were amplified from *P. fici* genomic DNA using the designed primers. The three PCR fragments were ligated into the T-vector p-Blunt, and then were amplified for transformation in *P. fici* strains.

3.3. Transformation in *P. fici*

For creation of deletion of *PfptaA* (PFICI_10824) mutants in *P. fici* wild type, ΔPfclA, and ΔPfidaA mutants, the deletion cassette was amplified with the template of pYYJ1.1 using primers 10824-5f-FL and 10824-3f-RL. The DNA cassette fragments were transformed into *P. fici* WT, TYXW7.1 and TYXW8.1 as described previously [29]. Then candidate colonies were singled out after culturing on PDA with G418 resistant at 28 °C for 5 days. The disruption mutants were verified using diagnostic PCR with primers inside and outside of the gene *PfptaA* (Figure 1a and Table S2).

3.4. Oxidative Stress Sensitivity Assays

Different oxidative reagents were used to estimate the stress sensitivity of the mutants according to the method described previously [35]. Moreover, the colony diameters are the indicator supplementing with the following stress-generating agents: 0.5 mM diamide, 0.5 mM menadione sodium bisulfite (MSB), and 1.8 mM tert-butyl hydroperoxide (tBOOH), respectively. The strains were incubated at 25 °C for 5 days, and colony diameters were measured on the 3rd day and 5th day. Three replicates were performed for each experiment.

3.5. Conidia Counting

The conidia counting in *P. fici* wild type and its mutants were performed as described previously [52]. These strains were grown at 25 °C on PDA medium in 90 mm plate for 14 days. Three replicates were performed for each culture sample. Then, we used ddH$_2$O with 0.1% (v/v) Tween to flush the plates repeatedly and remove the hyphae and impurities through Miracloth. The filtrate containing conidia was centrifuged and concentrated to 1 mL of volume. The number of conidia from different mutants and wild type was determined using a blood-cell-counting plate. Values are means of three replicates for each culture are presented. Data were analyzed using the GraphPad Prism 8.0 performing Tukey–Kramer multiple comparison test at $p \leq 0.05$. Asterisks indicate statistically significant differences in mean values.
3.6. Analytical Methods for HPLC and LC-MS

Analysis of secondary metabolites was performed on a Waters HPLC system (Waters 2998, Photodiode Array Detector) with an ODS column (C18, 250 × 4.6 mm, Waters Pak, 5 µm). Water (A) and acetonitrile (B), both with 0.1 % (v/v) formic acid, were used as solvents at a flow rate of 1 mL/min. The substances were eluted with a linear gradient from 5–100% B in 40 min, then washed with 100 % (v/v) solvent B for 5 min and equilibrated with 5 % (v/v) solvent B for 5 min. UV absorptions at 236 nm were illustrated. LC-MS analyses of secondary metabolites was determined using an Agilent 1200 Accurate-Mass QTOF LC/MS system (Agilent Technologies, Santa Clara, CA, USA) with Agilent ZORBAX Eclipse column (C18 Plus, 2.1 × 4.6 mm, 3 µm) and an electrospray ionization (ESI) source. Water (A) and acetonitrile (B), both with 0.1 % (v/v) formic acid, were used as solvents at a flow rate of 1 mL/min. The substances were eluted with a linear gradient from 5–100% B in 40 min, then washed with 100 % (v/v) solvent B for 5 min and equilibrated with 5 % (v/v) solvent B for 5 min.

3.7. Isolation and Identification of New Compound

The ∆PfptaA ∆PfhdaA mutant was cultivated in flasks. Each flask contained 80 g rice and 120 mL distilled water and was sterilized by autoclave. A total of 10 kilograms of rice media were made, and static cultured at 25 °C for 20 d. The rice culture was extracted three times with ethyl acetate. The organic phase was evaporated to dryness under reduced pressure to afford the residue (7.6 g). The crude residue was applied on a C-18 ODS column using a stepped gradient elution of MeOH-H₂O yielding 10 subfractions (fractions 1–10). Fraction 2 (eluting with MeOH:H₂O = 25:75) was chromatographed on Sephadex LH-20 (MeOH) and the targeted fraction 2.1 was further separated by HPLC (C-18ODS) using a stepped gradient elution of MeOH-H₂O (5:95 to 100:0, 120 min) to furnish eight subfractions 2.1.1–2.1.8. The subfraction 2.1.1 was further purified by semi-preparative HPLC with a gradient of H₂O and CH₃CN (linear gradient of 15 % to 45 % CH₃CN over 30 min at 3 mL/min) to afford compound 1 (1.7 mg tR = 5.4 min). The assignments of 11 compounds were based on the published data of proton LC-MS. The related figures were shown individually, in the same order as they are referred to in the manuscript.

4. Conclusions

In summary, we developed a combined approach to modulate the secondary metabolic profile in filamentous fungi. We deleted the epigenetic regulators PfclA or PfhdaA in a host lacking major SM pestheic acid. This led to significant changes in secondary metabolic profiles. We discovered 1 novel SM named pestaloficiol X (1) as well as 11 other known compounds with obvious yield changes in ∆PfptaA ∆PfclA or/and ∆PfptaA ∆PfhdaA mutants. Compound 1 was only produced in the ∆PfptaA ∆PfhdaA mutant strain, suggesting a successful activation by modulation of histone acetylation and Pfpta dereplication. Moreover, the deletion of PfptaA in ∆PfclA or ∆PfhdaA background host did not overproduce conidia as seen in the single mutant ∆PfptaA. We also found that the ∆PfptaA ∆PfhdaA mutant is generally not sensitive to oxidative stressors in comparison with the single mutant ∆PfptaA or the parental strains, whereas the ∆PfptaA ∆PfclA mutant was more sensitive. Our findings support that combination strategy of genetic dereplication and manipulation of epigenetic regulators is an efficient approach to discover novel SMs in plant endophytic fungi P. fici as well as is a valuable strategy to be applied for new natural product discovery in filamentous fungi.

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References
1. Keller, N.P.; Turner, G.; Bennett, J.W. Fungal secondary metabolism—from biochemistry to genomics. Nat. Rev. Microbiol. 2005, 3, 937–947. [CrossRef] [PubMed]
2. Pfannenstiel, B.T.; Keller, N.P. On top of biosynthetic gene clusters: How epigenetic machinery influences secondary metabolism in fungi. Biotechnol. Adv. 2019, 37, 107345. [CrossRef] [PubMed]
3. Lyu, H.N.; Liu, H.W.; Keller, N.P.; Yin, W.B. Harnessing diverse transcriptional regulators for natural product discovery in fungi. Nat. Prod. Rep. 2020, 37, 6–16. [CrossRef] [PubMed]
4. Chiang, Y.M.; Ahuja, M.; Oakley, C.E.; Entwistle, R.; Asokan, A.; Zutz, C.; Wang, C.C.; Oakley, B.R. Development of genetic dereplication strains in Aspergillus nidulans results in the discovery of aspercryptin. Angew. Chem. Int. Ed. Engl. 2016, 55, 1662–1665. [CrossRef]
5. Chen, L.; Wu, H.; Liu, H.; Li, E.; Ren, J.; Wang, W.; Wang, S.; Yin, W.B. Genetic dereplication of Trichoderma hypoxylon reveals two novel polycyclic lactones. Bioorg. Chem. 2019, 91, 103185. [CrossRef]
6. Liu, H.; Pu, Y.H.; Ren, J.W.; Li, E.W.; Guo, L.X.; Yin, W.B. Genetic dereplication driven discovery of a tricinoloniol acid biosynthetic pathway in Trichoderma hypoxylon. Org. Biomol. Chem. 2020, 18, 5344–5348. [CrossRef]
7. Kim, W.; Liu, R.; Woo, S.; Kang, K.B.; Park, H.; Yu, Y.H.; Ha, H.-H.; Oh, S.-Y.; Yang, J.H.; Kim, H.; et al. Linking a gene cluster to atranorin, a major cortical substance of Lichens, through genetic dereplication and heterologous expression. mbio 2021, 12, e011121. [CrossRef]
8. Wei, Q.; Bai, J.; Yan, D.; Bao, X.; Li, W.; Liu, B.; Zhang, D.; Qi, X.; Yu, D.; Hu, Y. Genome mining combined metabolic shunting and OSMAC strategy of an endophytic fungus leads to the production of diverse natural products. Acta. Pharm. Sin. B. 2021, 11, 572–587. [CrossRef]
9. Lee, I.; Oh, J.H.; Shwab, E.K.; Dagenais, T.R.; Andes, D.; Keller, N.P. HdaA, a class 2 histone deacetylase of Aspergillus fumigatus, affects germination and secondary metabolite production. Fungal Genet. Biol. 2009, 46, 782–790. [CrossRef]
10. Studt, L.; Schmidt, F.J.; Jahn, L.; Sieber, C.M.; Connolly, L.R.; Niehaus, E.M.; Humpf, H.U.; Tudzynski, B. Two histone deacetylases, FfHda1 and FfHda2, are important for Fusarium fujikuroi secondary metabolism and virulence. Appl. Environ. Microbiol. 2013, 79, 7719–7734. [CrossRef]
11. Niu, X.; Hao, X.; Hong, Z.; Chen, L.; Yu, X.; Zhu, X. A putative histone deacetylase modulates the biosynthesis of pestalotioliolide B and conidiation in Pestalotiopsis microspora. J. Microbiol. Biotechnol. 2015, 25, 579–588. [CrossRef] [PubMed]
12. Shwab, E.K.; Bok, J.W.; Tribus, M.; Galehr, J.; Graessle, S.; Keller, N.P. Histone deacetylase activity regulates chemical diversity in Aspergillus. Eukaryot. Cell. 2007, 6, 1656–1664. [CrossRef] [PubMed]
13. Maeda, K.; Izawa, M.; Nakajima, Y.; Jin, Q.; Hirose, T.; Nakamura, T.; Koshino, H.; Kanamaru, K.; Ohshato, S.; Kamakura, T.; et al. Increased metabolite production by deletion of an HDA1-type histone deacetylase in the phytopathogenic fungi, Magnaporthe oryzae (Pyricularia oryzae) and Fusarium asiaticum. Lett. Appl. Microbiol. 2017, 65, 446–452. [CrossRef] [PubMed]
14. Li, X.; Pan, L.; Wang, B.; Pan, L. The histone deacetylases HosA and HdaA affect the phenotype and transcriptomic and metabolic profiles of Aspergillus niger. Toxins 2021, 11, 520. [CrossRef]
15. Ding, Z.; Zhou, H.; Wang, X.; Huang, H.; Wang, H.; Zhang, R.; Wang, Z.; Han, J. Deletion of the histone deacetylase hdaA in Aspergillus fumigatus Penicillium chrysogenum Fes1701 induces the complex response of multiple bioactive secondary metabolite production and relevant gene cluster expression. Molecules 2020, 25, 3657. [CrossRef]
16. Mao, X.M.; Xu, W.; Li, D.; Yin, W.B.; Chooi, Y.H.; Li, Y.Q.; Tang, Y.; Hu, Y. Epigenetic genome mining of an endophytic fungus leads to the pleiotropic biosynthesis of natural products. Angew. Chem. Int. Ed. Engl. 2015, 54, 7592–7596. [CrossRef]
17. Guzman-Chavez, F.; Salo, O.; Samol, M.; Ries, M.; Kuipers, J.; Bovenberg, R.A.L.; Veenken, R.J.; Driessen, A.J.M. Deregulation of secondary metabolism in a histone deacetylase mutant of Penicillium chrysogenum. Microbiology 2018, 7, e00598. [CrossRef]
18. Bok, J.W.; Chiang, YM.; Szewczyk, E.; Reyes-Dominguez, Y.; Davidson, A.D.; Sanchez, J.F.; Lo, H.C.; Watanabe, K.; Strauss, J.; Oakley, B.R.; et al. Chromatin-level regulation of biosynthetic gene clusters. Nat. Chem. Biol. 2009, 5, 462–464. [CrossRef]
19. Palmer, J.M.; Bok, J.; Lee, S.; Dagenais, T.R.T.; Andes, D.R.; Kontoyiannis, D.P.; Keller, N.P. Loss of ClaA, required for histone 3 lysine 4 methylation, decreases growth but increases secondary metabolite production in Aspergillus fumigatus. PeerJ 2013, 1, e4. [CrossRef]

20. Shinohara, Y.; Kawatani, M.; Futamura, Y.; Osada, H.; Koyama, Y. An overproduction of astelolides induced by genetic disruption of chromatin-remodeling factors in Aspergillus oryzae. J. Antimicrob. [Tokyo] 2016, 69, 4–8. [CrossRef]

21. Dallery, J.F.; Adelin, E.; Le Goiff, G.; Pigne, A.; Auger, A.; Ouazzani, J.; O’Connell, R.J. H3K4 trimethylation by Ccla regulates pathogenicity and the production of three families of terpenoid secondary metabolites in Colletotrichum higginsianum. Mol. Plant Pathol. 2019, 20, 831–842. [PubMed]

22. Liu, Y.; Liu, N.; Yin, Y.; Chen, Y.; Jiang, J.; Ma, Z. Histone H3K4 methylation regulates hyphal growth, secondary metabolism and multiple stress responses in Fusarium graminearum. Environ. Microbiol. 2015, 17, 4615–4630. [CrossRef] [PubMed]

23. Studt, L.; Janevska, S.; Arndt, B.; Boedi, S.; Sulyok, M.; Humpf, H.U.; Tudzynski, B.; Strauss, J. Lack of the COMPASS component Ccl1 reduces H3K4 trimethylation levels and affects transcription of secondary metabolite genes in two plant-pathogenic Fusarium species. Front. Microbiol. 2017, 7, 2144. [CrossRef] [PubMed]

24. Harting, R.; Bayram, O.; Laubinger, K.; Valerius, O.; Braus, G.H. Interplay of the fungal sumoylation network for control of multicellular development. Mol. Microbiol. 2013, 90, 1125–1145. [CrossRef]

25. Harting, R.; Bayram, O.; Laubinger, K.; Valerius, O.; Braus, G.H. Interplay of the fungal sumoylation network for control of multicellular development. Mol. Microbiol. 2013, 90, 1125–1145. [CrossRef]

26. Liu, L.; Liu, S.; Chen, X.; Guo, L.; Che, Y. Pestalofones A-E, bioactive cyclohexanone derivatives from the plant endophytic fungus Dracaena sp. with new skeleton and its activity. J. Antibiot. (Tokyo) 2017, 70, 703–711. [CrossRef]

27. Dallery, J.F.; Adelin, E.; Le Goiff, G.; Pigne, A.; Auger, A.; Ouazzani, J.; O’Connell, R.J. H3K4 trimethylation by Ccla regulates pathogenicity and the production of three families of terpenoid secondary metabolites in Colletotrichum higginsianum. Mol. Plant Pathol. 2019, 20, 831–842. [PubMed]

28. Xu, X.; Liu, L.; Zhang, F.; Wang, W.; Li, J.; Guo, L.; Che, Y.; Liu, G. Identification of the first diphenyl ether gene cluster for pestheic acid biosynthesis in plant endophyte Pestalotiopsis fici. ChemBioChem 2014, 15, 284–292. [CrossRef]

29. Wang, X.; Zhou, H.; Zang, P.; Wang, X.; Li, W.; Zhang, W.; Liu, X.; Liu, H.W.; Keller, N.P.; An, Z.; et al. Polyskeletal production of pestalofolios and macrodiolide ficiolides revealed by manipulations of epigenetic regulators in an endophytic fungus. Org. Lett. 2016, 18, 1832–1835. [CrossRef]

30. Zheng, Y.; Ma, K.; Lyu, H.; Huang, Y.; Liu, H.; Liu, L.; Che, Y.; Liu, X.; Zou, H.; Yin, W.B. Genetic manipulation of the COP9 signalosome subunit PfCsnE regulates Pestalotiopsis fici precursors from pestaloficiols and macrodiolide ficiolides revealed by manipulations of epigenetic regulators in an endophytic fungus. J. Nat. Prod. 2019, 82, 642–646. [CrossRef]

31. Liu, J.; Liu, S.; Niou, S.; Guo, L.; Chen, X.; Che, Y. Isoprenylated chromone derivatives from the plant endophytic fungus Pestalotiopsis fici. Org. Lett. 2018, 20, 450–453. [CrossRef]

32. Liu, L. Bioactive metabolites from the plant endophyte Pestalotiopsis fici. Mycol. Int. J. Fungal Biol. 2011, 2, 37–45. [CrossRef]

33. Liu, L.; Liu, S.; Jiang, L.; Chen, X.; Guo, L.; Che, Y. Chloropupukeananin, the first chlorinated pupukeanane derivative, and its precursors from Pestalotiopsis fici. Org. Lett. 2009, 11, 3936–3939. [CrossRef] [PubMed]

34. Zheng, Y.; Wang, X.; Zhang, F.; Liu, X.; Guo, L.; Che, Y. Chloropropylpyrrole, the first chlorinated pyrrolepyrrole derivative, and its precursors from Pestalotiopsis fici. Org. Lett. 2008, 10, 1397–1400. [CrossRef] [PubMed]

35. Wang, X.; Wu, F.; Liu, L.; Liu, X.; Che, Y.; Keller, N.P.; Guo, L.; Yin, W.B. The bZIP transcription factor PfZipA regulates secondary metabolism and oxidative stress response in the plant endophytic fungus Pestalotiopsis fici. Fungal Genet. Biol. 2015, 81, 221–228. [CrossRef]

36. Liu, L.; Liu, S.; Chen, X.; Guo, L.; Che, Y. Pestalofones A–E, bioactive cyclohexane derivatives from the plant endophytic fungus Pestalotiopsis fici. Bioorg. Med. Chem. 2009, 17, 606–613. [CrossRef]

37. Liu, L.; Liu, S.; Niou, S.; Guo, L.; Chen, X.; Che, Y. Isoprenylated chromosome derivatives from the plant endophytic fungus Pestalotiopsis fici. J. Nat. Prod. 2009, 72, 1482–1486. [CrossRef]

38. Liu, S.; Liu, X.; Guo, L.; Che, Y.; Liu, L. 2H-pyran-2-one and 2H-furan-2-one derivatives from the plant endophytic fungus Pestalotiopsis fici. Chem. Biodivers. 2013, 10, 2007–2013. [CrossRef]

39. Kupka, J.; Anke, T.; Steglich, W.; Zechlin, L. Antibiotics from Basidiomycetes. XI. The biological activity of siccayne, isolated from the marine fungus Halocynthia rufescens] & E. Kohlmeyer. J. Antibiot. (Tokyo) 1981, 34, 298–304.

40. Feng, J.; Zhang, P.; Cui, Y.L.; Li, K.; Qiao, X.; Zhang, Y.T.; Li, S.M.; Cox, R.J.; Wu, B.; Ye, M.; et al. Regio- and stereospecific O-glycosylation of phenolic compounds catalyzed by a fungal glycosyltransferase from Mucor hiemalis. J. Agric. Food Chem. 2017, 65, 2370–2371. [CrossRef] [PubMed]

41. Liu, S.; Guo, L.; Che, Y.; Liu, L. Pestaloficiols Q–S from the plant endophytic fungus Pestalotiopsis fici. Fitoterapia 2013, 85, 114–118. [CrossRef] [PubMed]

42. Zhao, H.; Chen, G.D.; Zou, J.; He, R.R.; Qin, S.Y.; Hu, D.; Li, G.Q.; Guo, L.D.; Yao, X.S.; Gao, H. Dimethylsucinoglycan: A new meroterpenoid dimer from Biscogniauxia sp. with new skeleton and its activity. Org. Lett. 2017, 19, 38–41. [CrossRef] [PubMed]

43. Moloney, R.J.; Mahoney, N.; Bayman, P.; Wong, R.Y.; Meyer, K.; Irelan, N. Eutypa dieback in grapevines: Differential production of acetylenic phenol metabolites by strains of Eutypa lata. J. Agric. Food Chem. 2002, 50, 1393–1399. [CrossRef] [PubMed]

44. Rukachaisirikul, V.; Rungsawattana, N.; Klaiaklay, S.; Phongpaichit, S.; Borzwornwiriyanup, K.; Sakayaroj, J. γ-Butyrolactone, cytochalasin, cyclic carbonate, eutypic acid, and phenalenone derivatives from the soil fungus Aspergillus sp. PSU-RSPG185. J. Nat. Prod. 2014, 77, 2375–2382. [CrossRef] [PubMed]

45. Pan, Y.; Liu, L.; Guan, F.; Li, E.; Jin, J.; Li, J.; Che, Y.; Liu, G. Characterization of a prenyltransferase for iso-A82775C biosynthesis and generation of new congeners of chloropostelolides. ACS Chem. Biol. 2018, 13, 703–711. [CrossRef]
46. Lv, J.M.; Gao, Y.H.; Zhao, H.; Awakawa, T.; Liu, L.; Chen, G.D.; Yao, X.S.; Hu, D.; Abe, I.; Gao, H. Biosynthesis of biscognienyne B involving a cytochrome P450-dependent alkylation. Angew. Chem. Int. Ed. Engl. 2020, 59, 13531–13536. [CrossRef]

47. Chen, Y.R.; Naresh, A.; Liang, S.Y.; Lin, C.H.; Chein, R.J.; Lin, H.C. Discovery of a dual function cytochrome P450 that catalyzes enyne formation in cyclohexanoid terpenoid biosynthesis. Angew. Chem. Int. Ed. Engl. 2020, 59, 13537–13541. [CrossRef]

48. Sordon, S.; Poplonski, J.; Tronina, T.; Huszczza, E. Regioselective O-glycosylation of flavonoids by fungi Beauveria bassiana, Absidia coerulea and Absidia glauca. Bioorg. Chem. 2019, 93, 102750. [CrossRef]

49. Tribus, M.; Galehr, J.; Trojer, P.; Brosch, G.; Loidl, P.; Marx, F.; Haas, H.; Graessle, S. HdaA, a major class 2 histone deacetylase of Aspergillus nidulans, affects growth under conditions of oxidative stress. Eukaryot. Cell 2005, 4, 1736–1745. [CrossRef]

50. Lan, H.; Wu, L.; Sun, R.; Keller, N.P.; Yang, K.; Ye, L.; He, S.; Zhang, F.; Wang, S. The HosA histone deacetylase regulates aflatoxin biosynthesis through direct regulation of aflatoxin cluster genes. Mol. Plant Microbe. Interact. 2019, 32, 1210–1228. [CrossRef]

51. Li, W.; Fan, A.; Wang, L.; Zhang, P.; Liu, Z.; An, Z.; Yin, W.B. Asperphenamate biosynthesis reveals a novel two-module NRPS system to synthesize amino acid esters in fungi. Chem. Sci. 2018, 9, 2589–2594. [CrossRef] [PubMed]

52. Zhang, P.; Zhou, S.; Wang, G.; An, Z.; Liu, X.; Li, K.; Yin, W.B. Two transcription factors cooperatively regulate DHN melanin biosynthesis and development in Pestalotiopsis fici. Mol. Microbiol. 2019, 112, 649–666. [CrossRef] [PubMed]