



Article

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

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**Abstract:** The strategies of genetic dereplication and manipulation of epigenetic regulators to activate the cryptic gene clusters are effective to discover natural products with novel structure in filamentous fungi. In this study, a combination of genetic dereplication (deletion of pesthetic acid biosynthetic gene, *PfptaA*) and manipulation of epigenetic regulators (deletion of histone methyltransferase gene *PfcclA* and histone deacetylase gene *PfhdaA*) was developed in plant endophytic fungus *Pestalotiopsis fici*. The deletion of *PfptaA* with *PfcclA* and/or *PfhdaA* led to isolation of 1 novel compound, pestaloficiol X (1), as well as another 11 known compounds with obvious yield changes. The proposed biosynthesis pathway of pestaloficiol X was speculated using comparative analysis of homologous biosynthetic gene clusters. Moreover, phenotypic effects on the conidial development and response to oxidative stressors in the mutants were explored. Our results revealed that the new strain with deletion of *PfcclA* or *PfhdaA* in  $\Delta PfptaA$  background host can neutralise the hyperformation of conidia in the *PfptaA* mutant, and that the  $\Delta PfptaA \Delta PfhdA$  mutant was generally not sensitive to oxidative stressors as much as the  $\Delta PfptaA \Delta cclA$  mutant in comparison with the single mutant  $\Delta PfptaA$  or the parental strains. This combinatorial approach can be applied to discover new natural products in filamentous fungi.

**Keywords:** genetic dereplication; epigenetic regulation; secondary metabolites; oxidative stress; filamentous fungi



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## 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 danggaardii* [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, phosphorylase, 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 microspore* [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 alkaloid production was upregulated by 84.8-fold in the *Penicillium chrysogenum*  $\Delta hdaA$  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*  $\Delta hosA \Delta hdaA$  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].

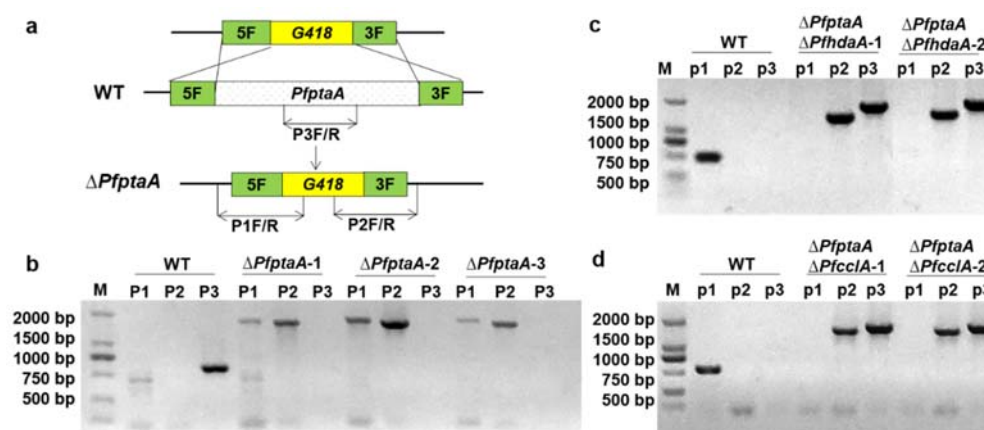
The plant endophytic fungi from *Pestalotiopsis* genus is well-studied for their SM production [25–27]. *Pestalotiopsis fici* has been reported to produce about 100 distinctive compounds by traditional isolation and genetic manipulation strategies [28–32]. Disruption of epigenetic regulators PfcclA and PfhdaA led to the identification of 15 new structures [29]. The diphenyl ether pestheic acid and its analogues are the major SMs produced in *P. fici* [33]. Disruption of pestheic acid biosynthesis gene (*pta*) could observably reduce the SMs background in this strain [28]. In this study,  $\Delta Pfpta \Delta PfcclA$  and  $\Delta Pfpta \Delta PfhdaA$  mutants in *P. fici* were constructed. One novel structure was characterized with 11 known compounds in both of the double deletion mutants, and the effect of these genetic manipulation on the growth, sporulation, and sensitivity to oxidative stress were evaluated.

## 2. Results and Discussion

### 2.1. Construction of PfptaA Deletion Mutants

In previous studies, the *pta* gene cluster for pestheic acid biosynthesis has been identified in *P. fici* [28], and both  $\Delta PfcclA$  and  $\Delta PfhdaA$  mutants has been obtained, respectively [29]. Here, we constructed the single knockout mutant of *PfptaA* (PFICI\_10824), double knockout mutants of *PfptaA* and *PfcclA* (PFICI\_05127), and *PfptaA* and *PfhdaA*

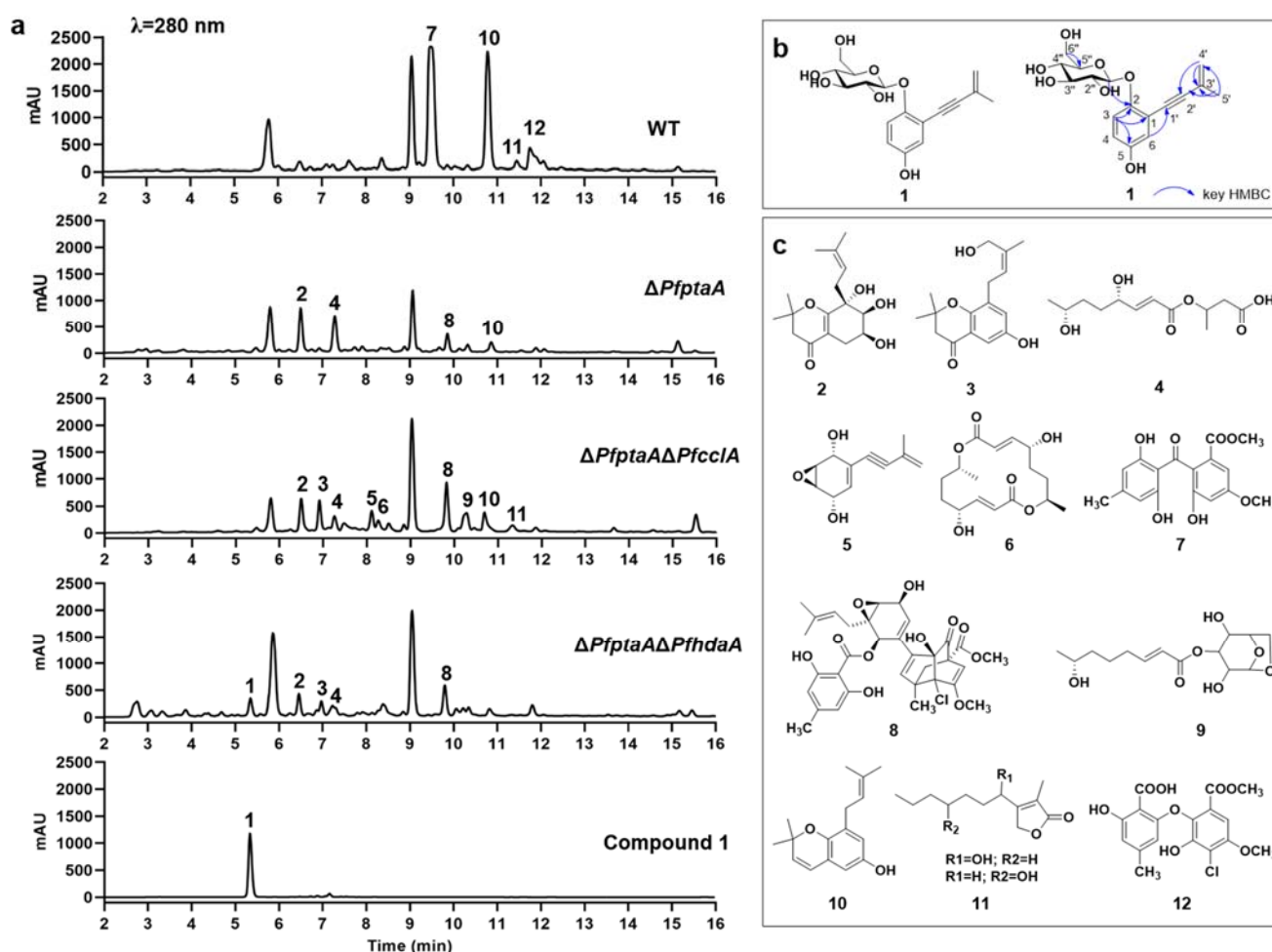
(*PFICI\_08988*) using the previously described transformation method, individually [29] (Figure 1 and Table S1). For deletion of *PfptaA*, we constructed a plasmid containing the upstream and downstream homologous arms of *PfptaA* with the resistance gene of G418 antibiotic. The fragments of deletion 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 ( $\Delta PfclA$ ) and TYXW8 ( $\Delta PfhdA$ ). 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.** 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  $\Delta PfptaA$  host; (d) PCR verification for *PfclA* gene deletion in  $\Delta PfptaA$  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  $\Delta Pfpta$ ,  $\Delta PfclA$  and  $\Delta Pfpta \Delta PfhdA$  mutants, compared with WT and  $\Delta Pfpta$  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  $\Delta Pfpta \Delta PfhdA$  mutant (Figure 2b). Compounds 7 and 12 produced in WT were disappeared in both  $\Delta Pfpta \Delta PfclA$  and  $\Delta Pfpta \Delta PfhdA$  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  $\Delta Pfpta$  mutant. The known SMs identified in *P. fici* wild type,  $\Delta PfcsnE$  (the fifth subunit of COP9 signalosome, CsnE), and  $\Delta PfhdA$  strains are pestaloficiol M (2), pestaloficin D (3), ficiolide J (4), asperpentyn (5), ficiolide C (6), isosulochrin (7), chloropupukeananin (8), ficiolide K (9), pestaloficiol 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).



**Figure 2.** Secondary metabolites analysis for *P. fici* wild type and mutants. (a) HPLC analysis of secondary metabolites; (b) structure and key HMBC correlation of pestaloficiol X (1); (c) known SMs produced by *P. fici* strains in this study.

### 2.3. Identification of Compound 1

The new compound **1** was only produced in  $\Delta Pfpta \Delta PfhdA$  mutant (Figures 2a and S7). To elucidate the structure of **1**, we made a scale-up fermentation and isolated it by a combination of UV-guided fractionation and retention time. Compound **1** was isolated as yellowish oil with the molecular formula  $C_{17}H_{20}O_7$ , which was deduced by the protonated molecule HR-ESI-MS ion peak with  $[M + Na]^+$  at  $m/z$  359 (Figure S7). The careful inspection of  $^1H$  and  $^{13}C$  NMR of **1** with previously identified compounds in fungal strains indicated that **1** was a 1,3-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 compound from the deuteromycete *Helminthosporium siccans* in 1981 [39]. The remaining NMR signals and unsaturation revealed that the hexose moiety is proposed. H-1''/C-5'' 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.**  $^1\text{H}$  (500 MHz) and  $^{13}\text{C}$  (125 MHz) NMR spectroscopic data for **1** in  $\text{DMSO-}d_6$ .

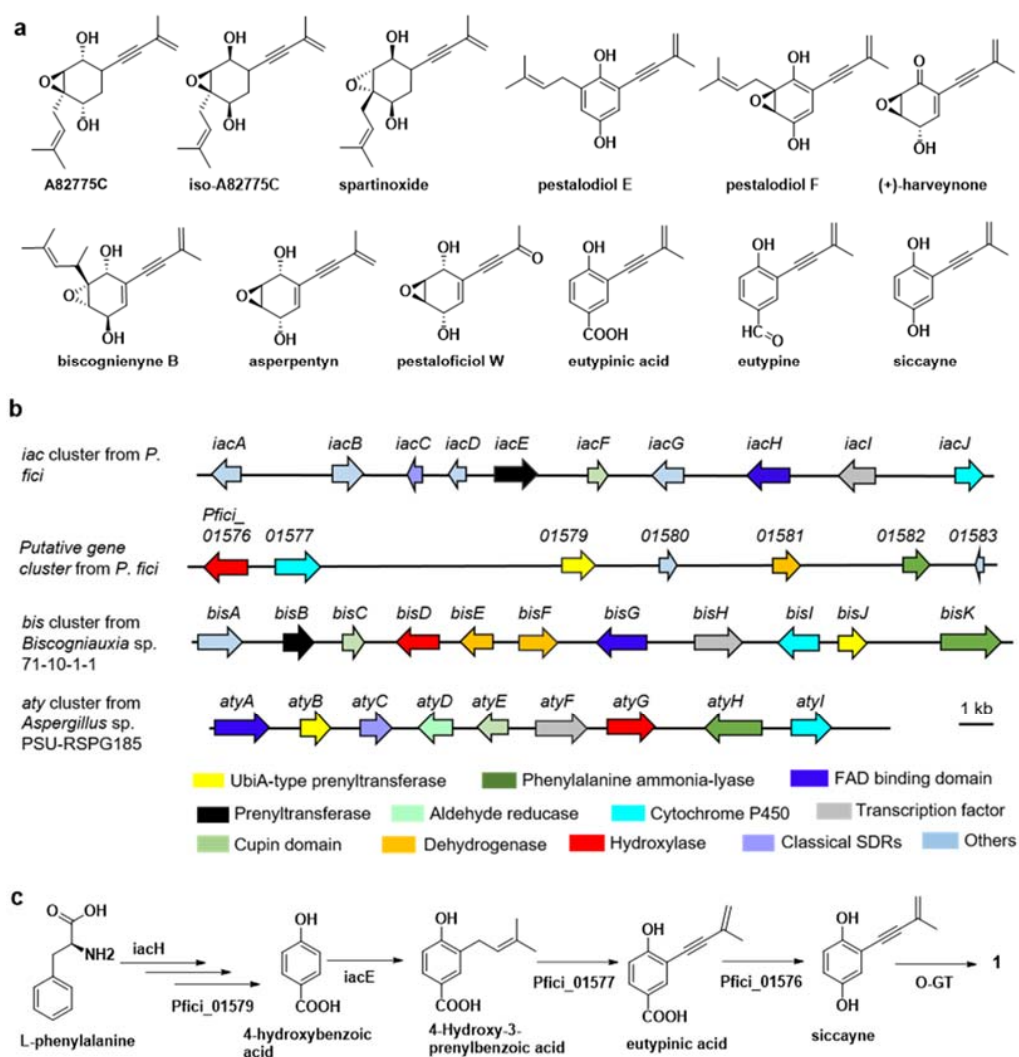
Position	$\delta_{\text{C}}$ , Type	$\delta_{\text{H}}$ , Multi., $J$ in Hz	HMBC Correlation	$^1\text{H-}^1\text{H}$ COSY
1	113.0 C	-	-	-
2	150.5 C	-	-	-
3	117.0 CH	6.99, d, 9.8	C-1', 1, 2, 5	H-4
4	116.8 CH	6.71, m, overlap	C-2, 6	H-3
5	151.7 C	-	-	-
6	118.2 CH	6.72, m, overlap	C-1', 4, 5	-
1'	85.3 C	-	-	-
2'	94.1 C	-	-	-
3'	126.7 C	-	-	-
4'	122.1 CH <sub>2</sub>	5.38, s 5.35, s	C-2', 3', 5'	H-5'
5'	23.2 CH <sub>3</sub>	1.93, s	C-2', 3', 4'	H-4'
1''	100.7 CH	4.81, d, 7.2	C-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 <sub>2</sub>	3.65, d, 12.0 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 *AtyI* 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 stereospecificity [48]. The several O-GTs predicted in *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 *P. fici*. The proposed biosynthetic pathways of pestaloficiol X (**1**) in *P. fici* was shown in Figure 3c.

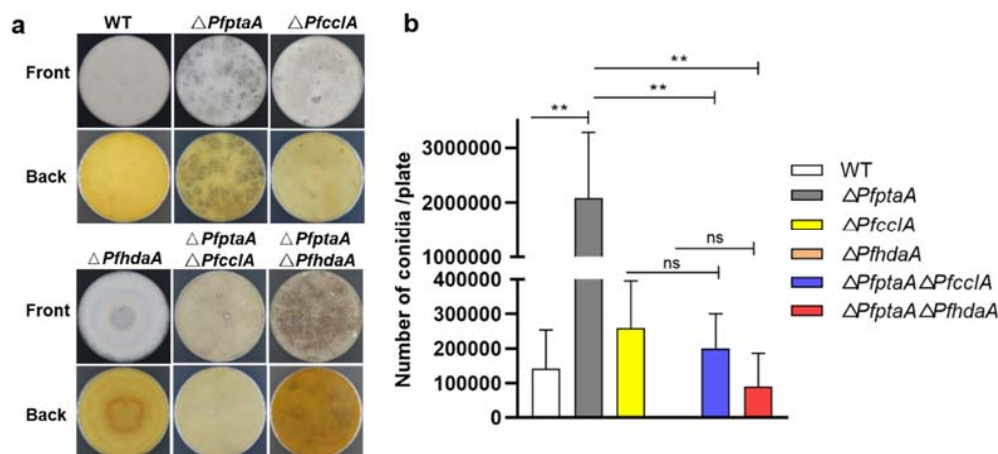


**Figure 3.** The structure analogues with alkynyl of **1** and biosynthesis of **1**. (a) The structure analogues of **1** produced in filamentous fungi; (b) the gene clusters related to biosynthesis of siccayne and **1** in filamentous fungi; (c) proposed biosynthetic pathway for **1** in *P. fici*.

### 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 *A. nidulans* [24]. To examine the effect on the morphology and conidia development, the strains of *P. fici* WT,  $\Delta PfpftaA$ ,  $\Delta PfcclA$ ,  $\Delta PfhdaA$ ,  $\Delta PfpftaA \Delta PfcclA$ , and  $\Delta PfpftaA \Delta PfhdaA$  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  $\Delta PfpftaA$  mutant, the conidia number increased about 10-fold compared with *P. fici* WT. Both deletion of *PfcclA* and *PfhdaA* led to no obvious change in conidia number compared with *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  $\Delta PfptaA$  mutant had no differential effect on the conidia number compared with  $\Delta PfcclA$  and  $\Delta PfhdaA$  mutants, respectively, but both double mutant strains had decreased numbers of conidia in comparison with  $\Delta PfptaA$  mutant. The results indicated that deletion of *PfcclA* or *PfhdaA* neutralises the enhancement of conidia formation in  $\Delta PfptaA$  host, suggesting interconnected regulatory network among these genes and products in conidia development and formation.

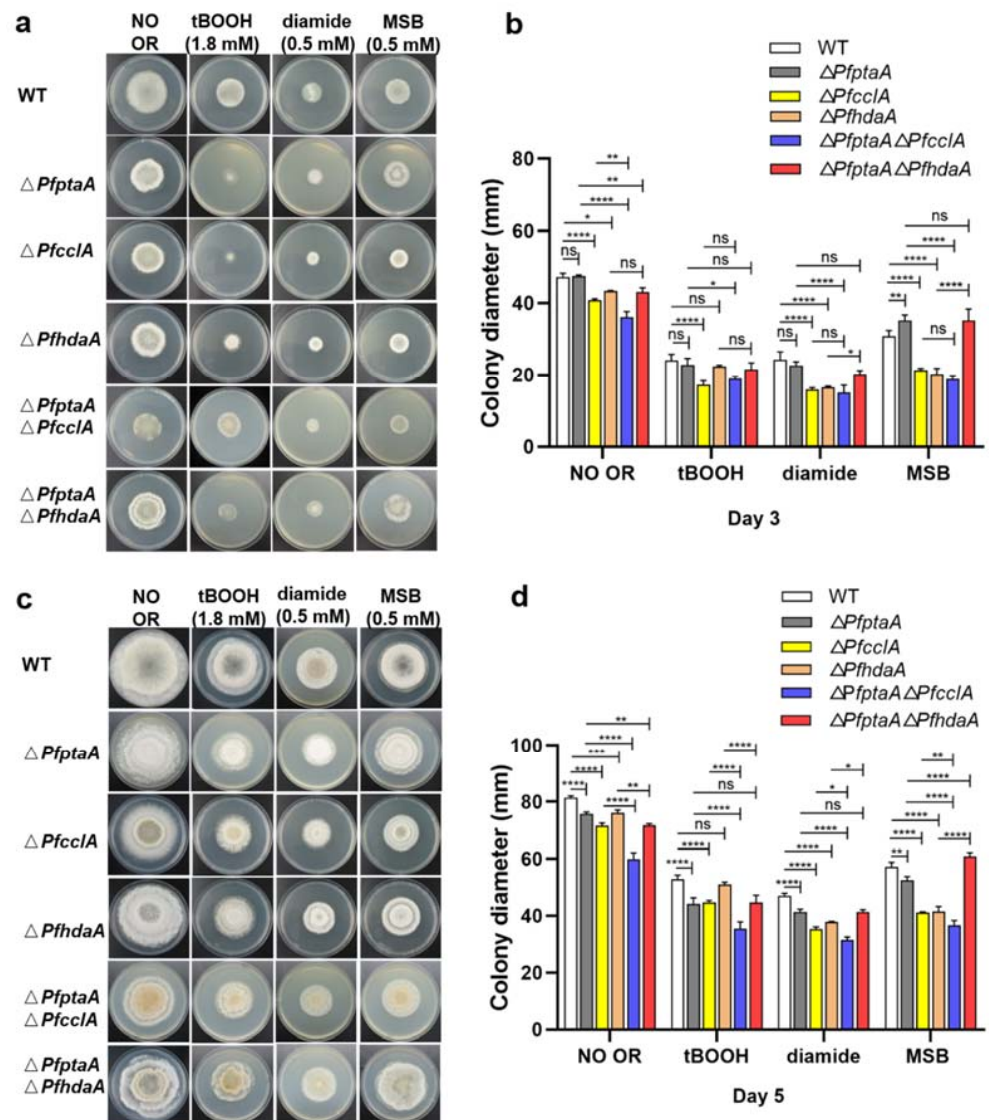


**Figure 4.** Phenotypic effect on conidia development in *P. fici* strains. (a) Phenotype observations of strains of *P. fici* WT,  $\Delta PfptaA$ ,  $\Delta PfcclA$ ,  $\Delta PfhdaA$ ,  $\Delta PfptaA \Delta PfcclA$ , and  $\Delta PfptaA \Delta PfhdaA$ ; (b) the difference of conidia production in strains of *P. fici* WT,  $\Delta PfptaA$ ,  $\Delta PfcclA$ ,  $\Delta PfhdaA$ ,  $\Delta PfptaA \Delta PfcclA$ , and  $\Delta PfptaA \Delta PfhdaA$ . 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*  $\Delta hdaA$  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 menadionesodium 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  $\Delta PfcclA$  mutant than the  $\Delta PfptaA$  and  $\Delta PfhdaA$  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  $\Delta PfptaA \Delta PfhdaA$  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  $\Delta PfptaA$  mutant. For the double genes-deficient mutants, the  $\Delta PfptaA \Delta PfcclA$  mutant was more sensitive to all of the stressors than the  $\Delta PfptaA$  mutant, and only more sensitive to tBOOH than  $\Delta PfptaA$ . The  $\Delta PfptaA \Delta PfhdaA$  mutant was not sensitive to MSB as well as  $\Delta PfptaA$  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. nidulans*  $\Delta hdaA$  mutant increased susceptibility to oxidative stress compared with the wild type [49], but the growth of the *A. fumigatus*  $\Delta hdaA$  mutant was not affected under oxidative stress conditions compared with the wild type [9]. Moreover, conidial production was indistinguishable between *A. fumigatus*  $\Delta hdaA$  and wild-type strains [9]. The *A. 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.



**Figure 5.** Comparison of oxidative stress tolerances of *P. 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* DH5 $\alpha$  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 $\times$  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,  $\Delta$ *PfclA*, and  $\Delta$ *PfidaA* 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 menadionesodium bisulfite (MSB), and 1.8 mM tert-butylhydroperoxide (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<sub>2</sub>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 statically 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  $\mu$ m). 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  $\times$  4.6 mm, 3  $\mu$ m) 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  $\Delta PfptaA \Delta PfhdaA$  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-H<sub>2</sub>O yielding 10 subfractions (fractions 1–10). Fraction 2 (eluting with MeOH:H<sub>2</sub>O = 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-H<sub>2</sub>O (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 H<sub>2</sub>O and CH<sub>3</sub>CN (linear gradient of 15 % to 45 % CH<sub>3</sub>CN 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 pesthelic 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  $\Delta PfptaA \Delta PfclA$  or/and  $\Delta PfptaA \Delta PfhdaA$  mutants. Compound **1** was only produced in the  $\Delta PfptaA \Delta PfhdaA$  mutant strain, suggesting a successful activation by modulation of histone acetylation and *Pfpta* dereplication. Moreover, the deletion of *PfptaA* in  $\Delta PfclA$  or  $\Delta PfhdaA$  background host did not overproduce conidia as seen in the single mutant  $\Delta PfptaA$ . We also found that the  $\Delta PfptaA \Delta PfhdaA$  mutant is generally not sensitive to oxidative stressors in comparison with the single mutant  $\Delta PfptaA$  or the parental strains, whereas the  $\Delta PfptaA \Delta PfclA$  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.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms23073686/s1>.

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