VerZ, a Zn(II)$_2$Cys$_6$ DNA-binding protein, regulates the biosynthesis of verticillin in Clonostachys rogersoniana

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Abstract

Verticillins are the dimeric epipolythiodioxopiperazines (ETPs) produced by the fungus Clonostachys rogersoniana. Despite their profound biological effects, they are commonly produced in rice medium as complex mixtures that are difficult to separate, limiting further study and evaluation for this class of metabolites. Therefore, there is an urgent need to understand the regulation of verticillin biosynthesis. Recently, we cloned the biosynthetic gene cluster of verticillin (ver), and identified the only regulatory gene verZ in this cluster. The deduced product of verZ contains a basic Zn(II)$_2$Cys$_6$ DNA-binding domain. Disruption of verZ significantly reduced the production of 11'-deoxyverticillin A (C42) and decreased the transcriptional level of the verticillin biosynthetic genes. To further reveal its function, a recombinant gene encoding the DNA-binding domain of VerZ was expressed in E. coli and the His$_6$-tagged VerZbd was purified to homogeneity by Ni-NTA chromatography. Electrophoretic mobility shift assays (EMSAs) showed that VerZbd bound specifically to the promoter regions of the verticillin biosynthetic genes. Bioinformatic analysis of the VerZbd-binding regions revealed a conserved palindromic sequence of (T/C)(A/G)CC(G/T)(A/G)C. Base substitution of the conserved sequence completely abolished the binding activity of VerZbd to its targets. These results suggested that VerZ controls verticillin production through directly activating transcription of the biosynthetic genes in C. rogersoniana.

INTRODUCTION

Verticillins belong to the epipolythiodioxopiperazine (ETP) class of fungal secondary metabolites. ETPs are characterized by a disulphide bridge across a diketopiperazine ring and are synthesized from two amino acids [1]. Gliotoxin is the best class of fungal secondary metabolites. ETPs are characterized ETP, and is involved in mammalian mycotoxicosis invasive aspergillosis [2, 3]. Gliotoxin is produced by several fungi, including the opportunistic human pathogen Aspergillus fumigatus, Trichoderma spp. [4] and Penicillium spp. [5]. The results from in vitro and in vivo experiments showed that gliotoxin was both antiangiogenic and antitumoric in a preclinical model of prostate cancer [6].

The dimeric ETPs showed more potent antimicrobial activities compared to the monomeric ones [7, 8]. In 1970, verticillins were first isolated from Verticillium sp. and showed antimicrobial activity and antitumour effects [9]. One of this class of metabolites, 11'-deoxyverticillin A (C42) produced by the Cordyceps-colonizing C. rogersoniana (formerly Gliocladium sp.) [10–12], was found to induce autophagy and apoptosis of human tumour cells [13]. Due to their fascinating architecture and profound bioactivities, tremendous effort has been devoted to the synthesis of this class of metabolites, and the first total synthesis of 11,11'-dideoxyverticillin A was achieved via a bio-inspired strategy [14].

Verticillins are commonly produced in rice medium as a complex mixture of related compounds, but in low yields [10–12]. Since further separation and purification of individual metabolites are challenging, further study and evaluation are normally impossible for this class of metabolites due to sample limitations. Therefore, targeted production and isolation of a certain compound are highly desirable, which means that it is an urgent need to...
understand the regulation of biosynthesis of this class of metabolites.

Generally, the biosynthesis of fungal secondary metabolites is strictly controlled by transcriptional activators or repressors [15], and the zinc-binding proteins comprise one of the largest families of transcription regulators in eukaryotes [16]. Based on their binding motifs, the zinc-binding proteins are classified into three main groups, which include Cys2His2, Cys4 and Zn(II)2Cys6 [17], while the Zn(II)2Cys6 proteins (hereafter C6) have only been found in fungi [18]. Due to the feature that the majority of zinc finger proteins can bind to DNA (and also to RNA in the case of TFIIIA), the zinc finger proteins play significant roles in transcriptional and translational processes [19]. The transcriptional activator Gal4 in Saccharomyces cerevisiae is the best studied C6 protein [20], and it interacts with targets consisting of conserved terminal trinucleotides, which are usually in a symmetrical configuration and are spaced by an internally variable sequence of defined length, ranging from 2 to 17 nucleotides [17, 21].

Recently, our group cloned the biosynthetic gene cluster of verticillin (ver; GenBank accession no. KY359203) in the Cordyceps-colonizing C. rogersoniana [12]. In the ver biosynthetic cluster, there are 13 genes. The deduced products of the ver genes are similar to those of the gliotoxin biosynthetic genes in A. fumigatus [1]. The product of verP is responsible for the formation of the nonribosomal peptide skeleton. Disruption of verP completely abolished the production of verticillin. Other genes in the ver cluster include verA encoding a transporter protein, verL encoding a cyclopropanecarboxylic acid synthetase, verF containing a dipeptide enzyme, verC and verL encoding cystochrome P450 monooxygenases, verG encoding a glutathione-S-transferase, verT encoding thioredoxin reductase, verM encoding a O-methyltransferase and verN encoding a methytransferase [12]. Among these genes, verZ is the only one encoding a putative Zn(II)2Cys6 protein. Gene disruption and transcription analysis revealed that verZ plays a key role in the verticillin biosynthesis of C. rogersoniana. Furthermore, the binding motif of VerZ was identified by electrophoretic mobility shift assays (EMSAs) and base substitution analysis. To the best of our knowledge, this is the first report on the regulation of dimeric ETP verticillin biosynthesis.

METHOD

Strains, plasmids, culture media and growth conditions

The strains and plasmids used in this study are listed in Table S1 (available in the online Supplementary Material). For sporulation, C. rogersoniana was grown at 28 °C for 7 d in LPE medium [22, 23]. Generally, the fungus was grown on TSA medium [22, 23]. TSA medium with 200 µg ml⁻¹ hygromycin B or 500 µg ml⁻¹ G418 was used to screen the transformants. For Agrobacterium tumefaciens-mediated transformation (ATMT) of C. rogersoniana, minimal medium (MM), induction medium (IM) and co-cultivation medium (CM) were used [24]. Escherichia coli DH5α and BL21 (DE3) were routinely grown at 37 °C in Luria–Bertani (LB) medium [25] with antibiotics when required. For RNA extraction, 1 x 10⁹ spores of C. rogersoniana were pre-grown in 100 ml of TSA liquid medium at 28 °C for 24 h. After filtration, the fungal mycelia were washed thoroughly with distilled water and tiled on the rice medium containing 20 g rice per 30 ml distilled water. The fungal mycelia were collected at appropriate time intervals and kept frozen at −80 °C. To detect the production of verticillin, the cultures were collected at appropriate time, soaked with ethyl acetate (EtOAc) and evaporated to dryness under vacuum to produce the extract.

DNA sequencing, protein alignment and polymerase chain reaction

The fungal genomic DNA was extracted as described previously [22, 23]. The verZ gene (GenBank accession no. KY359203) was cloned from C. rogersoniana using specific primers designed via Primer Premier 5.0. The DNA and cDNA of verZ were amplified with verZ-F/verZ-R primers, and cloned into the vector pEASY-Blunt (TransGene, People’s Republic of China). The DNA sequencing was performed by the Biosune Company (Beijing, People’s Republic of China). To predict the domain architecture, the sequence analysis of VerZ and SirZ (GenBank accession no. AY553235) from Letosphaeria maculans, and GliZ (GenBank accession no. AY838877) from A. fumigatus was performed manually using InterProScan on the EBI web server (http://www.ebi.ac.uk/interpro/). The domains were then visualized using IBS (version 1.0.2) [26].

All of the primers used in this study are listed in Table S2. PCR analysis was performed using either the EasyTag DNA polymerase (TransGen Biotech) or KOD FX (TOYOBO, Japan). For PCR, an initial denaturation at 94 °C for 5 min was followed by 30 cycles of amplification (94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min), and an additional 10 min at 72 °C (or 68 °C for KOD FX). When considering different DNA templates and primers, the annealing temperature and elongation time were changed in some cases.

Constructions of the disruption mutant, the complemented strain and the verZ-overexpressed strain

In order to disrupt verZ, the 2.0 and 2.1 kb DNA fragments corresponding to the upstream and downstream of verZ were amplified from the genomic DNA of C. rogersoniana with the primers verZLB-F/verZRB-R and verZRB-F/verZRB-R, respectively. The amplified DNA fragments were inserted into pEASY-Blunt to generate pEASY-verZL and pEASY-verZ-R, respectively. After being verified by sequencing, pEASY-verZ was digested with KpnI/XhoI, and the digested DNA fragment containing the upstream of verZ was ligated into the corresponding site of pAgHG418 to give pAgHG418-verZ. Meanwhile, pEASY-verZ was digested with Ascl/PacI, and the digested DNA fragment...
containing the downstream of verZ was ligated into the corresponding site of pAgHG418-verZL. The resulting pAg-verZDM was introduced into the C. rogersoniana wild-type (WT) strain via ATMT as described previously [12]. The transformants were co-incubated on TSA at 28 °C for 3 d and selected on the TSA plates containing 200 µg ml⁻¹ hygromycin B and 400 µg ml⁻¹ cefotaxime. The transformants were further checked on the TSA plates containing 500 µg ml⁻¹ G418. Finally, the G418-sensitive and hygromycin-resistant transformants were selected and one verZ disruption mutant (ΔverZ) was confirmed by PCR with the outer primers Out-F/Out-R and the inner primers In-F/In-R. Southern hybridization was performed for further confirmation of ΔverZ with a non-radioactive digoxigenin DNA labelling and detection kit (Roche, Germany) as described previously [22]. A 500-bp DNA fragment was amplified from C. rogersoniana with the primers Probe-f/Probe-r and used as a probe. For Southern hybridization, the fungal genomic DNA was digested with EcoRI.

To complement ΔverZ, the intact verZ with its putative promoter region was amplified from the C. rogersoniana WT strain with the CverZ-F/CverZ-R primers, and the amplified 3313-bp DNA fragment was ligated into pEASY-Blunt to give pEASY-verZC. After being verified by sequencing, the Ascl/PacI-digested DNA fragment containing verZ was ligated into the same site of pAgG418. Then, the resulting pAgG418-verZC was introduced into ΔverZ by ATMT. After co-incubation on TSA at 28 °C for 3 d, the transformants were transferred to the TSA plates supplemented with 500 µg ml⁻¹ G418 and 400 µg ml⁻¹ cefotaxime, and the G418-resistant strains were selected. The complemented strain (verZC) was further verified by PCR with the primers RTverZ-F/RTverZ-R and Southern hybridization.

To overexpress verZ, pAgG418-verZC was introduced into the C. rogersoniana WT strain by ATMT. The G418-resistant strains were selected. The verZ overexpressed strains (VerZOE1, VerZOE2 and VerZOE3) were further verified by real-time RT-PCR. VerZOE3 was chosen for further experimentation.

**RNA isolation and real-time RT-PCR analysis**

The mycelia from WT, ΔverZ and VerZOE were collected after 1–7 d fermentation. The total RNA was extracted with TRIzol reagent (Invitrogen, USA) according to the manufacturer’s protocol. RT-PCR analysis was carried out as described previously [22, 23].

**Heterologous expression of verZ and protein purification**

The DNA fragment encoding the DNA-binding domain of VerZ was amplified from the cDNA of C. rogersoniana with the verZdb-F/verZdb-R primers. After being verified by sequencing, the amplified DNA fragment was digested with Ndel/XhoI and inserted into the corresponding sites of the plasmid pET30a (Novagen) to generate pET30a::verZdb. The resulting plasmid was introduced into E. coli BL21 (DE3), and gene expression was induced by the addition of 1 mM Isopropyl β-D-thiogalactopyranoside (IPTG) at OD₆₀₀=0.6, while cultivation of the culture continued at 28 °C in 200 ml LB with 100 µg ml⁻¹ kanamycin for an additional 3 h. Cell pellets were collected by centrifugation (6000 g, 5 min, 4 °C) and washed with the binding buffer (20 mM Tris–HCl, 500 mM NaCl, 5 mM imidazole; pH 7.9) and resuspended in 20 ml of the same buffer. The cells were lysed by ultrasonication on ice, and the lysate was cleared by centrifugation (12 000 g, 20 min, 4 °C). Then, the VerZdb-His₉ protein was purified by chromatography on nickel-nitrilotriacetic acid resin as described previously [22, 27]. The concentration of VerZdb-His₉ was measured using the BCA protein assay reagent (Novagen). Protein purity was determined by Coomassie blue staining after SDS-polyacrylamide gel electrophoresis (PAGE) on a 15 % polyacrylamide gel. The purified protein was stored with 5 % glycerol at −70 °C until it was used in the subsequent experiments.

**Electrophoretic mobility shift assays**

The interaction between DNA probes and VerZ was monitored by EMSAs as described previously [28]. In brief, DNA fragments containing the promoter regions of verA, verM, verK, verF, verG, verZ and verB, and the intergenic regions of verT-verL, verN-verI and verC-verP, were generated by PCR with the primers listed in Table S2. The probe actin was amplified by PCR with the actinPF/actinPR primers and used as the negative control. According to the manufacturer’s instructions, EMSAs were performed using a DIG gel shift kit, second generation (Roche) as described previously [22]. Varying quantities of VerZ-His₉ (0, 40, 60, 80, 100 and 120 nM) and 2 ng of the DIG-ddUTP-labelled oligonucleotide probes were used in the EMSAs.

**Bioinformatic analysis of the promoter regions of the verticillin biosynthetic genes in C. rogersoniana**

The nucleotide sequences upstream of the biosynthetic genes were examined to screen the palindrome motif using the MEME motif discovery tool [29–31]. Multiple analyses were performed by varying the width of the motif and the search was performed on both strands. The other parameters were not changed. The MEME program was run with the frequencies included in the parameters to calculate the probability of the motif occurring by chance [29]. Inclusion of the nucleotide and dinucleotide frequency across all the promoters increased the sensitivity of MEME program. The identified motif was processed using the BLOCKS multiple alignment processor and the logo program was used to generate the graph of the nucleotide frequency. This motif was then used in the MAST program, again including the nucleotide and dinucleotide frequency background file, to search for the occurrence of the motif in the promoters of the biosynthetic genes in C. rogersoniana. All of the settings were left as default, including the e-value cut-off of 10.

**Mutational analysis of the VerZ-binding sites**

To evaluate the identified binding motif of VerZ, DNA probes containing the mutagenized promoter regions of verC-P-1 and verC-P-2 were generated by PCR, and the
amplified DNA probes were ligated into pEASY-Blunt to give pEASY-verC-verP-1M and pEASY-verC-verP-2M, respectively. The conserved sequence of (T/C)(C/A)(G/T)GN3CC(G/T)(A/G)(G/C) at the promoter region of verC-verP was changed to CTTAAG and performed as described previously [32] with the primers listed in Table S2. After being verified by DNA sequencing, the binding ability of VerZ-His6 to the mutagenized probes was measured by EMSAs.

Chemical analysis

WT, ΔverZ, verZC and verZOE were grown on rice medium at 25 °C for 7 d. The production of 11'-deoxyverticillin A (C42) was analysed as described previously [12]. The fold differences in the production of 11'-deoxyverticillin for WT, ΔverZ, verZC and verZOE were measured according to the following formula: [area(sample)/area(blank)]/[area(sample)/area(Blank)].

Statistical analysis

For statistical analyses, the data were analysed using the GraphPad InStat software package version 5.01 (GraphPad Software, Inc.) according to the Tukey–Kramer multiple comparison test at P≤0.05.

RESULTS

verZ encodes a putative regulator in the verticillin biosynthetic gene cluster

The ver biosynthetic cluster contains 13 genes (Fig. 1). Based on sequence analysis, verZ consists of 1152 bp (GenBank accession no. KY359203), and no intron was found in its open reading frame through comparison of the DNA sequences with the cDNA of verZ. Sequence analysis showed that the deduced protein of verZ contains 418 amino acids and belongs to the Zn6Cys6 protein family (Fig. S1). The DNA-binding domain of VerZ was predicted using protein sequence analysis and classification software (http://www.ebi.ac.uk/interpro/). Since VerZ shows no end-to-end similarity with any known proteins, the DNA-binding domain of VerZ was used to effect alignment (Fig. 2). The DNA-binding domain of VerZ showed 45% amino acid identity with the regulator SirZ for sirodesmin biosynthesis in L. maculans [35], and 41% amino acid identity to the regulator GliZ for gliotoxin biosynthesis in A. fumigatus [1]. To determine whether verZ is related to the production of verticillin, the transcription of verZ was measured. In WT, the transcript of verZ reached the maximum level after 7 days of fermentation when verticillin production began (Fig. 3). Combined with the bioinformatic analysis, this implies that verZ is the regulatory gene responsible for the biosynthesis of verticillin in C. rogersoniana.

Disruption of verZ significantly decreased verticillin production in C. rogersoniana

To address its function, verZ was disrupted via homologous recombination (Fig. 4a). The plasmid pAg-verZDM was constructed and transformed into the WT C. rogersoniana by ATMT. Four disruption mutants of verZ were obtained from 600 transformants, and one of them was selected at random and confirmed by Southern hybridization and PCR (Fig. 4b-d). As shown in Fig. 4(a, b), a distinctive band of 6683 bp was amplified with the outer primers in the verZ disruption mutant (ΔverZ) and a 5514 bp band appeared in WT, as predicted. When using the inner primers, a 941-bp DNA fragment was amplified from the coding region of verZ in WT, but no fragment was amplified from ΔverZ. Southern hybridization showed a 2.7 kb band in ΔverZ and a 5.1 kb band in WT, confirming that verZ was replaced by the hygromycin phosphotransferase gene (hph) in ΔverZ. The complemented strain (verZC) was constructed by introducing the entire verZ gene into ΔverZ and confirmed by PCR and Southern hybridization analysis (Figs 4b–d and S2). After 7 d fermentation in rice medium, the production of verticillin in WT, ΔverZ, verZC and verZOE was detected by HPLC/MS (Figs 5 and S3). The production of 11’-deoxyverticillin A (C42) in ΔverZ decreased significantly, whereas verZC almost restored verticillin production. To further confirm its function in vivo, verZ was overexpressed and verified by real-time RT-PCR (Fig. S4). VerZOE3 was selected for fermentation because its expression was highest. The results demonstrated that overexpression of verZ increased the production of verticillin (Fig. 5).

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**Fig. 1.** The gene organization in the ver biosynthetic cluster, which spans a 32-kb DNA fragment and contains 13 genes. verA encodes a transporter protein; verT encodes a thioether reductase; verL encodes a cytochrome P450 monoxygenase; verM encodes an O-methyltransferase; verN encodes a methyltransferase; verL encodes a cytochrome P450 monoxygenase; verP encodes a non-ribosomal peptide synthetase; verK encodes a unknown protein; verJ encodes a dipeptide enzyme; verG encodes a glutathione-S-transferase; verZ encodes a zinc finger transcription factor; verB encodes an unknown protein.
Disruption of verZ significantly decreased the transcription of the verticillin biosynthetic genes

Since the production of C42 decreased in ΔverZ, the transcription level of the verticillin biosynthetic genes was measured by real-time RT-PCR. The highest transcriptional level of verZ was observed during 7-day fermentation in rice medium (Fig. 3). Disruption of verZ resulted in a significant decrease in transcription for all of the verticillin biosynthetic genes (Fig. 6). By contrast, their transcription level increased remarkably in verZOE, especially for verT. The transcription of verT increased 100-fold compared with that of WT. verT encodes a thioredoxin reductase that might be responsible for detoxification when verticillin production increases, since disruption of verT increased the fungal sensitivity to verticillin [12]. This indicated that the excessively increased production of C42 in verZOE is due to the transcriptional increase of verticillin biosynthetic genes. Therefore, verZ positively regulates verticillin production by enhancing the transcription of ver genes in C. rogersoniana.

VerZ directly bound to the upstream regions of the verticillin biosynthetic genes

To determine whether VerZ plays a direct role in controlling the biosynthesis of verticillin, EMSAs were performed as described previously [28]. Probes covering the upstream regions of verA, verM, verK, verJ, verG, verZ and verB, and the intergenic regions of verT-verL, verN-verl and verC-verP, were used in EMSAs. The upstream regions of verA, verC-verP, verZ and verB were divided into two or three parts as probes, and the length of each probe used in the EMSAs was around 500 bp (Fig. 7a). The recombinant gene (verZbd) encoding the VerZ DNA-binding domain was expressed in E. coli BL21 (DE3) and the His6-tagged VerZbd was purified by chromatography on nickel-nitrilotriacetic acid resin (Fig. 7b). Binding activity was enhanced with increased VerZbd-His6 (up to 120 nM) (Fig. 7c–l). The binding of VerZbd-His6 to the intergenic regions of verA, verG, verB and verT-verL and the upstream regions of verK, verN-verl and verC-verP formed one or two stable complexes, respectively, while VerZbd-His6 failed to bind to the upstream region of verJ (Figs 7c–l and S5). The specificity of the binding ability of VerZbd-His6 was further examined by adding excessive unlabelled probes, and excess in unlabelled specific probes clearly abolished the binding ability of VerZbd-His6 (120 nM) to the corresponding labelled fragments, but excessive actin probes did not abolish the binding ability of VerZbd-His6 (Fig. 7c–l). Viewing this in combination with the results from the transcription analysis, we can state that VerZ activates the transcription of the verticillin biosynthesis genes via direct interaction with their upstream regions in C. rogersoniana.

Identification of the VerZ-binding motif in the ver cluster

To further identify the specific binding motif of VerZ, sequences of the binding probes located upstream of the biosynthetic genes were searched as a single group for statistically overrepresented palindromic binding sites using the MEME motif discovery tool. A consensus motif \((\text{T/C})(\text{C/A})(\text{G/T})\text{GN}_3\text{CC}(\text{G/T})(\text{A/G})(\text{G/C})\) was
identified using different combinations of input sequences with motif width parameters (Fig. 8a). The motif was identified in the promoters of \textit{verA}, \textit{verT-verL}, \textit{verM}, \textit{verN-verI}, \textit{verC-verP}, \textit{verK}, \textit{verG}, \textit{verZ}, and \textit{verB} (Fig. 8b), but was not found in the promoters of \textit{verJ}, which was consistent with the results from EMSAs.

To evaluate the identified VerZ-binding motif, the conserved binding sequence of VerZ was mutagenized to CTTAAG. Base substitution analysis showed that the binding activity of VerZ to the mutagenized probes was completely abolished compared to their corresponding intact targets (Fig. 8c, d), indicating that the conserved motif is essential for the binding activity of VerZ.

**DISCUSSION**

Although the dimeric ETPs such as verticillin (C42) show profound bioactivity, little is known about their biosynthesis and

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**Fig. 4.** Construction of the \textit{verZ} disruption mutant (Δ\textit{verZ}). (a) Strategy for construction of the \textit{verZ} disruption mutant (Δ\textit{VerZ}) via homologous recombination. kb, kilobase pairs; bp, base pairs; \textit{hph}, the hygromycin phosphotransferase gene. (b) Confirmation of Δ\textit{verZ} by Southern hybridization. A 500-bp DNA fragment was amplified from \textit{C. rogersoniana} with the Probe-f/Probe-r primers and used as the probe in Southern hybridization. WT, the \textit{C. rogersoniana} wild-type strain; Δ\textit{VerZ}, the disruption mutant of \textit{verZ}; \textit{verZC}, the complemented strain of Δ\textit{verZ}. (c) Confirmation of Δ\textit{verZ} by PCR analysis. PCR1 and PCR2 were performed with the gene-inside (In-F/In-R) and the gene-outside (Out-F/OUT-R) primers, respectively. NC, the negative control. (d) Transcription of \textit{verZ} in WT, Δ\textit{verZ} and \textit{verZC}. All of the strains were grown in TSA liquid medium at 28 °C for 1 d, and tiled on rice medium at 28 °C for 7 d. The total RNA extraction and cDNA synthesis were performed as described in the Materials section. Transcription of \textit{verZ} and \textit{actin} was detected by RT-PCR with the RT\textit{verZ}-F/RT\textit{verZ}-R and actin-F/actin-R primers, respectively.

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regulation. Even for the monomeric ETPs, only the gene clusters for sirodesmin PL and the gliotoxin biosynthesis have been characterized [1]. Recently, a verticillin biosynthetic gene cluster (ver) was cloned in *C. rogersoniana* [12].

Low yield, difficult separation and a complex chemical synthesis process limit the further study of C42. It is important to regulate the production level of compound in this strain. The regulation of fungal secondary metabolism is very complex and operates on different regulatory levels, including pathway-specific and global regulators, and epigenetic control. Pathway-specific regulation is the most direct and effective method [36]. Like the gene clusters for sirodesmin PL and gliotoxin biosynthesis, the *ver* biosynthetic cluster contains a putative regulatory gene (*verZ*) that encodes a putative Zn(II)$_2$Cys$_6$ DNA-binding protein. However, amino acid identity among VerZ, SirZ and GliZ is only found in the DNA-binding domain. The binding motifs of SirZ and GliZ were only predicted by the MEME motif discovery tool [37]. In this study, the binding motifs of *verZ* were not only predicted by MEME, but also proved by EMSA and alterations of the VerZ-binding sequence. Our results demonstrated that *verZ* plays a positive role in regulating the biosynthesis of verticillin, e.g. *sirZ* in *L. maculans* and *gliZ* in *A. fumigatus*. *verZ*, as the only transcription factor in the verticillin biosynthetic gene cluster, is an ideal candidate for improving the production of C42 in *C. rogersoniana*.

As mentioned above, VerZ, SirZ and GliZ contain the Zn(II)$_2$Cys$_6$ DNA-binding domain. The DNA-binding motif (T/C)
(C/A)(G/T)GN3CC(G/T)(A/G)(G/C) of VerZ was similar to the DNA-binding motif (consensus TCGGN3CCGA) of SirZ. In L. maculans, ΔSirZ did not produce detectable levels of sirodesmin PL [37]. In A. fumigatus, ΔgliZ also lost the production of gliotoxin [38]. However, ΔVerZ still produced 11′-deoxyverticillin A, although at a very low yield compared with WT. These differences imply that the regulation of verticillin biosynthesis is complicated, and other unknown regulatory factors related to verticillin production may exist in C. rogersoniana.

By searching the statistically overrepresented palindromic binding sites, the potential binding sites for several pathway-specific regulators, such as SirZ, GliZ and PglGliZ, in the
 promoter regions of the ETP biosynthetic genes were predicted in *L. maculans*, *A. fumigatus* and *Penicillium lilacinoechinulatum*, respectively [37]. Although the identified sites were similar to those commonly found for the Zn(II)$_2$Cys$_6$ proteins, their presence was not always consistent with the regulation pattern of the genes. For example, the palindromic element, consensus (T/C)(C/A)(G/T)GN$_3$CC(G/T)(A/G)(G/C), was found in the promoters of all the *ver* genes, but not in *verJ*. This is unsurprising, as the DNA-binding capacity of transcription regulators typically deviates from a core consensus, and is likely dependent on other sequences and proteins presented on the promoter. It has been demonstrated that AflR might actually recognize several binding sites with fairly significant deviation from the core sequence [39].

In conclusion, VerZ was demonstrated to positively regulate the transcription of the verticillin biosynthetic genes by binding directly to their promoter regions in *C. rogersoniana*. The illustration of the mechanism for regulating the biosynthesis of verticillin could provide insights into increasing the production of verticillin in *C. rogersoniana*.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


**Fig. 8.** Putative Zn(II)$_2$Cys$_6$ DNA-binding motif in the promoter regions of the biosynthetic genes in *C. rogersoniana*. (a) A logo diagram of the predicted palindromic element shows the frequency of nucleotides in the statistically overrepresented sequences. (b) A logo diagram of the element was predicted using the sequences of *C. rogersoniana* with a nucleotide and dinucleotide frequency background model derived from the upstream regions of all the biosynthetic genes. The locations of the DNA-binding motif are shown in the upstream regions of the biosynthetic genes. (c, d) EMSAs with the probe containing a mutagenized VerZ-binding site. BS, probe without mutagenesis as a control; M, probe with base substitution.
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