Antifungal Activity of Chaetoviridin A from *Chaetomium globosum* CEF-082 Metabolites Against *Verticillium dahliae* in Cotton

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Cotton Verticillium wilt (CVW) is a severe soilborne disease caused by the pathogen *Verticillium dahliae*, and it has a great impact on cotton production. Previous studies found that the biocontrol agent *Chaetomium globosum* CEF-082 and its metabolic filtrate could reduce the incidence of CVW; however, the underlying mechanism remains unclear. The metabolic crude extract of CEF-082 increased the sensitivity of *V. dahliae* to stress, degraded the cell wall of *V. dahliae*, and increased the emergence and plant height of cotton. Through separation and purification of the metabolic crude extract of CEF-082, chaetoviridin A was identified and found to be highly active against *V. dahliae*. The compound caused cell necrosis and mycelial deformation, increased the production of reactive oxygen species and nitrous oxide, and inhibited the germination of microsclerotia of *V. dahliae*, enhancing the cotton plant defense response. In addition, CEF-082 also colonized cotton plants.

Keywords: activity, biocontrol, chaetoviridin A, cotton, *Verticillium dahliae*

Cotton Verticillium wilt (CVW) is a soilborne fungal disease caused by *Verticillium dahliae* Kleb., which can cause decreases in cotton yield and quality (James 2003). At present, CVW is the most important cotton disease in the world (Z. Zhang et al. 2016). There is a lack of resources with natural disease resistance in cotton breeding, and there are no effective fungicides to

control the disease. Biological control agents (BCAs) are environmentally derived and safe and have become important means of CVW control. There is also an urgent need to find an effective and environmentally friendly fungicide for controlling plant diseases in general (Zhao et al. 2017).

Chaetomium spp. have been documented as potential BCAs for plant diseases. *Chaetomium globosum* CDW7 exhibited strong inhibitory activity against rape sclerotinia rot caused by *Sclerotinia sclerotiorum* (Zhao et al. 2017). Nanosilver produced by *C. globosum* can control Fusarium wilt of tomato (Madbouly et al. 2017). *C. globosum* was used for the biocontrol of potato late blight disease (Shanthiyaa et al. 2013). *C. globosum* strain 22-10 significantly inhibited the growth of *Bipolaris sorokiniana*, causal agent of common root rot of wheat (Yue et al. 2018). The main mechanisms by which *C. globosum* controls pathogenic fungi are the induction of disease resistance (Istifadah and McGee 2006), the secretion of bioactive metabolites (Yan et al. 2018), and the promotion of host growth (Tarafdar and Gharu 2006). The inhibition of pathogen growth by antifungal substances is a very important mechanism of action of *C. globosum*, and many diverse bioactive metabolites such as trichoglobulin, azaphenanthrone, xanthone, and steroids have been identified from *C. globosum*. The application of active antifungal substances produced by microorganisms has become a research hotspot (Yan et al. 2020).

Chaetoviridins are usually called azaphilones and contain a pyrone-quinone structure (Park et al. 2005). Chaetoviridin A has been separated from *C. globosum*, and it can inhibit the growth of *S. sclerotiorum*, *Rhizoctonia solani*, *Magnaporthe grisea*, and *Pythium ultimum* (Awad et al. 2014; Park et al. 2005; Yan et al. 2018). McMullin et al. (2013) found that chaetoviridin A was separated from the filtrates of 19 of 25 strains of *C. globosum*. However, the biological effects of chaetoviridin A on *V. dahliae* still remain untested.

In past research, we also isolated *C. globosum* CEF-082, which has a certain control effect against CVW (Y. Zhang et al. 2016). CEF-082 could induce the defense response of cotton and improve resistance to CVW (Zhang et al. 2020). The non-volatile compounds of CEF-082 also suppressed the growth of *V. dahliae*, and the inhibition rate was 100% (Y. Zhang et al. 2016). To study the active substances and the antifungal mechanism involved in CEF-082 control of *V. dahliae*, the active substance chaetoviridin A in the fermentation broth of CEF-082 was isolated and identified.

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RESULTS

Antagonistic effect of CEF-082 on *V. dahliae*.

In the dual culture, CEF-082 inhibited the colony expansion of *V. dahliae* strains Vd080, Vd129, and Vd147 and promoted the production of microsclerotia of Vd080 and Vd147 (Fig. 1). However, CEF-082 did not promote the production of microsclerotia of Vd129.

The stability of CEF-082 crude extract under high temperature.

In order to determine the stability of these compounds, the CEF-082 crude extract was autoclaved. Two days after the crude extract was added to the Oxford cups, an inhibition zone could be observed, and the inhibition zone of the 121°C treatment was significantly smaller than that of the 25°C treatment. The diameters of the inhibition zones in the 121°C, 25°C, and control (CK) groups were 1.4, 2.6, and 0.8 cm, respectively (Fig. 2). This indicates that high temperature leads to deactivation of some substances in the CEF-082 crude extracts.

Effect of CEF-082 crude extract on the internal structure of *V. dahliae* Vd080.

In the treatment group, the spores of *V. dahliae* Vd080 were treated with the CEF-082 crude extract and were compared with the CK (Fig. 3A). Their cell walls were incomplete, black particles were attached to the cell walls, the inner color of the cells became lighter, the structure was incomplete, and the organelles and other structures were degraded (Fig. 3B). The results showed that the crude extract could destroy the cells and internal structures of *V. dahliae* Vd080.

Crude extract increased the sensitivity of *V. dahliae* Vd080 to stress.

The more sensitive *V. dahliae* is to stress, the less likely it is to survive. To study whether the crude extract of CEF-082 can affect the sensitivity of *V. dahliae* to stress, the crude extract of CEF-082 was applied to a plate of complete medium (CM) supplemented with sodium dodecyl sulfate (SDS), sorbitol, and Congo red or to CM alone; then, a 2- μ l spore suspension of *V. dahliae* Vd080 was dripped onto the plate. Methanol was used to replace the crude extract of CEF-082 in the CK group. After 2 days of culturing at 28°C, the growth of *V. dahliae* Vd080 on the CM only was almost unaffected by treatment with the CEF-082 crude extract, whereas the concentration required for the growth of *V. dahliae* Vd080 in the treatment group increased by at least one order of magnitude compared with that of the CK group without the CEF-082 crude extract. The concentration required for the growth of *V. dahliae* Vd080 on the plate

amended with SDS changed from 1×10^3 spores/ml to 1×10^5 spores/ml, that on the plate amended with sorbitol changed from 1×10^5 spores/ml to 1×10^6 spores/ml, and that on the plate amended with Congo red changed from 1×10^4 spores/ml to 1×10^5 spores/ml (Fig. 4). The results showed that the low concentration group of *V. dahliae* Vd080 could not grow after treatment with the CEF-082 crude extract, and the sensitivity of *V. dahliae* Vd080 to osmotic stress and cell wall inhibition stress was obviously increased.

CEF-082 crude extract promotes cotton growth.

The emergence rates of cotton in the CK and treatment groups were 53.3 and 60.0%, respectively. No negative effect of the crude extract on the emergence, plant height, root length and fresh weight of cotton was found, and the extract increased the emergence and plant height (Table 1).

CEF-082 colonizes cotton plants.

No fungal growth occurred on the CK plate, and fungi similar to CEF-082 grew on the treatment plates (Fig. 5A). The fungal strains were purified, their DNA was extracted, and the fragments were amplified with the universal primers internal transcribed

Fig. 2. Stability of CEF-082 crude extract under high temperature. After 121°C, the activity of the crude extract decreased obviously but the inhibition zone still formed. CK = control.

Fig. 1. Inhibition effect of CEF-082 on *Verticillium dahliae*. A, *V. dahliae* Vd147; B, *V. dahliae* Vd129; C, *V. dahliae* Vd080. The red arrow refers to microsclerotia.

spacer (ITS)4 and ITS5 (Fig. 5B). Bands of 500 to 750 bp were generated, and the sequencing results were compared with the sequences of CEF-082 (Supplementary Fig. S2); the similarity among these sequences was 100%. These results indicated that CEF-082 recolonizes cotton plants.

Extracellular enzyme activity of CEF-082.
 Proteins are present in the cell wall of *V. dahliae*, and cellulose and pectin are present in the cell wall of plants. Pectin lyase can enhance cotton resistance to *Verticillium* wilt and induce cell apoptosis of *V. dahliae* strain Vd080 (Zhang et al. 2021).

Fig. 3. Spore cross sections. **A**, Control and **B**, spores of *Verticillium dahliae* Vd080 treated with CEF-082 crude extract. The red arrow refers to the damaged cell wall and the green arrow refers to the normal cell wall. Cell contents of treated spores were also affected by treatment.

Fig. 4. Sensitivity of *Verticillium dahliae* to crude extract. CM = complete medium and SDS = sodium dodecyl sulfate. Concentrations of *V. dahliae* from left to right were 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , and 1×10^3 spores/ml, respectively.

Table 1. Crude extracts of *Chaetomium globosum* CEF-082 increased cotton growth^z

Treatment	Emergence rate (%)	Plant height (cm)	Root length (cm)	Fresh weight (g)
Control	53.3 b	6.5 ± 0.3533 b	10.3 ± 2.4159 a	0.661 ± 0.0292 a
CEF-082	60.0 a	7.4 ± 0.4524 a	10.3 ± 2.3758 a	0.671 ± 0.0399 a

^z Different lowercase letters indicate significant difference at the level of $P < 0.05$.

Fig. 5. Isolation and identification of CEF-082. **A**, Numerals 1 and 2 indicate strains similar to CEF-082. **B**, Lanes 1, 2, and 3 are the isolated strain A-2, CEF-082, and negative control, respectively. Lane M indicates the DL2000 marker.

Fig. 6. Extracellular enzyme activity of CEF-082. **A**, Protease activity: the transparent circle indicates the protease degrading the protein on the plate; and **B**, cellulase activity: the transparent circle indicates cellulase degrading the cellulose on the plate.

Fig. 7. Chaetoviridin A inhibited the growth of *Verticillium dahliae*. **A**, Purification of chaetoviridin A by high-performance liquid chromatography; **B**, structure of chaetoviridin A (Phonkerd et al. 2008); and **C**, active substance inhibited colony formation of *V. dahliae*. Control (CK) = methanol + spores of *V. dahliae* and Treatment = chaetoviridin A (2 mg/ml, dissolved in methanol) + spores of *V. dahliae*.

These enzymes are of great value for colonization, survival, and reproduction of beneficial microorganisms in the field (Liu et al. 2012). In order to study the relationship between extracellular enzyme activity and antagonistic effect and plant colonization of CEF-082, the extracellular protease, cellulase, and pectinase activities of *C. globosum* CEF-082 were detected. CEF-082 was cultured on plates containing protein, pectin, and cellulose. Five days later, transparent circles were produced on plates containing protein and cellulose (Fig. 6), whereas no transparent circles were produced on plates containing pectin. The results showed that CEF-082 has protease activity (Fig. 6A) and cellulase activity (Fig. 6B).

Purification and identification of CEF-082 metabolites.

After separation and purification, a pure compound was obtained (Fig. 7A). The compound contained crystals that were reddish brown, needle-like, and soluble in methanol and chloroform. The compound was identified as a known compound, chaetoviridin A, and its structure was established by integrated interpretation of electrospray ionization-mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR) spectra (Phonkerd et al. 2008) (Fig. 7B; Table 2). Spores of *V. dahliae* Vd080 did not form colonies 5 days after treatment with this compound (Fig. 7C). After 12 days, no colonies of *V. dahliae* were observed. The results showed that chaetoviridin A suppressed the growth of *V. dahliae*.

Chaetoviridin A inhibited the germination of *V. dahliae* Vd080 microsclerotia.

Three days after treatment with chaetoviridin A, the germination rates of *V. dahliae* Vd080 microsclerotia in the CK group and the 75- μ g/ml and 150- μ g/ml treatment groups were 72.02, 50.24, and 7.33%, respectively (Fig. 8). The germination rates of the two treatment groups were significantly lower than that of the CK group. Therefore, chaetoviridin A inhibited the germination of microsclerotia of *V. dahliae*.

Inhibitory mechanism.

After *V. dahliae* was treated with chaetoviridin A, its mycelial growth was inhibited (Supplementary Fig. S1), and the mycelium

on the edge of inhibition zone appeared shrunken (Fig. 9A), which may be indicative of damage to the internal structure of the mycelium. Comparing the number of apoptotic cells with the number of necrotic cells, almost all cells were found to be necrotic after the treatment with chaetoviridin A (Fig. 9B). The results showed that chaetoviridin A destroyed the integrity of the cell membrane. After treatment with chaetoviridin A for 12 h, the production of reactive oxygen species (ROS) and nitrous oxide (NO) was significantly increased compared with that of the CK (Fig. 10).

Expression of genes related to the cell wall in *V. dahliae* Vd080.

After chaetoviridin A treatment, the related genes of cell wall synthesis, mainly glucanase and chitinase genes, were affected to varying degrees. These genes were mostly downregulated at 1 and 2 h after treatment, indicating that cell wall synthesis was inhibited. After 6 h, three of five chitin-related genes were upregulated (Fig. 11). The results indicated that chaetoviridin A destroyed the cell wall integrity of *V. dahliae*.

Chaetoviridin A enhanced the defense response of cotton plants.

After cotton leaves were treated with chaetoviridin A (150 μ g/ml) for 24 h, they were inoculated with *V. dahliae*. After 24 h, no significant difference in ROS accumulation was found between the chaetoviridin A + water and chaetoviridin A + *V. dahliae* groups. ROS accumulation in the water + *V. dahliae* group was decreased compared with that of the two groups treated with chaetoviridin A (Fig. 12). This experiment showed that chaetoviridin A enhanced the defense response of cotton plants.

DISCUSSION

C. globosum is beneficial to many plants. *Chaetomium* spp. can control root rot diseases of black pepper, strawberry, and citrus and late blight disease of potato (Shanthiyaa et al. 2013; Tomilova and Shternshis 2006). *C. globosum* TAMU 520 negatively affects both above- and belowground herbivores in cotton (Zhou et al. 2016); *C. globosum* D38 promotes the accumulation of bioactive constituents and root production in *Salvia miltiorrhiza* (Zhai et al. 2018). *C. globosum* strain TAMU 520 was isolated from cotton (*Gossypium hirsutum*), successfully reinoculated onto cotton, and also found to systemically colonize cotton plants via seed treatment (Zhou et al. 2016); in this study, CEF-082 was also reinoculated onto cotton and found to systemically colonize the plants by root irrigation. Additionally, the cellulase activity of CEF-082 may promote its colonization of cotton plants because the cell wall of plants mainly contains cellulose and pectin. The protease activity may have an antagonistic effect on *V. dahliae*, because the cell wall of *V. dahliae* contains structural proteins and the protease can degrade the proteins in the cell wall. In our previous study, *C. globosum* CEF-082 could control CVW (Y. Zhang et al. 2016). CEF-082 colonization and protease activity are speculated to promote the control of CVW.

C. globosum has been found for a long time to control plant diseases, and it is an important source of living secondary metabolites. The fermentation broth of *C. globosum* CDW7 significantly inhibited the germination of *S. sclerotiorum* and inhibited the development of *S. sclerotiorum* on rape leaves in vitro (Zhao et al. 2017). In this study, the fermentation broth crude extracts of CEF-082 suppressed the growth of *V. dahliae*, destroyed the cell wall of *V. dahliae*, and increased the sensitivity of *V. dahliae* to osmotic stress and cell wall inhibition stress. Therefore, the crude extract very likely increased the sensitivity

Table 2. Hydrogen nuclear magnetic resonance ($^1\text{H-NMR}$, δ_{H}) and carbon nuclear magnetic resonance ($^{13}\text{C-NMR}$, δ_{C}) data for the compound in CDCl_3

Position	δ_{C}	δ_{H}
1	151.8	8.78 (s)
3	157.1	—
4	105.6	6.55 (s)
4a	139.8	—
5	109.1	—
6	183.5	—
7	87.8	—
8	163.1	—
8a	110.6	—
9	119.9	6.09 (d)
10	148.3	6.61 (dd)
11	39.2	2.30 (quint)
12	29.3	1.42 (m)
13	11.9	0.92 (t)
14	19.5	1.10 (d)
15	26.5	1.71 (s)
16	168.1	—
17	125.0	—
18	201.3	—
19	51.1	3.62 (t)
20	71.1	3.88 (t)
21	13.8	1.18 (d)
22	21.7	1.18 (d)

of *V. dahliae* to oxidative stress. Chaetoviridin A also induced the ROS produced in cotton leaves. Overall, the crude extract may enhance plant defense by making *V. dahliae* more sensitive to the ROS produced by its plant host. In addition, a part of the crude extract is resistant to high temperature (121°C), and it is easily applied in the field.

Chaetoglobosin A is produced by *C. globosum* NK102, and it has activity against *Meloidogyne incognita* (Hu et al. 2013). In this study, we found that chaetoviridin A produced by CEF-082 suppressed the growth of *V. dahliae*. Chaetoviridins were first characterized from *C. globosum* var. *flavo-viridae* (Takahashi et al. 1990). Chaetoviridin A exhibits inhibitory activity against various plant pathogens (Table 3) and strong in vivo activity against the rice blast and wheat leaf rust pathogens (Park et al. 2005). Chaetoviridin A exhibits antifungal activity against *S. sclerotiorum*, *Botrytis cinerea*, *Fusarium graminearum*, *Phytophthora capsici*, and *F. moniliforme* (Yan et al. 2018). In

this study, we found for the first time that chaetoviridin A can control *V. dahliae*, inhibiting the germination of microsclerotia and degrading the cell walls of the pathogen.

Tomoda et al. (1999) found that chaetoviridin A inhibits the cholesteryl ester transfer protein. In this study, after treatment with chaetoviridin A, NO production, ROS accumulation, and cell wall degradation were induced. In addition, cell wall degradation was consistent with the corresponding changes in gene expression. This result was similar to that of Han et al. (2015), who found that treatment of *V. dahliae* with iturins resulted in cell necrosis and the inability of the pathogen to germinate and grow.

MATERIALS AND METHODS

Fungal strain culture.

The endophytic fungus *C. globosum* CEF-082 was isolated from healthy cotton plants, inoculated on potato dextrose agar

Fig. 8. Chaetoviridin A inhibited the germination of microsclerotia of *Verticillium dahliae*. **A**, Germination of the microsclerotia after treatment with chaetoviridin A. CK = control. **B**, Germination rates of the microsclerotia after treatment with chaetoviridin A. **C**, Appearance of microsclerotia after germination. Bar = 50 µm.

(PDA) plates, and cultured in darkness at 25°C for 7 days. The edge of a colony was picked and placed into a 1,000-ml Erlenmeyer flask. Each flask was filled with 400 ml of potato dextrose broth (PDB), brought to a total of 50 liters, and cultured at 25°C and 150 rpm for 15 days. Then, the mycelium was removed by filtration with four layers of gauze, and the filtrate was extracted three times by an equal volume of ethyl acetate and evaporated to dryness at 37°C to obtain a crude extract.

V. dahliae Vd080, Vd129, and Vd147 were provided by the Institute of Cotton Research of Chinese Academy of Agricultural Sciences. They were inoculated on PDA plates and cultured in darkness at 25°C for 7 days. Then, Vd080 was inoculated into liquid Czapek-Dox medium (Zhang et al. 2020) and placed at 25°C and 150 rpm for 7 days. The mycelia were filtered out and removed, and the filtrate was subsequently diluted to spore suspensions of 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , and 1×10^3 spores/ml.

A spore suspension of CEF-082 was obtained by adding sterile water to the plates, rubbing a sterile spatula over the colonies, and, finally, filtering the suspensions through sterile cheese cloth and diluting the suspensions to form a spore suspension of 1×10^5 spores/ml (Zhang et al. 2020).

Dual-culture antagonism bioassay against *V. dahliae*.

Plugs (5 ml in diameter) were taken from the edges of colonies of CEF-082 and Vd129 with a sterilized perforator and were inoculated onto the same PDA plate at an interval of 3 cm. Vd147 and Vd080 were treated in the same way. The inhibitory effect was observed after culturing at 25°C for 12 days. The experiment was repeated three times.

Determination of CEF-082 crude extract activity under high temperature.

CEF-082 crude extract (0.025 g/ml, dissolved in methanol) was autoclaved (121°C) for 30 min in the treatment group; in the CK group, the crude extract were placed at 25°C for 30 min and filtered for sterilization. The spore suspension of Vd080 of 1×10^7 spores/ml was applied to the PDA plates. Three Oxford cups were symmetrically placed on the PDA, and 100 µl of crude extract treated at 121 and 25°C was added to the Oxford cups. Methanol was used as a CK. Then, the PDA plates were cultured at 28°C for 2 days, and the inhibition zones were observed.

Effect of crude extract on *V. dahliae* Vd080 cell structure.

The spores of *V. dahliae* Vd080 were treated with sterile water containing CEF-082 crude extract at 5 mg/ml (final concentration) (dissolved in methanol, 10 µl), and 10 µl of methanol instead of crude extract was used as a CK. The spores were cultured in darkness at 28°C for 2 h, then washed three times with PBS. The spores were fixed with 3% glutaraldehyde for 2 h at 4°C and washed with 0.1 M PBS three times, for 10 min each time. The spores were immersed in 1% osmic acid solution; placed at 4°C for 1 h; washed with 0.1 M PBS three times, each time for 10 min; dehydrated with a 50, 70, and 90% ethanol gradient for 15 min; and dehydrated with 100% acetone three times, each time for 10 min. Epon 812 epoxy resin was used as the embedding agent. After embedding, fixed spores were put into a constant temperature box for polymerization, and ultrathin sections were cut and stained with uranyl acetate and lead citrate for 30 min. The samples were observed and recorded with a transmission electron microscope.

Fig. 9. Effect of chaetoviridin A on mycelia and spores of *Verticillium dahliae*. **A**, Effect of chaetoviridin A on mycelia. The green arrow refers to the normal mycelium and the red arrow refers to the shrunken mycelium. CK = control. **B**, Cell necrosis after treatment with chaetoviridin A. Bar = 10 µm. LFM = light-field microscopy; FITC = Annexin V-fluorescein isothiocyanate, where green fluorescence indicates apoptotic + necrotic cells; and PI = propidium iodide, where red fluorescence indicates necrotic cells.

The effect of CEF-082 crude extract on the sensitivity of *V. dahliae* Vd080.

Crude extract (50 μ l, 8 mg/ml) was coated on CM plates containing 0.002% SDS, 1 M sorbitol, and 0.02% Congo red. Methanol was used as a CK, and the CK medium was CM. After air drying, the plates were inoculated with the *V. dahliae* Vd080 spore suspension. From left to right, the five concentrations were 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , and 1×10^3 spores/ml. Each concentration (2 μ l) was applied (Han et al. 2015), the application was repeated three times, and the cultures were maintained in the dark at 28°C for 2 days.

Safety test of CEF-082 crude extract to cotton.

Jimian 11 was provided by the Institute of Cotton Research of Chinese Academy of Agricultural Sciences. The cotton seed were sterilized with 70% alcohol for 1 min and with 1.05%

sodium hypochlorite for 10 min, then washed with sterile water five times. Then, cotton seed were soaked in CEF-082 crude extract (4 mg/ml) for 5 h. The cotton seed were planted in vermiculite at 60 seeds/treatment and cultured at 25°C; three repetitions were used for each treatment. After 10 days, cotton seedling emergence was investigated, and the plant height, root length, and fresh weight of the cotton seedlings were measured 20 days later.

Colonization of cotton plants with CEF-082.

Jimian 11 cotton seed were sterilized with 70% alcohol for 1 min and with 1.05% sodium hypochlorite for 10 min, then washed with sterile water five times. The cotton seed were planted in vermiculite and nutrient-amended soil (2:1, vol/vol). Twenty plants were cultured per pot for each treatment. Plants were inoculated with CEF-082 spore suspension of 1×10^5

Fig. 10. Nitrous oxide (NO) and reactive oxygen species (ROS) production after treatment with chaetoviridin **A**, ROS production after treatment with chaetoviridin A. Green fluorescence indicates the production of NO. CK = control, LFM = light-field microscopy, DCFH-DA = 2,7-dichlorodihydrofluorescein diacetate, and bar = 10 μ m. **B**, ROS production after treatment with chaetoviridin A. Green fluorescence indicates the production of ROS. DAF-FM DA = diaminofluorescein-FM diacetate and bar = 10 μ m.

spores/ml at 200 ml/pot when the first two true leaves became flattened, and sterile water was used as a CK. Every treatment was repeated three times. Cotton plants were cultured at 25°C (16 h of light and 8 h of darkness) in 70% relative humidity for 24 h. Samples were randomly collected 2 weeks postinoculation with CEF-082, and each treatment was repeated three times. Cotton stem tissues were cut into approximately 1-cm-long pieces, soaked in 75% alcohol for 1 min, soaked in 1.05% sodium hypochlorite for 30 min, washed with sterile water five times, then put into PDA (with kanamycin). After washing, the stems were placed on PDA for 10 min; then, the stem tissue was removed as a negative CK. The plates were cultured at 25°C for 10 days. A strain similar to CEF-082 was purified. After PDB culture, mycelia were collected for DNA extraction, and the fragments were amplified with the universal primers of *C. globosum* ITS4 and ITS5 (Khan and Bhaduria 2018). PCR products were analyzed by 1% agarose gel electrophoresis and observed under UV light after purification with an Extract DNA purification kit (catalog DP1501; Biotek Corporation, Beijing, China), then sequenced at Beijing Genomics Institute (Shanghai, China). The ITS sequences were compared with those of CEF-082 by Clustalx (v1.83).

Extracellular enzyme activity of CEF-082.

To detect the extracellular enzyme activity of CEF-082, media for cellulase, pectinase, and protease detection were prepared (Han et al. 2015). Then, the plates were poured, and a 5-mm-diameter plug of CEF-082 was inoculated onto the centers of the plates. After 5 days, the plates were soaked in Congo red

(1 mg/ml) for 20 min, then washed with NaCl (1 mol/liter) three times, and transparent circles were observed.

Purification and structural identification of CEF-082 metabolites.

After rotary evaporation, 35 g of crude extract of CEF-082 was obtained. Crude extract was separated using silica gel column chromatography eluted with a mixture of CH₂Cl₂ and MeOH (100:0 and 100:1, vol/vol) to obtain one fraction (2.6 g). The fraction was subjected to silica gel column chromatography and eluted with a mixture of petroleum ether and ethyl acetate (1 liter each of 15:1, 10:1, 5:1, and 3:1, vol/vol) to yield seven fractions (F-1 = 179 mg, F-2 = 60 mg, F-3 = 854 mg, F-4 = 242 mg, F-5 = 282 mg, F-6 = 359 mg, and F-7 = 212 mg). Only F-2 had good activity against *V. dahliae*. F-2 was eluted with MeOH using a Sephadex LH-20 column to obtain two fractions. Then, the two fractions were purified by preparative high-performance liquid chromatography (column: Kromasil 100-5-C18 [Akzonobel, Bohus, Sweden], 250 by 10 mm, mobile phase: acetonitrile-H₂O [80:20]) to yield chaetoviridin A (11 mg, retention time = 11.773 min).

The PDA plate was perforated, 20 µl of chaetoviridin A (2 mg/ml, dissolved in methanol) was added, and the plate was air dried; then, 20 µl of Vd080 spore suspension was added. After air drying, the plate was cultured in the dark at 28°C and the colony size was observed. The experiment was repeated three times.

The molecular structure was identified by integrated interpretation of ESI-MS and NMR spectra.

Fig. 11. Expression of genes related to the cell wall. CK = control and bars represent standard error of the mean. Different letters indicate significant difference at the level of $P < 0.05$.

Detection of *V. dahliae* Vd080 cell necrosis.

In the treatment group, the spores of *V. dahliae* Vd080 were treated with chaetoviridin A (sterile water containing chaetoviridin A dissolved in methanol) at 150 µg/ml for 12 h, and the CK group was treated with sterile water containing methanol. The spores were collected by centrifugation and washed with PBS three times, then gently resuspended in 195 µl of Annexin V-fluorescein isothiocyanate (V-FITC). Then, 5 µl of Annexin V-FITC and 10 µl of propidium iodide staining solution were added for double staining, and the solution was mixed gently. The samples were incubated at room temperature (20 to 25°C) in the dark for 10 to 20 min, then placed in an ice bath. During the incubation process, the spores were resuspended two to three times to improve the staining effect. The spores were collected after centrifugation at $1,000 \times g$ for 5 min. The spores were gently resuspended with 50 to 100 µl of Annexin V-FITC and observed under a laser confocal microscope.

The effect of chaetoviridin A on the germination of *V. dahliae* Vd080 microsclerotia.

Vd080 was inoculated on PDA for 20 days; then, the microsclerotia of *V. dahliae* were collected from the plate with a toothpick, while trying to avoid removing any attached PDA. The microsclerotia were placed into a 1.5-ml centrifuge tube, ground with a grinding rod, and diluted with sterile water. Thus, a suspension of microsclerotia was obtained. In new 1.5-ml centrifuge tubes, chaetoviridin A dissolved in methanol was added, and methanol was used as a CK. Then, the chaetoviridin A in the tubes was diluted to concentrations of 75 and 150 µg ml⁻¹

with the suspension of microsclerotia. The tubes were cultured at 4°C for 48 h, washed with sterile water three times, added to 1 ml of PDB, and cultured at 28°C and 150 rpm for 3 days. Then, the germination of microsclerotia was observed under the microscope; microsclerotia that produced hyphae ≥ 50 µm long were considered to be germinating, and 100 microsclerotia were observed in each treatment, which was performed in triplicate.

ROS burst in *V. dahliae* Vd080 induced by chaetoviridin A.

The *V. dahliae* Vd080 spore suspension (1 ml) was placed into a 1.5-ml centrifuge tube, and the sediment was collected by centrifugation at 5,000 rpm for 1 min. This process was repeated three times, and 3 ml of the *V. dahliae* spore suspension was collected in total. The sterile water containing chaetoviridin A at 150 µg/ml dissolved in methanol was added to the centrifuge tube, and the contents were cultured in darkness at 25°C for 12 h. Sterile water containing methanol was used as the CK. The spores were collected by centrifugation and washed three times with PBS. 2,7-Dichlorodi-hydrofluorescein diacetate (DCFH-DA) was diluted with PBS at 1:1,000 to a final concentration of 10 µmol/liter. The spores were suspended in diluted DCFH-DA at a concentration of 20 million/ml, then incubated at 37°C for 20 min. The spore solutions were mixed by being turned upside down every 5 min to ensure that the probe fully contacted the cell. The cells were washed three times with PBS to remove the DCFH-DA, which did not enter the cells. The cells were observed with a laser confocal microscope; the excitation wavelength was 488 nm and the emission wavelength was 525 nm.

Fig. 12. Reactive oxygen species (ROS) accumulation in cotton leaves. The concentration of chaetoviridin A was 150 µg/ml. The dark color on the leaves represents ROS production. Chaetoviridin A + water = the leaves were pretreated with chaetoviridin A, then inoculated with water; chaetoviridin A + *Verticillium dahliae* = the leaves were pretreated with chaetoviridin A, then inoculated with *V. dahliae*; and water + *V. dahliae* = the leaves were pretreated with water and then inoculated with *V. dahliae*.

Table 3. Antimicrobial spectrum of chaetoviridin A^y

Pathogen	MIC (µg/ml) ^z	Inhibition efficiency(%)	Reference
<i>Sclerotinia sclerotiorum</i>	—	97.8	Yan et al. 2018
<i>Botrytis cinerea</i>	—	69.1	Yan et al. 2018
<i>Fusarium graminearum</i>	—	77.0	Yan et al. 2018
<i>Phytophthora capsici</i>	—	60.7	Yan et al. 2018
<i>Fusarium moniliforme</i>	—	59.2	Yan et al. 2018
<i>Magnaporthe grisea</i>	1.23	>80	Park et al. 2005
<i>Puccinia recondita</i>	—	>80	Park et al. 2005
<i>Phytophthora infestans</i>	—	50	Park et al. 2005
<i>Rhizoctonia solani</i>	—	—	Awad et al. 2014
<i>Pythium ultimum</i>	—	—	Awad et al. 2014
<i>Alternaria mali</i>	33.3	—	Park et al. 2005
<i>Colletotrichum gloeosporioides</i>	33.3	—	Park et al. 2005
<i>Fusarium oxysporum</i>	33.3	—	Park et al. 2005

^y The concentration of chaetoviridin A used for *S. sclerotiorum*, *B. cinerea*, *F. graminearum*, *Phytophthora capsici*, and *F. moniliforme* was 20 µg/ml; the concentration of chaetoviridin A used for *M. grisea* and *Puccinia recondita* was 62.5 µg/ml; and the concentration of chaetoviridin A used for *Phytophthora infestans* was 125 µg/ml.

^z MIC = minimum inhibitory concentration.

NO production of *V. dahliae* Vd080 induced by chaetoviridin A.

The *V. dahliae* Vd080 spore suspension (1 ml) was placed into a 1.5 ml-centrifuge tube, and the sediment was collected by centrifugation at 5,000 rpm for 1 min. This process was repeated three times, and 3 ml of the *V. dahliae* spore suspension was collected in total. The sterile water containing chaetoviridin A at 150 µg/ml dissolved in methanol was added to the centrifuge tube and cultured in darkness at 25°C for 12 h. Sterile water containing methanol was used as the CK. The spores were collected by centrifugation and washed three times with PBS. Diaminofluorescein-FM diacetate (DAF-FM DA) was diluted with DAF-FM DA diluent at a ratio of 1:1,000 to a final concentration of 2.5 µmol/liter. Spores were resuspended with diluted DAF-FM DA at a concentration of 1 to 20 million/ml and incubated at 37°C for 20 min. The solution was mixed every 5 min to ensure that the probe fully contacted the cell. The cells were washed with PBS (pH 7.4) three times to remove the DAF-FM DA, which did not enter the cells. The cells were observed with a laser confocal microscope; the excitation wavelength was 495 nm and the emission wavelength was 515 nm.

Quantitative reverse-transcription PCR analysis of *V. dahliae* Vd080.

V. dahliae Vd080 spores were treated with chaetoviridin A at 150 µg/ml (the dilution method was the same as that described above) for 1, 2, or 6 h, then collected for RNA extraction. For the CK group, sterile water containing methanol was applied instead of chaetoviridin A. Three experimental replicates were used. The RNA was reverse transcribed into cDNA. The expression levels of genes related to the cell wall were detected. Quantitative reverse-transcription PCR was performed via a Bio-Rad CFX96 Real-Time System (Bio-Rad), and each PCR mixture (20 µl) consisted of 10 µl of SuperReal PreMix PlusSYBR Green (Tiangen), 0.4 µl of each primer, 2 µl of cDNA, and 7.2 µl of sterile water. Each sample involved at least three technical repeats. The PCR cycle was the same as that used by Zhang et al. (2020). The *V. dahliae* β -tubulin gene was used as the internal reference, and relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The primers used were the same as those described by Han et al. (2015) (Supplementary Table S1).

Chaetoviridin A enhanced the defense response of cotton plants.

Jimian 11 plants with a large true leaf were used. Treatment 1: Chaetoviridin A (150 µg/ml) was applied to true leaves of cotton plants with a brush; then, *V. dahliae* Vd080 spore suspension was inoculated onto the wound with a 1-ml syringe (with the needle removed) after 24 h. Treatment 2: Chaetoviridin A (150 µg/ml) was applied to cotton true leaves with a brush; then, sterile water was inoculated onto the wound with a 1-ml syringe after 24 h. Treatment 3: Cotton true leaves were smeared with sterile water. After 24 h, a 1-ml syringe was used to inoculate the wound with *V. dahliae* spore suspension. After 24 h, the ROS accumulation in the cotton true leaves was detected. The production and accumulation of ROS in cotton leaf cells were detected by 3,3'-diaminobenzidine (DAB) tissue staining according to Kumar's method (Kumar et al. 2009). The leaves were washed with distilled water, placed in a dish, treated with DAB (1 mg/ml), and stained at 25°C for 8 h. Then, the dye solution was removed, and 95% ethanol solution was added to remove the chlorophyll from the leaves. The leaves were boiled for 20 min and suspended in clear water. Photos were taken under a stereomicroscope.

Data analysis.

The statistical software Statistix (8.1) was used to analyze the significance of the data, and means were separated by Tukey's honestly significant difference test.

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