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Authors

Zhang, Tao
Zhao, Yun-Long
Zhao, Jian-Hua
[et al.](#)

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Cotton plants export microRNAs to inhibit virulence gene expression in a fungal pathogen

Tao Zhang^{1,2†}, Yun-Long Zhao^{1,2†}, Jian-Hua Zhao^{1†}, Sheng Wang¹, Yun Jin¹, Zhong-Qi Chen¹, Yuan-Yuan Fang¹, Chen-Lei Hua¹, Shou-Wei Ding³ and Hui-Shan Guo^{1,2*}

Plant pathogenic fungi represent the largest group of disease-causing agents on crop plants, and are a constant and major threat to agriculture worldwide. Recent studies have shown that engineered production of RNA interference (RNAi)-inducing dsRNA in host plants can trigger specific fungal gene silencing and confer resistance to fungal pathogens^{1–7}. Although these findings illustrate efficient uptake of host RNAi triggers by pathogenic fungi, it is unknown whether or not such an uptake mechanism has been evolved for a natural biological function in fungus–host interactions. Here, we show that in response to infection with *Verticillium dahliae* (a vascular fungal pathogen responsible for devastating wilt diseases in many crops) cotton plants increase production of microRNA 166 (miR166) and miR159 and export both to the fungal hyphae for specific silencing. We found that two *V. dahliae* genes encoding a Ca²⁺-dependent cysteine protease (*Clp-1*) and an isotrichodermin C-15 hydroxylase (*HiC-15*), and targeted by miR166 and miR159, respectively, are both essential for fungal virulence. Notably, *V. dahliae* strains expressing either *Clp-1* or *HiC-15* rendered resistant to the respective miRNA exhibited drastically enhanced virulence in cotton plants. Together, our findings identify a novel defence strategy of host plants by exporting specific miRNAs to induce cross-kingdom gene silencing in pathogenic fungi and confer disease resistance.

V. dahliae Kleb. is a hemibiotrophic fungus that causes vascular wilt disease in more than 400 plant species, including cotton. Cotton wilt disease is one of the most important diseases in most cotton-growing countries^{8–10}. *V. dahliae* grows preferably in vascular tissues of host plants from parasitic phase to saprophytic phase when conidia and microsclerotium are produced, probably resulting in the blockage of host xylem vessels and the wilt symptom of the infected plants¹¹. Because of the long-term existence of microsclerotium as its resting structure in host xylem vessels, this fungus is difficult to control and becomes a major threat to cotton production¹².

To investigate the role of RNA silencing in *V. dahliae* pathogenesis, we deep sequenced the total small RNAs from *V. dahliae* (strain V592, a virulent defoliating isolate) cultured from hyphae recovered from infected cotton plants (*Vda*^{Cotton}) (Fig. 1a). We found that *Vda*^{Cotton} contained large amount of small RNAs that could not be mapped to the sequenced genomes of *V. dahliae* (strains VdLs.17 and Vd JR2) or *V. albo-atrum* (strain VaMs.102)^{13,14}. Instead, 28 of these small RNAs were identical in sequence to cotton miRNAs^{15,16}, among which miR166 was the most abundant (Supplementary Fig. 1a). Northern blot analysis showed that four of these miRNAs identified by deep sequencing accumulated to readily detectable levels in *V. dahliae* recovered from infected cotton, but

were all undetectable in V592 cultured *in vitro* without prior cotton colonization (Fig. 1b and Supplementary Fig. 1a). Notably, we found that both miR166 and miR159 accumulated to higher levels in the roots of both cotton and *A. thaliana* plants after *V. dahliae* infection (Fig. 1c). These findings indicate that high levels of specific host miRNAs were exported into the fungal hyphae after host infection.

We next combined computational prediction with 5' rapid amplification of cDNA ends (5' RACE) to identify the genes of *V. dahliae* targeted by the imported cotton miRNAs. Twenty-one of the 28 sequenced cotton miRNAs were predicted to target V592 genes using the transcript sequences of the VdLs.17 strain¹³; most of the 21 target genes selected were not verified by 5' RACE (Supplementary Table 1), possibly because of the sequence variation between VdLs.17 (a lettuce isolate) and V592 (a cotton isolate) strains. However, we detected specific cleavages at the predicted binding sites of *VDAG_09736* and *VDAG_09950* mRNAs targeted respectively by miR166 and miR159 in *V. dahliae* V592 after cotton colonization (*Vda*^{Cotton}), but not in V592 cultured *in vitro* (Fig. 2a and Supplementary Fig. 1b). *VDAG_09736* encodes the Ca²⁺-dependent cysteine protease calpain *clp-1* (designated *Clp-1*) whereas *VDAG_09950* encodes isotrichodermin C-15 hydroxylase (designated *HiC-15*). Northern blotting and reverse transcription quantitative real time (RT-qPCR) showed that the transcript level of both *Clp-1* and *HiC-15* significantly decreased in *Vda*^{Cotton} compared with that in *V. dahliae* without colonization (Fig. 2b,c). We also detected the import of miR166 and the suppression of *Clp-1* mRNA accumulation in *V. dahliae* hyphae recovered from the infected *A. thaliana* plants (*Vda*^{Arabi}) (Fig. 2d).

To verify the specific targeting of the fungal mRNAs by plant miRNAs, we first transiently expressed wild type and miRNA-resistant *Clp-1* and *HiC-15* (*Clp-1m* and *HiC-15m*) in *Nicotiana benthamiana* plants known to produce miR166 and miR159. Both of the miRNA-resistant fungal mRNAs consistently accumulated to higher levels than the wild-type mRNAs in *N. benthamiana* (Supplementary Fig. 2a,b). Second, we transformed the V592 strain with *Clp-1m* and *HiC-15m* under the endogenous promoter and examined the expression of the wild type and miRNA-resistant fungal mRNAs before and after cotton infection. Both northern blotting and RT-qPCR demonstrated expression of both the wild-type and miRNA-resistant *Clp-1* and *HiC-15* mRNAs in the transformed fungal strains cultured *in vitro* (Supplementary Fig. 2c,d). However, specific silencing induced on cotton infection was detected for the wild type, but not the miRNA-resistant mRNA of both *Clp-1* (Fig. 2e,f) and *HiC-15* (Fig. 2g). These findings together demonstrate that cotton host plants export miRNAs to direct specific gene silencing in a fungal pathogen.

¹State Key Laboratory of Plant Genomics, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China. ²College of Life Sciences, University of the Chinese Academy of Sciences, Beijing 100049, China. ³Department of Plant Pathology and Microbiology, Institute for Integrative Genome Biology, University of California, Riverside, California, 92521, USA. [†]These authors contributed equally to this work. *e-mail: guohs@im.ac.cn

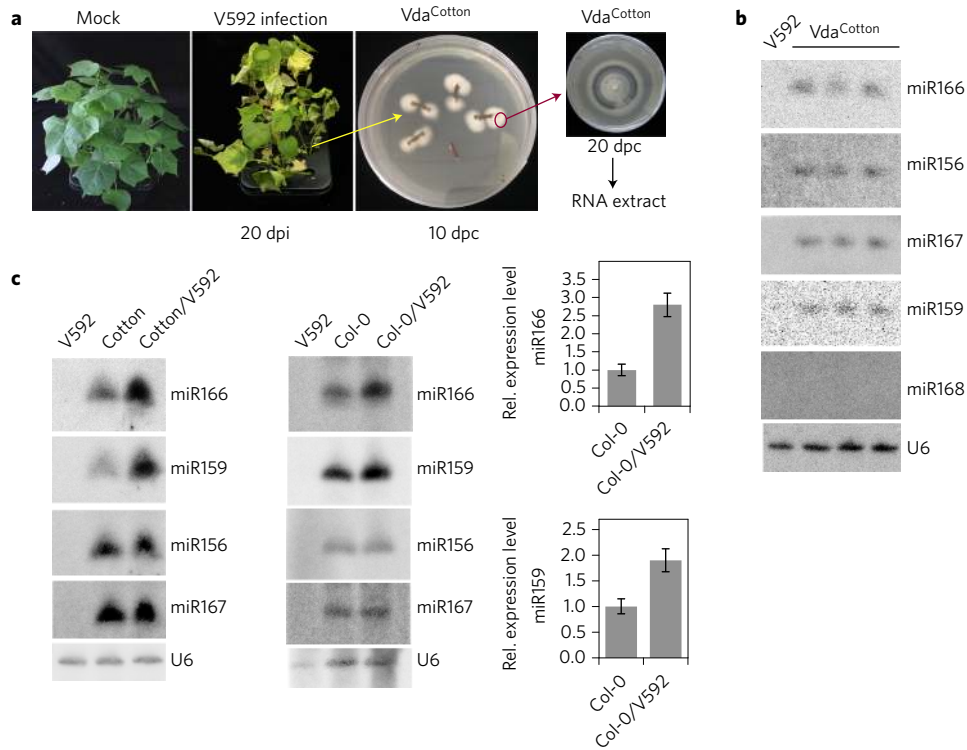


Figure 1 | Host miRNAs are induced in response to fungal infection and exported into fungal hyphae. **a**, Cotton wilt disease symptoms caused by infection with *V. dahliae* strain V592 and growth of hyphae recovered from infected cotton (Vda^{Cotton}). Hyphae grow from two ends of the cut stem. Recovered hyphae growing far away from stem on the PDA plate were cut (purple circle) and inoculated on new plates for another 20 days for RNA isolation. dpc, day post-culture. **b**, Cotton miRNAs were detected in three individual Vda^{Cotton} colonies using low molecular weight RNA gel blots. miR168 was not detected in Vda^{Cotton}. The snRNA gene U6 was used as a loading control. **c**, miR166 and miR159 were induced in V592-infected cotton and *Arabidopsis* roots (cotton/V592 and Col-0/V592). miR156 and miR167 were not induced after V592 infection. Induction of miR166 and miR159 in Col-0/V592 was confirmed by RT-qPCR. Similar results were obtained from three biological repeats. Error bars indicate the s.d. of three replicates.

We hypothesized that cotton plants export miRNAs to confer host resistance to the fungal pathogen. Calpain is required for alkaline stress tolerance in fungi and yeasts¹⁷ whereas isotrichodermin C-15 hydroxylase is a flavoprotein that is important for the production of trichothecene metabolites, which are sesquiterpenes produced by various species of Fungi Imperfecti and have a wide range of biological properties ranging from highly toxic to antileukemic¹⁸. We found that mutant *V. dahliae* knocked out for *Clp-1* showed normal colony morphology, but the microsclerotia formation was inhibited (Supplementary Fig. 3b). *HiC-15* knockout mutant *V. dahliae* showed strong inhibition of hyphal growth with normal microsclerotium formation (Supplementary Fig. 3b). Consistently, both of the *V. dahliae* knockout mutants, without significantly altering fungal biomass, exhibited markedly reduced virulence in cotton plants and no longer induced the wilt symptoms in contrast to the wild-type *V. dahliae* (Fig. 3a,b and Supplementary Fig. 4). These results show that both of the fungal genes targeted by host miRNAs are essential for the fungal virulence in cotton plants. Strikingly, the wilt symptoms appeared earlier and disease severity increased in cotton seedlings infected with *V. dahliae* expressing the miRNA-resistant mutant *Clp-1m* or *HiC-15m* (Fig. 3c,b; Supplementary Fig. 4). *V. dahliae* expressing the control vector VdaGFP did not influence fungal aggressiveness compared with wild-type V592 infection (Supplementary Fig. 4). These findings demonstrate that the fungal virulence genes are specifically targeted by the miRNAs exported from the infected host plants for silencing to confer resistance to the fungal pathogen.

To further verify the role of host miRNAs in the specific gene silencing and virulence of the fungal pathogen, we compared V592 infection in wild-type *Arabidopsis* plants and the short tandem

target mimic (STTM) line STTM166, in which miR166 is degraded¹⁹. We found that miR166 remained undetectable in STTM166 plants both before and after V592 infection in contrast to miR159, which was induced on V592 infection in STTM166 plants (Fig. 4a) as in wild-type plants (Fig. 1c). Consistent with their accumulation levels in the infected STTM166 plants, miR166 was undetectable, whereas miR159 was highly abundant in *V. dahliae* hyphae recovered from the infected STTM166 plants (Vda^{STTM166}) (Fig. 4b). These findings indicate that miR166 detected in the fungal hyphae was from the host plants and was not encoded by the fungal genome induced after host infection. Moreover, the accumulation of *Clp-1* transcripts targeted by miR166 was reduced in *V. dahliae* after colonization in wild-type plants, but not in STTM166 plants (Fig. 4b). In contrast, the abundance of *HiC-15* transcripts targeted by miR159 was reduced in *V. dahliae* after infection of both wild-type and STTM166 plants (Fig. 4b). Although STTM166 plants exhibited developmental defects¹⁹ because of the depletion of miR166, the wilt symptoms in leaf margins became clearly visible two days earlier and spread more extensively in STTM166 plants than in wild-type plants (Fig. 4c). Taken together, our data show that both the specific silencing of the fungal virulent genes and the disease tolerance of the infected plants are mediated by miRNAs produced in response to the fungal infection by host plants and exported into the fungal hyphae.

We report here a novel plant defence strategy against pathogenic fungi by specific silencing of fungal virulence genes with miRNAs exported from the colonized host plants. We show that *Cpl-1* and *HiC-15* targeted by the host miRNAs are essential for microsclerotium formation and hyphal growth, respectively. Microsclerotium formation is critical for long-term survival in soil as well as for

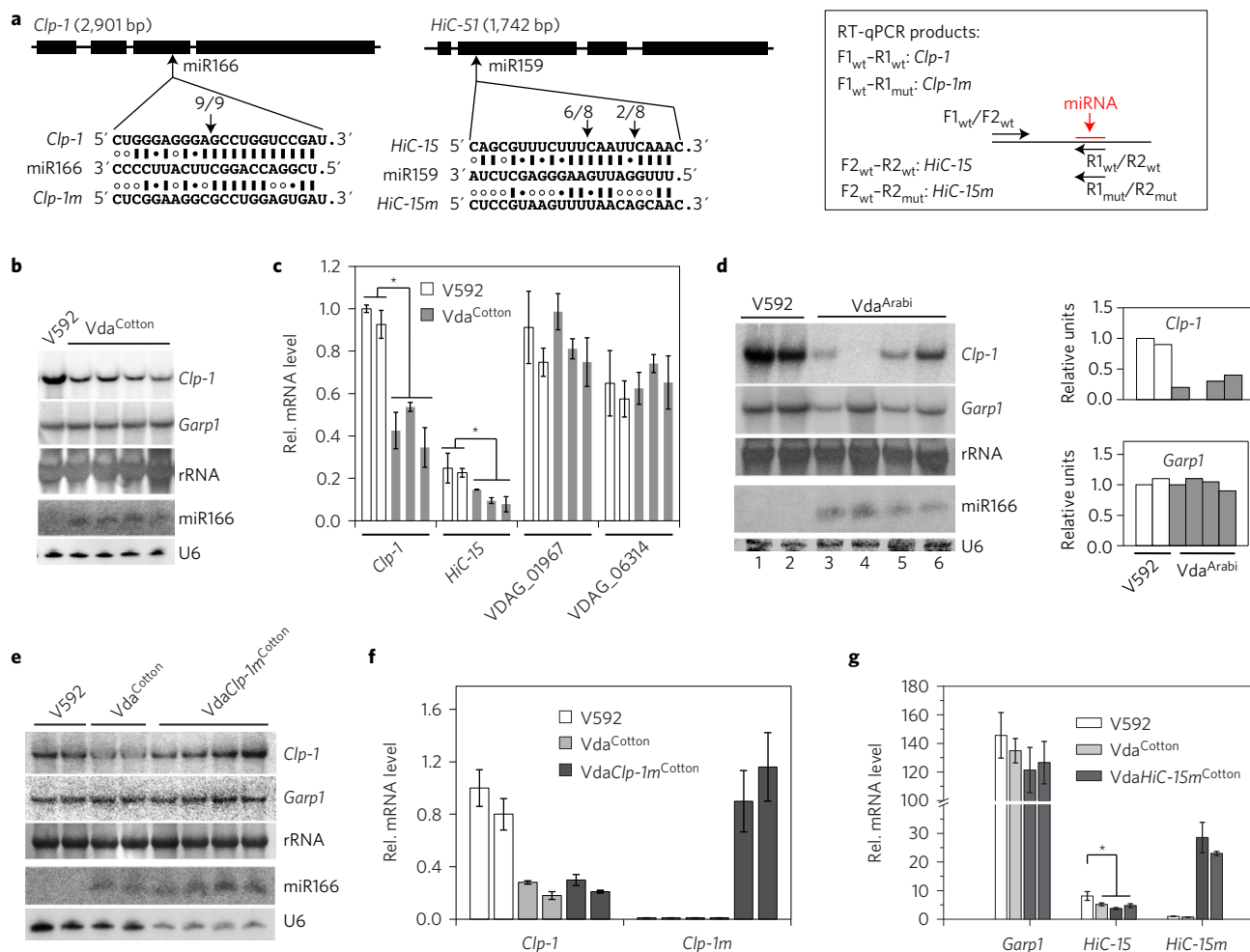


Figure 2 | Fungal *Clp-1* and *HiC-15* genes are targets of plant miRNAs. **a**, Alignments of miR166 and miR159 with fungal targets (*Clp-1* and *HiC-15*) and the miRNA-resistant mutated version at the predicted binding sites (*Clp-1m* and *HiC-15m*). The arrows indicate the cleavage sites between two nucleotides of target mRNAs detected with the 5' RACE assay. Fractions above the arrows indicate the numbers of clones with an identical 5' end detected in the total sequenced clones. **b–g**, *Clp-1* and *HiC-15* were suppressed in individual *Vda*^{Cotton} (**b,c,e–g**) and *Vda*^{Arabi} (**d**) colonies. The suppression was inhibited in each individual *VdaClp-1m*^{Cotton} and *VdaClp-1m*^{Cotton} colony, carrying miR166-resistant *Clp-1m* or miR159-resistant *HiC-15m* (**e–g**). Three *V. dahliae* genes, *Garp1*, *VDAG_01967* and *VDAG_06314*, were used as controls. RT-qPCR was performed using *Elf* (*Verticillium* elongation factor 1- α) as an internal gene. **f,g**, RT-qPCR with specific primers, illustrated as a schematic in the rectangle in **a**. Similar results were obtained from three biological repeats. Error bars indicate the s.d. of three replicates. The asterisk indicates significant differences ($P < 0.05$, one-way ANOVA). Quantification of *Clp-1* and *Garp1* mRNA relative to total fungal RNA (rRNA) in **d** is shown at the right part of the panel. The value of lane 1 mRNA in **d** was arbitrarily designated as 1. miR166 was detected in each individual *Vda*^{Cotton}, *Vda*^{Arabi} and *VdaClp-1m*^{Cotton} colony (**b,d,e**).

the infection and the saprophytic development of *V. dahliae*. *V. dahliae* mutants knocked out for either *Clp-1* or *HiC-15* displayed significantly reduced disease severity in cotton plants, further verifying their virulence functions in *V. dahliae*. We note that the sequences of *Clp-1* and *HiC-15* targeted respectively by miR166 and miR159 are highly conserved, especially in miRNA-binding regions, among *V. dahliae* VdLs.17 (ref. 13), Vd JR2 (ref. 14) and V592 as well as *V. albo-atrum* VaMs.102 (ref. 13) (Supplementary Figs 5 and 6). Infection of tomato plants with VdLs.17 also enhanced the accumulation of miR166 in the infected plants and induced export of the host miR166 into the fungal hyphae to direct specific cleavage of *Clp-1* mRNA and *Clp-1* silencing (Supplementary Fig. 7). Together, our data suggest that *Verticillium* might have preserved or even evolved such miRNA-dependent regulation to prevent hypersensitive response-like responses of the host and maintain its host alive during the biotrophic phase of the infection. In view of coevolution during a long-term specific host–pathogen interaction, it is also possible that these mRNAs are not targeted for cleavage by the identified

host miRNAs. Changes in the abundance of host miRNAs, including miR166, were previously observed in plants after infection with fungal and oomycete pathogens^{20,21}. The miR166 gene is expressed in the root–hypocotyl junctions, the cotyledon vasculature, leaf veins and root tissues²², which may explain why miR166 is the most abundant host miRNA detected in hyphae recovered from infected cotton plants. The efficient uptake of host miRNAs into the fungal hyphae proliferated extensively in plant vascular tissues²³ is consistent with the observed spread of plant RNA silencing/small RNA signal molecules throughout the entire plant via the vascular system^{24,25}. Since efficient uptake of engineered host RNAi triggers by distinct pathogenic fungi has been documented^{1–5}, we propose that the export of small silencing RNAs to downregulate virulence gene expression of pathogens represents a conserved host defence strategy against fungal and oomycete pathogens.

Methods

Fungal isolates, culture conditions, fungal recovery and infection assays.

A virulent defoliating *V. dahliae* strain (V592) isolated from cotton in Xinjiang, China,

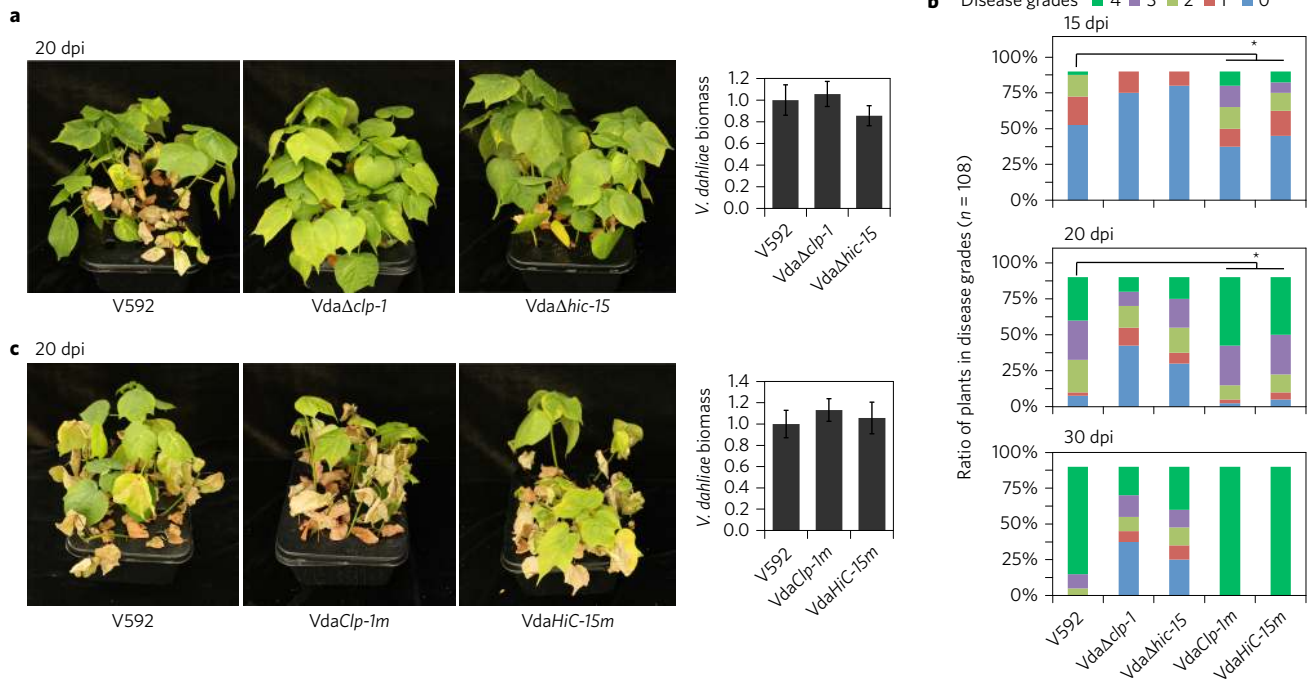


Figure 3 | Cotton miRNAs target fungal virulence factors to confer disease resistance. **a**, The *Clp-1* and *HiC-15* knockout mutants *VdaΔclp-1* and *VdaΔhic-15* exhibited reduced virulence in cotton compared with wild-type V592 at 20-dpi. **b**, Disease grades for all pathogen infection assays at 15, 20 and 30 days post inoculation (dpi). The asterisks indicate significant differences of the hypervirulence phenotype of *VdaClp-1m* and *VdaHiC-15m* vs. wild-type V592 at 15 dpi and 20 dpi ($P < 0.05$, chi-square test). **c**, *V. dahliae* *VdaClp-1m* and *VdaHiC-15m* carrying the miRNA-resistant *Clp-1m* or *HiC-15m* displayed enhanced virulence in cotton. **a,c**, Fungal biomass at 20 dpi was examined by RT-qPCR using *Elf*-specific primers. The value of V592 was arbitrarily designated as 1. Similar results were obtained from three biological repeats. Error bars indicate the s.d. of three replicates. Record of disease progress/grades is described in the Methods.

and a lettuce isolate (VdLs.17) were used in this study. The culture conditions and conidia production for infection assays have been previously described²⁶. The fungus was recovered from infected cotton: the stem sections immediately under cotyledons of cotton plants were taken 20 days after inoculation with V592 and surface-sterilized for 1 min in 70% ethanol followed by 15 min in 30% sodium hypochlorite. The samples were then rinsed three times with sterile water, cut into 1 cm slices and cultured at 26 °C on potato dextrose agar (PDA) medium. *V. dahliae* hyphae that grew from cotton stems after 5 days of culture were cut (away from the stems) and transferred to PDA medium to continue growth for another 20 days for morphology observation or RNA extraction (below section). For the cotton infection assays, the 'laboratory unimpaired root-dip inoculation method' described previously²⁶ was used. Ten-day-old seedlings of wild-type *Arabidopsis* and a STTM166 (STTM165/166-31) line in Columbia background, in which miR166 is degraded and the plants exhibited developmental defective¹⁹, were transferred to soil that was mixed with 1×10^6 cfu ml⁻¹ spores of V592 for infection. Samples at 16 days post inoculation were collected for RNA extraction or hyphae isolation. Five-week-old seedlings of tomato (MM82) were transferred to soil that had been mixed with 1×10^6 cfu ml⁻¹ spores of VdLs.17 for infection. Samples at 18 days post inoculation were collected for RNA extraction or hyphae isolation. Disease progression in cotton was recorded over time for at least 1 month during the experimental period. The infection assay was repeated at least three times. The disease grades were classified on cotton leaves according to one of five levels of symptoms severity during fungal invasion: 0, no visible wilting or yellowing symptoms; 1, one or two cotyledons wilted or dropped off; 2 and 3, one or two true leaves wilted or dropped off; and 4, all leaves dropped off or the whole plant has died²⁷.

RNA extraction, RNA gel blotting, quantitative real-time PCR and 5' RLM-RACE assay. To extract fungal RNAs, *V. dahliae* strains (colonies in PDA medium) were grown at 26 °C in liquid Czapek-Dox medium for 3 days with 200 rpm shaking in the dark. The resulting mycelia were harvested, and the RNAs were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For high molecular weight RNA gel blots, 20 μg of total RNA was separated on 1.2% agarose gels containing 6% formaldehyde, and transferred to a nylon N+ membrane. The DNA probes were labelled with [α -³²P]dCTP using the Rediprime II system (Amersham). For low molecular weight RNA gel blots, 40 μg of total RNA was separated by electrophoresis on 17% PAGE gels, and electrically transferred to a nylon N+ membrane. [α -³²P]UTP-labelled transcript or [γ -³²P]ATP-labelled specific oligonucleotide probe sequences were used (New England Biolabs). For RT-qPCR, the residual DNA was removed from the total RNA using DNase I

(Takara), and 5 μg of total RNA was then reverse-transcribed into cDNA using SuperScript III reverse transcriptase (Promega). The RT-qPCR analysis was performed with a 1,000 series Thermal Cycling Platform (Bio-Rad) using a One-Step RT-PCR kit (abm). The 5' RACE assay was performed using the First Choice RLM-RACEkit (Ambion). The PCR fragments obtained from 5' RACE were inserted into the pMD18-T vector (Takara), and individual clones were selected for DNA sequencing.

Illumina hiseq data analysis. *V. dahliae* recovered from V592-infected cotton (*Vda*^{Cotton}) and the V592 strain cultured on PDA medium without prior cotton colonization were grown in liquid Czapek-Dox medium and harvested as described above for RNA extraction. sRNA library construction and sRNA sequencing were carried out by BGI (<http://www.bgitechsolutions.com/>). All clean reads were mapped to the sequenced genomes of *Verticillium*^{13,14} using SOAP (<http://soap.genomics.org.cn/>). A Perl script was used to search for known miRNAs in cotton^{15,16}. The expression levels of miRNAs were normalized by the reads per million (rpm) value. The targets of miRNAs were predicted using psRNA target (<http://plantgrn.noble.org/psRNATarget/>) with default parameters.

Cloning and constructs. To generate the *Clp-1* and *HiC-15* deletion constructs, the 1-kb genomic sequences upstream and downstream of *Clp-1* and *HiC-15* were amplified from V592 DNA with the following specific primer pairs: *Clp-1*up-s/*Clp-1*up-a, *Clp-1*dn-s/*Clp-1*dn-a and *HiC-15*up-s/*HiC-15*up-a, *HiC-15*dn-s/*HiC-15*dn-a using the Pfu enzyme (Agilent). The PCR products were each ligated to pRF-HU2²⁸ with USER enzyme as described previously²⁸, producing pRF-*Clp-1*del and pRF-*HiC-15*del. These constructs were then used to transform *V. dahliae* and generate the *VdaΔclp-1* and *VdaΔhic-15* mutated strains.

To obtain the *Clp-1* and *HiC-15*, and the *Clp-1m* and *HiC-15m*, the *Clp-1* and *HiC-15* were amplified from V592 DNA with the primers *Clp-1*gene-s/*Clp-1*gene-a and *HiC-15*gene-s/*HiC-15*gene-a the PCR products were ligated to the pGEM-T vector (Tiagen) to generate T-*Clp-1* and T-*HiC-15*. For the *Clp-1m* and *HiC-15m*, oligonucleotide-directed mutagenesis was introduced into *Clp-1* and *HiC-15* with two pairs of primers in opposite orientations: *Clp-1m*-F/*Clp-1m*-R and *HiC-15m*-F/*HiC-15m*-R using a Site-Directed Mutagenesis Kit (NEB) according to the manufacturer's instructions to generate T-*Clp-1m* and T-*HiC-15m*.

To perform transient expression assays in *Nicotiana benthamiana*, *Clp-1*, *Clp-1m*, *HiC-15* and *HiC-15m* were amplified from T-*Clp-1*/T-*Clp-1m* and T-*HiC-15*/T-*HiC-15m*, respectively, with the following specific primers: 1300-*Clp-1*-s/1300-*Clp-1*-a and 1300-*HiC-15*-s/1300-*HiC-15*-a. The resultant products

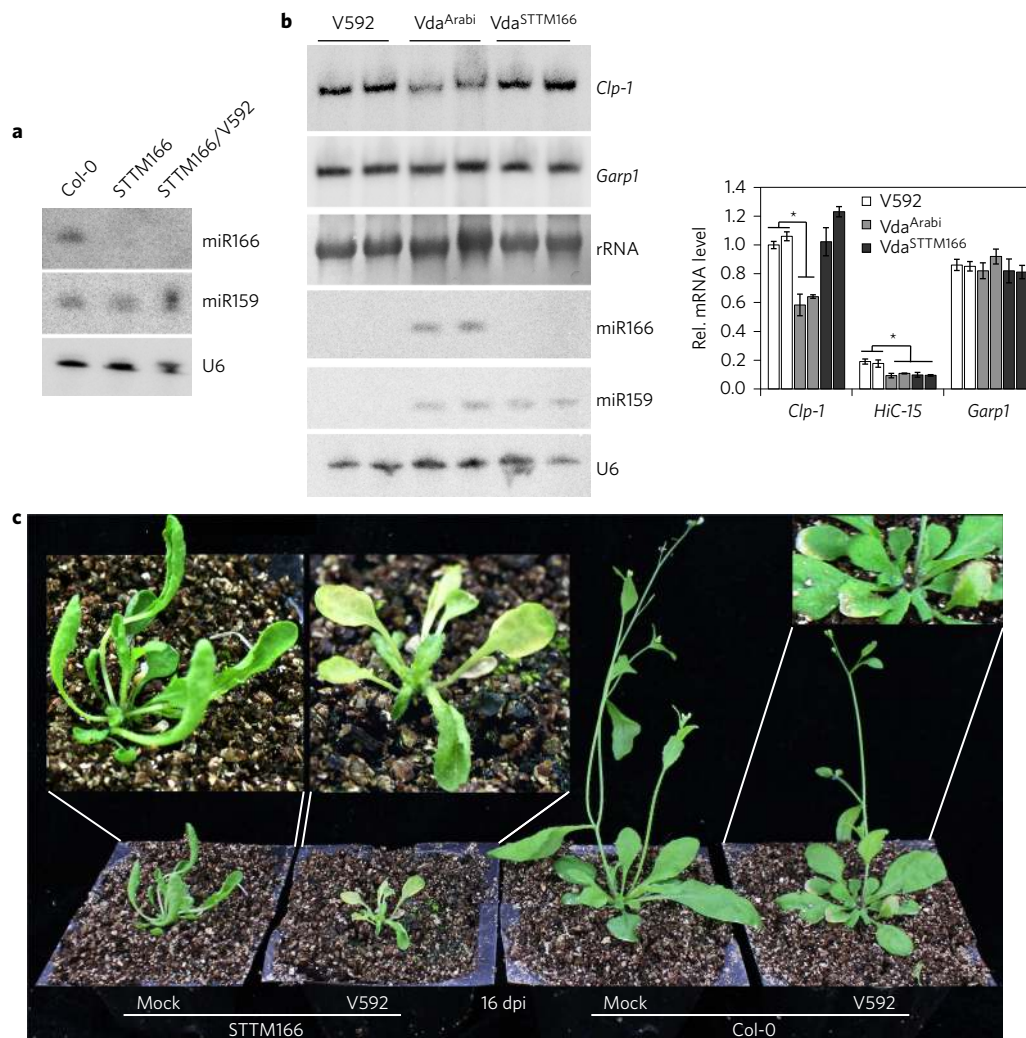


Figure 4 | Increased specific host miR166 and decreased fungal target *Clp-1* were impeded in STTM166 plants in response to *V. dahliae* infection.

a, miR166 was undetectable in STTM166 plants both before and after V592 infection. miR159 was induced upon V592 infection. U6 was used as a loading control. **b**, Detection of miRNAs and fungal targets in individual *Vda*^{Arabi} and *Vda*^{STTM166} colonies. miR166 accumulation and reduced *Clp-1* mRNA detected in *Vda*^{Arabi} colonies was not obtained in *Vda*^{STTM166} colonies. miR159 and reduced *HiC-15* mRNA were detected in both *Vda*^{Arabi} and *Vda*^{STTM166} colonies. *Garp-1* was used as a control. Change in mRNA levels was confirmed by RT-qPCR, similar results were obtained from three biological repeats. Error bars indicate the s.d. of three replicates. The asterisks indicate significant differences ($P < 0.05$, one-way ANOVA). **c**, STTM166 plants with defective developmental phenotype exhibited more severe wilt symptoms than wild-type *Arabidopsis* Col-0 plants upon V592 infection. A similar disease phenotype was observed in eight plants of each genotype used for infection. Photographs of representative plants were taken at 16 dpi. Enlarged images corresponding to each plant are linked with white lines.

were cloned into the binary vector pCambia-1300221, yielding constructs 35S-*Clp-1*, 35S-*Clp-1m*, 35S-*HiC-15* and 35S-*HiC-15m*.

For the fungi transformation construct with endogenous promoters, *Clp-1m* and *HiC-15m* were amplified from T-*Clp-1m* and T-*HiC-15m* with the following primers: *Clp-1m* gene-s/*Clp-1m* gene-a and *HiC-15m* gene-s/*HiC-15m* gene-a that contain the appropriate enzyme digestion sites (BamHI/EcoRI). The BamHI-EcoRI fragments of *Clp-1m* and *HiC-15m* were individually ligated into BamHI/EcoRI-digested binary vector pNEO^{tef}-GFP, derived from pSULPH-GFP²⁹ in which the chlorimuron-ethyl resistance had been replaced by a *neo* resistance cassette from pKOV21 and TEF promoter from pPN94 plasmid³⁰, to produce pNEO^{tef}-*Clp-1m* and pNEO^{tef}-*HiC-15m*. The promoters of *Clp-1* and *HiC-15* were amplified from V592 DNA with the following primers *Clp-1* pro-F/*Clp-1* pro-R and *HiC-15* pro-F/*HiC-15* pro-R, the promoter PCR fragments were individually ligated into pNEO^{tef}-*Clp-1m* and pNEO^{tef}-*HiC-15m* to replace the TEF promoter to produce pNEO-*Clp-1m* and pNEO-*HiC-15m*. These constructs were then used to transfer *V. dahliae*, generating the *VdaClp-1m* and *VdaHiC-15m* mutation strains. pNEO^{tef}-GFP was also transferred to *V. dahliae* for generation of vector control strain *VdaGFP* in infection assay.

Agrobacterium tumefaciens-mediated *Clp-1* and *HiC-15* genes deletion and transient expression of fungal genes in plants. The *Clp1* gene and *HiC-15* gene deletion mutants (*VdaΔclp-1* and *VdaΔhic-15*) were generated by transforming the pRF-*Clp-1*del and pRF-*HiC-15*del constructs, respectively, into the V592 strain

according to the *A. tumefaciens*-mediated transformation method described previously²⁹. The individual transformants were transferred to PDA medium containing hygromycin B and incubated until conidiogenesis. The homology of the recombinants was further verified by PCR analysis. The conidia of individual transformants were suspended in sterile water and plated on PDA medium. Spores from these monoconidial cultures were stored in 20% glycerol at -80°C until further analysis.

For *Agrobacterium*-mediated transient expression of fungal genes in *N. benthamiana*, the 35S-*Clp-1*, 35S-*Clp-1m*, 35S-*HiC-15* or 35S-*HiC-15m* construct was transformed into the EHA105 strain of *Agrobacterium* by electroporation and selected on Luria-Bertani medium containing kanamycin at 50 mg/l and rifampicin at 10 mg l⁻¹. *Agrobacterium* cultures ($A_{600} = 1$) were infiltrated into leaves of *N. benthamiana*.

Data availability. The data that support the findings of this study are available from the corresponding author on request.

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References

- Nowara, D. et al. HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *Plant Cell* **22**, 3130–3141 (2010).

2. Tinoco, M. L., Dias, B. B., Dall'Asta, R. C., Pamphile, J. A. & Aragao, F. J. *In vivo* trans-specific gene silencing in fungal cells by *in planta* expression of a double-stranded RNA. *BMC Biol.* **8**, 27 (2010).
3. Panwar, V., McCallum, B. & Bakkeren, G. Host-induced gene silencing of wheat leaf rust fungus *Puccinia triticina* pathogenicity genes mediated by the Barley stripe mosaic virus. *Plant Mol. Biol.* **81**, 595–608 (2013).
4. Panwar, V., McCallum, B. & Bakkeren, G. Endogenous silencing of *Puccinia triticina* pathogenicity genes through *in planta*-expressed sequences leads to the suppression of rust diseases on wheat. *Plant J.* **73**, 521–532 (2013).
5. Koch, A. *et al.* Host-induced gene silencing of cytochrome P450 lanosterol C14 alpha-demethylase-encoding genes confers strong resistance to *Fusarium* species. *Proc. Natl Acad. Sci. USA* **110**, 19324–19329 (2013).
6. Baulcombe, D. Small RNA—the secret of noble rot. *Science* **342**, 45–46 (2013).
7. Weiberg, A. & Jin, H. Small RNAs—the secret agents in the plant–pathogen interactions. *Curr. Opin. Plant Biol.* **26**, 87–94 (2015).
8. Zhengjun, X., Achar, P. N. & Benkang, G. Vegetative compatibility groupings of *Verticillium dahliae* from cotton in mainland China. *Eur. J. Plant Pathol.* **104**, 871–876 (1998).
9. Bowman, D. Public cotton breeders—do we need them? *J. Cotton Sci.* **3**, 139–152 (1999).
10. Koroleva, O. V., Stepanova, E. V., Gavrilova, V. P., Biniukov, V. I. & Pronin, A. M. Comparative characterization of methods for removal of Cu(II) from the active sites of fungal laccases. *Biochemistry (Moscow)* **66**, 960–966 (2001).
11. Gerik, J. & Huisman, O. Study of field-grown cotton roots infected with *Verticillium dahliae* using an immunoenzymatic staining technique. *Phytopathology* **78**, 1174–1178 (1988).
12. Bell, A. A. *Verticillium* wilt. *Cotton Dis.* 87–126 (1992).
13. Klosterman, S. J. *et al.* Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. *PLoS Pathog.* **7**, e1002137 (2011).
14. de Jonge, R. *et al.* Extensive chromosomal reshuffling drives evolution of virulence in an asexual pathogen. *Genome Res.* **23**, 1271–1282 (2013).
15. Gong, L., Kakrana, A., Arikat, S., Meyers, B. C. & Wendel, J. F. Composition and expression of conserved microRNA genes in diploid cotton (*Gossypium*) species. *Genome Biol. Evol.* **5**, 2449–2459 (2013).
16. Liu, N. *et al.* Small RNA and degradome profiling reveals a role for miRNAs and their targets in the developing fibers of *Gossypium barbadense*. *Plant J.* **80**, 331–344 (2014).
17. Sorimachi, H., Hata, S., Ono, Y. Calpain chronicle—an enzyme family under multidisciplinary characterization. *Proc. Jpn Acad. Ser. B Phys. Biol. Sci.* **87**, 287 (2011).
18. Zamir, L. O., Devor, K. A., Nikolakakis, A. & Sauriol, F. Biosynthesis of *Fusarium culmorum* trichothecenes. The roles of isotrichodermin and 12, 13-epoxytrichothec-9-ene. *J. Biol. Chem.* **265**, 6713–6725 (1990).
19. Yan, J. *et al.* Effective small RNA destruction by the expression of a short tandem target mimic in *Arabidopsis*. *Plant Cell* **24**, 415–427 (2012).
20. Campo, S. *et al.* Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the *Nramp6* (*Natural resistance-associated macrophage protein 6*) gene involved in pathogen resistance. *New Phytol.* **199**, 212–227 (2013).
21. Wong, J. *et al.* Roles of small RNAs in soybean defense against *Phytophthora sojae* infection. *Plant J.* **79**, 928–940 (2014).
22. Jung, J. H. & Park, C. M. *MIR166/165* genes exhibit dynamic expression patterns in regulating shoot apical meristem and floral development in *Arabidopsis*. *Planta* **225**, 1327–1338 (2007).
23. Zhao, P., Zhao, Y.-L., Jin, Y., Zhang, T. & Guo, H.-S. Colonization process of *Arabidopsis thaliana* roots by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. *Protein Cell* **5**, 94–98 (2014).
24. Kalantidis, K. Grafting the way to the systemic silencing signal in plants. *PLoS Biol.* **2**, E224 (2004).
25. Molnar, A. *et al.* Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* **328**, 872–875 (2010).
26. Zhou, B. J., Jia, P. S., Gao, F. & Guo, H. S. Molecular characterization and functional analysis of a necrosis- and ethylene-inducing, protein-encoding gene family from *Verticillium dahliae*. *Mol. Plant Microbe Interact.* **25**, 964–975 (2012).
27. Xu, L. *et al.* Functional characterization of cotton genes responsive to *Verticillium dahliae* through bioinformatics and reverse genetics strategies. *J. Exp. Bot.* **65**, 6679–6692 (2014).
28. Frandsen, R., Frandsen, M. & Giese, H. Targeted gene replacement in fungal pathogens via *Agrobacterium tumefaciens*-mediated transformation. *Methods Mol. Biol.* **835** (2012).
29. Gao, F. *et al.* A glutamic acid-rich protein identified in *Verticillium dahliae* from an insertional mutagenesis affects microsclerotial formation and pathogenicity. *PLoS ONE* **5**, e15319 (2010).
30. Takemoto, D., Tanaka, A. & Scott, B. A p67Phox-like regulator is recruited to control hyphal branching in a fungal-grass mutualistic symbiosis. *Plant Cell* **18**, 2807–2821 (2006).

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Author contributions

H.S.G. and T.Z. designed experiments. T.Z., Y.L.Z. and S.W. performed experiments. J.H.Z. performed sRNA computational informatics analysis. J.Y. assisted with the 5'-RACE assay. Y.Y.F. and Z.Q.C. provided technical support. H.S.G., J.H.Z., T.Z. and Y.L.Z. analysed data. H.S.G., S.W.D., C.L.H. and T.Z. discussed the results and wrote the paper.

Additional information

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Competing interests

The authors declare no competing financial interests.