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In vitro RNA-mediated gene silencing of *Fusarium oxysporum* f.sp. *cubense* from Ecuador and assessment of RNAi molecule stability in banana plants

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Abstract

Objective Fusarium wilt poses a significant threat to banana production, caused by diverse clonal *Fusarium* lineages. Given the lack of curative measures, developing effective treatments is crucial. RNA interference (RNAi) technology, utilizing double-stranded RNA (dsRNA) molecules, offers a promising solution. In this study, RNAi was evaluated by silencing the activity of the *Beta-tubulin* (*Focβ-tub*), *C5 Sterol desaturase (FocERG3)* and *Chitin synthase 1 (FocChs1)* genes in a pathogenic *Fusarium* strain. Furthermore, we study the potential of dsRNA translocation in 3 months old banana plants at early hours of been spray under greenhouse conditions.

Results In vitro results demonstrated that dsRNA-FocChs1 was more effective in inhibiting spores, with an average IC_{50} of 156.84 mg/L, compared to dsRNA-Focβ-tub (IC_{50}: 532.7 mg/L), dsRNA-FocERG3 (IC_{50}: 635.59 mg/L), and a positive control (IC_{50}: 243.91 mg/L). A greenhouse test was conducted to evaluate the translocation of dsRNA in banana plants. The results demonstrated that the dsRNA remained on the applied leaf without degradation up to 48 h post-application (hpa). However, no translocation to other plant tissues was detected until the last time point. Further time points should be evaluated to ascertain the dsRNA translocation to other banana plant tissue.

Keywords Banana, RNA interference, *Fusarium oxysporum* F.sp. *Cubense*

Introduction

Bananas are one of the most important fruits worldwide, with a high demand for consumption [1, 2]. Ecuador is the prime exporter, with a volume of 6.2 million tons in 2023 [1]. Banana industry is facing a significant threat from *Fusarium oxysporum* f. sp. *cubense* (Foc), a fungus that causes Fusarium wilt of banana [2, 3]. All commercial banana varieties are currently threatened by a new Foc genetic variant, commonly referred to as *Fusarium* tropical race 4 (FocTR4) [4–6]. Foc leads to plant death by blocking the vascular bundles [7–9]. Currently, most fungicides are ineffective in controlling Foc [10–12]. Nonetheless, a new compound may be able to control

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Fusarium species [13]. In Ecuador, there are no reports of the presence of FocTR4 [14], but has been detected in neighboring countries [15–17]. RNAi is a biological process that silences virulence-inducing genes in pathogens [18]. The RNAi process involves the formation of the RNA-induced silencing complex (RISC), which degrades mRNA or suppresses target gene expression [19, 20]. RNAi-mediated host-induced gene silencing (HIGS) has been used to develop disease-resistant transgenic bananas [21]. But legal restrictions in Ecuador prohibit their cultivation [22]. Alternative, Spray-induced gene silencing (SIGS) offers an efficient, sustainable method for diseases control, reducing agrochemicals use and preventing resistance [23]. The RNAi method has been proven effective in inhibiting the growth of banana pathogenic fungi [24–26]. This study was conducted on the Foc race 1 strain (FocR1) from Ecuador. Notably, there is a high similarity in the sequence of target genes used between FocR1 and Foc TR4 (Additional file 3).

Table 1 Primers F.r obtaining double-stranded RNA F.r *Fusarium oxysporum* F. Sp. *Cubense* race 1 and RT-qPCR reaction

Target gene		Secuence	Size of fragment
Beta tubulin (Focβ-tub)- for dsRNA	forward 5'- 3'	*GCGTAATACGACTCACTATAGGGAGA*CTTGAGCCTGGTACCATGGA	
	Reverse 5'- 3'	*GCGTAATACGACTCACTATAGGGAGA*GGAGCAAAGCCAACCATGAA	*614 bp*
C5 - Sterol desaturase (FocERG3) - for dsRNA	forward 5'- 3'	*GCGTAATACGACTCACTATAGGGAGA*TGGATCTTTGGGCTTCTCGT	
	Reverse 5'- 3'	*GCGTAATACGACTCACTATAGGGAGA*ATCGGCCCTCTGACATCTTC	*622 bp*
Chitin synthase 1 (FocChs1) - for dsRNA	forward 5'- 3'	*GCGTAATACGACTCACTATAGGGAGA*GGACAAGCCTCTCGAGTCTT	
	Reverse 5'- 3'	*GCGTAATACGACTCACTATAGGGAGA*GCATGACGAAGGCCAAGTAG	*620 bp*
Adenylate cyclase (FocAdcy) - for dsRNA	forward 5'- 3'	*GCGTAATACGACTCACTATAGGGAGA*TGCACGAAAATTTTGGTCACATATTCGC	
	Reverse 5'- 3'	*GCGTAATACGACTCACTATAGGGAGA*CCATAATTTTGCCCGAGGCGC	*660 bp*
Chitin synthase 1 (FocChs1)– for RT-qPCR	forward 5'- 3'	*GAATGCTGTCCAGCCTCAGT*	*127 bp*
	Reverse 5'- 3'	*AGGTCGCAAATCGTAACCGT*	
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)– for RT- qPCR [29]	forward 5'- 3'	*CGTCGATCTTACTGTCCGCC*	*110 bp*
	Reverse 5'- 3'	*CCTCAGTGTAGGCCAGAACG*	

Main text

Materials and methods

Inoculate obtention

Assays was performed using FocR1 isolate "EC35-G-GM1" (obtained from CIBE-ESPOL collection, Ecuador) [14]. The fungus was grown and maintained at 28° C in PDA culture medium (Difco, US). To obtain spores, three 0.5 mm diameter discs were punched from the strains and transferred to 50 ml of sterile PDB medium (Difco, US) and kept for 6 days at 28° C in a shaker incubator (Innova 40, New Brunswick, Germany) at 150 rpm. The spore suspension was filtered to remove the mycelial structure and an aliquot was used to estimate the concentration using a Neubauer chamber (1/10 mm deep, Boeco, Germany), under a microscope (ZIESS, Axioskop 2 plus, Germany) at 10 X magnification.

Target genes selection

The target genes selected for RNAi were *beta tubulin* (*Focβ-tub*), *C5-esterol desaturase (FocERG3)* and *chitin synthase 1 (FocChs1).* These genes were selected according to their importance in cell function as described in Additional file 4. Furthermore, *adenylate cyclase* sequence from Mumbanza et al. (2013) was included as a positive control target [26]. The complete coding DNA sequence (CDS) of each target gene was obtained from "Ensembl fungi" online database [27]. Primers were designed using "Primer 3" software [28]. The T7 RNA polymerase promoter sequences (underlined in each primer in Table 1) were added to primers to enable double-stranded RNA synthesis.

Synthesis of DsRNA

Total RNA was extracted from the mycelium of FocR1 using Trizol® method (Cat. 15596-018, Invitrogen, USA). Next, complementary DNA (cDNA) was obtained with the "Maxima First Strand cDNA Synthesis kit with dsDNAse" (Ref. K1642, Thermo Scientific, USA). Subsequently, RT-PCR was performed with all the target genes as described in Additional file 1. A total of 5 µl of each RT-PCR reaction was loaded for analysis via electrophoresis using a 1.5% agarose gel. The gel was run for 30 min at 10 V/cm and visualized using the GelDoc XR+ (Bio-RAD, USA) (Additional file 5). The dsRNA synthesis for inhibition and translocation assays was carried out following the instructions provided in the "MEGAscript™ T7 Kit" (Ref. AM1334, Invitrogen, USA). 5 µl of each RT-PCR product was used as a template, with a final reaction

volume of 20 µl. dsRNA was quantified using the Nanodrop 2000 (Invitrogen, USA).

FocR1 DsRNA spore Inhibition bioassays

To study the antifungal effects of dsRNA-FocChs1, dsRNAFocβ-tub, dsRNAFocERG3 and dsRNAFocAdcy on fungal growth, assays were performed to quantify the reduction in the number of FocR1 colonies. The methodology described by Bailey et al. (2010), Mumbanza et al. (2013) [26, 30] were followed as described in Additional file 1. The dsRNA was tested at concentrations of 100, 250, 500, 1000 and 1500 mg/L with nine replicates for each target gene. Data from inhibition and survival percentages were used to independently calculate the IC_{50} for nine replicates, which were then plotted and fitted to the *IC50%.FUN* model using Kyplot 6.0 software (KyensLab Inc., Tokyo, Japan) (Additional file 1) [31, 32]. A gene expression assay was conducted with *FocChs1*, examining the interaction between FocR1 and dsRNA-FocChs1 in PDB medium for 24 and 48 h of Foc grow (Additional file 1).

DsRNA translocation bioassay in banana plants

A greenhouse assay was conducted to study the translocation of the dsRNA in banana plants. The dsRNA-FocAdcy was synthesized using the MEGAscript™ T7 Kit (Ref. AM1334, Invitrogen, USA). The solution, comprising 500 mg/L dsRNA-FocAdcy, 1% Triton X (Sigma, USA) and 1% DMSO (Sigma, USA), was sprayed on the upper and lower surfaces of leaf number 3 of 3-month-old banana plants of the Williams variety (Genotype AAA), which were approximately 30 cm in height. Two biological replicates (due to limited dsRNA solution) were used. Samples were collected at 1-, 12-, 24-, and 48-hours post-application (hpa) from pseudostem, corm, root, and leaf number 2, 3, and 4. Upon collection, the samples were immediately frozen with liquid nitrogen and stored at -80 °C until processing. For total RNA extraction, frozen samples were pulverized in liquid nitrogen using a MM400 mixer-mill (Retsh, Hann, Germany). From each ground tissue, 100 mg was used for total RNA extraction with the "Spectrum™ Plant Total RNA Kit" (Ref. STRN50-1KT, Sigma, USA). Total RNA was quantified using the Nanodrop 2000 (Invitrogen, USA).

Northern blot detection of DsRNA in different tissues of banana plants

The Northern blot technique was used to detect the mobility of the dsRNA using total RNA from all tissue samples. This highly sensitive method was developed following the manufacturer's instructions for the "Dig Northern Starter Kit" (Cat. No 12039672910, Roche Switzerland). Probes generated are described in Additional file 2 [33–35]. To detect luminescence, immunological detection of RNA via probe hybridization on a membrane was performed using the Stella 3200 (Raytest, Germany) with 20 min of exposure in dark conditions. The resulting image was analyzed with the "AIDA Image Analyzer" software (Raytest, Germany).

Statistical analysis

Data analysis and plotting were performed using R Studio [36]. To compare IC_{50} values and spore inhibition percentages, statistical analyses were conducted to an analysis of variance (ANOVA), followed by Fisher's Least Significant Difference (LSD) test. A significance level of $p < 0.05$ was used to assess differences between treatment. Student's T-test at $P < 0.05$ was use for the significance analysis results of RT-qPCR.

Results and discussions

Inhibition of FocR1 spores

All dsRNA were able to inhibit FocR1 growth (Additional file 7). At a concentration of 1000 mg/L dsRNA-Chs1 and dsRNA-FocAdcy achieved 100% inhibition approximately, while dsRNA-Focβ-tub and dsRNA-ERG3 showed 70.64% and 81.3% inhibition, respectively. At 1500 mg/L, there was no significant difference in inhibition for dsRNA-Focβ-tub and dsRNA-ERG3, with both showing 75.87% and 80.18% inhibition, respectively (Fig. 1a and b). Considering the role of the genes in the host organism, we analyzed the inhibition behavior of each dsRNA independently.

The *FocAdcy* gene was chosen as a control for inhibition based on previous research [26]. However, our results differ significantly from those described by Mumbanza et al. (2013), where, an inhibition percentage of 93% was reported with a dsRNA concentration of 0.6 mg/L, which is significantly lower than that observed in our investigation. However, in a study conducted by Fei et al. (2018), the inhibition of three target genes by RNAi was found to halt the virulence and spore growth of Fo5176 affecting *Arabidopsis thaliana* at higher concentrations. The study demonstrated a significant reduction in spore germination, with 118-fold, 153-fold, and 100-fold decreases for the target genes *GenA*, *GenB* and *GenC*, respectively, compared to the control using 2.5 µg of dsRNA in 25 µl (100 mg/L). The authors suggest these genes may offer more control than those in Mumbanza's study, though the dsRNA concentration differed significantly between the studies [25]. It should be noted that the strains or *forma specialis* used in the studies are different from those we used, which may explain the variability.

According to the IC_{50} calculation of dsRNA-FocChs1, dsRNA-FocAdcy, dsRNA-Focβ-tub and dsRNA-ERG3, the mean concentrations were 156.84 mg/L, 243.91 mg/L, 536.7 mg/L and 635.59 mg/L respectively. Fisher's mean comparison analysis revealed the presence

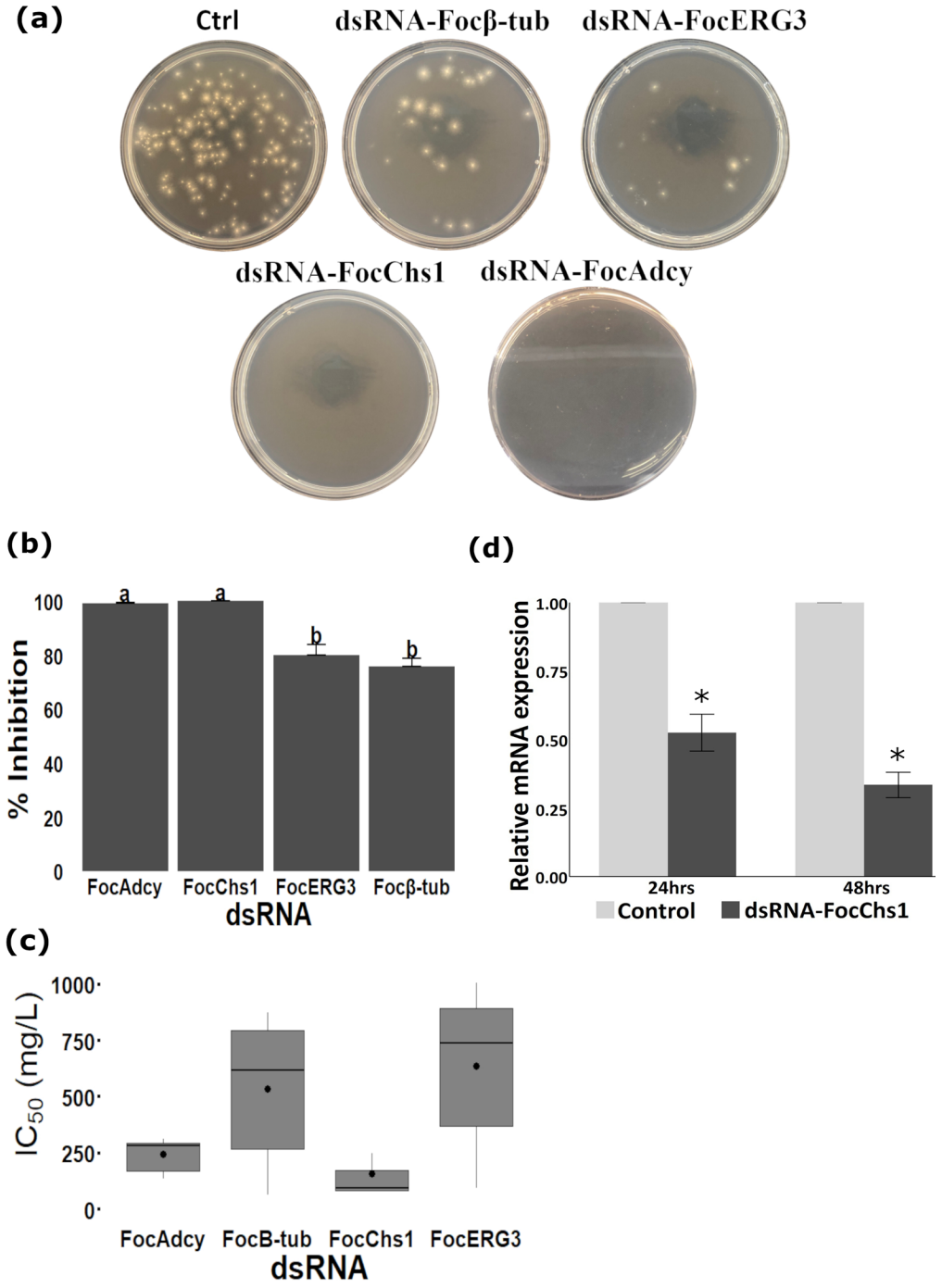

Fig. 1 Spore inhibition of *Fusarium oxysporum* f. sp. *cubense* race 1 with dsRNA. **(a)** Inhibition with dsRNA at 1000 mg/L on isolate EC35-G-GM1 of FocR1 with dsRNA-FocChs1, dsRNA-FocB-tub, dsRNA-FocERG3 and dsRNA-FocAdcy. **(b)** Bar graph of spore inhibition with dsRNA-FocChs1 (100%), dsRNA-FocB-tub (75.87%), dsRNA-FocERG3 (80.18%) and dsRNA-FocAdcy (99.37%) at concentrations of 1500 mg/L on spores of isolate EC35-G-GM1 of FocR1. Bars represent standard errors. Equal letters above bars are not significantly different at $p > 0.05$. **(c)** Box plot of the mean inhibitory concentration (IC_{50}) in mg/L with dsRNA-FocChs1, dsRNA-FocAdcy, dsRNA-FocB-tub and dsRNA-FocERG3, on isolate EC35-G-GM1 of FocR1. 9 observations were included for each gene. **(d)** Expression level of *FocChs1* gene following Foc spore growth with dsRNA-FocChs1 at 24 and 48 h (hrs). The expression level of *FocChs1* was normalized to the expression of Foc GAPDH. Expression level was performed by RT-qPCR in three technical replicates and three biological replicates independently. Statistical significance (Student's t-test) between the treatment and the control: *, $P < 0.05$

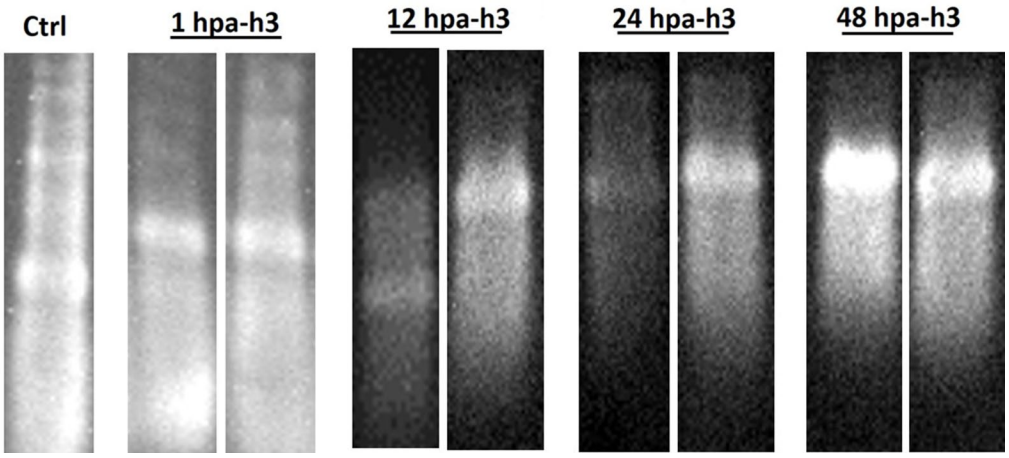

Fig. 2 Detection of dsRNA by Northern blot in banana tissue samples. Samples were collected at 1, 12, 24 and 48 h post-application, h3 corresponds to leaf #3 of banana the plant. Ctrl, corresponds to the dsRNA control (500 mg/L) with 1% Triton + 1% DMSO. Samples from each time point evaluated were processed individually, except those corresponding to 24 and 48 h post-application (hpa), which were analyzed together in a single Northern blot experiment

of two statistically different homologous groups mark group A comprising dsRNA-Chs1 and dsRNA-FocAdcy and group B comprising dsRNA-Focβ-tub and dsRNA-ERG3. Furthermore, dsRNA-Chs1 had the lowest IC_{50} value with the strain tested, indicating a higher efficiency. dsRNA-Chs1 was slightly more efficient than the control gene dsRNA-FocAdcy (Additional file 6). In Fig. 1c, the box plot illustrates the behavior of all the observations of the IC_{50} calculation with respect to each dsRNA, showing a greater variability with dsRNA-Focβ-tub and dsRNA-ERG3 than with dsRNA-Chs1 and dsRNA-FocAdcy, which show a high and stable inhibition in the analysis. Additionally, the relative mRNA expression level of *FocChs1* was reduced in the dsRNA-FocChs1 in vitro interaction at 24 and 48 h of Foc growth, with expression levels of 0.53 and 0.33, respectively, compared to a control (Fig. 1d).

Several genes are involved in the chitin biosynthetic process. However, a study showed that these genes lack compensatory mechanisms [37]. Therefore, the inhibitory effect observed in this study is likely due to the effective degradation of the *FocChs1* mRNA. Conversely, with *FocERG3*, 25 other genes are related to the metabolic pathway of ergosterol production participate in a regulatory compensation mechanism utilizing alternative ergosterol production routes, suggesting that ERG3 is a non-essential gene [38–40]. This would explain the suboptimal efficacy of dsRNA-ERG3 silencing. In relation to *Focβ-tub*, at least four genes related to its function are known: *α1- tubulin*, *α2- tubulin*, *β1- tubulin* and *β2- tubulin.* The sequence used in this study is related to *β2- tubulin*, which colocalizes with *β1- tubulin*, indicating that they share a specific region. Studies on the functional roles of these genes suggest that *β1- tubulin* can compensate for the growth function in a Δ*β2- tubulin* mutant [41]. This may partly explain the partial inhibition of dsRNA-Focβ-tub observed in our assay.

Translocation of the DsRNA molecule

The results showed that dsRNA-FocAdcy was only detected in leaf number 3, corresponding to the tissue of application (Fig. 2). In contrast, the dsRNA was not observed in the other evaluated tissues during the analyzed time points. This finding suggests that the dsRNA-FocAdcy did not translocate to other plant tissues in the timeframe tested. Currently, there is limited information available on the uptake and translocation of dsRNA in banana plants. Nevertheless, studies conducted on barley, *Arabidopsis thaliana*, and grape have demonstrated that the RNAi can be transported through phloem to other tissues, thereby inhibiting the target pathogen that affects these crops [42–44]. However, Qiao et al. (2021) demonstrate using confocal microscopy laser scanner (CMLS) that dsRNA can be taken up by fungi, but the efficacy of silencing depends on the eukaryotic fungal species and cell types [45].

A possible reason for the inability to detect translocated dsRNA in banana plants assay could be the reduction in dsRNA concentration influenced by factors such as greenhouse conditions, physical barriers on the banana leaf surface and the presence of nucleases [46–48]. Even with the addition of a surfactant to the solution during spraying, some dsRNA may still be lost to the environment [46]. Conversely, the detection of dsRNA translocation may require a longer evaluation period, as the molecule's quantity may be reduced in other tissues. However, our results demonstrate that the dsRNA remains stable at the application site under greenhouse

conditions, at least during the first 48 h of evaluation. Northern blotting detection of the dsRNA-FocAdcy molecule in the leaves where the solution was applied showed that the dsRNA remained intact at all evaluated time points.

Limitation

dsRNA was not detected in tissues other than those to which it was applied. To improve detection, further translocation bioassays are being conducted with higher dsRNA concentrations, direct application to the banana corm and longer evaluation period.

Abbreviations

RNAi	RNA interference
dsRNA	Double stream RNA
mRNA	Messenger RNA
IC_{50}	Half maximal inhibitory concentration

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-025-07253-9.

Supplementary Material 1

Supplementary Material 2: Description of probes generated for the Northen blot.

Supplementary Material 3: Sequence alignment of Target genes from FocR1 and FocTR4 results in NCBI database.

Supplementary Material 4: Description of target genes for silencing of ***Fusarium oxysporum*** f. sp. c***ubense*** race 1.

Supplementary Material 5: RT-PCR amplification of target genes from isolate EC35-G-GM1 of ***Fusarium oxysporum*** f. sp. c***ubense*** race 1.

Supplementary Material 6: Fisher's least significant difference (LSD) test with the IC_{50} of target genes for EC35-G-GM1 inhibition of ***F. oxysporum*** f. sp. c***ubense*** race 1.

Supplementary Material 7: Inhibitory curve ***of*** dsRNA-FocChs1, dsRNA-Focβ-tub, dsRNA-FocERG3, dsRNA-FocAdcy.

Supplementary Material 8: Raw Data of Northern Blot Results.

Supplementary Material 9: Raw image of the first section of the Northern blot.

Supplementary Material 10: Raw image of the second section of the Northern blot.

Supplementary Material 11: Raw image of the third section of the Northern blot.

Supplementary Material 12: ***FocChs1 g***ene expression data.

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Author contributions

R.P. Wrote the original draft, prepared the figures, and did the experiments and data analysis. A.P. help with the in vitro experiments and data analysis. R.P., J.B., A.P., F.M. and P.Ch. review and edited the article. P.Ch. was the project administrator and find the resources and funding for the research and P.Ch. and J.B., did the conceptualization and validations of the experiments.

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Data availability

The authors declare that all relevant data generated is available in the Tables and Figures of the manuscript. The sequences of the target genes used in this study are found in the EmseblFungi database (Link: https://fungi.ensembl.org/index.html) under the following Ensembl ID codes and links: FOC1_g10012728 (Chs1) (link: https://fungi.ensembl.org/Multi/Search/Results?species=all;idx=;q=FOC1_g10012728;site=ensemblunit), FOC1_g10011241 (β-tub) (link: https://fungi.ensembl.org/Multi/Search/Results?species=all;idx=;q=FOC1_g10011241;site=ensemblunit), FOC1_g10010052 (ERG3 - Putative C-5 sterol desaturase) (link: https://fungi.ensembl.org/Multi/Search/Results?species=all;idx=;q=FOC1_g10010052;site=ensemblunit), FOC1_g10015438 (AdcyFocR1) (link:https://fungi.ensembl.org/Multi/Search/Results?species=all;idx=;q=FOC1_g10015438;site=ensemblunit), and FOC4_g10015430 (AdcyFocTR4) (link: https://fungi.ensembl.org/Multi/Search/Results?species=all;idx=;q=FOC4_g10015430;site=ensemblunit). The gene expression of FocChs1 data supporting this study have been provided as Additional File 12.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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