UNIVERSITY OF THESSALY

FACULTY OF HEALTH SCIENCES

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

Postgraduate studies: *ADVANCED EXPERIMENTAL AND COMPUTATIONAL BIOSCIENCES*

DIPLOMA THESIS

RNA biology of a plant-fungus interaction

Afroditi-Maria Katsaouni

Larissa 2022

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

Διπλωματική Εργασία

RNA βιολογία μιας αλληλεπίδρασης φυτού-μύκητα

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

THESIS EXAMINATION COMMITTEE:

Dr. Kalliope K. Papadopoulou, Professor, Plant Biotechnology

Dr. Athanasios Dalakouras, Agronomist/Biotechnologist, HELLENIC AGRICULTURAL ORGANIZATION – DEMETER

Dr. Antonios Giakountis, Assistant Professor, Molecular Biology - Genomics

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to Professor Kalliope K. Papadopoulou, Professor of Plant Biotechnology, under whose supervision this work was carried out, for her guidance, her trust and her unwavering support during the experimental process as well as the writing of this thesis. I also would like to express my gratitude to Dr. Athanasios Dalakouras, my co-supervisor and Agronomist/Biotechnologist of the HELLENIC AGRICULTURAL ORGANIZATION – DEMETER, for his guidance and firm support and encouragement he provided during my experiments as well as its writing. Without the guidance of my supervisors, and the valuable knowledge they provided, this work would never be possible. Additionally, I would like to express my gratitude to Professor Antonios Giakountis, Assistant Professor of Molecular Biology – Genomics, who guided me through the writing and helped shape my thesis into its final form. Finally, I would like to thank as well all the members of the Plant and Environmental Biotechnology laboratory for the pleasant and fruitful cooperation we had and for the help they offered me during my thesis.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

TABLE OF CONTENTS

Abstract **6**

Introduction **7**

Plant RNAi 7

Fungal RNAi 8

Cross-kingdom RNAi 9

Fusarium Solani strain K 10

Aim **12**

Materials and Methods **13**

Biological material (strains and plants) 13

In vitro transcription of sGFP dsRNA 14

In vitro RNAi assay 14

Nucleic acid isolations 14

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) 15

Trans-kingdom RNAi in *Nicotiana benthamiana* (Nb)-GFP plants 15

Quantification of fungal colonization and GFP silencing levels by RT-qPCR 16

Bisulfite sequencing 16

Small RNA sequencing 17

Results **18**

1. Plate reader assay; *in vitro* RNAi in FsK-sGFP 18

2. Characterization of FsK's RNAi machinery 19

3. RNA directed DNA Methylation (RdDM) in FsK 21

4. Translocation of RNAi signals from FsK to the host plant 22

5. Epigenetic modifications in the host plant after translocation of RNAi signals 25

Discussion **27**

Future Vision **30**

Conclusions **31**

Literature **32**

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

ABSTRACT

Fusarium solani strain K (FsK) is a soil-borne beneficial endophytic fungus, which confers resistance and/or tolerance to biotic and abiotic stress in tomato and, according to recent studies, promotes plant growth in *Nicotiana benthamiana*. The mechanistical details governing the interaction between this endophyte and its hosts are still elusive, but a growing body of evidence suggests that RNA interference (RNAi)-related processes underlie the onset of symbiosis, similarly to the communication between plants and pathogenic fungi, where a bi-directional cross-kingdom RNAi is established to the advantage of either the host or the pathogen. Here, we report on an RNAi-based mechanism of communication between the beneficial fungal endophyte FsK and its host plant. After characterizing the core RNAi machinery of FsK, we demonstrate that the fungus's DCLs cleave double stranded RNAs (dsRNAs) into short interfering RNAs (siRNAs) of primarily 21-nt in size, which lead to the degradation of homologous mRNAs in the fungal hyphae. Importantly, we show that root colonization of *N. benthamiana* by FsK led to the induction of systemic silencing and DNA methylation of a host reporter gene. This study, overall, reflects a so far uncharacterized mechanism in which beneficial fungal endophytes translocate RNAi signals to their hosts upon symbiosis to regulate gene expression, suggesting that trans-kingdom RNAi phenomena may govern symbiotic relationships to a greater extent than previously thought.

Keywords: Endophytes, epigenetics, *Fusarium solani*, *Nicotiana benthamiana*, RNA interference, small RNAs.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

INTRODUCTION

Plant RNAi

RNA interference (RNAi) is a conserved eukaryotic gene regulatory mechanism, triggered by small RNAs (sRNAs) of approximately 20-25 nucleotides (nt) (Gutbrod and Martienssen, 2020)Baulcombe, 2004; Hung and Slotkin, 2021). RNAi was first discovered in *C. elegans* and it was defined as sequence-specific mRNA degradation induced by long double-stranded RNAs (dsRNAs) (Fire *et al.,* 1998). In plants this process has diversified into mechanisms to defend the plant against viruses, protect the genome from transposons and regulate gene expression (Baulcombe 2004). **RNAi in plants has diverse roles with small RNAs being involved in a variety of phenomena that are essential for genome stability, development, and adaptive responses to biotic and abiotic stresses**. The mode of action of sRNAs is also diverse, and despite the complexity of RNAi pathways and the numerous sRNA classes, there are essentially two types of sRNAs: the small interfering RNAs (siRNAs) and the microRNAs (miRNAs) (Borges and Martienssen, 2015; Vaucheret, 2006).

In plants, miRNA biogenesis begins with PolII-transcription of an endogenous MIR gene; the occurring stem-loop hairpin RNA transcript is processed by Dicer-like endonuclease 1 (DCL1) into 21-nt miRNA that are loaded on Argonaute 1 (AGO1) and mediate mRNA cleavage and/or translational arrest of endogenous targets (Voinnet, 2009). In contrast siRNA biogenesis is initiated by the occurrence of perfect dsRNAs, that may form upon viral replication or PolII transcription of inverted repeats. In plants, long dsRNAs are processed by DCL4, DCL2 and DCL3 into 21-, 22- and 24-nt siRNAs, respectively (Dalakouras et al., 2020) (Figure 1). DCL4-produced 21-nt siRNAs are loaded on AGO1 and mediate mRNA cleavage and degradation, a process often termed Post-Transcriptional Gene Silencing (PTGS) (Vaucheret, 2006). DCL2-produced 22-nt siRNAs are also loaded on AGO1 but, instead of mediating mRNA cleavage, recruit RNA-dependent RNA polymerase 6 (RDR6) to the mRNA target, transcribing it into dsRNA and thus leading to the amplification and spreading of silencing, a phenomenon termed transitivity (Voinnet, 2008). Finally, DCL3-produced 24-nt siRNAs are loaded on AGO4 and are transported to the nucleus, where they recognize cognate DNA sequences (or their corresponding nascent transcripts), recruiting the Domains Rearranged methyltransferase 2 (DRM2) to methylate the corresponding cytosines in CG, CHG and CHH context, a mechanism termed RNA-directed DNA methylation (Wassenegger and Dalakouras, 2021). Notably, RdDM may further ensue chromatin modifications such as histone methylation and deacetylation,

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

eventually leading to heterochromatinization and Transcriptional Gene Silencing (TGS) (Gallego-Bartolomé, 2020).

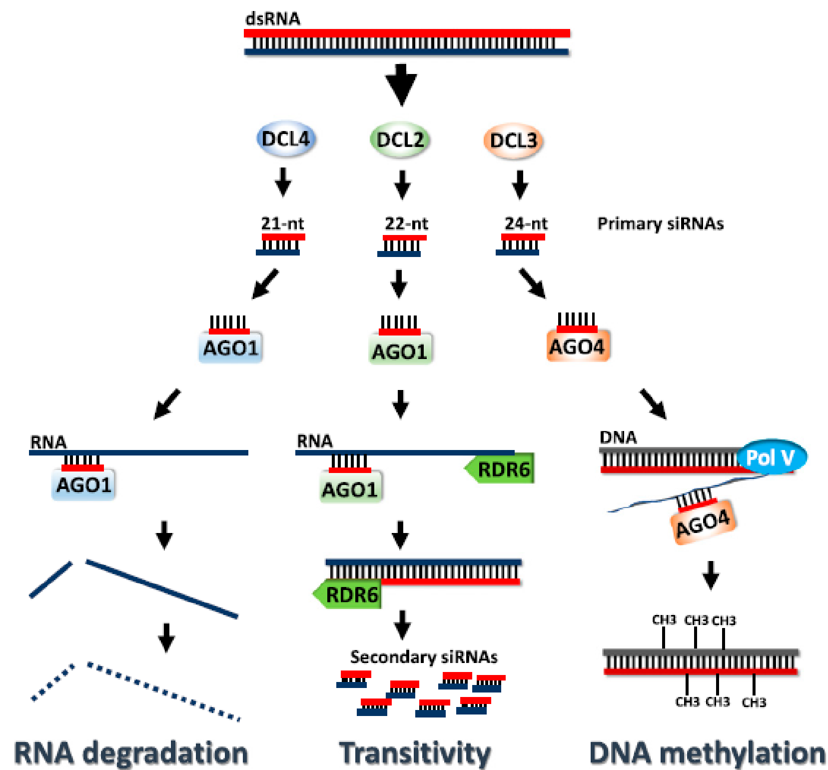

Fig 1. The siRNA pathway in plants (Dalakouras *et al.,* 2020)

Fungal RNAi

Fungal RNAi was initially described as 'quelling' in the vegetative tissue of the filamentous fungus *Neurospora crassa* and is mediated by sRNAs (Romano and Macino, 1992). The mechanistic details and role of RNAi in the fungal kingdom is diverse, and it has been observed that not even all fungi encode for important components of the RNAi pathway, despite being conserved among eukaryotic organisms. However, it is known to have a two-fold role: (a) siRNAs generated from (usually RDR-transcribed) dsRNA precursors are involved in genome defense and maintenance of genome integrity as well as fighting against transposons, viruses and transgenes (Lax *et al.*, 2020); (b) miRNAs (also called miRNA-like, milRNAs), generated by Pol III-transcribed primary miRNA transcripts, regulate gene

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

expression during vegetative and sexual development, while also responding to different types of stresses (Torres-Martinez and Ruiz-Vazquez, 2017; Lax et al., 2020). It has been reported, however, that *Saccharomyces cerevisiae* and *Ustilago maydis* lack DCLs, AGOs and RDRs proteins (Drinnenberg et al., 2009), while miRNAs have been identified in most fungal species but not in the basal fungus *Mucor circinelloides* (Torres-Martinez and Ruiz-Vazquez, 2017). Generally, the fungal kingdom depicts a remarkable diversity of RNAi pathways and functions and the numbers of RNA silencing proteins differ considerably among fungal species.

Cross-kingdom RNAi

Plants are constantly subjected to biotic stressors, such as invasions by parasitic plants, nematodes, insects, and microorganisms (Atkinson and Urwin, 2012), and these plants and their invaders exchange a large number of molecules such as nutrients, proteins and nucleic acids (Horbach *et al.,* 2011). During plant-microbe interactions, plants can identify conserved microbial patterns (microbe associated molecular pattern) with membrane-bound receptors (pattern recognition receptor) and consequently trigger their immunity to these infections (Nurnberger *et al.,* 2004; Zhang *et al.,* 2014), while the microbes release effector proteins to suppress the host's immunity and effectively colonize the host. Some plants have additionally acquired resistance (R) proteins as an additional level of defense, in order to control pathogen effectors (de Jonge *et al.,* 2010; Thomma *et al.,* 2011). During viral infections, dsRNA molecules from the virus are recognized by host DCL or AGO proteins and cleaved into virus-derived small interfering RNAs (viRNAs), acting as viral effectors by targeting host genes (Shimura *et al.,* 2011; Smith *et al.,* 2011). Similarly, during interactions between fungal pathogens and plants, effector translocation and effector-triggered immunity have been extensively studied while the mechanisms related to sRNA translocation and RNAi-triggered immunity remain elusive.

A growing body of recent evidence suggests that RNAi has an important role in the communication of fungi with their hosts. SRNAs implicated in gene silencing, are reported to be transmitted bidirectionally between fungal pathogens and their hosts. *Botrytis cinerea* has been found to deliver sRNA molecules in *Arabidopsis* and tomato targeting members of the mitogen-activated protein kinases (MAPKs) which function in plant immunity. In addition, this interaction seems to also take place in reverse, where tomato and *Arabidopsis* plants transmit sRNAs to in *B. cinerea* targeting the fungal dicer-like 1 and dicer-like 2 and thus attenuating fungal pathogenicity and growth (Wang et al., 2016), (Weiberg et al., 2013)**.** Similarly, cotton plants transmit sRNAs to the vascular pathogen *Verticillium dahliae* upon infection, targeting

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

the isotrichodermin C-15 hydroxylase and Ca(2+)-dependent cysteine protease, both of which are required for fungal pathogenicity (Zhang *et al.* 2016). One of the most severe wheat pathogens, *Puccinia striiformis* (*Ps*) delivers sRNAs to its host, like a novel microRNA-like RNA1 (milR1), targeting the wheat *Pathogenesis-related 2* gene in the defense pathway (Wang *et al*. 2017). Likewise, *Fusarium graminearum* translocates sRNAs to target defense genes in *Hordeum vulgare* and *Brachypodium distachyon*. However, the exact mechanism of this cross-talk of sRNAs between fungi and their hosts has not been established yet and there has been **no clear observation of a similar cross-talk between plants and beneficial fungi**. Indeed, some recent *in silico* studies about the AMF strain *Rhizophagus irregularis* and the beneficial root endophyte *Serendipita indica* and their hosts, predict the production of sRNAs with candidate targets in the host plant, and also vice versa in the case of *Serendipita indica* (Silvestri *et al*. 2019, Secic *et al.* 2021). However, in these studies, the transfer of sRNAs and the reciprocal RNAi- mediated targeting of mRNAs and transcript degradation were not established.

Fusarium solani strain K

The ascomycete *Fusarium solani* is the anamorph state of *Nectria haematococca* and a member of the '*F. solani* species complex (FSSC)' (Coleman et al., 2009, Coleman, 2016). *Fusarium solani* strain K (FsK) is an endophytic, non-pathogenic strain, previously isolated from the roots of tomato plants in suppressive compost (Kavroulakis et al., 2007) and according to recent studies, it can colonize also other plant species, including legumes (Skiada et al., 2019). FsK has been observed to be capable of protecting tomatoes against root and foliar pathogens, as well as against spider mites and zoophytophagous insects (Kavroulakis *et al.*, 2007; Pappas *et al.*, 2018; Garantonakis *et al.*, 2018). The endophyte can also enhance its host tolerance against abiotic stresses, like drought stress (Kavroulakis *et al.*, 2018). Interestingly, it has been demonstrated that the ethylene signaling pathway of the host is important for the mode of action of the endophyte in order to confer resistance to abiotic and biotic stresses (Kavroulakis et al., 2007). This observation suggests that the endophyte can induce systemic responses to the plant.

Recent studies demonstrated that the fungus can also effectively colonize another member of the Solanaceae, *Nicotiana benthamiana,* a widely used model plant for RNAi studies (Philips et al., 2017), while upon its colonization the endophyte demonstrated an ability to promote plant growth in *Nicotiana benthamiana* plants (Dalakouras, Katsaouni *et al,* unpublished data) (Fig. 2b, 2c).

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

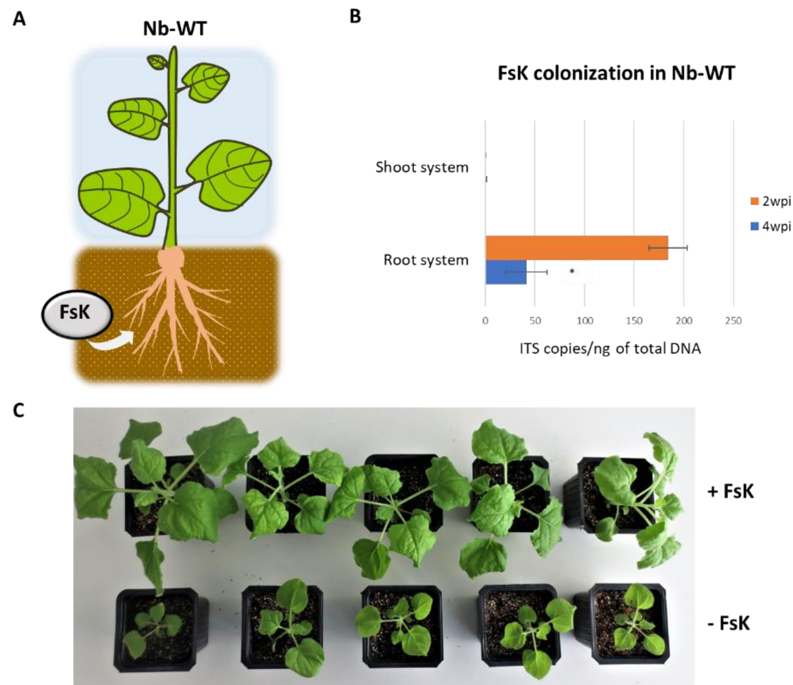

Fig. 2. Colonization of Nb-WT by FsK. (A) Schematic representation of the colonization assay. (B) Quantification of fungal colonization in shoot and root system at 2 and 4 wpi. (C) Impact of the FsK in growth of Nb-WT 4 wpi (unpublished data).

Furthermore, in both tomato and *N. benthamiana* plants, the endophyte can colonize effectively the root system but fails to expand to the shoot system (Fig. 2b). These results reflect on an ability of FsK to establish communication and initiate a beneficial symbiotic relationship with its hosts, although its effects and mode of action in different hosts remain to be elucidated.

Additionally, although not all fungi encode for the core RNAi pathway, as mentioned, recent studies of the Plant and Environmental Biotechnology group (unpublished data), demonstrated through transcriptome-validated genome annotation that FsK encodes for core RNAi components. Two DCLs (FsKDCL1 and FsKDCL2), two AGOs (FsKAGO1 and FsKAGO2) and four RDRs (FsKRDR1-4) were identified with their corresponding domains, suggesting the possibility that FsK has a functional RNAi machinery, further enhancing our interest towards the role of RNAi during FsK-host symbiosis (Fig. 3).

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

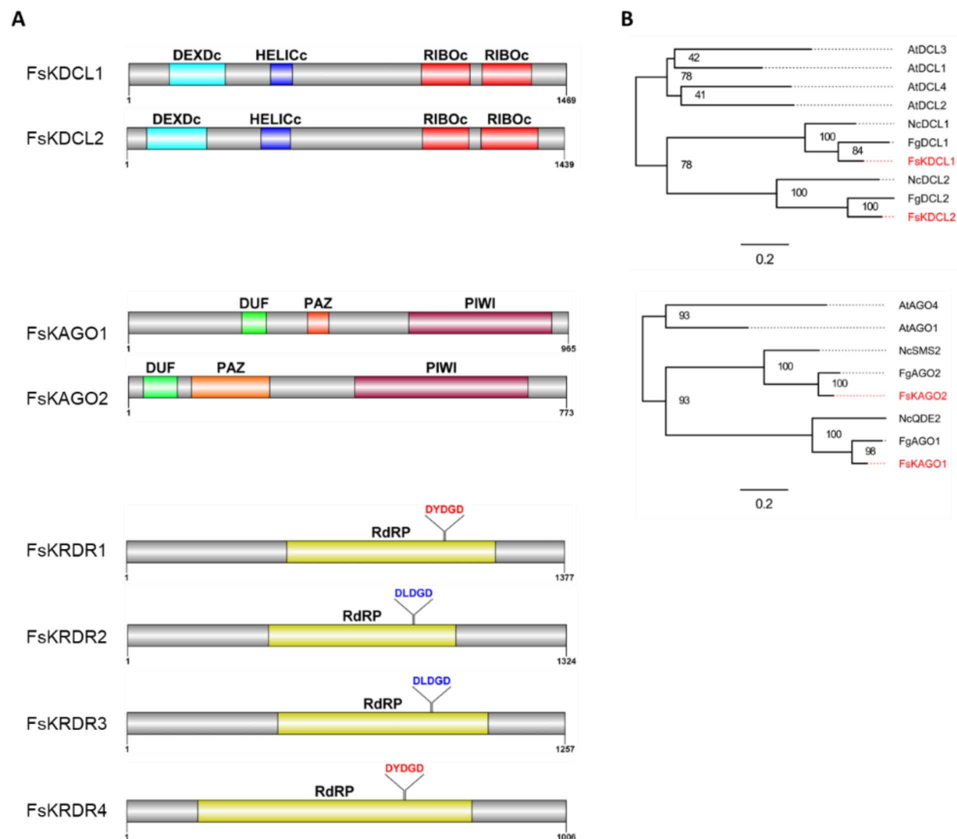

Fig. 3 Identification of FsK RNAi core machinery; (A) Schematic representation of FsK DCL, AGO and RDR proteins using DOG1.0 software (Ren *et al.,* 2009). (B) Maximum likelihood phylogenies of the FsK (indicated red), Fusarium graminearum, *Neurospora crassa* and *Arabidopsis thaliana* (as an outgroup member) DCL, AGO and RDR proteins using the LG model matrix and 100 bootstrap replicates for assessing branch support.

Overall, the mechanistic details of the mode of action of FsK and how it communicates with its hosts upon this symbiosis are elusive, and there is a great need for further characterization of the interaction and its effects on different hosts.

AIM

The purpose of this study was to characterize FsK's core RNAi machinery and establish whether the endophyte is able to transmit RNAi signals to its host plant upon symbiosis to control expression of a host reporter gene, and if these signals can trigger epigenetic modifications of the reporter gene.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

MATERIALS AND METHODS

Biological material (strains and plants)

Nicotiana benthamiana plants

Nicotiana benthamiana seeds used for the experiments, were prepared via sterilization with 10% chlorine solution for 5 minutes and washes with sterilized ddH_2O. Subsequently, the seeds were spread in ½ Murashige and Skoog (MS) agar plates and covered with aluminum foil for ~7 days and then grown in the light in RT conditions for ~7-15 more days. The seedlings were then planted in 250ml pots using sterilized sand mix as the growth medium. The mix contained a 2:1:1 ration of coarse sand, fine-grained sand and vermiculite. After planting of the seedlings, the plants were grown for 4 more days, until the emergence of their true leaves, in 25,5°C, 60% humidity and 16/8 h light-dark cycle.

FsK strains

The FsK strains used for the experiments were following:

- FsK-sGFP, i.e. an sGFP-expressing FsK transformant, available in the lab, which contains a GFP variant with a serine-to-threonine substitution at amino acid 65, optimized for use in fungi (Sesma and Osbourn, 2004) (Fig 4a, 4b).
- FsK-hpGF+GFP, i.e. FsK transformed with a transgene comprised of a full length green fluorescent protein (GFP) corresponding to mGFP5-ER (Haseloff and Siemering, 2006) and a hairpin (hp) construct of the first 332 bp of GFP (hpGF). Three independent transformants were available in the lab, with the sole difference that each one of them was a separate transformation event, thus the only difference was the integration site. These different transformants were named “6”, “7”, and “27” (Dalakouras, Katsaouni *et al.*,2022 unpublished results).

Isolation of fungal conidia.

FsK was routinely cultured for 4 days in potato dextrose broth (PDB) (26 °C, 160 rpm). Following removal of mycelium fragments by sieving through sterile cheesecloth, conidia were recovered from the filtrate by centrifugation at 6,500 rpm, counted using a haemocytometer and suspended in an appropriate volume of 0.85% NaCl to achieve the desired inoculum

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

concentration. Approximately 100 conidia were used to inoculate *N. benthamiana* plants at the cotyledon stage.

In vitro transcription of sGFP dsRNA

For the generation of the *in vitro* transcribed sGFP dsRNA, genomic DNA was extracted from FsK-sGFP (Sesma and Osbourn, 2004) and used as template for PCR with KAPA Taq DNA Polymerase (www.sigmaaldrich.com) with the T7 promoter-containing primers 5'-taa tac gac tca cta tag gga gaC GTA AAC GGC CAC AAG TTC AGC-3' and 5'-taa tac gac tca cta tag gga gaG TGG CGG ATC TTG AAG TTC ACC-3' (T7 promoter sequence with lowercase). The T7 promoter-containing 491 bp amplicon was then used as template in the MEGAscript™ RNAi Kit (www.thermofisher.com) for the generation of a 445 bp sGFP dsRNA.

In vitro RNAi assay

In 24 wells of a 96-well plate, FsK-sGFP conidia were added (in each well, 6 conidia diluted in 100 μl PDB/100). *In vitro* transcribed sGFP dsRNA was added (100 μl, 1 ng/μl) in 12 wells (dsRNA application samples). In the remaining 12 wells containing FsK-sGFP conidia, 100 μl water was added (control samples). The 96-well plate was covered with a removable membrane and incubated at 25° C. At timepoints 0-24-48-hour post application (hpa), the plate was subjected to fluorometric analysis using the Varioskan™ LUX multimode microplate reader (www.thermofisher.com). The ratio of sGFP-indicative fluorescence (excitation 488 nm, emission 515 nm) to growth-indicative absorbance (wavelength 595 nm) was calculated as relative fluorescence unit (RFU).

Nucleic acid isolations

- Genomic DNA from plant and fungal tissue was isolated with DNeasy Plant Pro (/www.qiagen.com) according to the manufacturer's instructions.
- The RNA extractions were performed using TRItidy G™ Reagent (www.thermofisher.com) according to the instructions provided by the company, where 100mg were used from each tissue sample each time.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

- For small RNA sequencing, the enriched for small RNAs fraction was isolated from the mycelium using mirVana™ miRNA Isolation Kit (www.thermofisher.com) according to the manufacturer's instructions.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

DNaseI-treated (www.thermofisher.com) RNA isolated from mycelium was quantified by Qubit Fluorometric Quantification (www.thermofisher.com). The DNA-free RNA (10 ng) was then subjected to RT-qPCR using the Luna® Universal Probe One-Step RT-qPCR Kit (www.neb.com) according to the manufacturer's instructions. Minor modifications include: the total volume of the reaction was reduced to 10μl and the cycling parameters consisted of incubation at 55˚C for 10 min for reverse transcription, 95˚C for 1 min followed by 39 cycles of 95˚C for 10 sec and 60˚C for 30 sec. Analysis was carried out using the geometric mean of FsK ITS and Tef-1a transcripts as a reference. For Tef-1a (120 bp amplicon), the primers 5'-TCG AAC TTC CAG AGG GCA AT-3' and 5'-CCA ACA ATA GGA AGC CGC TG-3' were used. For ITS (108 bp amplicon), the primers 5'-TAG GGT AGC TGG GTC TGA CT-3' and 5'-ACC AAG TCT AAC CCG CCT AC-3' were used. For GFP (133 bp amplicon), the primers 5'-TCC CAG CAG CTG TTA CAA AC-3' and 5'-AAT ACT CCA ATT GGC GAT GG-3' were used.

The relative expression of GFP gene was calculated from two to three technical replicates for every sample as described in the corresponding figure legend. Data were analyzed using the student's two-tailed homoscedastic t-test.

Trans-kingdom RNAi in *Nicotiana benthamiana* (Nb)-GFP plants

For the *in vivo* silencing experiment, mGFP-expressing *Nicotiana benthamiana* plants of the "16C" line (Nb-GFP, Voinnet and Baulcombe, 1997) were inoculated with fungal conidia from the available hpGF+GFP expressing FsK strain (i.e. FsK transformation event "27", Dalakouras, Katsaouni *et al.,* 2022 unpublished*)*. Twently (20) Nb-GFP plants were inoculated with FsK-hpGF+GFP, 10 Nb-GFP plants were used as the silencing "negative" control and 10 Nb-WT plants were used as the silencing "positive" control. The experiment was repeated with no significant differences; results are presented from one experiment only.

N. benthamiana plants were handled as mentioned above and on the 4th day after planting, the roots were inoculated with 10^2 conidia of the FsK-hpGF+GFP strain. UV observations of the stems and leaves took place every 7 days post inoculation (dpi). Samples of the roots and

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

leaves were taken from the plants at 6 weeks post inoculation (wpi). Samples were grinded with liquid nitrogen using sterile a mortar and pestle.

Quantification of fungal colonization and GFP silencing levels by RT-qPCR

DNaseI-treated (www.thermofisher.com) RNA isolated from the plants was quantified by Qubit Fluorometric Quantification (www.thermofisher.com). The DNA-free RNA (10 ng) was then subjected to RT-qPCR using the the Luna® Universal Probe One-Step RT-qPCR Kit (www.neb.com) according to the manufacturer's instructions. Essentially, the total volume of the reaction was reduced to 10μl and the cycling parameters consisted of incubation at 55˚C for 10 min for reverse transcription, 95˚C for 1 min followed by 39 cycles of 95˚C for 10 sec and 60˚C for 30 sec. Analysis was carried out using Nb-Actin transcript as a reference gene, the mGFP transcript for the measurement of fluorescence differences (silencing) between samples, and finally, the FsK ITS transcript as a measurement of colonization levels in the roots. For, Actin (169 bp amplicon) the primers 5'- CGA GCG GGA AAT TGT TAG GG -3'and 5'-AAC TTC TGG GCA GCG GAA TC-3' were used. For ITS (108 bp amplicon), the primers 5'-TAG GGT AGC TGG GTC TGA CT-3' and 5'-ACC AAG TCT AAC CCG CCT AC-3' were used. For GFP (133 bp amplicon), the primers 5'-TCC CAG CAG CTG TTA CAA AC-3' and 5'-AAT ACT CCA ATT GGC GAT GG-3' were used. To create the bar plots of the results, we used the ggplot2 package via RStudio (Wickham H. 2016, RStudio Team 2022).

Bisulfite sequencing

Genomic DNA from the fungus (20 ng) or the plant (100 ng) was used for bisulfite sequencing analysis using the EZ DNA Methylation-Gold Kit (www.zymoresearch.com) according to the manufacturer's instructions and as previously described (Dalakouras et al., 2016). First, the samples were treated with the EZ DNA Methylation-Gold Kit according to instructions and the samples were the used to isolate the desired DNA fragment for analysis (TA-cloning and sequencing). Essentially, for the cis-RdDM bisulfite analysis on FsK, the primers 5'-AAT CTC CAR TRR RTA CAC TAT TC-3' and 5'-CCT CCT TRA AAT CRA TTC CCT TAA-3' were used, whereas for the trans-RdDM bisulfite analysis on FsK and Nb-GFP the primers 5'-AGT GGA GAG GGT GAA GGT GAT G-3' and 5'-CCT CCT TRA AAT CRA TTC CCT TAA-3' were used in a PCR reaction with ZymoTaq PreMix (www.zymoresearch.com) according to the manufacturer's instructions. The occuring 262 bp and 311 bp amplicons for cis-RdDM and trans-RdDM,

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

respectively, were cloned into pGEM®-T Easy Vector (worldwide.promega.com) and for each analysis 10-12 clones were subjected to Sanger sequencing.

Small RNA sequencing

Sequencing of small RNAs from fungal RNA (small RNA fraction) was performed by GenXPro (https://genxpro.net/) as previously described (Dalakouras *et al.,* 2016). Essentially, for the preparation of sRNA libraries, 300 ng RNA (sRNA fraction) was spiked with 1 fmol *Caenorhabditis elegans* miR-39 RNA and ligated using the Next Small RNA Library Prep (New England Biolabs) with modified 3' and 5' adapters (TrueQuant RNA adaptors, GenXPro). Adapter-ligated RNA was reverse transcribed (First-Strand Synthesis System; ThermoFisher Scientific), purified with SPRI beads (SPRIselect; Beckman Coulter, http://www.beckmancoulter.com) and PCR-amplified with nine cycles (KAPA HiFi Hot-Start Polymerase; Kapa Biosystems, http://www.kapabiosystems.com). The PCR products were purified (Amicon Utracel-10; EMD Millipore, http://www.emdmillipore.com) and size-selected by polyacrylamide gel electrophoresis. The sRNA library was sequenced on an Illumina HiSeq2000 with 1950 bps. SRNA reads from 15 up to 40 nt were considered. TABLET software was used for reading the data and sRNA mapping depictions (Milneet al., 2013).

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

RESULTS

1. Plate reader assay; *in vitro* RNAi in FsK-sGFP

As mentioned, not all fungi encode for core components of the RNAi machinery, and not all fungi have a functional RNAi machinery. However, our recent data suggest that FsK encodes for core components of the RNAi machinery (Dalakouras, Katsaouni *et al*. 2022, unpublished). More specifically, after transcriptome-validated genome annotation, two DCLs (FsKDCL1 and FsKDCL2), two AGOs (FsKAGO1 and FsKAGO2) and four RDRs (FsKRDR1-4) were identified in FsK. In accordance, our first aim was to establish the functionality of FsK's predicted RNAi machinery, and to achieve that we utilized the FsK-sGFP strain provided by the lab (Fig. 4a, 4b).

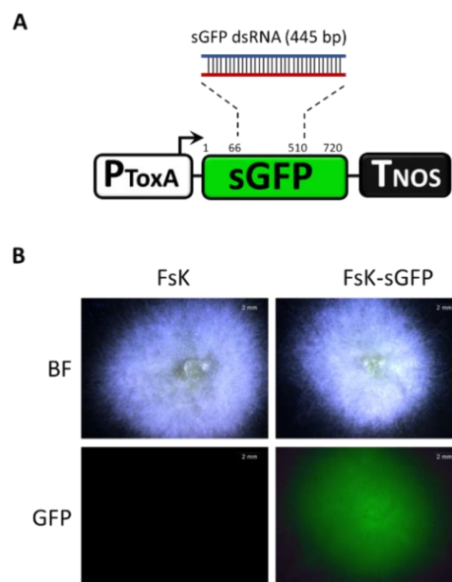

Fig.4 sGFP-expressing FsK; **(A)** Graphical representation of the the sGFP transgene PToxA: promoter *from Pyrenophora tritici-repentis* ToxA gene; sGFP: GFP variant with a serine-to-threonine substitution at amino acid 65; TNOS: terminator for the nopaline synthase gene. The 445 bp fragment chosen for in vitro transcription of dsRNA is included. **(B)** Stereoscopic observation of sGFP fluorescence.

In order to determine the functionality of the RNAi machinery of FsK, we aimed at establishing whether FsK is able to take up this dsRNA from its environment and process it via its RNAi machinery leading to a downregulation of the fungus' sGFP. To this end, we applied exogenously an *in vitro* transcribed dsRNA that targets the sGFP transgene. A plate reader assay was designed where an *in vitro*-transcribed 445 bp sGFP dsRNA was applied to isolated conidia of the FsK-sGFP strain (Figs 4a, 4b). After fluorometric analysis at 0, 24 and 48 hours post application (hpa), the sGFP expression levels dropped to almost 50% at 24 hpa (Fig. 5).

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

This suggests that the externally applied dsRNA targeting the sGFP mRNA, was processed by fungal DCLs into siRNAs, which in turn mediated the cleavage of the sGFP mRNA. However, later timepoints (48 hpa) revealed no additional decrease in sGFP levels.

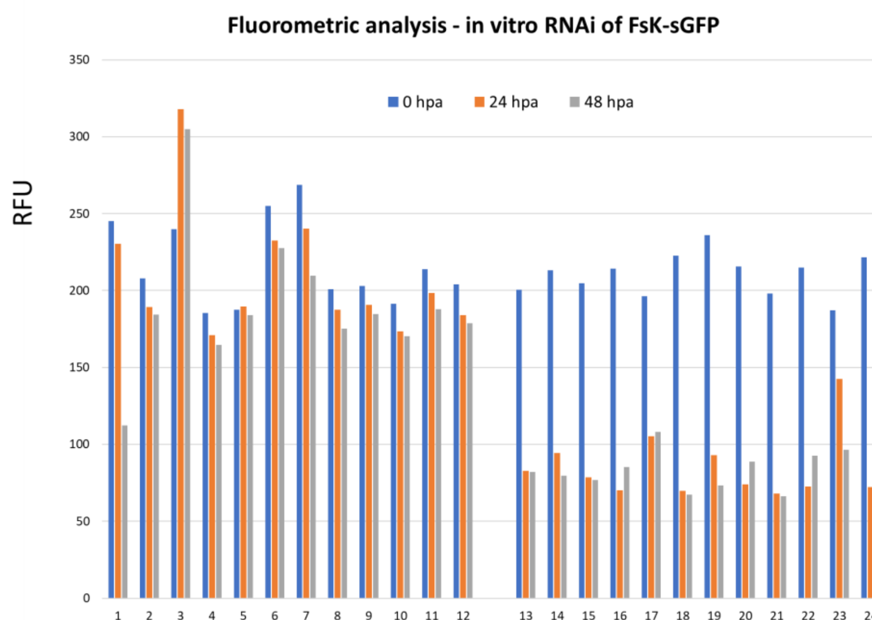

Fig.5 Fluorometeric analysis for in vitro RNAi in FsK-sGFP. Vertical axis: RFU: relative fluorescence unit, calculated as the ratio of sGFP-indicative fluorescence (excitation 488 nm, emission 515 nm) to growth-indicative absorbance (wavelength 595 nm). Horizontal axis: 1-12: 12 wells containing FsK-sGFP conidia. 13-24: 12 wells containing FsK-sGFP conidia plus 100 ng (each well) sGFP dsRNA.

Overall, these findings imply that FsK's RNAi machinery is not only functional, but FsK is also able to take up RNAi molecules from its environment and process them accordingly.

2. Characterization of FsK’s RNAi machinery

In order to examine the mode of dsRNA processing in the endophyte, FsK was previously transformed with a transgene comprised of a full length green fluorescent protein (GFP) corresponding to mGFP5-ER (Haseloff and Siemering, 2006) and a hairpin (hp) construct of the first 332 bp of GFP (hpGF) (Dalakouras, Katsaouni et al 2022, unpublished) (Fig. 6). With this design, an “RNAi-trigger” (represented by the hpGF locus) and a “RNAi-target” (represented by the GFP locus) were combined in one final construct.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

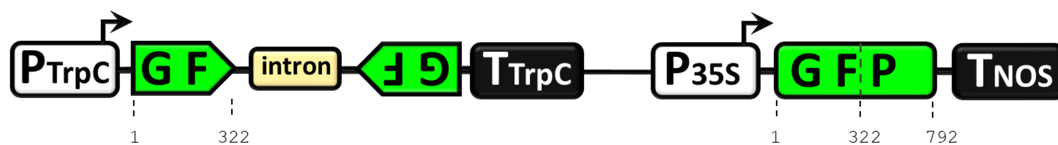

Fig.6 Schematic representation of the hpGF+GFP transgene. PTrpC: promoter for the *Aspergillus nidulans* trpC gene; GF: 322 bp fragment of the GFP; intron: *Magnaporthe grisea* cutinase gene intron; TTrpC: terminator for the *Aspergillus nidulans* trpC gene, P35S: Cauliflower mosaic virus 35S promoter; GFP: full-length (792 bp) green fluorescent protein (mGFP-ER version); TNOS: terminator for the nopaline synthase gene. FsK-hpGF+GFP transformants contain the full length hpGF+GFP transgene, whereas FsK-GFP transformants contain only the P35S-GFP-TNOS part of the transgene

Three independent FsK-hpGF+GFP transformants (#6, #7, #27) were used to isolate enriched for small RNAs fractions and, then, for small RNA sequencing (sRNA-seq). As shown in Fig. 7, the sRNA-seq revealed the abundant accumulation of siRNAs matching the GF region of the GFP transgene. Only a very small fragment of these reads was mapped in the remaining sequence of GFP (P region) (Fig. 7a), clearly showing the absence of RDR-dependent transitivity in the fungal hyphae. The siRNAs had variable sizes, mostly of 21-nt, 22-nt and 24-nt (Figs 7a, 7b).

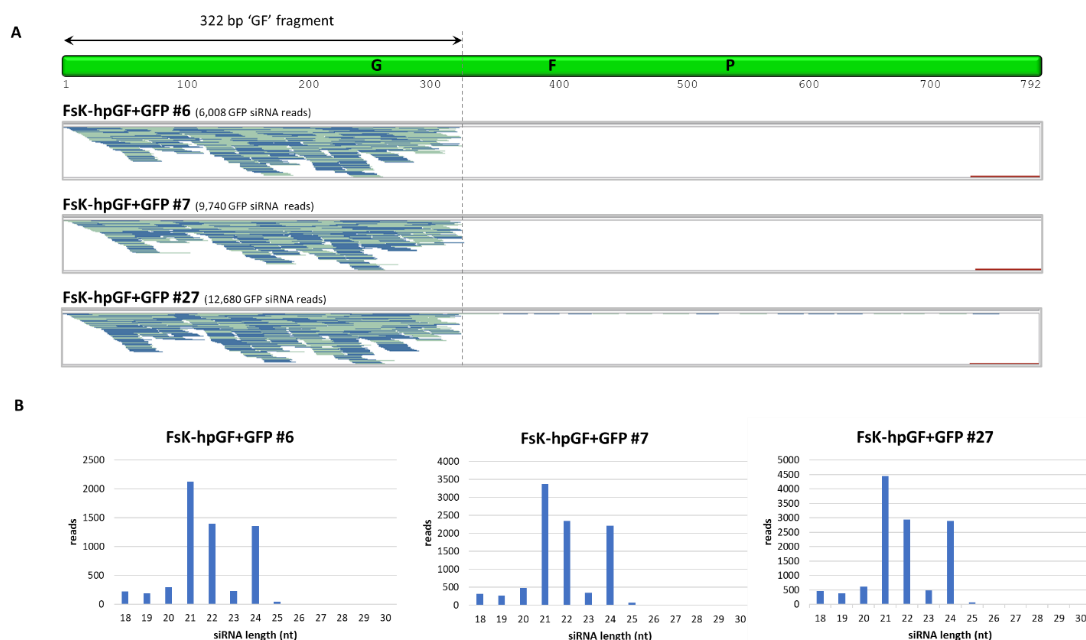

Fig. 7 Small RNA sequencing; sRNA-seq in FsK-hpGF+GFP #6, #7 and #27. All sRNA reads of 18-30-nt fully matching to GFP region are depicted. **With light blue the siRNA reads in plus polarity, with dark blue the siRNA reads in minus polarity.** The Tablet software (Milne *et al.,* 2013) was used for visualization of the sRNA reads. (B) Bar graphs depicting the different lengths (18-30 nt) of GFP siRNAs found in FsK-hpGF+GFP#6, #7 and #27 in relation to their number of reads.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

As a next step, we sought to determine if these siRNAs indeed have a biological activity. In order to achieve that, we measured the GF siRNA-mediated downregulation of the GFP mRNA in all three independent FsK-hpGF+GFP transformants (#6, #7, #27) when compared to FsK-GFP (transformed with a cassette lacking the hpGF transgene) (Fig. 8). As observed, in all three independent transformants, we had a significant decrease of the mGFP expression indication that these siRNAs resulting from the sRNA-seq are indeed products of the RNAi machinery of FsK (DCLs) and have a biological activity. **Overall, these observations further confirm the presence of a functional RNAi machinery in FsK, able to process hpRNAs into siRNAs and lead to a downregulation of the target transcript in the fungal hyphae.**

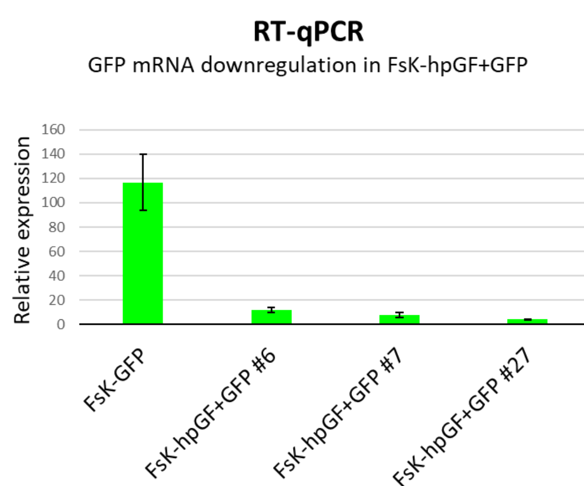

Fig. 8 RT-qPCR for the estimation of GFP mRNA downregulation in FsK-hpGF+GFP compared to FsK-GFP.

3. RNA directed DNA Methylation (RdDM) in FsK

Although 24-nt siRNA-triggered RNA directed DNA Methylation (RdDM) is a common phenomenon in plants (Dalakouras and Vlachostergios, 2021), only recently sRNA-dependent RdDM-like phenomena were reported in some fungal species (Zhang *et al.*, 2018, Sperschneider *et al.*, 2021). Given the abundant accumulation of 24-nt GF siRNAs in FsK-hpGF+GFP, our next goal was to determine whether these siRNAs could trigger RdDM events of cognate DNA sequences in the FsK transformant. Since the results of the three transformants so far gave us almost identical results, we chose to continue with transformant #27. To analyze cis-RdDM (at the locus generating the siRNAs), we chose a 262 bp fragment of the hpGF transgene (Fig. 9a). For trans-RdDM (at a locus that does not generate siRNAs but is homologous to them), we chose a 311 bp fragment of the GFP transgene (Fig. 9a). In order to obtain a detailed overview of the cytosine methylation

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

status of these two fragments in base pair resolution, bisulfite sequencing was performed. Treatment of DNA with sodium bisulfite results in the conversion of non-methylated cytosine to uracil, and during PCR amplification uracil is replaced by thymine. Thus, sequences of PCR products from bisulfite-treated DNA exhibit thymines for non-methylated cytosines (Clark *et al.*, 2006). Accordingly, after bisulfite sequencing, we observed no methylated cytosines in any sequence context (CG, CHG, CHH), at neither cis (Fig. 9b) nor trans (Fig. 9c) loci, suggesting that no RdDM takes place in FsK, despite the presence of 24-nt siRNAs.

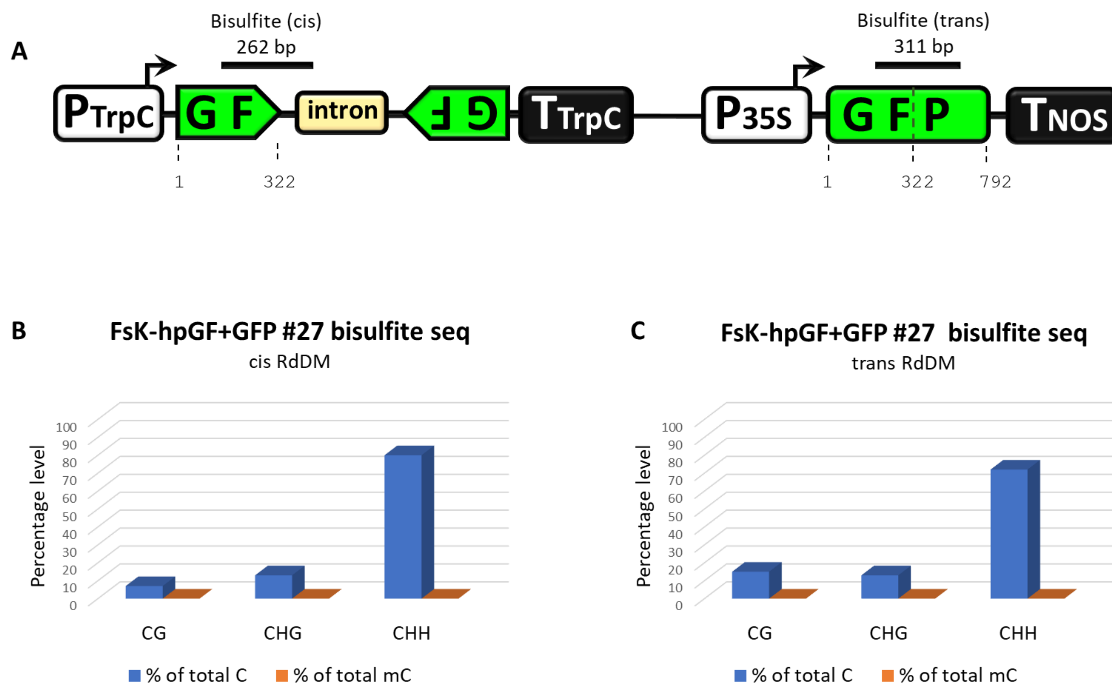

Fig. 9 Bisulfite sequencing results; (A) Schematic representation of the hpGF+GFP transgene. Visualization of the fragments chosen for cis-RdDM and trans-RdDM analysis. (B) Bisulfite sequencing for cis RdDM. (C) Bisulfite sequencing for trans RdDM.

4. Translocation of RNAi signals from FsK to the host plant

Recently small RNAs involved in gene silencing, have been found to be transmitted bidirectionally between fungal pathogens and their hosts, thus establishing a cross-kingdom RNA communication between host and pathogen (Hua *et al.*, 2018). It is very likely that, similar to fungal pathogens, beneficial fungal endophytes also display an RNA-based communication with their hosts. However, **so far there have been no clear observations of RNAi molecule translocation and subsequent cross-kingdom RNAi between beneficial fungal endophytes**

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

and their hosts. Having demonstrated that FsK has a fully functional RNAi machinery, we examined the capacity of FsK for translocation of RNA signals and cross-kingdom RNAi communication with its host, by utilizing the GFP-expressing *N. benthamiana* plant line "16C" (Nb-GFP) (Voinnet and Baulcombe, 1997), as an RNAi sensor system. Specifically, Nb-GFP is a well characterized RNAi model system with a mGFP5-ER transgene controlled by the 35S promoter (Fig. 10) and can be used for monitoring of systemic RNAi by observing the GFP expression under ultraviolet light, in the stem and leaves of the plant.

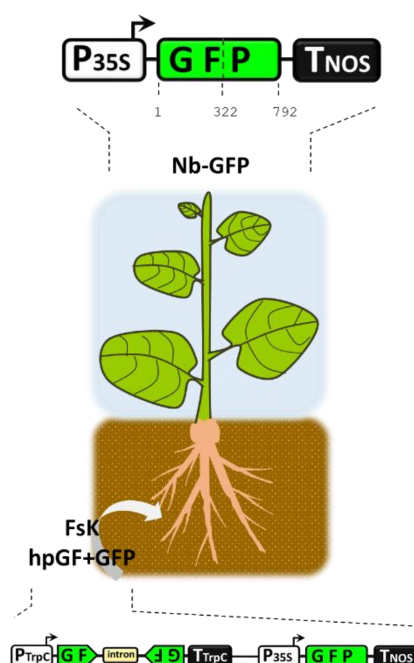

Fig 10. FsK-hpGF+GFP colonization of Nb-GFP. Schematic overview of the colonization assay. And the GFP transgene of 16C (Nb-GFP) plants. P35S: Cauliflower mosaic virus 35S promoter; GFP: full-length (792 bp) green fluorescent protein (mGFP5-ER version); TNOS: terminator for the nopaline synthase gene.

Specifically, in our experiments we inoculated Nb-GFP plants, grown in sterile conditions, with FsK-hpGF+GFP and, using the appropriate controls (Nb-WT, Nb-GFP non-inoculated), we recorded GFP expression (fluorescence) under UV light in multiple time-points (0 weeks post-inoculation (wpi), 1wpi, 2wpi, 3wpi, 4wpi, 5wpi, 6wpi). Inoculation of Nb-GFP plants with FsK-hpGF+GFP (Fig. 10) resulted in the following outcomes within an RNAi context:

(i) no visible RNAi events (45% of the plants, 6 wpi)

(ii) spot-like RNAi (45% of the plants, 4 wpi)

(iii) vein-restricted RNAi (5% of the plants, 4 wpi)

(iv) full-tissue RNAi (5% of the plants, 4 wpi) (Fig. 11)

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

Overall, we observed the onset of systemic RNAi of the GFP transgene in Nb-GFP plants colonized with FsK-hpGF+GFP, and more specifically, we observed different levels of silencing of the transgene, ranging from spots to full-tissue silencing (Fig.11).

Colonization of Nb-GFP plants with non-transformed FsK and/or FsK-sGFP failed to trigger any visible silencing phenotype of GFP on the leaves or the stem (not shown). This further establishes that the RNAi molecules produced by the endophyte, and not merely the presence of the endophyte itself, are responsible for the RNAi phenotypes observed in its host.

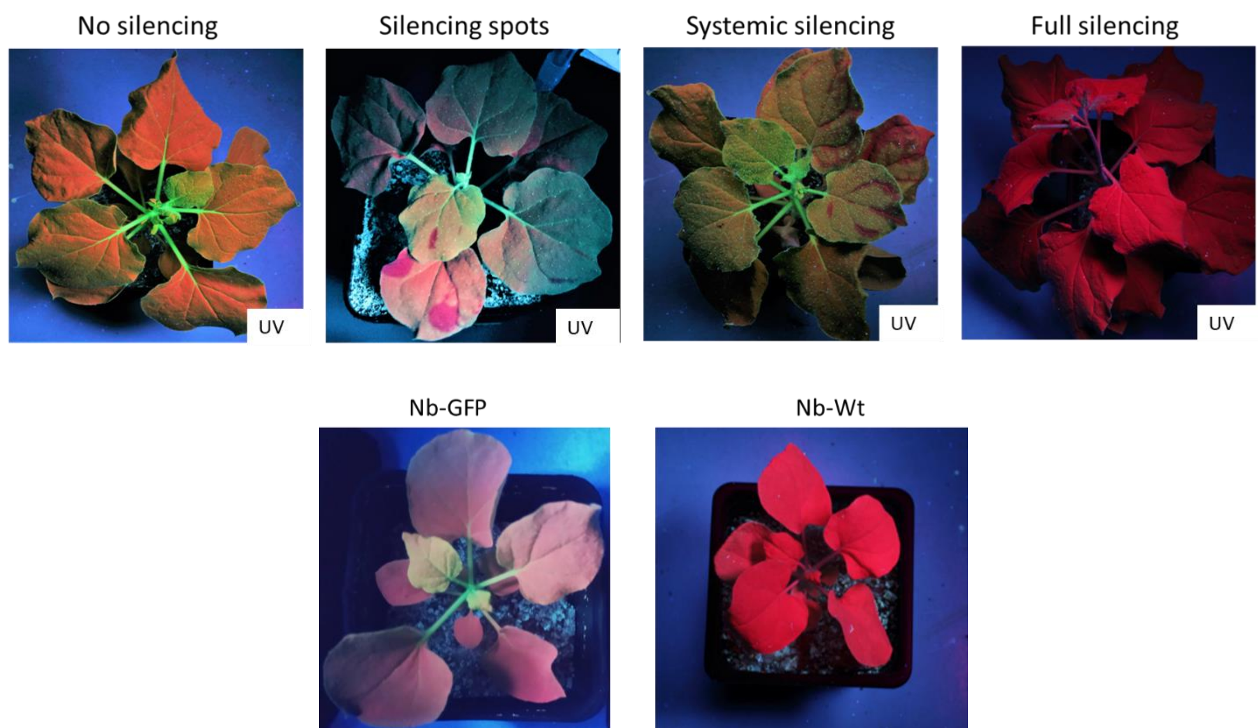

Fig 11. RNAi sensor system; Systemic silencing phenotypes under ultraviolet light 4-6 wpi. Under UV, red colour (chlorophyll autofluorescence) denotes absence/silencing of GFP expression.

Taking into consideration the different phenotypes observed and the different silencing levels observed, our hypothesis was that there could be a link between the colonization levels of the fungus on the roots with the different silencing levels. We analyzed by RT-qPCR the expression levels of mGFP in the silenced plants and verified the reduction in the abundance of RNA levels in the silenced plants. In addition, the extent of FsK colonization in the roots of these plants was also estimated. We were able to observe a trend linking the colonization levels of FsK inversely with the relative expression of GFP

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

on the roots and directly with the systemic silencing phenotype (Fig. 12). This will be established in the future with more biological repeats for every observed phenotype, to establish statistical significance, since the number of plants representing every silencing phenotype were limited.

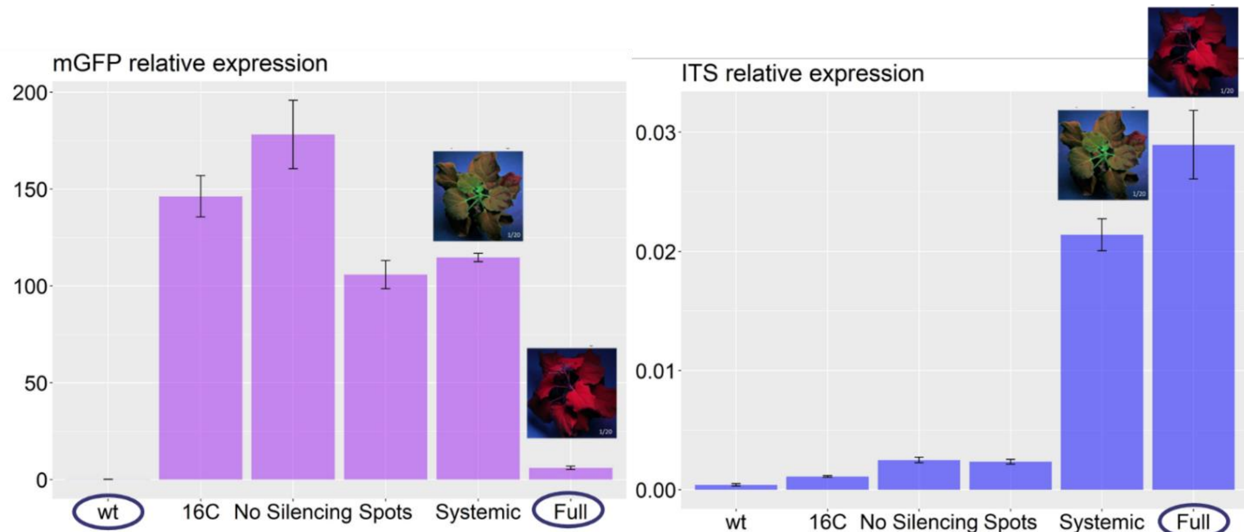

Fig. 12 Bar plot; Relative expression qPCR for the plant mGFP transgene and fungal ITS gene normalized with Nb Actin gene expression levels. Bar plot created with ggplot2 package in RStudio.

5. Epigenetic modifications in the host plant after translocation of RNAi signals

DNA methylation, small RNAs, and post-translational histone modifications have all been discovered to be important modulators of plant responses to the environment in general, and in plant biotic interactions (Alonso *et al.,* 2019). Hence, according to our previous results and based on the knowledge that RNAi/PTGS in plants is linked with RdDM events (Jones et al., 1999; Dalakouras and Vlachostergios, 2021), we sought to examine the presence of putative changes in DNA methylation in Nb-GFP plants, inoculated with FsK-hpGF+GFP, with a systemic silencing phenotype (full-tissue RNAi). We implemented a bisulfite sequencing analysis in root and leaf tissue samples from the fully silenced plants and mock conditions (Nb-GFP non-inoculated), as detailed in the Materials and Methods section, using as a target for analysis, the GFP transgene of Nb-GFP. The analysis revealed extensive (100 %) DNA methylation in the GFP region (Fig. 13a) in every sequence context: CG, CHG and CHH (Fig. 13b).

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

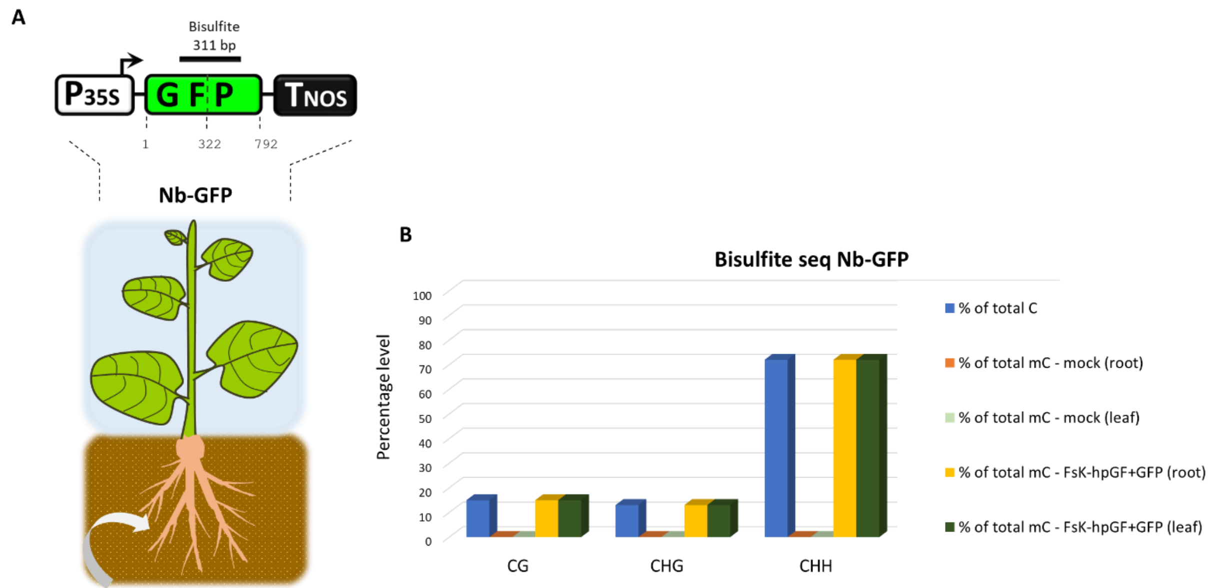

Fig. 13 Bisulfite sequencing analysis; (A) Graphic representation of the GFP transgene in Nb-GFP used as a target for the Bisulfite sequencing analysis. (B) Bisulfite sequencing in the host GFP transgene in both roots and leaves in silenced Nb-GFP plants.

Overall, these data clearly show that the endophyte triggered not only mRNA degradation but also DNA methylation of the GFP host reporter gene.

*Generally, these results suggest that FsK, as a root endophyte, upon colonization of its host, translocated RNAi signals to the roots initiating local RNAi***,** *after which, the primary/secondary siRNAs triggered a RDR6-mediated generation of (host-derived) tertiary siRNAs, ensuring the efficient establishment of GFP mRNA degradation and DNA methylation*.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

DISCUSSION

Fusarium solani strain K (FsK) is a soil-borne beneficial endophytic fungus, previously isolated from the roots of tomato plants, proven able to protect them against biotic and abiotic stresses (Kavroulakis *et al.,* 2007, Kavroulakis *et al.,* 2018). According to recent studies in the Laboratory of Plant and Environmental Biotechnology (Dalakouras, Katsaouni *et al,* unpublished data), it has been established that FsK is able to colonize another member of the Solanaceae, *Nicotiana benthamiana,* a widely used model plant for RNAi studies (Philips et al., 2017). Upon root inoculation FsK colonizes the root system but fails to expand to the shoot system, a colonization pattern that resembles the one observed in tomato plants. Interestingly, the endophyte also seems to promote plant growth in *Nicotiana benthamiana.* Overall, the mechanistical details governing the interaction between this endophyte and its hosts are still elusive.

Fungi seem to establish complex and dynamic interactions with their plant hosts, with RNAi serving as a way to achieve that and recent research reveals that small RNAs implicated in gene silencing, travel bidirectionally between fungal pathogens and their hosts, like in the cases of *Botrytis cinerea* (Wang et al., 2016); (Weiberg et al., 2013)**,** *Verticillium dahliae* (Zhang *et al.* 2016), *Puccinia striiformis* (*Ps*) (Wang *et al*. 2017), and *Fusarium graminearum (Werner et al., 2021)*. However, the exact mechanism of this cross-talk is not determined and more importantly there are not clear data to demonstrate such an activity in **beneficial fungi**.

FsK is able to take up RNAi molecules from his environment and process them via its RNAi machinery leading to a downregulation of the target mRNA, which hasn't been recorded before, to the best of our knowledge, in a beneficial strain. Considering that not all fungi are able to take up RNAi molecules from their environment, like in the cases of *Colletrotrichum gloesporiodes, Trichoderma virens,* and *Phytophtora infestans,* these results alone are a significant addition to the characterization of the RNAi machinery of the endophyte (Qiao *et al.,* 2021). The significance of such a function lies in the fact that fungi with the ability to absorb RNAi molecules from their environment are suspected to be promising candidates for successful Host – induced gene silencing (HIGS) or Spray-induced gene silencing (SIGS) mediated fungal control (Šečić and Kogel, 2021) but also to likely be capable of RNAi-based cross-kingdom communication with their host (He *et al*., 2021).

In our experimental set-up, under *in vitro* assays, no additional decrease in sGFP levels was measured at later timepoints (48 hpa) (Fig. 6). This is consistent with previous findings in *F.*

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

asiaticum and may well suggest an absence of RDR-mediated self-reinforcing mechanism of RNAi which could ensure that RNAi continues even if the initial dsRNA input is lost or degraded.

Our sRNA-seq analysis in three independent FsK-hpGF+GFP transformants (#6, #7, #27) revealed the abundant accumulation of GF siRNAs having variable sizes from 18-30 nt but mainly of 21-nt, 22-nt and 24-nt (Fig. 7). Interestingly this result is very similar to what happens in plants where DCLs process hpRNAs into 21-, 22- and 24-nt siRNAs (Guo *et al.,* 2016). Although there is no knowledge of similar specifically addressed sRNA-seq analyses in other fungi, seeking to show the mode of processing of a given hpRNA/dsRNA, genome-wide sRNA-seq analyses in fungi reported various lengths of sRNAs from 19-22-nt in *Penicillium chrysogenum* (Dahlmann and Kück, 2015), to 22-25-nt in *S. pombe* (Djupedal et al., 2009), to 27-28-nt in *F. graminearum* (Chen et al., 2015), and two different clusters sRNAs with various sizes in the AMF strain *R. irregularis* during its symbiosis with *M. truncatula* (Silvestri *et al.* 2019).

Furthermore, we observe that only a very small fragment of the siRNAs was mapped in the remaining sequence of GFP (P region) that could had potentially resulted from FsKRDR processing on the GF siRNA-targeted GFP mRNA. These results suggest the absence of an RDR-dependent amplification of the RNAi signal in FsK, despite their validated presence, as also reported before in *F. asiaticum* (Song et al., 2018) (Fig. 7a).

From our analysis and the phylogenetic analysis of the predicted RNAi components of FsK, it is suggested that FsKDCL2 generated the majority of the sRNA reads, since it is related to *F. graminearum* DCL2, which has a prominent role in RNAi by processing of dsRNAs into siRNAs (Chen et al., 2015). Moreover, it seems that 21-nt sRNAs are the most prevalent ones. In order to confirm their biological activity, we investigated whether these transformants had indeed resulted in a downregulation of the target GFP mRNA through relative expression RT-qPCR analysis, and indeed, we established their biological activity after observing that GFP expression in all three independent FsK-hpGF+GFP transformants was almost eliminated (Fig. 8).

The accumulation of siRNAs and the initiation of the RNAi machinery is commonly linked in plants with the RNA-directed DNA Methylation (RdDM) pathway, which has been reported to participate in different plant processes (Dalakouras and Vlachostergios, 2021). In fungi, however, DNA methylation is not that common, while it usually occurs in repetitive sequences, and was considered to be independent of siRNAs accumulation and the RNAi

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

pathway (Nai et al., 2020). Yet, sRNA-dependent RdDM-like events have been observed in *Pleurotus tuoliensis* and *P. eryngii var. eryngii* (Basiodiomycetes) (Zhang *et al.,* 2018) and *Puccinia graminis* (Ascomycetes) (Sperschneider *et al.,* 2021). Two regions for cis- and trans-RdDM were analysed in this work. Although CG and CHG methylation is able to be maintained in an RNAi-independent manner (Law and Jacobsen, 2010), CHH methylation requires the presence of siRNAs and is a signature for ongoing de novo RdDM (Pelissier et al., 1999). Both the cis and trans fragments chosen were rich in asymmetric CHH context (80% for cis and 72% for trans) (Figs 9b, 9c). However, our results suggested that no methylation occurred in FsK on a DNA level, in any sequence context (CG, CHG, CHH) for the targeted regions, hence suggesting that no RdDM takes place in FsK hyphae. There is recent evidence that, in fungi, RdRM-like pathways may involve fungal proteins **with de novo methyltransferase (DNMT) and/or helicase-like Snf2 family domains** (Nai et al., 2020). Such genes were not detected in FsKs genome, which further supports our conclusion for the absence of an active RdDM mechanism in FsK.

Finally, in this study we provide evidence that the endophyte triggered not only mRNA degradation but also DNA methylation of a host reporter gene.

The onset of systemic RNAi in the above-ground, non-colonized by the endophyte, part of *N. benthamiana*, suggests a root- to-shoot movement of RNA molecules through the phloem. Our analysis did not allow us to establish whether the GFP RNAi signals that the endophyte transmitted to the plant were siRNAs or non-DCL-processed dsRNAs, although the currently available evidence is in favor of siRNA movement (Weiberg et al. 2013; Zhang et al. 2016) These mobile siRNAs that had to move to the shoot of the plants may be originated either as primary siRNAs from the endophyte or/and as secondary siRNAs from an RDR6-dependent amplification of the signal by the root cells. Generally, siRNAs are known to be mobile and, in plants, they move cell-to-cell through the plasmodesmata in local silencing events and through the phloem to distant parts of the plant in systemic silencing events (Voinnet and Baulcombe, 1997; Voinnet, 2022). **Taking into consideration that for the onset of systemic silencing, a certain quantitative siRNA threshold needs to be achieved (Kalantidis et al., 2006), it is reasonable to assume that in their majority, the RNA molecules that are responsible for the systemic silencing are plant-derived secondary siRNAs.**

As regards the RdDM phenomenon observed, it's unclear whether the endophyte-derived primary or host-derived secondary siRNAs caused the RdDM in the root tissues. However, the presence of RdDM not only in the GF but also in the P region of the Nb-GFP transgene (Fig 9a,

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

311 bp bisulfite fragment covering both GF and P regions) definitely suggests that RdDM is imposed by transitive host-derived secondary siRNAs.

Hence, we propose that **FsK-hpGF+GFP translocated RNAi signals (dsRNAs but most likely siRNAs) to the roots of Nb-GFP initiating local RNAi of the host GFP, triggering an RDR6-mediated amplification of the siRNAs by the plant cells, ensuring that way the efficient establishment of GFP mRNA degradation and DNA methylation.**

FUTURE VISION

In the future, further analysis will be required to pinpoint and confirm endogenous fungal sRNAs (siRNAs and/or miRNAs) with a biological significance and their respective targets to the host plants.

Datasets of fungal small RNAs are already available in the lab, hence after analysis and comparisons with data from predicted mRNAs produced by the host plant, we could create a dataset containing all fungal sRNAs with putative targets to a host plant e.g., tomato. Then, further validations could confirm their production in the fungus and their targets on the host;

- Stem-loop RT-qPCR could be used to validate the sRNAs' production on the fungus
- Rapid amplification of cDNA ends (RACE) on the putative host-derived target mRNAs, during symbiosis, would depict whether the target mRNA in the presence of the fungus is indeed cleaved in the predicted position (The 10th -11th nt from the start of the recognition site of each sRNA)
- RT-qPCR analysis on the predicted target mRNAs of the host in the presence/absence of the endophyte could provide further validation of a translocation and functionality of the predicted sRNAs from the endophyte to the host.
- Further functional analysis could give more details about the biological significance of such a translocation (dcl/ago FsK mutants phenotypical analysis).
- Bisulfite sequencing of the respective target DNA regions or even whole-genome Bisulfite sequencing during, and in the absence of, symbiosis could give more information about the effect of the endophyte in the plasticity of the host epigenome.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

CONCLUSIONS

In this study, we have characterized the core RNAi machinery of the beneficial fungal endophyte *Fusarium solani* strain K (FsK) and we provide evidence that it is able to translocate RNAi molecules/signals to its host and result in systemic silencing and epigenetic modifications of a host transgene. As a proof-of-principle, we used a synthetic RNAi sensor system based on the model plant. In the future, further analysis will be required to identify and confirm endogenous fungal sRNAs (siRNAs and/or miRNAs) with a biological significance, and their respective targets to the host plants. Our results could point to a thus far unrecognized pathway in which endophytes form symbiotic relationships and/or exert beneficial effects on their hosts by translocating RNA molecules that influence host gene expression and alter the plasticity of the host epigenome. RNAi-mediated communication between plants and their hosts could be far more prevalent than previously assumed, and it might explain why plants function better in the presence of particular microbiota.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

LITERATURE

- Agrawal, N, Dasaradhi, PV, Mohmmed, A, Malhotra, P, Bhatnagar, RK and Mukherjee, SK. 2003. RNA interference: biology, mechanism, and applications. Microbiol Mol Biol Rev 67, 657–685.
- Alonso, C., Ramos-Cruz, D. and Becker, C. 2019. The role of plant epigenetics in biotic interactions. New Phytol 221, 731-737.
- Atkinson NJ, and Urwin PE. 2012. The interaction of plant biotic and abiotic stresses: from genes to the field. J. Exp. Bot. 63. 3523–3543
- Baulcombe D. 2004. RNA silencing in plants. Nature 431, 356-363.
- Borges F, Martienssen RA. 2015. The expanding world of small RNAs in plants. Nature Reviews Molecular Cell Biology 16, 727-741.
- Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, Voinnet O. 2008. Widespread translational inhibition by plant miRNAs and siRNAs. Science 320, 1185-1190.
- Chen Y, Gao Q, Huang M, Liu Y, Liu Z, Liu X, Ma Z. 2015. Characterization of RNA silencing components in the plant pathogenic fungus Fusarium graminearum. Sci Rep 5, 12500.
- Clark SJ, Statham A, Stirzaker C, Molloy PL, Frommer M (2006) DNA methylation: bisulphite modification and analysis. Nat Protoc 1: 2353-2364
- COGONI C, IRELAN JT, SCHUMACHER M, SCHMIDHAUSER TJ, SELKER EU, MACINO G. 1996. Transgene silencing of the al-1 gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. EMBO J. 15, 3153–63.
- Coleman, JJ. 2016. Fusarium solani species complex. Molecular Plant Pathology, 17: 146-158.
- Coleman JJ, Rounsley SD, Rodriguez-Carres M, Kuo A, Wasmann CC, Grimwood J, et al. 2009. The Genome of Nectria haematococca: Contribution of Supernumerary Chromosomes to Gene Expansion. PLoS Genet 5(8): e1000618.
- Dahlmann TA, Kück U. 2015. Dicer-Dependent Biogenesis of Small RNAs and Evidence for MicroRNA-Like RNAs in the Penicillin Producing Fungus Penicillium chrysogenum. PLoS One 10, e0125989.
- Dalakouras A, Vlachostergios D. 2021. Epigenetic approaches to crop breeding: current status and perspectives. Journal of Experimental Botany 72, 5356-5371
- Dalakouras A, Dadami E, Wassenegger M, Krczal G, Wassenegger M (2016) RNA-directed DNA methylation efficiency depends on trigger and target sequence identity. Plant J 87: 202-214
- Dalakouras A, Wassenegger M, Dadami E, Ganopoulos I, Pappas ML, Papadopoulou K. 2020. Genetically Modified Organism-Free RNA Interference: Exogenous Application of RNA Molecules in Plants. Plant Physiol 182(1), 38-50.
- de Jonge R, van Esse HP, Kombrink A, Shinya T, Desaki Y, Bours R, van der Krol S, Shibuya N, Joosten MH, and Thomma BP. 2010. Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. Science 329, 953–955.
- de Felippes FF, Waterhouse PM. 2020. The Whys and Wherefores of Transitivity in Plants. Frontiers in Plant Science 11.
- Djupedal I, Kos-Braun IC, Mosher RA, Söderholm N, Simmer F, Hardcastle TJ, Fender A, Heidrich N, Kagansky A, Bayne E, Wagner EGH, Baulcombe DC, Allshire RC, Ekwall K. 2009. Analysis of small RNA in fission yeast; centromeric siRNAs are potentially generated through a structured RNA. The EMBO journal 28, 3832-3844.
- Drinnenberg IA, Weinberg DE, Xie KT, Mower JP, Wolfe KH, Fink GR, Bartel DP. 2009. RNAi in budding yeast. Science 326, 544-550.
- Fire A, Xu S, Montgomery M, Kostas S, Driver S, Mello C. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806–811.
- Gallego-Bartolomé J (2020) DNA methylation in plants: mechanisms and tools for targeted manipulation. New Phytol
- Garantonakis N, Pappas ML, Varikou K, Skiada V, Broufas GD, Kavroulakis N, Papadopoulou KK. 2018. Tomato Inoculation With the Endophytic Strain Fusarium solani K Results in Reduced Feeding Damage by the Zoophytophagous Predator Nesidiocoris tenuis. Frontiers in Ecology and Evolution 6.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

- Guo Q, Liu Q, Smith NA, Liang G, Wang MB. 2016. RNA Silencing in Plants: Mechanisms, Technologies and Applications in Horticultural Crops. Curr Genomics 17(6), 476-489.
- Gutbrod MJ, Martienssen RA (2020) Conserved chromosomal functions of RNA interference. Nat Rev Genet 21: 311-331
- Ha M and Kim VN. 2014. Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol 15, 509–524
- Hamilton AJ, Baulcombe DC. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286, 950-952.
- Haseloff J, Siemering KR. 2006. The uses of green fluorescent protein in plants. Methods Biochem Anal 47, 259-284
- He B, Hamby R, Jin H. 2021. Plant extracellular vesicles: Trojan horses of cross-kingdom warfare. FASEB Bioadv 3, 657-664.
- Horbach R, Navarro-Quesada AR, Knogge W, and Deising HB. 2011. When and how to kill a plant cell: infection strategies of plant pathogenic fungi. J. Plant Physiol 168, 51–62.
- Hua C, Zhao JH, Guo HS. 2018. Trans-Kingdom RNA Silencing in Plant-Fungal Pathogen Interactions. Mol Plant. 11(2):235-244.
- Hung Y-H, Slotkin RK. 2021. The initiation of RNA interference (RNAi) in plants. Current Opinion in Plant Biology 61, 102014.
- Iwakawa HO, Tomari Y. 2022. Life of RISC: Formation, action, and degradation of RNA-induced silencing complex. Mol Cell 82, 30-43.
- Jones L, Hamilton AJ, Voinnet O, Thomas CL, Maule AJ, Baulcombe DC. 1999. RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. Plant Cell 11, 2291-2301
- Kalantidis K, Tsagris M, Tabler M. 2006. Spontaneous short-range silencing of a GFP transgene in Nicotiana benthamiana is possibly mediated by small quantities of siRNA that do not trigger systemic silencing. Plant J 45, 1006-1016
- Kavroulakis N, Doupis G, Papadakis IE, Ehaliotis C, Papadopoulou KK. 2018. Tolerance of tomato plants to water stress is improved by the root endophyte Fusarium solani FsK. Rhizosphere 6, 77-85.
- Kavroulakis N, Ntougias S, Zervakis GI, Ehaliotis C, Haralampidis K, Papadopoulou KK. 2007. Role of ethylene in the protection of tomato plants against soil-borne fungal pathogens conferred by an endophytic Fusarium solani strain. J Exp Bot 58, 3853-3864.
- Lam JK, Chow MY, Zhang Y, Leung SW. 2015. siRNA Versus miRNA as Therapeutics for Gene Silencing. Mol Ther Nucleic Acids 4(9):e252.
- Laurie JD, Linning R, Bakkeren G. 2008. Hallmarks of RNA silencing are found in the smut fungus Ustilago hordei but not in its close relative Ustilago maydis. Curr Genet 53, 49-58
- Law JA, Jacobsen SE. 2010. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat Rev Genet 11, 204-220.
- Lax C, Tahiri G, Patiño-Medina JA, Cánovas-Márquez JT, Pérez-Ruiz JA, Osorio-Concepción M, Navarro E, Calo S. 2020. The Evolutionary Significance of RNAi in the Fungal Kingdom. Int J Mol Sci 21.
- Matzke MA, Matzke AJM. 2004. Planting the Seeds of a New Paradigm. PLoS Biol 2(5): e133.
- Meister G, Tuschl T. 2004. Mechanisms of gene silencing by double-stranded RNA. Nature 431, 343–349
- Milne I, Stephen G, Bayer M, Cock PJ, Pritchard L, Cardle L, Shaw PD, Marshall D. 2013. Using Tablet for visual exploration of second-generation sequencing data. Brief Bioinform 14, 193-202.
- Nai YS, Huang YC, Yen MR, Chen PY. 2020. Diversity of Fungal DNA Methyltransferases and Their Association With DNA Methylation Patterns. Front Microbiol 11, 616922.
- Nurnberger T, Brunner F, Kemmerling B, and Piater L. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol. Rev 198, 249–266.
- Palomar VM, Garciarrubio A, Garay-Arroyo A, Martínez-Martínez C, Rosas-Bringas O, Reyes JL and Covarrubias AA. 2021. The canonical RdDM pathway mediates the control of seed germination timing under salinity. Plant J 105, 691-707.
- Pappas ML, Liapoura M, Papantoniou D, Avramidou M, Kavroulakis N, Weinhold A, Broufas GD, Papadopoulou KK. 2018. The Beneficial Endophytic Fungus Fusarium solani Strain K Alters Tomato Responses Against Spider Mites to the Benefit of the Plant. Front Plant Sci 9, 1603.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

- Pelissier T, Thalmeir S, Kempe D, Sanger HL, Wassenegger M. 1999. Heavy de novo methylation at symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation. Nucleic Acids Res 27, 1625-1634.
- Philips JG, Naim F, Lorenc MT, Dudley KJ, Hellens RP, Waterhouse PM. 2017. The widely used *Nicotiana benthamiana* 16c line has an unusual T-DNA integration pattern including a transposon sequence. PLoS One 12, e0171311.
- Qiao L, Lan C, Capriotti L, Ah-Fong A, Nino Sanchez J, Hamby R, Heller J, Zhao H, Glass NL, Judelson HS, Mezzetti B, Niu D, Jin H. 2021. Spray-induced gene silencing for disease control is dependent on the efficiency of pathogen RNA uptake. Plant Biotechnology Journal 19, 1756-1768.
- Romano N, Macino G. 1992. Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. Mol Microbiol 6, 3343-3353.
- Sakurai Y, Baeg K, Lam AYW, Shoji K, Tomari Y, Iwakawa H-o. 2021. Cell-free reconstitution reveals the molecular mechanisms for the initiation of secondary siRNA biogenesis in plants. Proceedings of the National Academy of Sciences 118, e2102889118.
- Schwach F, Vaistij FE, Jones L, Baulcombe DC. 2005. An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. Plant Physiol 138, 1842-1852.
- Šečić E, Kogel KH. 2021. Requirements for fungal uptake of dsRNA and gene silencing in RNAi-based crop protection strategies. Curr Opin Biotechnol 70, 136-142.
- Secic E, Zanini S, Wibberg D, Jelonek L, Busche T, Kalinowski J, Nasfi S, Thielmann J, Imani J, Steinbrenner J, Kogel KH. 2021. A novel plant-fungal association reveals fundamental sRNA and gene expression reprogramming at the onset of symbiosis. BMC Biol 19, 171.
- Sesma A, Osbourn AE. 2004. The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. Nature 431, 582-586
- Skiada V, Faccio A, Kavroulakis N, Genre A, Bonfante P, Papadopoulou KK. 2019. Colonization of legumes by an endophytic Fusarium solani strain FsK reveals common features to symbionts or pathogens. Fungal Genet Biol 127, 60-74.
- Silvestri A, Fiorilli V, Miozzi L, Accotto GP, Turina M, Lanfranco L. 2019. In silico analysis of fungal small RNA accumulation reveals putative plant mRNA targets in the symbiosis between an arbuscular mycorrhizal fungus and its host plant. BMC Genomics 20, 169.
- Shimura H, Pantaleo V, Ishihara T, Myojo N, Inaba J, Sueda K, Burgya´ n J, and Masuta C. 2011. A viral satellite RNA induces yellow symptoms on tobacco by targeting a gene involved in chlorophyll biosynthesis using the RNA silencing machinery. PLoS Pathog 7, e1002022.
- Smith NA, Eamens AL, and Wang MB. 2011. Viral small interfering RNAs target host genes to mediate disease symptoms in plants. PLoS Pathog 7, e1002022.
- Song XS, Gu KX, Duan XX, Xiao XM, Hou YP, Duan YB, Wang JX, Yu N, Zhou MG. 2018. Secondary amplification of siRNA machinery limits the application of spray-induced gene silencing. Mol Plant Pathol 19, 2543-2560
- Sperschneider J, Jones AW, Nasim J, Xu B, Jacques S, Zhong C, Upadhyaya NM, Mago R, Hu Y, Figueroa M, Singh KB, Stone EA, Schwessinger B, Wang M-B, Taylor JM, Dodds PN. 2021. The stem rust fungus Puccinia graminis f. sp. tritici induces centromeric small RNAs during late infection that are associated with genome-wide DNA methylation. BMC Biology 19, 203
- Thomma BP, Nurnberger T, and Joosten MH. 2011. Of PAMPs and effectors: the blurred PTI-ETI dichotomy. Plant Cell 23, 4–15.
- Tomari Y, Zamore PD. 2005. Perspective: machines for RNAi. Genes Dev 19, 517–529.
- Torres-Martinez S, Ruiz-Vazquez RM. 2017. The RNAi Universe in Fungi: A Varied Landscape of Small RNAs and Biological Functions. Annu Rev Microbiol 71, 371-391.
- Torres-Martinez S, Ruiz-Vazquez RM. 2017. The RNAi Universe in Fungi: A Varied Landscape of Small RNAs and Biological Functions. Annu Rev Microbiol 71, 371-391.
- Vaucheret H. 2006. Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes Dev 20, 759-771
- Vaucheret H. 2008. Plant ARGONAUTES. Trends Plant Sci 13, 350-358.
- Voinnet O. 2008. Use, tolerance and avoidance of amplified RNA silencing by plants. Trends Plant Sci 13: 317-328

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211

- Voinnet O. 2009. Origin, biogenesis, and activity of plant microRNAs. Cell 136: 669-687
- Voinnet O. 2022. Revisiting small RNA movement in plants. Nat Rev Mol Cell Biol.
- Voinnet O, Baulcombe DC. 1997. Systemic signalling in gene silencing. Nature 389, 553
- Wang B, Sun YF, Song N, Zhao MX, Liu R, Feng H, Wang XJ, Kang ZS. 2017. Puccinia striiformis f. sp tritici microRNA-like RNA 1 (Pst-milR1), an important pathogenicity factor of Pst, impairs wheat resistance to Pst by suppressing the wheat pathogenesis-related 2 gene. New Phytol 215, 338-350
- Wang M, Weiberg A, Lin FM, Thomma BP, Huang HD, Jin H. 2016. Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. Nat Plants 2, 16151.
- Wassenegger M, Dalakouras A. 2021. Viroids as a Tool to Study RNA-Directed DNA Methylation in Plants. Cells 10, 1187.
- Wassenegger M, Heimes S, Riedel L, Sanger HL. 1994. RNA-directed de novo methylation of genomic sequences in plants. Cell 76, 567-576.
- Weiberg A, Wang M, Lin FM, Zhao H, Zhang Z, Kaloshian I, Huang HD, Jin H. 2013. Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. Science 342, 118-123.
- Werner B, Koch A, Šečić E, Engelhardt J, Jelonek L, Steinbrenner J, Kogel K-H. 2021. Fusarium graminearum DICER-like-dependent sRNAs are required for the suppression of host immune genes and full virulence. bioRxiv, 2021.2005.2017.444440.
- Wickham H (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. ISBN 978-3-319-24277-4
- Zhang L, Kars I, Essenstam B, Liebrand TW, Wagemakers L, Elberse J, Tagkalaki P, Tjoitang D, van den Ackerveken G, and van Kan JA. 2014. Fungal endopolygalacturonases are recognized as microbe-associated molecular patterns by the Arabidopsis receptor-like protein RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES1. Plant Physiol 164, 352–364.
- Zhang T, Zhao YL, Zhao JH, Wang S, Jin Y, Chen ZQ, Fang YY, Hua CL, Ding SW, Guo HS. 2016. Cotton plants export microRNAs to inhibit virulence gene expression in a fungal pathogen. Nat Plants 2, 16153.
- Zhang Z, Wen J, Li J, Ma X, Yu Y, Tan X, Wang Q, Liu B, Li X, Li Y, Gong L. 2018. The evolution of genomic and epigenomic features in two Pleurotus fungi. Sci Rep 8, 8313.

Institutional Repository - Library & Information Centre - University of Thessaly
09/06/2024 10:06:31 EEST - 18.118.0.211