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Developing RNA-Interference Based Antifungals for Plant Disease Management in Agricultural Settings

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The text of Chapter 1 in full, is a reprint of the material as it appears in L Qiao*, J Niño-Sànchez*, R Hamby*, L Capriotti, A Chen, B Mezzetti and H Jin. “Artificial nanovesicles for dsRNA delivery in spray induced gene silencing for crop protection.” *Plant Biotechnology Journal*. 2023. * denotes equal contribution. H.J. conceived the idea, designed the experiments and supervised the study. L.Q., R.H., J.N.S., L.C. and A.C. performed the experiments. L.Q. performed the experiments in Figure 1.1 A and B, Figure 1.2, Figure 1.3, Figure 1.4 and Supplementary Figure 1.1, did the data analysis associated with these experiments, and assisted in drafting and revising the manuscript. J.N.S performed replicates needed for Figure 1.6 and did qPCR work in Supplementary Figure 1.2, did the data analysis associated with these experiments and assisted in drafting and revising the manuscript. R.H. led the writing and drafting of the manuscript, revised the manuscript, and took the confocal images in Figure 1.1 C, assisted in the grape leaf assays in Figure 1.4, performed experiments in Figure 1.5 A-C, designed the alternative AV formulations and performed replicates for the experiments in Figure 1.6. L.C. led the grape leaf work shown in Figure 1.4. A.C. took the confocal images shown in Figure 1.5. B.M. revised the manuscript and was advisor to L.C. All authors read and approved of its content. I thank Philippe Rolshausen for providing the grape plants used in this chapter. This work was supported by grants from National Institute of Health (R35GM136379), National Science Foundation (IOS 2020731), United State Department of Agriculture (2021-

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Dedication

Dedicated to all my friends who supported me through graduate school.

ABSTRACT OF THE DISSERTATION

Developing RNA-Interference Based Antifungals for Plant Disease Management in Agricultural Settings

by

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Fungal pathogens pose a significant threat to global food security, as resistance to conventional fungicides becomes increasingly widespread. RNA-based antifungals have emerged as promising new technologies for combatting this microbial threat. In one specific approach, Spray-Induced Gene Silencing (SIGS), fungal gene-targeting RNAs are topically applied to plant materials where they silence important fungal genes, limiting their virulence. This practical deployment of SIGS, however, is severely limited by the instability of RNA in the environment, especially in field settings where rainfall, UV light, and high humidity can quickly degrade RNA. Further, many fungal pathogens are soilborne, and the rhizosphere is an even more inhospitable place for RNAs than plant surfaces. In order to become more feasible for agricultural use, RNA-based antifungals need to be improved for stability on both plant surfaces and in the rhizosphere. To address these issues, I worked with three different RNA delivery systems in this work. First, the lipid-based nanoparticle, artificial nanovesicles (AVs), were developed to shield RNAs from environmental degradation. These AVs effectively

protected RNAs, and significantly extended the duration of RNA-mediated protection against the foliar pathogen *Botrytis cinerea*. Three different formulations of AVs with different lipid compositions were all found to be effective in stabilizing and delivering RNA. Next, to address the issue of soilborne fungi and decrease the costs of RNA synthesis, an innovative bacterial delivery platform was developed by engineering two plant-beneficial bacteria, *Bacillus subtilis* and *Pseudomonas putida*, to produce and excrete fungal gene-targeting RNAs via extracellular vesicles (EVs). These bacterial platforms conferred protection to tomatoes and *Arabidopsis thaliana* against both *B. cinerea* and the soil-borne pathogen *Verticillium dahliae*. Finally, another nanoparticle-based solution, layered double hydroxide (LDH) clay nanosheets, were investigated for their ability to stabilize and prolong the efficacy of RNA applications. A variety of BioClay™ formulations enhanced RNA durability and efficacy, further demonstrating the potential for nanoparticles to be optimized in their formulations to decrease production costs and toxicity while increasing antifungal effects. Further refinement of RNA targets and formulations will help optimize SIGS for field deployment, offering sustainable and effective tools for fungal disease management.

TABLE OF CONTENTS

List of Figures

Introduction

Chapter 1

Chapter 2

Chapter 3

List of Supplementary Figures

Chapter 1

Chapter 2

List of Tables

Chapter 3

Introduction

The text of this chapter is adapted from two published reviews, Hamby, Cai and Jin, “RNA communication between organisms inspires innovative eco-friendly strategies for disease control.” *Nature Reviews Molecular Cell Biology*. 2024, and Niu*, Hamby*, Niño-Sanchez, Cai, Yan, and Jin, “RNAs - a new frontier in crop protection.” *Current Opinion in Biotechnology*. 2021. * denotes equal contribution.

Summary

Crops are constantly under siege by pathogens and pests in both the pre- and post-harvest stages, leading to the loss of approximately 30% of crops worldwide (Savary et al. 2019) . Currently, these diseases and pests are largely controlled by chemical pesticides and fungicides, which can leave harmful residues in the environment. Further, overuse of fungicides has led to the development of resistant fungal strains against every major fungicide used in both agricultural and clinical applications (Fisher et al. 2018). Therefore, there is an urgent need to develop innovative, effective, and environmentally friendly crop protection strategies to safeguard both global food security and human health.

Novel disease management strategies often come from better understanding the molecular mechanisms underpinning host-pathogen communication. Although protein and metabolite exchange during infection is well established, RNA exchange has recently been recognized as a key regulatory mechanism for host-microbe/parasite communication across many pathosystems

(Weiberg et al. 2013; Cai, Qiao, et al. 2018; Buck et al. 2014; Halder et al. 2021; S. Wang et al. 2024). However, the full suite of RNA classes transported, the mechanisms of RNA secretion and uptake, and their modes of action in recipient organisms still require additional investigation. Further, the mechanisms for selective RNA secretion, trafficking, and uptake remain largely unknown.

While multiple pathways for cross-kingdom and cross-species RNA transport likely exist, extracellular vesicles (EVs) are utilized by all kingdoms of organisms, including mammals(Halder et al. 2021), plants(Cai, Qiao, et al. 2018; S. Wang et al. 2024), fungi(B. He et al. 2023), parasites(B. He et al. 2023; Buck et al. 2014), insects (Cui et al. 2019), and even prokaryotes (Koeppen et al. 2016; Mills et al. 2024), as carriers to protect and transport RNAs between cells and organisms. EVs are membrane-bound structures that can originate either from plasma membrane shedding, in the form of microvesicles, or from the fusion of multivesicular bodies with the plasma membrane, releasing intraluminal vesicles known as exosomes (van Niel, D'Angelo, and Raposo 2018). EVs can be utilized clinically as disease biomarkers and as carriers for RNA-based therapeutics (O'Brien et al. 2020). The widespread ability of organisms to engage in RNA communication underscores the significance of investigating RNA transport via EVs and other mechanisms. This research has the potential to lead to the development of transformative new eco-friendly disease control strategies in both agricultural and clinical applications.

Emerging discoveries have revealed that sRNAs, in addition to their endogenous functions, are also transported between hosts and their pests/pathogens, where they can induce “cross-kingdom or cross-species RNAi” in the counterparty (Weiberg et al. 2013; Knip, Constantin, and Thordal-Christensen 2014; Weiberg, Bellinger, and Jin 2015; Buck et al. 2014). This makes it possible to apply host-induced gene silencing (HIGS) for plant disease control (Intro Figure C). In HIGS, host plants are genetically engineered to express pathogen- or pest-gene targeting double-stranded RNAs (dsRNAs) or sRNAs. These RNAs are then transported into the pest or pathogen via cross-kingdom RNAi, where they target and silence pest or pathogen genes, conferring protection to the plant host (Nunes and Dean 2012).

Additionally, recent research has found that some eukaryotic pathogens, such as some nematodes and many aggressive fungal pathogens, are capable of taking up RNAs from the environment (M. Wang et al. 2016; Whangbo and Hunter 2008; Qiao et al. 2021). The transferred dsRNAs and sRNAs that have complementary sequences to the genes in the organism can potentially induce silencing of the target genes, a phenomenon named “Environmental RNAi” (Whangbo and Hunter 2008). This discovery prompted the development of Spray-Induced Gene Silencing (SIGS), where artificially synthesized pathogen or pest gene-targeting dsRNAs or sRNAs are sprayed directly onto plant material. These RNAs then target and silence pathogen genes through Environmental RNAi, inhibiting disease development (Qiao et al. 2021; M. Wang and Jin 2017; Cai et al.

2019). Current research efforts are focusing on utilizing nanomaterials to stabilize the RNA on plant material and enhance the delivery of these RNAs to the target pathogens (Landry and Mitter 2019).

Cross-Kingdom/Species RNA Trafficking and RNAi

Cross-kingdom RNA trafficking was first observed over a decade ago in the *Arabidopsis thaliana-Botrytis cinerea* plant-fungal pathosystem (Weiberg et al. 2013) *B. cinerea*, the causative agent of gray mold in hundreds of plant species, transports small RNAs (sRNAs) into its plant hosts (Weiberg et al. 2013). Once inside the plant cell, these fungal sRNAs hijack host Argonaute (AGO) proteins, particularly AGO1, a key component of RNAi machinery, to silence plant immunity-related genes, contributing to its aggressive infection (Intro Figure A). These fungal sRNAs serve as a novel class of pathogen effector molecules to suppress host immunity. *Arabidopsis*, in turn, co-evolved mechanisms to send sRNAs into *B. cinerea* to silence virulence-related genes(Cai, Qiao, et al. 2018). Since its initial discovery, many additional examples of cross-kingdom sRNA communication between plants have fungi have been observed, including in pathogens such as *Verticillium dahliae (M. Wang et al. 2016), Puccinia striiformis (B. Wang et al. 2017)*, and *Fusarium oxysporum(Ji et al. 2021)*.

Beyond plant-fungal communications, cross-kingdom/species RNA interference (RNAi) has been observed in a variety of interaction systems (Intro Figure B). For example, the parasitic plant, *Cuscuta campestris*, sends miRNAs

into its host plants to silence plant defense genes(Shahid et al. 2018). Similarly, Arabidopsis plants deliversRNAs into the oomycete pathogen *Phytophthora capsici* and suppress expression of *P. capsici* genes, leading to a decrease in mycelial growth and defective sporangia development (Cheng et al. 2022). Outside of plant systems there are also abundant examples of this phenomenon. Specifically, the mosquito fungal pathogen *Beauveria bassiana* delivers an miRNA to mosquito cells and employs mosquito AGO1 to silence mosquito gene *Toll receptor ligand Spätzle 4* (Cui et al. 2019), while the gastrointestinal nematode *Heligmosomoides polygyrus* secretes microRNAs into mammalian hosts to modulate immune responses (Buck et al. 2014). An interaction between human immune cells and *Candida albicans* reveals that fungal cells induce human monocytes to release exosomes containing microRNAs that silence fungal cell cycle inhibitor genes, promoting fungal growth (Halder et al. 2021).

Cross-kingdom RNAi also exists in mutualistic interaction systems. For example, mycorrhizal fungi can transport sRNAs to their plant hosts (Wong-Bajracharya et al. 2022). Interestingly, despite bacteria lacking traditional RNAi machinery, the plant symbiotic bacterium Rhizobium sends transfer RNA (tRNA)-derived RNA fragments into soybeans to silence nodulation-related genes using host AGO1(Ren et al. 2019). The mode-of-action for microbial sRNAs in the host cells seems to be conserved in at least some of these interactions. Similarly, the human bacterial pathogen *Pseudomonas aeruginosa* transports tRNA-derived sRNAs via outer membrane vesicles (OMVs) into human airway epithelial cells to

reduce IL-8 mRNA abundance and IL-8 cytokine secretion (Koeppen et al. 2016). Prokaryotes also participate in cross-kingdom RNA communication, as mammalian gut epithelial cells release microRNAs that may modulate gene expression in gut bacteria (Liu et al. 2016). These studies further implicate prokaryotes as participants in cross-kingdom RNA communication.

While sRNAs are the best-characterized trafficked RNAs (Weiberg et al. 2013; Cai, Qiao, et al. 2018; Buck et al. 2014; Halder et al. 2021), recent discoveries have shown that other RNA classes, including messenger RNAs (mRNAs) and non-coding RNAs, can also be transported between organisms (S. Wang et al. 2024; Mills et al. 2024). For instance, *Arabidopsis* mRNAs are delivered via EVs into *B. cinerea* cells, where they are translated by fungal ribosomes to produce proteins that reduce infection (S. Wang et al. 2024). A halophilic archaeon, *H. volcanii*, can package non-coding RNAs in EVs for intercellular communication (Mills et al. 2024). Collectively, these studies demonstrate that a variety of RNA types are trafficked between organisms.

The Role of Extracellular Vesicles (EVs) in Cross-Kingdom/Species RNA Trafficking

Cross-kingdom RNA trafficking requires RNAs to remain intact as they move from their origins to recipient cells or organisms, often through extracellular environments rich in various proteases and ribonucleases (Gruner and McManus 2021). Extracellular vesicles (EVs) have emerged as key vehicles for protecting

RNAs during this transport process. Studies indicate that more than 60% of *Arabidopsis* mRNAs and 70% of sRNAs transferred into fungal cells are enclosed within plant EVs (Cai, Qiao, et al. 2018; S. Wang et al. 2024). Similarly, the fungal pathogen *B. cinerea* uses EVs to deliver its sRNAs into plants, with plant vesicles taken up efficiently by fungal cells (B. He et al. 2023). EVs and lipid-based nanoparticles also promote RNA delivery into mammalian target cells (O'Brien et al. 2020), suggesting that in addition to their protective role, EVs may actively facilitate RNA uptake in recipient organisms.

Despite the diversity of organisms and RNA types involved in cross-kingdom RNA communication, the precise mechanisms for RNA selection, secretion, and transport within recipient cells are not fully understood. EVs appear to be the major pathway for this inter-organismal RNA exchange, utilized widely across different domains of life(Cai, Qiao, et al. 2018; Cai et al. 2021; Cai, He, and Jin 2019). Tetraspanin-positive exosomes, a specific class of EVs, have been identified as significant contributors to sRNA transport between organisms (Cai et al. 2019). Research suggests that specific RNA-binding proteins within EVs, such as AGO1, DEAD-box RNA helicases, and annexins, are involved in both the selective loading and stabilization of sRNAs, enhancing cross-kingdom RNA-based communication (B. He et al. 2021). Understanding these mechanisms is advancing the development of innovative RNA interference (RNAi) strategies for crop protection, leveraging these natural RNA trafficking pathways to boost plant defenses against pathogens.

Applications Inspired by Cross-Kingdom RNA Communication

Understanding cross-kingdom RNA communication mechanisms has opened new avenues for innovative strategies in disease control and crop protection. Pests and pathogens contribute to 10-40% of annual crop losses, and climate change is exacerbating the spread of pathogens while resistant strains to conventional treatments emerge (Chen et al. 2023). The development of eco-friendly disease management strategies is crucial for ensuring global food security.

Host-Induced Gene Silencing (HIGS)

Host-Induced Gene Silencing (HIGS) uses cross-kingdom RNAi to protect crops by genetically engineering plants to produce pathogen- or pest-targeting sRNAs or dsRNAs. These sRNAs are transferred to the pest or pathogen to silence virulence-related genes (M. Wang, Thomas, and Jin 2017). HIGS approaches are effective against a wide variety of pests and pathogens, including viruses, viroids, fungi, insects, and nematodes (Nunes and Dean 2012; Koch and Kogel 2014; Yadav, Veluthambi, and Subramaniam 2006). Further, HIGS has been successfully utilized in crops like wheat, barley, and soybean to combat pathogens such as *Blumeria graminis (Nowara et al. 2010)*, *Puccinia triticina (Panwar et al. 2018)*, *Fusarium graminearum (F. He et al. 2019)*, and *Phakopsora pachyrhizi (D. Hu et al. 2020)*. These examples illustrate that HIGS is a promising tool to limit chemical-based pesticide applications.

Additionally, HIGS is a versatile tool, as the engineered RNA constructs can be designed to target multiple pathogens simultaneously. One example is *Arabidopsis* plants engineered to produce sRNAs targeting Dicer-like genes in *Botrytis cinerea* and *Verticillium dahliae*, providing protection from both pathogens (M. Wang et al. 2016). Importantly, sRNAs do not require complete complementarity to their target mRNAs (Amarzguioui et al. 2003), which (McDougall 2011) reduces the likelihood of resistance arising from point mutations in the target gene.

However, the development of genetically modified (GM) crops for HIGS is still challenging, time-consuming, and expensive due to regulatory hurdles (McDougall 2011; Head et al. 2017). Despite these challenges, in 2017 the EPA approved a GM corn variety, SmartStax Pro, expressing dsRNA against the Western corn rootworm, which could soon be available in the U.S (Head et al. 2017).

Spray-Induced Gene Silencing (SIGS)

Because of the lengthy and costly process of generating GM crops, there is high demand for a plant-disease management strategy not reliant on transgenic crops. Some fungal pathogens can take up RNAs directly from the environment. These dsRNAs or sRNAs can induce silencing of fungal genes with complementary sequences (M. Wang et al. 2016). This discovery prompted the development of an eco-friendly, GM-free, RNAi-based plant protection strategy,

Spray-Induced Gene Silencing (SIGS) (M. Wang and Jin 2017) (Intro Figure C) . In SIGS applications, pathogen-gene targeting RNAs are sprayed directly onto plant materials in order to confer protection. Externally applied sRNAs and dsRNAs targeting *B. cinerea* DCL1 and DCL2 can effectively inhibit *B. cinerea* disease on a variety of post-harvest plant materials, including vegetables, fruits, and flowers, as well as on pre-harvest *Arabidopsis* and tomato plants (M. Wang et al. 2016; Qiao et al. 2021). Results in barley demonstrated that the application of *F. graminearum* gene-targeting dsRNA prevents the growth of the pathogen(Koch et al. 2016)**.** SIGS approaches can also inhibit infection of *Brassica napus* by the pathogens *Sclerotinia sclerotiorum* or *B. cinerea* (McLoughlin et al. 2018). More recently, results have demonstrated that SIGS approaches can reduce biomass accumulation of fungal pathogen *P. pachyrhizi* in soybean by 75% (D. Hu et al. 2020). Remarkably, dsRNA applications could control *F. graminearum* growth and infection not only at the local application site, but also in the distal untreated part of the leaf, suggesting that dsRNAs on plant surfaces can be taken up and transported within plant tissues, and that the silencing molecules are transmitted to the distal part (Koch et al. 2016).

Early successes of SIGS approaches demonstrate the potential for a new class of RNA-based fungicides to be developed. An RNA-based fungicide could offer many key advantages over traditional fungicides. Specifically, because RNA is already present in most food, it is likely to be safe for consumption. Additionally, like HIGS, RNAs developed for SIGS can be designed to target multiple pathogens

simultaneously, and because complete base-pairing is unnecessary for effective silencing (Amarzguioui et al. 2003), fungicide-resistance strains are less likely to develop. Another key advantage of RNA-based fungicides is that, unlike traditional fungicides which can leave harmful residues in ecosystems, RNAs rapidly degrade in the soil (Dubelman et al. 2014). In fact, this rapid environmental degradation is a major hurdle in the practical application of SIGS to control soil-borne pathogens.

The efficacy of SIGS approaches is dependent on the RNA uptake efficiency of the pathogen (Qiao et al. 2021). Many aggressive fungal pathogens can take up RNAs from the environment very efficiently, even as quickly as within a couple of hours, which makes it possible to apply SIGS for plant protection against these pathogens (Qiao et al. 2021). In order to improve both RNA stability and RNA uptake efficiency, current efforts are focused on nanoparticle technology to improve the application system and the limited durability of the RNAi effect (Chen et al. 2023).

Key Considerations for SIGS Strategies

dsRNA Fragment Properties

The effectiveness of SIGS is heavily influenced by the properties of the dsRNA sequence. When designing dsRNA for gene silencing, secondary structures within the target sequence must be considered, as complex RNA structures can hinder effective silencing by preventing base-pairing with the target

(Holen et al. 2002; Bohula et al. 2003; Vickers et al. 2003). Additionally, siRNA design must avoid secondary structures in the guide RNA, as they can reduce silencing strength (Patzel et al. 2005). In HIGS applications, RNA sequences that favorably bind to AGO1 have a higher chance of being selectively loaded into EVs and transported to the pathogen or pest (B. He et al. 2021).

Biosafety concerns exist regarding off-target silencing, which could affect non-target organisms such as beneficial microbes or plants. As few as 11 contiguous nucleotides, or 15 out of 19 base pairs of complementarities can lead to off-target silencing (Jackson et al. 2003). To minimize this risk, the designed SIGS RNA constructs should be highly specific to the target organism, avoiding conserved regions and ensuring that sequences in both sense and antisense strands are thoroughly analyzed for potential off-target effects. Genome-wide base-pairing analysis should be performed to avoid any base pairing regions longer than 15 nucleotides within the genomes of the host and other beneficial microbes. This design approach has been shown to be successful in honeybees, which had no sensitivity to ingested RNAs specifically designed to target insect pests, *Bactrocera dorsalis* and *Varroa destructor (Arpaia, Smagghe, and Sweet 2021)(Arpaia, Smagghe, and Sweet 2021; Christiaens, Dzhambazova, et al. 2018)*. Though these results are encouraging, ultimately more research is needed to comprehensively understand biosafety risks associated with plant RNAi systems, as this is still an emerging field (Christiaens, Dzhambazova, et al. 2018).

A final consideration in SIGS RNA construct design is to optimize the length of the RNA construct, as the optimal length has been shown to vary across pathosystems (W. He et al. 2020; Höfle et al. 2020; Tenllado and Díaz-Ruíz 2001).

Pathogen RNA Uptake Efficiency

The RNA uptake efficiency of the pathogen plays a crucial role in determining the success of SIGS strategies. Some fungal pathogens, such as *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Fusarium graminearum*, are highly efficient at taking up RNA, making them prime candidates for SIGS-based disease control (M. Wang et al. 2016; Koch et al. 2016; Qiao et al. 2021). Conversely, pathogens like *Colletotrichum gloeosporioides* exhibit no RNA uptake, limiting SIGS effectiveness (Qiao et al. 2021). Understanding the RNA uptake efficiency of specific pathogens is vital before applying SIGS in crop protection.

Moreover, the RNAi machinery of pathogens and the presence of RNases can impact the success of RNAi-based strategies. Some fungal species have lost their RNAi pathways over time (Dang et al. 2011), making them unsuitable for RNAi-based management. In pests like *Nezara viridula*, a dsRNase impedes RNAi efficiency (Sharma et al. 2021), further complicating the use of SIGS. Understanding the RNAi pathways which exist within a pathogen are critical for applying SIGS approaches successfully.

Plant RNA Uptake Efficiency

RNA can also be absorbed by plants (M. Wang et al. 2016; Koch et al. 2016), enhancing the scope of SIGS applications. The efficiency of RNA uptake varies across different plant tissues. Research has shown that high-pressure spraying is an effective method for transferring exogenous siRNAs into plant cells, even more so that petiole absorption of trunk injection (Dalakouras et al. 2018). Additionally, plant surfaces with damage (Song et al. 2018) or open stomata show increased RNA uptake, which could be exploited to enhance SIGS efficacy (Koch et al. 2016).

Nanoparticles as Carriers of RNAi for Crop Protection

The main challenge in SIGS technology is the instability of RNA in the environment. To overcome this, research is focusing on utilizing nanoparticles to enhance RNA stability and pathogen RNA uptake (Joga et al. 2016). A diverse range of nanoparticles have been deployed for this purpose, including layered double hydroxide (LDH) clay nanosheets, guanine containing polymers, and liposome complexes (Mitter, Worrall, Robinson, Xu, et al. 2017; Christiaens, Tardajos, et al. 2018; C. N. T. Taning et al. 2016). Some nanoparticles previously developed for transporting nucleic acids into plant cells may also serve as potential candidates for use in SIGS applications. Effective delivery of nanoparticles and their cargo is dependent upon its size and charge (P. Hu et al. 2020). For example, charged nanoparticles are more likely to be absorbed by plant cell or chloroplast

membranes. Further, as size decreases, a larger zeta potential is needed for the nanoparticle to effectively pass through the plant cell wall and membranes(P. Hu et al. 2020).

One of the first such nanotechnologies adopted for agricultural RNA delivery was “BioClay,” where dsRNAs are loaded onto layered double hydroxide (LDH) clay nanosheets. BioClay has shown sustained RNA release and protection against virus infection for up to 20 days against viruses (Mitter, Worrall, Robinson, Xu, et al. 2017; Mitter, Worrall, Robinson, Li, et al. 2017), insects(Jain et al. 2022), and fungi (Niño-Sánchez et al. 2022). Other nanoparticles, such as carbon quantum dots (CQDs) and chitosan, have been tested for RNA delivery in various organisms, with CQDs showing the most efficient silencing in mosquitoes(Das et al. 2015). Carbon nanotubes have also been explored as RNA carriers, providing RNA protection from nucleases and delivering siRNA with high silencing efficiency in plant cells (Zhang et al. 2019; Demirer et al. 2020). However, the toxicity of carbon nanotubes to mammals makes them less ideal for agricultural use (Francis and Devasena 2018). Another potential RNA delivery tool could be carbon dots. Carbon dots have been utilized to deliver siRNAs into plant cells (Schwartz et al. 2020). Because carbon dots can successfully infiltrate plant cell walls, they could also likely be taken up by walled plant pathogens such as fungi. Peptide-based carriers have also shown promise, such as fusion proteins that increase RNA uptake in insect pests like *Spodoptera exigua* and *Anthonomus grandis(Martinez et al. 2021; De Schutter et al. 2021)*. These carriers could be adapted for use in

plant protection against pests, but optimization for specific species and tissues is needed to achieve the highest silencing efficiency.

To mimic natural RNA transport pathways, lipid-based nanovesicles are being developed for RNA delivery. Plant cells secrete EVs containing sRNAs that pathogens, such as *Botrytis cinerea*, can take up(M. Wang et al. 2016). Mimicking this mechanism with lipid-based nanoparticles could improve RNA delivery to plant pathogens. Lipid-based nanoparticles are already being utilized in clinical settings, here they are used to deliver therapeutic agents, including siRNAs, to mammalian cells (Q. Wang et al. 2013; Tatiparti et al. 2017). Perhaps the most famous example of this approach is the use of liposomes to package and deliver mRNAs in COVID vaccinations(Forni, Mantovani, and COVID-19 Commission of Accademia Nazionale dei Lincei, Rome 2021). Additionally, co-delivering RNA-binding proteins (RBPs) like AGO1 in these artificial vesicles could further enhance the stability and silencing efficiency of SIGS RNAs in pathogens, especially those lacking their own RNAi machinery.

Other RNA Delivery Strategies

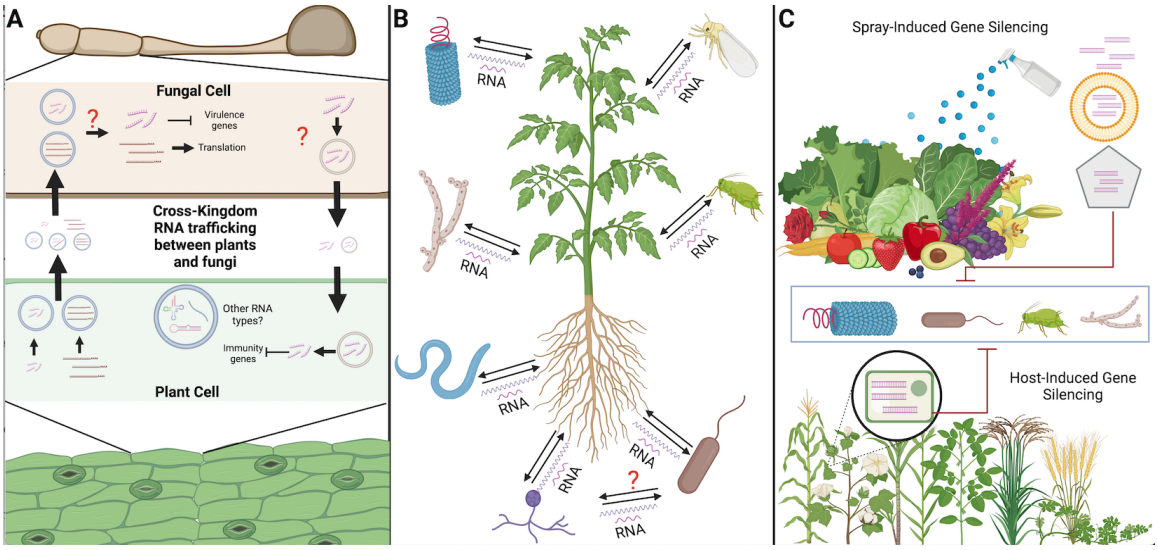
Another potential strategy is the genetic engineering of microbes or viruses to produce and deliver dsRNAs. Viruses have been proposed as RNAi delivery vectors (Kolliopoulou et al. 2017), and dsRNA production and delivery by bacteria has been demonstrated in insects (Cagliari et al. 2019; Dyson et al. 2022), and nematodes (Timmons, Court, and Fire 2001). Recent work has even demonstrated

that the RNaseIII-null mutant strain of *Escherichia coli* could generate dsRNA molecules that successfully induced RNAi in *Aspergillus flavus* (Niño-Sánchez et al. 2021). Microbial biocontrol agents can be engineered to enhance RNA production and delivery, offering a synergistic approach when combined with RNAi strategies. However, further studies are required to fully understand the mechanisms of RNA transfer between bacteria and eukaryotic cells.

Conclusion

Overall, while RNAi based crop protection offers promising solutions to modern problems facing agriculture, new innovations are needed to make it more practical in actual applications. First, RNA needs to be stabilized so it can persist in the environment longer before delivery to fungal pathogens. Next, a platform for delivering RNA to soilborne pathogens needs to be developed. I aim to address both these challenges in this dissertation. In my first chapter, I work to develop a lipid-nanoparticle based RNA delivery system to enhance RNA stability in the environment and prolong SIGS-mediated plant protection. In my second chapter, I work to develop a bacterial-based RNA production and delivery platform. Finally, in my third chapter I work on optimizing another nanoparticle based RNA delivery system, Biolclay™. Overall, this work serves to bring RNA-based disease management strategies closer to the point of application.

Figure



Introduction Figure 1: RNA-based Cross-Kingdom Communication in Plant Systems.

(A) Plants send mRNAs and sRNAs into their fungal pathogens using extracellular vesicles to translate antifungal proteins directly in fungal cells and silence fungal virulence genes, respectively. In turn, fungi send sRNAs into plant cells via EVs, which are taken up via clathrin-mediated endocytosis, to silence plant immune response genes. Question marks on the figure denote pathways whose mechanisms remain unknown. (B) Cross-kingdom RNA communication has been observed between plants and a wide variety of microbes and pests, including viruses, fungi, nematodes, insects, and bacteria. These pests are responsible for 10-40% of annual losses of crops crucial for food security (Savary, S. et al. The global burden of pathogens and pests on major food crops. Nature ecology & evolution 3, 430-+, doi:10.1038/s41559-018-0793-y (2019). Whether soil fungi and bacteria also exchange RNA in the rhizosphere remains to be discovered. (C) The principles of cross-kingdom RNA communication have been leveraged to produce innovative disease management strategies. In Host-Induced Gene Silencing, plants are engineered to produce pathogen gene targeting RNAs which limit virulence and enhance plant resilience to disease. Another approach, Spray-Induced gene silencing, relies on directly spraying RNAs onto plant material. These RNAs can be packaged in a variety of nanoparticles to enhance their stability on the plant and uptake by pathogens.

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CHAPTER 1

Artificial Nanovesicles for dsRNA Delivery in Spray Induced Gene Silencing for Crop Protection

The text of this chapter in full, is a reprint of the material as it appears in L Qiao*, J Niño-Sànchez*, R Hamby*, L Capriotti, A Chen, B Mezzetti and H Jin. “Artificial nanovesicles for dsRNA delivery in spray induced gene silencing for crop protection.” *Plant Biotechnology Journal*. 2023. * denotes equal contribution. H.J. conceived the idea, designed the experiments and supervised the study. L.Q., R.H., J.N.S., L.C. and A.C. performed the experiments. L.Q. performed the experiments in Figure 1.1 A and B, Figure 1.2, Figure 1.3, Figure 1.4 and Supplementary Figure 1.1, did the data analysis associated with these experiments, and assisted in drafting and revising the manuscript. J.N.S performed replicates needed for Figure 1.6 and did qPCR work in Supplementary Figure 1.2, did the data analysis associated with these experiments and assisted in drafting and revising the manuscript. R.H. led the writing and drafting of the manuscript, revised the manuscript, and took the confocal images in Figure 1.1 C, assisted in the grape leaf assays in Figure 1.4, performed experiments in Figure 1.5 A-C, designed the alternative AV formulations and performed replicates for the experiments in Figure 1.6. L.C. led the grape leaf work shown in Figure 1.4. A.C. took the confocal images shown in Figure 1.5. B.M. revised the manuscript and was advisor to L.C. All authors read and approved of its content.

Abstract

Fungal pathogens are a major threat to global food security, and current management strategies are limited to mainly chemical control, which is harmful to human health and the environment. Therefore, innovative, eco-friendly, strategies for combating fungal pathogens must be developed. Recent advances have shown that Spray-Induced Gene Silencing (SIGS) via topical application of pathogen gene-targeting RNAs can inhibit plant diseases caused by fungal pathogens that can effectively take up RNAs from the environment. These antifungal RNAs can be versatilely designed to be species-specific and to target multiple genes simultaneously. Though promising, a major drawback to SIGS approaches is the instability of RNA in the environment, which can be rapidly degraded when exposed to rainfall, high humidity, and UV light. Previously, we discovered that plant hosts utilize extracellular vesicles to protect and deliver fungal-gene suppressing small RNAs into fungal cells. Inspired by this naturally occurring pathway, we examined three artificial nanovesicle (AV) formulations to shield RNA from degradation for topical application to control the fungal pathogen, *Botrytis cinerea*. All three formulations are effective in both RNA delivery and protection, and greatly extend the length of RNA-mediated plant protection.

Introduction

Plant pests and pathogens are a major threat to global food security(Godfray, Mason-D'Croz, and Robinson 2016; Delgado-Baquerizo et al. 2020), causing crop yield losses of up to 20%, and postharvest product losses of up to 10% worldwide(Bebber and Gurr 2015). Of these biotic threats, fungi represent some of the most aggressive and pervasive pathogens(Bebber and Gurr 2015). For example, the causal agent of gray mold disease in over 1000 plant species, *Botrytis cinerea*, alone causes billions of dollars in annual crop yield losses(Petrasch et al. 2019; Fillinger and Elad 2016; Hua et al. 2018). Alarmingly, this threat is projected to increase as rising temperatures associated with global climate change favor fungal pathogen growth(Delgado-Baquerizo et al. 2020; Nnadi and Carter 2021). Currently, the most widely used plant pathogen control practices require routine application of fungicides which threaten the environment(Wightwick et al. 2010) and can lead to the development of fungicide resistant pathogens(Fisher et al. 2018). To safeguard global food security, an alternative, environmentally friendly fungal control method must be developed. Recent studies have shown that many aggressive fungal pathogens can take up RNAs from the environment(M. Wang et al. 2016; Qiao et al. 2021; Koch et al. 2016; Werner et al. 2020; McLoughlin et al. 2018). These RNAs can then induce silencing of fungal genes with complementary sequences. This discovery led to the development of Spray-Induced Gene Silencing (SIGS), where fungal virulence gene-targeting RNAs are topically applied to plant material to control fungal

pathogens. SIGS can provide safe and powerful plant protection on both pre-harvest crops and post-harvest products against fungal pathogens that have high RNA uptake efficiency(Niu et al. 2021; Cai et al. 2019; Cai, He, et al. 2018; C. N. Taning et al. 2020; Rosa et al. 2022). SIGS RNAs can be versatilely designed to be species-specific, minimizing the risk of off-target effects on other organisms, and to target multiple genes and pathogens at once. Furthermore, because RNAi can tolerate multiple mismatches between small RNAs (sRNAs) and target RNAs(Neumeier and Meister 2020), fungal pathogens are less likely to develop resistance to SIGS RNAs than to traditional fungicides. Unlike host-induced gene silencing (HIGS), SIGS does not require the generation of transgenic plants, which remains technically challenging in many crops and necessitates overcoming expensive and complicated regulatory hurdles(Capriotti et al. 2020). To date, SIGS has effectively been used to control a wide range of insect pests(Y. Wang et al. 2011; Killiny et al. 2014; Li et al. 2015; San Miguel and Scott 2016), viruses(Mitter, Worrall, Robinson, Li, et al. 2017; Worrall et al. 2019), oomycetes(Cheng et al. 2022; Kalyandurg et al. 2021) and pathogenic fungi including *Fusarium graminearum* infection in barley(Koch et al. 2016; Werner et al. 2020), soybean rust(Saito et al. 2022; D. Hu et al. 2020), powdery mildew(Ruiz-Jiménez et al. 2021) and gray mold disease on fruits, vegetables and flowers(M. Wang et al. 2016; Nerva et al. 2020; McLoughlin et al. 2018).

One major drawback of SIGS is the relative instability of RNA in the environment, particularly when subjected to rainfall, high humidity, or UV light(San

Miguel and Scott 2016). Thus, improving environmental RNA stability is critical for successful SIGS applications. One strategy is to dock RNAs in synthetic inorganic materials. Specifically, dsRNAs targeting plant viruses have been loaded into layered double hydroxide (LDH) nanosheets to protect dsRNA from nuclease activity(Mitter, Worrall, Robinson, Li, et al. 2017; Worrall et al. 2019) and increase the stability and the durability of the RNAi effect. Ultimately, this strategy can provide RNAi-based systemic protection against several plant viruses for at least 20 days after topical application(Mitter, Worrall, Robinson, Li, et al. 2017; Mitter, Worrall, Robinson, Xu, et al. 2017). This technology still needs to be tested against fungal pathogens.

In nature, plants and animals encapsulate RNAs in extracellular vesicles (EVs) for safe transportation between cells or interacting organisms(Cai, Qiao, et al. 2018; Buck et al. 2014; B. He, Hamby, and Jin 2021; Cai et al. 2021; O'Brien et al. 2020). We have recently shown that plant EVs not only protect sRNAs from degradation but also enhance fungal uptake efficiency(Cai, Qiao, et al. 2018). Recent advances in RNA-based pharmaceuticals have utilized liposomes, synthetic spherical lipid-based nanoparticles for drug delivery, notably in both mRNA COVID-19 vaccines(Yan, Ren, and Shen 2021; Ahmadzada, Reid, and McKenzie 2018; Mahmoodi Chalbatani et al. 2019; Dong, Siegwart, and Anderson 2019; Tenchov et al. 2021; Thi et al. 2021). Liposome formulations are often cationic to facilitate the binding of dsRNA through electrostatic interactions(Mahmoodi Chalbatani et al. 2019; Podesta and Kostarelos 2009;

Huang and Liu 2011), which protects the dsRNA from degradation. In one example, continuous feeding to the insect *Blatella germanica* of liposome-encapsulated dsRNA targeting the *α-tubulin* gene in the midgut, significantly reduced *α-tubulin* expression in the midgut cells, leading to 60% insect mortality(Lin et al. 2017). This study presents evidence that liposomes could be used in agricultural applications. Drawing inspiration from both naturally occurring vesicles and the clinical use of liposomes, here, we package fungal gene-targeting RNAs in liposomes, termed artificial nanovesicles (AVs), to mimic the plant's natural RNA delivery strategy for use in SIGS applications.

In this study, we demonstrate that double-stranded RNA (dsRNA)-packaged in AVs can be successfully utilized in crop protection strategies. Three types of AVs were synthesized and found to confer protection to loaded dsRNA, which remained detectable in large amounts on plant surfaces over a long period of time. When applied to plants, AV-dsRNA can extend the length of fungal protection conferred by fungicidal dsRNA to crops by over 10-fold. Overall, this work demonstrates how organic nanoparticles can be utilized to strengthen SIGS-based crop protection strategies.

Results

Artificial nanovesicles protect and efficiently deliver dsRNA to the fungal pathogen Botrytis cinerea

PEGylated AVs were synthesized using the lipid film hydration method for cationic liposomes(Podesta and Kostarelos 2009). Specifically, AVs were generated using a mixture of the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG2000)(Podesta and Kostarelos 2009). This AV composition had previously been demonstrated to deliver dsRNA to mammalian cells so we hypothesized that it could also be ideal for antifungal RNAi applications(Podesta and Kostarelos 2009). We then established the loading ratio necessary for the AVs to completely encapsulate dsRNAs of interest. Previous studies have shown that the fungal Dicer-like proteins play a critical role in fungal virulence and can be used as ideal targets for SIGS(Weiberg et al. 2013; M. Wang et al. 2016; Werner et al. 2020; Qiao et al. 2021; Haile et al. 2021). Exogenous treatment of *Bc-DCL1/2*-dsRNA, a dsRNA integrating fragments of the Dicer-like 1 (252 bp) and Dicer-like 2 (238 pb) sequences from *Botrytis cinerea,* on the plant leaf surface can efficiently inhibit fungal disease(M. Wang et al. 2016). Thus, several charge ratios (N:P where N = # of positively-charged polymer nitrogen groups and P = # of negatively-charged nucleic acid phosphate groups) between AVs and the *Bc-DCL1/2*-dsRNA, from 1:1 to 4:1, were examined to identify the minimum amount of AVs required to bind

all the dsRNA present in the solution. We concluded that a 4:1 (AV:dsRNA) ratio was the minimum ratio needed for dsRNA loading as *Bc-DCL1/2*-dsRNA loaded into AVs at this ratio could not migrate from the loading well due to complete association with the AVs (Figure 1.1A).

The ability of the AVs to prevent nuclease degradation was then validated under different enzymatic hydrolysis conditions. Naked and AV-loaded *Bc-DCL1/2*-dsRNA were both treated with Micrococcal Nuclease (MNase). As seen in Figure 1.1 B, the naked-*Bc-DCL1/2*-dsRNA exhibited greater degradation after MNase treatment as compared to the *Bc-DCL1/2*-dsRNA released from the AV-*Bc-DCL1/2*-dsRNA using 1% Triton X-100. Thus, the AVs provide protection for dsRNA against nuclease degradation. To further confirm that the dsRNA is encapsulated and protected by the AVs, we used Fluorescein-12-UTP to label both naked-*Bc-DCL1/2*-dsRNA and AV-*Bc-DCL1/2*-dsRNA. The Fluorescein-labeled naked-*Bc-DCL1/2*-dsRNA showed a diffused fluorescent signal when examined by confocal laser scanning microscopy (CLSM), while the Fluorescein-labeled AV-*Bc-DCL1/2*-dsRNA showed a punctuated fluorescent signal after MNase treatment, indicating encapsulation in the AVs (Figure 1.1C). However, no fluorescent signal was observed when MNase was applied after rupturing the AVs by application of 1% Triton X-100 (Figure 1.1C). Therefore, these results demonstrate that dsRNA can be efficiently encapsulated inside AVs, conferring nuclease protection.

Finally, we assessed the ability of the AVs as an efficient vehicle for dsRNA delivery to *B. cinerea* fungal cells. Previously, we discovered that naked dsRNA is effectively taken up by *B. cinerea* (Wang et al. 2016). Here, we compared fungal uptake of naked and AV-encapsulated Fluorescein-labeled dsRNA using CLSM. Fluorescent dsRNA was detected inside the fungal cells after application of either naked- or AV- *Bc-DCL1*/*2*-dsRNA to *B. cinerea* spores cultured on PDA plates (Figure 1.1D). The CLSM analysis was carried out after Triton X-100 and MNase treatment to eliminate any fluorescent signals coming from dsRNA or AV-dsRNA not inside the fungal hyphae. Under these conditions, a strong fluorescent signal was found on the hyphae edges after AV-dsRNA application, suggesting that the AV-dsRNA were taken up by the fungal cells (Figure 1.1D).

External AV-dsRNA application triggers RNAi in *B. cinerea*

After demonstrating that the AVs could be loaded with dsRNA and taken up by fungal cells, we next examined if external AV-dsRNA application triggered RNAi in *B. cinerea.* Naked- and AV-dsRNA were externally applied to a variety of agriculturally relevant plant materials, including tomato and table grape fruits, lettuce leaves and rose petals, and a reduction of *B. cinerea* virulence was observed (Figure 1.2A). Two fungal-gene targeting dsRNA sequences were used, both of which have previously been successfully used in SIGS applications against *B. cinerea*. One was the above-mentioned BcDCL1/2 sequence, for which we had previously reported that fungal disease was reduced on *Arabidopsis Bc-DCL1/2*

RNAi transgenic plants using the HIGS system (Wang et al. 2016). The other was a sequence of 516 bp containing three fragments of *B. cinerea* genes involved in the vesicle-trafficking pathway: *VPS51* (*BC1G_10728*), *DCTN1* (*BC1G_10508*), and *SAC1* (*BC1G_08464*). These fungal genes were previously described by our group as natural targets in the cross-kingdom RNAi interaction between *Arabidopsis* and *B. cinerea(Cai, Qiao, et al. 2018)*, and have been proven to be effective for SIGS-mediated inhibition of fungal pathogens, such as *B. cinerea, Sclerotinia sclerotiorum,* and *Aspergillus niger* (Qiao et al. 2021).

Consequently, three dsRNA sequences were generated by *in vitro* transcription for loading into AVs: two of them specifically targeting *B. cinerea* virulence-related genes (*Bc-DCL1/2* and *Bc-VPS51+DCTN1+SAC1* (*Bc-VDS*)), while the third one was a non-specific target sequence (*YFP*) used as a negative control. All plant materials treated with naked- or AV- fungal gene targeting-dsRNA (*Bc-DCL1/2* or -*VDS)* had reduced disease symptoms in comparison to the water treatment and *YFP*-dsRNA controls (Figure 1.2A, 1.2B). Further, both naked- and AV-*Bc-VDS* treatments decreased expression of the three targeted fungal virulence genes (Figure 1.2C). Taken together, these results demonstrate how externally applied AV–dsRNA can inhibit pathogen virulence by suppression of dsRNA target genes and improve RNAi activity as compared to naked dsRNA.

AV-dsRNA extends RNAi-mediated protection against gray mold disease due to enhanced dsRNA stability and durability

The instability of naked dsRNA currently limits the practical applications of SIGS. Though we demonstrated that AVs can protect dsRNA from nuclease degradation, environmental variables can also influence RNA stability, including leaf washing caused by rainfall events. Thus, in addition to enhancing RNAi efficiency in comparison to naked dsRNA, we were interested in evaluating if using the AV-dsRNA would prolong and improve the durability of the RNAi effect on *B. cinerea*.

To assess the influence of washing on the stability and adherence of the AV-dsRNA to plant leaves, we analyzed the intact dsRNA content on the leaf surface using Fluorescein-labeled *Bc-VDS*-dsRNA and Northern blot analysis after water rinsing. The same concentration of Fluorescein-labeled naked- or AV-*Bc-VDS*-dsRNA (20 ng μl^{-1}) was applied to the surface of *Arabidopsis* leaves. After 24 h of incubation, the treated leaves were rinsed twice with water by vigorous pipetting. Immediately after, we found by CLSM that the naked-dsRNA treated leaves showed a drastic decrease in fluorescence compared with AV-dsRNA treated leaves (Figure 1.3A). These results suggest that most of the naked-dsRNA was washed off, whereas the AV-dsRNA largely remained on the leaves after rinsing (Figure 1.3A). The effect of the AVs on dsRNA stability over time was also assessed. We observed a strong fluorescence signal after 10 days on *Arabidopsis* leaves that were treated with Fluorescein-labeled AV-dsRNA,

indicating that AVs confer stability to dsRNA (Figure 1.3B). By contrast, the naked-dsRNA application showed an undetectable fluorescent signal (Figure 1.3B) and a weak hybridization signal on the Northern blot analysis, compared to AV-*Bc*-*VDS*-dsRNA treated leaves, which retained *Bc*-*VDS*-dsRNA (Figure 1.3C). We further examined whether the AV-dsRNA remained biologically active over time and prolonged protection against *B. cinerea* compared to naked dsRNA. To this end, *Arabidopsis* leaves were inoculated with *B. cinerea* 1, 3, and 10 days post RNA treatment (dpt). Both naked- and AV-*Bc*-*VDS*-dsRNA treatments led to a clear reduction in lesion size over the time points assessed (Figure 1.3D). However, the efficacy of the naked-*VDS*-dsRNA was reduced at a much faster rate than that of the AV-*VDS*-dsRNA, demonstrating that AVs can enhance the longevity of the RNAi effect of the loaded dsRNAs (Figure 1.3E).

To examine if AV-dsRNAs could be similarly effective on economically important crops, we repeated these experiments using tomato fruits, grape fruits (*V. lambusca* var. Concord) and grape (*V. vinifer*a) leaves. We applied naked- or AV-*Bc*-*VDS*-dsRNA on the surface of tomato and grape fruits and on the surface of grape leaves. Both the naked and AV-*Bc*-*VDS*-dsRNA applications led to weaker disease symptoms on tomato and grape fruits at 1, 5 and 10 dpt, as well as on detached grape leaves at 1, 7, 14 and 21 dpt, compared to the water or empty AV treatments (Figure 1.4A). As we had observed in the *Arabidopsis* interactions, the AV-*Bc*-*VDS*-dsRNA applications greatly prolonged and improved the RNAi activity as compared to the naked-dsRNA over time for all plant

materials (Figure 4B). While the naked treatment lost the majority of its efficacy at 5-dpt in tomato fruits, 10-dpt in grape fruits, and 21-dpt in grape leaves, the AV-dsRNA treatments significantly reduced lesion sizes across all timepoints and plant material tested (Figure 1.4B). These trends were also reflected in experiments on rose petals after the naked- and AV-*Bc-VDS*-dsRNA treatments (Supplementary Figure 1.1). The enhanced reduction in lesion size observed specifically at the longer time points (i.e 5, 10, 14, and 21 dpt) after AV-*Bc-VDS*-dsRNA application clearly demonstrates how AVs protect loaded dsRNA from degradation to extend the duration of plant protection against *B. cinerea.* Together, these results strongly support the ability of AVs to confer higher RNAi activity over time, effectively enhancing dsRNA stability for SIGS applications.

Cost-effective AV formulations also provide strong RNAi activity

Our discovery that AVs can lengthen dsRNA mediated plant protection opens the door for its practical use in agricultural applications. Cost is a critical consideration for any crop protection strategy, so we next tested if more cost-effective AV formulations could be used for dsRNA delivery and RNAi activity. First, we removed the PEG, an expensive reagent in the formula, from our original DOTAP+PEG formulation, resulting in DOTAP AVs composed only of DOTAP and cholesterol in a 2:1 ratio. Additionally, we used a cheaper cationic lipid, 1,2-dioleyloxy-3-dimethylaminopropane (DODMA), in a 2:1 ratio with cholesterol to form DODMA AVs. DODMA has previously been utilized in drug

delivery formulations, but has a tertiary amine and is an ionizable lipid compared to DOTAP, which could result in changes in RNA loading and activity. The DOTAP AVs were fully loaded with *Bc-VDS* dsRNA at a 1:1 N:P ratio (Figure 1.5A), requiring the use of 4x fewer lipids than the DOTAP+PEG AVs, or the DODMA AVs, which were completely loaded at a 4:1 N:P ratio (Figure 1.5B). Both DOTAP and DODMA formulations could effectively protect *Bc-VDS* dsRNA from nuclease degradation (Figure 1.5C). The size distribution data for each AV formulation can be found in Table S1. As expected, the z-average sizes of the DOTAP-derived AVs are similar, while the use of DODMA increases the z-average size (Supplementary Table 1.1).

Next, we examined if the different AV formulations influenced fungal dsRNA uptake or RNAi activity. After application of the different AV formulations, the fungal dsRNA uptake was tracked over 16 hours using CLSM. After 16 hours, all three AV formulations showed a similar amount of fungal RNA uptake, however, the uptake of DOTAP AVs was slower than that of DOTAP+PEG, or DODMA AVs, as evidenced by the weaker signal at the 90 minute and 3 hour time points (Figure 1.5E). This could be due to differences in the AV chemistry. To confirm that the lower cost AV formulations have similar RNAi activity on *B. cinerea* over time as our original AV formulation, we performed treatments on tomato fruits. Both the DOTAP and DODMA formulations in complex with *Bc-VDS*-dsRNA trigger a steady RNAi effect on *B. cinerea* over time (Figure 1.6), significantly reducing lesion sizes at all time points (1, 5 and 10 dpt). In addition, fungal biomass

quantification indicated that the treatments with *Bc-VDS*-dsRNA encapsulated in DOTAP and DODMA AV formulations resulted in a statistically significant reduction of the fungal biomass at all time points. All AV-*VDS*-dsRNA treatments were also able to reduce expression of the targeted *B. cinerea* genes at all time points (Supplementary Figure 1.2). Overall, these experiments demonstrate how new AV formulations that are more economical, but equally as effective, can be developed.

Discussion

Liposomes have been extensively researched for their applications in clinical contexts (Tenchov et al., 2021), in fact, they have been utilized for drug delivery to human fungal pathogens (Meagher et al., 2021; Voltan et al., 2016) and are able to transit across the fungal cell wall (Walker et al., 2018). Here, we provide the first demonstration that lipid-based nanovesicles can also be used in agricultural contexts, to deliver dsRNA to plant pathogens. The primary advantage that AV-dsRNA offers for SIGS over naked dsRNA is increased dsRNA stability. This is crucial for extending the shelf-life of dsRNA products, since extracellular RNases and other ribonucleases have been identified on fruits and the leaves of important economic crops such as tomato or tobacco (Galiana et al., 1997; Parry et al., 1997), and for increasing the length of time needed between RNA applications. In fact, utilizing AV-dsRNAs could extend necessary treatment intervals up to a few weeks, as we demonstrated on both grapes and tomatoes (Figure 1.4), making SIGS a much more agriculturally feasible crop protection strategy. This is similar to the extended protection provided by inorganic dsRNA complex formulations against viruses on *Nicotiana tabacum* cv. Xanthi leaves and fungal pathogens on tomato plants (Mitter et al., 2017a; Niño-Sánchez et al., 2022). Another key advantage of utilizing AV-dsRNA technology for crop protection, especially post-harvest products, is that the success of similar liposome formulations in clinical applications (Tenchov et al., 2021) suggests that the AVs will be safe for human consumption.

With agricultural applications in mind, we tested two more cost-effective AV formulations. By removing the PEG from DOTAP-AVs, we can reduce the cost of AV synthesis. PEG is used in liposome preparations in clinical contexts to protect liposomes from immune cell recognition and prolonged circulation time (Shen et al., 2018), however, this is not a concern in agricultural applications. Additionally, in our DODMA formulation, we used the lipid DODMA in place of DOTAP, which can further reduce costs. Surprisingly, our DOTAP only formulation was able to load dsRNA at a 1:1 N:P ratio, in comparison to a 4:1 N:P ratio observed in other formulations. At this lower loading ratio, the cost of DOTAP AV formulations can be even further reduced. The decreased costs of the DODMA and DOTAP AVs potentially make these formulations more suitable for agricultural use.

In summary, we have provided the first example of utilizing a lipid-based nanoparticle, AVs, for the delivery of dsRNAs in SIGS applications. The AV organic formulations used here confer protection to dsRNA that results in an effective and more durable RNAi effect against the fungal pathogen *B. cinerea* in a wide range of plant products, overcoming the main limitation of SIGS to date. This is one key step forward in the development of RNAi-based fungicides which will help reduce the volume of chemical fungicides sprayed on fields and offer a sustainable option to limit the impact of fungal pathogens on crop production and food security.

Materials and Methods

Plant Materials

Lettuce (iceberg lettuce, *Lactuca sativa*), rose petals (*Rosa hybrida* L.), tomato fruits (*Solanum lycopersicum* cv. Roma), and grape berries (*Vitis labrusca* cv. Concord) were purchased from a local supermarket.. Host plants, including *Arabidopsis thaliana*, tomato (money maker), and grape plants were grown in the greenhouse in a 16/8 photoperiod regime at 24±1°C before use in SIGS experiments.

Botrytis cinerea Culture and Infection Conditions

B. cinerea strain B05.10 was cultured on Malt Extract Agar (MEA) medium (malt extract 20 g, bacto protease peptone 10 g, agar 15 g per liter). Fungal mycelia used for genomic DNA and total RNA extraction were harvested from cultures grown on MEA medium covered by a sterile cellophane membrane. For *B. cinerea* infection, the B. cinerea spores were diluted in 1% Sabouraud Maltose Broth infection buffer to a final concentration of 104 spores ml-1 on tomato leaves and 105 spores ml-1 for drop inoculation on the other plant materials 56, 10 µl of spore suspension was used for drop inoculation of all plant materials used, except tomato fruits, in which 20 µl was used. Infected leaf tissues were cultured in a light incubator at 25 °C for 72 h and fruits for 120 h preserving constant and high humidity. Fungal biomass quantification was performed following the methods described by Gachon and Saindrenan 57. The p-values were calculated using

Student's t-test for the comparison of two samples and using one-way ANOVA for the comparison of multiple samples.

Synthesis and Characterization of Artificial Vesicles

PEGylated artificial vesicles were prepared following previously established protocols46. In brief, PEGylated artificial vesicles were prepared by mixing 260 µl of 5% dextrose-RNase free dH2O with the lipid mix and re-hydrating overnight on a rocker at 4°C. The re-hydrated lipid mix was then diluted 4-fold and extruded 11 times using a Mini-Extruder with a 0.4 µm membrane (https://avantilipids.com/divisions/equipment-products/mini-extruder-extrusion-technique). PEGylated artificial vesicles-dsRNA (20 ng µl-1) were prepared in the same manner by adding the appropriate amount of dsRNA to the 5% dextrose-RNase free dH2O before combining with the lipid mix. The average particle size of the artificial vesicles was determined using dynamic light scattering. All measurements were conducted at 25°C using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Malvern, Worcestershire, UK) and the samples were measured after 10-fold dilution in water. Data reported is the average of three independent measurements.

In Vitro Synthesis of dsRNA

In vitro synthesis of dsRNA was based on established protocols14. Following the MEGAscript® RNAi Kit instructions (Life Technologies, Carlsbad, CA), the T7 promoter sequence was introduced into both 5’ and 3’ ends of the

RNAi fragments by PCR, respectively. After purification, the DNA fragments containing T7 promoters at both ends were used for in vitro transcription.

In Vitro Naked- and AV-dsRNA Fluorescence Labeling for Confocal Microscopy

In vitro synthesis of dsRNA and labeling was performed based on established protocols 58. Briefly, Bc-DCL1/2-dsRNA was labeled using the Fluorescein RNA Labeling Mix Kit following the manufacturer's instructions (MilliporeSigma, St. Louis, MO). For confocal microscopy examination of fluorescent dsRNA trafficking into B. cinerea cells, 20 μl of 20 ng μl-1 fluorescent RNAs, either naked or loaded into AVs were applied onto 5 μl of 105 spores ml-1. Germinating spores were grown on PDA medium and placed on microscope slides. The mycelium was treated by KCl buffer or 75 U Micrococcal Nuclease enzyme (Thermo Scientific, Waltham, MA) at 37°C for 30 minutes, The fluorescent signal was analyzed using a Leica SP5 confocal microscope.

External Application of RNAs on the Surface of Plant Materials

All RNAs were adjusted to a final concentration of 20 ng μl-1 with RNase-free water before use. 20 μl of RNA (20 ng μl-1) were used for drop treatment onto the surface of plant materials, or, approximately 1 mL was sprayed onto grape leaves before inoculation with B. cinerea.

Stability of dsRNAs Bound to AVs

The potential environmental degradation of dsRNA was investigated by exposure of naked-Bc-VPS51+DCTN+SAC1-dsRNA (200 ng) and AV-Bc-VDS-dsRNA (200 ng/2.5 µg) to Micrococcal nuclease enzyme (MNase) (Thermo Fisher) treatment in four replicate experiments. Samples were treated with 0.2 U µL-1 MNase for 10 min at 37 °C, and dsRNAs were released using 1% Triton X-100. All samples were visualized on a 2% agarose gel. The persistence of sprayed naked-Bc-VDS-dsRNAs and AV-Bc-VDS-dsRNAs (4:1) on leaves was assessed in two replicate experiments by total RNA extraction followed by northern blot analysis. 4-week old Arabidopsis plants were treated at day 0 with either a 20µl drop of Bc-VPS51+DCTN1+SAC1-dsRNAs (20 ng µl-1) or AV-Bc-VDS-dsRNAs (400:100 ng µl-1) and maintained under greenhouse conditions. Single leaf samples were collected at 1, 3, 7, and 10 dpt. Total RNA was extracted using TRIzol and subjected to northern blot analysis as described above.

Figures

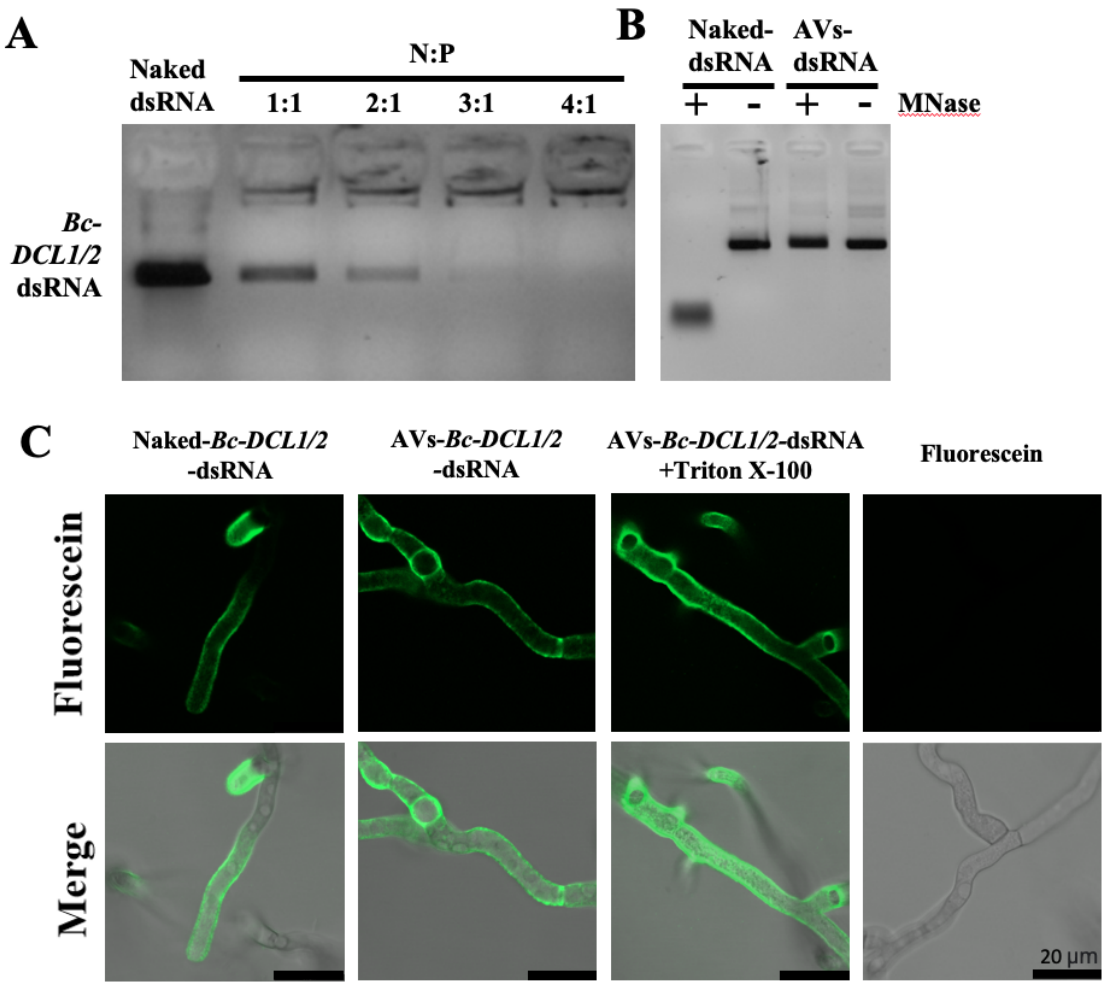


Figure 1.1: dsRNA loading to AVs form AV-dsRNA and protect dsRNA from degradation.

(A) AV-Bc-DCL1/2-dsRNA lipoplexes were formed at a range of indicated charge ratios (N:P) and incubated for 2 h at room temperature before being loaded onto a 2% agarose gel. Complete loading was achieved at an AV:dsRNA mass ratio of 4:1. (B) The stability of naked- and AV-Bc-DCL1/2-dsRNA was tested after MNase treatment. Bc-DCL1/2-dsRNA was released from AVs using 1% Triton X-100 before gel electrophoresis. (C) Fluorescein-labeled naked-Bc-DCL1/2 dsRNA, AV-Bc-DCL1/2-dsRNA, and AV-Bc-DCL1/2-dsRNA + Triton and MNase. (D) Fluorescein-labeled naked- or AV-Bc-DCL1/2-dsRNA were added to B. cinerea spores and fluorescent signals were detected in B. cinerea cells after culturing on PDA medium for 10 h. MNase treatment was performed 30 min before image acquisition. Fluorescence signals remained visible in B. cinerea cells treated with AV-Bc-DCL1/2-dsRNA using Triton X-100 and MNase treatment before observation. Scale bars, 20 μm.

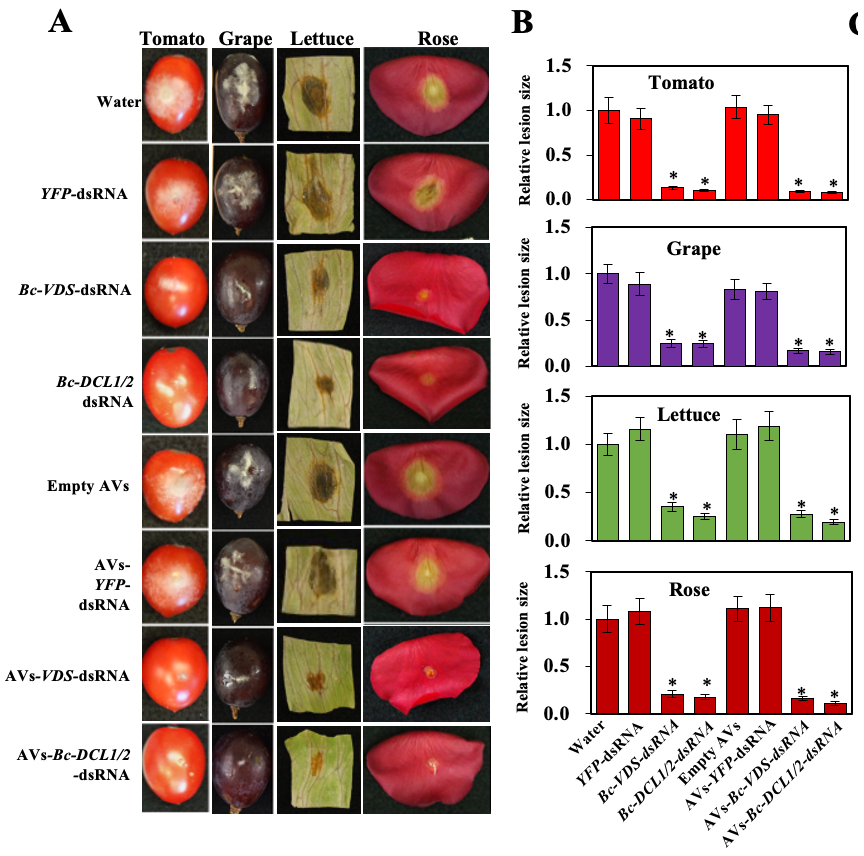


Figure 1.2: Externally applied naked-dsRNAs or AVs-dsRNA inhibited pathogen virulence.

(A) External application of naked- and AV-Bc-VDS-dsRNA, as well as the application of naked- and AV-Bc-DCL1/2-dsRNA (20 µl at a concentration of 20 ng µl-1 of synthetic RNAs), inhibited B. cinerea virulence on tomato fruits, grape berries, lettuce leaves and rose petals compared to the water, AVs empty, naked- or AV-YFP-dsRNA treatments. (B) Relative lesion sizes were measured at 5 dpi on tomato and grape fruits, and at 3 dpi on lettuce leaves and rose petals, and with the help of ImageJ software. Error bars indicate the SD of 10 samples, and three technical repeats were conducted for relative lesion sizes. Statistical significance (Student's t-test): *, $P < 0.05$.

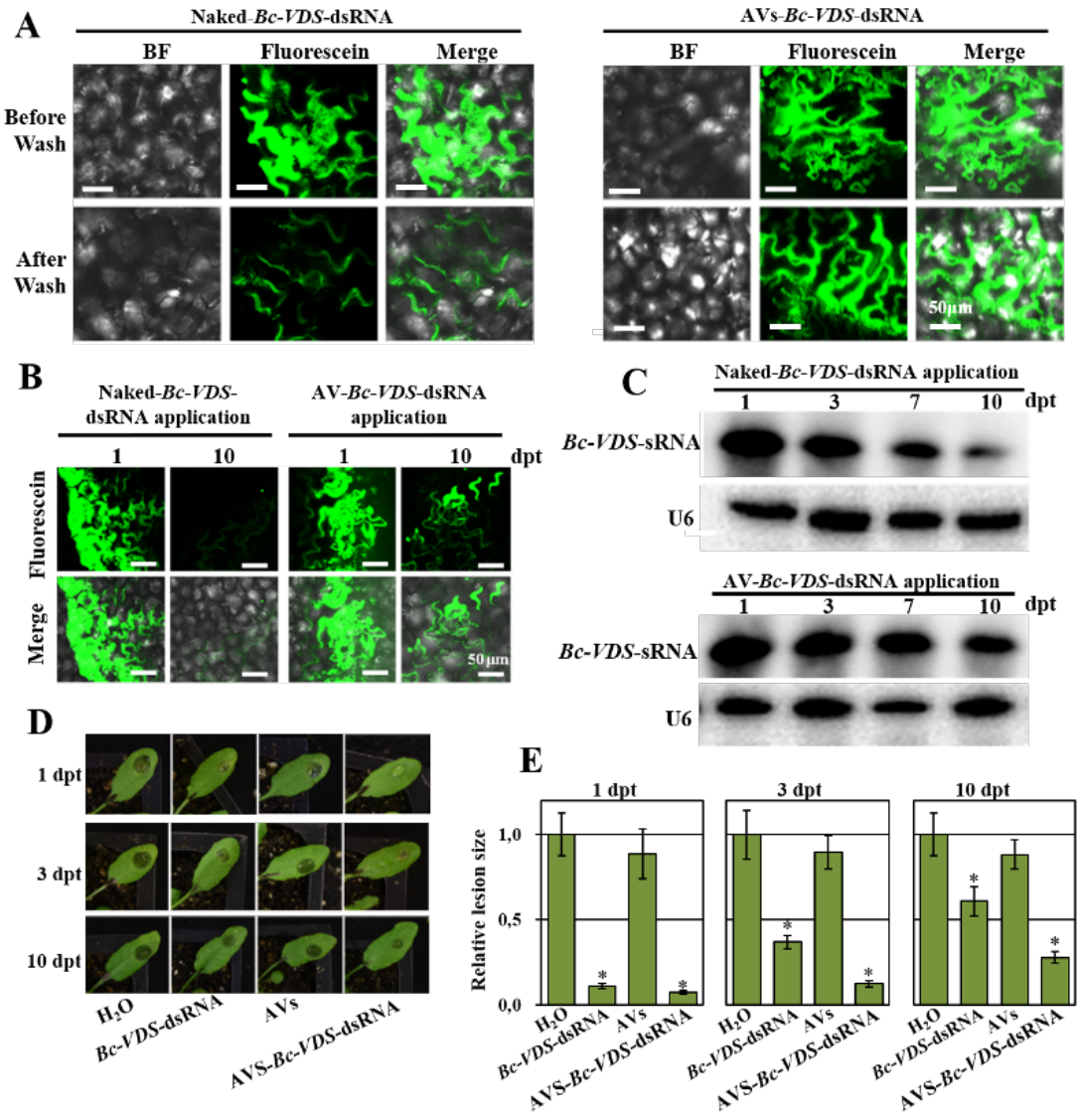


Figure 1.3: Adherence and stability of dsRNA loaded into AVs on Arabidopsis leaves.

(A) CLSM analysis of Arabidopsis leaves 1 dpt before and after a water rinsing treatment shows the capability of AVs to protect dsRNA molecules from the mechanical action exerted by the water. Scale bars, 50 μm. (B) Arabidopsis leaves were treated with Fluorescein-labeled naked- or AV-dsRNA for 1 and 10 days. The fluorescent signals on the surface of leaves were observed using CLSM. Scale bars, 50 μm. (C) The AV-Bc-VDS-dsRNA is highly stable compared with naked-Bc-VDS-dsRNA on Arabidopsis leaves at 10 dpt, as detected by Northern Blot. (D) Lesions on Arabidopsis leaves inoculated with B. cinerea at 1, 3, and 14 dpt. (E) Relative lesion sizes were measured 3 dpi with the help of ImageJ software. Error bars indicate the SD. Statistical significance (Student's t-test): *, $P < 0.05$.

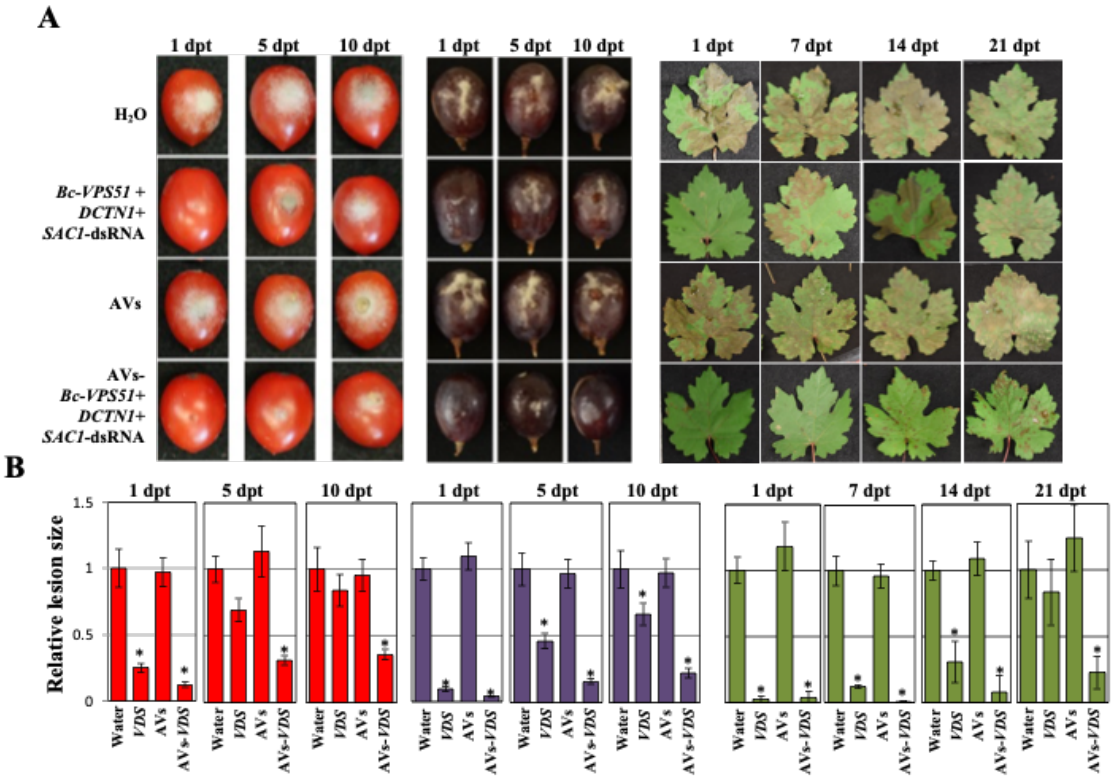


Figure 1.4: **Treatment with AV-dsRNA provides prolonged protection against *B. cinerea* in tomato fruits, grape berries and *V. vinifera* leaves.**

(A) Tomato fruits and grape berries, as well as grape leaves were pre-treated with naked- or AV-Bc-VDS-dsRNA, for 1, 5, and 10 days; or 1, 7, 14, and 21 days respectively, then inoculated with B. cinerea. Pictures were taken at 5 days post infection. (B) Relative lesion sizes were measured with the help of ImageJ software. Error bars indicate the SD. Statistical significance (Student's t-test): *, $P < 0.05$.

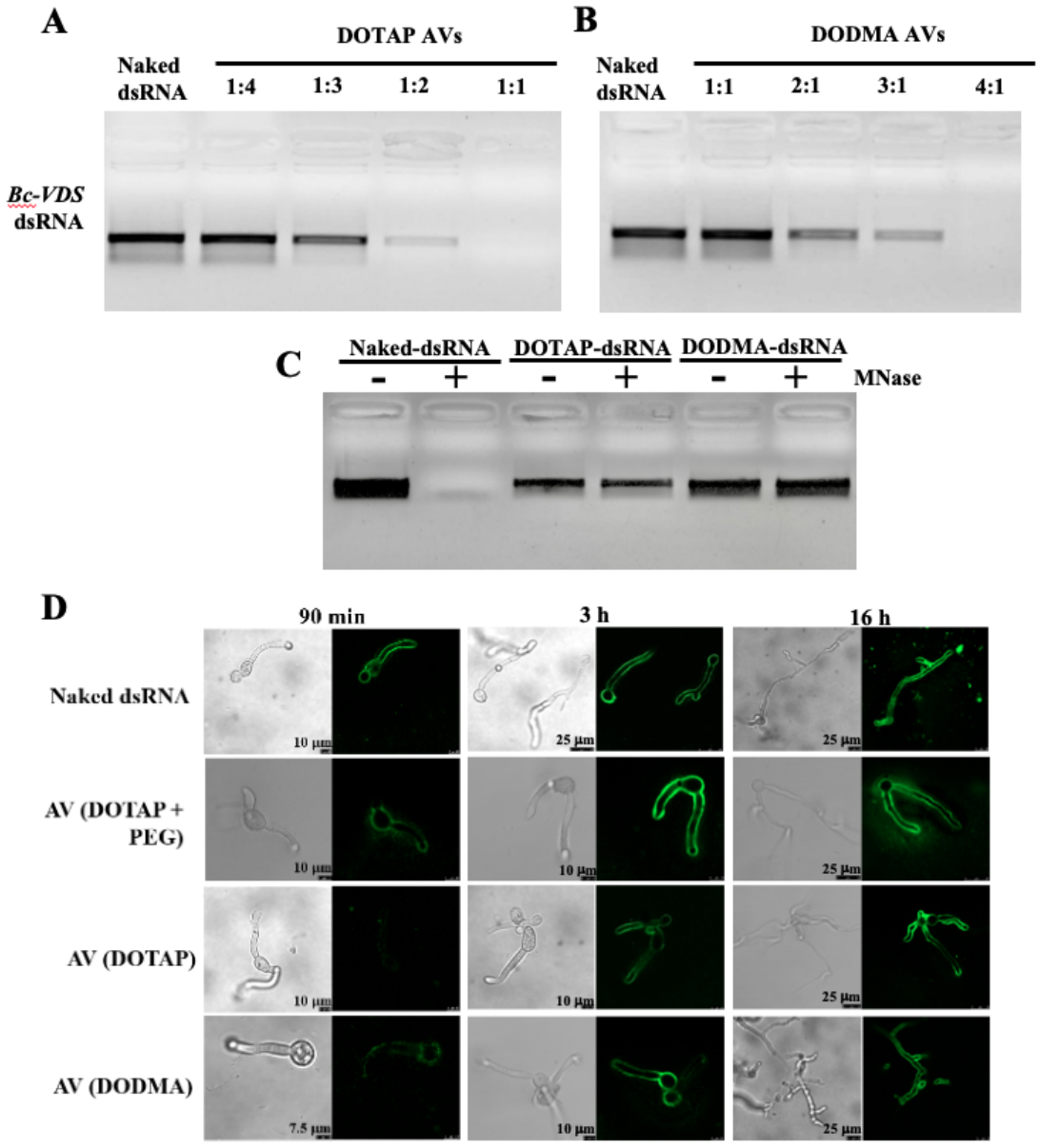

A
DOTAP AVs
Naked dsRNA
1:4
1:3
1:2
1:1
Bc-VDS dsRNA
B
DODMA AVs
Naked dsRNA
1:1
2:1
3:1
4:1
C
Naked-dsRNA
DOTAP-dsRNA
DODMA-dsRNA
-
+
-
+
-
+
MNase
D
90 min
3 h
16 h
Naked dsRNA
AV (DOTAP + PEG)
AV (DOTAP)
AV (DODMA)
10 µm
25 µm
7.5 µm

Figure 1.5: Alternative AV formulations protect dsRNA from nuclease degradation and are easily taken up by *B. cinerea.*

(A) DOTAP AV-Bc-DCL1/2-dsRNA lipoplexes were formed at a range of indicated charge ratios (N:P) and incubated for 2 h at room temperature before being loaded onto 2% agarose gel. Complete loading was achieved to an AV:dsRNA mass ratio of 1:1. (B) DODMA AV-Bc-DCL1/2-dsRNA lipoplexes were formed at a range of indicated charge ratios (N:P) and incubated for 2 h at room temperature before being loaded onto 2% agarose gel. Complete loading was achieved to an AV:dsRNA mass ratio of 4:1. (C) The stability of naked-, DOTAP-, and DODMA-Bc-DCL1/2-dsRNA was tested after MNase treatment. Bc-DCL1/2-dsRNA was released from AVs using 1% Triton X-100 before gel electrophoresis. (D) The size distributions of the dsRNA-loaded AV formulations were determined using dynamic light scattering. Data shown is the average of three individual measurements. (E) Analysis of B. cinerea uptake of fluorescein-labeled dsRNA encapsulated in three different AV formulations (DOTAP+PEG, DOTAP and DODMA) after 3 and 16 hours of incubation. Fluorescence signals are visible in the B. cinerea cells treated with the three AV-Bc-DCL1/2-dsRNA using Triton X-100 and MNase treatment before observation.

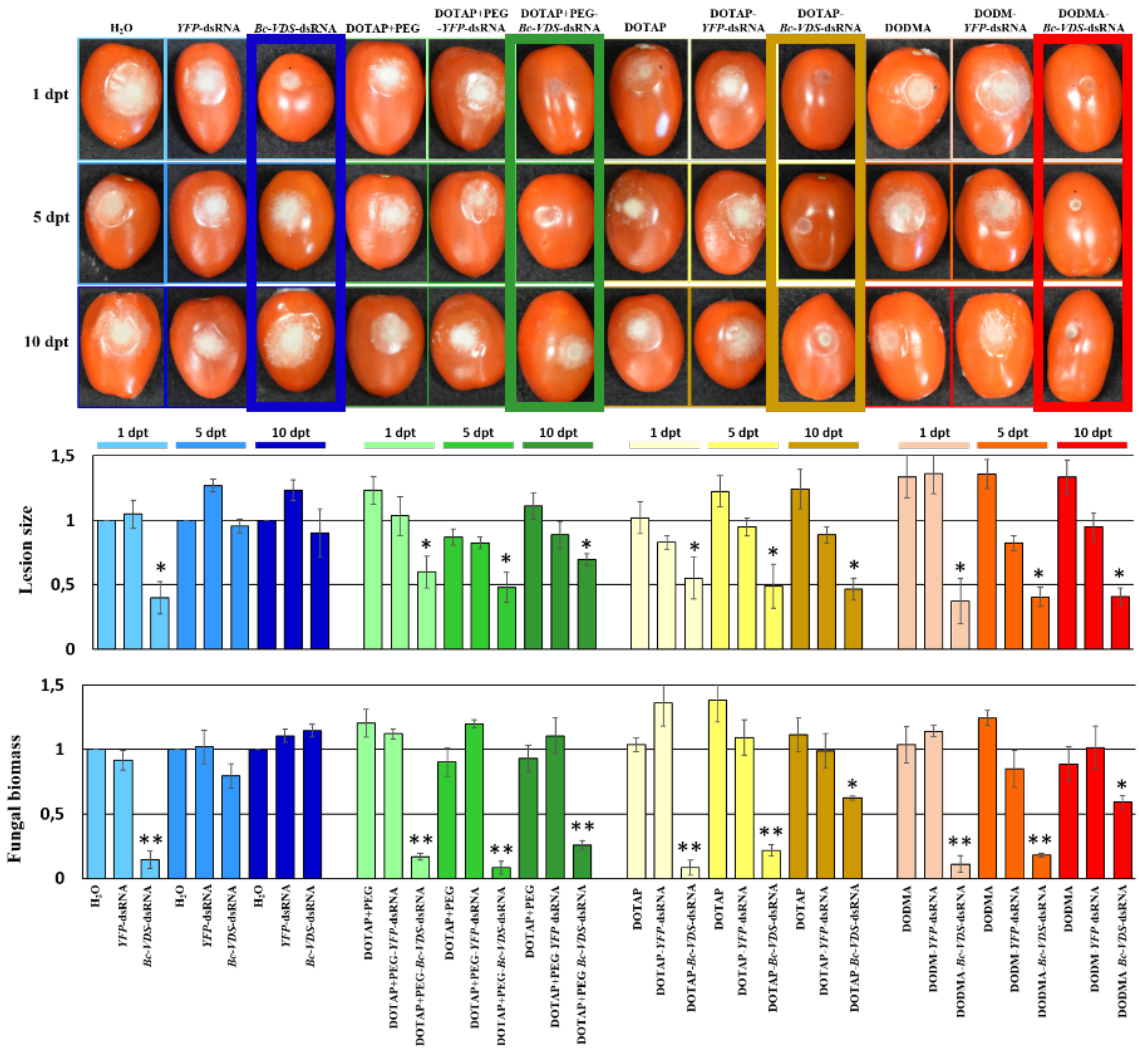
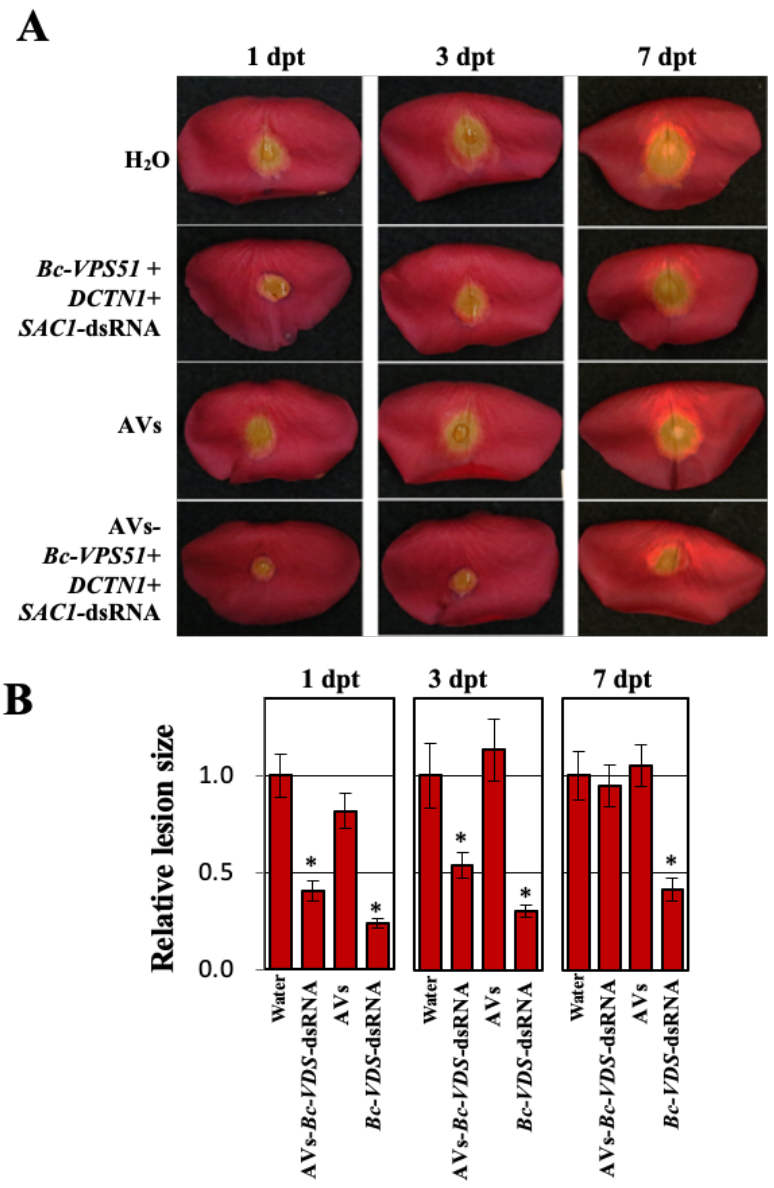


Figure 1.6: Treatment with all DOTAP+PEG, DOTAP and DODMA AV-dsRNA formulations provide prolonged protection against *B. cinerea* in tomato fruits.

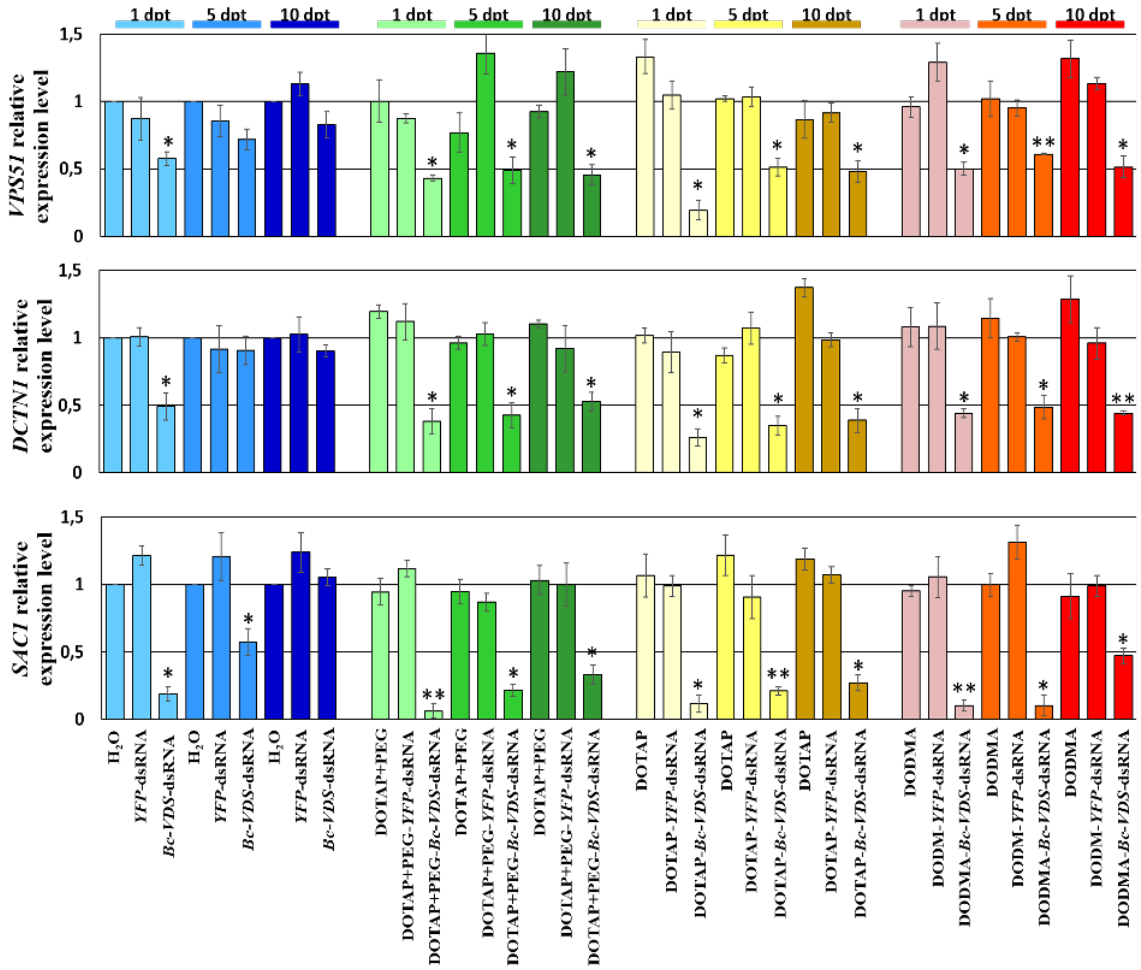
(A) Tomato fruits were pre-treated with naked- or AV(DOTAP+PEG)-Bc-VDS-dsRNA, AV(DOTAP)-Bc-VDS-dsRNA and AV(DODMA)-Bc-VDS-dsRNA, for 1, 5, and 10 days, then inoculated with B. cinerea. Pictures were taken at 5 dpi. (B) Relative lesion sizes were measured with the help of ImageJ software. Error bars indicate the SD. Statistical significance (Student's t-test): *, $P < 0.05$. (C) Relative fungal biomass was quantified by qPCR. Fungal RNA relative to tomato RNA was measured by assaying the fungal actin gene and the tomato actin gene by qPCR using RNA extracted from the infected fruits at 5 dpi. Statistical significance (Student's t-test): *, $P < 0.05$; **, $P < 0.01$.

Supplementary Figures



Supplementary Figure 1.1: Treatment with AV-dsRNA provides prolonged protection against *B. cinerea* in rose petals.

(A) Rose petals were pre-treated with naked- or AV-Bc-VDS-dsRNA, for 1, 3, and 7 days, then inoculated with B. cinerea. Pictures were taken at 3 dpi. (B) The relative lesion sizes were measured with the help of ImageJ software. Error bars indicate the SD. Statistical significance (Student's t-test): *, $P < 0.05$.



Supplementary Figure 1.2: Treatment with AV-dsRNAs reduces expression of targeted genes in *B. cinerea*.

From tomato treatments shown in Figure 4, relative gene expression of the three targeted genes in Botrytis, VPS51, DCTN1, and SAC1 was quantified by qPCR using RNA extracted from the infected fruits at 5 dpi. Statistical significance (Student's t-test): *, $P < 0.05$; **, $P < 0.01$.

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CHAPTER 2

Cross-kingdom RNA trafficking from bacteria to fungi enables plant protection against fungal pathogens

The text of this chapter in full, is part of a soon to be submitted work, J Niño-Sanchez*, H Wu*, R Hamby*, A Chen, M Zhang and H Jin. “Cross-kingdom RNA trafficking from bacteria to fungi enables plant protection against fungal pathogens.” *In progress.* * denotes equal contributions. H.J. conceived the idea, designed the experiments and supervised the study. J.N.S. constructed the RNA-expressing *B. subtilis* and the OpuaC-YFP tagged *B. subtilis* lines, performed experiments in Figure 2.2B, 2.2D, 2.3A, and Figure 2.4. H.W. constructed the 3WJ-Broccoli tagged RNA lines of *B. subtilis*, performed the experiments in Figure 2.1, performed replicates for Figure 2.2E, 2.3B, 2.5B, and 2.6B and performed data analysis throughout the manuscript. R.H. led the drafting, writing, and revising of the manuscript, designed and led tomato leaf pathogenicity assays in Figures 2.2E, 2.3B, 2.5E, and 2.6B, and assisted with pathogenicity experiments in 2.2D, 2.3A, 2.4, 2.5D, 2.6A, and 2.6A-F. A.C. constructed all *P. putida* constructs and performed experiments in 2.5D, 2.6 A,C,D,E, and Supplementary Figures 2.1 and 2.2. All authors read and approved of its content.

Abstract

Fungal pathogens are a growing threat to global food security, however, resistance is emerging to nearly every commercial fungicide used in agriculture. RNA-based antifungals represent an innovative and eco-friendly strategy for disease control, however, it is limited by the instability of RNA in the environment, particularly the soil. Here, we engineered two plant beneficial soil bacteria, a gram-positive bacterium, *Bacillus subtilis,* and a gram-negative bacterium, *Pseudomonas putida,* to produce and deliver fungal gene-targeting RNAs to the destructive foliar and postharvest fungal pathogen *Botrytis cinerea*, and the soil-borne pathogen *Verticillium dahliae*. Both species of bacteria secrete RNA via extracellular vesicles (EVs), and cross-kingdom trafficking of RNA and EVs from bacterial cells to fungal cells was observed. Treatment of plant leaves with these RNA-containing bacterial EVs conferred protection against *B. cinerea* infection. Further, direct treatment with either species of engineered bacteria could protect both tomatoes and *Arabidopsis* plants from *B. cinerea* and *V. dahliae*. These results show that bacterial-based platforms are a potent and cost-effective strategy for producing & delivering antifungal RNAs to fungal pathogens.

Introduction

In the face of worldwide population growth and climate change, plant scientists and farmers are now tasked with growing more food than ever, on less space, with ever increasing abiotic and biotic threats to plant health(Delgado-Baquerizo et al. 2020). Plant pathogens can cause devastating yield losses in crops both pre- and post-harvest(Savary et al. 2019), with fungi among the most severe and devastating of plant pathogens. Current fungal disease management strategies rely heavily on the application of chemical fungicides, which can leave behind residues harmful to both the environment and human health(Van de Wouw et al. 2021). More alarmingly, this overuse of fungicides in agriculture has contributed to the rise of fungicide resistance. In fact, resistant strains of fungi have been identified against nearly every commercially available fungicide used in both agriculture and healthcare(Fisher et al. 2018). To safeguard global food security, the environment, and human health, it is essential that novel, eco-friendly fungal disease management strategies are developed.

Recent discoveries revealed that many fungi can uptake double-stranded (ds)RNA or small RNAs (sRNAs) from the environment(Qiao et al. 2021). Once inside the fungal cells, through the process of RNA interference (RNAi), this dsRNA can utilize existing fungal machinery to be processed into sRNAs and target complementary fungal messenger (m)RNAs for gene silencing(Wang et al. 2016). This naturally occurring pathway can be leveraged for plant protection, by treating plant material (often via spray) with RNA constructs which target and

silence essential fungal virulence genes, in a strategy termed Spray-Induced Gene Silencing (SIGS)(Niu et al. 2021). This technique is effective against a large variety of fungal pathogens, including *Botrytis cinerea*(Wang et al. 2016), *Fusarium graminearum*(Koch et al. 2016), and *Sclerotinia sclerotiorum*(McLoughlin et al. 2018).

SIGS has several major drawbacks, however. Primarily, dsRNA lacks stability in the environment, breaking down in 5-7 days on plant surfaces(Qiao et al. 2023; Niño-Sánchez et al. 2022) and within 2 days in soil(Dubelman et al. 2014). This necessitates labor intensive and costly reapplication of treatments, and eliminates dsRNA's viability as an antifungal agent against soil borne pathogens. Additionally, though the cost of RNA production has been reduced by the advent of RNA vaccines in human medicine(Kis et al. 2020), it remains expensive. In naturally occurring RNA-based communication between plants and fungi, RNA is packaged and protected from environmental degradation inside lipid-enclosed nanoparticles called extracellular vesicles (EVs)(He et al. 2023; Cai et al. 2018). Similarly, nanoparticles have been utilized in SIGS approaches to package and enhance the stability of dsRNA, including liposomes(Qiao et al. 2023) and layered-hydroxide clay nanosheets. These approaches, however, still fail to address the significant challenges of combatting soilborne pathogens and the cost associated with RNA production.

Plants exist alongside rich microbial communities in both the rhizosphere and on plant surfaces(Trivedi et al. 2020). Many of these microbes are beneficial

to plant growth and have been leveraged as biocontrol agents against plant pathogens(El-Saadony et al. 2022). These microbes could be engineered to consistently produce RNAs directly in the soil or on plant tissue, replacing RNA as it is degraded and circumventing the high costs associated with RNA production. Additionally, their own biocontrol properties could have an additive effect to the RNA, leading to a robust fungal disease management strategy.

Here, we engineered two plant-beneficial bacteria, the gram-positive bacteria *Bacillus subtilis,* and the gram-negative *Pseudomonas pudita*, to produce and excrete fungal-gene targeting dsRNAs. We demonstrate that both these bacteria can be used to protect *Arabidopsis* and tomato plants from the foliar fungal pathogen, *Botrytis cinerea*, and the root fungal pathogen, *Verticillium dahliae*. Further, we demonstrate that both species excrete these RNAs in EVs, and that these EVs alone were sufficient in protecting both *Arabidopsis* and tomato leaves from *Botrytis cinerea* infection. Overall, we found that harnessing bacteria for RNA production and excretion is an effective method for fungal disease control, significantly enhancing the utility of RNAi-based disease management strategies.

Results

B. subtilis can be engineered to produce RNAs and secrete them in EVs

To generate bacteria which produce fungal-gene targeting RNAs, we first obtained a strain of *B. subtilis,* BG322, lacking RNaseIII (*ΔrncS*) which cleaves dsRNA, so it can be engineered for the production of dsRNA. We generated lines of BG322 expressing dsRNAs targeting *B. cinerea* genes, DCL1/2 and VDS (a fusion construct targeting vesicle-trafficking genes VPS51, DCTN1, and SAC1), and expressing dsRNAs targeting *V. dahliae* genes DCL1/2 upon IPTG induction. All these dsRNA constructs have previously shown effectiveness in targeting pathogen genes and reducing virulence(Wang et al. 2016; Qiao et al. 2023; Niño-Sánchez et al. 2022). To confirm that these strains of bacteria were producing the dsRNAs, we performed a Northern blot analysis. This analysis clearly shows signals of our dsRNAs IPTG-inducible production in the engineered strain BG322_VDS compared to the empty vector (Figure 2.1A).

Production of dsRNA alone, however, is not sufficient for transport of dsRNAs into fungal pathogens. The RNAs also need to be secreted into the environment. Extracellular vesicles (EVs) have been identified as vehicles of RNA transport in a variety of organisms, including plants, fungi and bacteria(Luz et al. 2021; Lécrivain and Beckmann 2020; Chronopoulos and Kalluri 2020). We hypothesized that our engineered *Bacillus* may also be secreting dsRNA within their bacterial (B)EVs. To test this, we isolated BEVs from *Bacillus* using ultracentrifugation as previous protocols established(Brown et al. 2014). We

confirmed the presence of our dsRNAs in these BEVs using Northern blot analysis (Figure 2.1A). Next, to confirm our BEV isolation procedure was successfully isolating intact bacterial EVs, we performed TEM analysis on the BEV samples and visualized the classic EV cuplike structure (Figures 2.1B) and confirmed that *Bacillus* produced EVs of the expected size (~100nm) using Nanoparticle Tracking Analysis (Supplementary Figures 2.1A).

To further demonstrate that the produced RNAs are associated with EVs, we generated bacterial strains expressing single and double-stranded VDS fused with a fluorescent RNA aptamer for RNA imaging called Three Way Junction-4 x Broccoli (3WJ-4xBro). Using confocal microscopy, we observed that 3WJ-4xBro tagged single or double-stranded VDS constructs colocalize with FM4-64-stained membranous vesicles in purified EV fractions (Figures 2.1D). This provides direct evidence that the produced RNAs are associated with membranous extracellular vesicles. We then treated bacteria EVs with nuclease both with and without the detergent, Triton-x, which disrupts and bursts membranous EVs. Our PCR results show most of the RNAs are protected by the EV and can only be degraded by nuclease after Triton-x disrupts the membranes (Figures 2.1C)**.** We also performed sucrose density gradient fraction to further purify collected BEVs. Through RT-PCR and immunoblotting, we show that both the EV marker Opuac-YFP and the produced RNAs are concentrated between 1.13g/cm3 and 1.21g/cm3, which is the density fraction in which exosomes or exosome-like EVs are typically found in mammalian, plant, fungal and bacteria EV samples (Figures 2.1A)**.** This provides

another line of evidence that RNA can be imported into or associate with bacterial EVs.

Bacillus EVs can facilitate cross-kingdom RNA trafficking between bacteria and fungi

After confirming our engineered RNAs were secreted in BEVs, we next sought to determine whether these BEVs could be internalized by fungi as a mechanism for RNA delivery. We incubated germinated *B. cinerea* spores with the Opuac-YFP marker strains of BG322 or BEVs isolated from these marker strains and using confocal microscopy observed the internalization of the fluorescent signal inside of fungal cells (Figure 2.2B). Next, we wanted to confirm that dsRNA is being transported into the fungal cells along with the EVs. To do this, we co-incubated the same Opuac-YFP BG322 strains expressing single and double-stranded VDS fused with 3WJ-4xBro with germinated fungal cells and observed uptake of the RNAs upon examination via confocal microscopy (Figure 2.2C). Taken together, these results demonstrate that fungi can uptake RNAs originating from bacteria via BEVs.

dsRNA-containing *Bacillus* EVs can protect plants from *B. cinerea* infection

After determining that our engineered bacteria secreted RNA in BEVs, and that these BEVs could be transported into fungal cells, we hypothesized that these dsRNA-containing BEVs could prevent fungal infection. To test this hypothesis, we

isolated BEVs from *Bacillus* strains and treated Arabidopsis leaves with 20uL droplets of resuspended BEVs before inoculating them with *B. cinerea* in the same spot. Leaves were kept in humid conditions and the infection lesions were observed and measured after 2-3 days. There was a clear reduction in the resulting fungal lesion size on the leaves treated with BEVs containing the fungal gene targeting constructs, DCL or VDS (Figure 2.2D). Next, to see if this strategy could be effective outside a model system on a crop plant, we repeated this assay on tomato leaves and obtained similar results (Figure 2.2E). Taken together, these results demonstrate that dsRNA containing BEVs are an effective treatment against foliar fungal pathogens.

To confirm that the mode of action of our BEV-contained fungal gene-targeting dsRNAs was via the targeting of fungal mRNAs, we cultured *B. cinerea* in the presence of BEVs. After 12 hours, we extracted the fungal RNA and measured the relative expression of the two genes targeted by the DCL1/2 construct and the three genes targeted by the VDS construct via qPCR. In the fungi treated with BG322 EVs (Supplementary Figure 2.2) we saw a clear reduction in expression of all five targeted genes. These results demonstrate that the BEV-contained dsRNAs function via the targeting and silencing of their complementary fungal mRNAs.

dsRNA producing bacteria can protect plants from *B. cinerea* infection

Though we found BEVs to be an effective treatment, EV isolation can be time consuming and costly. In some commercial settings, it may be more practical to treat plants directly with the bacteria. To determine whether this could be an effective antifungal treatment, we repeated the experiments above, but instead of treating plant leaves with BEVs, we sprayed them with a suspension of our dsRNA expressing *Bacillus*. In both Arabidopsis (Figure 2.3A), and tomato (Figure 2.3B), we saw a clear reduction in fungal lesion size on plant leaves treated with either species of bacteria producing fungal-gene targeting dsRNAs. These results demonstrate that our bacteria can provide protection against foliar fungal pathogens when directly sprayed onto plant material.

Next, to demonstrate that the bacteria could reduce expression of targeted genes in fungal cells, we co-cultured our engineered *Bacillus* with *B. cinerea*. We collected RNA from these co-cultures at three time points, 12, 24, and 36 hours after starting the co-culture. For all five targeted genes, we found a reduction in gene expression at all three timepoints, though the reduction was markedly increased at 24 and 36 hours in comparison to 12 (Supplementary Figure 2.2). These experiments offer further evidence that the dsRNA constructs we engineered the bacteria to produce are being transported into fungal cells where they target and silence complementary genes.

dsRNA producing bacteria can protect plants from *V. dahliae* infection

While the above experiments clearly demonstrate the utility of our engineered bacteria in preventing foliar fungal disease, one of our biggest goals here was to establish a protocol for utilizing RNA-based antifungals against *soil-borne* fungal pathogens. To test this, we utilized the aggressive soil-borne pathogen, *Verticillium dahliae* as our model, and generated lines of BG322 which expressed the dsRNA construct VdDCL1/2, which targets *V. dahliae* genes VdDCL1 and VdDCL2. Because we were unsure of the best way to introduce the bacterial treatments, we utilized two different treatment methods, bacterial-fungal spores co-culture at infection time, and bacterial suspension soil treatment. For the co-culture infection treatment, we mixed the bacterial strains with *V. dahliae* before root-dip inoculation of 10-day old Arabidopsis seedlings in the co-culture suspension. At 21 dpi infection, *Bacillus* producing VdDCL1/2 RNA showed significantly reduced infection based on disease index and fungal biomass in comparison to the controls (Figure 2.4A,B).

For the soil treatment, we treated soil directly with bacteria suspension before transplanting 10-day old Arabidopsis inoculated with *V. dahliae*.At 7 dpi additional bacterial suspension was applied right at the base of the seedlings. At 21 dpi Arabidopsis plants grown in soil treated with either species of bacteria producing VdDCL1/2 RNA showed significantly reduced infection based on disease index and fungal biomass in comparison to the controls (Figure 2.4C, 4D).

Once establishing the efficacy of our bacteria in reducing *V. dahliae* infection in Arabidopsis, we wanted to test whether this technique would work in a crop system. To do this, we utilized our engineered bacteria to protect tomato plants against *V. dahliae* infection. To more closely mirror practical commercial application techniques, we only used the soil treatment technique described above. After three weeks, disease severity in tomato plants was determined by measuring the canopy area and weight of the tomato plants. Additionally, to measure the colonization of the xylem by *V. dahliae*, we performed a fungal outgrowth assay by taking cross sections of the tomato stems from just above the roots and plating them on potato dextrose agar. We observed that *V. dahliae* inoculated tomatoes planted in soil treated with bacteria producing VdDCL1/2 RNA had a restoration of canopy area and weight resembling that of the uninfected control tomato plants which was not observed in tomato plants grown in soil treated with empty vector or non-specific RNA producing bacteria (Figure 2.4E,F,H). Further, while fungal outgrowth was nearly ubiquitous in the infected plants grown in untreated soil or soil treated with control bacteria, only one stem of eight tomato plants grown in soil treated with VdDCL1/2 producing bacteria showed xylem colonization (Figure 2.4G). Overall, we found that utilizing bacteria as an RNA-production platform is an effective method of preventing fungal disease in tomatoes.

P. pudita can be engineered to produce RNAs and secretes EVs

After confirming we could successfully engineer the gram-positive bacterium, *Bacillus*, for dsRNA generation and delivery, we next wanted to see if this technique would work in a gram-negative bacterium. We chose *Pseudomonas pudita,* a known beneficial soil microbe. Similar to our work in *Bacillus*, we used *P. pudita* strain KT2440 with an RNase III mutation, CMA701, and generated strains of *Pseudomonas* expressing dsRNAs targeting *B. cinerea* genes, BcDCL1/2 and BcVDS and expressing dsRNAs targeting *V. dahliae* genes BcDCL1/2. We isolated EVs from our *Pseudomonas* strains as described above and performed TEM analysis on the EV samples and visualized the classic EV cuplike structure (Figures 2.5A). Using Nanoparticle Tracking Analysis we confirmed *Pseudomonas* produced EVs of the expected size (~100nm) (Supplementary Figure 2.1). Similar to Bacillus EVs, we performed confocal microscopy, Triton-x treatment followed by RT-PCR and density gradient to demonstrate RNA produced by our engineered *Pseudomonas pudita* strain is present in extracellular vesicles (Supplementary 2.3).

Bacterial EVs can facilitate cross-kingdom RNA trafficking between bacteria and fungi

To confirm that *Pseudomonas* EVs can also be internalized by *Botrytis*, we labeled known EV-marker protein OprH with GFP. We were able to visualize fungal uptake of the marked EVs using confocal microscopy (Figure 2.5B). Next,

we generated lines of *Pseudomonas* expressing VDS fused to the RNA aptamer 3wJ. We co-incubated the 3wJ expressing *Pseudomonas* with germinated *Botrytis* spores and observed uptake of the RNAs upon examination via confocal microscopy. These results show that fungi can take up RNA secreted by *Pseudomonas* (Figure 2.5C).

dsRNA-containing *Pseudomonas* can protect plants from foliar and root fungal pathogens

Next, to demonstrate that our engineered *Pseudomonas* could also be effective in crop protection strategies, we challenged Arabidopsis with *Botrytis* after *Pseudomonas* EV treatment similar to *Bacillus* as described above. There was a clear reduction in the fungal lesion size on the leaves treated with BEVs containing the fungal gene targeting constructs, DCL or VDS (Figure 5D). We repeated this assay on tomato leaves and obtained similar results (Figure 5E). To confirm that the mode of action of our BEV-contained fungal gene-targeting dsRNAs was via the targeting of fungal mRNAs, we cultured *B. cinerea* in the presence of BEVs. In the fungi treated with *Pseudomonas* EVs (Figure S2), we saw a clear reduction in expression of all five targeted genes. These results demonstrate that the *Pseudomonas* EV-contained dsRNAs function via the targeting and silencing of their complementary fungal mRNAs.

Next, we challenged Arabidopsis and tomato with *Botrytis* after treatment with a suspension of our dsRNA expressing *Pseudomonas*. We saw a clear

reduction of fungal lesion size on plant leaves treated with *Pseudomonas* producing fungal-gene targeting dsRNAs in both Arabidopsis (Figure 2.6A), and tomato (Figure 2.6B). Taken together, these results demonstrate that dsRNA producing *Pseudomonas* can be an effective tool against foliar fungal pathogens.

Pseudomonas can protect plants from *V. dahliae* infection

We next wanted to test the effectiveness of our engineered *Pseudomonas* against *V.dahliae.* Similar to our *Bacillus* experiments, we utilized two different treatment methods, bacterial-fungal spores co-culture, and bacterial soil treatment. The Arabidopsis plants inoculated with *V. dahliae* and both *Pseudomonas* producing VdDCL1/2 RNA treatments showed significantly reduced infection in comparison to the controls (Figure 2.6D and 2.6E). Next, we co-cultured *Pseudomonas* with *V. dahliae* to determine if the dsRNA construct VdDCL1/2 was effectively silencing gene targets in the fungi. Expression of both targeted genes, VdDCL1 and VdDCL2, was reduced at 6 hours, and this reduction markedly increased by the 24 hour time point (Supplementary Figure 2.2).

Discussion

While the advent of RNA-based antifungals presents a promising advancement of fungal disease management in agriculture, their commercial viability remains limited by RNA stability in the environment, particularly in the rhizosphere, and the cost of RNA production. Previous efforts have been made to enhance the stability of RNA used in both agricultural and clinical applications utilizing nanoparticles. Specifically, lipid-based nanoparticles such as liposomes have been used to package and deliver dsRNA to plant pathogens(Qiao et al. 2023), as well as for the delivery of RNA molecules in clinical contexts(Ahmadzada, Reid, and McKenzie 2018), perhaps most famously in the mRNA covid vaccines(Thi et al. 2021). Additionally, layered-hydroxide nanosheets, termed Bioclay, have been successfully utilized to package and deliver RNAs to viruses(Worrall et al. 2019), fungal pathogens(Niño-Sánchez et al. 2022), and whiteflies(Jain et al. 2022) in agricultural applications. These nanoparticle platforms, however, still do not address the problem of soilborne pathogens, nor the cost of RNA production.

Here, we have developed a bacterial based dsRNA production platform to circumvent both the issues of stability and cost by continuously synthesizing fungal-gene targeting RNA directly on plant surfaces or in the soil. We demonstrated this could be done with two plant beneficial bacteria, the gram-negative *Bacillus subtilis,* and the gram-positive *Pseudomonas pudita*. Our engineered bacteria effectively produce fungal gene-targeting dsRNAs, which they

can excrete and deliver into fungal cells via EVs. Both engineered bacteria species were effective at reducing both foliar and soilborne fungal infections on both the model plant, *Arabidopsis thaliana*, and the important crop species, tomato. This greatly expands the utility of RNAi based fungal control strategies.

Notably, while another recent study utilized a beneficial plant fungus, *Trichoderma*, for delivery of dsRNAs to soil pathogens(Wen et al. 2023), our work presented here offers mechanistic evidence for how the microbe-produced RNA is transported-EVs. This adds to a growing body of work demonstrating the key role EVs play in the transport of RNAs between organisms, notably between plants and fungus(Cai et al. 2018; He et al. 2023) and mammalian hosts and their parasites(Buck et al. 2014). Here, we show the first example of cross-kingdom trafficking of RNA from bacteria to fungi, adding another dimension to the cross-kingdom RNA communication occurring in the rhizosphere. It remains to be discovered whether bacteria communicate with fungi in the natural ecosystem via transport of endogenous RNAs.

Plant fungal pathogens are a growing threat to global food security. In addition to developing robust strategies to combat them, it is essential to better understand how these fungi communicate with both their plant hosts and the microbial communities that they exist within. Our research here furthers both these goals, first by creating a bacterial-based platform the generation and delivery of antifungal RNAs, and notably, unveiling a new direction for cross-kingdom RNAi research between bacteria and fungi.

Materials and Methods

Bacteria strains, culture conditions and EV isolation

RNaseIII-deficient bacteria strains, *P. putida* KT2440-CMA702 and *B. subtilis* BG322, were utilized for dsRNA and BEV production. *B. subtilis* strain BG322 (*ΔrncS SpR*) was kindly provided by Dr. Bechhofer (Mount Sinai School of Medicine of New York University, New York, NY, USA) (Herskoviz and Bechhofer, 2000). *P. putida* strain CMA702 (KT2440 *Δrnc* derivative) was kindly provided by Dr. Sandra Viegas (Universidade Nova de Lisboa, Lisbon, Portugal). BG322 was used for plasmid construction were incubated in Luria-Bertani (LB) liquid medium (10 g Bacto™ Tryptone (Difco Laboratories, Detroit, NI, USA), 10 g NaCl, 5 g Bacto™ Yeast Extract (Difco Laboratories, Detroit, NI, USA), per liter, adjusted to pH 7.0) and treated with ampicillin (100 mg/ml) or kanamycin (10 mg/ml for *B. subtilis* transformants) when required at 37°C at 250 rpm. For dsRNA production, HT115(DE3) and BG322 transformants where cultured to reach OD_{600} ~ 0.8, then isopropyl-b-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) was added at 1.0 mM final concentration to the cultures. Bacterial cultures were incubated until they reached the stationary phase (at least 4 hours).

BEV isolation was performed with minor modifications to the method followed by Brown et. al., 2014. Briefly, 100 ml of overnight (16 h) bacterial culture wA spun for 15 min at 4°C at 4,000 x g and the resultant supernatant was filtered through a 0.22 mm Nalgene™ Rapid-Flow™ (Thermo Fisher Scientific, Waltham,

MA, USA). We then cultured a sample of the supernatant on an LB plate and observed no colony formation, confirming this was cell free. The filtrate was then concentrated using 100 KDa Amicon® ultrafiltration system (Millipore, Burlington, MA, USA) which also removed larger cellular debris or aggregated material. The concentrate was then eluted in PBS and ultracentrifuged at 174,900 x g (maximum speed) for 90 min at 4^{o}C in a Beckman SW 32 Ti rotor. BEVs were washed twice in 12 ml of PBS and finally resuspended on 300 ml of PBS at 4^{o}C. All the experiments used fresh isolated BEVs.

Plasmid construction

The plasmid pDG148-Stu (Joseph et. al., 2001) was modified for dsRNA expression in *B. subtilis* BG322 strain. pDG148-Stu was provided by *Bacillus* Genetic Stock Center (Columbus, OH, USA). It is a shuttle vector capable of replicating in HT115(DE3) from pBR322 origin and in BG322 from the pUB110 origin. It was developed for the inducible expression by IPTG of a foreign insert cloned into its unique *Stu*I restriction site after a P*spac* promoter.

The plasmid pJOE771.1 (Hoffman 2015) was modified for dsRNA expression in *P. putida* strain CMA702 (KT2440 *Δrnc* derivative). In addition, a *EcoRI* restriction site was added in between pMtlE promoters and the specific insert sequence for dsRNA production so that the sequence could easily be exchanged by *EcoR*I and ligase treatment.

Fungal strains and culture conditions

Fungal cultures of *B. cinerea* BO5.10 and *V. dahlia* JR2 were established from frozen mycelia stored on 15% glycerol V/V at -80°C. *B. cinerea* B05.10 was cultured on malt extract agar (MEA) medium (20 g of malt extract, 10 g of Bacto™ proteose peptone (Difco Laboratories, Detroit, NI, USA), and 15 g of agar per liter for 10 days to obtain spores. Then, the fungal spores were diluted in 1% Saboraud maltose liquid medium to inoculation procedure. *V. dahliae* was routinely cultured on PDA medium (24 g of potato dextrose broth (Difco Laboratories, Detroit, NI, USA), and 15 g of agar per litre), however to quickly obtain spores, *V. dahliae* was cultured for 3 days at 120 rpm in GOX liquid medium (60 g sucrose, 7 g $NaNO_3$, 3 g Bacto™ Peptone (Difco Laboratories, Detroit, NI, USA), 1 g de KH_2PO_4, 0.5 g de $MgSO_4.7H_2O$, 0.5 g KCl per liter adjusted to pH 7.0 with NaOH). For the inoculation, spores were diluted in milli-Q autoclaved water after two steps of spore wash through a double-layered cheese cloth with milli-Q autoclaved water at 2500 x g for 15 min.

Pathogenicity test

To test efficacy of dsRNA producing bacteria or BEVs associated with these bacteria in preventing fungal disease, plants were pretreated with a bacterial or BEVs solution. For the *B. cinerea* inoculation on *A. thaliana* leaves, the plants were sprayed with a bacterial suspension of ~1.5 x 10^{10} CFU/ml of HT115(DE3) and ~5 x 10^9 CFU/ml of BG322 (~0.5 ml per plant) or treated by 10 ml droplets of BEVs

solution. Also, the bacterial suspension in milli-Q autoclaved water was supplemented with 0.1 mM of IPTG when required. Once bacterial solution dried (~2 hours), three leaves per plant were inoculated by a 10 ml drop of the *B. cinerea* spore suspension at 10^5 spores/ml. In case of BEVs treatment, the spore suspension was placed at the same point that BEVs were placed. After inoculation, *A. thaliana* plants were incubated for 3 days inside a translucent plastic box at 22^{o}C. Then, lesion areas were measured by diameter measurements using an electronic caliper.

The bacterial treatment for *V. dahliae* inoculations consisted of i) soil treatment based on 1 ml of bacterial suspension 2 hours before the inoculated plants were transplanted at the same point of treatment application and another 1 ml of bacterial application on the soil at 7 dpi. ii) a mixture of bacterial suspension and fungal spores suspension at the fungal inoculation step. The bacterial suspension consisted in 3.75 x 10^8 CFU/ml for HT115(DE3) and 1.25 x 10^8 CFU/ml for BG322 strains. For *V. dahliae* inoculations in *A. thaliana*, 2-week-old plants were uprooted, and the roots were rinsed in milli-Q autoclaved water, then they were dipped for 3 min in a 10^6 spore/ml suspension. After replanting in fresh or bacterial treated soil, disease was evaluated at 21 dpi by quantification of symptom development as percentage of diseased rosette leaves in the non-treated plants, similarly to Fradin et. al., 2011. For *V. dahliae* inoculations in *S. lycopersicum* was followed the protocol as described previously Fradin et. Al., 2009 with minimal modifications. Briefly, ten days-old plants of the Money Maker cultivar were

uprooted, and roots were rinsed in water. ~0.5 cm of the root was streamed to standardize the fungal inoculation process, subsequently they were dipped for 5 min in a suspension of 10^6 or 10^7 spores/ml and replanted in bacterial treated soil. After 3 weeks of the inoculation (after 2 weeks for the 10^7 spores/ml inoculation), relative canopy area was calculated with the help of ImageJ software from overhead pictures. Also, tomato plants were mowed and weighed at that time. Finally, we carried out the fungal recovery assay from the inoculated tomato plants, which is a measure of the susceptibility of the plant, as described in Frandin et. al., 2009, e.g. a stem section immediately above the cotyledons was taken. The surface was sterilized for 15 min in 70% ethanol, followed by 15 min in 10% hypochlorite and at least rinsed three times in water. Then, samples were sliced with a sterile scalpel and placed onto PDA plates supplemented with chloramphenicol (34 mg/ml).

Total RNA extraction and Northern Blot assay

A. thaliana leaves of plant material inoculated with *V. dahliae* were collected and frozen at -80°C. Total RNA was extracted using TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA was eluted in DEPC-treated water (Sigma-Aldrich, St. Louis, MO, US), and treated with DNaseI according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Then, RNA was tested for integrity in 1.2% agarose

gels and quantified using a Nanodrop Spectrophotometer. The absence of DNA was confirmed by the lack of conventional PCR amplification of *A. thaliana* and *V. dahliae* genes.

For total RNA extraction from the BEVs, the only modification was the incorporation of the MNase enzyme treatment at the first step according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, US) in order to remove acidic nuclei content outside of the BEVs before of BEVs RNA extraction in the strict sense.

Total RNA from BEV were separated on 1.1% agarose gel containing 1XMOPS and 0.5% formaldehyde. RNA on the gel was transferred to membrane via salt bridge overnight in 20xSSC buffer. The membrane was baked under 80 Celsius degrees for 2 hours after the transfer process. The membrane was then probed with ^{32}P end labeling probe which signal is captured in a Typhoon 9410 GE Healthcare machine.

RT-qPCR for fungal biomass quantification and RT-PCR for dsRNA detection in BEVs.

After RNA extraction, cDNA was synthesized using the Superscript™ III First-Strand Synthesis System (Thermo Fisher, Waltham, MA, USA) following the manufacturer's instructions. The SYBR Green mix (Bio-Rad Laboratories, Hercules, CA, USA) was used to carry out RT-qPCR reactions in a CFX384 (Bio-Rad laboratories, Hercules, CA, USA) with the following thermal profile: 95°C for

15 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and testing single amplification by a specific peak in the dissociation melting curve (0.5^{o}C increments every 10 s from 65^{o}C to 95^{o}C). Fungal biomass in the inoculated samples was estimated as the relative quantity of *actin* transcript of *V. dahliae* using Vd-actin 2F and Vd-actin 2R primers (Table S1) normalized to the *actin* transcript of *A. thaliana* using At-actin F and At-actin R (Table S1) by the $2^{-\Delta\Delta Ct}$ method (Livak et. al., 2001).

cDNA synthesized from BEVs RNA was carried out by specific primers (both strands separately) and random hexamer primers (control) (Thermo Fisher, Waltham, MA, USA) using Superscript™ III First-Strand Synthesis System (Thermo Fisher, Waltham, MA, USA) following the manufacturer's instructions in order to assure the presence of both strands of RNA in the BEVs. The RT-PCR was completed taking the specific cDNA as template for the PCR step using Phusion™ High-Fidelity DNA Polymerase following the manufacturer's instructions (Thermo Fisher, Waltham, MA, USA) and the amplicons were observed in 1.2% agarose gels.

Density gradient fractionation of BEVs

Bacterial EVs were also purified by discontinuous sucrose density gradient centrifugation. Using 10–90% sucrose stocks (w/v), including 10, 16, 22, 28, 34, 40,46, 52, 58, 64, 70 and 90%, the discontinuous gradient was prepared by layering 1 ml of each solution in the 15-ml ultracentrifuge tube. The bacterial EV

was premixed with 1 ml of 10% sucrose stock then top loaded onto the discontinuous gradient. Samples were then centrifuged in a swinging-bucket rotor for 16 h at 100,000g, 4 °C and six fractions (2 ml each) were collected. Collected fractions were transferred to new ultracentrifuge tubes and each sample was diluted to 12 ml using PBS, followed by a final centrifugation for 2 h at 174,900g, 4 °C to obtain pellet for further analysis.

Fungal uptake efficiency

To evaluate fungal uptake efficiency BEVS, uptake of YFP-labeled BEVs by *B. cinerea* cells was quantified using confocal microscopy, following the methodology basis in (Hamby et. al., 2020). Briefly, labeled-YFP BEVs were isolated from 100 ml culture and resuspended in 300 ml of PBS. 10 ml of isolated BEVs was added to germinated fungal spores on a glass microscope slide with 3 ml of PDA medium. The mix was incubated for 3 hours and then treated with 1% Triton X-100 to disrupt no integrated BEVs in fungal cells. The analysis was carried out by confocal laser scanning microscopy (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany). Excitation was provided by an argon laser (514 nm) and the emission signal of fluorescein was detected at 515-560 nm.

Visualization of Fungal uptake bacterial expressed RNA

Overnight cultures of *Bacillus* strainsBG332-pDG148-Stu, BG332-pDG148-VDS3WJ4xBroccoli and BG332-pDG148-VDS3WJ4xBroccoliSDV grown at 37C in LB media with kanamycin (25nM) were diluted to 0.2 OD and incubated at 37C until the OD reached 0.8. Once the OD was 0.8, IPTG was added to a final concentration of 0.8mM and the cultures were incubated for another 3 hours. Then, 0.5ml of the culture was centrifuged at 5000 x g for 5 minutes and resuspended in 500µl of YEPD media with germinated *Botrytis cinerea* spores and incubated overnight. DFHBI-1T was then added to the samples (1:500 dilution) and incubated in the dark for 30 minutes before confocal imaging.

Figures

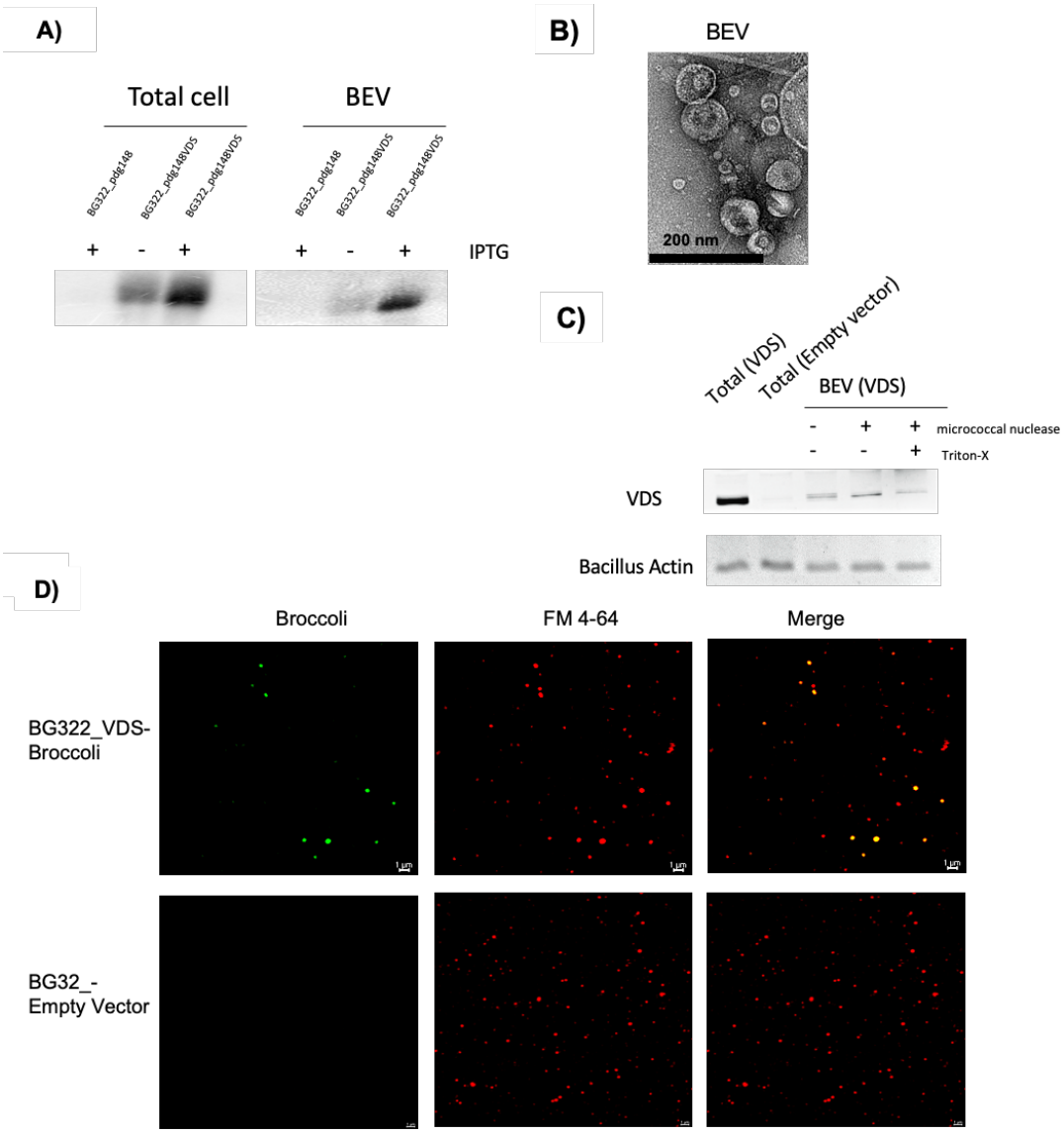


Figure 2.1: *Bacillus subtilis* can be engineered to secrete fungal-gene targeting RNA in extracellular vesicles (BEVs)

(A) Northern blots of VDS RNA in total cell and purified BEVs from BG322-BcVDS. The empty vector strain BG322_pdg148 was used as a negative control. (B) Transmission Electron Microscopy of BEVs purified from B. subtilis (C) VDS RNA produced by *Bacillus* can be detected and protected from micrococcal nuclease treatment by BEVs. Total RNA from BG322_pdg148 was used as a control. (D) Confocal microscopy showed that VDS-Broccoli RNA signals overlap with FM4-64 stained BEVs in BEV samples from BG322-BcVDS-Broccoli. BEV samples from the empty vector strain were used as a control.

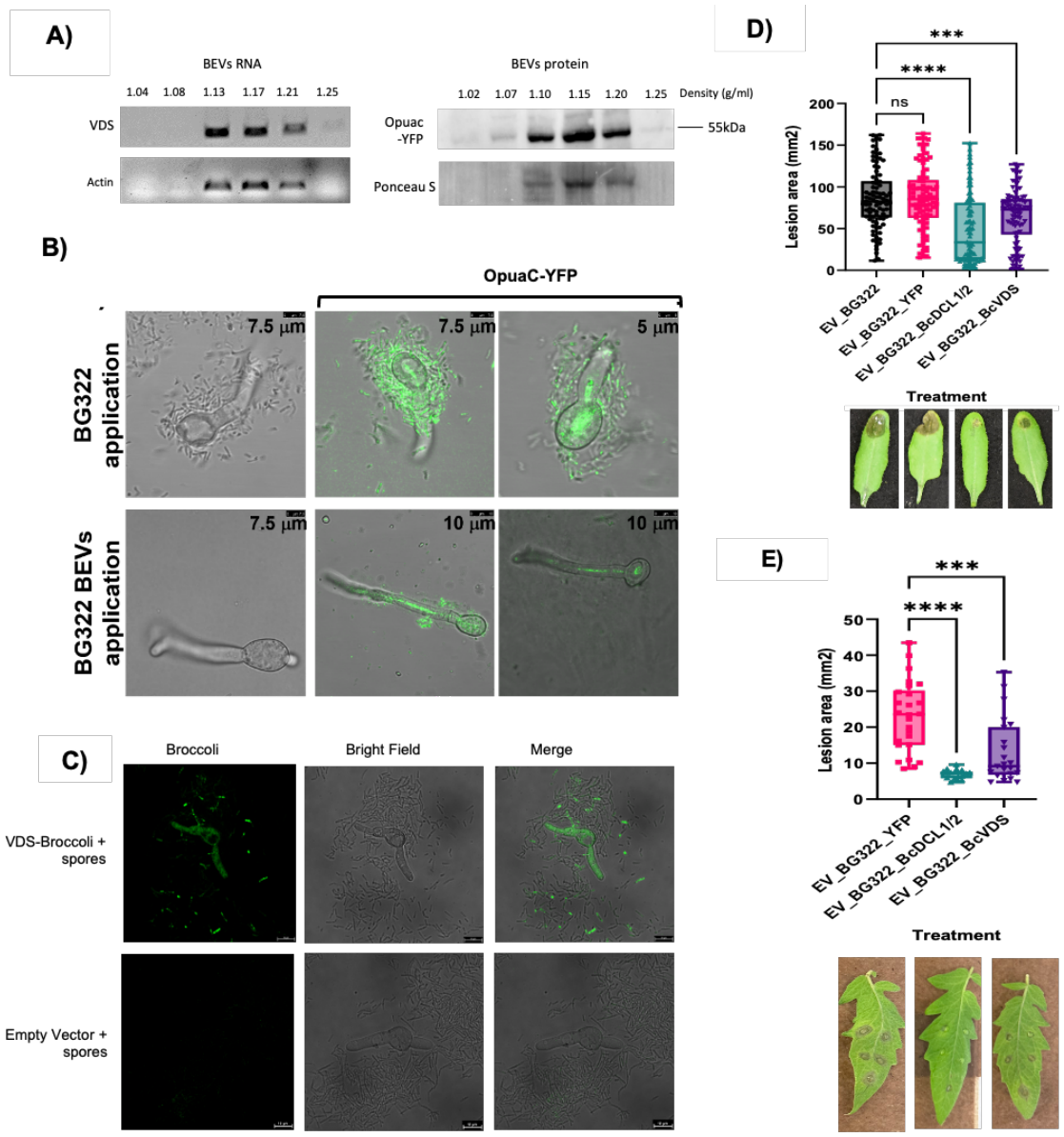

A)
BEVs RNA
1.04 1.08 1.13 1.17 1.21 1.25
VDS
Actin
BEVs protein
1.02 1.07 1.10 1.15 1.20 1.25 Density (g/ml)
Opuac -YFP
55kDa
Ponceau S
B)
OpuaC-YFP
BG322 application
BG322 BEVs application
7.5 µm
7.5 µm
5 µm
7.5 µm
10 µm
10 µm
C)
Broccoli
Bright Field
Merge
VDS-Broccoli + spores
Empty Vector + spores
D)
***
****
ns
Lesion area (mm2)
200
150
100
50
0
EV_BG322
EV_BG322_YFP
EV_BG322_BcDCL1/2
EV_BG322_BcVDS
Treatment
E)
***
****
Lesion area (mm2)
50
40
30
20
10
0
EV_BG322_YFP
EV_BG322_BcDCL1/2
EV_BG322_BcVDS
Treatment

Figure 2.2: *Bacillus* EVs can facilitate cross-kingdom trafficking between bacteria and fungi

(A) Confocal microscopy showed that the BEV marker OpuAC-YFP overlaps with FM4-64 stained BEVs. (B) VDS RNA and BEV marker OpuAC were examined in BEVs isolated through sucrose gradient fractionation. (C) YFP-tagged OpuAC was observed inside B. cinerea cells after incubation with either Opuac-YFP expressing Bacillus strain (BG322_Opuac-YFP) or BEVs isolated from this strain. The empty vector strain BG322_pdg148 was used as control. (D) BG322-BcVDS-Broccoli RNA was visualized in Botrytis cells after incubation with BG322-BcVDS-Broccoli. BG322_pdg148 was used as a control. (E) Lesions caused by B. cinerea infection was reduced in Arabidopsis leaves that were treated by BG322-BcVDS or BG322-BcDCL (F) Lesions produced by B. cinerea infection was reduced in tomato leaves that were treated by BEVs isolated from BG322-BcVDS or BG322-BcDCL. Statistically significant differences ((* P< 0.05; ** P< 0.01;; *** P<0.001; **** P<0.0001) were determined by one-way ANOVA, ANOVA, followed by Dunnet's test. Pictures were taken at 3dpi.

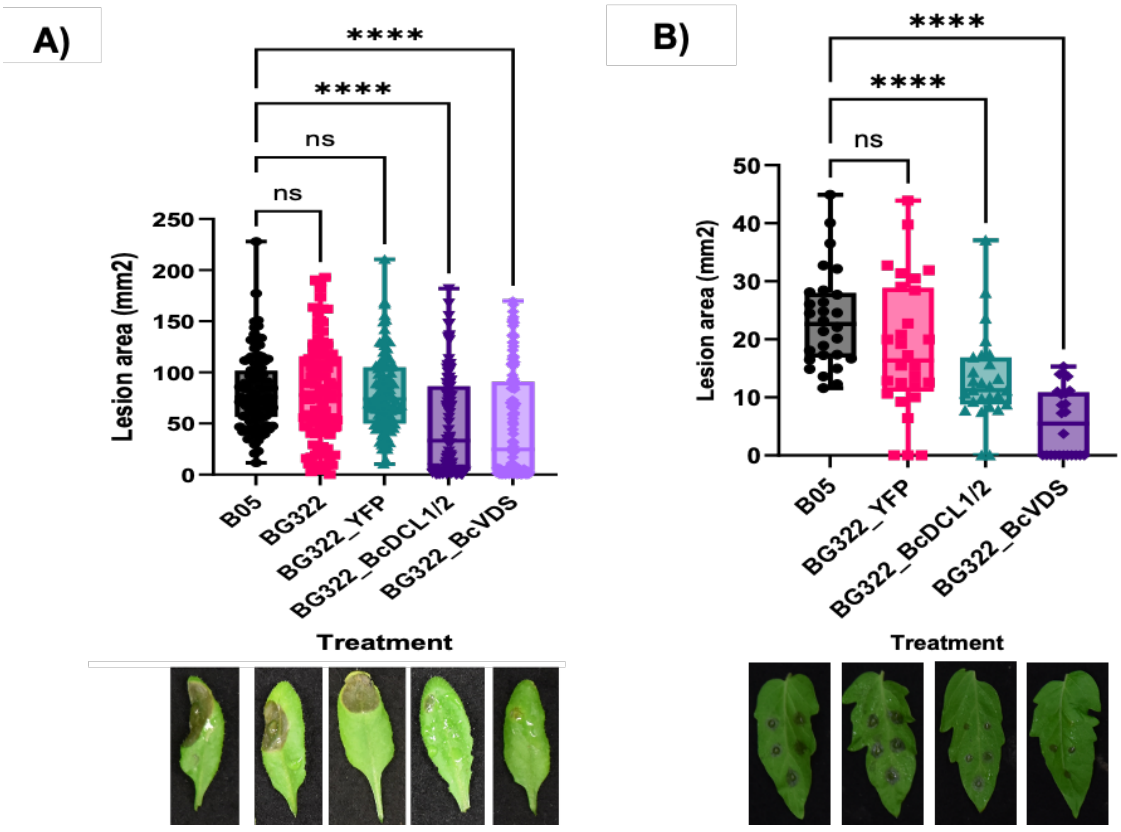


Figure 2.3: Direct application of *B. subtilis* provides protection against *B. cinerea*

(A) Lesions caused by *B. cinerea* infection was reduced on Arabidopsis leaves that were treated by BG322-BcVDS or BG322-BcDCL. (B) Lesion size caused by *B. cinerea* infection was reduced on tomato leaves that were treated by BG322-BcVDS or BG322-BcDCL. Statistically significant differences ((* P< 0.05; ** P< 0.01; *** P<0.001; **** P<0.0001) were determined by one-way ANOVA, followed by Dunnet's test. ((* P< 0.05; ** P< 0.01; *** P<0.001; **** P<0.0001) were determined by one-way ANOVA, followed by Tuckey's test. Pictures were taken at 3dpi.

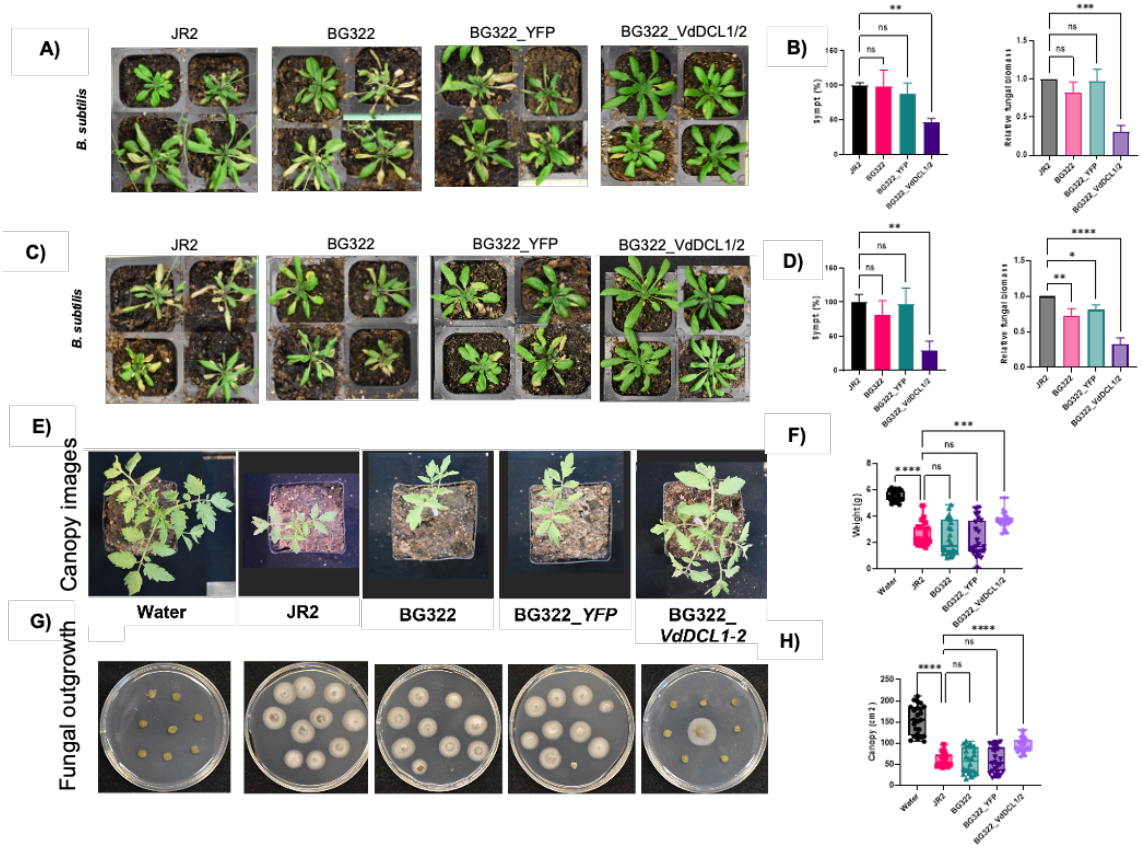


Figure 2.4: Engineered bacteria can be used to provide protection against *V. dahliae*

(A) Disease produced in *A. thaliana* by *V. dahliae* at 21 dpi, when fungal spores were co-incubated with BG322-VdDCL or BG322-YFP at plant infection time. (B) Relative disease index and fungal biomass data from assay A were calculated using the disease observed in the untreated JR2 *V. dahliae* inoculation as a positive control reference. (C) Disease produced in *A. thaliana* by *V. dahliae* at 21 dpi, when soil was treated by the bacterial *B. subtilis* solution 2 hours before *V. dahliae* infection and 7 days after the inoculation. (D) Relative disease index and fungal biomass data from assay C were calculated using the disease observed in the untreated JR2 *V. dahliae* inoculation as the reference. (E) Overhead picture of inoculated tomato MoneyMaker plants in bacterial treated soil at 21 dpi. (F) Weight (in grams) of inoculated tomato plants in bacterial treated soil at 21 dpi. (G) Fungal outgrowth at 6 days after plating of stem sections harvested at 21 dpi. (H) Canopy area of inoculated tomato plants were measured in bacterial treated soil at 21 dpi. Error bars in (B,D, F and H) indicate standard deviation obtained from three biological replicates. Statistically significant differences ((* P< 0.05; ** P< 0.01; *** P<0.001; **** P<0.0001) were determined by one-way ANOVA, followed by Dunnet's test.

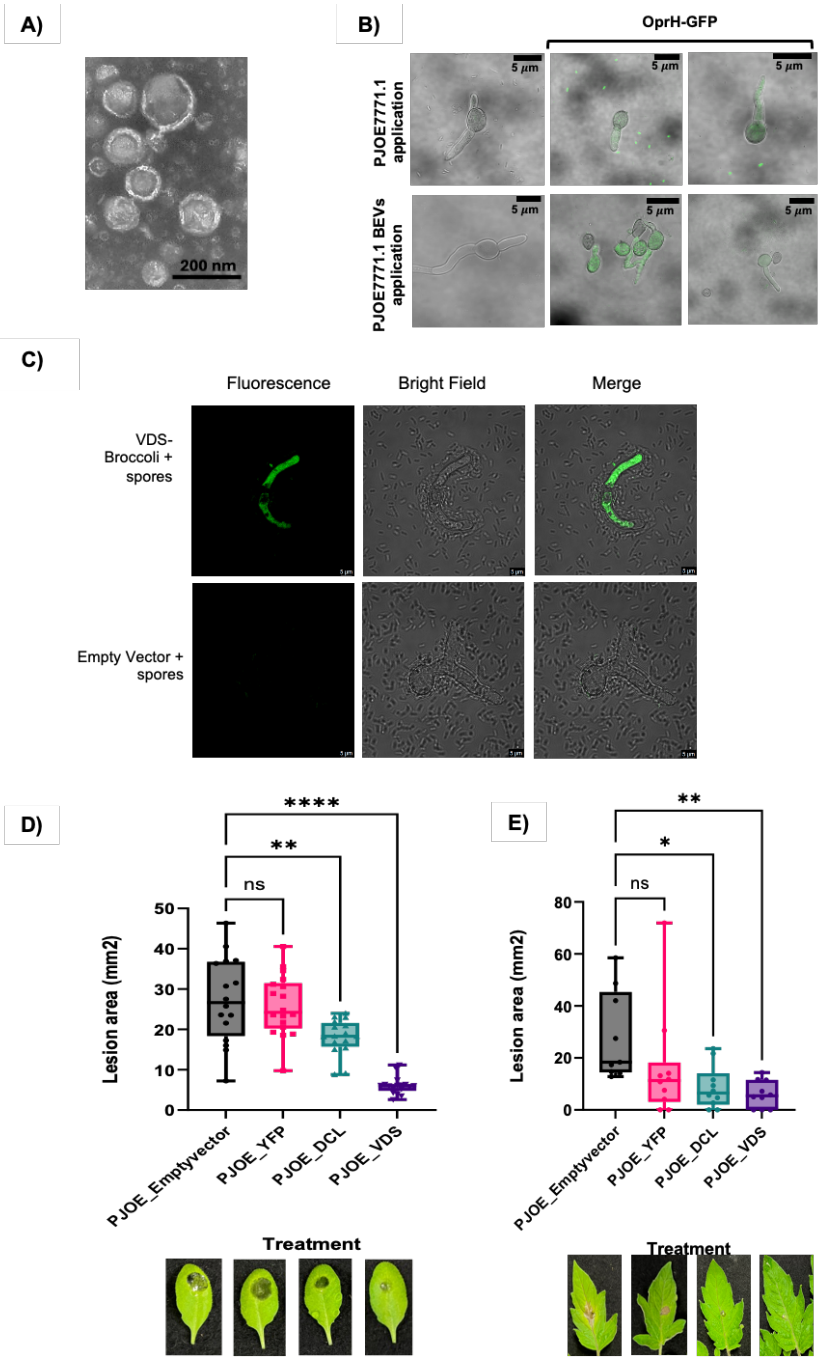

A)
200 nm
B)
OprH-GFP
PJOE7771.1 application
PJOE7771.1 BEVs application
5 µm
C)
Fluorescence
Bright Field
Merge
VDS-Broccoli + spores
Empty Vector + spores
D)
****
**
ns
Lesion area (mm2)
50
40
30
20
10
0
PJOE_Emptyvector
PJOE_YFP
PJOE_DCL
PJOE_VDS
Treatment
E)
**
*
ns
Lesion area (mm2)
80
60
40
20
0
PJOE_Emptyvector
PJOE_YFP
PJOE_DCL
PJOE_VDS
Treatment

Figure 2.5: *Pseudomonas* OMVs can facilitate cross-kingdom trafficking between bacteria and fungi

(A) Transmission Electron Microscopy of OMVs from *P. putida*. (B) GFP-tagged OprH was observed inside *B. cinerea* cells after incubation with either KT2440-Oprh-GFP or OMVs from this strain. (C) Broccoli-labeled VDS RNA were visualized in *B. cinerea* cells after incubation with KT2440-VDS-Broccoli. The empty vector strain was used as a negative control. (D) Lesions produced by *B. cinerea* infection was reduced on the Arabidopsis leaves that were treated by dsRNA-producing KT2440-BcVDS or KT2440 BcDCL. (E) Lesions produced by *B. cinerea* infection after 2 dpi was reduced on tomato leaves that were treated by KT2440-BcVDS or KT2440 BcDCL. Statistically significant differences (* $P< 0.05$; ** $P< 0.01$; *** $P<0.001$; **** $P<0.0001$) were determined by one-way ANOVA, followed by Dunnet's test.

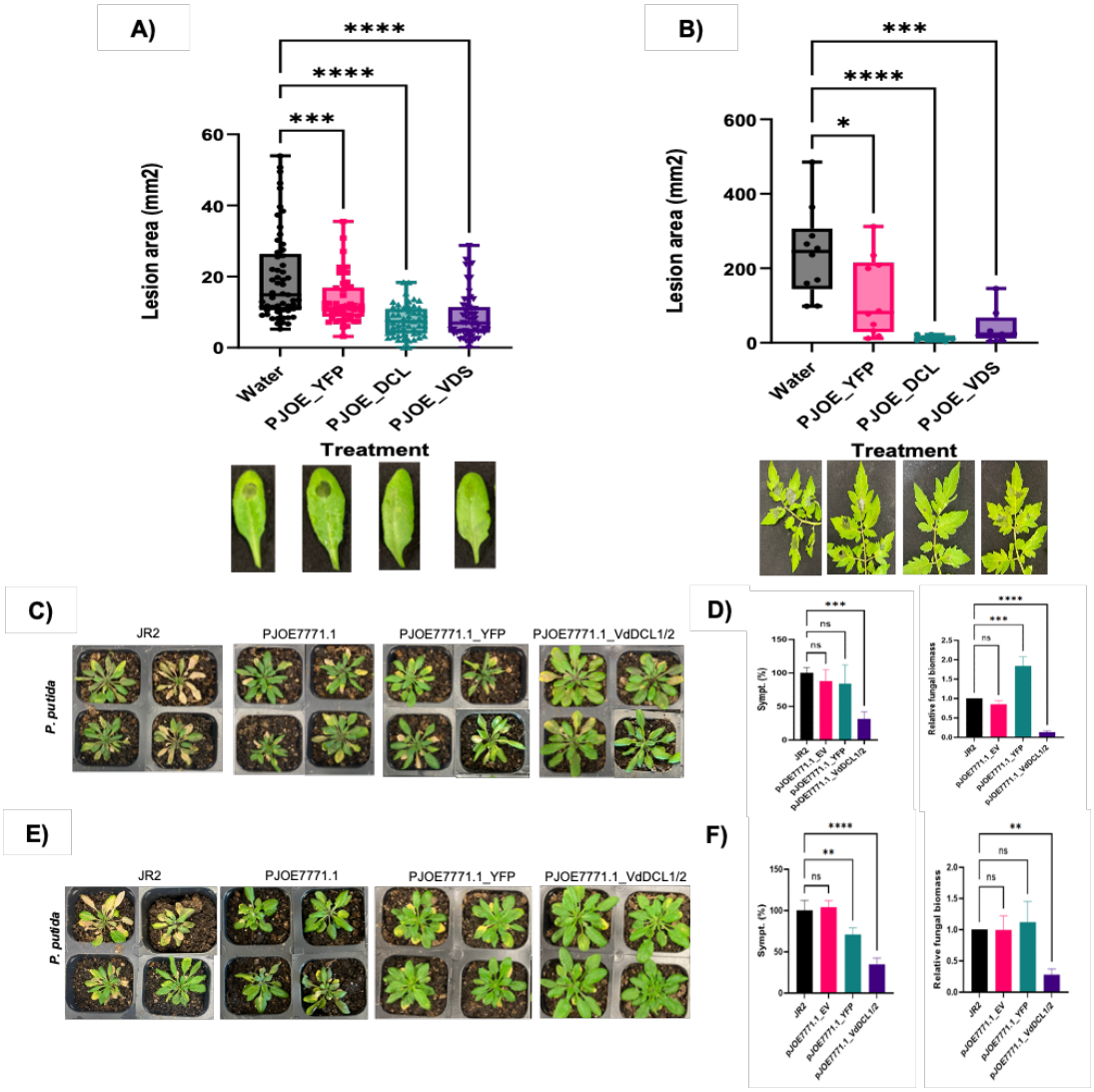
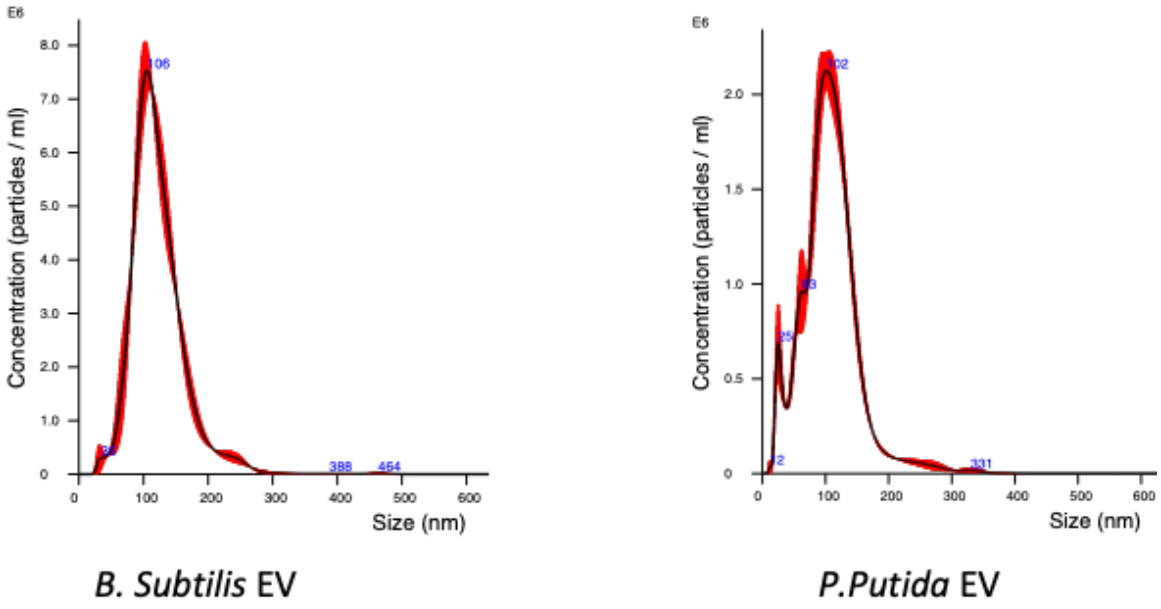


Figure 2.6: Direct application of dsRNA-expressing *Pseudomonas putida* provides protection against *B. cinerea*

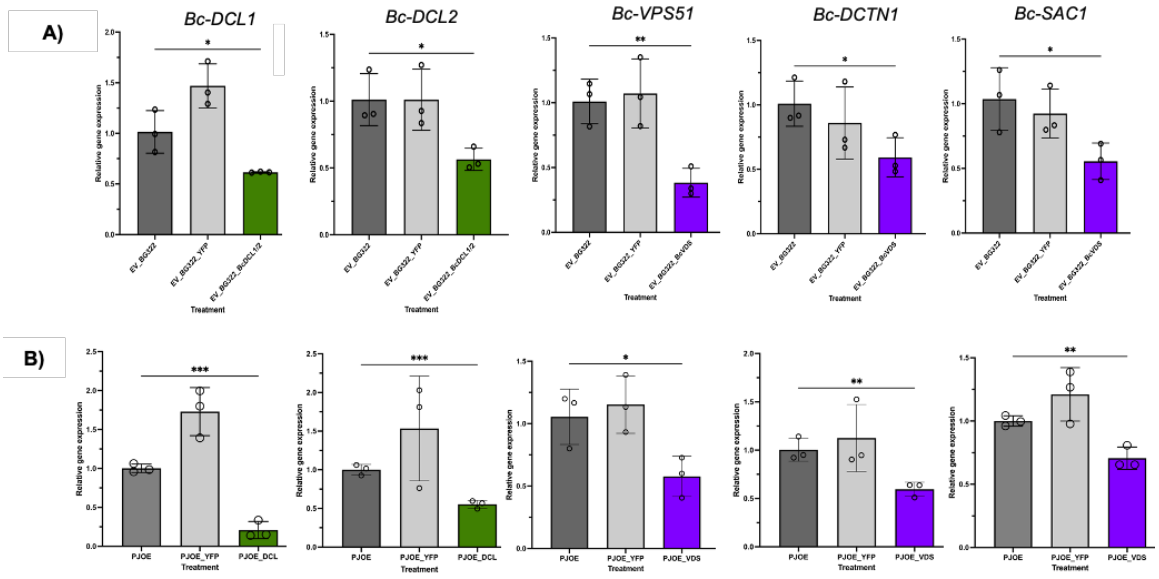
Lesions caused by *B. cinerea* infection were reduced on Arabidopsis leaves (A) and tomato leaves (B) that were treated by KT2440-BcVDS or KT2440-BcDCL. Pictures were taken at 3 dpi. (C) Disease caused by *V. dahliae* infection on Arabidopsis plants, when fungal spores were co-incubated with KT2440-VdDCL or KT2440-YFP at plant infection time. (D) Relative disease index and fungal biomass were measured using the disease observed in the untreated JR2 *V. dahliae* inoculated plants as the reference. (E) Disease produced by *V. dahliae* infection, when soil was treated directly with KT2440-VdDCL or KT2440-YFP 2 hours before transplanting and 7 days after the inoculation. Pictures were taken at 21dpi. (F) Relative disease index and fungal biomass were calculated using the disease observed in the untreated JR2 *V. dahliae* inoculation as the reference. (D, F) Relative fungal biomass was calculated by the expression of actin genes in *V. dahliae* and *A. thaliana.* Statistically significant differences ((* P< 0.05; ** P< 0.01; *** P<0.001; **** P<0.0001) were determined by one-way ANOVA, followed by Dunnet's test.

Supplementary Figures



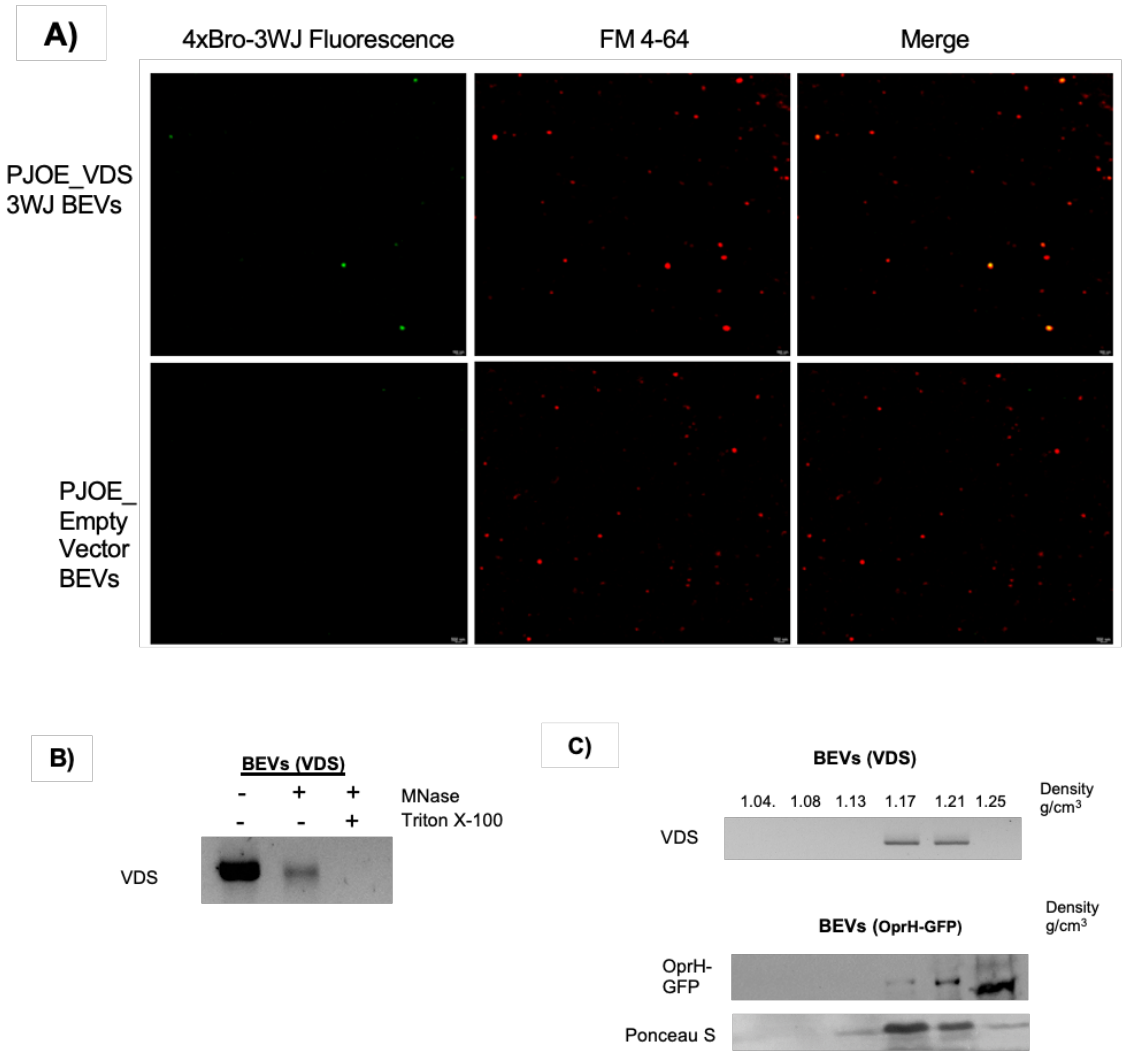
Supplementary Figure 2.1: Nanoparticle tracking analysis of bacterial EVs

Nanoparticle tracking analysis of purified EVs from *B. subtilis* and *P. putida* showing size distributions and concentrations.



Supplementary Figure 2.2 EV-contained fungal gene-targeting dsRNAs silence the targeted genes in fungi

(A) BEVs from *Bacillus subtilis* containing fungal gene-targeting dsRNAs were added to *B. cinerea* cultures. Expression of targeted genes in *B. cinerea* were detected through RT-qPCR after 12 hours. (B) OMVs from *Pseudomonas putida* containing fungal gene-targeting dsRNAs were added to *B. cinerea* cultures. Expression of targeted genes in *B. cinerea* were detected through RT-qPCR after 12 hours. Significant differences ((* P< 0.05; ** P< 0.01; *** P<0.001) were determined by one-way ANOVA.



Supplementary Figure 2.3: *Pseudomonas putida* can secrete fungal-gene targeting RNA in extracellular vesicles

(A) Confocal microscopy showed that VDS-Broccoli signals overlap with FM4-64 stained EVs in EV samples from VDS-Broccoli producing *P. putida* strain. EV samples from the empty vector strain were used as control. (B) VDS RNA produced by *P. putida* can be detected and protected from micrococcal nuclease treatment by EVs. (C) VDS RNA and BEV marker OprH were examined in BEVs isolated from *P. putida* through sucrose gradient fractionation.

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CHAPTER 3

Optimizing SIGS strategies: Testing new RNA targets and BioClay™ formulations to prolong RNA interference-mediated crop protection against *Botrytis cinerea*

The text of this chapter uses material which appears in J Niño-Sanchez, Sambasivam, Sawyer, R Hamby, A Chen, Czislowski, Li, Manzie, Gardiner, Ford, Xu, Mitter, and H Jin. "BioClayTM prolongs RNA interference-mediated crop protection against *Botrytis cinerea." Journal of Integrative Plant Biology.* 2022. Specifically, Figures 3.1-3.4 appeared in this publication. J.N.S led the experiments in Figures 3.2, 3.3 and 3.4. RH led, designed, and performed the experiments in Figure 3.1, 3.5, 3.6, and 3.7. R.H. assisted with experiments in 3.2, 3.3, and 3.4. R.H. performed experiments in 3.8 alone. A.C. led, designed, and performed experiments in 3.9, and assisted in experiments in 3.5, 3.6, and 3.7.

Abstract

While spray-induced gene silencing (SIGS) offers a promising new method for fungal disease control, it still needs to undergo optimization before it can be deployed in the field. In SIGS, RNA targeting pathogen genes are externally applied to plant materials to silence targeted genes, inhibiting fungal growth and disease. SIGS, however, is currently limited by the unstable nature of RNA in the environment. One strategy to enhance RNA stability is utilizing nanoparticles. Layered double hydroxide (LDH) particles can be used as RNA carriers, in a technology termed BioClayTM. Bioclay can enhance RNA durability on plants and prolong its effects against pathogens. A variety of Bioclay formulations, designed for low-toxicity and affordability, can effectively protect and deliver RNAs. Further development of SIGS approaches also requires understanding how RNA behaves once it is inside plant material. Here, we found that RNA can provide protection to distal plant leaves that were not directly treated with RNA. Another consideration of SIGS approaches is developing new fungal gene targets. Here, 8 RNA target candidates were tested and 2 promising gene targets were identified. In total, the work done in this chapter takes further steps to optimize SIGS approaches for field deployment by testing nanoparticle formulations, RNA dynamics and RNA targets.

Introduction

Though effective, the utility of AV technology is still limited by high costs of cationic lipids. Bioclay technology, developed by the Mitter Lab at the University of Queensland, utilizes layered double hydroxide (LDH) clay nanosheets to protect pathogen-targeting dsRNA from environmental degradation. Initially used to prevent and reduce the symptoms of plant viral diseases (Mitter et al. 2017), these bioclays can increase the length of dsRNA effectiveness for up to 21 days (Mitter et al. 2017). Previous work in both has utilized Aluminum based bioclays, but there are concerns about aluminum's toxicity and persistence in the environment (Alasfar and Isaifan 2021). To circumvent these issues, the Mitter lab has developed a new Bioclay formulation using iron in place of aluminum. In collaboration with the Mitter lab, I will be testing the efficacy of these Iron Bioclays in comparison to the Aluminum Bioclay at reducing fungal disease in both pre- and post-harvest plant material. Two formulations have been provided to the Jin lab, FeLDH (washed), which is the more purified product, and FeLDH (raw), which forgoes a final purification step and is therefore cheaper to manufacture.

Understanding RNA dynamics once RNA gets into plant material is an important step in further developing SIGS approaches. In work with barley leaves, it was found that RNA treatments could provide protection to distal untreated parts of the leaf (Koch et al. 2016). I was curious as to whether we would see a similar effect in tomato plants with both naked RNA and the Bioclay formulations. In this chapter, I observed whether treatments of Bioclay or naked RNA directly on tomato

leaves could provide protection to newly emerged leaves which were not directly treated with RNA.

Additionally, SIGS approaches benefit from a diversity of potential RNA targets to circumvent the issue of pathogens developing resistance to specific targets. In this chapter, in addition to testing new formulations of Bioclays, I also tested new RNA targets which target important fungal genes. The selected targets were Cyp51, which is a common target of a class of conventional fungicides also used as a SIGS target in *Fusarium (Koch et al. 2016)*, alpha tubulin, an important housekeeping gene (Ren et al. 2017), atg4, an autophagy related gene known for playing a role in fungal virulence (Woo et al. 2024), NoxR, a regulator of NADPH dehydrogenase complexes (An et al. 2016), BcCCC2, a copper transporting ATPase necessary for *B. cinerea* pathogenicity (Saitoh et al. 2010)**,** Ste50, an adaptor protein for map kinase signaling (Schamber et al. 2010), Pls1, a tetraspanin protein(Schamber et al. 2010; He et al. 2023), and sdhA, succinate dehydrogenase subunit A, another common target of conventional fungicides (Liu, Lee, and Sang 2024). These targets were tested both *in-vitro* and *in-vivo* on plant leaves on their ability to reduce fungal virulence.

Results

Fungal cells can take up dsRNA from BioClayTM after dsRNA release.

For SIGS approaches to be effective, RNA molecules need to enter fungal cells. While Bioclay itself does not enter fungal cells, their slowly released RNA cargo should be taken up by fungal cells. To confirm that *B. cinerea* can uptake dsRNA associated with LDH, germinated spores were incubated with BioClay containing fluorescently labeled dsRNA on PDA medium and then examined by confocal microscopy for fluorescence. While naked dsRNA was rapidly taken up within 3 hours, the spores were unable to take up the labeled BioClay-associated dsRNA by 16 hours (Figure 3.1). However, using a rapid dsRNA release protocol to dissociate the LDH and dsRNA in BioClay, the spores were able to take up the labeled RNA within 3 hours, and at 16 hours, similar levels of fluorescence were observed as with the naked dsRNA (Figure 3.1).

BioClayTM application provides a steady inhibition of B. cinerea infection on tomato leaves and fruit.

To determine whether BioClay can extend the protection window against *B. cinerea*, we used a detached tomato leaf assay and two dsRNAs that had previously been shown to provide protection against B. cinerea in post-harvest fruit assays: BcDCL1/2 and BcVDS (Wang et al. 2016; Qiao et al. 2021). BcDCL1/2 targets both *B. cinerea* dicer-like (DCL) genes, while BcVDS targets three genes involved in fungal vesicle trafficking (Cai et al. 2018). Leaves on intact plants were

sprayed with naked dsRNA or BioClay and subsequently detached for inoculation 1 to 21 days post treatment (dpt). Water, LDH and non-specific dsRNA (cucumber mosaic virus 2b (CMV2b)) were included as controls.

In comparison to the control treatments, both naked dsRNA treatments (BcDCL1/2 and BcVDS) were able to effectively reduce *B. cinerea* lesion sizes when applied either 1 or 7 days prior to inoculation, but this protection effect was lost when plant material was inoculated 14 days post treatment (Figure 3.2A, 3.2B). Conversely, both BcDCL1/2 BioClay and BcVDS BioClay treatments led to a prolonged protection effect compared to naked dsRNA. (Figure 3.2A, 3.2B). To further quantify these results, fungal biomass analysis was performed on the inoculated tomato leaflets. Overall, the biomass results reflected those found by directly measuring fungal lesion sizes (Figure 3.2C). Taken together, these results strongly suggest that BioClay provides protection with enhanced durability compared to naked dsRNA in detached leaf assays.

BioClay™ confers a steady inhibition of B. cinerea infection on tomato fruit.

Next, we tested the ability of MgAl and MgFe BioClay to enhance post-harvest disease protection on tomato fruit. Similar to the leaf assays, treatment with BcDCL1/2 and BcVDS MgAl BioClay led to effective and long-lasting protection against *B. cinerea* disease development. Unlike previous experiments, treatments were applied as 20 µL droplets, instead of as a spray, and fruits were inoculated with fungal spores at 1, 5 and 10 dpt. Results were consistent with the

assays conducted in tomato leaves. *B. cinerea* lesion size was only reduced in the naked dsRNA treatments when inoculation occurred 1 dpt (Figure 3.4A, 3.4B), and this trend was reflected in the fungal biomass assays (Figure 3.4C). While the naked dsRNA did not reduce lesion size at 5 dpt (Figure 3.4B), it still reduced fungal biomass (Figure 3.4C) Application of MgAl LDH or BioClay containing a non-specific dsRNA sequence had no effect on *B. cinerea* lesion size or fungal biomass (Figure 3.4). In contrast, treatment with BcDCL1/2 or BcVDS BioClay significantly reduced fungal lesion size and fungal biomass at all three time points (Figure 3.4A, B, C). Because of concerns with Aluminum toxicity, LDH formulations using Iron in place of aluminum were developed. These formulations were also tested on tomato fruits. Similar to previous results obtained with Aluminum Bioclays, MgFe Bioclay loaded with either BcDCL1/2 or BcVDS were able to significantly reduce fungal disease up to 10 days post treatment.

Raw formulations of MgFe BioClay™ outperformed washed formulations in inhibiting B. cinerea infection on tomato fruits, rose petals and tomato leaves

As our collaborators at the University of Queensland were continuously making improvements for cost to their LDH formulations, we were sent a new MgFe LDH formulation, referred to here as MgFe LDH raw, which is a preparation that forgoes a final wash step to reduce production costs. Both this MgFe LDH raw formulation, the MgFe LDH washed formulation, and the original Aluminum LDH formulation were loaded with VDS dsRNA and used to treat rose petals and tomato

fruits one day before inoculation with *B. cinerea*. In tomato fruits, all Bioclay formulations were effective at reducing infection. Intriguingly, the empty formulations, specifically the Raw FeLDH formulation also provided some protection against *B. cinerea*. (Figure 3.5). Similar results were obtained on rose petals, but in this case the empty Washed FeLDH formulation completely blocked lesion formation.

After testing these formulations on post-harvest products, we next wanted to test these new formulations on tomato plants. The detached leaf assays were performed as previously described and are outlined in Figure 3.6A. In brief, tomato plant leaves were sprayed with the RNA or Bioclay treatments. After 7 days, leaves were collected and inoculated with *B. cinerea*. Both the Raw and Washed MgFe LDH formulations loaded with BcVDS RNA outperformed the naked RNA in reducing fungal lesion size (Figure 3.6B).

Raw and washed formulations of MgFe BioClay™ confer lasting protection to indirectly treated tomato leaves.

Next, we sought to determine how mobile these Bioclay RNAs were inside plant tissue by performing pathogenicity assays on untreated tomato leaves. These assays are similar to previous assays, however, instead of harvesting the leaves that were treated with the RNA treatments, we instead harvested leaves 7-days post RNA treatment (Figure 3.7C). We found that naked Bc-VDS, Raw MgFe-LDH Bc-VDS, and Washed MgFe-LDH Bc-VDS treatments could confer protection

against *B. cinerea* to distal untreated tomato leaves (Figure 3.7A,B), suggesting the applied RNA can travel through plant tissue.

Testing novel dsRNA constructs potential for use in SIGS applications

Finally, to further optimize SIGS approaches, we wanted to identify new potential RNA targets in *B. cinerea*. To do this, we tested a total of 8 new RNA constructs (Table 3.1) using *in-vitro* and *in-vivo* approaches. In the *in-vitro* system, fungal spores were mixed with 800 ng of one of the 8 RNA constructs, non-specific constructs 92a or GFP, or previously tested constructs Bc-VDS and Bc-DCL1/2, before being plated on low-nutrient media. The diameter of fungal growth was then measured at 3, 5, and 7 days post inoculation. At 5 days post inoculation, dsRNA constructs 92a, Bc-6, Bc-8, Bc-9, Bc-10 and Bc-13 significantly reduced fungal growth. At 7 days post inoculation, dsRNA constructs 92a, Bc-6, Bc-8, Bc-9, Bc-10 and Bc-13 significantly reduced fungal growth.

Next, these constructs were tested on *Arabidopsis* leaves for their ability to reduce fungal infection. *Arabidopsis* leaves were treated with a 10uL drop of 200ng/uL RNA of one of the 8 new constructs or the non-specific control GFP one day before inoculation with fungal spores. After 3 days lesion sizes were measured. The constructs targeting Cyp51, atg4, and Pls1 were found most effective in reducing fungal growth (Figure 3.9). These results are summarized in table 3.1

Discussion

As I have continued to harken back to, in order to deploy SIGS in the field it is so crucial that we develop ways for it to be more stable in the environment. Further, it is of critical importance that whatever methods used to stabilize it in the environment are non-toxic. A variety of BioClay™ formulations demonstrated enhanced RNA stability and prolonged protection against *B. cinerea*. These results show that Bioclay is a robust and flexible nanoparticle tool that can be readily optimized for cost reduction and non-toxicity without losing its protective properties. Additionally, Bioclay formulations demonstrated effectiveness on a variety of plant and post-harvest materials, including rose petals, tomato fruits, *Arabidopsis* and tomato plants, further highlighting their versatility.

A potentially exciting application of SIGS approaches is the ability to confer protection against pathogens throughout the plant, not just where the RNA solution is directly applied. This has previously been demonstrated in barley plants, but these were cut leaves and the RNA could have potentially entered the vascular system through this cut in the leaf (Koch et al. 2016). Here, we found that treating leaves on a tomato plant could confer protection against *B. cinerea* in new leaves that had not yet emerged when the old leaves were treated. This provides evidence that RNA applied to plant leaves is able to get into the plant itself and travel systematically through plant vasculature.

Finally, through screening new potential RNA targets, 3 promising new constructs were identified. *Cyp51*, *atg4*, and *Pls1*. These constructs demonstrated

significant reductions in fungal growth both in vitro and in vivo, expanding the repertoire of effective SIGS targets. The potential of these targets to complement existing ones, such as *Bc-DCL1/2* and *Bc-VDS*, can mitigate the risk of resistance development by fungal pathogens, enhancing the robustness of SIGS-based strategies. Additionally, the targets Cyp51 is already a common target of common fungicides (Arnold et al. 2024), opening up the potential for mixing RNA-based disease management strategies, to disrupt RNA and protein function, for more robust disruption of fungal biology.

Overall, this chapter makes strides towards optimizing Spray-Induced Gene Silencing (SIGS) as a strategy for plant protection against fungal pathogens, specifically *Botrytis cinerea*.

Materials and Methods

Layered double hydroxide synthesis and BioClay loading

MgAl and MgFe LDH were prepared as detailed in previous work (Mitter et al., 2017; Jain et al., 2022). To determine the ratio at which naked dsRNA was completely complexed with the LDH, 300 ng of BcDCL1/2 or BcVDS dsRNA was combined with varying amounts of LDH (dsRNA: LDH from 1:1 to 1:5 (w/w)) and incubated at room temperature for 30 min. Complete loading of dsRNA onto LDH was confirmed when the dsRNA-LDH complex was retained in the well and unable to migrate through a 1% agarose gel. Following loading, sodium phosphate was added to the MgAl BioClay to a final concentration of 20 mM and ammonium sulfate was added to the MgFe BioClay to a final concentration of 10 mM.

dsRNA material

dsRNA material used in this chapter was synthesized by Genolutions (Korea) using their Agriculture Grade 2 service.

Fungal dsRNA uptake

Fluorescently-labeled YFP and DCL1/2 dsRNA was synthesized in vitro using the MEGAscript RNAi Kit (Life Technologies, Carlsbad, CA, US) following the manufacturer's instructions but with the addition of Fluorescein RNA labeling mix, a nucleotide mixture containing fluorescein-12-uridin-triphosphate (Sigma-Aldrich, St. Louis, MO, US) for a final concentration of 1 mM NTP (including 0.35

mM fluorescein-12-UTP). Fluorescein-labeled YFP or DCL1/2 dsRNA (100 ng/µL) was added to germinated fungal spores on a glass microscope slide with 3 mL of PDA medium. The rapid release protocol used PDA media adjusted to pH 4 by addition of 10% citric buffer (50 mM, pH: 3), from the original pH 5.8. The mix was incubated at room temperature in the dark for 3 and 16 hours and then treated with 1 l micrococcal nuclease (MNase) (Thermo Fisher Scientific, Waltham, MA, USA) before analysis by confocal laser scanning microscopy (Leica TCS SP5, Lecia Microsystems, Wetzlar, Germany). Excitation was provided by an argon laser (488 nm) and the emission signal of fluorescein was detected at 495-545 nm.

Pathogenicity Assays

B. cinerea strain B05 was incubated on Malt Extract Agar (MEA) (Sigma-Aldrich, St. Louis, MO, USA) plates from frozen glycerol stock vials (-80oC). The fungus was incubated at 22°C for 10 days before spores were isolated from the plate using a sterile loop and incubated in 1% DifcoTM Sabouraud Maltose Broth (BD Biosciences, Sparks, MD, USA). Spores were counted using a hematocytometer and spore concentration was adjusted in 1% DifcoTM Sabouraud Maltose Broth.

For foliar inoculation of the tomato plants, commercial Money maker seeds were sown on soil and maintained at a controlled temperature (25°C) and high relative humidity (>70%) for 7 to 10 days until germination (relative humidity was stabilized at 70%). After 2 weeks, plants were transferred to 1-gallon pots with a

mix of soil:sand (3:1 ratio). Plants were grown in a greenhouse with controlled temperature (25°C), and photoperiod (8-hour night/16-hour day) conditions, and were manually bottom watered every 2-4 days as needed. Tomato plants were incubated for another 3 weeks until 3 completely extended real leaves were present (identified as the first leaf with at least 5 leaflets is the third leaf of the plant). Afterwards, 3 real leaves from each plant were treated by spraying approximately 5 mL of a suspension of 100 ng/µL dsRNA or BioClay. We established 4 time points (1, 7, 14 and 21 days post treatment (dpt)) to harvest leaves and inoculate them with B. cinerea spores. Treated leaves were detached and placed into plastic boxes that were previously disinfected with a 10% bleach solution. Two pieces of wet filter paper were placed at the bottom of each box to maintain humidity. Each leaflet (5 per leaf) was inoculated with a 10 µL drop of the B. cinerea spore suspension at 2x103 spores/mL.

For foliar inoculation of the *Arabidopsis thaliana* plants, Col-0 *Arabidopsis* seeds were sown on soil and maintained at a controlled temperature (25°C) and high relative humidity (>70%) for 7 to 10 days until germination (relative humidity was stabilized at 70%). After 10 days, plants were grown in a greenhouse with controlled temperature (25°C), and photoperiod (8-hour night/16-hour day) conditions, and were manually bottom watered every 2-4 days as needed. *Arabidopsis* plants were grown for another 3 weeks until 4-5 completely extended real leaves were present. Afterwards, 4-5 real leaves from each plant were treated by dropping a 20uL suspension of 100 ng/µL dsRNA or BioClay onto the leaf. Each

leaflet (5 per leaf) was inoculated with a 10 µL drop of the B. cinerea spore suspension at 2x103 spores/mL.

On fresh local organic tomato fruits, the treatments were applied as 20 µL droplets of a solution with 100 ng/µL of dsRNA. Subsequently, tomato fruit were inoculated at 1 , 5 and 10 dpt with 10 µL droplets of a B. cinerea spore suspension (106 spores/mL).

After inoculation, the detached leaves and tomato fruits were incubated for 4 days and 6 days, respectively, inside the plastic box at 22ºC with continuous light. Then, lesions were photographed to accurately determine size with the help of ImageJ software, and/or were measured by two perpendicular diameter measurements using an electronic calibrator OriginCal® Digital Caliper, (iGAGING, San Clemente, CA, US). The diameter measurements were used to calculate the area of the ellipse contained in the lesion using the formula A = (D1x0.5) x (D2x0.5) x π.

In-vitro fungal growth assay

1 x 10^5 fungal spores were mixed with 800 ng of dsRNA in 20uL of distilled water. This suspension was then plated on a 60 x 15 mm plate containing low nutrient agar. The diameter of fungal growth (mm) was measured at 3, 5, and 7 days post inoculation.

Figures

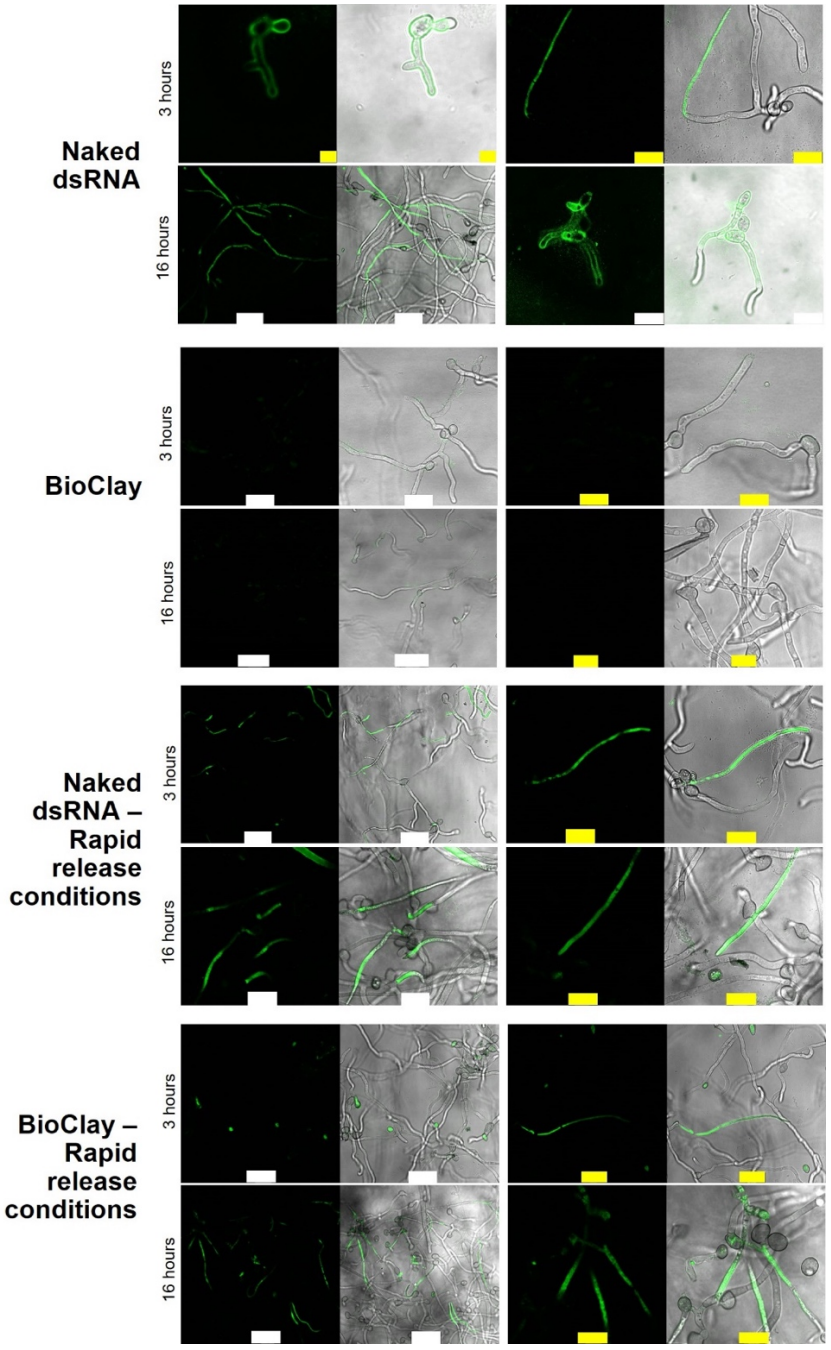
Naked dsRNA
3 hours
16 hours
BioClay
3 hours
16 hours
Naked dsRNA – Rapid release conditions
3 hours
16 hours
BioClay – Rapid release conditions
3 hours
16 hours

Figure 3.1: *B. cinerea* can internalize dsRNA from BioClay™ after dsRNA release.

Confocal laser scanning microscopy (CLSM) analysis of *B. cinerea* 3 and 16 hours after treatment with fluorescein-labeled dsRNA (*YFP* or *BcDCL1/2*). The top group of rows show that *B. cinerea* internalizes labeled dsRNA at pH ~ 5.8 after 3 and 16 hours. The second group of rows show that dsRNA is not internalized when the dsRNA is in the BioClay complex after 3 hours. The third group of rows show the rapid release protocol does not affect dsRNA uptake when the dsRNA is in a naked form. The fourth group of rows show that dsRNA can be internalized rapid release from BioClay complex conditions. Micrococcal nuclease (MNase) was added 30 min before imaging to remove any dsRNA external to the fungal cells. Scale white bars indicate 25 mm, yellow indicate 10 mm

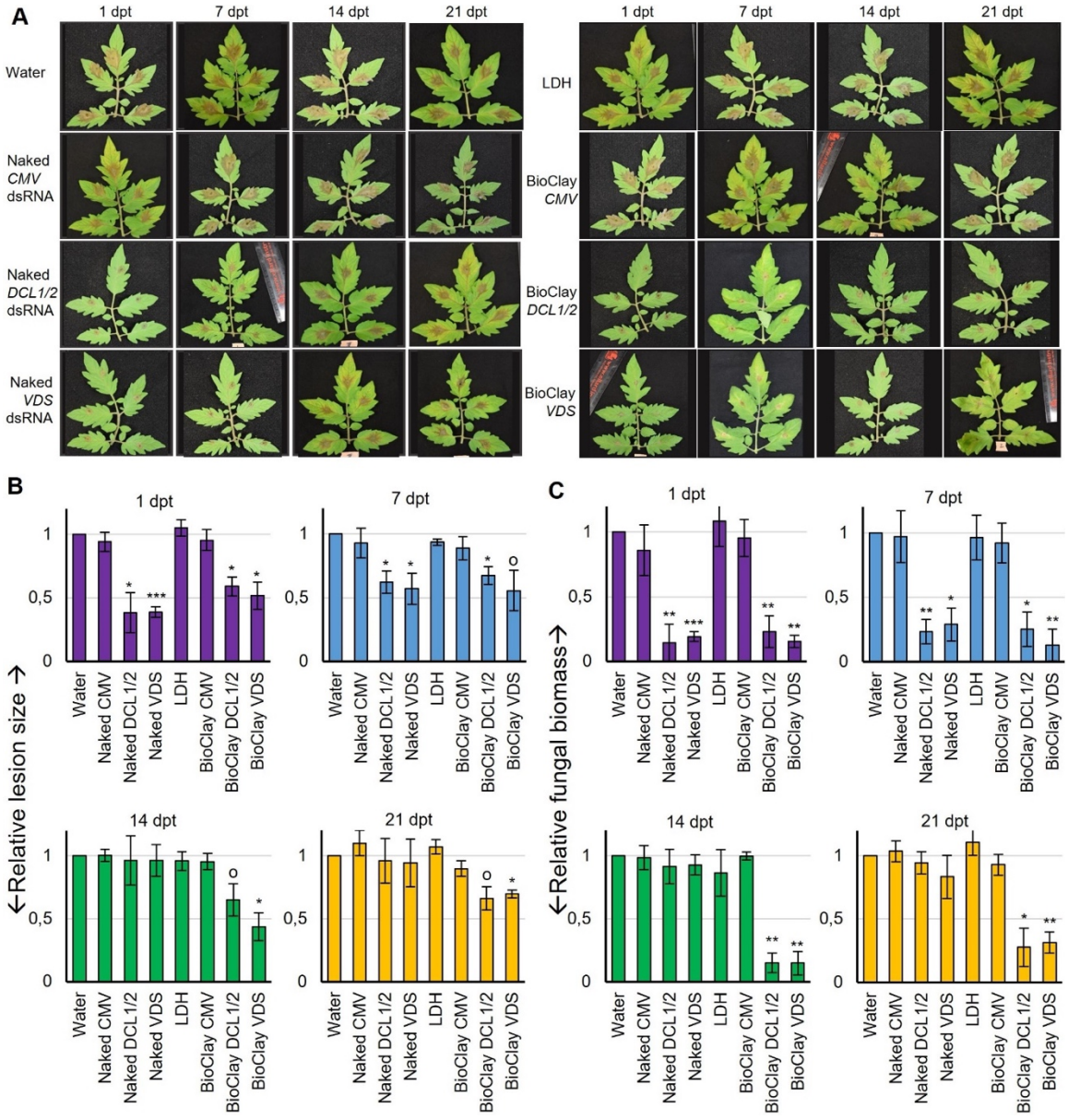
A
1 dpt
7 dpt
14 dpt
21 dpt
Water
LDH
Naked CMV dsRNA
BioClay CMV
Naked DCL1/2 dsRNA
BioClay DCL1/2
Naked VDS dsRNA
BioClay VDS
B
C
←Relative lesion size →
←Relative fungal biomass→
Water
Naked CMV
Naked DCL1/2
Naked VDS
LDH
BioClay CMV
BioClay DCL1/2
BioClay VDS

Figure 3.2: BioClay™ application provides a steady inhibition of B. cinerea infection on tomato detached leaves.

(A) Tomato detached leaves were pre-treated with water, naked dsRNA, LDH and BioClay for 1, 7, 14 and 21 days, then inoculated with *B. cinerea* spores. Pictures were taken at 4 days post inoculation. Representative inoculated detached leaves showed similar lesion sizes in the water control treatment, LDH treatment, CMV treatment (naked or BioClay) and both *BcDCL1*/2- and *BcVDS*-targeting naked dsRNA treatments at the later time points (14 and 21 days post treatment (dpt)). Smaller lesion sizes were observed at all time points with BioClay treatments (either *BcDCL1/2* or *BcVDS*) and at the earlier time points (1 dpt and 7 dpt) for the naked dsRNA treatments. **(B)** Relative lesion size areas were measured 4 days post inoculation on detached tomato leaves with the help of an electronic calibrator, assigning a value of 1.0 to the average lesion size area in the water treatment. **(C)** Relative fungal biomass was quantified by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in detached leaves inoculated with *B. cinerea*. Fungal RNA relative to tomato RNA was measured with the fungal *actin* gene and the tomato *actin* gene by RT-qPCR using total RNA extracted from each time point (1, 7, 14 and 21 dpt). All measurements were referred to the value 1.0 obtained for the water treatment. Error bars indicate standard deviations (SD) obtained from 4 to 7 biological replicates for the relative measurement of the lesion size area (B) and three biological replicates for relative quantification of fungal biomass (C). Levels of significant differences were determined by a one-way ANOVA followed by Tukey's HSD test and are indicated above the bars by (^{0} $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

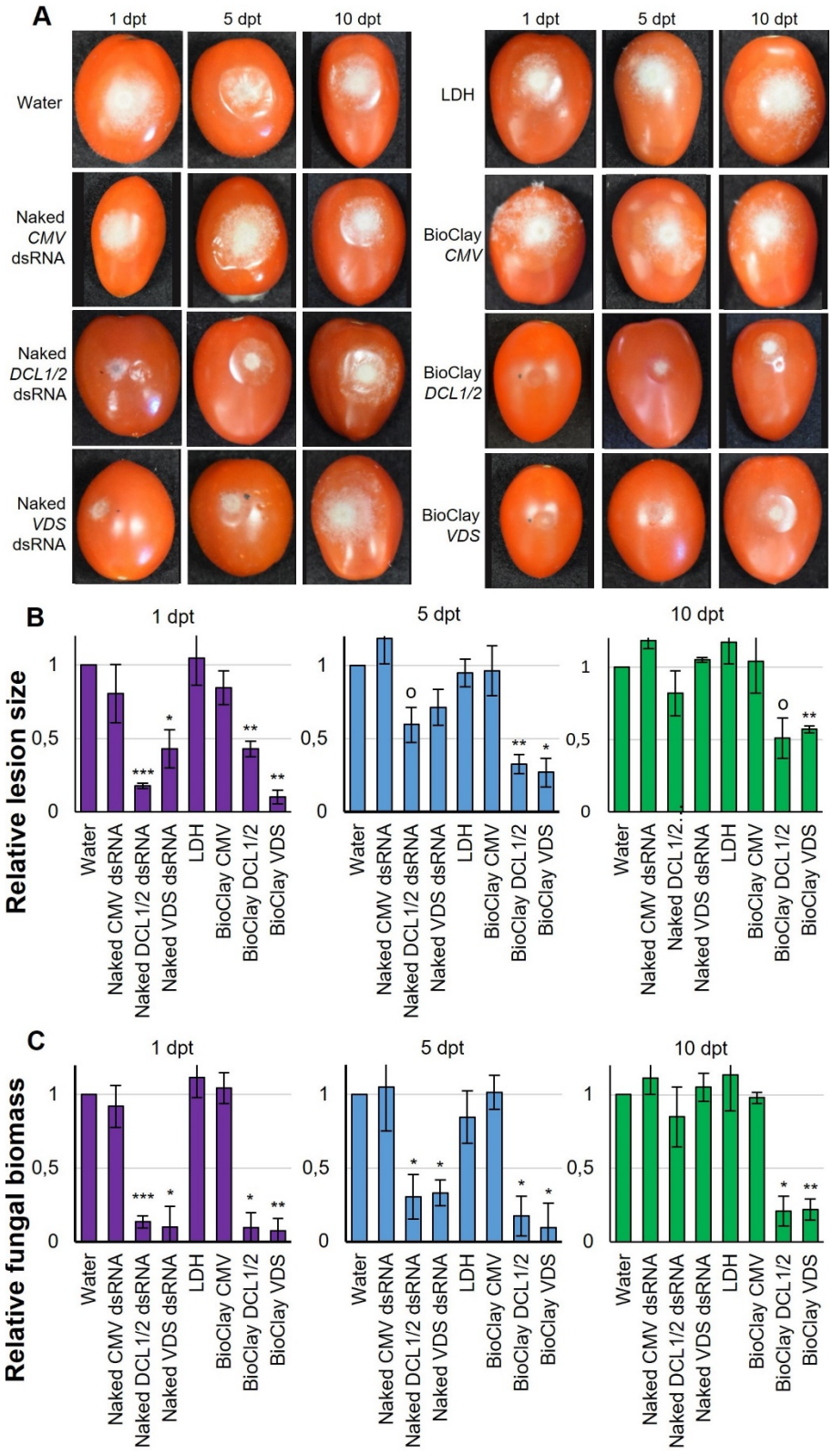
A
1 dpt
5 dpt
10 dpt
Water
Naked CMV dsRNA
Naked DCL1/2 dsRNA
Naked VDS dsRNA
LDH
BioClay CMV
BioClay DCL1/2
BioClay VDS
B
Relative lesion size
C
Relative fungal biomass

Figure 3.3: BioClay™ application provides a steady inhibition of *B. cinerea* infection on tomato fruit.

(A) Tomato fruit were treated with water, naked dsRNA, LDH or BioClay and were then inoculated with *B. cinerea* spores 1, 5 or 10 days post treatment (dpt). Pictures were taken at 6 days post inoculation. Representative inoculated tomato fruit showed similar lesion sizes in the water control treatment, LDH treatment, *CMV* treatment (naked or BioClay) and both *BcDCL1*/2- and *BcVDS* naked dsRNA treatments at the last time point (10 dpt). Smaller lesion sizes were observed at all time points with BioClay treatments (either *BcDCL1/2* or *BcVDS*) and at 1 dpt in the naked dsRNA treatments. **(B)** Relative lesion size areas were measured 6 days post inoculation on tomato fruits with the help of an electronic calibrator, assigning a value of 1.0 to the average lesion size area in the water treatment. **(C)** Relative fungal biomass was quantified by RT-qPCR in tomato fruits inoculated with *B. cinerea*. Fungal RNA relative to tomato RNA was measured with the fungal *actin* gene and the tomato *tubulin* gene by RT-qPCR using total RNA extracted from each time point (1, 5, 10 dpt). All measurements were referred to the value 1.0 obtained for the water treatment. Error bars indicate standard deviations (SD) obtained of at least three biological replicates. Levels of significant differences were determined by a one-way ANOVA followed by Tukey's HSD test and are indicated above the bars by (^{0} $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

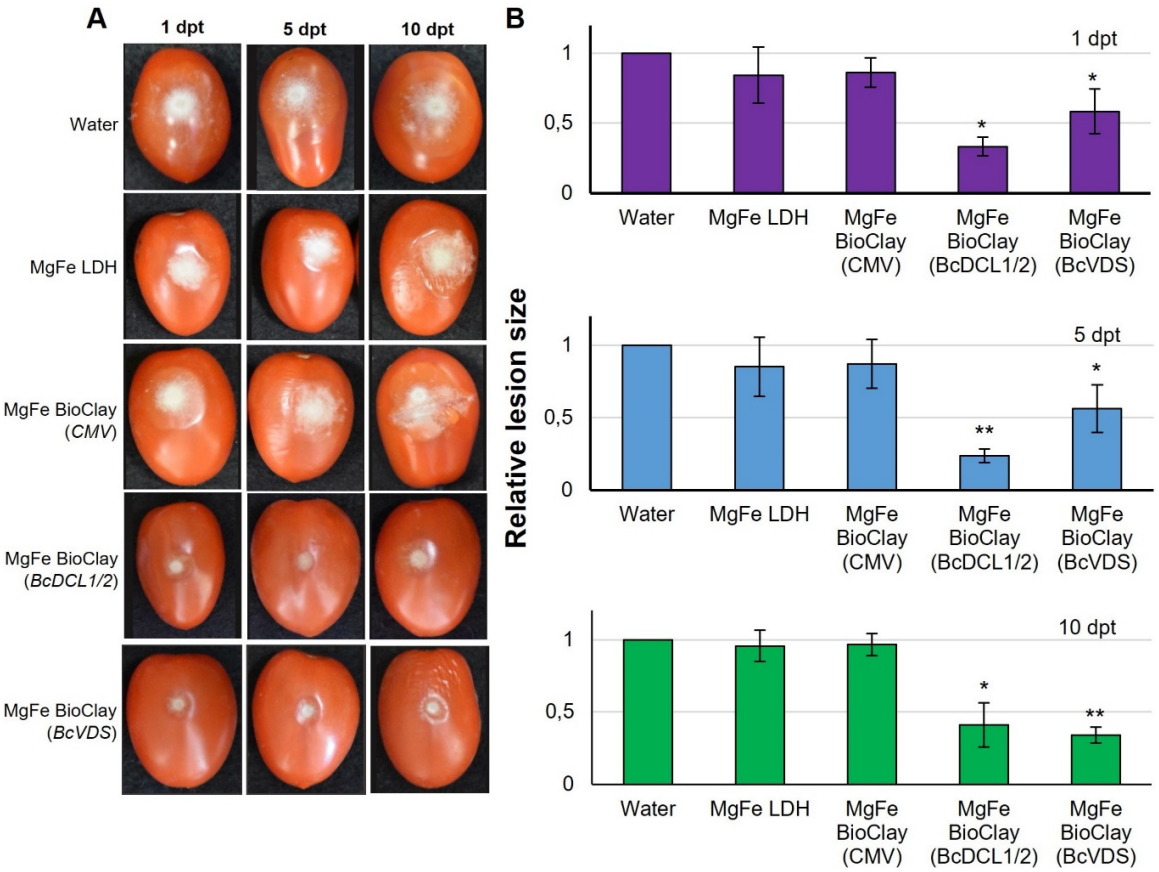

Figure 3.4: MgFe BioClay™ confers a steady inhibition of *B. cinerea* infection on tomato fruit.

(A) Tomato fruit were pre-treated with water, MgFe-based LDH and MgFe-based BioClay™ for 1, 5 and 10 days, then inoculated with *B. cinerea* spores. Pictures were taken at 6 dpi. Representative inoculated tomato fruits showed similar lesion sizes in the water control treatment, LDH treatment, and *CMV* BioClay. Smaller lesion sizes were observed at all time points with BioClayTM treatments (either *BcDCL1/2* or *BcVDS*) **(B)** Relative lesion size areas were measured 6 dpi on tomato fruits with the help of an electronic calibrator, assigning a value of 1.0 to the average lesion size area in the water treatment. Error bars indicate standard deviations (SD) obtained of at least three biological replicates. Levels of significant differences were determined by a one-way ANOVA followed by Tukey's HSD test and are indicated above the bars by (*$P < 0.05$, ** $P < 0.01$).

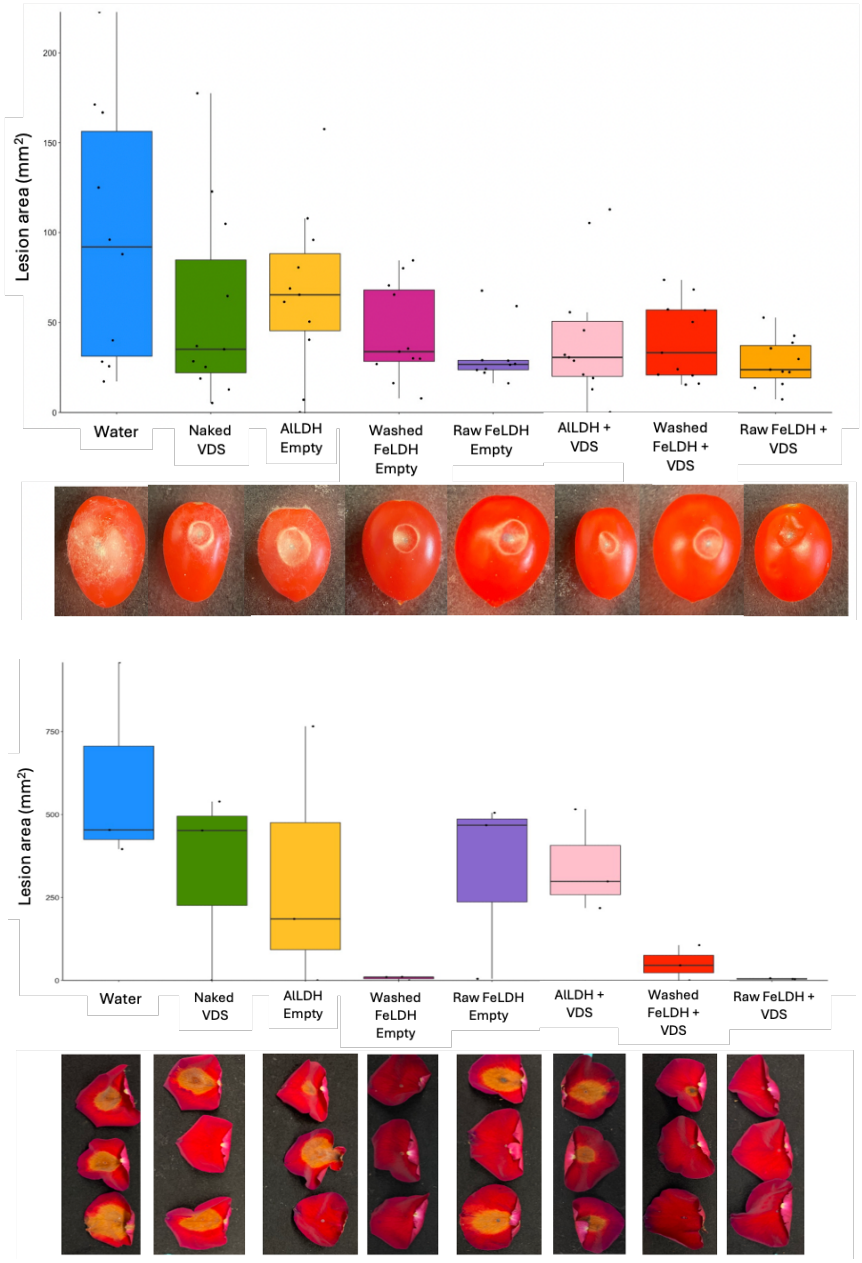

Figure 3.5: Raw formulations of MgFe BioClay™ outperformed washed formulations in inhibiting *B. cinerea* infection on tomato fruits and rose petals.

(A) Tomato fruit were pre-treated with water, MgFe-based LDH and MgFe-based BioClay™1 day before f,then *B. cinerea* inoculation. Pictures were taken at 6 dpi. Smaller lesion sizes were observed with all BioClayTM treatments **(B)** Rose petals were pre-treated with water, MgFe-based LDH and MgFe-based BioClay™1 day before f,then *B. cinerea* inoculation. Pictures were taken at 3 dpi. Smaller lesion sizes were observed with all BioClayTM treatments.

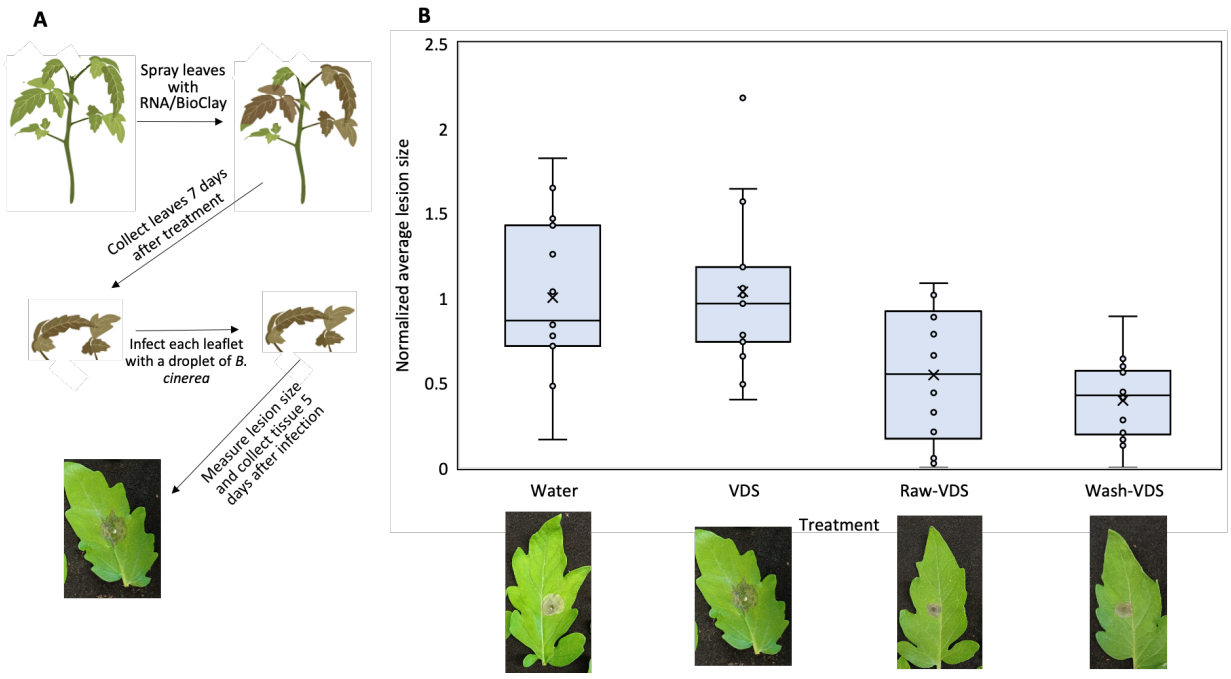

Figure 3.6: Raw and washed formulations MgFe BioClay™ confer lasting protection to directly treated tomato leaves.

(A) An outline of experimental design. Tomato plants in the greenhouse were sprayed with the BioClay treatment. After 7 days, sprayed leaves were collected and inoculated with *Botrytis cinerea*. After 5 days, leaves were collected, lesions were measured, and images were taken. **(B)** Both formulations of the MgFe Bioclay outperformed the naked dsRNA treatment after 7 days.

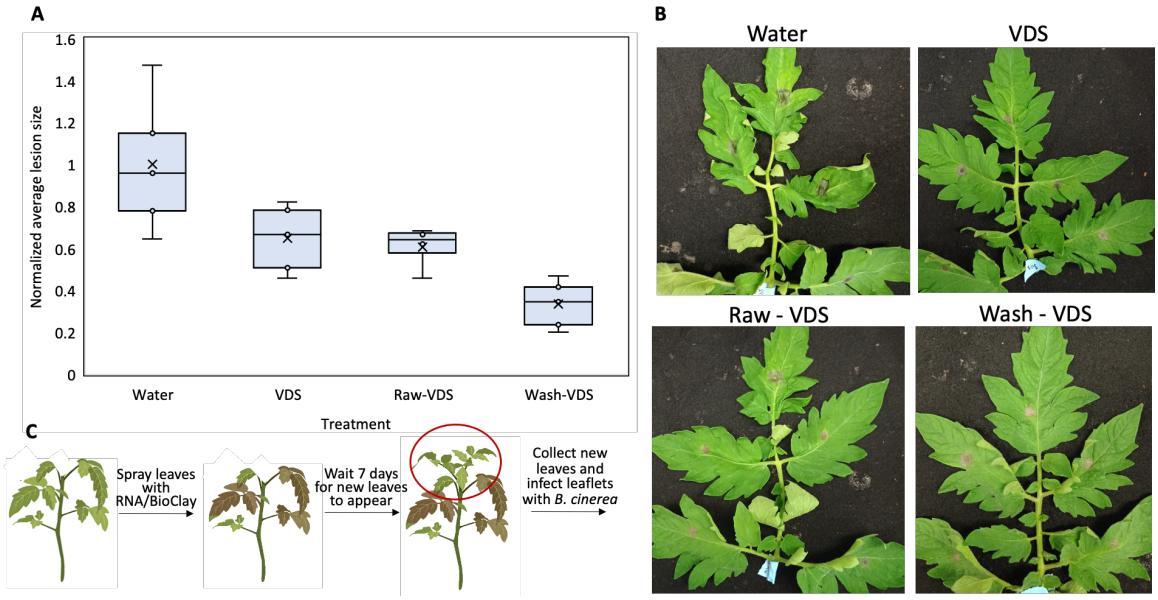

Figure 3.7: Raw and washed formulations MgFe BioClay™ confer lasting protection to distal untreated leaves on tomato plants

(A) New leaves were collected from tomato plants 7 days post treatment and challenged with *B. cinerea*. All treatments confered some protection against infection, with the washed MgFe Bioclay providing the greatest protective effect. **(B)** Photos of tomato leaves taken 3 days post infection. **(C)** Workflow of experiment. Tomato plants in the greenhouse were sprayed with treatment. After 7 days, new leaves emerged and were collected. These new leaves were then challenges with *Botrytis cinerea*. After 3 days, photos were taken and lesion sizes measured.

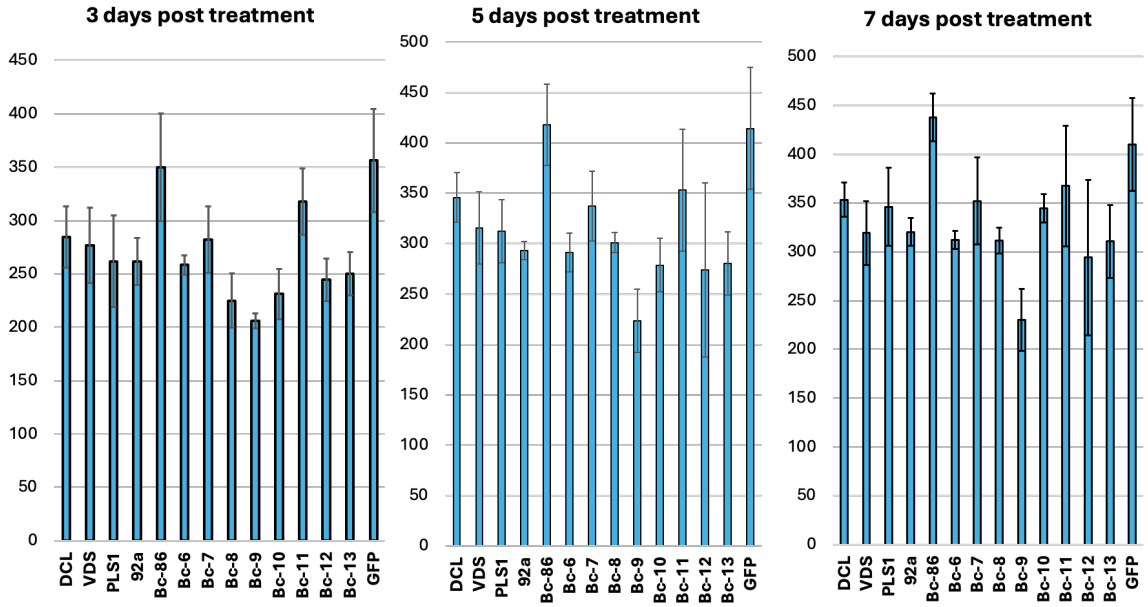

Figure 3.8: Screening of dsRNA constructs in an in-vitro fungal growth assay identified new potential candidates for SIGS applications.

Botrytis cinerea spores were mixed with 800 ng of specific dsRNA constructs then plated on low nutrient media. The diameter of fungal growth (mm) was measured at 3, 5, and 7 days post inoculation. At 3 days post inoculation, dsRNA constructs 92a, Bc-6, Bc-8, Bc-9, Bc-10, Bc-12, and Bc-13 significantly reduced fungal growth. At 5 days post inoculation, dsRNA constructs 92a, Bc-6, Bc-8, Bc-9, Bc-10 and Bc-13 significantly reduced fungal growth. At 7 days post inoculation, dsRNA constructs 92a, Bc-6, Bc-8, Bc-9, Bc-10 and Bc-13 significantly reduced fungal growth. Statistical significance (Student's t-test): *, $P < 0.05$

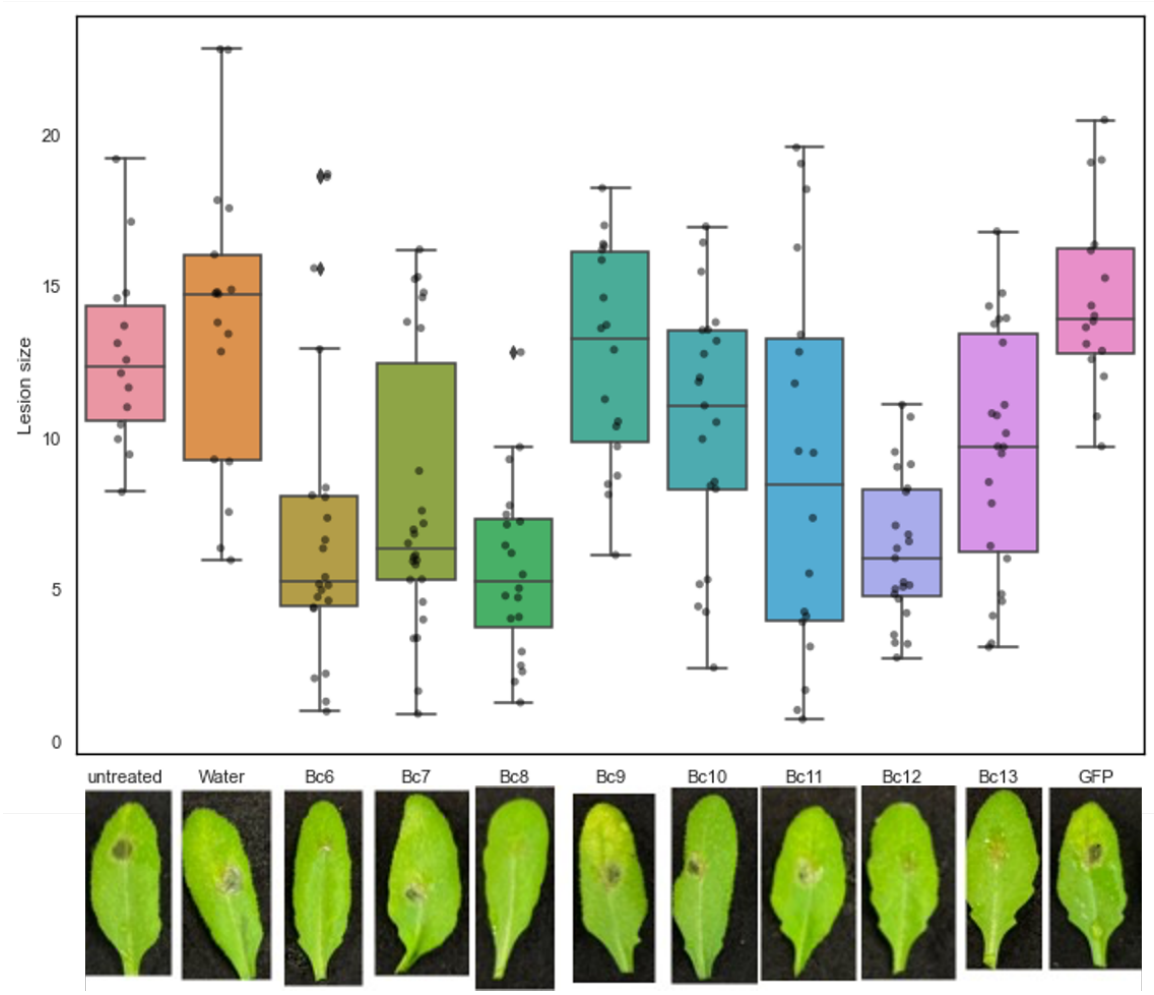

Figure 3.9: Screening of dsRNA constructs efficacy in SIGS approaches on Arabidopsis leaves.

Arabidopsis leaves were treated with a 20uL drop of 20ng/uL dsRNA. 1 day later the leaves were inoculated with *B. cinerea* spores. Lesions were measured and images were taken after 3 days.

Tables

RNA construct	Target	*In vitro*	*In planta*
Bc6	Cyp51	inhibition	Strong reduction in lesion size
Bc7	alpha tubulin	none	Moderate reduction
Bc8	atg4	inhibition	Strong reduction in lesion size
Bc9	NoxR	Strong inhibition	No reduction
Bc10	Copper transporting atpase (BcCCC2)	Moderate inhibition	No reduction
Bc11	Ste50 (adaptor protein for map kinease sig)	none	No reduction
Bc12	Tetraspanin (PLS1)	Weak inhibition	Strong reduction in lesion size
Bc13	Succinate dehydrogenase subunit A (sdhA)	inhibition	No reduction

Table 3.1: Results of *In-vitro* and *In-planta* assays to test the efficacy of dsRNA construct in SIGS approaches.

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Conclusion and Future Directions

I watched the Ponderosa pine trees that have proudly stood in my parents backyard for decades succumb to a native bark beetle in recent years. Why? The beetles have always been there. The trees have always been there. The ultimate culprit is, of course, climate change. As ponderosa pine trees become stressed by increasingly extreme drought conditions, they lose their ability to successfully defend themselves against the insect pests (Robbins et al. 2022).

This is not an isolated story. As abiotic stressors increase, plants everywhere, in wild ecosystems and croplands, become more susceptible to the attacks of native pathogens and pests (Singh et al. 2023). Globalization further exacerbates the problem by increasing the occurrence of invasive parasites, eager to predate upon plants that lack the defenses built through thousands of years of coevolution (Ristaino et al. 2021). Perhaps the most tragic example of this was the chestnut blight, a disease caused by an invasive fungal pathogen that led to the functional extinction of the once dominant American chestnut tree (Powell, Newhouse, and Coffey 2019).

Plant scientists and growers are faced with an increasingly impossible task: continue increasing the yield of crop plants to feed a growing population as land area for farming decreases (Brain et al. 2023), and abiotic and biotic threats to plant health increase annually(Singh et al. 2023). While I may mourn the loss of

my favorite childhood tree, the stakes are far higher. Pathogens and pests pose an immediate threat to our ability to continue feeding the human population.

Fungi represent some of the most devastating plant pathogens (Petrasch et al. 2019), and, alarmingly, to continue piling on the bad news, fungicide resistance is rampant and widespread (Fisher et al. 2018). Current large-scale chemical fungicide applications are only increasing the emergence of resistance, and can leave residues in the environment harmful to native ecosystems and human health (Van de Wouw et al. 2021). Novel disease management strategies are urgently needed!

In this dissertation work, my goal was to take an innovative, and sustainable approach to crop protection-- RNA interference based antifungals-- and bring them closer to deployment in actual, practical, real-world applications. To do this, a few major things needed to be done. First, antifungal RNAs need to be stabilized so they persist longer in the environment. Second, a method for delivering antifungal RNAs to soilborne pathogens needed to be developed. Finally, optimization work in nanoparticle formulations and RNA target design can help lead to a more robust and flexible antifungal defense strategy.

The first strategy I utilized here to increase the stability and efficacy of RNA-based antifungals were lipid based nanoparticles, Artificial Vesicles. These AVs mimic a naturally occurring communication pathway between plants and fungi, encapsulating RNA and shielding it from degradation by environmental RNases. This increased stability will extend the necessary treatment intervals, making AV-

dsRNA applications more practical for growers. However, AVs are not without limitations. While the cost of production can be reduced by tweaking formulations, such as removing PEG or using more economical lipids like DODMA, these savings might still fall short for large-scale agricultural use in low-resource settings. Despite these challenges, AVs still represent a significant step toward creating more durable and scalable RNAi-based crop protection strategies.

Bacterial platforms for RNA production combine the production and delivery of dsRNA into a single, self-sustaining system. Engineered strains of *Bacillus subtilis* and *Pseudomonas putida* demonstrated the ability to produce fungal-gene-targeting dsRNA continuously on plant surfaces or in the soil, excreting it via extracellular vesicles for delivery to fungal pathogens. This dual capability addresses two critical barriers: cost and RNA stability. Unlike traditional methods requiring the external synthesis and application of dsRNA, bacterial platforms essentially act as “living factories” for antifungal RNAs. Moreover, this approach expands RNAi applications to soilborne pathogens, an area that other SIGS approaches have been unable to address. However, significant drawbacks remain. The use of genetically modified organisms (GMOs) in agriculture is heavily regulated and controversial, posing challenges for widespread adoption. Additionally, there is uncertainty about the long-term ecological impact of introducing engineered bacteria into soil microbiomes. The engineered bacteria could potentially reproduce with native bacteria via conjugation, introducing transgenes into wild populations. I predict that this technology would be best first

utilized in controlled environmental agricultural systems, where there is a low risk of escape of engineered bacteria.

Another approach, Bioclay™, uses layered-hydroxide nanosheets to stabilize and deliver RNA molecules to target pathogens. This method has been successfully applied against a range of threats, including plant viruses (Mitter et al. 2017), fungal pathogens (Niño-Sánchez et al. 2022), and whiteflies (Jain et al. 2022), making it an incredibly versatile tool. Bioclay's primary strength lies in its ability to release RNA gradually, providing sustained protection over time while reducing the frequency of applications. In this work here, I found that a variety of Bioclay™ formulations are effective in delivering RNA to pathogens and protecting plant tissue, showing this tool is robust and flexible. However, in some of my experiments I noticed that Bioclay™ formed a thin film over plant material which was hard to wash off and created an actual physical barrier to pathogen infection. This could be a major drawback in utilizing Bioclay™ on post-harvest products, as consumers may not want to eat tomato fruits coated in a rusty film.

Looking ahead, there are several ways to improve SIGS for broader agricultural adoption. All the nanoparticles tested here should be tested for the length of time they can be stored. In agricultural settings, pesticides need to be able to sit in long term storage at ambient conditions in order to be practical. Increasing RNA uptake efficiency will allow for lower dosages per treatment, making the approach more cost-effective. Additionally, advances in RNA manufacturing—leveraging technologies developed for COVID-19 vaccine

production—could significantly reduce the costs associated with RNA synthesis, overcoming one of the major barriers to commercialization. Finally, combining RNA-based treatments with traditional fungicides or insecticides, or formulating mixtures targeting multiple pathogens simultaneously, could further enhance the robustness and reliability of SIGS in the field.

There aren't that many Ponderosa pine trees left at my parents' house. A testament to the devastating power of pathogens and pests as we move forward into an uncertain climate future. The solutions to combating the growing suite of abiotic and biotic threats facing native plants and crops will certainly be multifaceted. But I believe that this dissertation demonstrates that RNA has the potential to be a key player in developing robust, sustainable plant protection strategies for the future.

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