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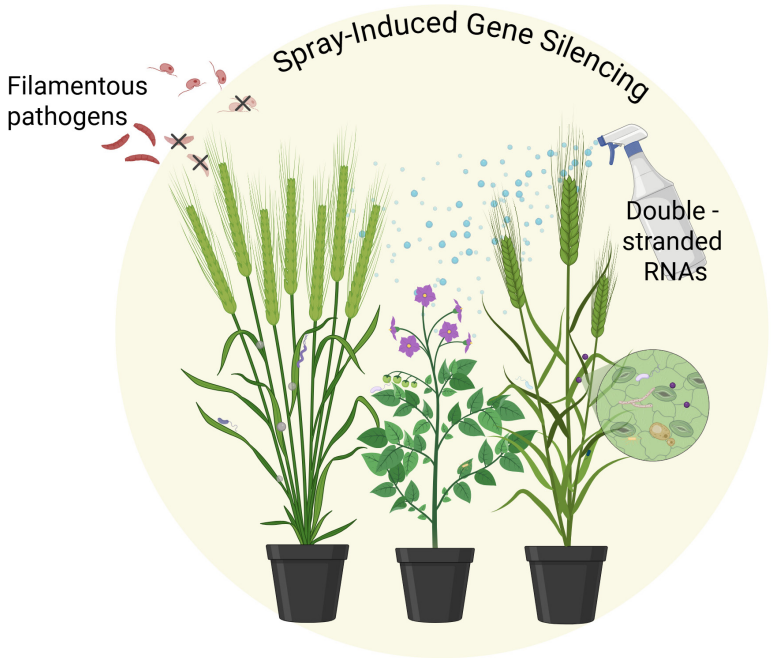
Spray-induced gene silencing as a promising tool for sustainable plant protection

Investigating disease control and microbial ecology

Poorva Sundararajan



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Poorva Sundararajan

Faculty of Landscape Architecture, Horticulture and Crop Production Science

Department of Plant Breeding

Alnarp

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Spray-induced gene silencing as a promising tool for sustainable plant protection: Investigating disease control and microbial ecology

Abstract

Triticum aestivum L. (wheat), *Hordeum vulgare* L. (barley) and *Solanum tuberosum* (potato) are major crops contributing to primary food production and food security worldwide. However, their cultivation is hampered by pathogens and pests. Existing methods for plant protection largely consist of cultural practices, using fungicides, and breeding for resistance and better agronomic traits. Such strategies can be variably successful, tedious, economically strenuous and harmful to human and environmental health. As the search for sustainable alternatives continues, solutions based on RNA interference (RNAi) have emerged as promising alternatives. RNAi is a natural defence mechanism in eukaryotic organisms through which targeted gene silencing leads to transcriptional and post-transcriptional gene regulation. Researchers have exploited RNAi for several applications using its characteristic components – double-stranded RNAs (dsRNA) and small RNAs. In the field of plant protection, RNAi and dsRNA sprays have shown promise for disease control, including the control of pathogens like *Fusarium graminearum* and *Phytophthora infestans*. This method of spraying dsRNAs for pathogen control was termed spray-induced gene silencing (SIGS). Despite promising initial results, several facets of SIGS still need development for its adoption for field use. Through this thesis, two such aspects have been addressed: the ability of SIGS to suppress disease under practical conditions and the impact of spraying dsRNA on the plant microbiome. We show that dsRNA targeting *F. graminearum* can reduce the progression of Fusarium head blight in intact wheat and barley spikes. Using amplicon sequencing, we show for the first time that spraying dsRNA does not alter the core bacterial and fungal constituents of the phyllosphere, while causing minor changes to the relative abundance of bacterial communities in wheat, barley and potato. These studies strengthen the potential of SIGS for managing filamentous phytopathogens and reinforce the target-specific nature of the methodology. Such a holistic understanding of the processes that underlie SIGS-based plant protection therefore enable smooth transition of the technology for large-scale use.

Keywords: SIGS, dsRNA, RNAi, *Fusarium graminearum*, *Phytophthora infestans*, Fusarium head blight, microbiome, phyllosphere, amplicon sequencing

Sprayinducerad gensläckning som ett lovande verktyg för hållbart växtskydd: studier av sjukdomsbekämpning och mikrobiell ekologi

Abstract

Triticum aestivum L. (vete), Hordeum vulgare L. (korn) och Solanum tuberosum (potatis) är viktiga grödor som bidrar till den globala livsmedelsproduktionen och livsmedelssäkerheten. Deras odling försvåras dock av mikrobiella patogener och insektsangrepp. Befintliga växtskyddsstrategier, såsom odlingstekniska åtgärder, förädling för resistens och användning av fungicider, är ofta resurskrävande, ekonomiskt betungande och kan ha negativa effekter på miljö och hälsa. Som ett hållbart alternativ har RNA-interferens (RNAi) uppmärksammats som ett lovande angreppssätt. RNAi är en naturlig försvarsmekanism hos eukaryota organismer där riktad gensläckning reglerar genuttryck på transkriptionell och post-transkriptionell nivå.

Inom växtskydd har exogen applicering av dubbelsträngat RNA (dsRNA) visat potential för kontroll av filamentösa patogener såsom Fusarium graminearum och Phytophthora infestans. Denna metod benämns också sprayinducerad gensläckning (spray-induced gene silencing, SIGS). Trots initialt lovande resultat kvarstår flera aspekter av SIGS som behöver utvecklas inför framtida användning i fält. I denna avhandling undersöks SIGS effektivitet under praktiska förhållanden samt dess påverkan på växternas mikrobiom. Vi visar att dsRNA riktat mot F. graminearum minskar utvecklingen av axfusarios i intakta vete- och kornax. Vidare visar amplikonsekvensering att dsRNA-besprutning inte påverkar de dominerande bakteriella och svampmässiga komponenterna i fyllosfären, medan endast mindre förändringar i bakteriell komposition observeras i vete, korn och potatis. Sammantaget stärker resultaten SIGS potential som ett målspecifikt och hållbart verktyg för växtskydd samt underlättar dess framtida storskaliga tillämpning.

Nyckelord: SIGS, dsRNA, RNAi, Fusarium graminearum, Phytophthora infestans, axfusarios, mikrobiom, fyllosfär, amplikonsekvensering

தாவர பாதுகாப்பிற்கான நிலையான கருவியாகத் தெளிப்பால் தூண்டப்படும் மரபணு மௌனப்படுத்தல்: நோய் கட்டுப்பாடு மற்றும் நுண்ணுயிரியல் சூழலியல் ஆய்வு

சுருக்கம்

கோதுமை (*Triticum aestivum* L.), பார்லி (*Hordeum vulgare* L.) மற்றும் உருளைக்கிழங்கு (*Solanum tuberosum*) ஆகியவை உலகளாவிய உணவு உற்பத்தி மற்றும் உணவுப் பாதுகாப்பிற்கு முக்கியமான பிரதான பயிர்களாகும். ஆனால், இப்பயிர்களின் சாகுபடி நுண்ணுயிரி நோய்க்கிருமிகள் மற்றும் பூச்சி தாக்குதல்களால் பாதிக்கப்படுகிறது. தாவர பாதுகாப்பிற்காக பயன்படுத்தப்படும் தற்போதைய முறைகள் பெரும்பாலும் சாகுபடி சார்ந்த நடைமுறைகள், நோயெதிர்ப்பு திறனுக்கான தாவர இனப்பெருக்கம் மற்றும் வேதியியல் பூஞ்சைநாசினிகள் ஆகியவற்றை உள்ளடக்கியவை. இம்முறைகள் செயல்திறன் கொண்டதாக இருந்தாலும், மனித ஆரோக்கியம் மற்றும் சுற்றுச்சூழலுக்கு தீங்கு விளைவிக்கக்கூடும். நிலைத்தன்மை கொண்ட மாற்று வழிகளைத் தேடும் முயற்சியில், RNA இடையீடு (RNA interference; RNAi) அடிப்படையிலான அணுகுமுறைகள் முக்கியத்துவம் பெற்றுள்ளன. RNAi என்பது யூகேரியோட்டிக் உயிரினங்களில் காணப்படும் இயற்கையான பாதுகாப்பு செயல்முறையாகும்; இதில் இரட்டை இழை RNA (dsRNA) மற்றும் சிறிய RNAகள் மூலம் குறிப்பிட்ட மரபணுக்களின் வெளிப்பாடு transcription மற்றும் post-transcription நிலைகளில் கட்டுப்படுத்தப்படுகிறது. தாவர பாதுகாப்பு துறையில், dsRNA-ஐ தெளிக்கும் RNAi முறைகள் *Fusarium graminearum* மற்றும் *Phytophthora infestans* போன்ற நூலிழை வடிவ நோய்க்கிருமிகளை கட்டுப்படுத்துவதில் நம்பிக்கைக்குரிய முடிவுகளை வழங்கியுள்ளன. இந்த முறை “spray-induced gene silencing” (SIGS) என அழைக்கப்படுகிறது. எனினும், வயல்வெளி பயன்பாட்டிற்கு முன் அதன் செயல்திறன் மற்றும் பாதுகாப்பு தொடர்பான மேலதிக ஆய்வுகள் அவசியமாகும். இத்தேர்வில், *F. graminearum*-ஐ குறிவைக்கும் dsRNA, முழுமையான கோதுமை மற்றும் பார்லி கதிர்களில் Fusarium head blight நோயின் முன்னேற்றத்தை குறைக்கக்கூடியது என்பதை காட்டுகிறோம். Amplicon sequencing மூலம், dsRNA தெளிப்பு கோதுமை, பார்லி மற்றும் உருளைக்கிழங்கின் இலைகளில் உள்ள முக்கிய பாக்டீரியா மற்றும் பூஞ்சை சமூகங்களை மாற்றவில்லை என்பதையும், பாக்டீரியா சமூகவமைப்பில் சிறிய மாற்றங்கள் மட்டுமே ஏற்பட்டுள்ளன என்பதையும் நிரூபிக்கிறோம். இந்த முடிவுகள் SIGS முறையின் குறிவைக்கும் தன்மையையும், நூலிழை வடிவ தாவர நோய்க்கிருமிகளை நிர்வகிப்பதில் அதன் சாத்தியத்தையும் வலுப்படுத்துகின்றன. SIGS அடிப்படையிலான தாவர பாதுகாப்பு செயல்முறைகளை இவ்வகையில் முழுமையாக புரிந்துகொள்வது இத்தொழில்நுட்பத்தை பெரிய அளவில் நடைமுறைப்படுத்துவதற்கு வழிவகுக்கிறது.

Dedication

To my parents, for always supporting me tirelessly.

கற்றது கை மண் அளவு கல்லாதது உலகளவு.

“What you have learned is a mere handful; what you have not is the size of the world.”

- ஔவையார் (Avvaiyar), Tamil poet

To a journey of forever learning!

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Poorva Sundararajan**, Katie Stevens, Anna Åsman, Stephen C. Whisson, Mukesh Dubey, Aakash Chawade & Ramesh R. Vetukuri. Spray-induced gene silencing to control Fusarium head blight in cereals. (Manuscript)

II. **Poorva Sundararajan***, Samrat Ghosh*, Bekele Gelena Kelbessa, Stephen C Whisson, Mukesh Dubey, Aakash Chawade & Ramesh R. Vetukuri (2025). The impact of spray-induced gene silencing on cereal phyllosphere microbiota. Environmental Microbiome, 20(1), 1. https://doi.org/10.1186/s40793-024-00660-8

III. **Poorva Sundararajan**, Pruthvi B. Kalyandurg, Qinsong Liu, Aakash Chawade, Stephen C. Whisson & Ramesh R. Vetukuri (2022). Spray-Induced Gene Silencing to Study Gene Function in *Phytophthora*. Methods in Molecular Biology (Clifton, N.J.), 2536, 459–474. https://doi.org/10.1007/978-1-0716-2517-0_27

IV. Samrat Ghosh*, **Poorva Sundararajan***, Bekele Gelena Kelbessa, Farideh Ghadamgahi, Stephen C. Whisson, Mukesh Dubey, Aakash Chawade & Ramesh R. Vetukuri. Effects on the leaf microbiota of potato plants arising from spray-induced gene silencing using double-stranded RNA. (Manuscript)

* Equally contributing authors

Paper II is published open access, and Paper III is reproduced with permission from Springer Nature.

The contribution of Poorva Sundararajan to the papers included in this thesis was as follows:

I. Designed the experiments together with co-authors. Conducted the Biotron experiments. Analysed disease scoring data. Wrote the manuscript with input from co-authors.

II. Designed the experiments together with co-authors. Conducted the plant experiments. Interpreted the results together with the shared first author. Wrote the manuscript with input from co-authors.

III. Performed and optimised protocols along with the second author. Wrote the manuscript together with co-authors.

IV. Designed the study together with co-authors. Carried out the plant experiments. Interpreted the results together with the shared first author. Wrote the manuscript with input from co-authors.

List of other publications

I. Kalyandurg, P. B., Sundararajan, P., Dubey, M., Ghadamgahi, F., Zahid, M. A., Whisson, S. C., & Vetukuri, R. R. (2021). Spray-Induced Gene Silencing as a Potential Tool to Control Potato Late Blight Disease. Phytopathology, 111(12), 2166–2175. https://doi.org/10.1094/PHYTO-02-21-0054-SC

II. Piombo, E., Vetukuri, R. R., Sundararajan, P., Kushwaha, S., Jensen, D. F., Karlsson, M., & Dubey, M. (2022). Comparative Small RNA and Degradome Sequencing Provides Insights into Antagonistic Interactions in the Biocontrol Fungus *Clonostachys rosea*. Applied and Environmental Microbiology, 88(13). https://doi.org/10.1128/AEM.00643-22

III. Piombo, E., Kelbessa, B. G., Sundararajan, P., Whisson, S. C., Vetukuri, R. R., & Dubey, M. (2023). RNA silencing proteins and small RNAs in oomycete plant pathogens and biocontrol agents. *Frontiers in Microbiology*, *14*. https://doi.org/10.3389/FMICB.2023.1076522

IV. Piombo, E., Vetukuri, R. R., Konakalla, N. C., Kalyandurg, P. B., Sundararajan, P., Jensen, D. F., Karlsson, M., & Dubey, M. (2024). RNA silencing is a key regulatory mechanism in the biocontrol fungus *Clonostachys rosea*-wheat interactions. *BMC Biology*, *22*(1), 1–27. https://doi.org/10.1186/S12915-024-02014-9

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Figure 1. Representative image showing the disease cycle of *Fusarium graminearum*, the causal pathogen of Fusarium head blight in cereals. The asexual and sexual spores of the pathogen are dispersed through wind and rain to infect inflorescences at anthesis. The pathogen proliferates slowly inside spike tissue and symptoms appear as brown, water-soaked lesions or premature bleaching in necrotized spikelets. The pathogen also produces mycotoxins that accumulate in grains. The sexual spore-bearing perithecia and asexual conidia act as overwintering inoculum on crop residue and carry the pathogen into the next growing season. Illustration created with Biorender.com. 25

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Abbreviations

AGO	Argonaute
ANCOM-BC	Analysis of compositions of microbiomes with bias correction
AUDPC	Area under the disease progression curve
ckRNAi	Cross-kingdom RNA interference
DCL	Dicer-like
DEG	Differentially expressed gene
DON	Deoxynivalenol
dpi	Days post inoculation
dsRNA	Double-stranded RNA
FHB	Fusarium head blight
hps	Hours post-spray
HIGS	Host-induced gene silencing
hpRNA	Hairpin RNA
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS	Internal transcribed spacer
MAPK	Mitogen-activated protein kinase
miRNA	MicroRNA
NIV	Nivalenol
NLR	Nucleotide-binding domain with leucine-rich repeats
Nsp	Non-specific dsRNA
PCoA	Principal coordinate analysis
phasiRNA	Phased secondary RNA
piRNA	PIWI-interacting RNA
PTGS	Post-transcriptional gene silencing
RdRP	RNA-dependent RNA polymerase
rRNA	Ribosomal RNA
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
SIGS	Spray-induced gene silencing
siRNA	Small-interfering RNA
snoRNA	Small nucleolar RNA
sRNA	Small RNA
tasiRNA	Trans-acting RNA

TE	Transposable element
VIGS	Virus-induced gene silencing
ZEA	Zearalenone

1. Introduction

The Agricultural Revolution helped bring farming to the world, facilitating and shaping the development of human civilization. Over the millennia, domestication of crops has reduced the diverse range of plants which were once utilized as food to just a handful. This handful of crops, now categorized broadly as cereals, sugar crops, vegetables, oil crops, fruits, and tubers, dominate food production and nutrient sufficiency to this day (FAO, 2024). The top food crops grown today are sugarcane, maize, wheat, rice, oil palm fruit and potatoes. Together, they account for 56 % of global primary crop production (FAO, 2024), enforcing the importance of cereals and starchy tubers in feeding the world. Crops like wheat, rice, maize, barley, sorghum and rye are grouped as cereals, while potatoes, sweet potatoes and cassava are members of starchy tubers or tuberous roots.

Cereals and tubers are food groups that form a major source of dietary energy, making wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and potato (*Solanum tuberosum* L.) food crops of high economic relevance. Both wheat and barley have been cultivated for over 10,000 years, and were domesticated from their respective wild relatives in the Fertile Crescent (modern-day southwestern Asia) (Badr et al., 2000; Feldman & Levy, 2015). Domestication and continuous selection of favourable traits (both unconsciously due to environmental conditions and deliberately by man) over several millennia have resulted in higher-yielding wheat and barley, leading to their extensive adoption and cultivation worldwide (Hafeez et al., 2021; Pourkheirandish & Komatsuda, 2007). Although cultivated in lesser quantities than wheat, potato accounts for four percent of primary crop production and is a food crop with high nutritional value (Lutaladio & Castaldi, 2009). Potatoes have also been cultivated for close to 10,000 years, with the cultivated potato *Solanum tuberosum* tracing back its origin to wild *Solanum spp.* from the Andean regions of South America (Spooner et al., 2005). It was introduced to Europe in the 16th century by Spanish conquistadors, spreading subsequently to other parts of the world over the next few centuries and becoming one of the most important crops of the modern world. In 2023, wheat, barley and potato cultivation was estimated to be 2.2 billion, 46.25 million and 16.79 million hectares worldwide, respectively (FAO, 2025a). Additionally, the three crops combined provide around 18 % of total calories and protein consumed by the world's

population (FAO, 2025b). Wheat, barley and potatoes are also some of the top crops grown in Sweden, occupying around 27 % of arable land (wheat 13.6 %, barley 12.3 % and potato 1 %) (Statistikmyndigheten SCB, 2008).

The increasing world population and the nutritional requirements for it have driven the need for an unprecedented intensification of food production in recent decades. However, the latest report from FAO shows that while production of crops has increased by 56% since the year 2000, the available arable land has decreased over the last two decades (FAO, 2024). Moreover, global agricultural land was estimated to be 4.78 billion hectares in 2022, accounting for 45 % of habitable land. Further increase in land use for agriculture will result in greater loss of biodiversity and accelerated global warming. It is therefore detrimental to intensify agriculture only through increased land use.

Global warming and climate change, on the other hand, have also led to more frequent extreme weather such as excessive rainfall or drought, and heat/cold waves. Erratic weather patterns have a negative effect on agricultural production and yield as they affect plant growth and development. The changing climate also impacts plant disease, as it can both worsen disease severity and lead to the emergence of new pests and pathogens resulting from changed environmental niches. Therefore, such plant disease epidemics pose a significant risk to food production, especially in the wake of climate change.

Farmers growing wheat, barley and potato are regularly met with disturbances from a range of abiotic and biotic stress factors. Savary et al., 2019 reported high interference of pests and pathogens to global wheat and potato production. Leaf rust, stripe rust, septoria tritici blotch, fusarium head blight, spot blotch, tan spots, aphids and powdery mildew were listed as top diseases in wheat, while late blight, brown rot, early blight and cyst nematodes were shown as major diseases in potato. Furthermore, several pests and pathogens of wheat can also use barley as a host, making this crop susceptible to these diseases as well. Among these, two major diseases – Fusarium head blight (FHB) in wheat and barley, and late blight in potatoes are discussed in more detail below in the context of this study.

2. Background

2.1 Filamentous pathogens and their devastating diseases

Members of the kingdom Mycota and phylum Oomycota that exhibit tubular threadlike growth are classified as filamentous fungi and oomycetes. Filamentous organisms are characterized by structures called hyphae and grow by extension of these hyphae as opposed to budding or fission in yeasts (Powers-Fletcher et al., 2016). These microorganisms can also be septate or aseptate (the cell wall separation that compartmentalizes spores and hyphae) and have branched or unbranched hyphae. Their branching patterns can vary between species and are impacted by environmental conditions. The hyphae of filamentous fungi and oomycetes also collectively form compound networks called mycelia. These organisms can also exhibit both asexual and sexual reproductive structures in addition to vegetative growth. The asexual structures are called conidiophores or sporangiophores depending on the genus, and these structures bear the asexual propagules called conidia or sporangia. In fungi, the sexual structures can vary between phyla and are called perithecia, basidia, zygosporangia or cleistothecia. In oomycetes, the sexual spores are known as oospores. These diverse morphological adaptations help filamentous organisms thrive in a wide range of habitats.

2.1.1 *Fusarium graminearum*

Fusarium graminearum Schwabe is a plant pathogenic fungus belonging to the genus *Fusarium* of ascomycete fungi (Sordariomycetes: Hypocreales: Nectriaceae). It is a filamentous pathogen and features both sexual and asexual life stages. Unlike some other pathogenic fungi belonging to the genus *Fusarium* that produce both macro- and micro-conidia, *F. graminearum* only produces macroconidia. Masses of cells called sporodochia support the growth of sickle-shaped macroconidia arising from conidiophores. The sexual spores of *F. graminearum* are known as ascospores. They are released from perithecia, a flask-shaped form of its fruiting body (ascocarp). The ascocarp is a distinct feature of fungi from the phylum Ascomycota, with the name denoting the sac-like structures called asci. In *F. graminearum*, both macroconidia and ascospores act as dormant

inoculum and aid the survival of the pathogen from year to year on crop residues. Previously, fungal binomial nomenclature depended on whether an asexual (anamorphic) or sexual (teleomorphic) stage was observed morphologically. Hence, *F. graminearum* has also been denoted as *Gibberella zeae* (teleomorph) throughout its history. This dual nomenclature is however no longer adopted to avoid confusion (Geiser et al., 2013) and the unique name of *F. graminearum* is now used to denote all sexual stages.

The infection cycle of *F. graminearum* starts when the macroconidia or ascospores infect cereal spikes during anthesis of the next crop cycle (Alisaac & Mahlein, 2023; Goswami & Kistler, 2004; Trail, 2009). Under favourable conditions of warm and humid weather, the pathogen spreads to other spikes through wind and rain **(Figure 1)**. The spores then germinate to produce germination tubes, following which fungal hyphae extend into the plant surfaces through natural openings and other susceptible sites. The pathogen then grows asymptomatically and intercellularly within the spikelet for a few days, first establishing a biotrophic phase where colonized host tissue remains alive. Soon after, the necrotrophic phase sets in when plant cells are killed, and the pathogen grows both intra- and inter-cellularly to rapidly colonize neighbouring spikelets. The fungus thus follows a brief biotrophic relationship with its host before switching to necrosis, thereby exhibiting a hemi-biotrophic lifestyle. The symptoms of *F. graminearum* infection show up during necrosis as browning and water-soaked lesions on developing spikelets and bleaching of colonized tissue. Premature bleaching has therefore been characterized as a key symptom of FHB in cereal spikes. FHB symptoms are sometimes also visible as salmon-orange or pink masses on the infected spikelets. At this phase of infection, the pathogen produces secondary metabolites which act as virulence factors and accumulate in the spike tissues and kernels (Bottalico & Perrone, 2002). The infected kernels appear small and shrivelled and carry the mycotoxins through harvest and post-harvest. Besides the asexual cycle, the sexual cycle of *F. graminearum* is also critical for its infection as the perithecia serve as overwintering inoculum, releasing ascospores in spring (McMullen et al., 2007). Occasionally, infected spikelets display bluish-black masses of perithecia.

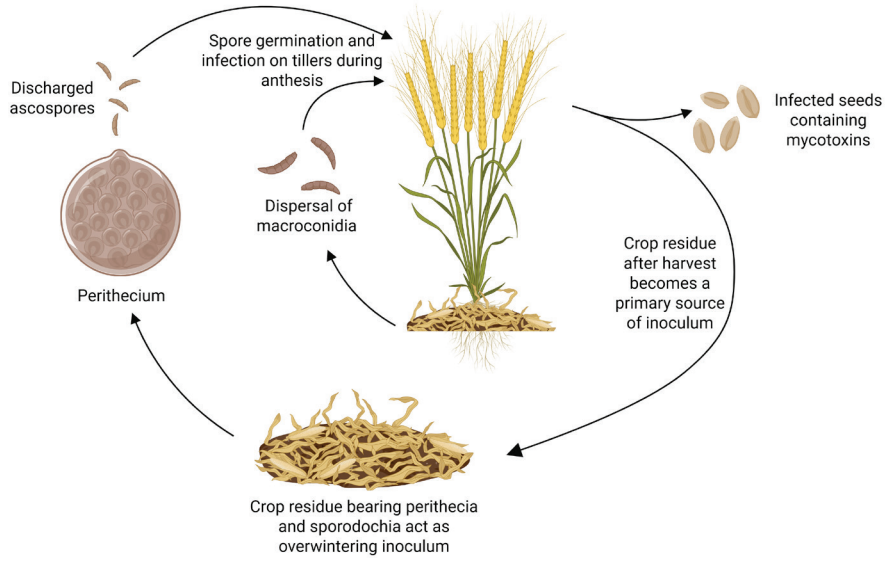

Figure 1. Representative image showing the disease cycle of *Fusarium graminearum*, the causal pathogen of Fusarium head blight in cereals. The asexual and sexual spores of the pathogen are dispersed through wind and rain to infect inflorescences at anthesis. The pathogen proliferates slowly inside spike tissue and symptoms appear as brown, water-soaked lesions or premature bleaching in necrotized spikelets. The pathogen also produces mycotoxins that accumulate in grains. The sexual spore-bearing perithecia and asexual conidia act as overwintering inoculum on crop residue and carry the pathogen into the next growing season. Illustration created with Biorender.com.

While certain species of *Fusarium* (*F. solani* and *F. oxysporum* complexes, and *Gibberella fujikuroi*) can be opportunistic human pathogens (Powers-Fletcher et al., 2016), *F. graminearum* mainly impacts human health through the mycotoxins it produces. Common *Fusarium* secondary metabolites detected worldwide include trichothecenes like deoxynivalenol (DON) and nivalenol (NIV) as well as zearalenone (ZEA) and fumosins, which are factors of fungal aggressiveness and aid progression of *Fusarium* infection (Langevin et al., 2004; Wu et al., 2014). Highlighting their importance in the context of Swedish agriculture, a study showed the wide prevalence of *Fusarium* mycotoxins in both spring and winter wheat across two different

years in Sweden (Lindblad et al., 2013). These metabolites act as naturally occurring toxins and are carried into food and feed products from infected grains during crop production and processing (Qu et al., 2024). Ingestion of these mycotoxins has been linked to acute and chronic adverse reactions in humans and animals. Trichothecenes can cause irritation to skin and intestinal mucosa in humans and immune suppression in animals, while ZEA and fumosins can have hormonal effects and cancer risks, respectively (WHO, 2023).

2.1.2 Fusarium head blight (FHB)

FHB is a disease that predominantly affects small grain cereals such as wheat, barley, rye, triticale, maize and rice. The disease is caused by a complex of fungi that includes 16 species from the genus *Fusarium* such as *F. culmorum*, *F. avenaceum* and *F. graminearum* (Aoki et al., 2012). Collectively, this is known as the Fusarium species complex. Of these 16 species, *F. graminearum* is considered predominant and more virulent in causing FHB (Parry et al., 1995). The pathogen complex infects developing inflorescences, grains, seedlings, roots and stems in cereals, causing Fusarium head blight (FHB) (**Figure 2**), crown rot and seedling blight (Karlsson et al., 2021). Warm and humid conditions during anthesis and early grain-filling provide conducive conditions for the spread of FHB (Kriss et al., 2012). It is a highly destructive pathogen which impairs both grain yield and quality in several cereal crops (Mielniczuk & Skwaryło-Bednarz, 2020). Most FHB symptoms are similar across the different plant hosts. However, in barley, infection can sometimes be asymptomatic (Goswami & Kistler, 2004).

Figure 2. Fusarium head blight symptoms in wheat (left) and barley (right) inflorescence. (Photograph: Poorva Sundararajan)

2.1.3 *Phytophthora infestans*

Oomycetes have long been considered a part of fungi since they possess morphological structures like mycelia and spores similar to filamentous fungi. However, these eukaryotes only superficially resemble members of the kingdom Mycota. Molecular studies of oomycete rRNA sequences described them as stramenopiles from the kingdom Chromista (Cooke et al., 2000). Hence, oomycetes are phenotypically closer to filamentous fungi but phylogenetically closer to diatoms and brown algae (Beakes et al., 2012; Gunderson et al., 1987; Thines, 2014). A cell wall composed more of cellulose and β 1-3-glucans than chitin, a deviated lysine biosynthesis pathway and possessing two flagella instead of one are some important attributes that differentiate oomycetes from true fungi (Bartnicki-Garcia, 1968; Judelson & Blanco, 2005; Vogel, 1960). Although lesser studied than fungi, oomycetes have colonized diverse ecosystems and are distributed globally (Thines, 2014; Y. Wang et al., 2025).

Phytophthora infestans is the best known oomycete (Oomycetes: Peronosporales: Peronosporaceae), causing diseases in solanaceous crops potato and tomato. The name is derived from a combination of Greek words

meaning plant (Phyton) destroyer (phthora) and a Latin word meaning to infest (infestare).

Like other filamentous organisms, *P. infestans* also exhibits morphological features like hyphae, mycelia and asexual and sexual spores (**Figure 3**). Asexual structures include sporangia and zoospores. Sporangia are multi-nucleate lemon-shaped structures that are formed on sporangiophores. Sporangia either germinate directly or release zoospores to invade host plants (Judelson & Blanco, 2005). Each sporangium contains six to eight uninucleate and biflagellate zoospores that are highly motile. The zoospores can encyst and form germ tubes when they find host surfaces. Both germinated sporangia and cysts can invade leaf tissue by forming specialized structures called appressoria (Latijnhouwers et al., 2003), which then slice into host cells (Bronkhorst et al., 2021) to form an infection vesicle, and subsequently intercellular hyphae with haustoria which extend into host cells. Haustoria are major sites of effector protein secretion and delivery to suppress plant defences (Kagda et al., 2020; S. Wang et al., 2017, 2018). Like *F. graminearum*, *P. infestans* also exhibits a hemi-biotrophic lifestyle, and the infection cycle is well studied (Boevink et al., 2020). The biotrophic phase of infection is established when the infection vesicle and haustoria help the pathogen invade and proliferate in plant tissue. During this stage, the plant remains asymptomatic. As the infection progresses, disease symptoms become more evident, characterizing the necrotrophic phase. Typical symptoms include brown necrotic lesions on the leaves and stems of plants (Avrova et al., 2008) (**Figure 4**). At this point, the pathogen also develops more sporangiophores at the plant surface, which then release secondary sporangia to further colonize neighbouring tissue and plants. Through this cycle, the pathogen is able to quickly proliferate and infect host plants in as few as four to five days, making *P. infestans* a plant pathogen of high concern (Fry, 2008). In addition, when the two mating types (called A1 and A2) co-occur in infected tissue, *P. infestans* also produces sexual spores called oospores that overwinter in soil and help with increased disease spread (Mayton et al., 2000).

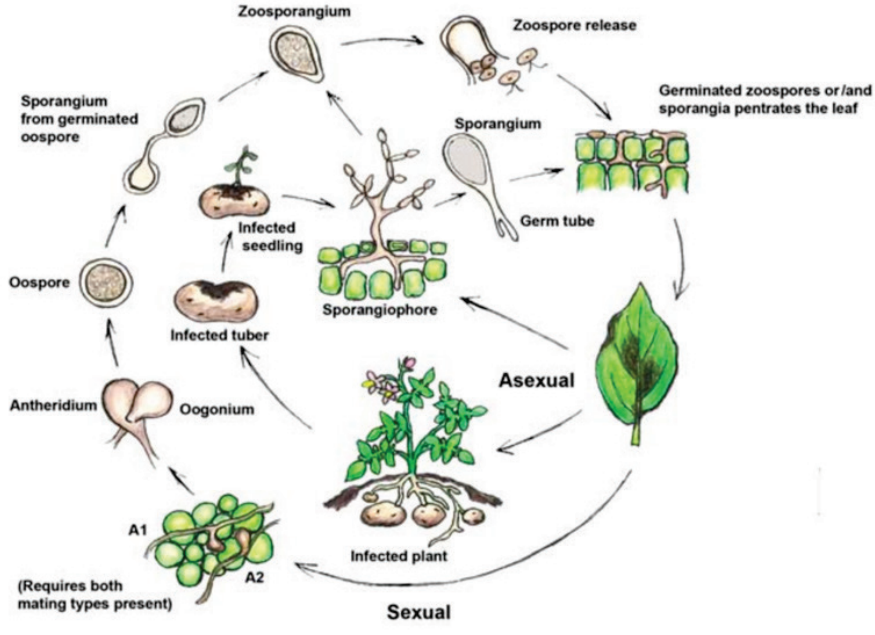

Figure 3. Disease cycle of *Phytophthora infestans* in potato plants. Both asexual and sexual spores germinate and penetrate host tissue upon contact. As infection progresses, the pathogen colonises neighbouring tissue and the symptoms appear as necrotic lesions. The disease progresses further to invade the whole plant, including stems and tubers. Infected tubers can act as sources of overwintering inoculum that carries the pathogen to the next growing season. Image adapted from Therése Bengtsson, 2013.

2.1.4 Potato late blight

Among oomycetes, *Phytophthora spp.* are extensively studied due to the expansive list of hosts they interact with and the magnitude of their economic impact. Several *Phytophthora spp.* are known to be highly invasive plant pathogens (Kamoun, 2003). The most notorious example of a plant disease caused by *Phytophthora spp.* is late blight disease in potato caused by *P. infestans*. Late blight was first reported in Europe in the nineteenth century, when entire potato crops were destroyed. The most severe impact of late blight resulted in the Irish Potato Famine. Since then, the world has seen repeated occurrences of the disease, including most major potato-growing countries in Africa, Asia and North America (Fry et al., 2015). The recurrent epidemics combined with a constant flux in *P. infestans* populations have led scientists to label late blight as a reemerging disease and *P. infestans* as a

catastrophic pathogen of the modern world (Fry et al., 2015; Kamoun et al., 2015).

Figure 4. Late blight symptoms visible as brown necrotic lesions on potato leaves. (Photograph: Ramesh Vetukuri)

2.2 The persistent need for alternative control

The world has experienced major FHB outbreaks causing huge economic losses (McMullen et al., 2012; Salgado et al., 2015; Savary et al., 2019). To date, cultural practices, developing resistant cultivars and application of fungicides are the common methods currently used to control FHB (Shah et al., 2018; Willyerd et al., 2012). *F. graminearum* overwinters in crop residues of most grasses, therefore tillage to bury crop residue and rotating with a non-host crop has shown promise for control of FHB (Shah et al., 2018). Efforts for resistance breeding using traditional and molecular methods are ongoing and both native and external sources of resistance have been identified (Wegulo et al., 2015). However, host resistance to *F. graminearum* is a complex trait in wheat, with multiple genetic loci across the different chromosomes contributing to it, making resistance breeding attempts harder (Buerstmayr et al., 2020). Additionally, the development of resistant cultivars can result in a yield penalty. On the other hand, fungicides

offer only partial control of FHB (Paul et al., 2008; Wegulo et al., 2011). Uneven flowering times, which are a common occurrence in cereal crops, can necessitate multiple applications of fungicides to achieve disease control. Even then, complete control is not achieved. Certain agronomic traits like plant height, lodging and cleistogamy can also have unfavourable effects on FHB infection. This therefore offers researchers an opportunity to additionally exploit plant agronomic traits for selecting varieties for FHB resistance (Wegulo et al., 2015).

Combining different strategies can prove to be beneficial. Wegulo et al., 2011 showed that combining cultivar resistance with fungicide treatment was 70% effective in controlling FHB and DON production. However, the development of these strategies can be time-consuming and economically costly, and their application can have lasting negative impacts on the environment. In addition, with climate change, conditions like increased temperature, rainfall and elevated carbon dioxide levels can create critical environmental factors that contribute to increased production of mycotoxins by *Fusarium spp*. (Qu et al., 2024). Therefore, there is a need to find quick and effective control strategies that can not only limit disease incidence but also mycotoxin production in cereals.

The major strategy currently employed to manage late blight in potato is the use of agrochemicals (Eriksson et al., 2016). Although the use of chemical plant protection can be highly effective, it poses a risk to human and animal health and includes the possibility that the pathogen develops fungicide resistance (Ivanov et al., 2021; Vleeshouwers et al., 2011). Therefore, other strategies to target the pathogen and fortify hosts against infection are being actively pursued (Y. Wang et al., 2025). This includes the use of host resistance genes (*R* genes) in plant breeding to develop cultivars with enhanced late blight resistance. *R* genes are plant genes that are part of the defence arsenal against pathogen infection (Hammond-Kosack & Jones, 1997). The *R* genes typically encode proteins with nucleotide-binding domains and leucine-rich repeats (called NLRs) that can detect pathogen effectors upon infection. Unfortunately, the pathogen can quickly evolve to circumvent these receptors, thereby limiting the use of single *R* genes in plant breeding for resistance. Other approaches that use *R* genes include the stacking of multiple *R* genes using cis- or trans-genesis to provide enhanced resistance (Haverkort et al., 2016). This is enabled both by the identification of numerous *P. infestans* effectors with potential *R* gene targets in potato and

by the discovery of R genes in several wild *Solanum* relatives (Haas et al., 2009; Vleeshouwers et al., 2011). However, the use of *R* genes and plant breeding for late blight control is a time-intensive process and includes the risk of the pathogen evolving to evade detection by the plant. Pathogen evasion can be attributed to the high plasticity of the *P. infestans* genome and the presence of many mobile transposable elements (TEs). TEs or transposons are genetic elements that can jump to different loci within the genome (Kamoun, 2003). In *P. infestans*, several effector-encoding genes are located in TE-rich regions, thereby providing the pathogen with the opportunity to generate different targets and alternative modes of action to manipulate host immunity. The hemibiotrophic lifestyle and short infection cycles, combined with the genomic instability of *P. infestans* makes effective management of potato late blight challenging. In addition, with climate change and increasingly unpredictable weather, there is a need to find effective and economical late blight control strategies that are sustainable in the longer term. To address these growing concerns, researchers have recently been pursuing RNA interference (RNAi) as an alternative route.

2.3 RNA Interference

RNAi is a natural cellular defence mechanism that is conserved through most eukaryotes. It is a form of post-transcriptional gene regulation using double-stranded RNAs (dsRNAs) or hairpin RNAs (hpRNAs) to direct the destruction of sequence-specific transcript accumulation. RNAi was first discovered in plants in 1990 when researchers observed that experimental introduction of a chimeric chalcone synthase gene in petunia plants caused co-suppression of both the transgene and homologous endogenous gene (Napoli et al., 1990). Following that, a similar phenomenon of "quelling" was observed in the fungus *Neurospora crassa* in another study (Romano & Macino, 1992). Subsequently, a study in the nematode *Caenorhabditis elegans* revealed that double-stranded RNA had the potential to cause sequence-specific gene interference (Fire et al., 1998). These studies led to the identification of the biological pathway of RNAi and characterization of the molecular components involved.

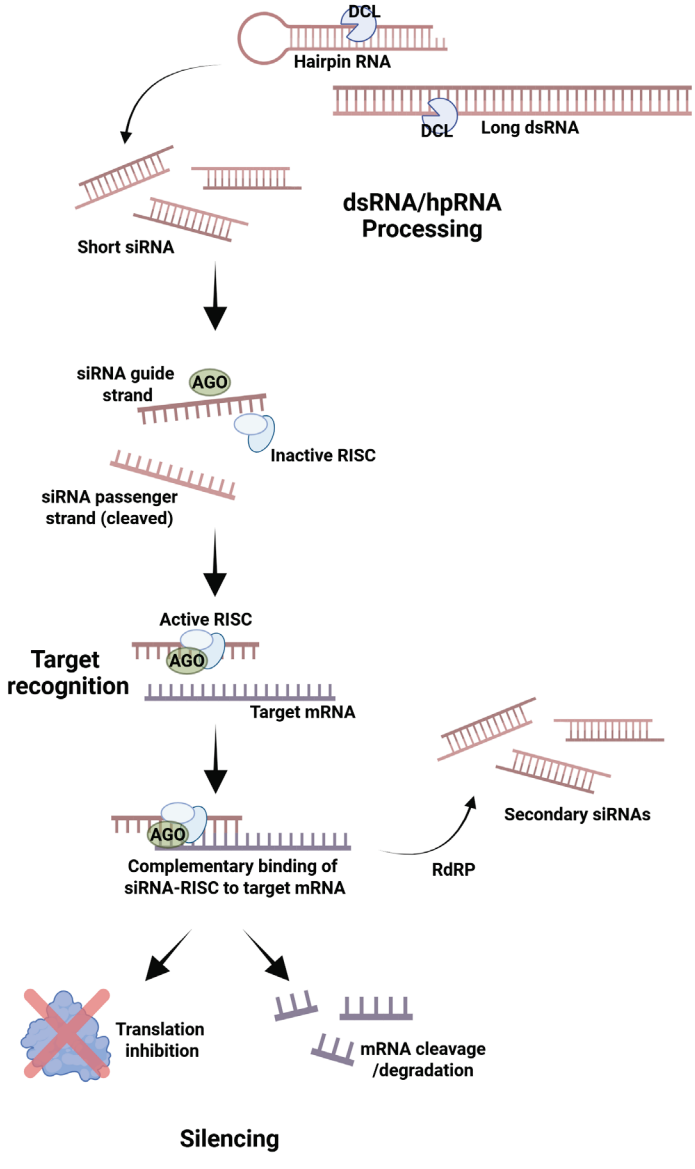

Figure 5. The RNA interference pathway. The mechanism is broadly divided into three steps: dsRNA/hpRNA processing, sRNA unwinding and target recognition, and gene silencing through sequence complementarity. Dicers and Argonautes are important proteins that facilitate these steps. Additionally, amplification of silencing is achieved by secondary sRNA production through RdRPs. Illustration created with Biorender.com

The mechanism of gene silencing through RNAi can be categorized into three broad steps: biogenesis of small RNAs through dsRNA/hpRNA processing, sRNA recognition and unwinding, and sequence-specific gene silencing (**Figure 5**). The first step is initiated when long dsRNAs or hpRNAs of foreign or endogenous origin are recognized by ribonucleases from the Dicer family of proteins (Q. Liu et al., 2009). Dicers and Dicer-like proteins (DCL) cleave the dsRNAs and hpRNAs into 21 – 24 nucleotide (nt) small-interfering RNAs (siRNA) and microRNAs (miRNA) (Dalakouras et al., 2020). These siRNAs and miRNAs, collectively called small RNAs (sRNA) then go on to trigger the next step of RNAi. sRNAs are recognized by another set of ribonuclease proteins called Argonautes (AGO), thereby activating the RNA-induced silencing complex (RISC). RISC, containing the AGO protein then unwinds the sRNA duplex into two strands – the guide strand and the passenger strand (Hutvagner & Simard, 2008). The guide strand is bound into the activated RISC and can then bind the mRNA sequence with base pair complementarity to the sRNA guide strand, thereby initiating the final step of RNAi: gene silencing. Upon binding, the complementary mRNA sequence is subject to either cleavage/degradation or translation repression (Vaucheret, 2008). This way, RNAi therefore achieves post-transcriptional gene-silencing (PTGS) in a target-specific manner.

Another intriguing part of RNAi is the secondary amplification leading to systemic spread of silencing (Mlotshwa et al., 2002; Pak & Fire, 2007; Tang et al., 2003). Upon unwinding of the sRNA duplex, the guide strand can prime dsRNA synthesis by RNA-dependent RNA polymerases (RdRP) to generate more siRNAs. These secondary sRNAs can therefore trigger further RNAi and amplification of dsRNA-triggered gene silencing in a process called transitive RNAi/transitivity (Sijen et al., 2001).

2.4 Role of small RNAs in biological processes

The collective term small RNA includes a diverse array of RNA types ranging between 20 - 40 nt in size. Besides siRNA and miRNA derived from dsRNA and hpRNA, small RNAs also include trans-acting small-interfering RNAs (tasiRNA), phased secondary siRNAs (phasiRNA), piwi-interacting RNAs (piRNA) and small nucleolar RNAs (snoRNA), among others (Borges & Martienssen, 2015; Chang et al., 2012; Zhan & Meyers, 2023). In plants, these sRNAs likely evolved as a defense mechanism to suppress viral

replication and mobilization of transposons, adapting over time to regulate endogenous gene expression and maintain structure and function of heterochromatin (Borges & Martienssen, 2015). Several studies have shown that plants can recognize and silence viral pathogens through their RNA-silencing machinery. This was initially supported by a study where transforming plants with a potyvirus homologous transgene made the plants resistant to the virus (Dougherty & Parks, 1995). Multiple studies have since shown the role of small RNAs in plant defense responses against viruses (Peláez & Sanchez, 2013; Xie et al., 2004). A study in *Arabidopsis thaliana* showed that 24-nt siRNAs might be involved in silencing highly repeated sequences (Kasschau et al., 2007), revealing the involvement of sRNAs in silencing of transposons. Furthermore, studies aiming to understand the roles of Dicer proteins in Arabidopsis and rice plants have shown the importance of DCLs and their small RNAs in plant development, morphogenesis and reproduction (Borges & Martienssen, 2015). Additionally, both short-distance and long-distance movement of small RNAs have been associated with roles such as cell-to-cell communication and inheritance of epigenetic signals trans-generationally (Melnyk et al., 2011; Slotkin et al., 2009).

Along similar lines, genes involved in sRNA biogenesis are found in most fungi and oomycetes (Nakayashiki et al., 2006; Piombo et al., 2023; Piombo et al., 2024) and have been associated with their growth and development (Carreras-Villaseñor et al., 2013; J. Zhou et al., 2012; Q. Zhou et al., 2012). Several sRNA molecules have also shown differential expression upon interaction of fungi/oomycetes with plant hosts, indicating their role in plant-microbe interactions and pathogenesis (Chang et al., 2012; Piombo et al., 2024). Furthermore, a study looking into two *P. infestans* isolates with varying pathogenicity identified sRNAs matching effector proteins, showing the role of oomycete sRNAs in virulence and induction of disease (Vetukuri et al., 2012). Fungal sRNAs can also target transcripts for transcription factors, increasing their regulatory role to a cascade of genes (Mueth & Hulbert, 2022; Silvestri et al., 2025). More interestingly, the transfer of sRNAs from plants to microorganisms and vice versa has been reported in both mutualistic and pathogenic plant-microbe contexts (Cai et al., 2018; Mueth & Hulbert, 2022; Piombo, et al., 2024; Ren et al., 2019; Silvestri et al., 2025; M. Wang et al., 2016; Weiberg et al., 2013; Werner et al., 2021). This bidirectional movement of sRNAs across organismal boundaries can trigger RNAi-mediated gene silencing in both the interacting host and

microbe, a phenomenon called bidirectional cross-kingdom RNA interference (ckRNAi).

Therefore, small RNAs undoubtedly possess multifaceted biological functions in plants and filamentous pathogens. Their diverse roles across different biological systems make sRNAs invaluable tools not only for biotechnological and biomedical applications such as genetic screens, development of antiviral drugs and cancer therapy (Kang et al., 2023; Pan et al., 2024), but also for plant trait development and pathogen/pest resistance in the field of agriculture (Vetukuri et al., 2021). This mechanistic basis underpins the application of RNAi for crop protection.

2.5 Why target *Fusarium graminearum* and *Phytophthora infestans* using RNAi?

While RNA silencing components have been identified in several fungi and oomycetes through phylogenetic analyses, some ascomycete and basidiomycete fungi like *Saccharomyces cerevisiae*, *Candida lusitaniae* and *Ustilago maydis* do not possess genes homologous to Dicers, AGOs and RdRPs, indicating the lack of a functioning RNAi pathway in these organisms (Nakayashiki et al., 2006). However, *F. graminearum* and *P. infestans* both possess functional RNAi pathways. Chen et al., 2015 identified two DCLs, two AGOs and five RdRP encoding genes in *F. graminearum*. These RNAi components have diverse roles in *F. graminearum* biological processes spanning from defense against mycoviruses and ascosporogenesis to asexual development and secondary metabolite production (Caihong Liu et al., 2024). Vetukuri et al., 2011 and Fahlgren et al., 2013 together identified two Dicers, five Argonautes and one RdRP encoding genes in *P. infestans* through comparative genomics and study of endogenous sRNA populations. The use of RNAi-based strategies in *F. graminearum* and *P. infestans* is therefore possible owing to the existence and characterization of the canonical RNAi pathway in these organisms.

2.5.1 SIGS to control FHB

Over the past decade, several studies have focused on testing the potential of RNA-based strategies for plant disease control, owing to the immense potential of RNAi in producing targeted gene-silencing and the growing need

to find alternative control methods to combat *Fusarium* diseases in cereals. Early on, researchers used plant transformation to constitutively express RNAi constructs of *F. graminearum* genes in host plants, in a method called host-induced gene silencing or HIGS. HIGS was successful in both silencing the genes of interest and reducing the progression of infection, as shown in W. Cheng et al., 2015 and Koch et al., 2013. While both studies targeted important genes in *F. graminearum*, the former targeted a virulence gene, chitin synthase 3b (*FgChs3B*), while the latter targeted three paralogous genes from the Cytochrome P450 family (*FgCyp51A, FgCyp51B* and *FgCyp51C* combined in one construct called CYP3RNA), genes that are essential for ergosterol biosynthesis. Wheat plants transformed with *FgChs3B* RNAi constructs displayed stable and consistent Type I and Type II resistance to both head blight and seedling blight under greenhouse and natural infection conditions. Similarly, Arabidopsis and barley plants harbouring the combined *FgCYP51* RNAi construct also showed increased resistance to *F. graminearum* infection on leaves.

Although highly effective, HIGS involves plant transformation and the generation of transgenic plants. Not all crops, or their cultivars, are amenable to transformation. In addition, public acceptance of genetically modified organisms (GMOs) is poor and the legislation surrounding them is limiting. This has provided the basis to develop alternative methods to execute RNAi-mediated gene silencing for plant protection.

To address these concerns, researchers tested whether dsRNA sprays would help achieve similar targeted pathogen suppression. This method of spraying dsRNA for disease control was hence termed spray-induced gene silencing (SIGS). To date, multiple studies have shown that spraying dsRNAs and sRNAs can be an effective tool to control different fungal and oomycete plant diseases including FHB (Bilir et al., 2019; Kalyandurg et al., 2021; Caihong Liu et al., 2024; Qiao et al., 2021; M. Wang et al., 2016; Y. Wang et al., 2023). It was found that the combined CYP3 dsRNA construct, which was previously used in an HIGS study, could also be employed through SIGS to inhibit *F. graminearum* growth on detached barley leaves (Koch et al., 2016). A later study using individual and double dsRNA constructs of the same *FgCYP51* genes in place of a combined one showed that spraying CYP51-dsRNAs reduced *F. graminearum* infection symptoms in detached barley leaves (Koch et al., 2019). These results together showed that inhibiting fungicide gene targets in the pathogen through methods other

than chemical fungicides can also produce desirable plant protection outcomes. Apart from CYP51 genes, another group of genes widely tested for *F. graminearum* control through SIGS include genes involved in the RNAi mechanism. A study showed that RNAi mutants of Dicers (DCL1, DCL2), Argonautes (Ago1, Ago2) and an Argonaute-interacting protein (QIP) in *F. graminearum* exhibited compromised virulence phenotypes in detached barley leaves sprayed with CYP3 dsRNA (Gaffar et al., 2019). A subsequent study showed that spraying these dsRNAs also offers protection from *F. graminearum* infection spread in detached barley leaves (Werner et al., 2020). These results together demonstrated that both generating RNAi mutant strains of *F. graminearum* and spraying dsRNA sequences targeting *Dicer* and *Argonaute* genes can provide FHB resistance in barley leaves.

Besides the adoption of *CYP51* and chitin synthase genes from HIGS studies, other genes that have been used as SIGS targets for controlling *F. graminearum* include different virulence genes, a glucan synthase and two different protein kinases. A study showed that dsRNA constructs targeting *FgGLS2*, *FgChs7* and *FgPkc* successfully reduced lesions in detached wheat leaves, both as independent dsRNAs or as a combination of two or three dsRNAs (Yang et al., 2021). Furthermore, the study demonstrated that spot injection of dsRNA on intact spikes also reduced spikelet infection significantly. A second study showed that dsRNA sprays targeting *FgCYP51C*, *FgCHS3b* and the mitogen-activated protein kinase *FgMGV1* resulted in reduced pathogenicity in detached wheat leaves and spikes, and in intact leaves under field conditions (Feng et al., 2025). Under natural infection conditions, spraying these dsRNAs also decreased FHB disease index and DON accumulation in wheat plants. Another study used reverse genetics and conditional promoter replacement to identify essential genes for addressing the limited availability of SIGS gene targets. The study identified 13 genes required for the establishment of SIGS-mediated *F. graminearum* control and analyzed the protective effects these dsRNA sprays yielded. They found that three of the 13 identified genes yielded greatly reduced disease lesions in detached barley leaves (Kim et al., 2023). More recently, a study tested whether dsRNA targeting *FgVE1*, a pathogenicity gene in *F. graminearum* leads to inhibition of growth, mycotoxin production and pathogenicity. They tested both naked and CaP nanoparticle-coated dsRNA on detached wheat leaves and found that naked dsRNA was effective in

inhibiting early stages of fungal growth while nanocomplexes provided a prolonged effect (Stakheev et al., 2025).

Genes involved in fungal secondary metabolite production have also been key targets for dsRNA-based plant protection. A SIGS study in wheat showed that spraying dsRNA targeting the transcription factor *FgTRI6* reduced disease spread and DON toxin accumulation in detached wheat spikes as well as intact wheat heads under greenhouse conditions (Hao et al., 2021).

Apart from *F. graminearum*, other phytopathogens from the genus *Fusarium* have also been targeted using SIGS and have shown promising results for disease suppression (Gu et al., 2019; Mosa & Youssef, 2021; Ouyang et al., 2023; Song et al., 2018; Tretiakova et al., 2022). Although these studies targeted *Fusarium* infection in tomato and wheat, the range of host plants affected by *Fusarium spp.* is wide, and studies showing gene silencing and disease control using alternative RNAi methods exist in diverse pathosystems (Caihong Liu et al., 2024). Therefore, there exists good promise to develop SIGS for combating *Fusarium* diseases in a wide variety of plants.

2.5.2 SIGS to study gene function

Through the years, researchers have developed several methods to study the function of genes. These studies enable us to understand the cellular functions of genes and their implications at the tissue/organism level. This information reveals insights into how biological systems operate and interact with their environment. Gene functional studies have therefore found applications in an extensive array of research fields, including drug discovery and development, disease detection in mammalian systems, improving plant productivity and tolerance to environmental stresses in crops, and plant disease control against microbial pathogens and insect pests (Borsani et al., 2005).

Much like plant/fungal transformation to silence/modulate gene expression, the ability to precisely control and silence their expression through RNAi provides the possibility to examine the functional effects of genes in eukaryotic systems through RNAi. Multiple SIGS studies have also investigated the effects of dsRNA on pathogen growth and morphology under *in-vitro* conditions (W. Cheng et al., 2015; Kalyandurg et al., 2021; Koch et al., 2013). These studies showed that dsRNA-mediated gene

silencing can alter fungal and oomycete morphology. These morphological changes associated with gene silencing can help assess its impact on pathogen virulence and thereby disease incidence and spread. A study in Arabidopsis and barley plants transformed with different RNAi constructs reported co-silencing of non-targeted paralogous genes and corresponding *F. graminearum* infection phenotypes (Koch et al., 2019). Information on co-silencing can hence offer mechanistic insights into how and why gene silencing produces varied phenotypes. Another study used VIGS to study co-silencing of two genes in tomato fruit production that do not have a visible silencing phenotype (Zhang et al., 2018). Further, *F. graminearum* DCL-dependent sRNAs share homology to host plant genes, meaning RNAi and SIGS can be used not only for disease control but also to help find molecular determinants of host immune suppression and pathogen virulence in plants (Werner et al., 2021). Additionally, RNAi-mediated gene silencing can also help improve plant traits by targeting negative regulators of desirable plant morphological and physiological traits (Vetukuri et al., 2021).

Spraying dsRNA therefore provides a practical tool to actualize the wide potential of RNAi-mediated gene silencing. While VIGS is a common methodology to functionally analyze the roles of targeted genes, the same principle can be applied through SIGS to bring about similar results. Since gene silencing through SIGS is transient, it offers a fast and simple process to study genes. In addition, SIGS is non-invasive as opposed to infiltration-based methods. Therefore, gene silencing through SIGS provides a practical approach to find functionally relevant genes for plant fitness, defense and pathogen control. With relevance to this study, SIGS can also help to gain insights into the morphological and molecular bases of the plant-microbe interactions in question.

2.6 Importance of microbiome for plant fitness and health

The microbiome of a plant is a complex environment comprised of a heterogeneous array of microorganisms. It is increasingly considered to be an extended layer of the plant due to the intricate relationship it shares with its host (Vandenkoornhuyse et al., 2015). Common microorganisms found in plant microbiomes include bacteria, protists, viruses, and microscopic fungi and nematodes (Trivedi et al., 2020; Turner et al., 2013). While several

mutual microbes are found in different plants, they can also be very distinct depending on the plant species and the environmental conditions that surround it (Agler et al., 2016; Matthews et al., 2019; Sapkota et al., 2015; Whipps et al., 2008). This is because diverse microorganisms possess varied nutritional requirements to colonize and thrive in specific ecological niches offered by the plant (Vorholt, 2012). Moreover, the microbiome also interacts within its members and with its macro-host through different positive strategies like mutualism, synergism, commensalism or negative interactions like antagonism, parasitism, competition and predation (Berg et al., 2020; Compant et al., 2025), resulting in varied microbial functions and assemblies. Domestication of plants also likely influenced the diversification of distinct microbial community assemblages and functions in different plant species (Pérez-Jaramillo et al., 2016). In addition, the microbial taxa inhabiting plant tissues vary significantly, with differences observed in communities of the phyllosphere, seeds, flowers, fruits, root endosphere, rhizosphere and rhizoplane (Compant et al., 2011, 2025).

The uniqueness of a plant's microbiome stems from the interdependency it shares with its host and the surrounding environment. The plant and its microbial residents influence each other for growth and development. Changes in microbial composition have previously been associated with better yield, more favourable phenotypes, and higher resilience and adaptation against abiotic and biotic stress factors (Compant et al., 2025; Singh et al., 2020; Trivedi et al., 2020). Bacteria in the phyllosphere and soil can transform and translocate important nutrients like nitrogen to increase their availability to plants (Trivedi et al., 2020; Whipps et al., 2008). Some rhizobacteria secrete and modulate hormones, secondary metabolites and antibiotics, thereby promoting plant growth (Backer et al., 2018). Soil microbiota also help the plant mitigate drought (Bashir et al., 2025; Canarini et al., 2021; Fitzpatrick et al., 2019). Furthermore, root-associated microbiota have been shown to sensitize the immune system of plants, triggering induced systemic resistance (Pieterse et al., 2014).

Alternatively, studies have shown that the plant and its environmental conditions specifically select its microbial inhabitants (Xiong et al., 2021). This is evident from different plant genotypes harbouring their own unique microbiomes (Agler et al., 2016; Sapkota et al., 2015). Under conditions such as drought or pathogen attack, plants recruit protective microbes to enhance resistance and suppress pathogens (Berendsen et al., 2012).

The microbiome of a plant can be broadly divided into the phyllosphere and the rhizosphere. The phyllosphere includes the microorganisms that inhabit the aboveground part of the plant, including the stem, leaves and flowering parts (inflorescence), while the rhizosphere consists of the microorganisms that inhabit the different root compartments and the soil surrounding it. Owing to the presence of decaying matter and root exudates that provide nutrients for microbial growth, soil and rhizospheres are hotspots of microbial activity and are therefore extensively studied. In comparison, the microbiome of the phyllosphere is still an underdeveloped field of research (Vorholt, 2012). Importantly, the phyllosphere occupies a large extent of the plant's surface area and is subject to a range of abiotic and biotic stress factors (Whipps et al., 2008). Due to the interdependency shared by the aerial plant parts and the phyllosphere microbiome, these microorganisms also have an impact on plant yield and fitness. The plant and its microbiome are also increasingly considered as inseparable entities, as the concept of the holobiont gains traction (Berg et al., 2020; Simon et al., 2019). It is therefore extremely insightful to know of possible changes to the phyllosphere microbiome when developing a new plant protection strategy.

3. Scientific Rationale

The global population is estimated to grow and reach 10 billion by 2050. Heavy reliance on a few crops to feed the world's growing population, combined with fluctuations in yield over seasons and increased uncertainty due to climate change has hence created the need to improve the efficiency of food production and meet global demands for food and nutrient security (DK Ray, 2012). Although a multitude of effective plant protection methods exist today, there is still a need for new and innovative methods which are also sustainable to combat plant diseases in the wake of climate change. This shift is also driven by legislative initiatives and regulations that limit the use of chemicals and promote alternative avenues for disease management. In that regard, EU directives such as 2009/128/EC and 2019/782 aim to reduce dependency on pesticides and promote the use of alternative approaches for plant protection. The common agricultural policy (CAP) in the European Union also aims to achieve economic, environmental and social sustainability through sustainable farming practices. The United Nations Sustainable Development Goals (UN SDGs) also encourage this new direction for greener alternatives in agriculture.

Except for Feng et al., 2025, most SIGS studies for *F. graminearum* control have only tested the effect of dsRNA synthesized through *in-vitro* transcription methods. The use of *in-vivo* produced dsRNA for SIGS is a relatively new development in the field of RNA-based plant protection that arose from the necessity to produce large quantities of dsRNA at a lower cost. A common method developed for large-scale production of dsRNA is IPTG-induced *in-vivo* production in HT115-DE3 *Escherichia coli* cells transformed with a T7-promoter expressing recombinant plasmid. This methodology for microbial production was initially developed for application in vertebrate and invertebrate systems, eventually being adopted to produce dsRNA for insect pest and microbial pathogen control in plants (Ahn et al., 2019; Newmark et al., 2003; Solis et al., 2009). Incorporating microbially produced dsRNA in this study therefore allows testing of whether dsRNA can cause pathogen reduction irrespective of production method.

Another critical aspect that this study aims to address is the ability of SIGS to reduce *F. graminearum* infection in intact spikes of wheat and barley. For the most part, previous SIGS studies for FHB control have

applied dsRNA sprays on detached plant tissues such as leaves, coleoptiles and spikes. While these provide essential proof-of-concept for SIGS against *F. graminearum*, the study of SIGS under conditions that closely mimic the natural spread of FHB is still largely lacking.

As elaborated in the previous section, the plant and its microbiome are inextricably intertwined with each other for growth and survival. Such hyper-dependent relationships shared by the plant with its microbiome, and especially the phyllosphere, mean that disturbing the microbiome can have great implications on plant health and the progression of disease. It is therefore imperative to understand what effect spraying dsRNA will have on the microbial communities of the plant host.

The results of this study aim to bridge these gaps in turning SIGS into a commercially viable technology for plant disease control.

4. Aims and Objectives

Against the mentioned background, the main aim of this thesis was to develop spray-induced gene silencing (SIGS) as a practical and sustainable disease management strategy for controlling economically relevant pathogens like *F. graminearum* and *P. infestans*. For this, we chose spring wheat and spring barley plants as hosts for *F. graminearum* and potato as the host for *P. infestans*.

The primary aim of the thesis was further divided into the following research objectives.

1. Evaluate the application of SIGS as an effective alternative strategy for Fusarium head blight control in intact wheat and barley spikes (Paper I)
2. Develop a protocol for *in-vitro* and *in-vivo* production of dsRNA for use in plant disease and gene function studies (Paper III)
3. Investigate the effect of dsRNA on host leaf microbiota in *F. graminearum*-wheat/barley and *P. infestans*-potato pathosystems. (Papers II and IV)

5. Results and Discussion

The specific research objectives of the thesis have been addressed in four papers. Paper I covers the first objective, which aims to evaluate the effectiveness of SIGS for Fusarium head blight control. Paper III covers the dsRNA production methods that were developed for use in the other three studies and corresponds to the second objective of this thesis. The third research objective concerning the microbiome changes associated with dsRNA spraying is addressed below in Papers II and IV.

5.1 *In-vivo* dsRNA targeting *F. graminearum* genes reduces FHB disease progression in intact wheat and barley spikes (Paper I)

Multiple studies have previously proved that spraying dsRNA targeting important *F. graminearum* genes can reduce pathogen infection in detached wheat and barley tissues. Building on that, we have shown in this study that *in-vivo* dsRNA targeting *F. graminearum* can cause FHB disease reduction in intact wheat and barley spikes (Paper I). To this end, dsRNAs targeting six *F. graminearum* genes were produced in HT115-DE3 *E. coli* cells following the protocol developed in Paper III. The target genes included *FgGT2*, *FgCON7*, *FgARB1*, *FgGCN5*, *FgPKS2* and *FgStuA*. *FgGT2* (FGSG_00702) is a gene that encodes a predicted type-2 glucosyltransferase. An orthologue of the gene has been characterized in *Zymoseptoria tritici* and identified in other filamentous ascomycetes. In a previous study, deleting the gene resulted in reduced radial growth of hyphae on solid agar and unsuccessful infection in wheat spikes. Thus, the gene plays a role in the extension of hyphae on solid surfaces, thereby aiding fungal pathogenicity on host plants. Moreover, the study found orthologues of the gene to be absent in ascomycete yeasts, making this gene target specific to *Fusarium spp.* and filamentous ascomycetes (King et al., 2017). *CON7* is a transcription factor initially characterized in *Magnaporthe oryzae* as a regulator of conidiogenesis and morphological changes associated with fungal infection. The deletion of the gene orthologue in *F. graminearum* (*FgCON7*, FGSG_04134) resulted in reduced asexual and sexual growth, defective conidial production and reduced virulence (Shin et al., 2024).

FgARB1 (FGSG_02025) is an ATP-binding cassette (ABC) transporter gene potentially involved in the regulation of the MAPK pathway in *F. graminearum*. Gene mutants displayed reduced infective growth within plant tissues caused by impaired cell wall integrity as well as reduced DON production. This highlights the importance of the gene in *F. graminearum* pathogenicity (Yin et al., 2018). *FgGCN5* (FGSG_00280) is a putative histone acetyltransferase. Gene mutants showed reduced asexual and sexual growth, conidiogenesis and DON production, emphasizing the central role of *FgGCN5* in *F. graminearum* growth and pathogenicity (Kong et al., 2018). *FgPKS2* (FGSG_04694) is a gene encoding a polyketide synthase and is expressed during active mycelial growth in *F. graminearum* (Gaffoor et al., 2005). Polyketide synthases are an important class of enzymes responsible for secondary metabolite production in *F. graminearum* and other fungi. *FgStuA* (FGSG_10129) is a transcription factor with a central role in developmental regulation in *F. graminearum* (Fan et al., 2020). Deletion of the gene previously resulted in impairment of conidiogenesis, pathogenicity and secondary metabolite production (Lysøe et al., 2010). The multifunctional roles of the selected target genes in *F. graminearum* growth and pathogenicity underscore their potential as successful pathogen control targets.

Figure 6. FHB disease progression in wheat spikes sprayed with *in-vivo* dsRNA. Images taken at 10 dpi. (Photograph: Poorva Sundararajan)

The whole plant assays were conducted under controlled climatic conditions in the Biotron facility at SLU Alnarp, as elaborated in Paper I. The experimental set-up included three controls: Water, an empty plasmid (L4440) and a non-specific dsRNA (Nsp), in addition to the six targeted dsRNAs. Plants were sprayed with *in-vivo* dsRNA, followed by spray inoculation with *F. graminearum* PH-1 conidia at 24 hours post-spray (hps). The experiment was performed with 6 – 7 replicates per treatment in each trial. The development of disease was followed on the main tiller from four to nine days post inoculation (dpi), and the progression of infection was then plotted as the area under the disease progression curve (AUDPC) to evaluate the effect of targeted dsRNA in reducing *F. graminearum* spread (Simko, 2021).

Spring wheat plants sprayed with dsRNA targeting *F. graminearum* displayed reduced disease for two of the six genes tested (Figure 1 in Paper I). FHB symptoms at 10 dpi and AUDPC plots revealed that dsRNA targeting *FgARB1* and *FgStuA* had significantly lower disease progression compared to the controls ($n = 20$, Tukey's HSD, $p < 0.05$) (**Figure 6**, Figure 1 in Paper I).

Figure 7. FHB disease progression in barley spikes sprayed with *in-vivo* dsRNA. Images taken at 10 dpi. (Photograph: Poorva Sundararajan)

Targeted dsRNA was also able to reduce *F. graminearum* disease in intact barley spikes. Plants sprayed with dsRNA *FgGT2* displayed fewer FHB symptoms and significantly lower disease progression compared to the controls ($n = 20$, Tukey's HSD, $p < 0.05$) (**Figure 7**, Figure 2 in Paper I).

The FHB restricting efficacy of dsRNA targeting *FgARB1* and *FgStuA* in wheat and *FgGT2* in barley together show promise for dsRNA spray-mediated *F. graminearum* control of disease spread in intact wheat and barley heads. However, the results of the whole plant assays revealed that not all targeted dsRNAs are equally effective in reducing disease. Other targeted dsRNAs showed AUDPC values comparable to controls in both hosts (Tukey's HSD, $p > 0.05$). This is in accordance with other RNAi studies that tested multiple dsRNA targets for pathogen and pest control and found variable control efficacies (H. Cheng et al., 2024; Kim et al., 2023; van Rijn et al., 2024).

This failure to suppress the pathogen by some of the selected gene targets could stem from a variety of factors. The choice of dsRNA target and sequence impacts the extent of disease control. Kalyandurg et al., 2021 reported no significant reduction in *P. infestans* growth on detached potato leaves treated with dsRNA *PiOSBP*, although downregulation of transcript accumulation was confirmed. It is interesting to note that *PiOSBP* is an agrochemical target with effectiveness against *P. infestans*, showing that targeting the same gene through dsRNA as opposed to chemical control may not always lead to favourable results. In another study, RNAi constructs targeting different regions of the beta-tubulin gene in *F. asiaticum* showed different effects on asexual growth and virulence (Gu et al., 2019), highlighting the importance of selecting optimal target sites in the gene of interest. Koch et al., 2019 reported that CYP-C dsRNA expressing Arabidopsis and barley plants did not display reduced disease lesions, while all other CYP single and double dsRNA construct expressing plants did, showing that the choice of targets impacts the silencing outcomes. Another factor that could influence disease control outcomes includes the size of the dsRNA tested. All dsRNAs targeted in this study have similar lengths of around 310 nt. However, a previous study showed that shorter dsRNAs (200-500 nt) can produce stronger silencing and disease suppression than longer (800 nt) or full-length constructs (Höfle et al., 2020). This raises the possibility that altering the lengths of the failed target genes in this study might improve disease suppression.

Another aspect possibly influencing the extent of dsRNA efficacy surrounds the factors influencing dsRNA uptake by the plant and/or pathogen. Qiao et al., 2021 reported significantly different RNA uptake efficiencies among different fungal and oomycete pathogens tested. In the future, it will therefore be intriguing to explore if the different dsRNAs tested in this study show diverse levels of uptake into the host and pathogen, thereby explaining the inconsistent disease control efficacies. Additionally, pathogens possess genes that usually have multiple functions during growth and infection, and are regulated by a tight network of genes. Such complex regulatory networks could therefore mean a disease reduction phenotype is not achieved even though the gene is silenced. Integrating the different considerations discussed above during target design can therefore potentially alleviate uncertainties surrounding the effectiveness of SIGS-based disease control.

Another important observation from the whole plant assays in this study was the difference in effectiveness between the different dsRNA targets in the two hosts. While dsRNA targeting *FgARB1* was effective in reducing FHB in wheat, it did not significantly reduce FHB in barley. Conversely, dsRNA targeting *FgGT2* was found to effectively reduce FHB in barley, but not in wheat. The dsRNAs used in this study were produced *in-vivo* through microbial production, meaning the bacterial background leftover in the dsRNA could influence the varied dsRNA efficacy in the two hosts. To ascertain if the *in-vivo* production had any impact, *in-vitro* synthesized dsRNA was tested instead to check if similar trends were observed. Spraying *in-vitro* dsRNA on intact wheat and barley spikes yielded similar differential efficacies (Figure 3 in Paper I). This consistent difference in efficacy observed between the hosts emphasizes that host biology and not the method of dsRNA production is the dominant explanatory factor. These findings therefore lead to the hypothesis that the plant host plays a role in determining dsRNA efficacy. The results also suggest that SIGS outcomes may not always be transferable across hosts.

5.1.1 The way forward

Results from the whole plant experiments using both *in-vivo* and *in-vitro* dsRNA (Paper I) showed that dsRNA efficacy is influenced by molecular components in the host. To investigate this further, an RNA-seq experiment was set up to look into transcriptomic changes associated with SIGS of both

effective and ineffective dsRNAs. Spikes and flag leaves of wheat and barley plants were subject to dsRNA spray and *F. graminearum* inoculation, followed by mRNA sequencing to reveal gene expression patterns.

Transcriptomic analysis (preliminary) further indicated that wheat and barley respond differently at the molecular level to dsRNA treatments during *F. graminearum* infection. The RNA-seq data revealed distinct host-specific gene expression patterns associated with different dsRNA candidates, suggesting that dsRNA application is not transcriptionally neutral in the host. Differential regulation of genes involved in defense signaling, stress responses, and primary metabolism points towards host-mediated processes contributing to disease outcomes following SIGS treatment. These differences were particularly evident when comparing dsRNA candidates that were effective in reducing disease symptoms with those that showed limited efficacy. Together, these observations support the notion that SIGS performance is influenced not only by the choice of pathogen target gene but also by host-specific molecular responses to dsRNA application. While the transcriptomic data are associative in nature and do not establish direct causality, they provide important insight into potential host factors that may shape SIGS efficacy and highlight the need for further mechanistic studies to disentangle host and pathogen contributions to RNA-based disease control.

5.2 Effects of dsRNA spray on the plant microbiome (Papers II and IV)

SIGS has been established as an effective and promising tool for controlling plant diseases caused by several filamentous pathogens. However, the translation of the methodology for practical applications and field adoption involves assessing several other facets of SIGS (C. Chen et al., 2025; Kalyandurg et al., 2021; Koch & Wassenegger, 2021; Šečić & Kogel, 2021; van Rijn et al., 2024). In this study, the impact of dsRNA sprays on the phyllosphere microbiome of plants was tested. The investigations were expanded to two pathosystems previously studied for SIGS – *F. graminearum* in cereal hosts wheat and barley, and *P. infestans* in potato.

Developments in the field of genomics have resulted in the use of high-throughput sequencing technologies that utilize the 16S rRNA and ITS gene sequences of bacteria and fungi/oomycetes to precisely distinguish and

identify microbial taxa. Changes to the bacterial and fungal microbes present in the phyllosphere were tested using 16S rRNA and ITS amplicon sequencing. The experimental set-up for the studies involved no dsRNA control treatments (mock and water), a non-specific dsRNA, and two targeted dsRNAs in each study. A subset of plants was also inoculated with the corresponding pathogen to ascertain changes in microbial interactions upon pathogen infection.

A variety of metrics are commonly used to understand microbial community characteristics (Y. X. Liu et al., 2021). A detailed explanation of the different metrics employed, and the changes observed are elaborated in Papers II and IV. A more concise summation highlighting results relevant to the third research objective of this thesis is detailed below.

5.2.1 Impact on phyllosphere bacteria

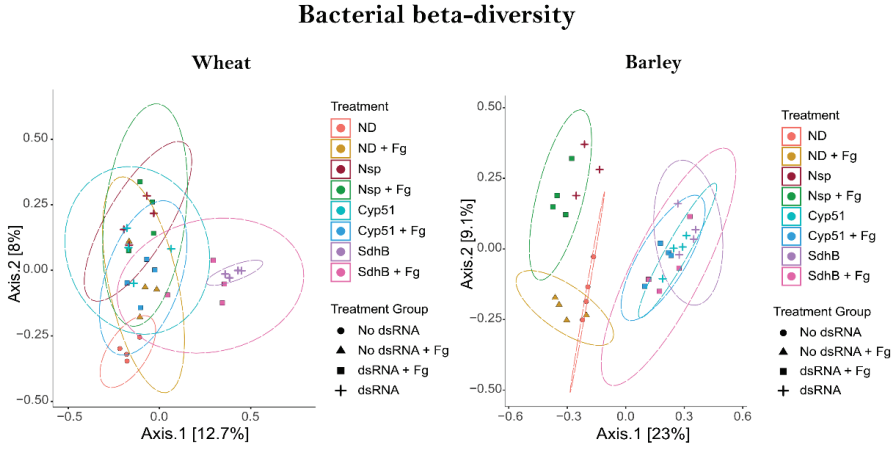

Figure 8. Beta-diversity of bacterial communities in wheat and barley based on Bray-Curtis dissimilarity distance. Treatments are indicated by colour and treatment groups are indicated by shape. PERMANOVA followed by Adonis showed significant differences ($p < 0.05$ in wheat and barley) between samples. (Adapted from Paper II)

Beta-diversity plots are common tools to visualize differences in composition structure between samples. PCoA plots based on Bray-Curtis dissimilarity distances showed differential effects in the three hosts tested. Clear separation of one (Cyp51) or both (Cyp51 and SdhB) targeted dsRNA samples was observed in wheat (**Figure 8**) (Adonis, $p = 0.001$) and barley

(**Figure 8**) (Adonis, p = 0.001), respectively, whereas both control and targeted dsRNA samples clustered together in potato (Figure 1 in Paper IV) (Adonis, p = 0.001).

To further understand the differences observed through diversity indices (Figures 3 and 4 in Paper II and Figure 1 in Paper IV), composition based on the relative abundance of observed taxa was plotted. The relative abundance of the genus *Pseudomonas* increased in all dsRNA-treated plants in wheat. Furthermore, *Chryseobacterium* and *Acinetobacter* showed increased abundance in SdhB-treated plants (**Figure 9**). Alternatively, genera *Acinetobacter* and *Massilia* displayed changed abundances in SdhB- and Cyp51-treated barley plants, respectively (**Figure 9**). Taken together, these findings show that changes in bacterial diversity and composition were influenced by the choice of dsRNA and the host studied in wheat and barley. In potato, however, shifts in diversity and composition were observed relating more to the time-point studied than the dsRNA targeted (Figure 1 and Supplementary Figure 1 in Paper IV).

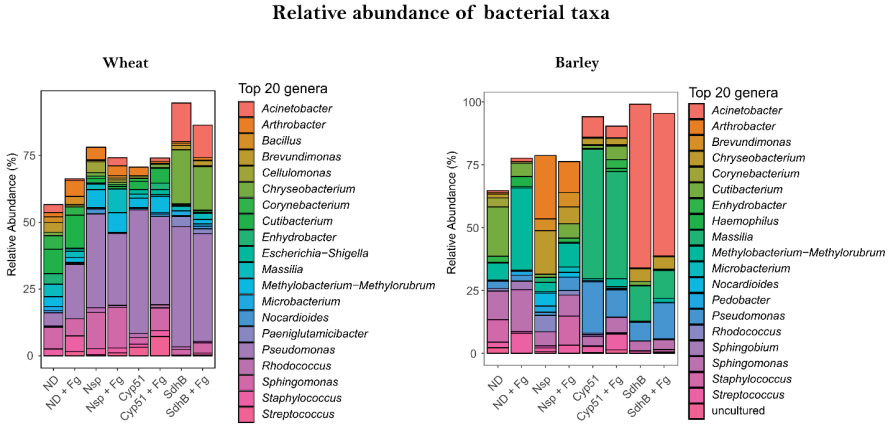

Figure 9. Composition of top wheat and barley bacterial communities across different treatments. The composition is based on the relative abundance of observed bacterial taxa. (Adapted from Paper II)

Combining the bacterial community characteristics from both Papers II and IV, it is evident that bacterial components in the phyllosphere of wheat, barley and potato are subject to only minor changes after dsRNA spraying. Additionally, bacterial taxa dominant in the phyllosphere showed less fluctuation under changed conditions and several ubiquitous microorganisms

found dominating the respective plant's indigenous phyllosphere were preserved in plants sprayed with dsRNA. Combined, these observations indicate minimal impact caused by dsRNA on the phyllosphere bacteria. Recently, wheat spikes infected with *F. graminearum* showed selective promotion of *Pseudomonas* isolates, suggesting a microbial response triggered for plant defence (Xu et al., 2025). Similarly, studies have shown that *Serratia* and *Acinetobacter* show biocontrol activity against *P. infestans* and other pathogenic *Phytophthora spp.* (Chunjiang Liu et al., 2025; Syed-Ab-Rahman et al., 2018). The enrichment of *Pseudomonas* in wheat, and *Serratia* and *Acinetobacter* in potato leaves sprayed with dsRNA point to the selection of plant beneficial bacteria that are fittest to survive the presence of infection.

However minor, the differential changes in the bacterial diversity and composition observed in the three different hosts can potentially be attributed to the stability and function of the microbiome in the individual hosts, the multi-dimensional processes that drive microbial assembly in these plants as well as short term microbiome shifts associated with response to environmental disturbances (Berg et al., 2021; de Vries et al., 2018). Such differences are also in accordance with previous studies that have reported the influence of host genotype and environmental conditions (including biotic and abiotic stress factors) in shaping microbial community assembly and ecosystem functioning (Agler et al., 2016; Berendsen et al., 2012; Sapkota et al., 2015). Further analysis using metagenomics can therefore help in understanding the basis of the dsRNA-, host- and time-dependent differences observed.

5.2.2 Impact on phyllosphere fungi

In stark contrast to the bacterial communities, significant changes to the phyllosphere fungal microbiota were not observed in wheat, barley and potato plants sprayed with dsRNA. Alpha- and beta-diversity metrics, combined with relative and differential abundance analyses, showed that fungal communities remain stable following dsRNA spray. The lack of changes could be attributed to heightened stability exhibited by the fungal microbiota when exposed to an external disturbance (de Vries et al., 2018). While dsRNA treatment did not impact fungal communities, a strong impact of pathogen infection was observed in fungal composition, as evident in **Figure 10** and Figure 2 in Paper IV. In wheat and barley leaves inoculated

with *F. graminearum*, the fungal composition changed irrespective of dsRNA treatment. In potato, all samples displayed comparable compositions, and ANCOM-BC identified taxa differentially abundant only in samples inoculated with *P. infestans*. These results are a clear indication that fungal shifts are associated with *F. graminearum* and *P. infestans* colonization and not with dsRNA.

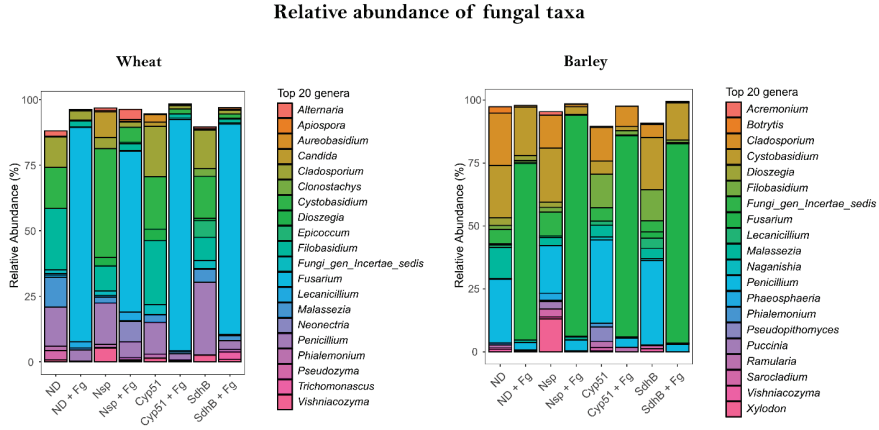

Figure 10. Composition of top wheat and barley fungal communities across different treatments. The composition is based on the relative abundance of observed fungal taxa. (Adapted from Paper II)

The data presented in Papers II and IV together paint a picture of minimal interference caused by dsRNA spray in the interactions and functioning of phyllosphere bacteria and fungi. The observations in the bacterial and fungal communities align with known differences in stability and resistance between plant-associated bacteria and fungi. The maintenance of microbial stability in these studies therefore supports the target-specificity and environmental safety of SIGS-mediated plant protection.

Continued explorations into the microbiome changes associated with dsRNA spray under varying environmental conditions and in multiple genotypes, combined with omics approaches like metagenomics and metatranscriptomics will further validate and extend the results observed in this study.

6. Conclusions

Spray-induced gene silencing is currently proposed as an effective and environmentally friendly strategy to combat pest and pathogen attack on plants. However, it is prudent to consider that SIGS and RNAi-based plant protection methods are still in their infancy. While several proof-of-concept studies demonstrating the effectiveness of SIGS for Fusarium head blight control exist, studies that closely mimic the natural conditions of disease spread are still largely lacking. In Paper I, using whole plant experiments, we have hence shown that dsRNA targeting the devastating filamentous pathogen *F. graminearum* can cause disease suppression in intact barley and wheat spikes. However, the efficacies of the tested dsRNAs vary, implying more is at play in determining desirable disease outcomes. These results support the feasibility of scaling SIGS for agricultural use and provide impetus towards a comprehensive understanding of SIGS-mediated FHB control.

The study detailed in Paper I would not have been possible without the establishment of SIGS methods as part of Paper III. The optimized methodologies for *in-vitro* synthesis and *in-vivo* production of dsRNA will therefore contribute to designing efficient SIGS studies for studying gene function and disease progression in the future.

A facet of SIGS that has long puzzled scientists is the assessment of microbiome changes associated with the methodology. Knowledge of possible microbiome changes and off-target effects will prove crucial in determining the target specificity of SIGS, thus providing more assurance for regulatory and public acceptance. In Papers II and IV, we therefore tested for the first time the impact of spraying dsRNA on the microbial communities of plants. Through studies in both monocot and dicot systems, we found that dsRNA sprays do not alter the dominant and ubiquitous bacterial and fungal inhabitants of the phyllosphere. These findings show that SIGS operates with a high degree of biological specificity and does not disrupt plant-associated microorganisms. Minor changes to the relative abundance of the bacterial communities were observed in both studies, possibly indicating a short-term response of bacterial communities to perturbations caused by dsRNA spray. These transient shifts indicate that microbiome responses to dsRNA application are limited and substantially smaller than those induced by pathogen infection itself. These findings help us decipher plant microbiome

functioning during SIGS, providing empirical support of its low risk for regulatory acceptance and advocating for its specificity and reliability in large-scale applications.

The studies making up this thesis therefore contribute to the holistic understanding of the processes that shape SIGS-mediated plant protection.

7. SIGS – Future directions

The long list of studies testing the potential of SIGS for plant disease control has paved the way to identify key knowledge gaps that are yet to be fully addressed.

- A major concern impacting the success of SIGS is the instability/degradation of dsRNAs or sRNAs sprayed. A variety of design elements, adjuvants and formulations have been proposed to tackle this question. However, studies report inconsistencies in their effect, revealing opportunities for more research in this field.
- Uptake of dsRNA by plants and pathogens can also skew SIGS disease outcomes. Nanotechnology strategies have been popular to create efficient dsRNA delivery systems, such as through the use of LDH nanosheets, carbon dots, carbon nanotubes, as well as chitosan- and silica-based nanoparticles. Studies show promise in such delivery methods extending the effect of SIGS. The use of nanotechnology therefore provides untapped potential that can be exploited to improve the efficacy and duration of SIGS.
- The molecular mechanisms that regulate the transport of dsRNA and sRNA cargo have also attracted attention recently. sRNAs can be transported bidirectionally (from plant to pathogen and vice versa) through encapsulation in extracellular vesicles (EVs) or through other apoplastic routes. The sRNAs are then transported bilaterally through clathrin-mediated endocytosis (CME). Additionally, mechanisms independent of CME exist in eukaryotes for the uptake of RNAs. Further experimental proof to characterize the CME-dependent and independent pathways in different plant pathosystems can better define the mechanisms of sRNA transport.
- The organismal and cellular locations of dsRNA processing into sRNAs are also unknown. More studies need to be conducted to draw a full picture of the processing of sprayed dsRNAs. This, in turn, will help design better dsRNA targets.

While all the questions mentioned above will help expand our knowledge on SIGS, addressing the degradation and delivery of dsRNAs will undeniably aid in the faster transition of SIGS to agricultural fields.

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Popular science summary

Wheat, barley and potato are important staple crops worldwide and in Sweden. However, their production is threatened by conditions such as heat, drought, and invasion by insect pests and harmful microbes. The increasing problem of food insecurity, combined with unpredictable weather caused by climate change, makes it vital to secure the production of our major food crops. This also involves diversifying and improving methods for plant disease control. Common methods currently used for plant protection against insects and harmful microbes include the use of chemical pesticides and fungicides. Although these treatments can be highly effective, extensive use of agrochemicals has been shown to pose risks to human and animal health. They can also cause biodiversity loss and soil degradation, thereby negatively affecting ecosystems. In addition, target organisms can also develop strategies to evade the effects of agrochemicals over time, making agrochemicals less impactful. Alternatively, plant breeding has been used to improve crop quality to increase resistance to diseases. While effective, breeding approaches are slow to develop and implement.

In the quest for more sustainable plant protection strategies, researchers have investigated whether RNA-based methods can be used to control pathogens. Both plants and pathogens naturally produce double-stranded RNA (dsRNA) and small RNA (sRNA) molecules that can suppress specific genes in the other organism. This bidirectional exchange of RNA molecules has been harnessed for plant protection by spraying dsRNA to silence pathogen genes, a method termed spray-induced gene silencing (SIGS).

Fusarium head blight (FHB) is a common disease in cereals like wheat, barley, rye and maize, and is caused by the fungus *Fusarium graminearum*. The pathogen causes bright-like symptoms that affect both grain yield and quality. In this thesis, we show that SIGS can successfully control FHB in wheat and barley.

The bacteria, fungi and other micro-organisms that inhabit plants and support them collectively form the plant microbiome. The microbiome plays a significant role in maintaining plant health through interactions among its members and with the plant, particularly during defence against pathogens. As part of developing SIGS into a sustainable disease control strategy, we investigated the effects of dsRNA spraying on the plant microbiome. Our results show that external dsRNA application does not alter the dominant

bacterial and fungal members of the leaf microbiome in wheat, barley and potato. Minor changes in relative abundance of bacterial communities were observed, while much stronger effects were caused by pathogen infection. This indicates that pathogen presence, rather than dsRNA treatment, has the greatest impact on the plant leaf microbiome.

Together, these findings demonstrate the effectiveness of SIGS for plant disease control and show that it has minor effects on the plant microbiome. This highlights the environemental safety of the method and brings it one step closer to practical use in agricultural fields.

Populärvetenskaplig sammanfattning

Vete, korn och potatis är viktiga basgrödor både globalt och i Sverige. Deras produktion hotas dock av faktorer som värme, torka samt angrepp av insekter och skadliga mikroorganismer. Den ökande livsmedelsosäkerheten, i kombination med mer oförutsägbart väder till följd av klimatförändringar, gör det nödvändigt att säkra produktionen av våra viktigaste grödor. Detta kräver också att växtskyddsmetoder utvecklas och breddas.

Vanliga metoder för att skydda grödor mot insekter och mikrobiella angrepp är kemiska bekämpningsmedel och fungicider. Även om dessa ofta är effektiva har omfattande användning av sådana kemikalier visat sig medföra risker för människors och djurs hälsa. De kan även bidra till förlust av biologisk mångfald och försämrad jordkvalitet, vilket i sin tur påverkar ekosystem negativt. Dessutom kan skadegörare med tiden utveckla resistens, vilket gör bekämpningsmedlen mindre effektiva. Ett alternativ är växtförädling, där grödor förbättras för att bli mer motståndskraftiga mot sjukdomar. Dessa metoder är effektiva, men ofta tidskrävande att utveckla och införa.

I arbetet med att ta fram mer hållbara växtskyddsstrategier har forskare undersökt om RNA-baserade metoder kan användas för att kontrollera växtpatogener. Både växter och patogener producerar naturligt dubbelsträngat RNA (dsRNA) och små RNA-molekyler (sRNA), som kan stänga av specifika gener hos den andra organismen. Detta ömsesidiga utbyte av RNA har utnyttjats inom växtskydd genom att spraya dsRNA för att hämma viktiga gener hos patogener, en metod som kallas sprayinducerad gensläckning (spray-induced gene silencing, SIGS).

Axfusarios (Fusarium head blight, FHB) är en vanlig sjukdom i spannmålsgrödor som vete, korn, råg och majs, och orsakas av svampen *Fusarium graminearum*. Sjukdomen ger axskador som försämrar både skördens mängd och kvalitet. I denna avhandling visar vi att SIGS effektivt kan användas för att kontrollera axfusarios i vete och korn.

De bakterier, svampar och andra mikroorganismer som lever på och i växter utgör tillsammans växtens mikrobiom. Mikrobiomet spelar en viktig roll för växtens hälsa genom samspel både mellan mikroorganismerna och med växten, särskilt vid försvar mot sjukdomar. För att utveckla SIGS till en hållbar strategi för sjukdomsbekämpning har vi därför undersökt hur dsRNA-besprutning påverkar växternas mikrobiom. Våra resultat visar att

dsRNA inte förändrar de dominerande bakterie- och svampgrupperna i bladens mikrobiom hos vete, korn och potatis. Mindre förändringar i bakteriesammansättningen observerades, medan betydligt större effekter orsakades av själva patogenangreppet. Detta visar att det främst är sjukdomsinfektioner, och inte dsRNA-behandlingen, som påverkar växternas bladmikrobiom.

Sammantaget visar dessa resultat att SIGS är en effektiv metod för sjukdomsbekämpning och att den har minimal påverkan på växternas mikrobiom. Detta understryker metodens säkerhet och innebär ett viktigt steg mot framtida användning i jordbruket.

பொதுஅறிவியல் சுருக்கம்

கோதுமை, பார்லி மற்றும் உருளைக்கிழங்கு ஆகியவை உலகம் முழுவதிலும், குறிப்பாக ஸ்வீடனிலும் பிரதானமான உணவுப் பயிர்களாகும். வெப்பம், வறட்சி, பூச்சி தாக்குதல்கள் மற்றும் தீங்கு விளைவிக்கும் நுண்ணுயிர்கள் போன்ற காரணிகள் இவ்வகைப் பயிர்களின் உற்பத்தியை அதிகமாகப் பாதிக்கின்றன. காலநிலை மாற்றத்தால் ஏற்படும் கணிக்க முடியாத வானிலை மாற்றங்களாலும், அதிகரித்து வரும் உணவுப் பாதுகாப்பு பிரச்சினைகளாலும், இந்த முக்கிய உணவுப் பயிர்களின் உற்பத்தியை பாதுகாப்பது அவசியமாகிறது. தற்போது தாவரங்களைப் பாதுகாக்க அதிகமாகப் பயன்படுத்தப்படும் முறைகளில் வேதியியல் பூச்சிக்கொல்லிகள் மற்றும் பூஞ்சை நாசினிகள் அடங்கும். இவை பல சமயங்களில் திறமையானவையாக இருந்தாலும், அதிக அளவில் பயன்படுத்தப்படும்போது, மனித மற்றும் விலங்குகளின் ஆரோக்கியத்திற்கு ஆபத்தை விளைவிக்கின்றன. மேலும், உயிரியல் பல்வகைமையைக் குறைக்கவும், மண்ணின் தரத்தைப் பாதிக்கவும், சுற்றுச்சூழல் சமநிலையில் எதிர்மறைத் தாக்கத்தையும் ஏற்படுத்துகிறது. காலப்போக்கில், பூச்சிகள் மற்றும் நோய்க்கிருமிகள் இந்த வேதியியல் மருந்துகளுக்கு எதிராக எதிர்ப்பு சக்தியை உருவாக்குவதால் அவைகளின் தாக்கம் குறைகிறது. இதற்கு மாறாக தாவர இனப்பெருக்கம் மூலம் தரமான மற்றும் நோய் எதிர்ப்புத திறன் கொண்ட பயிர்களை உருவாக்கும் முறைகளும் பயன்படுத்தப்படுகின்றன. ஆனால் இவை பயனுள்ளதாக இருந்தாலும், உருவாக்கவும் நடைமுறைப்படுத்தவும் அதிக காலம் எடுக்கின்றது. நிலைத்தன்மை கொண்ட தாவர பாதுகாப்பு முறைகளை உருவாக்கும் நோக்கில், ஆராய்ச்சியாளர்கள் RNA அடிப்படையிலான அணுகு முறைகளை ஆராய்ந்துள்ளனர். தாவரங்களும், நோய்க்கிருமிகளும் இயற்கையாகவே தங்களது இரட்டை இழை RNA (dsRNA) மற்றும் சிறிய RNA களை (sRNA) உற்பத்தி செய்கின்றன. இவை ஒருவருக்கொருவரின் குறிப்பிட்ட மரபணுக்களின் செயல்பாட்டை அடக்க முடியும். இந்த இருதிசை இயற்கை செயல்முறையைப் பயன்படுத்தி, நோய்க்கிருமி மரபணுக்களை அடக்க dsRNA–ஐ தெளிக்கும் முறை, “spray-induced gene silencing” (SIGS) என அழைக்கப்படுகிறது.

Fusarium head blight (FHB) என்பது கோதுமை, பார்லி, கம்பு மற்றும் மக்காச்சோளம் போன்ற தானியப் பயிர்களில் காணப்படும் பொதுவான நோயாகும். Fusarium graminearum என்ற பூஞ்சையால் இந்நோய் ஏற்படுகிறது. மேலும் இது கதிர்களை பாதித்து, விளைச்சலின் அளவையும் தரத்தையும் குறையச் செய்கிறது. இந்த ஆய்வில், SIGS முறையைப் பயன்படுத்தி இந்த நோயை கோதுமை மற்றும் பார்லியில் வெற்றிகரமாக கட்டுப்படுத்த முடியும் என்பதைக் காட்டியுள்ளோம்.

தாவரங்களில் வாழும் பாக்டீரியா, பூஞ்சைகள் மற்றும் பிற நுண்ணுயிர்கள் அனைத்தும் சேர்ந்து தாவர மைக்ரோபையோம் (Microbiome) என அழைக்கப்படுகின்றன. இந்த மைக்ரோபையோம் தங்களுடைய இணைந்த செயல்பாடுகளால் தாவர ஆரோக்கியத்தைப் பேணுவதிலும், நோய்க்கிருமிகளுக்கு எதிரான பாதுகாப்பை அளிப்பதிலும் முக்கிய பங்கு வகிக்கிறது.

ஆகவே SIGS ஐ ஒரு நிலையான நோய்க்கட்டுப்பாட்டு முறையாக உருவாக்கும் நோக்கில், dsRNA தெளிப்பு தாவர மைக்ரோபையோமில் ஏற்படுத்தும் விளைவுகளை ஆய்வு செய்தோம். dsRNA தெளிப்பு, கோதுமை, பார்லி மற்றும் உருளைக்கிழங்கின் இலை மைக்ரோபையோமில் உள்ள முக்கிய பாக்டீரியா மற்றும் பூஞ்சை சமூகங்களை மாற்றவில்லை என்பதையும், பாக்டீரியா பல்வகைமையில் சிறிய மாற்றங்கள் மட்டுமே ஏற்பட்டுள்ளன என்பதையும் முதல் முறையாக எங்கள் முடிவுகள் காட்டுகின்றன. ஆனால் நோய்க்கிருமி தொற்றுகளில் குறிப்பிடத்தக்க மாற்றங்களை ஏற்படுத்தின. இதன் மூலம் dsRNA தெளிப்பான்களை விட நோய்க்கிருமி தொற்றுகளே தாவர மைக்ரோபையோமை அதிகமாக பாதிக்கிறது என்பது தெளிவாகிறது. இந்த முடிவுகள், SIGS முறையின் நோய்க்கட்டுப்பாட்டு திறனையும், தாவர மைக்ரோபையோமில் ஏற்படுத்தும் குறைந்த தாக்கத்தையும் ஒருங்கே வெளிப்படுத்துகின்றன. இதன் மூலம், இந்த முறை சுற்றுச்சூழலுக்கு பாதுகாப்பானது என்பதும், எதிர்காலத்தில் விவசாய வயல்வெளிகளில் பயன்படுத்துவதற்கான ஒரு முக்கிய முன்னேற்றப்படியாக உள்ளது என்பதும் முன்னிலைப்படுத்தப்படுகிறது.

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The impact of spray-induced gene silencing on cereal phyllosphere microbiota

Poorva Sundararajan[1†], Samrat Ghosh[1†], Bekele Gelena Kelbessa[1], Stephen C. Whisson[2], Mukesh Dubey[3], Aakash Chawade[1] and Ramesh Raju Vetukuri[1*]

Abstract

Background Fusarium head blight (FHB) is a major disease affecting cereal crops including wheat, barley, rye, oats and maize. Its predominant causal agent is the ascomycete fungus *Fusarium graminearum*, which infects the spikes and thereby reduces grain yield and quality. The frequency and severity of FHB epidemics has increased in recent years, threatening global food security. Spray-induced gene silencing (SIGS) is an alternative technique for tackling this devastating disease through foliar spraying with exogenous double-stranded RNA (dsRNA) to silence specific pathogen genes via RNA interference. This has the advantage of avoiding transgenic approaches, but several aspects of the technology require further development to make it a viable field-level management tool. One such existing knowledge gap is how dsRNA spraying affects the microbiota of the host plants.

Results We found that the diversity, structure and composition of the bacterial microbiota are subject to changes depending on dsRNA targeted and host studied, while the fungal microbiota in the phyllosphere remained relatively unchanged upon spraying with dsRNA. Analyses of fungal co-occurrence patterns also showed that *F. graminearum* established itself among the fungal communities through negative interactions with neighbouring fungi. Through these analyses, we have also found bacterial and fungal genera ubiquitous in the phyllosphere, irrespective of dsRNA treatment. These results suggest that although rarer and less abundant microbial species change upon dsRNA spray, the ubiquitous bacterial and fungal components of the phyllosphere in wheat and barley remain unchanged.

Conclusion We show for the first time the effects of exogenous dsRNA spraying on bacterial and fungal communities in the wheat and barley phyllospheres using a high-throughput amplicon sequencing approach. The results obtained further validate the safety and target-specificity of SIGS and emphasize its potential as an environmentally friendly option for managing Fusarium head blight in wheat and barley.

Keywords Microbiome, Amplicon sequence variants (ASVs), *Fusarium graminearum*, Spray induced gene silencing (SIGS), Double-stranded RNA (dsRNA), Phyllosphere, Wheat, Barley

[†]Poorva Sundararajan and Samrat Ghosh contributed equally to this work.

*Correspondence:
Ramesh Raju Vetukuri
Ramesh.Vetukuri@slu.se

[1]Department of Plant Breeding, Swedish University of Agricultural Sciences, Alnarp, Sweden
[2]Cell and Molecular Sciences, The James Hutton Institute, Invergowrie, Dundee, UK
[3]Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden

Background

Wheat (*Triticum* spp.) and barley (*Hordeum vulgare*) are major cereal crops grown for food and feed worldwide [1, 2]. In 2022, 154 million tonnes of barley and 808 million tonnes of wheat were produced around the world, underscoring their importance as primary crops [3]. Unfortunately, their production is hampered by several diseases and pests [4] including Fusarium head blight (FHB). It is mainly caused by the ascomycete fungus *Fusarium graminearum* Schwabe [5], which grows best in warm and humid or semi-humid regions [6, 7]. FHB is one of the most destructive fungal crop diseases and causes billions of dollars of losses of wheat and barley [8–11]. In addition to yield losses, FHB-causing fungi promote the accumulation of toxic secondary fungal metabolites (mycotoxins) such as deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZEA) that significantly reduce grain quality [12, 13]. The mycotoxin DON is most frequently detected in food wheat and barley, and is harmful to human, animal, and ecosystem health [14–16]. Due to ongoing global climate change and changes in cropping systems, the frequency and severity of FHB epidemics have increased in recent years, posing challenges to human food security, animal nutrition, and the international grain trade [5, 17, 18].

Several disease control strategies have been used to mitigate the increasing threat of FHB and mycotoxin accumulation in grains, including cultural practices, biological control [19], induction of host resistance [5, 20], precision genome editing with CRISPR/Cas9 [21], and foliar spraying with fungicides [22]. RNAi-based strategies such as host-induced gene silencing (HIGS) have been reported to reduce crop losses caused by fungi, oomycetes, nematodes, and insect pests [23–27]. However, because HIGS involves the host-expression of hairpin RNAs (hpRNAs) or small RNAs (sRNAs) targeting genes in the interacting pathogen, its practical utility is limited by several factors including the limited transformability of various crops and the poor acceptance of genetically modified (GM) crops by many consumers [28]. These problems motivated the development of an alternative strategy that requires no genetic modification: spray-induced gene silencing, or SIGS [29]. This strategy involves spraying leaves with double-stranded RNAs (dsRNA) or sRNAs to specifically silence selected pathogen genes. The potential of SIGS as a tool for managing fungal and oomycete diseases and insect pests has been successfully demonstrated through several studies [26, 28, 30–36]. For example, one study showed that *F. graminearum* can take up exogenous dsRNA and that spraying detached barley leaves with dsRNA targeting the *F. graminearum CYP51A*, *CYP51B*, and *CYP51C* genes reduced the incidence and severity of infection [37]. Another study showed that using SIGS to target *TRI6*, a transcription factor involved in DON biosynthesis in *F. graminearum*, reduced FHB infection and DON levels in wheat heads inoculated under greenhouse conditions [38]. A third study demonstrated that targeting key components of the fungal RNAi machinery with SIGS reduced barley infection by *F. graminearum* [39]. SIGS has thus shown great potential for minimizing crop losses caused by filamentous pathogens.

Despite these promising results, to turn it into a practical disease management strategy, several facets of SIGS still need to be understood. Besides the disease reduction, the broader effects of spraying dsRNA on the host, such as the effect on the phyllosphere microbiome have received little attention. The phyllosphere (aerial habitat) is influenced by the plant and houses an intricate, dynamic and heterogeneous microbial community consisting primarily of bacteria, filamentous fungi, yeasts, algae and protozoans [40, 41]. The diversity and composition of these microbial communities are also sensitive to several factors that interact over space and time, including crop protection measures (e.g., pesticide treatment), synthetic fertilizers, environmental factors and host genotypes [42–45]. Conversely, several studies have also illustrated that microbial communities can enhance the host-plant's growth, health, and tolerance to abiotic and biotic stresses. This is achieved through various mechanisms, including secretion of growth-promoting phytohormones, enhancement of nutrient availability, secretion of secondary metabolites that are toxic to pathogenic microbes, and induction of systemic acquired resistance [46–49]. Therefore, it is imperative to ascertain changes to the host microbiome when developing new plant protection approaches. This can be done by exploiting recent advances in high-throughput sequencing and other meta-omic techniques that have facilitated the profiling of microbial communities and their functions in various crops, including wheat and barley [50, 51]. In this study, we sought to assess if dsRNA affects the microbial communities of the phyllosphere and how its effects on these interactions change upon *F. graminearum* infection. Our initial hypothesis was that dsRNA would not significantly alter the phyllosphere microbial communities in wheat and barley. To test this hypothesis, we used high-throughput amplicon sequencing techniques to characterize the diversity, structure and composition of the phyllosphere microbiota before and after spraying plants with dsRNA. For this purpose, two *F. graminearum* genes that are essential for FHB disease progression and are targeted by fungicides were utilized to synthesize dsRNA: cytochrome P450 lanosterol C-14α-demethylase (*FgCyp51A*, *FgCyp51B* and *FgCyp51C*) [52] and succinate dehydrogenase B subunit (*FgSdhB*) [53]. We also assessed the effects of *F. graminearum* inoculation on phyllosphere microbial composition after dsRNA spraying by

comparing diversity metrics for the microbial communities of non-inoculated and inoculated plants.

Materials and methods

Plant and fungal material

Seeds of the spring wheat breeding line SW141580 (Lantmännen) and spring barley market cultivar Tellus were germinated in Petri dishes lined with damp Whatman filter paper to induce uniform germination. The germinated seedlings were transplanted into 9×9×8 cm pots filled with well-draining soil and grown under controlled climatic conditions with 16 h of 200 µmol/m^2/s daylight and 8 h of darkness, and day/night temperatures of 22/21°C. *F. graminearum* PH-1 was grown on potato dextrose agar (PDA) (VWR International) in Petri dishes and incubated at 19 °C for seven days. Carboxymethyl cellulose (CMC) media (7.5 g of carboxymethyl cellulose, 0.5 g of yeast extract, 0.25 g of $MgSO_4.7H_20$, 0.5 g of NH_4NO_3 and 0.5 g of KH_2PO_4 dissolved in 1 l of distilled water) was used for conidiation. Agar plugs from seven-day-old PDA cultures were used to inoculate CMC media. The inoculated CMC media was incubated at 28 °C for seven days with constant shaking and illumination to produce conidia. They were then collected by passing the culture through two layers of cheesecloth followed by centrifugation to remove media, and subsequently resuspending in sterile water. The concentration of conidia was calculated using a Fuchs-Rosenthal chamber and adjusted to 20,000 conidia/ml for plant infection.

Table 1 Primers used for in-vitro transcription of dsRNA and amplicon sequencing

Primer type	Primer Name	Primer Sequence
In-vitro dsRNA synthesis	T7 Cyp51A FW	GTAATACGACTCACTATAGGG CGGTCCATTGACAATCCCCG
	T7 Cyp51A RV	GTAATACGACTCACTATAGGG GCAGCAAACTCGGCAGTGAG
	T7 Cyp51B FW	GTAATACGACTCACTATAGGG CAGCAAGTTTGACGAGTCCC
	T7 Cyp51B RV	GTAATACGACTCACTATAGGG AGAGTTCATAAGGTGCTTCA
	T7 Cyp51C FW	GTAATACGACTCACTATAGGG ATTGGAAGCACCGTACAATA
	T7 Cyp51C RV	GTAATACGACTCACTATAGGG CATTGGAGCAGTCATAAACA
	T7 Fg SdhB FW	GTAATACGACTCACTATAGGG GGACCTTGTCCCTGATCTGA
	T7 Fg SdhB RV	GTAATACGACTCACTATAGGG GCTTCTTGATCTCGGCAATC
Amplicon sequencing	Bac_799F	AACMGGATTAGATACCCKG
	Bac_1115R	AGGGTTGCGCTCGTTG
	Fun_ITS1Kyo2F	TAGAGGAAGTAAAAGTCGTAA
	Fun_ITS86R	TTCAAAGATTCGATGATTCA

In-vitro dsRNA synthesis

RNA was extracted from mycelia collected from seven-day-old PDA plates using the RNeasy Plant Mini kit (Qiagen). First-strand synthesis was then carried out using the iScript cDNA Synthesis kit (Bio-Rad) with one microgram of the extracted RNA as the template. Primers containing the T7 promoter sequence were designed for the *FgCyp51A* (FGSG_04092), *FgCyp51B* (FGSG_01000), *FgCyp51C* (FGSG_11024) and *FgSdhB* (FGSG_05610) gene sequences using NCBI Primer-BLAST (Table 1) [54]. Polymerase chain reaction was performed using Phusion polymerase (ThermoFisher Scientific) with *F. graminearum* cDNA as the template and the dsRNA-specific T7 primers, and following the reaction conditions recommended by the manufacturer. The PCR product was purified using the QIAquick PCR Purification kit (Qiagen) before proceeding with in-vitro transcription. Double-stranded RNA was synthesized using the MEGAscript RNAi Kit (ThermoFisher Scientific) and the appropriate PCR-amplified products as templates. In addition, the control template provided with the kit was used to synthesize non-specific dsRNA to serve as a control in subsequent experiments. The control template consisted of a linearized TRIPLEscript plasmid containing the 1.85 kb Xenopus elongation factor 1α gene under the transcriptional control of tandem SP6, T7, and T3 promoters. Gel electrophoresis was performed in a 1% agarose gel to confirm synthesis of appropriate dsRNA products. The concentration of the purified dsRNA was measured using a nano-drop spectrophotometer.

Plant assay – dsRNA treatment, *F. graminearum* infection and sample collection

The *FgCyp51* dsRNA was obtained by mixing equal concentrations of the individually synthesized *FgCyp51A*, *FgCyp51B* and *FgCyp51C* dsRNAs. Four-week-old spring wheat and barley plants were sprayed with *FgCyp51*/*FgSdhB* dsRNA (10 µg of dsRNA per plant) using an airbrush and compressor (CoCraft and Biltema, respectively). Untreated plants and plants sprayed with 10 µg of non-specific dsRNA were included as experimental controls. Twenty-four hours after spraying, half the plants from each treatment were drop inoculated with 20 µl of 20,000 *F. graminearum* conidia/ml. Four biological replicates were established for each treatment. Leaf samples were collected four days after spraying using three punches from a 1.5 ml microcentrifuge tube each measuring 10.8 mm diameter and stored at -80 °C.

DNA extraction

DNA was extracted from the collected leaf samples using a modified protocol of the DNeasy PowerSoil Pro kit (Qiagen) as mentioned below. The frozen leaf samples were ground to a powder in a pre-chilled mortar and

pestle filled with liquid nitrogen, before proceeding with the recommended protocol from the manufacturer. The washing step using solution EA was repeated three times to ensure the removal of phenolic compounds.

Amplicon sequencing

Extracted DNA was sent for Amplicon sequencing (LGC Genomics). The 799F-1115R primer pair targeting the 16 S rRNA gene and the ITS1Kyo2F-ITS86R primer pair targeting the ITS gene sequence were used for bacterial and fungal amplification [55, 56], respectively. The 799F and ITS1Kyo2F primers used in this study are discriminating primers and avoid amplification of host DNA during sequencing [57, 58]. In total, 126 samples were used for amplicon sequencing. Of these, 64 samples were from wheat (32 for bacterial amplification and 32 for fungal amplification) and 62 were from barley (with 31 samples for bacteria and 31 for fungi). Each treatment consisted of four biological replicates, except the non-specific dsRNA (Nsp) treatment in barley, where only three replicates were included due to poor DNA quality leading to no amplification. The PCR reactions were performed with 1–10 ng of DNA extract in a total volume of 1 µl, 15 pmol of the appropriate forward and reverse primers (Table 1) in a 20 µL volume of 1 x MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline GmbH, Luckenwalde, Germany), and 2 µl of BioStabII PCR Enhancer (Sigma-Aldrich Co.). All forward and reverse primers contained the same 10-nt barcode sequence. PCRs were performed for 30–40 cycles (30–33 cycles for samples amplified with 799F-1115R and 35–40 cycles for samples amplified with ITS1Kyo2F-ITS86R) using the following parameters: pre-denaturation at 96 °C for 1 min, denaturation at 96 °C for 15 s, annealing at 55 °C for 30 s and extension at 70 °C for 90 s. No template reactions were included as negative controls during PCR as part of standard procedure at LGC Genomics.

The DNA concentration of the amplicons was assessed by gel electrophoresis. In these experiments, amplicon pools representing up to 48 samples were created by mixing roughly 20 ng of amplicon DNA from each sample, each of which carried a unique barcode. The amplicon pools were purified by using one volume of Agencourt AMPure XP beads (Beckman Coulter, Inc., IN, USA) to remove primer dimers and other small mispriming products, and further purification was performed with MiniElute columns (QIAGEN GmbH, Hilden, Germany). The purified amplicon pool DNA (100 ng each) was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1–96 (NuGEN Technologies, Inc., CA, USA). Illumina libraries (Illumina, Inc., CA, USA) were pooled and size selected by preparative gel electrophoresis. Sequencing was done on an Illumina MiSeq using V3 Chemistry.

Processing of amplicon data

Raw Illumina paired-end reads were demultiplexed with Sabre2 [59] and adapters were trimmed with the bbduk.sh script [60]. Next, demultiplexed and adapter trimmed data were imported into the QIIME2-2022.8 pipeline [61]. Primers were trimmed with cutadapt plugin of QIIME2, and the demux plugin was used for quality checking. The DADA2 [62] plugin of QIIME2 was used for quality trimming, dereplication, chimera removal and generation of amplicon sequence variants (ASVs). The QIIME2-compatible SILVA v138.9 [63] and UNITEv9 [64] databases were used for bacterial and fungal taxonomy annotation, respectively. Three standard output files obtained from the DADA2 plugin (the count table, fasta file and assigned taxonomy data) and one external sample metadata file were merged into a phyloseq object using the R package "phyloseq v1.44" [65]. Before generating the phyloseq object, unassigned ASVs and ASVs assigned to the chloroplasts and mitochondria were filtered out.

Statistical analysis

After generating the phyloseq object, all statistical analyses were performed in R v 4.2.0 [66]. Data from the phyloseq object were first rarefied using the lowest sequencing depth (wheat −16173 and 11398 reads per sample for bacteria and fungi, respectively; barley −12663 and 4961 reads per sample for bacteria and fungi, respectively). Package UpSetR v1.4.0 [67] was used for generating UpSet plots. For core-microbiome analysis the microbiome package [68] was used. Normality of the data was checked using the shapiro.test() function of the stats v3.6.2 package. The alpha diversity metric Shannon index (H) and the statistical significance test (one-way ANOVA followed by pairwise t-test) were computed with the vegan v 2.6-4 package [69]. Beta diversity was evaluated using Bray-Curtis distance-based principal coordinate analysis (PCoA). Statistical significance (PERMANOVA) for distance matrices was computed using the adonis() function of the vegan v 2.6-4 package [69], while significance for pairwise comparisons was calculated using the pairwise.adonis() function of the pairwiseAdonis v 0.4 package [70]. Simultaneously, rarefied data were normalized to obtain relative abundance values (%), and taxon compositions based on these values were plotted using the plot_bar() function. The R package phylosmith [71] was used for microbial network analysis. Network construction was done using the Spearman rank correlation method with the p-value and rho cut-off set at 0.05 and 0.8, respectively. Network topology was calculated using the igraph v 1.5.1 package [72] and a customised script was used for the ZiPi plots. Based on standard criteria, all ASVs were categorized into four groups: peripherals ($Zi<2.5$ and $Pi<0.62$), connectors ($Pi>0.62$), module hubs ($Zi>2.5$) and network hubs ($Zi>2.5$ and $Pi>0.62$).

The package microbiomeMarker v 1.6.0 [73] was used for linear discriminatory analysis effect size computation (LefSe) [74] with a linear discriminatory score (LDA) and p-value cut-off of 3.0 and <0.05, respectively.

Results

High-throughput amplicon sequencing characterizes bacterial and fungal communities

Figure 1 depicts the experimental set-up used in this study. Samples from each host consisted of eight different control and dsRNA treatments - no dsRNA (ND), no dsRNA+Fg (ND+Fg), non-specific dsRNA (Nsp), non-specific dsRNA+Fg (Nsp+Fg), dsRNA Cyp51 (Cyp51), dsRNA Cyp51+Fg (Cyp51+Fg), dsRNA SdhB (SdhB), dsRNA SdhB+Fg (SdhB+Fg). Amplicon sequencing produced a total of 1,896,748 bacterial and 1,898,726 fungal filtered reads from wheat and 1,953,614 bacterial and 2,334,856 fungal filtered reads from barley, respectively (Additional file 1: Table S1). A plateau was observed in the rarefaction curves of all the sequenced samples, indicating that the samples provided adequate diversity and coverage for the tested conditions (Additional file 2: Fig. S1). A total of 1018 bacterial and 460 fungal ASVs were obtained from wheat as well as 548 bacterial and 333 fungal ASVs from barley (Additional file 1: Table S1; Additional file 3: Table S2; Additional file 4: Table S3). The proportion of artefactual bacterial reads (ASVs) was 1018/1252 (filtered/unfiltered) in

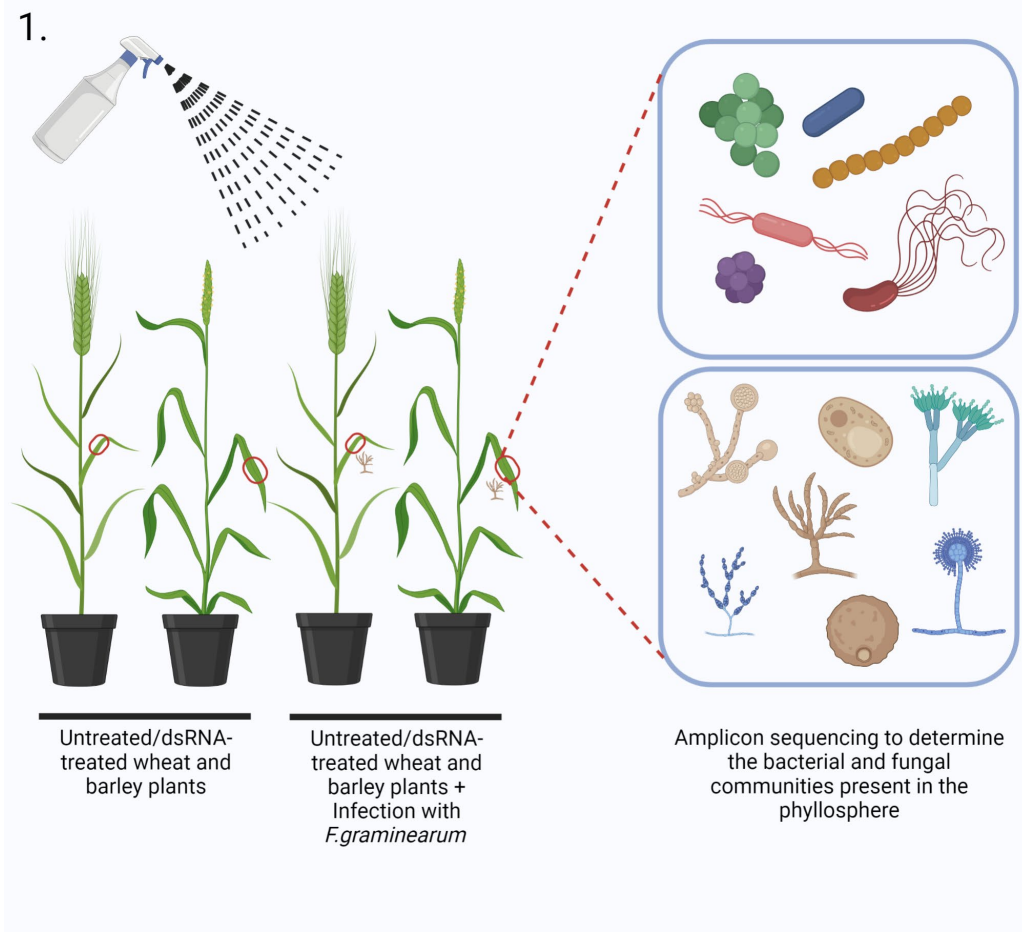


Fig. 1 Schematic depiction of the experimental set-up used to study the effects of dsRNA spraying on the phyllosphere microbiota. Created with BioRender.com

wheat and 548/943 (filtered/unfiltered) in barley. No artefactual fungal reads were found in both barley & wheat. To elucidate the changes induced by the dsRNA spray treatments, the eight treatments were further grouped into four treatment groups: no dsRNA (ND), no dsRNA + Fg (ND + Fg), dsRNA (Nsp, Cyp51 and SdhB), and dsRNA + Fg (Nsp + Fg, Cyp51 + Fg and SdhB + Fg). Details of the shared and unique ASVs found in the different treatment groups are shown in Fig. 2a-d, Additional file 5: Table S4 and Additional file 6: Table S5. For both bacterial and fungal ASVs and across both hosts, a higher number of unique ASVs than common ASVs were found in most of the treatment groups, with most of the unique ASVs being found in lower abundance or belonging to rarer taxa. This indicates that both dsRNA spray and *F. graminearum* inoculation selectively affect rare taxa. Fusarium abundance across the different treatments was quantified by plotting the number of reads that correspond to the genus Fusarium in the eight different treatments (Additional file 2: Fig. S2).

The diversity of phyllosphere microbial communities after double-stranded RNA spraying

The alpha diversity measures of the bacterial and fungal taxa were plotted for all eight treatments using the Shannon diversity index (Fig. 3a-d) (Additional file 7: Table S6). Similar bacterial taxonomic evenness was observed across all the treatments in both wheat and barley (Fig. 3a, b), except the SdhB-sprayed samples that showed significantly lower evenness in barley. Further pairwise comparisons revealed the diversity of the dsRNA SdhB-sprayed samples to be significantly different from the no dsRNA (ND) samples in both hosts (pairwise t-test, $p < 0.05$). In both wheat and barley, the fungal

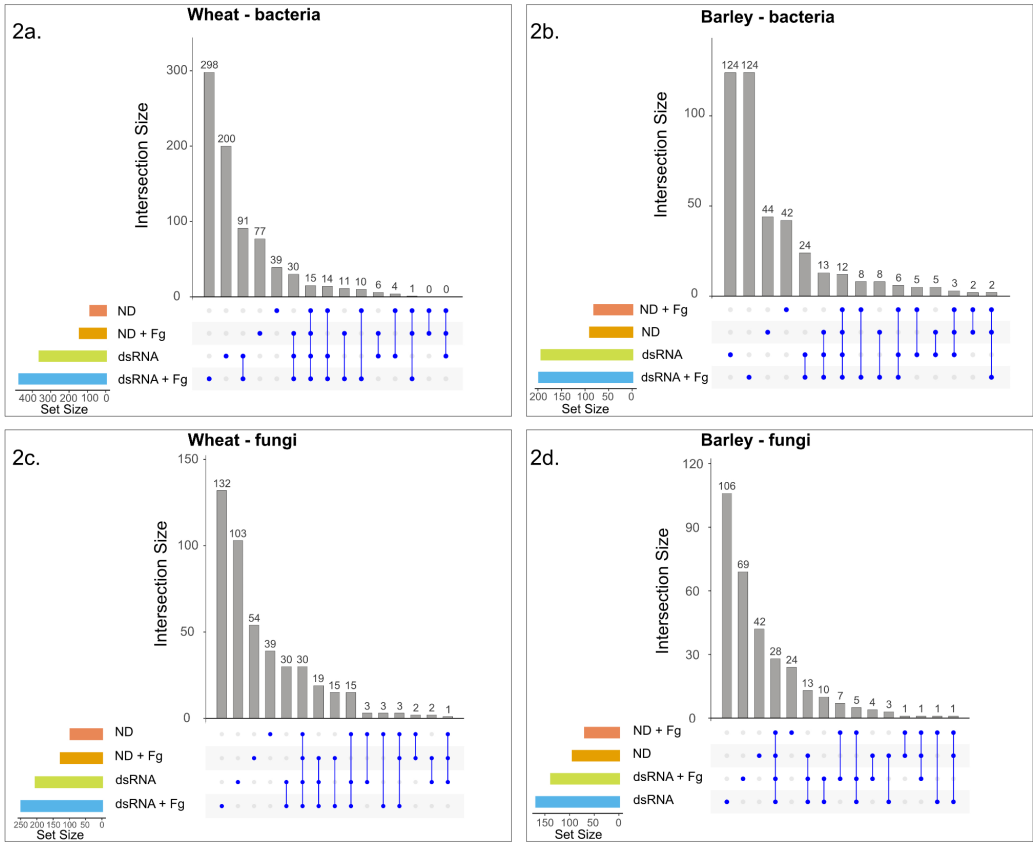


Fig. 2 UpSet plots showing the set sizes of the different treatment groups (ND, ND + Fg, dsRNA, dsRNA + Fg), and the shared and unique bacterial (**a** and **b**) and fungal (**c** and **d**) ASVs identified in wheat and barley, respectively. The blue dots represent the individual sets of the different treatment groups and the blue lines represent the intersecting sets

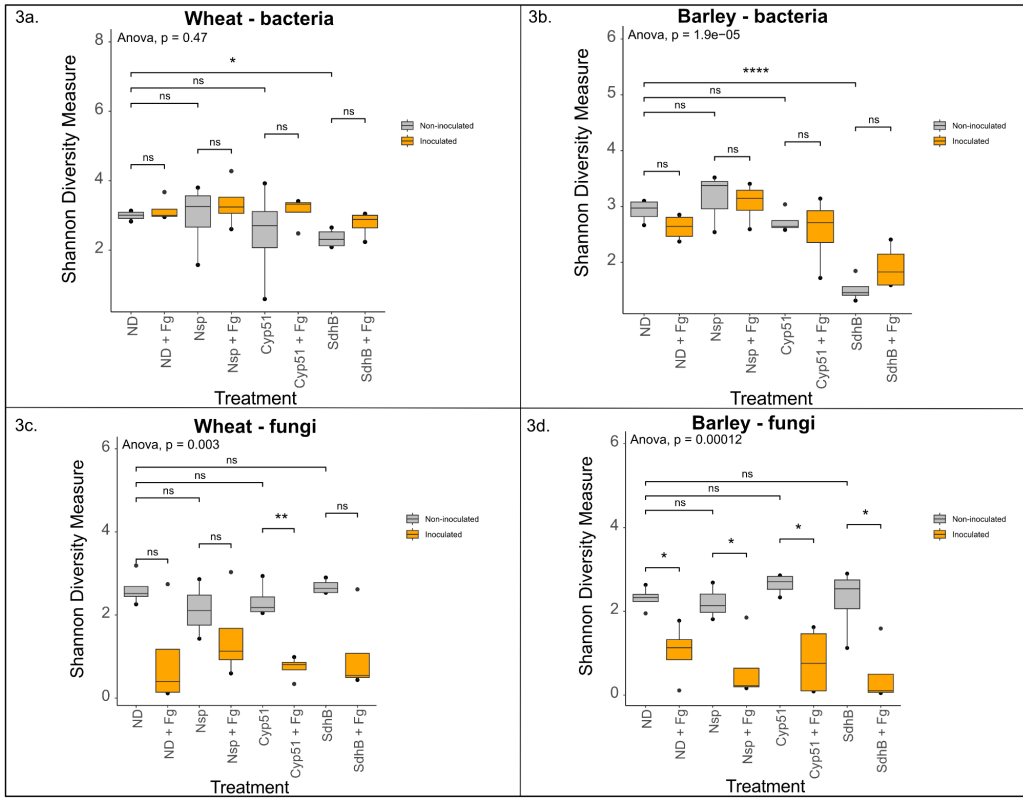


Fig. 3 Visualization of alpha diversity measures. Box plots show the Shannon alpha diversity metrics for bacterial (**a** and **b**) and fungal (**c** and **d**) communities in wheat and barley, respectively. The non-inoculated treatments (ND, Nsp, Cyp51 and SdhB) are marked in grey and the *F. graminearum* inoculated treatments (ND + Fg, Nsp + Fg, Cyp51 + Fg, SdhB + Fg) are marked in orange. Statistical significance was determined using one-way ANOVA followed by pairwise t-tests

taxonomic evenness remained similar between the native state in ND and the dsRNA treatments (Nsp, Cyp51 and SdhB) (Fig. 3c, d), indicating that the stability of the fungal communities is maintained upon dsRNA spray. However, irrespective of the treatment used, inoculation with *F. graminearum* resulted in lower fungal taxonomic evenness (ND + Fg, Nsp + Fg, Cyp51 + Fg, SdhB + Fg) in both hosts. In particular, significant differences were observed upon pairwise comparisons of the following: Cyp51 vs. Cyp51 + Fg in wheat (Fig. 3c), and ND vs. ND + Fg, Nsp vs. Nsp + Fg, Cyp51 vs. Cyp51 + Fg and SdhB vs. SdhB + Fg in barley (Fig. 3d) (pairwise t-test, $p < 0.05$) (Additional file 7: Table S6).

Principal coordinate analyses (PCoA) were performed using the Bray-Curtis dissimilarity and ordination plots based on the first two principal coordinates (PCs) were created to visualize differences and similarities in microbial community diversity between the treatments (Fig. 4a-d). In wheat, partial differentiation of the ND, SdhB and SdhB + Fg bacterial communities was observed, while a considerable overlap was observed between the rest of the treatments (Fig. 4a). This showed that the bacterial composition of the ND, SdhB and SdhB + Fg treatments varied from the bacterial composition of the rest of the treatments. In barley, clear clustering of the bacterial communities between the different treatment groups was observed (Fig. 4b). There was also a noticeable overlap in the bacterial community structure of the targeted-dsRNA samples (Cyp51, Cyp51 + Fg, SdhB and SdhB + Fg). This pointed to dissimilarities in the composition of bacterial communities between the control and targeted-dsRNA treatments. These observations were consistent with a permutational multivariate analyses of variance (PERMANOVA/Adonis; Number of permutations = 999) of the Bray–Curtis distance matrix. The overall Adonis values for the wheat and barley bacterial

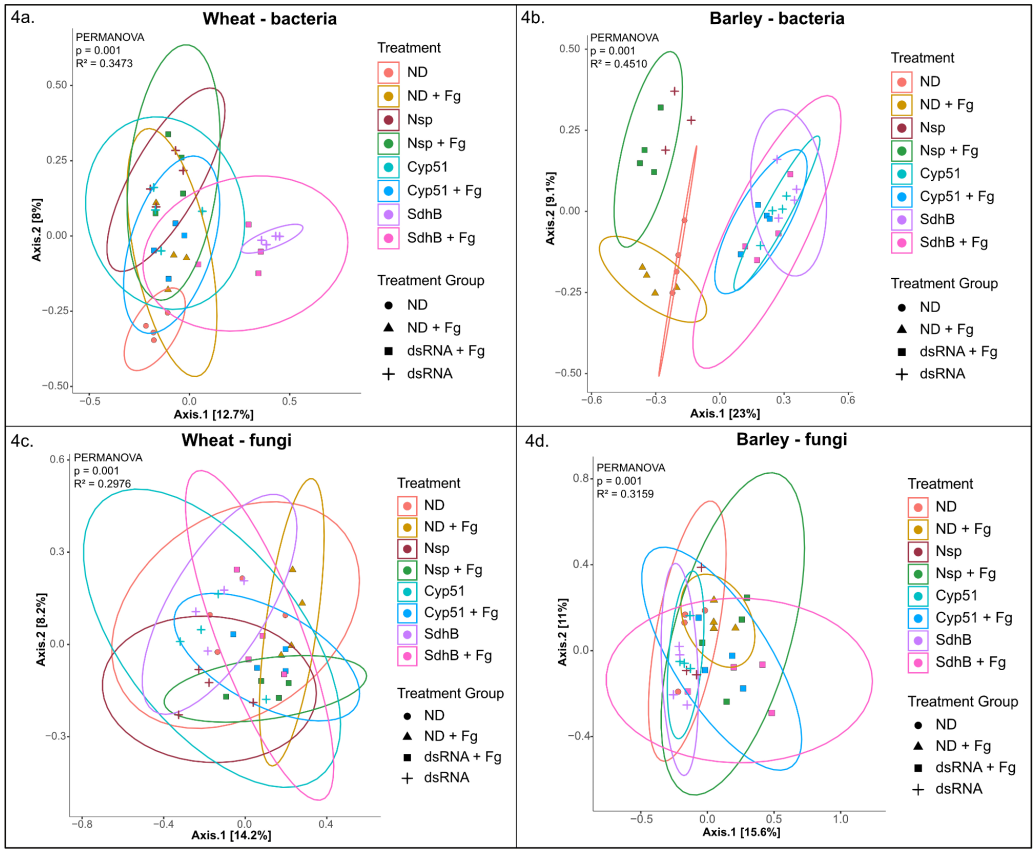


Fig. 4 Visualization of beta diversity using principal coordinate analysis (PCoA) plots based on Bray-Curtis distances for bacterial (**a** and **b**) and fungal (**c** and **d**) samples in wheat and barley, respectively. The colours distinguish the eight different treatments, while the shapes distinguish the treatment groups. The confidence level = 0.95 of different treatments are denoted by the confidence ellipsoids. Statistical significance was determined by PERMANOVA/Adonis (number of permutations: 999)

communities were $p<0.001$, R2=0.3473 and $p<0.001$, R2=0.4510, respectively. However, pairwise comparisons of individual treatments revealed no significant differences (Pairwise Adonis test, $p>0.05$) (Additional file 8: Table S7). Partial clustering of the fungal communities between the non-inoculated and inoculated samples was observed in both wheat and barley, indicating dsRNA spray resulted in fungal community structures similar to the hosts' native state, but *F. graminearum* inoculation caused shifts in the community structure (Fig. 4c, d). The overall Adonis values were $p<0.001$, R2=0.2976 for wheat and $p<0.001$, R2=0.3159 for barley. Pairwise comparisons of individual treatments also revealed no significant differences (Pairwise Adonis test, $p>0.05$) (Additional file 8: Table S7).

Composition of the phyllosphere microbial communities before and after dsRNA spraying

The relative abundance of the ASVs present in the different treatments and treatment groups was plotted to characterize the composition of the bacterial and fungal communities (Additional file 9: Table S8 and Additional file 10: Table S9). At the phylum level, the bacterial communities in both hosts were dominated by Proteobacteria (wheat: 46 -73%, barley: 29 −91%) and Actinobacteria (wheat: 9 −34%, barley: 2 −50%) (Additional file 2: Fig. S3a, b). The fungal communities were dominated by Ascomycota (wheat: 35 −95%, barley: 43 −95%) and Basidiomycota (wheat: 5 −63%, barley: 4 −47%) (Additional file 2: Fig. S3c, d). The relative abundances of the top 20 bacterial and fungal ASVs at the genus level are shown in Fig. 5a-d.

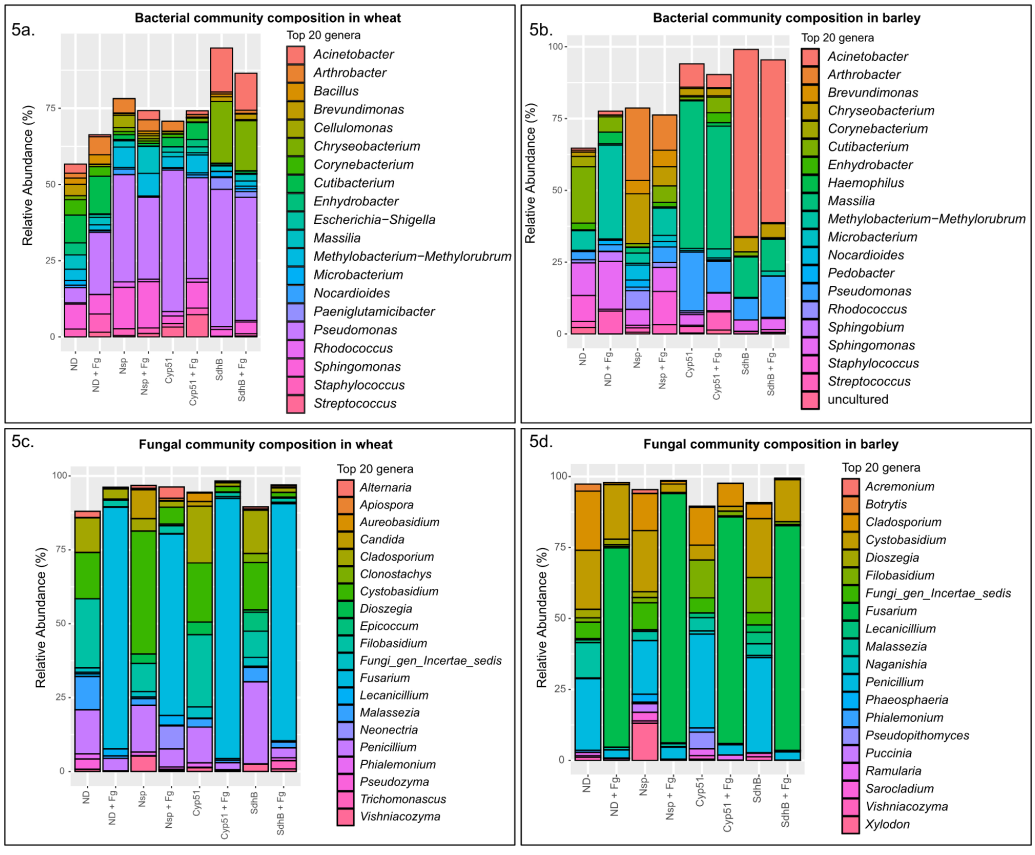


Fig. 5 Microbial community composition plots at the genus level. The relative abundance of the top 20 bacterial (**a** and **b**) and fungal (**c** and **d**) genera identified in different treatments in wheat and barley, respectively

Pseudomonas was the most abundant genus among the wheat bacterial communities for all treatments except the no-dsRNA treatment (ND). Compared to the plant's native state in ND, the relative abundance of *Pseudomonas* increased upon both dsRNA spray and *F. graminearum* inoculation. Other genera ubiquitous across all treatments in wheat include *Sphingomonas*, *Cutibacterium*, *Corynebacterium*, *Staphylococcus*, *Arthrobacter*, *Massilia*, *Methylobacterium-Methylorubrum*, *Brevundimonas*, *Microbacterium* and *Nocardioides*. In addition, an increase in the relative abundance of *Acinetobacter* and *Chryseobacterium* was observed in the dsRNA SdhB samples (SdhB, SdhB+Fg) (Fig. 5a, Additional file 2: Fig. S4a). In barley, while the genera that were most abundant remained roughly the same across treatments, differences in the relative abundance of individual genera were observed. The bacterial genera *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Massilia*, *Cutibacterium*, *Methylobacterium-Methylorubrum* and *Streptococcus* were present in all the treatments. *Acinetobacter* predominated in SdhB and SdhB+Fg samples, while *Massilia* was in high relative abundance in Cyp51 and Cyp51+Fg samples (Fig. 5b, Additional file 2: Fig. S4b). In both no dsRNA (ND) and non-specific dsRNA (Nsp) samples, *F. graminearum* inoculation shifted the relative abundance of bacterial communities, while this was not observed in targeted dsRNA samples.

Among the fungal communities identified in wheat, the genera *Cladosporium*, *Cystobasidium*, *Filobasidium* and *Penicillium* were relatively abundant in all treatments, whereas *Lecanicillium*, *Apiospora*, *Fungi_gen_Incertae_sedis*, *Phialemonium*, *Vishniacozyma* and *Malassezia* were ubiquitous across all treatments in varying amounts (Fig. 5c, Additional file 2: Fig. S4c). Similar to wheat, the genera *Cladosporium*, *Cystobasidium*, *Filobasidium* and *Penicillium* were relatively abundant across all

treatments in barley as well, while *Lecanicillium*, *Fungi_gen_Incertae_sedis*, *Vishniacozyma* and *Malassezia* were ubiquitous across all treatments but in varying amounts (Fig. 5d, Additional file 2: Fig. S4d). In both hosts, we observed a significant shift upon *F. graminearum* inoculation in the relative abundances of ascomycetes and basidiomycetes across the treatment groups (Additional file 2: Fig. S3c, d). Additionally, the genus *Fusarium* dominated the samples inoculated with *F. graminearum* in both hosts, showing a clear change in composition of the fungal communities upon pathogen inoculation and colonization. However, no obvious changes in composition were observed between the no dsRNA (ND) and dsRNA (Nsp, Cyp51, SdhB) samples or between their corresponding inoculated treatments (ND+Fg, Nsp+Fg, Cyp51+Fg, SdhB+Fg), indicating a change in fungal composition only upon *F. graminearum* inoculation.

Discriminatory analysis reveals taxa that shape the phyllosphere microbiota in wheat and barley

Linear discriminant analysis Effect Size (LEfSe) was used to identify differentially abundant ASVs in each treatment (Fig. 6a-d). The discriminatory value or LDA score was used to evaluate the extent to which individual ASVs could be used to distinguish treatments. A cut-off of LDA score >=3.0 and a p-value threshold of $p<0.05$ was used to identify the ASVs characteristic of each treatment, and the results of the analysis were plotted in a dot plot format. Three bacterial and four fungal ASVs exhibited differential abundance between treatment groups in wheat (Fig. 6a, c), while two bacterial and three fungal ASVs were differentially abundant in barley (Fig. 6b, d).

The differentially abundant bacterial ASVs in wheat included ASV16 (*Cutibacterium*) in ND+Fg samples, ASV8 (*Pseudomonas*) in Nsp samples and ASV1 (*Pseudomonas*) in Cyp51 samples (Fig. 6a). Similarly, the differentially abundant bacterial ASVs in barley included ASV12 (*Methylobacterium-Methylorubrum*) in ND+Fg samples and ASV10 (*Chryseobacterium*) in Nsp samples (Fig. 6b). Among the fungal communities in wheat, ASV2 (*Cystobasidium*) was differentially abundant in the Nsp samples (Nsp), ASV3 (*Filobasidium*) in Cyp51 samples, ASV1 (*Fusarium*) in Cyp51+Fg samples and ASV4 (*Penicillium*) in SdhB samples (Fig. 6c). In barley, ASV2 (*Cystobasidium*) and ASV12 (undefined genus) were differentially abundant in the Nsp samples while ASV8 (*Penicillium*) defined the Cyp51 samples (Fig. 6d).

F. graminearum infection alters bacterial and fungal co-occurrence patterns in leaves sprayed with double-stranded RNA

For microbial co-occurrence network analysis, ninety-four (48 from wheat and 46 from barley) of the 126 samples sequenced, belonging the dsRNA (Nsp, Cyp51, SdhB) and dsRNA+Fg (Nsp+Fg, Cyp51+Fg, SdhB+Fg) treatment groups were utilized for bacterial and fungal network construction.

At the genus level, smaller and sparse network clusters with strong internal relationships were observed for bacterial communities in wheat and barley (Additional file 2: Fig. S5a-d). In addition, the relationships detected between the bacterial ASVs were mostly positive but also included negative relations ($r=0.8$, $p<0.05$). The dsRNA-treated group had 27 bacterial nodes in wheat and 29 bacterial nodes in barley (Additional file 11: Table S10), but upon inoculation with *F. graminearum* (dsRNA+Fg), the number of bacterial nodes stayed relatively the same in wheat (26), while it increased in barley (75) (Additional file 11: Table S10). Contrary to observations in the bacterial networks, dense clusters were observed for the fungal communities in both hosts. In addition, both positive and negative interactions were observed between the fungal ASVs. The number of fungal nodes increased from 58 to 60 upon *F. graminearum* inoculation in wheat (Fig. 7a, b) but decreased from 48 to 42 in barley (Fig. 8a, b). Overall, inoculation with *F. graminearum* lowered bacterial community interactions and increased fungal interactions in dsRNA-sprayed wheat and barley leaves, as evident from the changes in the number of edges between the groups (Figs. 7c and 8c). The co-occurrence patterns were further characterized by computing average node degree and modularity (Figs. 7c and 8c) (Additional file 12: Table S11). Inoculation with *F. graminearum* increased the modularity of both bacterial and fungal networks in dsRNA-sprayed wheat leaves, whereas it increased bacterial modularity and lowered fungal modularity in dsRNA-treated barley leaves. Additional topological features from the bacterial and fungal networks are catalogued in supplementary file (Additional file 12: Table S11).

The Zi and Pi scores were then computed to evaluate the significance of each node in the network and categorize identified ASVs into the roles of peripherals, connectors, module hubs and network hubs, thereby revealing potential key taxa. All the nodes from the bacterial networks in wheat and barley were categorized as peripherals (Zi<2.5 and Pi<0.62), revealing that the identified nodes are only connected to other nodes within their own modules and thus do not play a significant role in maintaining the bacterial networks upon dsRNA spray (Additional file 2: Fig. S6). The fungal nodes classified from the co-occurrence patterns revealed mostly peripherals, a few connectors and one module hub. In wheat, ASV14 (*Penicillium*) and ASV26 (*Vishniacozyma*) from the dsRNA group and ASV5 (*Cladosporium*), ASV48 (*Candida*) and ASV64 (*Phialemonium*) from the dsRNA+Fg group were identified as connectors (Zi<2.5 and Pi>0.62), while ASV19 from the dsRNA+Fg group

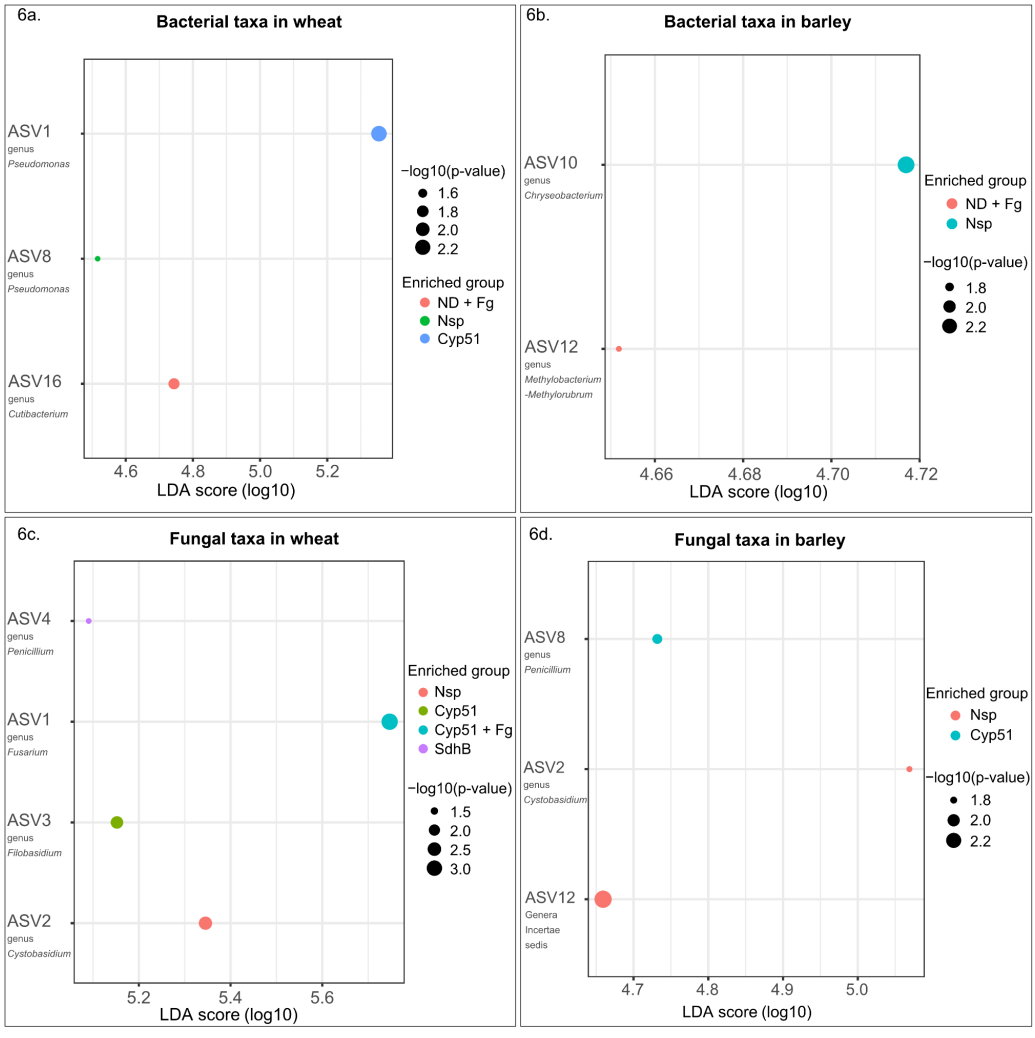
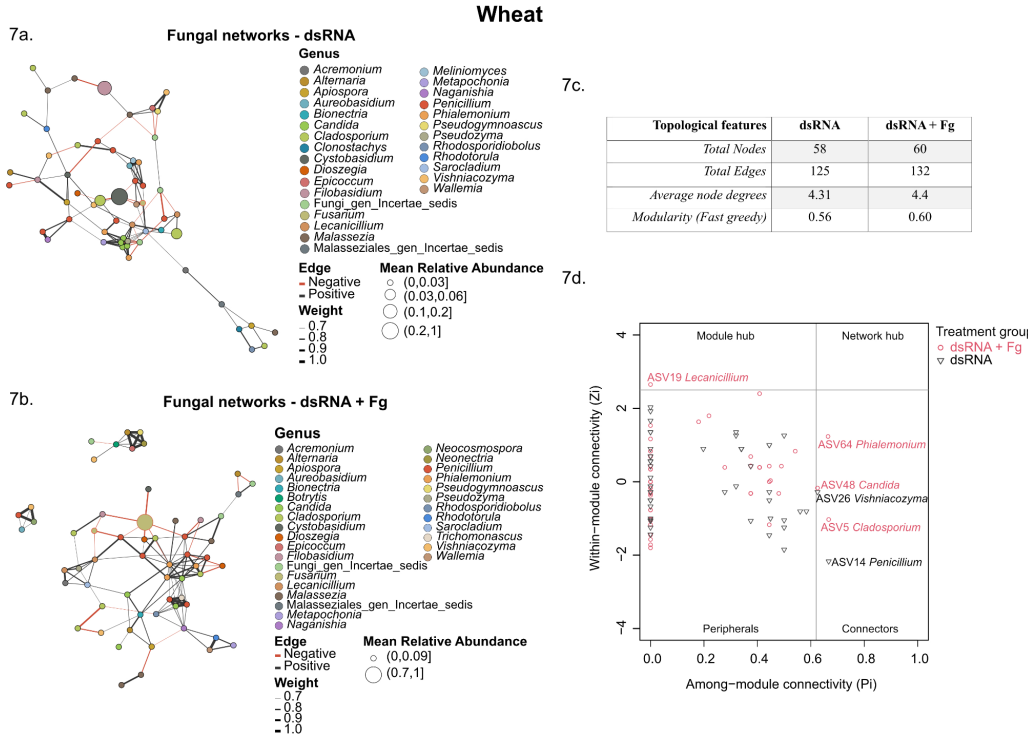


Fig. 6 Linear discriminant analysis Effect Size (LEfSe) plots representing the bacterial and fungal ASVs distinguishing the different treatments in wheat and barley. Plots **a** and **b** represent bacterial ASVs, while **c** and **d** represent the fungal ASVs. Linear discriminatory (LDA) score and p-value cut-offs are >= 3.0 and < 0.05, respectively

and assigned to the genus *Lecanicillium* was identified as a module hub (Zi > 2.5 and Pi < 0.62) (Fig. 7d). In barley, ASV12 (undefined genus) was identified as a connector from the dsRNA + Fg group (Fig. 8d).

Discussion

The microbial communities of the phyllosphere are predominated by bacteria [75]. Our findings also support this conclusion since more bacterial than fungal ASVs were identified by sequencing. For all eight treatments examined, the most abundant bacterial phyla in the phyllosphere were *Proteobacteria*, *Actinobacteria*, *Bacteroidota* and *Firmicutes*. This is consistent with previous studies on bacterial communities in wheat leaves [76, 77]. Other studies have also shown the dominance of these microbial taxa in the phyllosphere and other plant organs of various crops and native plants, although the relative abundance of individual taxa may vary depending on host genotype, human intervention, and geographic location [78–80]. The genus *Pseudomonas*, which was identified



Topological features	dsRNA	dsRNA + Fg
Total Nodes	58	60
Total Edges	125	132
Average node degrees	4.31	4.4
Modularity (Fast greedy)	0.56	0.60

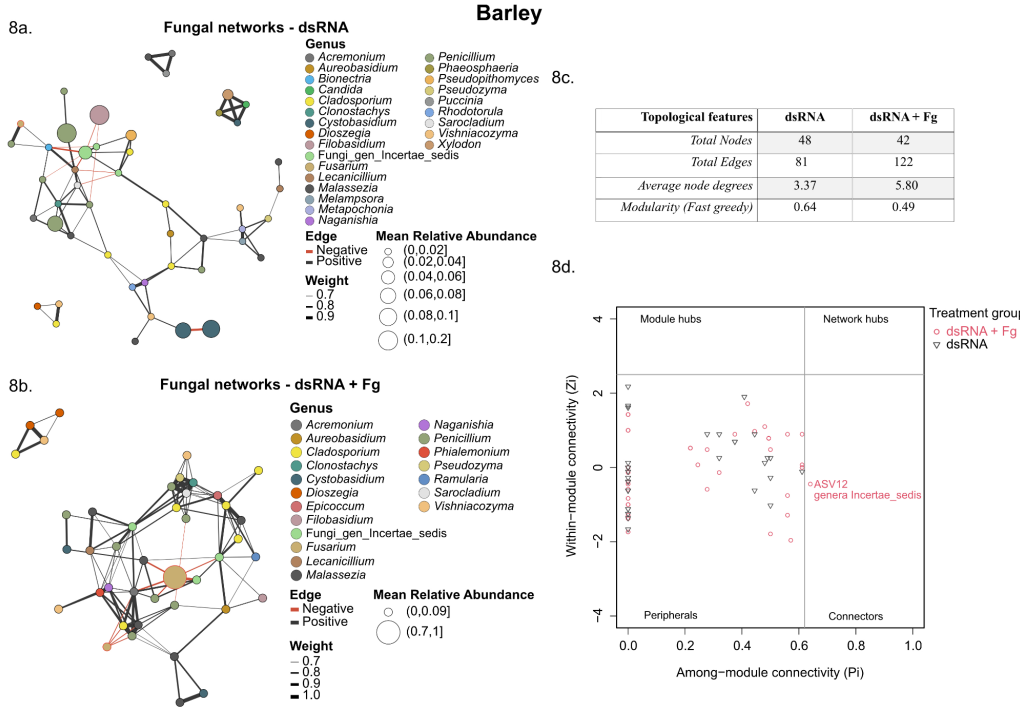
Fig. 7 Genus-level fungal co-occurrence networks in dsRNA-treated wheat. The ASVs are represented as nodes. The size of each node is proportional to the relative abundance of the corresponding ASV. Nodes belonging to the genus Fusarium are highlighted with red borders. The connections denote a strong and significant correlation ($r > 0.8$, $P < 0.05$). Black lines or edges indicate positive interactions and red lines or edges indicate negative interactions. The thickness of the lines are proportional to the weight. Panels **a** and **b** show fungal co-occurrence networks in the dsRNA and dsRNA + Fg treatments in wheat, respectively. Panel **c** summarizes the main topological features observed in the aforementioned networks. Panel **d** shows the ZiPi plot for the fungal ASVs in wheat, revealing the importance of the different ASVs within and among modules in the network. A cut-off of Zi = 2.5 and Pi = 0.62 was used to distinguish the different roles. The genera *Penicillium*, *Vishniacozyma*, *Cladosporium*, *Candida* and *Phialemonium* were identified as connectors while *Lecanicillium* was identified as a module hub

across all treatments in wheat and barley, is ubiquitous in the phyllosphere [81].

Of the fungal phyla identified in the eight treatments, most belonged to *Ascomycota* and *Basidiomycota*. The fungal communities in the phyllosphere exhibit high species diversity and contribute to plant growth and metabolism via complex relationships [82, 83]. They also play essential roles in driving carbon and nitrogen cycling in agronomic crops and forest environments [84, 85]. Taxa identified in this study such as *Cladosporium* sp., *Alternaria* sp., *Dioszegia* sp. and *Vishniacozyma* sp. have previously been identified as integral parts of the wheat and barley phyllosphere mycobiome [76, 86–88]. Species from the genera *Filobasidium* and *Cystobasidium*, which were prevalent in all treatments in both wheat and barley, have previously been identified in wheat flag leaf and leaf samples [76, 89] .

Spraying dsRNA differentially affects bacterial communities while maintaining fungal diversity and composition in wheat and barley

The Shannon diversity measures for bacterial communities in both wheat and barley were similar in all treatments except SdhB and SdhB + Fg, which displayed lower diversity. Beta diversity plots also revealed separate clustering of bacterial communities from SdhB samples in wheat. In barley though, the different control and dsRNA treatments clustered separately, with an overlap only between dsRNA Cyp51- and dsRNA SdhB- sprayed samples. These PCoA plots based on the Bray-Curtis distance therefore revealed dissimilarities in the composition of bacterial communities between the different treatments. In addition, dsRNA-specific and host-specific differences were also identified. Composition plots showed no major changes in the composition of the top 20 bacterial genera



Topological features	dsRNA	dsRNA + Fg
Total Nodes	48	42
Total Edges	81	122
Average node degrees	3.37	5.80
Modularity (Fast greedy)	0.64	0.49

Fig. 8 Genus-level fungal co-occurrence networks in dsRNA-treated barley. The ASVs are represented as nodes. The size of each node is proportional to the relative abundance of the corresponding ASV. Nodes belonging to the genus Fusarium are highlighted with red borders. The connections denote a strong and significant correlation ($r > 0.8$, $P < 0.05$). Black lines or edges indicate positive interactions and red lines or edges indicate negative interactions. The thickness of the lines are proportional to the weight. Panels **a** and **b** show fungal co-occurrence networks in the dsRNA and dsRNA + Fg treatments in barley, respectively. Panel **c** summarizes the main topological features observed in the aforementioned networks. Panel **d** shows the ZiPi plot for the fungal ASVs in barley, revealing the importance of the different ASVs within and among modules in the network. Scores of Zi = 2.5 and Pi = 0.62 were used to distinguish the different roles. An undefined genus was identified as a connector

in wheat. However, the relative abundance of the candidates from the top 20 genera increased upon dsRNA spray. In barley, changes in the relative abundance varied depending on the dsRNA sprayed. In particular, the relative abundance of *Methylobacterium-methylorubrum* increased significantly in dsRNA Cyp51- sprayed samples, while *Acinetobacter* increased significantly in dsRNA SdhB- sprayed samples. However, genera such as *Pseudomonas*, *Sphingomonas*, *Cutibacterium* and *Methylobacterium-Methylorubrum* were found to be ubiquitous across all treatments and in both hosts, indicating that spraying dsRNA does not impair the survival/existence of bacteria ubiquitous to the wheat and barley phyllosphere. Interestingly, it was observed that the relative abundance of the genus *Pseudomonas* increased upon both dsRNA spray and *F. graminearum* inoculation in wheat, but not as much in barley. This difference could be attributed to the significance of this genus in shaping the native microbial communities in the specific cultivars of wheat and barley chosen in this study. Further analysis using metagenomic and meta-transcriptomic approaches will help gain a deeper understanding of the genes and pathways that govern such intricate microbial community assemblies. Together, these results indicate that the effects of dsRNA on the diversity and structure of the bacterial communities of the phyllosphere varied depending on the gene targeted and the host studied.

The diversity, structure and composition of the fungal communities, on the other hand, were more uniform across both hosts. No obvious differences in the alpha- and beta- diversity measures were observed between the no dsRNA and dsRNA samples, indicating dsRNA spray did not impact the diversity of fungal communities in both wheat and barley. Studies have reported that high species richness and the presence of direct competitors can positively influence plant health, as other microorganisms compete for space and resources, increasing competition for the pathogen as a result [90, 91]. In

addition, the composition plots and heat maps revealed the ubiquitous presence of highly abundant fungal genera like *Cladosporium*, *Cystobasidium*, *Filobasidium* and *Penicillium* in both the no dsRNA and dsRNA samples. This similarity in fungal composition could be attributed to the fungal communities being more stable and displaying resistance in response to disturbance (dsRNA spray) in their environment [92]. These observations together underpin that spraying dsRNA does not alter the native fungal communities of the phyllosphere in wheat and barley.

F. graminearum inoculation alters fungal co-occurrence patterns in dsRNA-sprayed plants

Network topology analyses can reveal important network nodes and edges while also facilitating comparisons between networks. In these analyses, the node degree indicates the number of direct connections for a specific ASV, the closeness centrality value indicates how quickly information spreads from a given node to other reachable nodes, and the betweenness centrality of a node reflects the effects of one microbe on the co-occurrence of other nodes [93]. In addition, the modularity may reflect biotic interactions between closely associated ASVs in an ecological community [94]. Our results showed that the bacterial and fungal networks for all of the studied treatments had comparable degrees, eigenvectors, and closeness centralities, indicating stable and uninterrupted networks. Network topology analyses revealed that the modularity of the fungal networks in the dsRNA treatments was comparatively higher or not appreciably different than the dsRNA + Fg treatments. This suggests that dsRNA provided a range of ecological niches to allow a greater diversity of fungi to flourish, whereas *F. graminearum* inoculation reduced the range of these available niches. Conversely, the modularity of the bacterial networks in the dsRNA + Fg treatments was greater than the dsRNA only treatments in both hosts. In addition, all of the bacterial modules were highly connected within themselves, while the different modules remained isolated from each other.

Bacteria and fungi identified through other analyses as defining the microbial communities of dsRNA-sprayed wheat and barley leaves were also represented in the co-occurrence patterns. Interactions within the bacterial communities were mostly positive, while there was a mix of both positive and negative interactions between fungal communities, with a noticeable increase in negative interactions upon inoculation with *F. graminearum*. This reveals that *F. graminearum* establishes itself within the fungal community by interacting negatively and reducing the relative abundances of the top genera.

Identifying microbial keystone or hub taxa is extremely valuable for the sustainable development of cereal ecosystems as they play vital roles in helping other microbes to maintain the dynamics of microbial networks [95]. Their importance is such that their disappearance can cause network collapse [96]. Co-occurrence network analyses were therefore performed to evaluate the complexity of the targeted microbiota [97], which revealed that the numbers of nodes, edges, and modules in both the bacterial and fungal networks were sensitive to both the host plant species and infection by the pathogen *F. graminearum*. It is important to note that microbial co-occurrence analyses do not always predict exact real-time networks and therefore require further omics- and culture-based strategies to obtain deeper insights into relationships within microbial communities.

The results of the analyses described above collectively indicate that foliar spraying with dsRNA has varied effects on the bacterial communities and negligible effects on the fungal communities of the phyllosphere. Previous studies on the microbiome have examined the phyllosphere and flag leaf samples in wheat [76, 77, 87, 89], and the phyllosphere fungal endophytes [88], the rhizosphere and grains [98, 99] in barley. This work further expands our understanding of plant microbial communities by characterizing those found in the barley and wheat phyllosphere. The number of samples per treatment in our experiments was limited because our study was greenhouse-based, so it would be desirable to conduct follow-up field studies to obtain additional insights into the effects of dsRNA on host microbial communities. Additionally, previous studies have shown that plant genotype and environmental conditions can have a considerable influence on the phyllosphere microbial communities [41, 75, 86]. Testing the effects of dsRNA spraying on the phyllosphere microbiota of different host cultivars and under varying environmental conditions could validate the results presented here and reveal potential genotype-specific effects. Overall, this pilot study shows that although rarer and less abundant ASVs change upon dsRNA spray, the ubiquitous bacterial and fungal components of the phyllosphere in wheat and barley remain unchanged.

Conclusion

Spray-induced gene silencing (SIGS) is attracting considerable interest as a plant protection strategy because it has the potential to be an efficient and environmentally friendly alternative to conventional chemical fungicides and transgenic crops. Studies on several agricultural and horticultural crops have proven SIGS effective against diverse plant pathogens and pests. However, despite its proven efficiency in reducing the incidence and severity of plant diseases, several aspects of SIGS require further study to make it a practical plant protection strategy. Leaves represent a large surface area of the plant and

can act as entry points for pathogens and other microbes [100]. Moreover, the aerial parts of plants also influence growth, fitness and yield. Therefore, an important aspect of spraying dsRNA is its effect on the microbial communities, particularly in the phyllosphere. Our results address this need by providing novel insights into the effects of SIGS on the phyllosphere microbiome in wheat and barley. Using amplicon sequencing, we have shown that the diversity, structure and composition of the phyllosphere bacterial communities are subject to subtle changes upon exogenous dsRNA application, while the fungal communities remain largely unaffected. We also show that dsRNA does not impact the fungal compositional changes induced by *F. graminearum* inoculation in wheat and barley leaves. Further validation of these results through large-scale field studies can help incorporate how host genotype and environmental conditions influence the effect of dsRNA on phyllosphere communities, and reinforce the safety of SIGS for practical use.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40793-024-00660-8.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7
Supplementary Material 8
Supplementary Material 9
Supplementary Material 10
Supplementary Material 11
Supplementary Material 12

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Author contributions
R.R.V, A.C, M.D and S.C.W conceptualized the study. R.R.V, P.S and S.G designed the study. S.G and P.S conducted the experiments and analyzed the results under the guidance of R.R.V and A.C. The initial draft of the manuscript was composed by P.S, S.G, and B.G.K. Funding for the research was secured by R.R.V and A.C. All authors participated in the manuscript's writing, editing and approved the final version for submission.

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Data availability
The data that support the study are in the article and supplementary materials. Raw sequences have been deposited at the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the BioProject accession PRJNA980286. All code created during this work can be obtained from the following GitHub repository: https://github.com/samratencode/amplicon_analysis.git.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Publisher's note

III

Chapter 27

Spray-Induced Gene Silencing to Study Gene Function in *Phytophthora*

Poorva Sundararajan, Pruthvi B. Kalyandurg, Qinsong Liu, Aakash Chawade, Stephen C. Whisson, and Ramesh R. Vetukuri

Abstract

RNA interference (RNAi) is a conserved cellular defense mechanism mediated by double-stranded RNA (dsRNA) that can regulate gene expression through targeted destruction of mRNAs (messenger RNAs). Recent studies have shown that spraying dsRNAs or small RNAs (sRNAs) that target essential genes of pathogens on plant surfaces can confer protection against pests and pathogens. Also called spray-induced gene silencing (SIGS), this strategy can be used for disease control and for transient gene silencing to study the function of genes in plant–pathogen interactions. Furthermore, as sRNAs can move locally, systemically, and cross-kingdom during plant–microbe interactions, SIGS allows quick detection and characterization of gene functions in pathogens and plants.

Key words Oomycetes, SIGS, Gene silencing, RNAi, Potato

1 Introduction

Spray-induced gene silencing (SIGS) is an RNAi-based strategy for plant trait improvement and disease control [1, 2] that does not require plant transformation. From a plant protection perspective, it involves inhibition of plant pathogens through exogenous application of dsRNAs or sRNAs targeting pathogen genes essential for survival and disease development. dsRNAs are sprayed directly on plant tissues and can either be directly taken up by the plant pathogen, triggering the pathogen RNAi machinery, and/or by the host plant RNAi machinery [1–4]. The pathogen RNAi machinery then targets the cognate mRNAs for destruction or inhibition. The use of SIGS as a tool for disease control and reverse genetics aimed at modulation of pathogen gene expression has been demonstrated with several plant pathogens, paving the way for developing SIGS as a transient silencing tool to study the function of genes [5].

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Here we demonstrate methods for using SIGS as a tool for gene knockdown studies, using the oomycete pathogen *Phytophthora infestans* and potato pathosystem. Oomycetes, also commonly called water moulds, superficially resemble fungi as they share features such as filamentous growth and reproduction via spores. However, they are more closely related to brown algae, diatoms, and protists [6]. *Phytophthora* species within the oomycetes are all plant pathogens that cause billions of dollars in losses to agriculture every year. The most notorious example is late blight disease on potato and tomato caused by *P. infestans*, made infamous by its role in the Irish potato famine in the mid-1800s. We recently applied SIGS for knockdown of genes in *P. infestans*. As a test case, we selected the guanine-nucleotide binding (G) protein β-subunit (*PiGPB1*; PITG_06376; XP_002998508). PiGPB1 is known to be involved in the signal transduction process and sporangial development during infection on potato [7] and could be used for host-induced gene silencing (HIGS) to control *P. infestans* infection. Through SIGS, it was demonstrated that targeting *PiGPB1* resulted in severe reduction in disease [8]. The dsRNAs for SIGS application can either be produced in vitro or in vivo. The most critical parameters to carefully consider for successful SIGS are the size and concentration of the dsRNA, and delivery methods which are discussed in detail in the method and notes sections.

2 Materials

2.1 *Propagation of* P. infestans

1. Rye grain (organic; produced without agrochemical application).
2. Distilled water.
3. Domestic blender.
4. Cheesecloth.
5. Strainer.
6. Sucrose.
7. Bacteriological agar (Saveen and Werner AB).
8. Autoclave.
9. Pimaricin (50-mg/mL stock suspension; Sigma-Aldrich).
10. Geneticin (100-mg/mL stock solution; Sigma-Aldrich).
11. Ampicillin (100-mg/mL stock solution; Sigma-Aldrich).
12. A scalpel with No. 11 or No. 21 blades.
13. Forceps – straight tip.
14. 20 °C incubator.
15. Sterile Milli-Q water.

16. 90-mm sterile Petri dishes.
17. Parafilm sealing film.

2.2 Plant Material

1. Potting compost.
2. 2.5-L plastic plant pots.
3. Potato tubers (e.g., susceptible cultivars such as Bintje or Désirée).
4. Climate control chambers.

2.3 dsRNA Synthesis

1. DNeasy Plant Mini Kit (Qiagen).
2. Phusion™ High-Fidelity DNA Polymerase (ThermoFisher Scientific).
3. 5× Phusion HF Buffer (ThermoFisher Scientific).
4. 10-mM dNTPs.
5. Forward and reverse primers.
   (a) In-Vitro Transcription:
   T7 PiGPB1 dsRNA.FOR: 5′-GTAATACGACTCAC TATAGGGATGTTTATTTCGGGCTCGTGTGA-3′
   T7 PiGPB1 dsRNA.REV: 5′-GTAATACGACTCAC TATAGGGTAGATATGCGCTCCGGAAGT-3′
   (b) Bacterial Cloning:
   PiGPB1.FOR: 5′- AAAAAAGCTTCTCTACGCTC CAGTTGGGTC-3′
   PiGPB1.REV: 5′- AAAAACTAGTGGTAGA TATGCGCTCCGGAA-3′
   (c) qRT-PCR:
   PiGPB1 qPCR.FOR: 5′- TTCCGGAGCGCATATC TACC-3′
   PiGPB1 qPCR.REV: 5′-TCTTGAGTAGCGTGTCC CAG-3′
6. QIAquick® PCR Purification Kit (Qiagen).
7. MEGAscript RNAi Kit (Invitrogen).
8. L4440 plasmid (Addgene Plasmid #1654).
9. FastDigest restriction enzymes – HindIII & SpeI (Thermo-Fisher Scientific).
10. HT115 (DE3) *Echerichia coli* competent cells.
11. Tetracycline (100-mg/mL stock solution; Sigma-Aldrich).
12. LB (Luria-Bertani) medium (Duchefa Biochemie).
13. GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific).
14. DreamTaq DNA Polymerase (ThermoFisher Scientific).

15. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (100-mM stock solution; filter sterilized through a sterile 0.2-μm membrane filter).
16. Direct-zol™ RNA Miniprep Plus Kit (Zymo).
17. qScript cDNA SuperMix (Quantabio).
18. FastDigest restriction enzymes – HindIII & SpeI (ThermoFisher Scientific).
19. T4 DNA Ligase (ThermoFisher Scientific).
20. Gel DNA recovery kit (Zymo).
21. DyNAmo Flash SYBR Green qPCR Kit (ThermoFisher Scientific).
22. 6-well culture plates (e.g., Sigma-Aldrich cell culture plate).
23. PCR plate, 96-well, low profile, skirted (ThermoFisher Scientific).
24. 1.5-mL microcentrifuge tubes.
25. 2-mL microcentrifuge tubes.
26. 50-mL centrifuge tubes (e.g., Falcon tubes).
27. Mortar and pestle.
28. Plastic boxes for detached leaf assays (DLAs).
29. 1-mL Cuvettes.
30. Glass microscopic slides.
31. Coverslips.
32. Liquid nitrogen canister.
33. 50× TAE Buffer (Tris–acetate-EDTA) (Thermo Scientific) – dilute 20 mL of 50× stock in 980 mL of milli-Q water to make 1× TAE.
34. Nuclease-free water.
35. Standard agarose.
36. Gel Red (Biotium).
37. DNA Gel loading dye (6×) (Thermo Scientific).
38. RNA loading dye (2×) (Thermo Scientific).
39. 1-kb DNA ladder.
40. Trypan Blue (Sigma-Aldrich).
41. Lactic acid 85% (w/v) (Sigma-Aldrich).
42. Glycerol (Sigma-Aldrich).
43. Buffer saturated phenol (Invitrogen).

2.4 Equipment

1. Automizer.
2. Fume hood.

3. Orbital shaker.
4. Spectrophotometer.
5. Laminar flow cabinet.
6. 37 °C incubator and shaker.
7. Nano-drop spectrophotometer.
8. CFX96 qPCR machine (Bio-Rad).
9. Water bath with temperature control.
10. Heat block with temperature control.
11. Thermo Cycler for PCR (VWR).
12. Airbrush (Cocraft) and compressor (Biltema Mini Compressor MC.90).

3 Methods

3.1 Phytophthora infestans – *Culture*

1. Weigh 60 g of rye grain and wash thoroughly with distilled water. Soak the grains overnight at 21 °C in a beaker with water such that the grain is entirely submerged.
2. The next day, coarsely blend the soaked grain using a blender for 30 s, followed by heating at 50 °C for 3 h using a water bath.
3. Place a cheesecloth over a strainer and pass the heated mixture through to strain out the rye liquid. Make up the liquid with distilled H_2O to 800 mL and split equally into 400 mL each into two 1-L bottles.
4. Measure 10 g of sucrose and 7.5 g of agar separately for each bottle.
5. Add the weighed sucrose to the respective bottles and adjust pH to 7.0 using a pH meter. Next, make up the liquid to 500 mL with distilled H_2O in each bottle before adding the agar (*see* **Note 1**).
6. Mix well by shaking and autoclave at 121 °C for 30 min.
7. After autoclaving, allow the media to cool to around 55 °C before adding antibiotics. For 500 mL of rye agar, add to a final concentration 100-μg/mL ampicillin and 12-μg/mL pimaricin. Under sterile conditions, add antibiotics to rye agar and pour approximately 25 mL into individual 90-mm diameter Petri dishes. For growing *P. infestans* expressing green fluorescent protein (*Pi*-GFP), add 10-μg/mL geneticin (*see* **Notes 2–4**).
8. Cut 5-mm^2 plugs using a sterile blade from 2–3 week-old GFP-tagged *Phytophthora infestans* (*Pi*-GFP) agar-culture plates. Place the cut agar plugs with the side containing

mycelium facing down onto the rye agar plates, cooled down to room temperature.

9. Incubate the Petri dishes containing GFP-tagged *P. infestans* at 20 °C in darkness. Collect spores for further experiments after 2 weeks. Subculture, as explained above, every 2 weeks to maintain material.
10. Sporangia should be collected from (maximum age) two-week-old plates. Under sterile conditions, wash the plate with 2–5 mL of sterile water, scraping the surface and sides evenly and pass the solution through a 40-micron cell strainer into a 50-mL centrifuge tube.

3.2 Plant Material

1. Grow potatoes (e.g., cv. Bintje) from tubers in 2.5-L pots containing well-drained fertilized compost under greenhouse conditions of 21/19 °C day/night temperature, 16-h photoperiod, and 60% humidity.
2. Use four- to five-week-old plants to collect leaves for DLAs.

3.3 Phytophthora infestans *DNA Extraction*

1. Grow *P. infestans* in 6-well cell culture plate containing rye broth with 100-μg/mL ampicillin, 12-μg/mL pimaricin. Incubate at 20 °C without shaking for a week.
2. Collect the mycelia into a 2-mL microcentrifuge tube using a pair of forceps and weigh 50 mg. Freeze the sample in liquid nitrogen and grind immediately using a mortar and pestle. Extract *Phytophthora infestans* DNA using DNeasy Plant Mini Kit and elute in 100 μL of elution buffer.
3. Use a Nano-Drop spectrophotometer to determine the concentration of extracted DNA and store at −20 °C.

3.4 dsRNA Synthesis: In Vitro Transcription

1. Using a primer design tool for double-stranded RNA (e.g., SnapDragon – dsRNA Design [https://www.flyrnai.org/snapdragon]), design forward and reverse primer pairs amended with the T7 promoter sequence (GTAATACGACT CACTATAGGG) for *Phytophthora infestans* GPB1 (*PiGPB1*; PITG_06376; XP_002998508; positive control), and *P. infestans* test gene(s) with an amplicon size of approximately 200–400 bp.
2. Polymerase Chain Reaction can be carried out using Phusion™ High-Fidelity DNA Polymerase (*see* **Note 5**). Prepare a master mix containing 5× Phusion buffer, 10 mM dNTP, Phusion High-Fidelity DNA Polymerase, and distilled water. Add the respective forward and reverse primers individually and use 5–10 ng of *Phytophthora infestans* DNA obtained from previous steps as respective templates for GPB1 and other test gene (s) (*see* **Note 6**).

3. Use the following thermal conditions for PCR: Initial denaturation at 98 °C for 30 s; followed by denaturation (98 °C for 10 s); annealing (60 °C for 30 s); and extension (72 °C for 30 s) for a total of 30 cycles before proceeding to a final extension at 72 °C for 5 min.
4. Verify amplification of PCR products through gel electrophoresis (1% agarose in 1× TAE buffer) by running the samples alongside a 1-kb DNA ladder for reference. Incorporate a nucleic acid stain in the gel (e.g., GelRed), or stain after electrophoresis. Use a UV transilluminator to visualize the bands under ultraviolet light.
5. Before proceeding with dsRNA synthesis, purify the PCR product by QIAquick® PCR Purification Kit, and elute the purified PCR product in 20 μL of nuclease-free water. Determine the concentration using a Nano-Drop spectrophotometer.
6. Synthesize dsRNA for *PiGPB1* and other test gene(s) using 2 μL each of T7 enzyme mix, four ribonucleotides (ATP, CTP, GTP, UTP), and 10× T7 reaction buffer supplied with the MEGAscript RNAi Kit. Use 1 μg of respective purified PCR products as templates for dsRNA synthesis and make up the volume to a total of 20 μL using nuclease-free water. Treat the dsRNA with DNaseI and RNase provided in the kit to remove any DNA or ssRNA, before proceeding for purification. Elute the purified dsRNA in 100 μL of nuclease-free water (preheated to 95 °C).
7. As a positive control, synthesize dsRNA using the control template ($dsRNA^{Ct}$) provided in the kit.
8. Verify dsRNA synthesis by running a sample of the purified dsRNA with 6× loading dye or RNA dye in agarose gel (1% agarose in 1× TAE buffer) alongside a 1-kb ladder.
9. Measure the concentration of synthesized dsRNA using the Nano-Drop spectrophotometer before storing the dsRNA at −80 °C.

3.5 dsRNA Synthesis: Bacterial Cloning

3.5.1 PCR for Inserts

1. Using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), design forward and reverse primer pairs for *P. infestans GPB1* and other test gene(s) containing an additional restriction site at the 5′ end. In this case, for *PiGPB1,* the restriction recognition sites corresponding to HindIII (AAGCTT) and SpeI (ACTAGT) are added to the forward and reverse primers, respectively. The amplicon size should be approximately 200–400 bp (*see* **Note 7**).
2. As a negative control, use a nonspecific gene sequence from an unrelated species.

3. Carry out PCR using Phusion™ High-Fidelity DNA Polymerase, as explained in Subheading 3.4, steps **2**–**3**. Use 5–10 ng of *P. infestans* DNA obtained from previous steps as the template.
4. Verify amplification of PCR products through gel electrophoresis (1% agarose) by running the samples alongside a 1-kb ladder for reference.
5. Under UV light, swiftly excise the band corresponding to *PiGPB1* and other genes to be tested and transfer to a 1.5-mL microcentrifuge tube. Proceed with gel extraction and purification using the Zymoclean Gel DNA Recovery Kit. Weigh the excised gel and dissolve by incubating at 50 °C along with three volumes of ADB for each volume of excised agarose gel. Transfer to spin column for washing before elution in 10–15 μL of nuclease-free water. Store the purified PCR product at −20 °C until further use.

3.5.2 Plasmid Extraction

- Culture *E. coli* containing the L4440 plasmid in sterile LB broth containing 100-μg/mL ampicillin overnight at 37 °C in a shaker and extract plasmid using a GeneJET Plasmid Miniprep Kit.

3.5.3 Restriction Digestion

- Carry out restriction digestion of insert DNA (PCR products) and vector (L4440 plasmid) using FastDigest Restriction Digestion enzymes.
- Run the digested plasmid in 1% agarose in 1× TAE buffer. Swiftly excise the corresponding bands under UV light and transfer to a 1.5-mL microcentrifuge tube. Proceed with gel extraction and purification as explained in Subheading 3.5.1, step **5**.

3.5.4 Ligation

- Perform ligation of digested products using T4 DNA Ligase and a plasmid to insert ratio of 1:3 (calculate using http://nebiocalculator.neb.com/#!/). Incubate at 22 °C for 5 min using a heat block with temperature control.

3.5.5 Transformation of E. coli

1. Take HT115-DE3 competent cells from −80 °C storage and thaw on ice for 10–15 min.
2. Mix 5 μL of the ligated plasmid with 50 μL of HT115-DE3 competent cells in a sterile 1.5-mL microcentrifuge tube and incubate on ice for 30 min followed by heat shock at 42 °C for 30–60 s.
3. As an additional control, transform HT115-DE3 competent cells with empty L4440 plasmid.
4. Incubate on ice for 2 min.

5. Add 300 μL of fresh LB broth without any antibiotics to the transformed bacterial cells.
6. Incubate at 37 °C in a shaker for 1 h and plate on LB agar plates supplemented with tetracycline and ampicillin to a final concentration of 12 μg/mL and 100 μg/mL, respectively (*see* **Note 8**).
7. Incubate overnight at 37 °C and check for single colonies the next day.
8. Screen for positive colonies with the target gene insertion by colony PCR. Under sterile conditions, pick a single colony using a sterile inoculation loop and inoculate into 50 μL of sterile water. Use 2 μL of this as template for colony PCR and save the other 48 μL for setting up overnight cultures after verification. Proceed with PCR using DreamTaq DNA Polymerase kit. Electrophorese the amplified PCR product in 1% agarose gel alongside a 1-kb ladder and check if the bands correspond to the length of the designed target gene constructs.

3.5.6 Verification of Target Insertion

1. Set up overnight cultures of the transformed HT115-DE3 bacterial cells in LB broth containing 12-μg/mL tetracycline and 100-μg/mL ampicillin.
2. Extract plasmid the next day using the GeneJET Plasmid Miniprep Kit. Check the concentration of extracted plasmid using a Nano-Drop spectrophotometer.
3. Sequence the plasmid DNA using Sanger sequencing with primers designed in silico for dsRNA synthesis.
4. Using DNA sequence editing software (e.g., Snapgene or Benchling), prepare an in silico vector map including target insert. Align the sequences obtained from Sanger sequencing using Snapgene/Benchling to verify precise insertion.

3.5.7 dsRNA Synthesis

1. Set up cultures of the positive colonies in 3 mL of LB broth with ampicillin and tetracycline (final concentration of 100 μg/mL and 12 μg/mL, respectively). Incubate with shaking at 37 °C overnight.
2. The next day, fill a cuvette with 1 mL of the overnight culture and check the absorbance at 600-nm wavelength (optical density at 600 nm; OD_{600}) using a spectrophotometer.
3. Dilute the culture with fresh LB with antibiotics to an OD_{600} of 0.05 (make up to 20 mL) and incubate for approximately 3–4 h until an OD_{600} of 0.2 is obtained.
4. To induce dsRNA synthesis, add 2 μL of 100 mM IPTG to 20 mL of HT115 culture (final concentration of 10 mM) and incubate with shaking at 37 °C for 3–4 h.

5. Centrifuge the induced bacterial culture at 3500 rcf for 10 min at 20 °C. Decant the supernatant and use the pellet for RNA extraction using Direct-zol™ RNA Miniprep Plus Kit. Elute the RNA in 100 μL of elution buffer/nuclease-free water. Repeat elution in a new collection tube with 100 μL of fresh elution buffer/nuclease-free water.
6. Store the eluted RNA at −80 °C.
7. Dilute 2 μL of the eluted RNA in 8 μL of H_2O and 2 μL of 6× loading dye. Run in 1% agarose gel alongside a 1-kb ladder for reference. Visualize under UV light to verify if the bands correspond to the size of the target dsRNA sequence.

3.6 DLA

- Collect potato leaves, with six leaves for each treatment, from 4 to 5 weeks old potato plants (*see* **Note 9**). Place the collected leaves in the boxes.
- Prepare in vitro synthesized dsRNA for DLA to a final concentration of 20 ng μL^{-1} by diluting with distilled water accordingly. Spray the leaves using an automizer or an airbrush. Experimental controls are set by spraying $dsRNA^{Ct}$.
- For bacterial in vivo synthesized dsRNA, spray the leaves with 10 μg of total RNA per leaf using an airbrush. Experimental controls are set by spraying with RNA from HT115-DE3 carrying empty L4440, and L4440 with a cloned nonspecific gene target.
- Place the boxes with sprayed leaves in climate-controlled chambers with a temperature of 21 °C during the day and 19 °C at night, for 24 h.
- Collect *Pi*-GFP spores as explained in Subheading 3.1, step **10**; count the number of spores under a microscope with the help of a Fuchs Rosenthal chamber (0.200 mm depth) (*see* **Note 10**). Adjust the concentration of spores to 50,000 spores/mL by diluting with distilled water accordingly.

  1. Twenty-four hours post spraying, infect the sprayed potato leaves by drop inoculation with 15 μL per leaf of freshly collected *Pi*-GFP spores (adjusted to 50,000 spores/mL). Carefully place them back in the climate chamber.
  2. Five days after infection, observe the detached leaves for *P. infestans* infection lesions. Compare and quantify the lesion area of dsRNA-treated leaves to control leaves using software such as ImageJ (Fiji), to determine if the selected dsRNA target reduces *P. infestans* disease progression (Fig. 1).

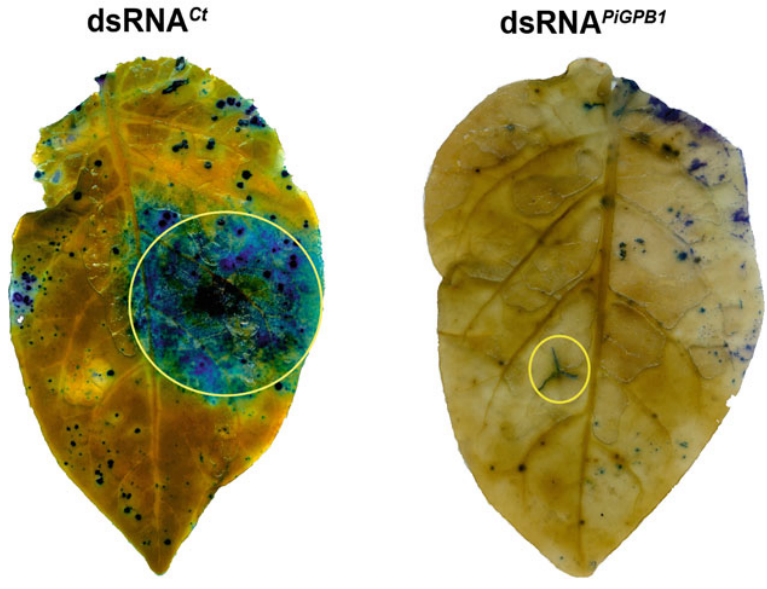

Fig. 1 Effect of control and test gene dsRNA sprays on *Phytophthora infestans* pathogenicity on potato leaves. Representative picture taken at 5-days postinoculation (dpi) of trypan blue stained potato leaves showing typical *P. infestans* infection on leaf sprayed with dsRNA^{Ct}, in comparison to decreased infection on leaf sprayed with dsRNA^{PiGPB1} (test). Yellow lines delineate the infected area on each leaf

3.6.1 Trypan Blue Staining

1. Prepare trypan blue staining solution containing 10 mL lactic acid, 10 mL buffer saturated phenol, 10 mL glycerol, 10 mL of distilled water, and 40 mg of trypan blue.
2. Five-days postinoculation, submerge infected potato leaves from each DLA treatment into individual 90-mm Petri dishes containing 20 mL of trypan blue stain solution for 30 min. Destain leaves with absolute ethanol in an orbital shaker overnight.
3. Transfer the destained leaves to 50% glycerol for 2 h to rehydrate. The rehydrated samples can be used for microscopy and long-term storage.

3.7 Confocal Microscopy

1. Preparation of samples for microscopy – Five-days postinoculation, take leaves from DLA to make 1 mm^2 cuts at the site of infection. Mount the cut leaf samples onto glass slides and add a drop of water before covering with a coverslip.
2. Using the confocal microscope (e.g., LSM880, Zeiss Microscopy GmbH, Germany), visualize disease lesions to confirm the effect of dsRNA^{PiGPB1} on *P. infestans* spore formation (or other test gene(s)) (Fig. 2). GFP and chlorophyll can be excited using lasers of 488 and 633 nm and detected at emission wavelengths of 499–552 and 647–721 nm, respectively.

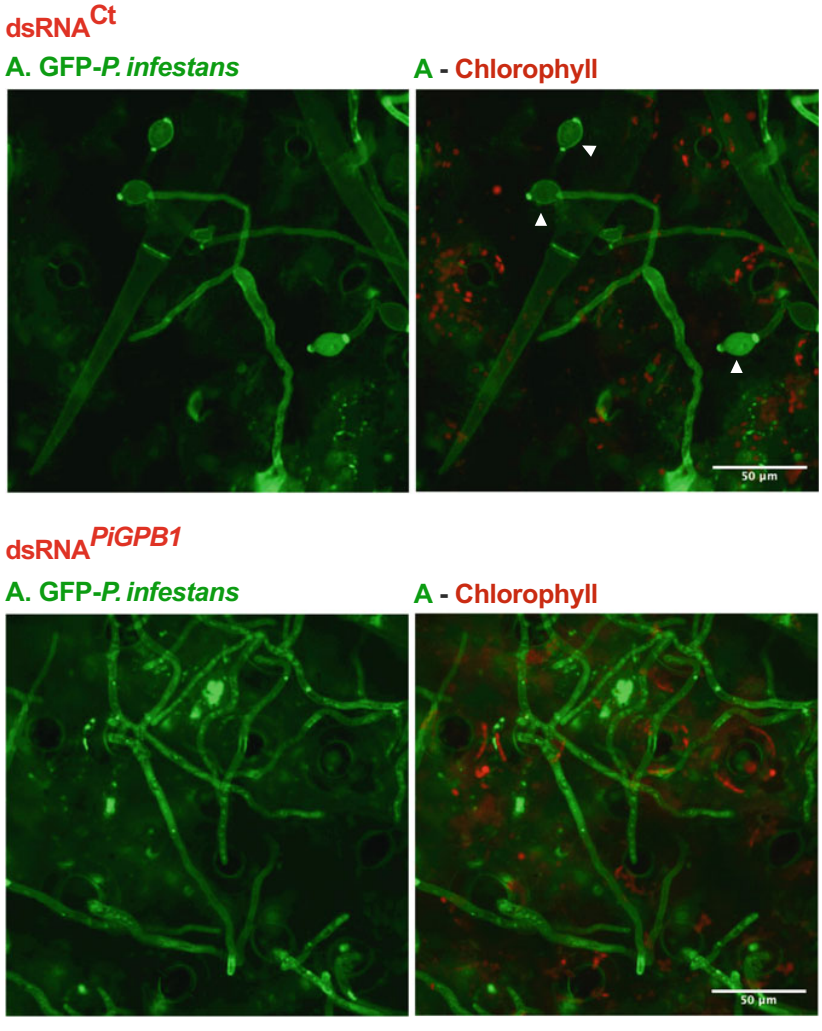

Fig. 2 Representative confocal microscopy images of *P. infestans* infection on potato leaves treated with dsRNA^{Ct} (control; **a**) or dsRNA^{PiGPB1} (**b**), taken at 5 dpi. Hyphal growth from the plant cells is visible in both control and treatment. Sporangia are formed in dsRNA^{Ct}-treated leaves (indicated as arrowheads) while there is no sporangia formation in the samples treated with dsRNA^{PiGPB1}, indicating possible gene knockdown of *GPB1* in *P. infestans*. Chloroplast autofluorescence is shown as red in these images; scale bars = 50 µm

3.8 RNA Extraction

1. Collect leaf discs (weighing 100 mg) from six infected detached leaf assay samples in individual 1.5-mL microcentrifuge tubes and freeze immediately using liquid nitrogen.
2. Grind the frozen samples into a fine powder in liquid nitrogen using mortar and pestle. Carry out RNA extraction using the Direct-zol™ RNA Miniprep Plus Kit. Once the ground samples are homogenized using TRIzol, centrifuge and transfer the

supernatant to a new 1.5-mL microcentrifuge tube (*see* **Note 11**). Elute RNA in 35 μL of nuclease-free water.

3. Measure the concentration of eluted RNA using a NanoDrop spectrophotometer before storing at −80 °C.

3.9 cDNA Synthesis

1. Generate cDNA from eluted RNA using qScript cDNA SuperMix. Combine 4 μL of the 5× cDNA SuperMix with respective RNA and make up to 20 μL using nuclease-free water. Use approximately 500 ng – 1 μg of extracted RNA as template for first-strand synthesis.
2. Incubate as follows: 25 °C for 5 min; 42 °C for 30 min; 85 °C for 5 min.
3. Store the cDNA at −20 °C.

3.10 qRT-PCR

- Design forward and reverse primers for qPCR using the Primer-BLAST tool from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (*see* **Notes 12** and **13**).
- Set up a 20 μL reaction using DyNAmo flash SYBR Green qPCR kit. The components are as follows.

Component	Volume
2× Master mix	10 μL
50× ROX dye	0.12 μL
Forward primer (0.5-μM final concentration)	1 μL
Reverse primer (0.5-μM final concentration)	1 μL
Template (cDNA) (20–25 ng)	variable
Water	Make up to 20 μL

- Use 20 ng of respective cDNA as template. Set up qPCR reactions targeting both reference and target genes individually; with four biological replicates each of cDNA synthesized from DLA samples (*see* **Note 14**). The thermal conditions are as follows.

Step	Temperature	Time	No of cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	10 s	40
Annealing/extension	60 °C	30 s	
Final extension and melt curve	65 °C to 95 °C in increments of 0.5 °C		

3.10.1 qRT-PCR Analysis

- Analyze the qPCR results using the $2^{-\Delta\Delta C}$ method [9, 10].
  - Obtain C_t values from the Bio-Rad CFX machine.
  - Calculate the average C_t value for each biological replicate.
  - Calculate $\Delta C_t = C_t$ (target) – C_t (reference) for the same biological replicate.
  - Calculate average ΔC_t of the control samples.
  - Calculate $\Delta\Delta C_t$.

    $\Delta\Delta C_t$ (control) = ΔC_t (each biological replicate, control sample) – Average ΔC_t of control sample.

    $\Delta\Delta C_t$ (test) = ΔC_t (each biological replicate, test sample) – Average ΔC_t of control sample.
  - Calculate $2^{-\Delta\Delta Ct}$.
  - To obtain fold change values, calculate geometric mean of $2^{-\Delta\Delta Ct}$ values of control and test samples individually.
  - Additionally, calculate standard deviation or standard error of the mean for the respective $2^{-\Delta\Delta Ct}$ values.

4 Notes

1. Exclude adding agar before autoclaving if preparing rye broth instead of rye agar.
2. When excited by light in the blue to ultraviolet range, the green fluorescent protein emits a bright green fluorescence, hence deriving its name.
3. Green fluorescent protein is expressed under the control of *Ham34* promoter [11].
4. Add antibiotics to the same final concentration if preparing rye broth.
5. For synthesis of dsRNA, several PCR reactions may be needed to generate enough amplified product for in vitro transcription.
6. GPB1 in *Phytophthora infestans* does not contain any introns and so genomic DNA can be used for PCR. For other *P. infestans* genes, check for presence of introns in FungiDB [https://fungidb.org/fungidb/app].
7. An additional 4 bp of random sequence needs to be added to the 5′ end of the primer to ensure correct restriction digestion after PCR.
8. Evenly spread transformed HT115-DE3 bacteria onto the prepared LB agar plates using a sterile L-shaped spreader.
9. Take airtight boxes and line the bottom with water-dampened tissue paper, overlaid with mesh. This maintains a microclimate

in the box, mimicking greenhouse conditions and preventing the leaves from drying and wilting quickly.

10. Load 10 μL of spore sample into each of the two chambers and cover using a coverslip. Each chamber has 16 squares of 1 mm length, represented by three grid lines and each of these 16 squares are further divided into 16 smaller squares. Count the number of sporangia in all 16 1-mm squares. Avoid including the spores on the triple-lined boundaries. Take an average of these numbers and use the formula below to determine the number of sporangia present in 1 mL of spore solution.

$$\text{Spores/mL} = (\text{Average per square} \times 16)/0.0032$$

11. This eliminates the chance of any particulate debris clogging the column and reducing the yield of extracted RNA.
12. Primers for qRT-PCR need to be outside the region used for generating the dsRNA.
13. Include primers for reference genes in *P. infestans* and *S. tuberosum* while designing qRT-PCR.
14. Use three technical replicates per biological replicate.

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Wheat, barley and potato production face increasing pressure from plant diseases, while conventional control methods often have economic and environmental drawbacks. This thesis evaluates spray-induced gene silencing (SIGS), an RNA-based plant protection strategy, as a sustainable alternative. The results show effective suppression of Fusarium head blight in wheat and barley using double-stranded RNA sprays, with no disruption of key leaf-associated microbial communities in cereals and potato. These findings support SIGS as a targeted and environmentally safe technology with potential for agricultural deployment.

Poorva Sundararajan received her doctoral education at the Department of Plant Breeding, Swedish University of Agricultural Sciences (SLU), Alnarp. She holds a Master of Science in Biology from SLU Alnarp and a Bachelor of Technology in Biotechnology from Vellore Institute of Technology (VIT), India.

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SLU generates knowledge for the sustainable use of biological natural resources. Research, education, extension, as well as environmental monitoring and assessment are used to achieve this goal.

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