Spray Induced Gene Silencing in *Phytophthora infestans*

– Investigating exogenous dsRNA application for the control of potato late blight

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Abstract

Phytophthora infestans is an oomycete pathogen and the causal agent of late blight disease in potato and tomato. Even today, it causes massive loss to crop and economy worldwide. Current disease control strategies include breeding for resistance and extensive fungicide spray. However, fungicides raise serious environmental concerns and include the risk of *P. infestans* developing resistance. Previously, RNA interference has been successfully employed to control the disease severity through a transgenic approach known as host-induced gene silencing. In this study, we exploit the RNAi mechanism in an alternative, non-transgenic way through a method called spray-induced gene silencing (SIGS). To achieve reduced disease progression, SIGS utilizes double stranded RNA (dsRNA) in the form of a foliar spray to target pathogen genes important for growth and infection. We targeted five genes in *P. infestans*. The guanine-nucleotide binding protein β-subunit (*PiGPB1*) is important in sporangia formation, the oxysterol binding protein (*PiOSBP*) is a fungicide target and the haustorial membrane protein (*PiHmp1*) is important to establish infection. In addition, the carbohydrate-active enzymes cutinase (*PiCut3*) & endo-1,3(4)-β-glucanase (*PiEndo3*) are essential for penetration and colonization of the host tissue. Through confocal imaging, we have provided a proof of concept that *in vitro* synthesized dsRNA is taken up by *P. infestans* sporangia from surrounding environment and dsRNA sprayed leaves. We then show that targeted dsRNA spray reduces *P. infestans* disease progression through detached leaf assay. The varying degree of disease reduction observed in the different dsRNA treatments indicated that the efficiency of SIGS relies on the selection of target genes. Thus, we conclude that target-specific gene silencing using SIGS can be a potential methodology to sustainably control potato late blight.

Table of contents

List of tables

List of figures

Abbreviations

dpi	Days post infection
DLA	Detached leaf assay
dsRNA	Double-stranded RNA
GFP	Green Fluorescent Protein
miRNA	Micro-RNA
PCR	Polymerase chain reaction
RT-qPCR	Quantitative reverse transcription PCR
SIGS	Spray Induced Gene Silencing
siRNA	Small-interfering RNA
sRNA	Small RNA

1. Introduction

1.1 Phytophthora infestans

Phytophthora infestans is an oomycete pathogen known for causing late-blight disease in potato and tomato crops. The devastating pathogen is of significant economic importance because of the huge crop losses associated with the late-blight disease (Haverkort et al., 2008). In potato plants, it infects leaves, stems and tubers, and can therefore effectively destroy the whole plant (Hardham, 2007; Judelson, 1997; Kamoun et al., 2015). The infected leaves and stems develop brownish necrotic lesions (Figure 1) that can also spread to the tubers. Infected seed tubers act as the main sources of inoculum and the pathogen can be carried this way to the next growing season (W. E. Fry et al., 2015). Apart from potato and tomato, the pathogen also has hosts in other members of the *Solanaceae* family including petunia, *S.nigrum, S. sisymbriifolium* and *S. dulcamara* (Becktell et al., 2007; Flier et al., 2003).

Figure 1: *Phytophthora infestans* disease lesions on potato leaves (Kamoun et al., 2015).

1.1.1 Oomycetes and taxonomy of *P. infestans*

Oomycetes were previously thought to be closely related to fungi as they form similar morphological structures like mycelia and spores (Ribeiro, 2013). However, more recent molecular studies based on their rRNA sequence (Cooke et al., 2000) describe oomycetes to be stramenopiles along with brown algae and diatoms (Beakes et al., 2012; Kamoun, 2003). Oomycetes are placed under the kingdom Chromista, alongside other fungal-like organisms (Cavalier-Smith, 1981). The main attributes distinguishing oomycetes from true fungi include differences in the composition of the cell wall, certain biochemical pathways and the type of flagellum produced (Bartnicki-Garcia, 1968; Judelson & Blanco, 2005; Vogel, 1960). The cell wall of oomycete mycelia is predominantly made of cellulose and β 1-3-glucans unlike the chitinous cell walls of fungal mycelia. Oomycetes also differ from fungi in using mycolaminarin, a carbohydrate as their energy storage molecule similar to the kelp and diatoms (Erwin et al., 1983). Phylogenetic analyses to understand the evolutionary relationships of filamentous oomycetes have led to their further classification into orders Peronosporales and Saprolegniales. The genus *Phytophthora* is placed under Peronosporales and includes over 120 species (Martin et al., 2014). The name *Phytophthora* is derived from a Greek word meaning plant (*Phyto*) destroyer (*phthora*). Over 60 species of *Phytophthora* are recognized as highly invasive plant pathogens globally (Kamoun, 2003). Some of the other plant pathogenic oomycetes include *Plasmopara viticola*, *Albugo candida* and various species from the genera *Hyaloperonospora* and *Pythium* (Agrios, 2005; Kamoun et al., 2015).

1.1.2 *P. infestans* biology

P. infestans is a hemibiotrophic organism meaning it exhibits an initial asymptomatic biotrophic phase followed by a destructive necrotrophic phase (Latijnhouwers et al., 2003). During the biotrophic phase, the pathogen infects and goes through a latent phase before displaying any disease symptoms. The biotrophic phase of *P. infestans* starts upon the landing of a spore on plant tissue and its subsequent germination.

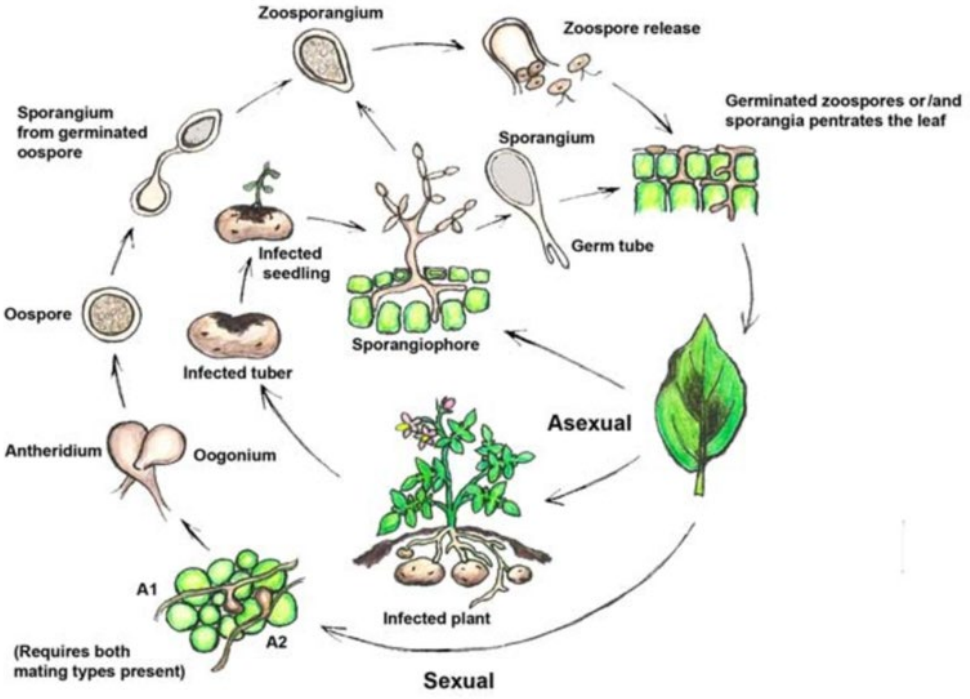

Figure 2: Life cycle of *Phytophthora infestans* depicting the different asexual and sexual phases involved. The asexual phase involves sporangia containing zoospores, both of which can germinate to form hyphae and appresorium. Appresoria are used to penetrate host cells and establish biotrophic infection alongside haustoria. The pathogen then feeds off the host cells before moving on to the necrotic phase and establishing secondary infection. Source: Illustration by H P Hovmalm, from (Therése Bengtsson, 2013).

As elucidated in Judelson & Blanco, 2005, the life cycle of *P. infestans* can involve both sexual and asexual phases. During the asexual phase, they can form two different kinds of spores known as sporangia and zoospores (Figure 2). Sporangia are multinucleate, lemon-shaped structures that are borne on stalk-like structures called sporangiophores. Sporangiophores in *Phytophthora* are branched and indeterminate, producing sporangia continuously. Direct germination occurs at higher temperatures, involving the formation of a germ tube from the sporangium itself. On the other hand, during low temperatures, sporangia release six to eight uninucleate, biflagellate and motile zoospores. Upon finding the host surface, zoospores then encyst and produce a germ tube. The germinated sporangia and cysts form a specialised structure called an appressorium (Figure 3) that helps to penetrate the surface of the leaf tissue (Latijnhouwers et al., 2003). Haustoria are structures that extend into host cells for the delivery of defence-suppressing effector proteins (Whisson et al., 2007). These structures together help the proliferation of *P. infestans* in host tissue and thus establish the biotrophic stage of infection. As the pathogen shifts from the biotrophic to necrotrophic phase, typical symptoms such as brown necrotic lesions on leaves and stems become visible (Avrova et al., 2008). The pathogen then produces sporangiophores at the exterior surface of the

tissue to generate new sporangia and further invade surrounding host tissue (Figure 3). The whole asexual cycle can happen in as short as four to five days, thereby making it a fast-spreading, devastating pathogen (W. Fry, 2008). In addition, the sexual spores or oospores can survive for months without a host in the soil thereby increasing the infection potential of *P. infestans* (Mayton et al., 2000; Widmark et al.,2007).

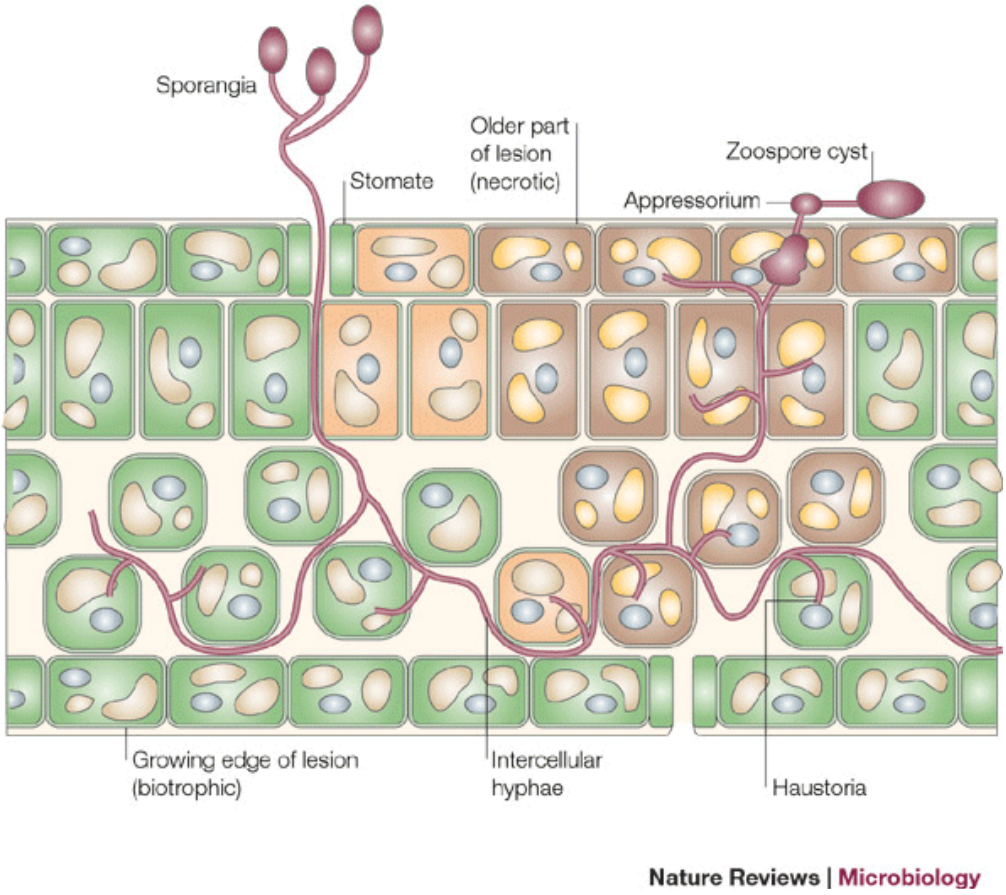

Figure 3: Representative image of *P.infestans* course of infection showing progression from biotrophic to necrotrophic phase and further invasion of new, surrounding host tissue (Judelson & Blanco, 2005).

1.2 Control strategies

At present, the control strategies to prevent and manage potato late blight rely heavily on spraying agrochemicals. Although effective, the use of such fungicides is expensive, alters the environmental conditions including the soil microbiota, poses a threat to animal and human health (Aktar et al., 2009) and also raises the question of the pathogen developing resistance against the fungicide (Ivanov et al., 2021).

Resistance genes (R-genes) are a part of the plant's defence arsenal against pathogen invasion (Hammond-Kosack & Jones, 1997). They encode for R proteins that contain specific domains capable of detecting pathogen effector molecules upon infection. Several wild relatives of *Solanum tuberosum* contain R-genes and display resistance against the pathogen (Vleeshouwers et al., 2011). Hence, they are an integral part of potato resistance breeding. Breeding for resistance using R-genes can help develop varieties that are resistant to the pathogen. More recent developments in potato resistance breeding include stacking several R genes either through cis- or trans- genesis to provide an increased resistance (Haverkort et al., 2016). However, such breeding programs are proving to be inadequately efficient due to several reasons. Both conventional and modern plant breeding techniques are tedious and time consuming. In addition, the genomic plasticity of *P. infestans* poses a risk of the pathogen overcoming the resistance (W. Fry, 2008; Haas et al., 2009; Haverkort et al., 2016). The genome of *P. infestans* is approximately 240 megabases and is larger compared to other oomycetes (Haas et al., 2009). It contains more gene sparse regions (approximately 74%) than gene rich regions (Haas et al., 2009). These gene sparse regions consist mostly of transposable elements (TE) and repetitive DNA sequences. Transposable elements or transposons are genetic elements that jump to different locations within the genome and are a common occurrence in oomycete genomes (Kamoun, 2003). Several effector-encoding genes important for establishing infection are located in these TE-rich regions. This contributes to the genomic plasticity of *P. infestans* and helps the pathogen generate more targets and alternative modes of action to better manipulate host immunity. Thus, breeding for resistance can be a challenging and insufficient way to control *P. infestans*.

Since it was first described in the 1870s, *P. infestans* has been a topic of numerous scientific studies owing to its historical importance and impact on the economy worldwide. But, several aspects of *P. infestans* biology and genetics are yet to be unravelled. The hemibiotrophic life style and short period of time for proliferation, combined with the genomic instability due to presence of transposons, makes finding sustainable control strategies against *P. infestans* challenging. Therefore, there is still a search for alternate control strategies to curb its devastating effects. It is unlikely that individual strategies will offer sustainable control of potato late blight. Hence, the concept of integrated pest management has taken precedence over the years. As the world population grows by the day, the need for improved food security grows along with it. Therefore, it is imperative now more than ever to find effective alternate control methods that are environmentally friendly and sustainable in the long run.

1.3 RNAi/ Gene Silencing

RNA silencing or RNA interference is a natural defense mechanism and is conserved through most eukaryotes. It was brought to light in 1990 when co-suppression of both the transgene and homologous endogenous gene was observed upon introduction of chimeric genes in petunia plants (Napoli et al., 1990). Subsequently, Fire et al., 1998 uncovered that double-stranded RNA has the potential to cause sequence-specific genetic interference in the nematode *Caenorhabditis elegans*. Hamilton & Baulcombe, 1999 then showed that antisense RNA derived from plant transgene was important in determining the specificity of gene silencing.

In RNAi, long double-stranded RNAs (dsRNAs) of cellular or exogenous origin (Dalakouras et al., 2020) are processed with the help of Dicer or Dicer-like endonucleases (DCL) into 21-24 nucleotide non-coding guide RNA molecules named as small interfering RNAs (siRNAs) (Liu et al., 2009). On the other hand, hairpin RNAs (hpRNAs) of endogenous origin can also be cleaved by DCL into micro RNAs (miRNAs) (X. Chen, 2009). siRNAs and miRNAs can be collectively termed small RNAs (sRNAs). The sRNAs generated subsequently trigger transcriptional or post-transcriptional silencing of genes via sequence-specific mRNA cleavage/degradation, translational repression; or chromatin modification (D. Baulcombe, 2004; D. C. Baulcombe, 2015) (*Figure 4*).

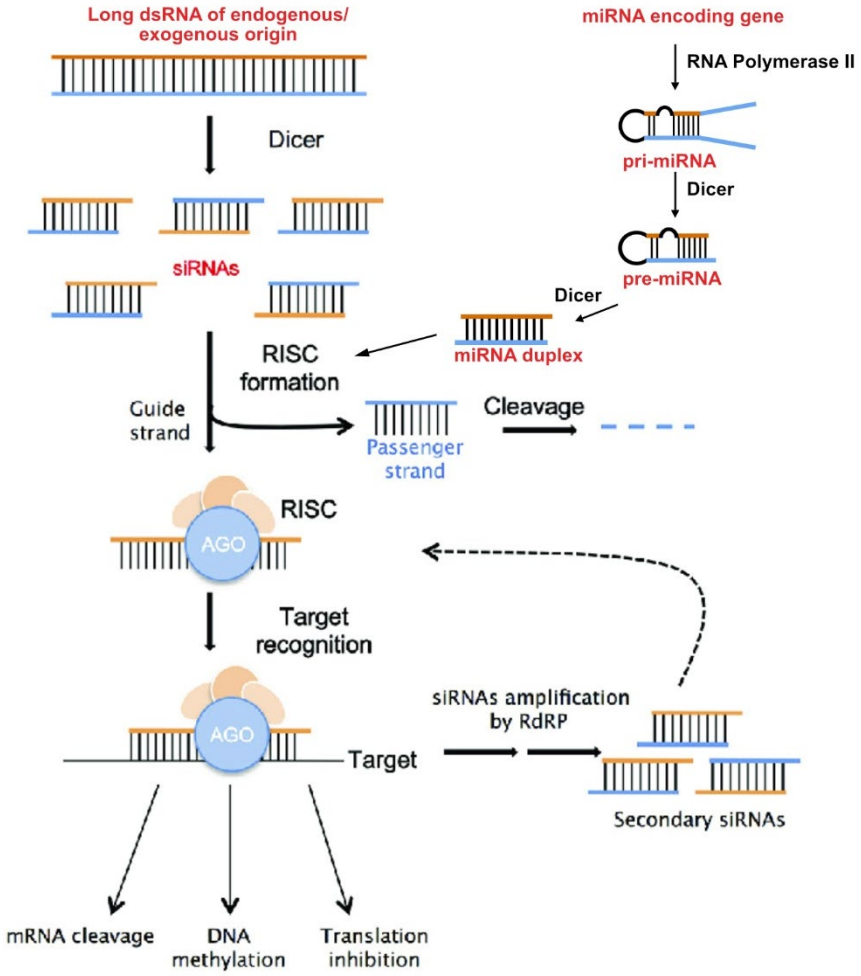

Figure 4: **The RNAi pathway**: Long dsRNAs or hairpin RNAs are spliced into short duplexes of siRNAs or miRNAs by Dicer/DCL in consecutive steps. The sRNAs thus generated are split into guide and passenger strands; and the guide strand is loaded onto the AGO containing complex RISC whereas the passenger strand is degraded. The activated RISC can then trigger post transcriptional gene silencing or generation of secondary sRNAs (modified from Limera et al., 2017).

1.3.1 Biogenesis of sRNAs

Although both siRNAs and miRNAs lead to transcriptional/post-transcriptional gene silencing, their biogenesis and precursor structures are different. The process is initiated when long strands of dsRNA/hpRNA are subsequently cleaved into mature sRNA by DCL proteins. miRNAs are derived from single-stranded hpRNA molecules cut at precise locations. The miRNA encoding genes undergo transcription by RNA Polymerase II to give rise to primary miRNA (pri-miRNA). With the help of Dicer complexes, pri-miRNA is then cleaved into precursor miRNA (pre-miRNA) and miRNA duplexes are formed in successive steps. These miRNA duplexes are then unwound by the RNA-induced silencing complex (RISC)

to produce mature miRNAs (Figure 4) (Sultana Nilufar Jahan, 2015; MacFarlane & R. Murphy, 2010).

1.3.2 DICER and DCL

In 2001, DICER was discovered as the enzyme responsible for producing putative guide RNAs (Bernstein et al., 2001). In plants, both siRNAs and miRNAs are cleaved by DICER/DCL (X. Chen, 2009). The DCL family belongs to a class of RNase-III enzymes and varies between species with 2-5 members in most higher plants and some fungi (Bologna & Voinnet, 2014; Liu et al., 2009). As discussed in Dalakouras et al. (2020), four Dicer-like paralogs have been identified in *Arabidopsis thaliana*. DCL2, DCL3 and DCL4 are responsible for cleaving dsRNA into 22-, 24- & 21- nucleotide siRNA, respectively, while DCL1 cleaves genome-encoded hairpin RNA into 21- or 22- nucleotide miRNA (Bologna & Voinnet, 2014; Borges & Martienssen, 2015; Dunoyer et al., 2005; Xie et al., 2004). DCL1 additionally plays a role in sRNA biogenesis from endogenous inverted repeats; DCL2 is involved in viral resistance and produces siRNAs from cis-regulatory antisense transcripts; DCL3 generates siRNAs leading to chromatin modification; and DCL4 is associated with trans-acting siRNA (tasiRNA) metabolism (Liu et al., 2009).

1.3.3 Argonaute

Once cleaved by DICER/DCL, sRNAs are loaded onto ARGONAUTE (AGO) containing RNA-induced silencing complex (RISC). sRNAs are characterized by a 2 nucleotide 3' overhang. The first nucleotide in the 5' end along with the 3' modification determines the next step in the RNAi pathway. Depending on the nature of the sRNA and the AGO involved, this process might lead to target cleavage and degradation, translational repression, or recruitment of additional co-factors. AGO1 preferentially binds to 21 - 22 nucleotide sRNAs. If the sRNA has a 5' Uridine and is of 21-nt length, it leads to recognition of complementary mRNA transcripts and subsequent cleavage/degradation. In the case of 22 nucleotide sRNAs, it leads to change in AGO1 conformation and transitivity/generation of secondary sRNAs through the RDR (RNA-directed RNA Polymerase) pathway (H. M. Chen et al., 2010; Dalmay et al., 2000; Mlotshwa et al., 2008; Moissiard et al., 2007). siRNAs of 24 nucleotides length are the most abundant in plants, and are recognized by AGO4 when with a 5' Adenosine. They trigger RNA-directed DNA methylation by recruitment of cognate DNA or its nascent transcript (Borges & Martienssen, 2015). miRNAs of 22 nucleotide length are processed in the same way as 22 nucleotide siRNAs and trigger the RDR pathway leading to transitivity. When the sRNA number passes a certain threshold through transitivity, it leads to

systemic resistance (Dalakouras et al., 2020). Transitivity thereby helps in enhancing the effect of RNA-mediated gene silencing.

1.4 Small RNAs in host-pathogen interactions.

sRNAs have been found to play significant roles in several plant developmental processes including developmental regulation, epigenetic changes and response to biotic stress/pathogen invasion (Kidner & Martienssen, 2005; Llave, 2004; Vaucheret, 2006; Zhang et al., 2006). They are thus critical components of post-transcriptional gene regulation. Mechanistic insights into the RNAi phenomenon provides new opportunities to exploit it as a plant protection strategy. Experimental systems have displayed host-induced gene silencing, wherein artificial sRNAs derived from plant transgenes effectively induce gene silencing in a wide range of pests/pathogens including nematodes, fungi and oomycetes. This is achieved by targeting specific genes in the pathogen (Sultana N. Jahan et al., 2015; Nowara et al., 2010). HIGS exploits the use of hairpin RNAs and sRNAs for gene silencing. Although promising as a prospective disease control strategy, HIGS is still a transgenic approach and is prone to heavy regulatory and public scrutiny. Another factor limiting the practical application of HIGS in plant protection is the absence of established transformation methods for several plants of agricultural importance.

To circumvent the use of transgenes, the external application of double-stranded RNA molecules was explored as a way to control *Botrytis cinerea* and *Fusarium graminearum* infections (Koch et al., 2016; Wang et al., 2016). Interestingly, the grey mould causing fungus *Botrytis cinerea* has also been shown to suppress plant defense response and establish infection by directing sRNAs into the plant and targeting the gene silencing mechanism in the host. This proved that gene silencing through sRNAs happens not only from host to pathogen but also the other way around, from pathogen to host (bidirectional cross-kingdom RNAi) (Wang et al., 2016). These promising prospects of spraying dsRNA/sRNA has resulted in a multitude of studies in the past few years (Rank & Koch, 2021) and the methodology has been termed as spray-induced gene silencing or SIGS. Studies exploring SIGS as a plant protection strategy have been carried out with varying degrees of success in fungal pathogens including *Aspergillus* niger, *Botrytis cinerea, Fusarium graminearum*, *Fusarium asiaticum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Verticillium dahliae* (Koch et al., 2019; Qiao et al., 2021; Song et al., 2018; Werner et al., 2020) and the oomycetes such as Hyaloperonospora arabidopsidis (Bilir et al., 2019).

SIGS is gaining popularity because of its sequence-specific ability to target only organisms of interest. It is also environment-friendly, as it does not employ

transgenes as in HIGS. In this project, we targeted five genes from *P. infestans* that played an important role in growth and development or infection. We then designed corresponding dsRNA in-vitro to study their effect in reducing the spread of the pathogen. The targeted genes include guanine-nucleotide binding protein β-subunit (*PiGPB1),* oxysterol binding protein *(PiOSBP),* haustorial membrane protein *(PiHmp1),* cutinase (putative, *PiCut3)* & endo-1,3(4)-β-glucanase *(PiEndo3).* Silencing of *PiGPB1* previously resulted in defective sporangia formation, indicating the gene is critical in sporangia development and vegetative growth (Latijnhouwers & Govers, 2003). *PiOSBP* is the molecular target of oxathiapiprolin, a recent fungicide effective against *Phytophthora* sp. (Miao et al., 2016; Pasteris et al., 2016). *PiHmp1* is essential for haustorium formation and therefore in establishing the biotrophic phase of *P. infestans* infection (Avrova et al., 2008). *PiCut3* and *PiEndo3* have been classified as potential carbohydrate-active enzymes (CAZymes). CAZymes are a class of enzymes that break down saccharides and include glucoside hydrolases such as endo- and exo- glucanases and carbohydrate esterases such as cutinases among others. These enzymes help in penetration of host outer tissue primarily comprising cutin and β-1,4-glucans and are therefore important in establishing *P. infestans* infection (Brouwer et al., 2014; Ospina-Giraldo et al., 2010).

2. Materials and Methods

2.1 Phytophthora infestans Cultures

P.infestans 88069 (Wildtype; Pi^{88069}) and a Green fluorescent protein-expressing *P.infestans* (Pi^{EGFP}) (Avrova et al., 2008) were grown by inoculating 5 mm^2 agar plugs in 90 mm diameter Petri plates containing rye agar with antibiotics ampicillin & pimaricin to a final concentration of 100 µg/ml and 12 µg/ml respectively. Additionally, geneticin was added to a final concentration of 10 µg/ml for growing Pi^{EGFP}. The plates were then incubated at 20 °C. For use in the experiments, spores were collected under sterile conditions from two-week-old plates by washing the agar plates with water, gently scraping the surface and sides to release spores and passing through a 40-micron cell strainer into a 50 ml centrifuge tube. The number of sporangia was counted under a microscope using a Fuchs-Rosenthal chamber and adjusted accordingly.

2.2 Growing Potato Plants

Potato plants (cv. Bintje) were propagated through tissue culture or grown from seed tubers. For tissue culture, under sterile conditions, explants were collected by cutting close to the nodes of existing tissue culture plantlets and inoculated on Petri plates containing Murashige-Skoog medium (Duchefa, full strength). Potato seed tubers or one-month-old tissue culture plantlets were transferred to 2.5 litre pots containing well-drained fertilized compost for growing in a climate-controlled chamber. The following conditions were used in the climate chamber - 21/19 °C day/night temperature, 16 hour photoperiod and 60 % humidity. The plants were watered regularly as required. Lateral leaflets from the same level across plants were collected from four to five-week-old plants for use in the experiments.

2.3 dsRNA Synthesis

2.3.1 DNA Extraction

In order to synthesize dsRNA for our target gene sequences, DNA was extracted from *P.infestans*. Pi^{88069} was grown in a 6 - well cell culture plate containing rye broth with ampicillin and pimaricin to a final concentration of 100 µg/ml and 12 µg/ml respectively. The plate was incubated at 20 °C without shaking for a week. Fifty milligrams of mycelia was collected; the sample was frozen using liquid nitrogen and lysed immediately using a mortar and pestle prior to extraction using QIAGEN DNeasy Plant Mini Kit. The DNA was eluted in 50 µl of nuclease-free water. The concentration of the extracted DNA was measured using the NanoDrop spectrophotometer and the extracted DNA was stored at -20 °C. Plasmid DNA previously extracted from the pTOR-eGFP plasmid was used to synthesize dsRNA corresponding to GFP sequence ($dsRNA^{GFP}$).

2.3.2 PCR

We selected target genes in *P.infestans* which were either reported as or likely to be essential for pathogenesis, expressed at different stages of the infection cycle, or was an agrochemical target. Using the SnapDragon primer design tool for double stranded RNA (https://www.flyrnai.org/snapdragon), forward and reverse primer pairs containing the T7 promoter sequence (GTAATACGACTCACTATAGGG) were designed for the target gene sequences with an amplicon size of 200 – 400 bp for shorter fragments and 700 bp for longer fragments.

Table 1: Target genes used in this study

S.No:	Target Gene	Accession number
1.	Guanine-nucleotide binding protein β-subunit (*PiGPB1*; PITG_06376)	XM_002998462.1
2.	Oxysterol binding protein *(PiOSBP;* PITG_10462*)*	XM_002902204.1
3.	Haustorial membrane protein *(PiHmp1; PITG_00375)*	XM_002908934.1
4.	Cutinase *(PiCut3*; PITG_12361*)*	XM_002900240.1
5.	Endo-1,3(4)-β-glucanase *(PiEndo3; PITG_13567)*	XM_002899724.1

Table 2: List of primers for dsRNA synthesis

Primers	**Sequence (5'-3')**
T7 *EGFP* dsRNA long F	**GTAATACGACTCACTATAGG**GGACGTAAACGGCCACAAGTT
T7 *EGFP* dsRNA long R	**GTAATACGACTCACTATAGG**GAGTTCACCTTGATGCCGTTC
T7 *PiGPB1* dsRNA short F	**GTAATACGACTCACTATAGGG**ATGTTTATTTCGGGCTCGTGTGA
T7 *PiGPB1* dsRNA long F	**GTAATACGACTCACTATAGGG**CTCTACGCTCCAGTTGGGTC
T7 *PiGPB1* dsRNA short/long R	**GTAATACGACTCACTATAGGG**TAGATATGCGCTCCGGAAGT
T7 *PiOSBP* dsRNA short F	**GTAATACGACTCACTATAGGG**CACCCGCCAATCAGTAACTT
T7 *PiOSBP* dsRNA short R	**GTAATACGACTCACTATAGGG**GCCCATACCAGACTTTGCAT
T7 *PiHmp1* dsRNA short F	**GTAATACGACTCACTATAGG**TTGTTGACCAGCTCGTTGAG
T7 *PiHmp1* dsRNA short R	**GTAATACGACTCACTATAGG**CCATCACCCTTCTTCTTCCA
T7 *PiHmp1* dsRNA long F	**GTAATACGACTCACTATAGG**TCCAGTAAGGGCTCTCCTGA
T7 *PiHmp1* dsRNA long R	**GTAATACGACTCACTATAGG**GCAGAAGTGTTAAAAGCGCC
T7 *PiCut3* dsRNA short/long F	**GTAATACGACTCACTATAGG**CAACCACGTCGTGTCTATCG
T7 *PiCut3* dsRNA short R	**GTAATACGACTCACTATAGG**GTTGCAGAACTCAATGGCCT
T7 *PiCut3* dsRNA long R	**GTAATACGACTCACTATAGG**CTAAGGCAGCAGCTTTCTCG
T7 *PiEndo3* dsRNA short F	**GTAATACGACTCACTATAGG**CGTCGAGGATCTAGGCAGTC
T7 PiEndo3 dsRNA long F	**GTAATACGACTCACTATAGG**GTCAAAGACGTGAACGCAGA
T7 *PiEndo3* dsRNA short/long R	**GTAATACGACTCACTATAGG**GACGAGAAGCAGCGATAACC

The target genes chosen in this project do not contain any introns and so genomic DNA was used for PCR. PCR was carried out using Phusion™ High–Fidelity DNA Polymerase. 10 ng of pTOR-eGFP/*P.infestans* 88069 DNA extracted in the

previous step was used as the template for GFP and *P.infestans* target genes, respectively. The thermal conditions used for PCR is listed below in *Table 3*.

Table 3: Thermal conditions for PCR

Step	Temperature	Time	No of cycles
Initial denaturation	98 °C	30 seconds	1
Denaturation	98 °C	10 seconds	
Annealing	60 °C	30 seconds	30
Extension	72 °C	30 seconds	
Final extension	72°C	5 minutes	1

Several PCR reactions were carried out to generate sufficient amplified product required for further in-vitro transcription. Amplification of products by PCR was verified by gel electrophoresis (1 % agarose in 1 x TAE buffer). A 1 kb ladder was run alongside for reference. The PCR products were then purified using QIAquick® PCR Purification Kit and eluted in 25 µl of nuclease-free water.

2.3.3 Synthesis of dsRNA

Double stranded RNA corresponding to the target gene sequences were synthesized and purified in-vitro using the Invitrogen MEGAscript RNAi Kit, with 1 µg of the respective purified PCR products as template. Additionally, Cyanine3-tagged UTP was used in in-vitro synthesis to label the dsRNA homologous to GFP (Cy3-dsRNA^{GFP}). A non-specific, control dsRNA (dsRNA^{Ct}) was also synthesized using the control template provided in the kit. Successful dsRNA synthesis was verified using gel electrophoresis (1 % agarose in 1 x TAE buffer) and quantified using the NanoDrop spectrophotometer. The dsRNA was stored at -80°C until further use.

2.4 Detached Leaf Assay

Leaves from the same level across plants were collected from four week old plants. 10 µg of dsRNA was sprayed on each leaf using an Airbrush and compressor setup (Cocraft and Biltema Mini Compressor MC.90, respectively). Experimental

controls were set by spraying water, dsRNA^{Ct} and dsRNA^{GFP}. The treatments included dsRNAs corresponding to *OSBP, GPB1, Hmp1, Cut3* and *Endo3*. The dsRNA sprayed leaves were placed in air-tight boxes in order to maintain a micro-climate and incubated for 24 hours in a climate chamber (22°C daytime and 20°C night-time temperature; 16 h photoperiod) before drop inoculation with *Pi*^{88069} (15 µl of 50,000 spores/ml). The boxes were returned to the climate chamber and the progression of infection measured in terms of lesion area was compared between the control and treatment samples at 5 dpi.

2.5 Trypan Blue Staining

Trypan blue staining was performed to visualize the extent of infection in the leaf samples. Trypan blue solution was prepared as follows: 40 mg of trypan blue, 10 ml lactic acid, 10 ml buffer saturated phenol, 10 ml glycerol and 10 ml of distilled water. Five days post infection, the infected potato leaves were transferred to a 90 mm diameter Petri plate and submerged in 20 ml of trypan blue solution for 30 minutes. The leaves were then submerged in absolute ethanol and placed on an orbital shaker overnight to de-stain. The next day, the leaves were transferred to 50% glycerol for two hours to rehydrate for visualization/microscopy and long-term storage. The leaves were then imaged using a scanner (V850Pro; Epson).

2.6 Quantification of lesion area and statistical analysis

The images of the trypan blue stained leaves were analysed using ImageJ software to quantify and compare the area of *P. infestans* disease progression. Statistical analyses of the area values thus obtained were performed using Jmp Pro software. Analysis of means (ANOVA) using Dunnett's test with Mock (No dsRNA) samples as control was used.

2.7 ChemiDoc Imaging

To verify the silencing of the targeted gene through dsRNA spray on detached leaves, leaves were sprayed with either dsRNA^{Ct} or dsRNA^{GFP} and infected with Pi^{EGFP} after 24 hours in the detached leaf assay set-up mentioned above. The infected samples were imaged at 5 dpi in a ChemiDoc MP imaging system (Bio-Rad Laboratories). Predefined settings for Alexa488 and Cy3 were used for visualizing GFP and Cy3 fluorescence, respectively.

2.8 Western Blot

Relative accumulation of GFP protein was quantified using immunoblot analysis with anti-GFP-HRP antibody. Equal amounts of the leaf samples were homogenized in the 4x Laemmli extraction buffer (Bio-Rad Laboratories), boiled for 95 °C for 10min and centrifuged at 13,000 rpm for 10 min. Proteins were separated in 12% SDS-PAGE gels and transferred to PVDF membrane. The membrane was then blocked w7ith EverBlot Blocking buffer (Bio-Rad Laboratories) The expression of GFP proteins were assayed using anti-GFP-HRP monoclonal antibodies (GF28R; Invitrogen) at a final dilution of 1:3000. The membrane was then washed with 1× TBS-T thrice and 1× TBS in successive steps. The membrane was the developed using ECL substrate (Amersham, GE Healthcare) before proceeding to view the bands using a ChemiDoc MP imaging system (Bio-Rad Laboratories). Predefined settings for chemiluminescence and colorimetry were used for visualizing the protein and ladder bands, respectively.

2.9 Quantification of transcript abundance

2.9.1 RNA Extraction

100 mg of infected leaf tissue from the detached leaf assay were collected 5 dpi, froze using liquid nitrogen and used for RNA extraction purposes. The extraction of total RNA was carried out using the Qiagen RNeasy Plant Mini Kit followed by DNase treatment using the Ambion Turbo DNA-free kit. The concentration of the extracted RNA was measured using the NanoDrop spectrophotometer before storing the samples at -80°C.

2.9.2 cDNA Synthesis

Reverse transcription to generate cDNA sequences was performed using the Quantabio qScript SuperMix, with 400 ng of respective RNA as template. The thermal conditions used is listed in *Table 4*.

Table 4: Thermal conditions for reverse transcription

Temperature	Time
22 °C	5 minutes
42 °C	30 minutes

85 °C	5 minutes

2.9.3 RT-qPCR

Quantitative reverse transcription PCR was performed using DyNAmo Flash SYBR Green kit (Thermo Scientific) to assess changes in target gene expression and confirm if the reduction in disease progression was indeed a consequence of dsRNA mediated gene silencing. 20 ng of respective cDNA obtained in the previous step was used as template. For each biological replicate, 3 technical replicates were used. The differential gene expression was normalized to the *PiActin* reference gene from *P.infestans*. Using the primer design tool from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), primers were designed for qPCR for the target and reference genes, with an amplicon size of 80-120 bp. The transcript levels of the *PiOSBP*, *PiGPB1*, *PiHmp1*, *PiCut3* and *PiEndo3* genes in the respective dsRNA treatments were compared to samples treated with dsRNA^{Ct}. The C_T values obtained from qPCR were analysed using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

Table 5: List of primers for RT-qPCR

qPCR Primers	**Primer sequence (5'-3')**
EGFP qPCR FOR	GACCACTACCAGCAGAACACC
EGFP qPCR REV	CGCTTCTCGTTGGGGTCTTT
PiGPB1 qPCR FOR	TTCCGGAGCGCATATCTACC
PiGPB1 qPCR REV	TCTTGAGTAGCGTGTCCCAG
PiOSBP qPCR FOR	TCTCCTCCACGTGAGATCTGT
PiOSBP qPCR REV	GCCACTCTGGAAGCTGTTGA
PiHmp1 qPCR FOR	GTGAAGGTGGTACCGAAGGA
PiHmp1 qPCR REV	TCTTGCCCTTGTTCTTGTCC
PiCut3 qPCR FOR	CCGACGTGCATGTTGTCTTC
PiCut3 qPCR REV	GGTTCGAGGTGATCCCACTG
PiEndo3 qPCR FOR	AACTATCGCAAGGGCAGGTT

PiEndo3 qPCR REV	CGTCCACCGATGTCTGAGTT

Table 6: Thermal conditions for RT-qPCR

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

2.10 Confocal Microscopy

2.10.1 dsRNA treatment with sporangia (Tube Assay)

10 µg Cy3-tagged dsRNA homologous to GFP (Cy3-dsRNA^{GFP}) was treated with 100 µl of undiluted *Pi*^{EGFP} sporangia in a micro-centrifuge tube. Cy3-tagged Control dsRNA (Cy3-dsRNA^{Ct}) provided in the MEGAscript kit was used as an experimental control. 24 hours post treatment, the samples were washed with nuclease-free water to remove non-specific fluorescence and imaged using an LSM 880 confocal microscope (Zeiss Microscopy). GFP and Cy3 were excited using lasers of 488 and 561 nm wavelength and detected at emission wavelengths of 499 to 552 and 570 to 624 nm, respectively.

2.10.2 dsRNA treatment on potato leaves

Potato leaves were sprayed with Cy3-dsRNA^{Ct} and infected with *Pi*^{EGFP} using the same experimental set-up as followed for detached leaf assay. 5 dpi, approximately 4- to 5-mm diameter leaf samples from the infected part of the leaf were mounted in aniline blue solution (0.1% aniline blue in phosphate buffered saline, pH 7) and incubated in the dark until imaged by confocal microscopy. In addition to GFP and Cy3 as used previously, aniline blue and chlorophyll were excited using lasers of

405 and 633 nm wavelength and detected at emission wavelengths of 410 to 496 and 647 to 721 nm, respectively.

2.11 Stereo Microscopy

To investigate if dsRNA spraying altered the morphology of *P.infestans* sporangia on infected potato leaves, the disease lesions from the detached leaf assay were imaged using a MDG41 Stereo Microscope (Leica Microsystems). Leaves sprayed with dsRNA targeting all 5 target genes were used in this experiment, with dsRNA^{Ct} as control.

3. Results

3.1 Sporangia take up dsRNA

3.1.1 Uptake from surrounding environment

To understand if *P. infestans* sporangia take up dsRNA from the surrounding environment, we co-inoculated Cy3-tagged dsRNA and sporangia in a tube assay. When the samples from the tube assay were visualized using the confocal microscope 24 hours after incubation, both Cy3-dsRNA^{Ct} and Cy3-dsRNA^{GFP} were taken up by sporangia (*Figure 5* a, b - Cy3 (dsRNA)), confirming that *Phytophthora infestans* sporangia are capable of taking up dsRNA from their environment. Furthermore, a significant reduction in the GFP fluorescence was also observed between the Cy3-dsRNA^{Ct} and Cy3-dsRNA^{GFP} treated samples (*Figure 5* b), indicating the occurrence of gene silencing through RNAi.

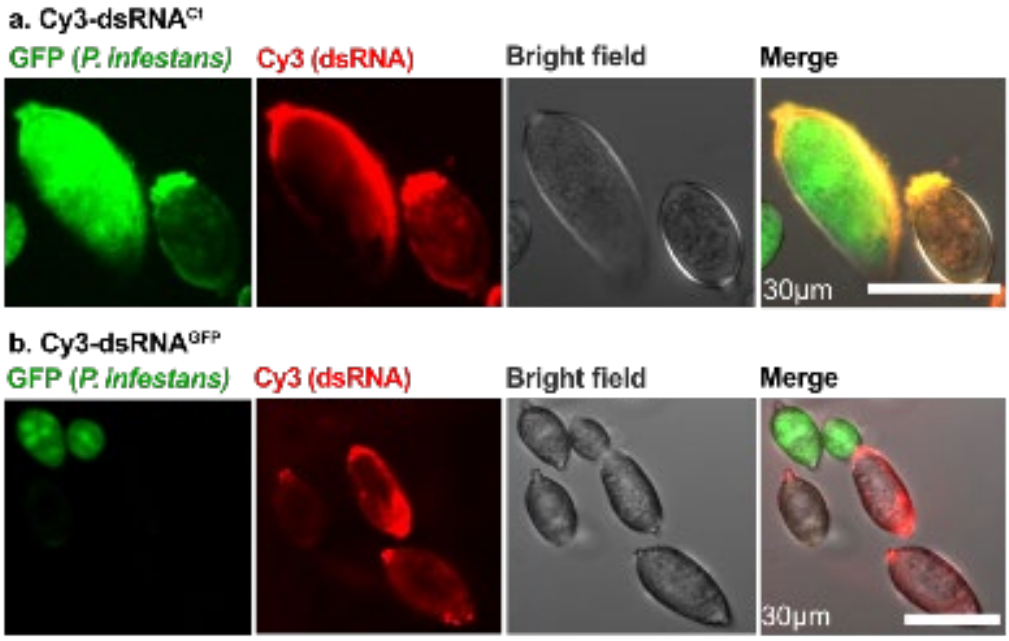

Figure 5: **Uptake of dsRNA by *P.infestans* sporangia:** Representative confocal microscopy images showing GFP & Cy3 fluorescence in *P. infestans* sporangia (first and second panel from left, respectively) and the corresponding merged image (panel to the right), indicating uptake of dsRNA from environment by sporangia. (b) Reduction in GFP fluorescence (first panel to the left) observed in sporangia treated with Cy3-dsRNA^{GFP} (second panel from left) and the corresponding merged image (right panel).

3.1.2 Uptake from sprayed leaves

To understand if *P. infestans* sporangia take up dsRNA from sprayed leaves, we conducted a detached leaf assay. When infected leaves from the DLA set-up were stained with aniline blue solution and viewed under the confocal microscope, co-localization of GFP and Cy3-dsRNA^{Ct} in *P. infestans* hyphae and sporangium was noted (*Figure 6* A,B). This indicates that *P.infestans* can take up dsRNA sprayed on leaves. Moreover, aniline blue staining demonstrated that β-1,3-glucan localization in sporangia was distinct from the co-localized GFP and Cy3-dsRNA^{Ct} (*Figure 6* C, A & B respectively).

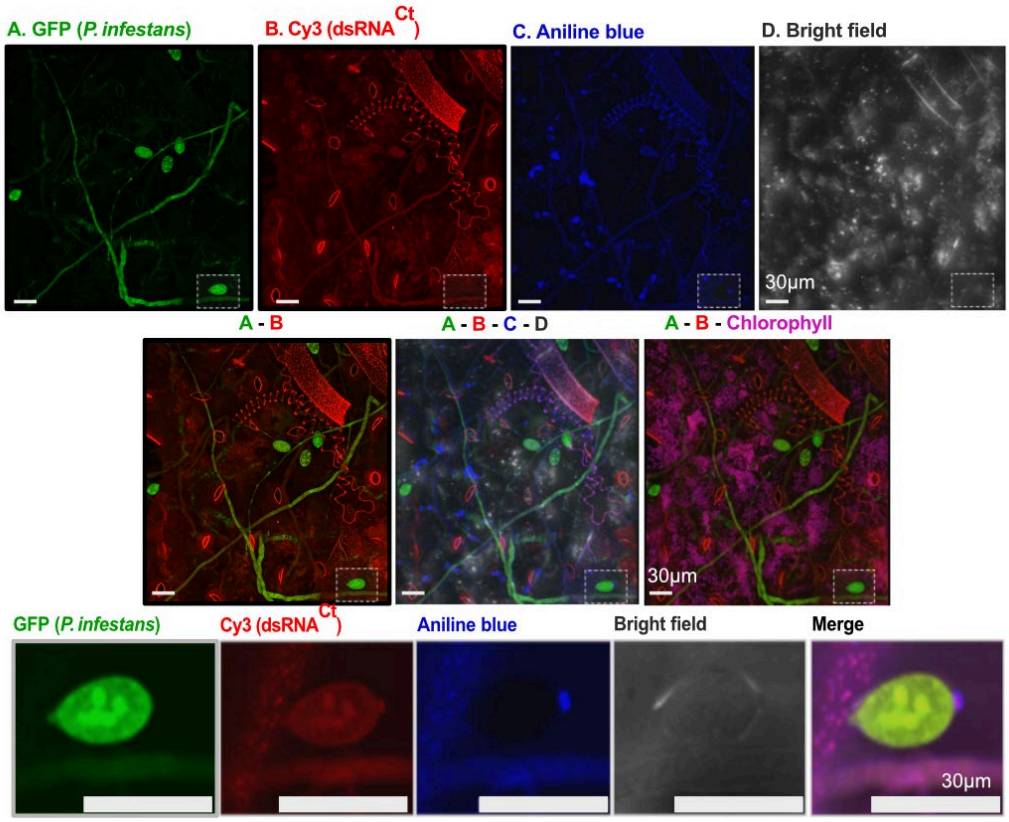

Figure 6: **Uptake of dsRNA sprayed on leaves by *P.infestans* sporangia:** Representative confocal microscopy images show accumulation of Cy3-dsRNA in the hyphae and sporangia of *P. infestans*. Trichome, stomatal guard cells, and epidermal cells of potato leaf also exhibit Cy3 fluorescence. Images were taken with wavelengths corresponding to GFP (A), Cy3 (B), and aniline blue stain (C, staining β-1,3glucans). The lower panel of images show a zoomed in image of highlighted region above, showing Cy3 accumulation in a single sporangium. Images were taken 5 dpi.

3.2 Confirming Exogenous dsRNA mediated Gene Silencing

The DLA also revealed if the silencing of targeted gene was triggered by dsRNA spraying. Potato leaves were collected and sprayed with either water (mock treatment) or Cy3-dsRNA^{Ct}/Cy3-dsRNA^{GFP}. 24 hours post spray, the leaves were drop inoculated with 15 µl of 50,000 spores/ml *Pi*^{EGFP} sporangia. The progression of infection was visualized 5 dpi using a fluorescence imaging system (Bio-Rad ChemiDoc) with respective wavelengths targeting Cy3 and GFP. Cy3 signal was observed in samples sprayed with Cy3-dsRNA^{Ct}/Cy3-dsRNA^{GFP} whereas it was absent in the samples with water (mock) treatment (*Figure 7*B). Gene silencing was visually confirmed by comparing the GFP fluorescence across the 3 treatments (*Figure 7*A). Although all 3 treatments showed comparable areas of infection, the Cy3-dsRNA^{GFP} treated leaves exhibited a reduced intensity of GFP fluorescence

while the mock and Cy3-dsRNA^{Ct} treated leaves exhibited similar and higher intensities of GFP fluorescence. Immunoblotting with anti-GFP-HRP antibody displayed reduced GFP protein levels in dsRNA GFP treated samples compared to mock treatment or dsRNA^{Ct} (*Figure 9*). RT-qPCR analysis showed downregulation of GFP expression in dsRNA GFP treated samples compared to mock treatment or dsRNA^{Ct} (*Figure 8*).

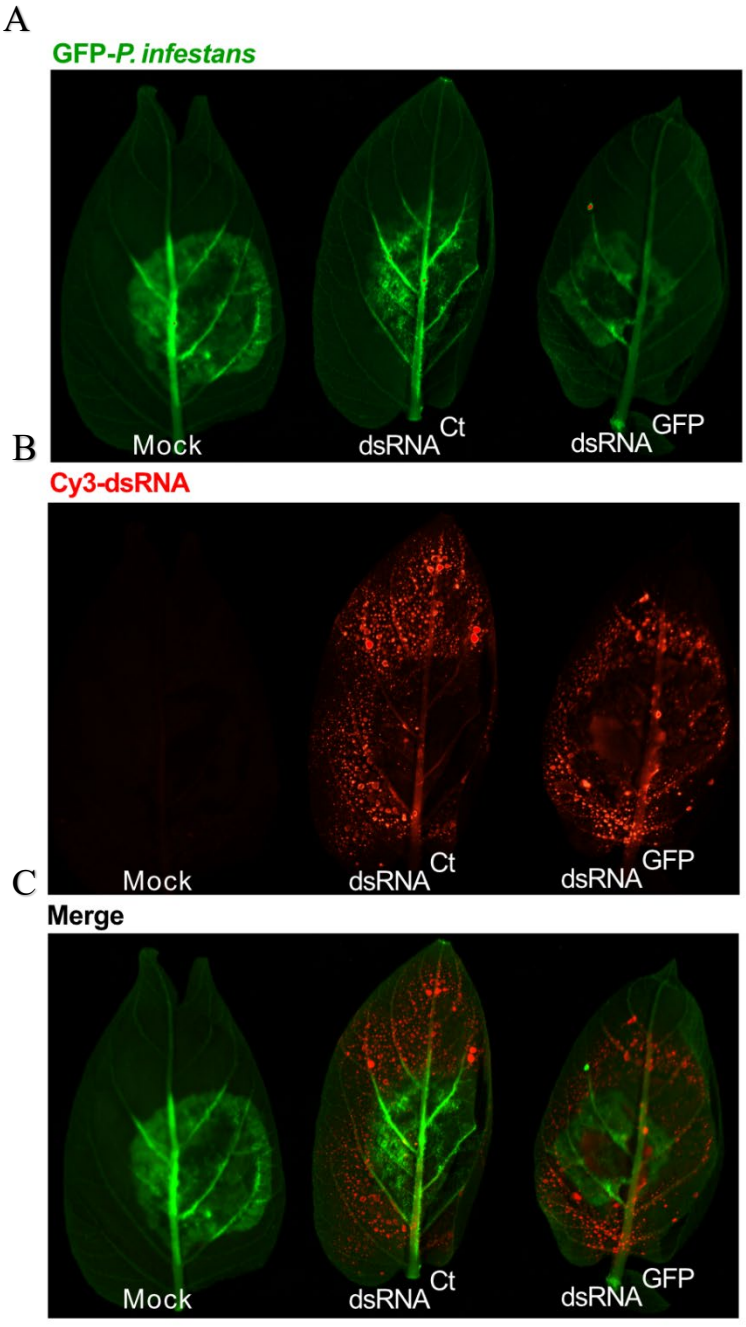

Figure 7: **Leaves from DLA display reduced GFP fkuorescence:** GFP **(A)** and Cy3 **(B)** fluorescence visualized using Alexa488 and Cy3 pre-defined settings, respectively, in leaves treated

with mock, Cy3-dsRNA^{Ct} or Cy3-dsRNA^{GFP}. Reduced GFP fluorescence observed in leaves sprayed with Cy3-dsRNA^{GFP}.

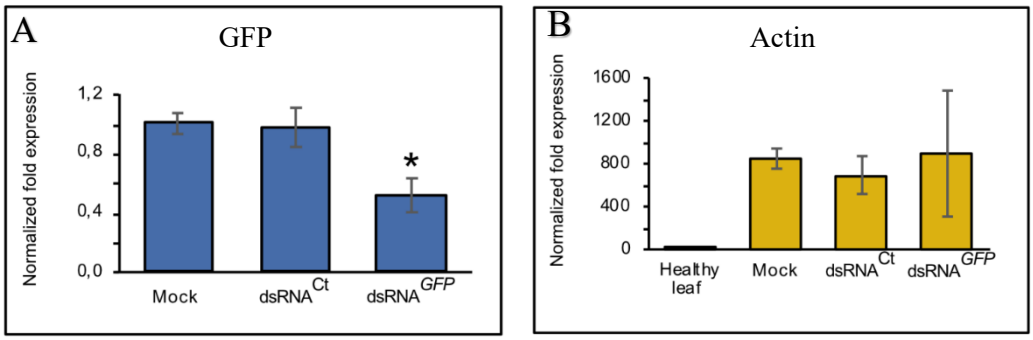

Figure 8: **RT-qPCR results showing relative expression of GFP and Actin: (A)** GFP gene expression showing significant decrease in Cy3-dsRNA^{GFP} treated samples when compared to mock/Cy3-dsRNA^{Ct} treated samples **(B)** Normalized Actin gene expression between *P.infestans* and potato showing no significant difference, thereby indicating dsRNA mediated silencing of GFP expression. Asterisks indicate statistically significant difference relative to mock treatment control, n = 6; *P < 0.01; Student's t-test.

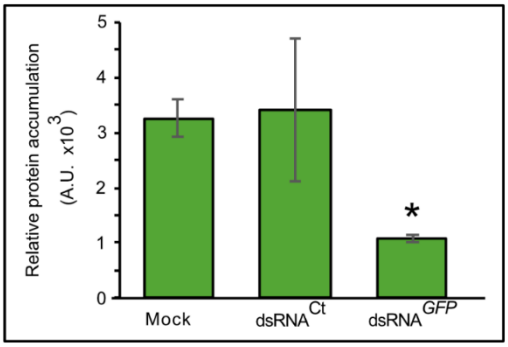

Figure 9: **Relative protein accumulation of GFP, as measured through western blot.** Significantly reduced GFP protein accumulation observed in samples treated with Cy3-dsRNA^{GFP} than in samples treated with mock/ Cy3-dsRNA^{Ct}. Asterisks indicate statistically significant difference relative to mock treatment control, n = 6; *P < 0.01; Student's t-test.

3.3 dsRNA spray reduces *P. infestans* infection

Detached leaf assays were performed to assess the effect of dsRNA spraying on *P. infestans* disease progression. The disease progression was compared between treatments following trypan blue staining performed at 5 dpi. The area of infection was comparable between the water and dsRNA^{Ct} treated samples. With the exception of samples treated with dsRNA^{OSBP}, all other dsRNA treatments namely dsRNA^{GPB1}, dsRNA^{Hmp1}, dsRNA^{Cut3}, and dsRNA^{Endo3} presented a significant reduction in area of infection (*Figure 10*). When the lesion area was quantified using the image analysis software ImageJ, the mean area of infection was 2.2 and 2.9 cm^2 in the water and control samples, respectively, whereas the dsRNA^{GPB1}, dsRNA^{Hmp1}, dsRNA^{Cut3}, and dsRNA^{Endo3} treated samples had mean lesion areas ranging from 0.6 to 1.24 cm^2. No significant change was observed in the mean lesion area for samples treated with dsRNA^{OSBP} (2.8 cm^2) (*Figure 11*). Hence, the DLAs were limited to four replications for dsRNA^{OSBP} treatment, while DLAs for each of the other targets were repeated at least five times with six leaves in each experiment (n = ~30; n = 24 for dsRNA^{OSBP}).

These observations demonstrate that SIGS can translate to reduced *P. infestans* infection on potato leaves depending on the gene targeted for silencing.

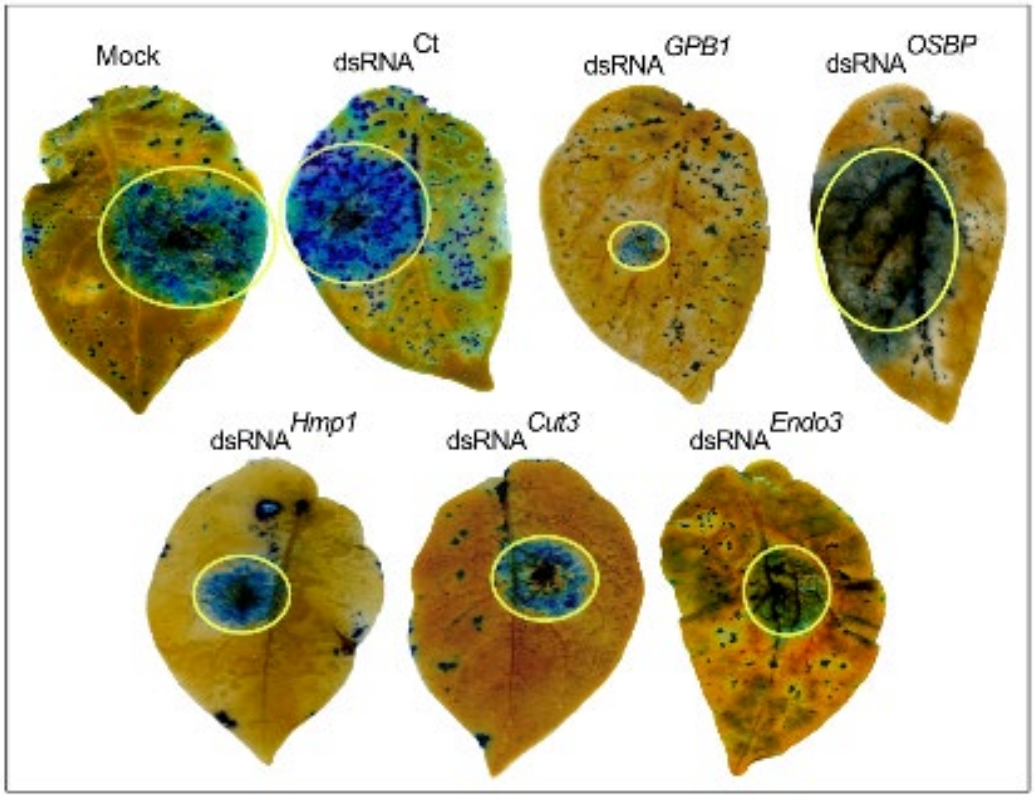

Figure 10: **Trypan blue staining of leaves from DLA show extent of *P.infestans* disease progression:** Comparison of *P. infestans* disease progression between control and target dsRNA treated samples showed that *P. infestans* infection was reduced significantly in samples treated with

dsRNA^{GPB1}, dsRNA^{Hmp1}, dsRNA^{Cut3} and dsRNA^{Endo3} but not in samples treated with dsRNA^{OSBP}. The area of infection is denoted using yellow elliptical rings.

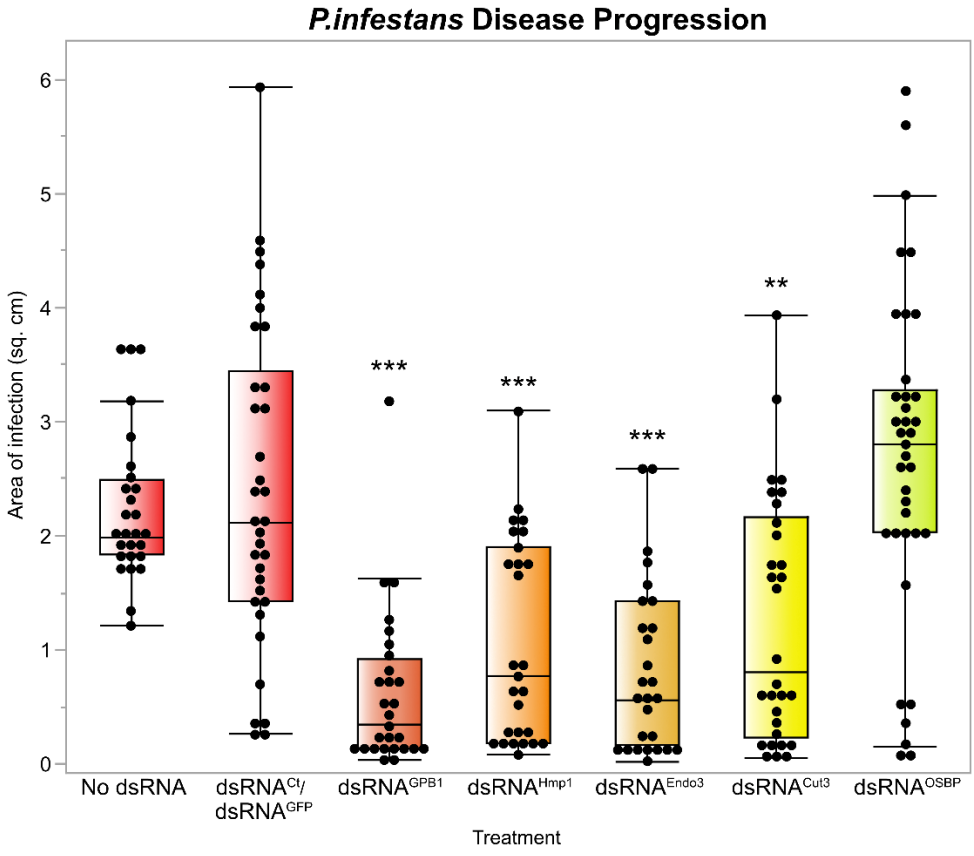

Figure 11: **Measured lesion area of *P.infestans* infection in control and dsRNA treated leaves:** Significantly reduced lesion area was observed in leaves treated with dsRNA^{GPB1}, dsRNA^{Hmp1}, dsRNA^{Cut3} and dsRNA^{Endo3} in comparison to mock/dsRNA^{GFP}/dsRNA^{Ct} treated leaves. dsRNA^{OSBP} treatment did not reduce lesion area. Asterisks signify p-value obtained from Dunnett's test. n = *~30;* n = *24 for dsRNA*^{OSBP} .

Table 7: Analysis of means using Dunnett's test with Mock (No dsRNA) as control.

dsRNA Treatment	**p-Value**
dsRNA^{OSBP}	0.6554
dsRNA^{Ct}/dsRNA^{GFP}	0.9828
No dsRNA (Mock)	1.0000
dsRNA^{Cut3}	0.0048*
dsRNA^{Hmp1}	0.0006*

dsRNA^{Endo3}	<.0001*
dsRNA^{GPB1}	<.0001*

3.4 Validating dsRNA spray mediated reduction in *P. infestans* infection

The occurrence of RNAi through SIGS in potato-*P.infestans* pathosystem and the ability of dsRNA to decrease *P.infestans* infection in potato leaves was validated by quantifying relative gene expression using RT-qPCR. Control and dsRNA treated leaves were collected from DLA experiments. After subsequent RNA extraction of cDNA synthesis, the generated cDNA was used for RT-qPCR. The transcript levels of the target genes were normalized to *P. infestans Actin* reference gene. Compared with the dsRNA^{Ct}-treated samples, a $\log_2$ fold-decrease of 2.5-, 1-, 1.5-, and 2 was observed in *PiGPB1*, *PiHmp1*, *PiCut3*, and *PiEndo3* transcript levels in each of the respective treatments. In contrast, the approximately one-fold decrease in *PiOSBP* transcript levels in samples treated with dsRNA^{OSBP} was insufficient to impact *P. infestans* infection (*Figure 12*). These results corroborate observations previously obtained from trypan blue staining.

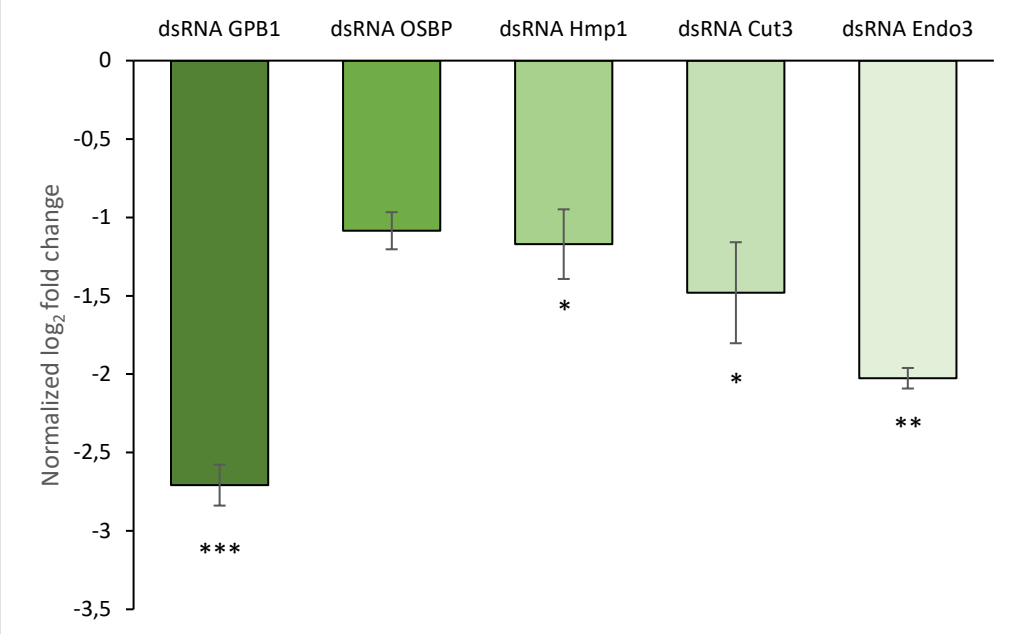

Figure 12: **RT-qPCR results show decreased expression of targeted genes:** Normalized $\log_2$ fold changes showing reduced *PiGPB1*, *PiOSBP, PiHmp1*, *PiCut3*, and *PiEndo3* gene expression levels in samples treated with respective dsRNA in comparison to control. The Cq values were normalized to that of *PiActin*. Asterisks indicate statistically significant difference relative to mock treatment

control using a Dunnett's test; ***P < 0.0001, **P < 0.005, *P < 0.05. Error bars represent standard error.

3.5 Effect of dsRNA spray on *P. infestans* morphology

To investigate if SIGS-mediated inhibition of disease progression was also associated with defects in sporulation, a microscopic examination of disease lesions using the stereo microscope was carried out. Analysis revealed that treatment with dsRNA^{GPB1} resulted in severe sporulation inhibition compared to the dsRNA^{Ct} treatment, but did not appear to disrupt the germination of the sporangia or mycelial progression, as mycelia were seen emerging from stomata (*Figure 13b, Figure 14*). Treatment with dsRNA^{Endo3} and dsRNA^{Cut3} also resulted in a significantly lower number of sporangia compared to dsRNA^{Ct} treatment (*Figure 13 e & f, Figure 14)*. Treatment with dsRNA^{OSBP} and dsRNA^{Hmp1} did not result in severe inhibition of sporulation (*Figure 13 c & d, Figure 14*).

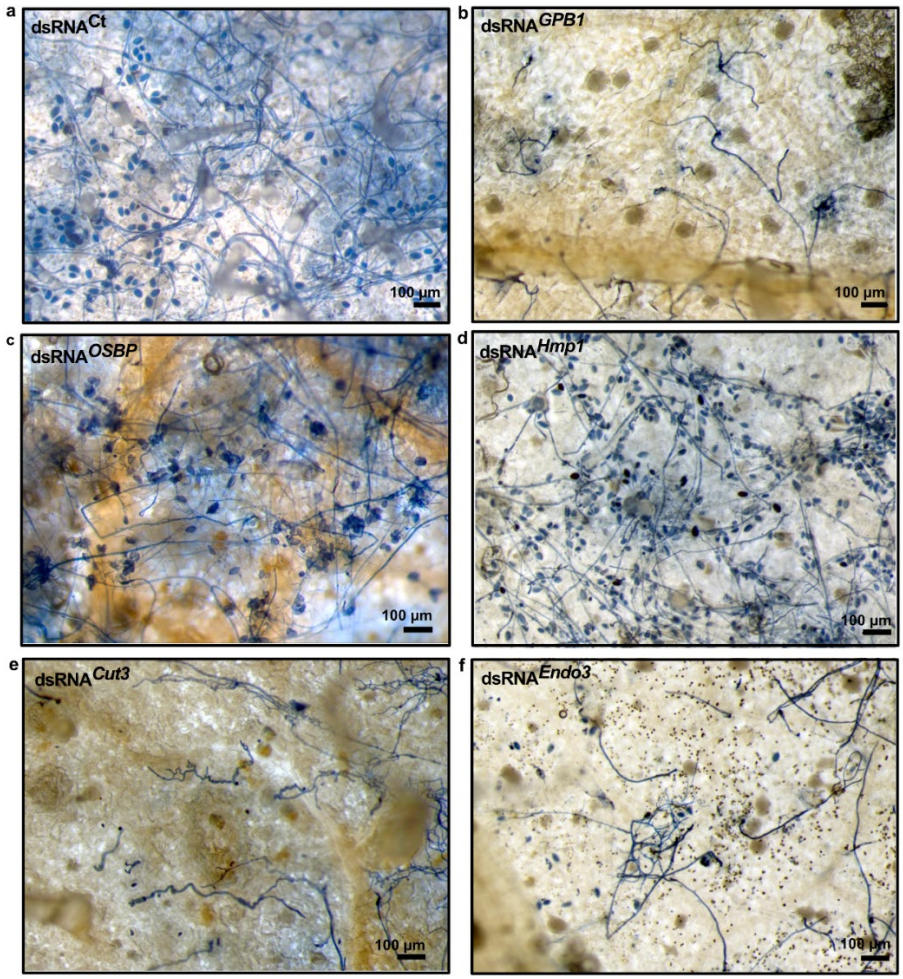

Figure 13: **Effect of dsRNA spray on *P.infestans* morphology:** dsRNA treated leaves were stained with trypan blue and observed under a microscope for changes in *P.infestans* morphology and

infection. Differences in sporulation, mycelial growth and extent of infection were observed in dsRNA^{GPB1}, dsRNA^{Cut3} and dsRNA^{Endo3} treated leaves while no significant change in morphology was observed in leaves treated with dsRNA^{OSBP} and dsRNA^{Hmp1}. All images were obtained using the 12 × objective in a Leica Stereo Microscope.

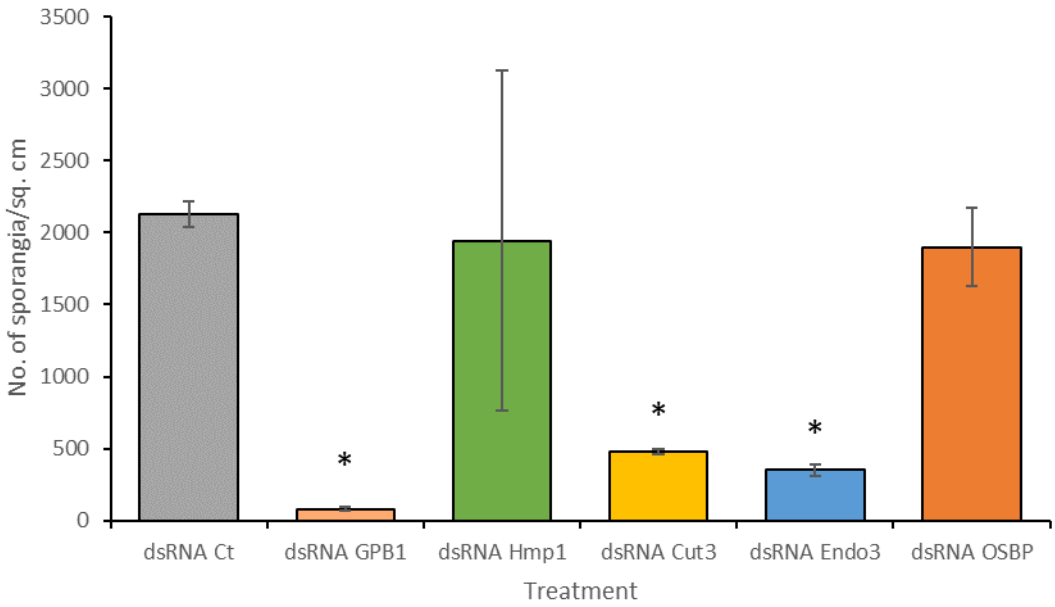

Figure 14: **Effect of dsRNA treatment on sporangial count:** Decrease in number of sporangia per sq cm observed in leaves sprayed with dsRNA^{GPB1}, dsRNA^{Cut3} and dsRNA^{Endo3}. Images were obtained using the 12 × objective in a Leica Stereo Microscope and the sporangia were counted manually. The graph represents mean sporangia count from three images taken from independent leaves. Asterisks represent p-value < 0.05 using a Student's t-test, error bars represent standard error.

4. Discussion

SIGS has previously been demonstrated in B. cinerea, F. graminearum and other pathogenic fungi (Koch et al., 2016; Wang et al., 2016). Oomycetes are speculated to exhibit SIGS in a similar fashion since RNAi is an evolutionarily conserved mechanism and has been used to successfully silence genes in *P. infestans* through HIGS (Sultana N. Jahan et al., 2015). A recent report by Qiao et al., 2021 explored uptake of dsRNA by P.infestans and its efficiency in curbing disease progression. They reported poor to no uptake of YFP-dsRNA by the different structures including sporangia, cysts and hyphae and no significant reduction in lesion area in dsRNA-sprayed leaves. The inability of dsRNA to control the disease severity was thus attributed to the inefficient uptake of dsRNA by the different *P. infestans* structures. This is in contrast with our results, where we show that dsRNA is readily taken up by *P. infestans* sporangia. Another study by Sundaresha et al., 2022 also demonstrated spraying dsRNA molecules from *P. infestans* for management of potato late blight in individual and different multigene combinations. Similar to our results from detached leaf assays, they reported reduced *P. infestans* growth and sporulation in both individual and combinatory dsRNA applications. Additionally, they also showed that incorporating nano-clay particles in the dsRNA application enhanced its disease controlling efficiency compared to naked dsRNA.

In this study, we have shown a proof of concept for SIGS in the potato-*P. infestans* pathosystem. Confocal microscopy studies revealed that dsRNA is taken up by *P.infestans* sporangia both from its surrounding environment and from sprayed potato leaves. RT-qPCR to quantify the GFP transcript levels displayed relative GFP expression levels reduced to half when normalized to *PiActin*. Moreover, the expression of Actin remained relatively unchanged between treatments, revealing that the changes in gene expression are specific to the targeted dsRNA. These results indicate that dsRNA spray triggers target gene silencing through RNAi**.** Detached leaf assays employing dsRNA targeting genes that are pathogenically important for *P. infestans* displayed reduced disease progression to varying degrees. While dsRNAs targeting the guanine-nucleotide binding protein β-subunit (*PiGPB1*), haustorial membrane protein (*PiHmp1*), cutinase (*PiCut3*) and endo-1,3(4)-β-glucanase (*PiEndo3*) caused significant reduction in *P. infestans* infection lesions, dsRNA targeting the important agrochemical target oxysterol binding

protein (*PiOSBP*) seemed to be ineffective in inhibiting *P. infestans* disease progression on detached leaves. These results were also supplemented by RT-qPCR using samples collected from the detached leaf assays. Thus, we speculate that the efficiency of SIGS in mitigating *P. infestans* infection on potato leaves is influenced by the selection of the target gene. It is still unclear what underlying mechanism governs if the targeted dsRNA will work or not.

An interesting aspect to spraying dsRNA is finding out if it causes any changes in *P. infestans* morphology. To that end, when trypan blue stained leaves from a detached leaf assay were observed under a microscope, changes in sporangial, hyphal growth and morphology were observed in leaves sprayed with dsRNA. The reduced sporulation effect observed in dsRNA^{GPB1} treated leaves is in accordance with Jahan et al., 2015; Latijnhouwers & Govers, 2003; where fewer and deformed sporangia were observed through silencing in hp-*PiGPB1* transgenic plants and transcriptional silencing in *P. infestans* stable transformants, respectively. The sporangial count or morphology was more or less unchanged upon treatment with dsRNA^{Hmp1}, although a significant decrease in overall disease progression was observed. This is in accordance with Avrova et al., 2008 where dsRNA-mediated transient silencing of *PiHmp1* produced a similar number of sporangia compared with the control when grown in agar culture, even though infection was suppressed compared with the control non-homologous dsRNA-treated lines. Smaller and deformed mycelia were observed in leaves treated with dsRNA^{Cut3}, as a possible result of erratic penetration and subsequent nutrient starvation. The decreased infection and sporangial count observed with dsRNA^{Endo3} treatment could be attributed to the inability to tear down host cell wall and successfully establish biotrophic infection.

Although we show that SIGS has promising potential as a plant protection strategy, several practical aspects of the technology and its underlying mechanisms are yet to be unravelled. While a high-pressure spraying system was used in this study to spray the dsRNA onto leaves, further research on improved application methods is required as not all sporangia uniformly took up the dsRNA when viewed using a confocal microscope. This could be credited to the sporangia being in different germination stages at the time of spray. The inclusion of adjuvants, nano-particle formulations and other carrier-based delivery systems has shown potential in improving the stability and durability of dsRNAs (Sundaresha et al., 2022; Yan et al., 2021). An array of nano-particle based delivery systems including the usage of chitosan, liposomes, layered double hydroxide clay nanosheets and carbon dots have been studied in relation to RNA based spray technologies to control viral and pest infections (Mitter et al., 2017; Pugsley et al., 2021; Yan et al., 2021). Incorporating such delivery systems could lead to increased disease protection against *P. infestans* as well. Another factor limiting the efficiency of SIGS is the

longevity of stable dsRNA present on leaves after spray. Further studies need to be conducted to evaluate the same. Little information is also available on how dsRNA is transported between the plant and the pathogen. Although this study shows proof that dsRNA is transferred to the pathogen by the use of a fluorescent dye (Cy3), several aspects of the transport mechanism is still widely unclear. As reported by Weiberg et al., 2013, sRNAs from the pathogen are transported to the host plant tissue, indicating the existence of bi-directional, cross-kingdom transport of sRNAs. However, it is yet unclear if the triggering of the RNAi pathway and the processing of dsRNA into sRNAs happens in the plant or if it happens in the pathogen after transfer as whole, long sequences of dsRNA. Thus, studying the transport of sRNAs between host and pathogen using omics strategies is a highly interesting future prospect. This study and many others have been successful in establishing the potential of SIGS for disease control using in-vitro and detached leaf experiments. In order to translate this technology to practical field use, cheap and large-scale production of dsRNA is required. While using the MEGAscript RNAi kit to synthesize dsRNA in-vitro produces dsRNA sufficient to conduct in-vitro and small-scale experiments, it can prove to be expensive for field scale use. Therefore, an alternate means for large scale dsRNA production using HT115 (DE3*) Escherichia coli* cells is currently being pursued. To ensure the safety and specificity of SIGS, it will also be worthwhile to evaluate changes to the microbiome caused by exogenous spray application of dsRNA.

The sequence specific nature of SIGS in addition to being a non-GMO technique make it a suitable alternative as a potential sustainable control strategy against potato late blight and several other economically important crop diseases. Several genes could also be stacked up in a “cocktail” mix to help provide improved and sustained protection against the deadly late blight pathogen. The ability to trigger RNAi through dsRNA spray also makes SIGS a prospective tool to study gene function and for plant trait improvement (Vetukuri et al., 2021).

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