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Spray-induced gene silencing to control plant pathogenic fungi: A step-by-step guide[oo]

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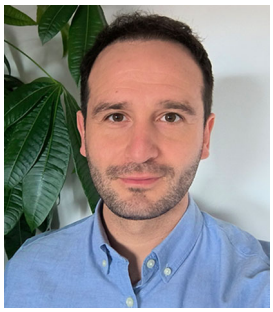

Jonatan Niño-Sánchez

ABSTRACT

RNA interference (RNAi)-based control technologies are gaining popularity as potential alternatives to synthetic fungicides in the ongoing effort to manage plant pathogenic fungi. Among these methods, spray-induced gene silencing (SIGS) emerges as particularly promising due to its convenience and feasibility for development. This approach is a new technology for plant disease management, in which double-stranded RNAs (dsRNAs) targeting essential or virulence genes are applied to plants or plant products and subsequently absorbed by plant pathogens, triggering a gene silencing effect and the inhibition of the infection process. Spray-induced gene silencing has demonstrated efficacy in laboratory settings against various fungal pathogens. However, as research progressed from the laboratory to the greenhouse and field environments, novel challenges arose, such as ensuring the stability of dsRNAs and their effective delivery to fungal targets. Here, we provide a practical guide to SIGS for the control of plant pathogenic fungi. This guide outlines the essential steps and considerations needed for designing and assessing dsRNA molecules. It also addresses key challenges inherent to SIGS, including delivery and stability of dsRNA molecules, and how nanoencapsulation of dsRNAs can aid in overcoming these obstacles. Additionally, the guide underscores existing knowledge gaps that warrant further research and aims to provide assistance to researchers, especially those new to the field, encouraging the advancement of SIGS for the control of a broad range of fungal pathogens.

Keywords: dsRNA delivery, dsRNA stability, dsRNA uptake, fungal plant pathogens, nanocarriers, spray-induced gene silencing

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INTRODUCTION

Plant pathogenic fungi impact agricultural and forest ecosystems, causing significant crop and economic losses (Fisher et al., 2020). Nearly 3.5 thousand tons of fungicides per year are applied globally to mitigate these losses (Sharma et al., 2019). The extensive use of fungicides represents environmental and health hazards as fungicides contaminate soil and water, and residues on crops can enter the food chain (Gikas et al., 2022; Pathak et al., 2022). In addition, this can lead to protection failure due to resistance development in some fungal pathogen populations (Zubrod et al., 2019).

Control alternatives based on the highly conserved gene silencing regulatory mechanism RNA interference (RNAi) could help to reduce the negative impacts of fungicides while

maintaining crop yields (Niu et al., 2021). Spray-induced gene silencing (SIGS) can effectively control fungal proliferation and reduce diseases for several plant pathogenic fungi (Song et al., 2018). It is considered a safe, eco-friendly alternative to fungicides due to the specificity of its control and the short persistence of double-stranded RNAs (dsRNAs) in the environment (Bachman et al., 2020; Qiao et al., 2021; Bocos-Asenjo et al., 2022; Niño-Sánchez et al., 2022; Spada et al., 2023). In addition, it is more convenient, faster, cheaper, and easier to develop than host-induced gene silencing (HIGS), as it does not require the transformation of the plant host and is thus not subjected to extensive GMO regulations (Herman et al., 2021; Rank and Koch, 2021).

The origin of SIGS dates back over two decades ago when it was observed that soaking the nematode *Caenorhabditis elegans* in a dsRNA solution or by feeding them with bacteria expressing dsRNAs was sufficient to induce silencing (Timmons and Fire, 1998; Timmons et al., 2001), a phenomenon later named "environmental RNAi" (Whangbo and Hunter, 2008). However, it was not until 2016 that it was demonstrated that the spray application of dsRNAs to plants conferred protection against the fungal pathogens *Botrytis cinerea* (Wang et al., 2016) and *Fusarium graminearum* (Koch et al., 2016), giving rise to the term SIGS. Since then, the number of SIGS-related studies targeting fungi and other plant pathogens has grown exponentially (Figure 1).

Spray-induced gene silencing consists of applying exogenous dsRNAs targeting disease-associated genes to crops (Koch et al., 2016; Wang et al., 2016). Once dsRNAs are taken up by fungal or plant cells they are cleaved into small interfering RNAs (siRNAs) by Dicer-like (DCL) proteins. siRNAs processed in plant cells are transferred to fungal cells. Then, siRNAs bind with Argonaute (AGO) proteins in the fungal cytoplasm and form the RNA-induced silencing complex (RISC). Last, RISC binds to and degrades the complementary mRNA target, thereby reducing disease progression. Interestingly, in some organisms such as *Mucor circinelloides* (Calo et al. 2012), RNA-dependent RNA polymerases (RDRPs) amplify the silencing signal using siRNAs to generate secondary siRNAs (Chang et al., 2012; Wilson and Doudna, 2013). However, this phenomenon is not compulsory for successful SIGS and in other fungi, such as *F. asiaticum*, amplification is absent (Song et al., 2018). In an attempt to encourage the development of SIGS against pathogenic fungi, this work aims to provide a practical guide for researchers who are new to the field of RNAi, especially those new to SIGS (Figure 2). It discusses the steps and considerations for designing and assessing dsRNA molecules and provides a handy list of resources and bioinformatics tools. It also describes challenges to consider when developing a SIGS strategy for the control of plant pathogenic fungi, including dsRNA delivery and stability, dsRNA nanoencapsulation, and the risk of an off-target effect (OTE). Finally, it highlights knowledge gaps and pitfalls that researchers might encounter.

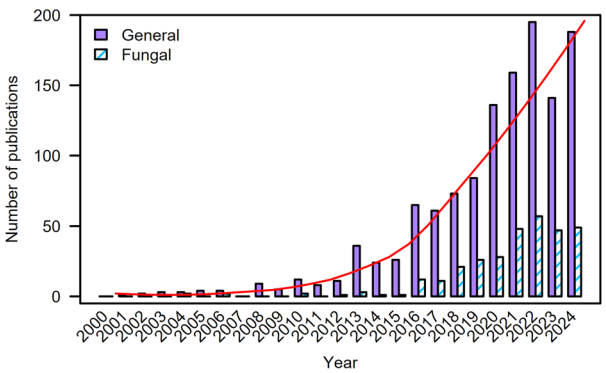

Figure 1. The number of spray-induced gene silencing (SIGS)-related studies targeting fungi (blue striped bars) and other organisms (purple solid bars) across the years according to the NCBI PubMed database

The red solid line represents the smoothed expected trend of general publications per year. Searching keywords (restricted to Title and Abstract) were: "spray-induced gene silencing," "RNAi external application," "dsRNA external application," "RNAi spray," "dsRNA spray," "exogenous dsRNA," "RNAi pest control," "dsRNA pest control," "RNAi crop protection," "dsRNA crop protection," "RNAi plant protection," "dsRNA plant protection," "RNAi-based plant protection," "dsRNA-based pesticides," "dsRNA-based fungicide," "dsRNA formulation." Results derived from every keyword were combined per year. Duplicated entries were removed. Fetched publications were further filtered out with the following fungi-related keywords: "fungicide," "fungi," "disease control," "fungal," and "phytopathogens." All analyses were performed on R v4.2.1 (R Core Team, 2021) using the package *rentrez* (Winter, 2017).

ASSESSING THE PRESENCE OF FUNCTIONAL RNAi MACHINERY IN THE PATHOGENIC FUNGI

The first step for developing a SIGS strategy to control plant pathogenic fungi is to check if the pathogen has functional RNAi machinery. Indeed, some fungi, such as the plant pathogen *Ustilago maydis* (Laurie et al., 2008), the human pathogen *Cryptococcus deuterogattii* (Feretzaki et al., 2016), and the yeast *Saccharomyces cerevisiae* (Drinnenberg et al., 2009), have lost some of their core components and, with them, their susceptibility to gene silencing. Therefore, initially checking if the targeted fungal pathogen possesses (i) DCL proteins, (ii) AGO proteins, and (iii) RDRPs is crucial.

The core components of the RNAi pathway and their functionality have been described for several plant pathogenic fungi, including *Neurospora crassa*, *Cryphonectria parasitica* (Chang et al., 2012), *Fusarium graminearum* (Chen et al., 2015), *Zymoseptoria tritici* (Kettles et al., 2019), *B. cinerea* (Weiberg et al., 2013), *Alternaria brassicicola* (Kwasiborski et al., 2022), *Magnaporthe oryzae* (Kadotani et al., 2003), *Colletotrichum higgisianum* (Campo et al., 2016), *Verticillium nonalfalfae* (Jeseničnik et al., 2019), and *Sclerotinia sclerotiorum* (Neupane et al., 2019). If the targeted fungus RNAi machinery is unknown, *in silico* analysis can be performed to identify protein homologs based on the information available for well characterized

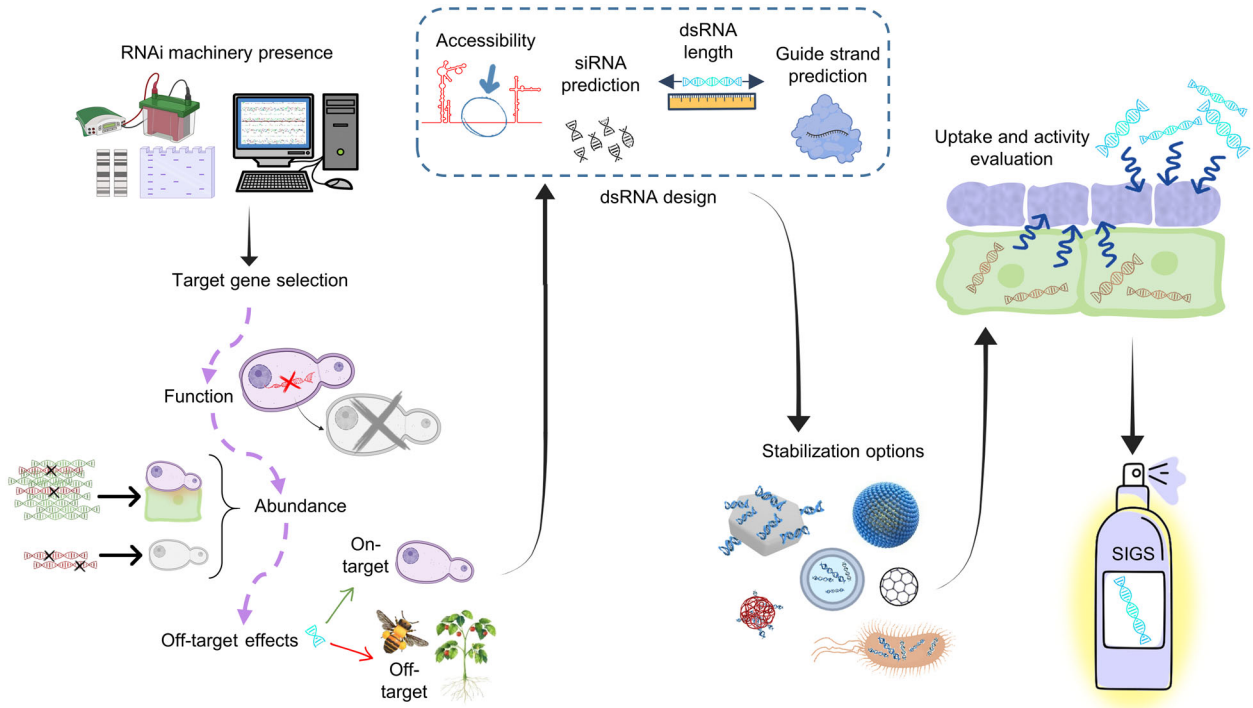

Figure 2. Practical guidelines indicating the relevant steps and considerations for developing a spray-induced gene silencing (SIGS) strategy for the control of plant pathogenic fungi

Developing a SIGS strategy begins by confirming the presence of RNAi machinery in the target fungal pathogen. Researchers must then select target genes based on factors such as function (essential and/or pathogenicity related), mRNA accessibility, and transcript abundance, while minimizing off-target effects in non-target organisms. These genes serve as the basis for designing active dsRNA molecules. Key considerations for dsRNA design include molecule length, the generation of putative siRNAs, and the guide strand preference by Argonaute proteins. Stabilization methods, such as nano-encapsulation, are critical to ensure effective application and should not be overlooked. The dsRNA formulation must then be tested for uptake and biological activity in the fungal pathogen, both *in vitro* and within the host plant, to identify the optimal RNA-based formulation for field testing.

fungi, preferably closely related (Jeseničnik et al., 2019; Kwasiborski et al., 2022). Interestingly, some fungi, such as *Schizosaccharomyces pombe*, have a single copy of DCL, AGO, and RDRP (Sigova et al., 2004), whereas many others have several copies (Wang et al., 2016; Qiao et al., 2021; Zm et al., 2021), which might be redundant, not functional, or not necessary for RNAi (see "Single-gene-target or multiple-gene-target dsRNAs?").

Yet, the presence of the core RNAi components might not entail susceptibility to silencing because some components might not be operative during infection (Kettles et al., 2019; Ma et al., 2020), or because the fungus might poorly take up dsRNAs (see "Fungal dsRNA uptake"). The *in silico* analysis of the fungal RNAi machinery must therefore be validated with experimental work (Chen et al., 2015; Kettles et al., 2019). This can be done *in vitro* by evaluating phenotypic changes or measuring differences in gene expression in the fungus after dsRNA exposure.

Nevertheless, the lack of phenotypic and/or gene expression might reflect the inefficacy of the dsRNA(s) used for the tests rather than the lack of sensitivity of the pathogen to RNAi. We recommend carefully considering the parameters described in the coming sections before discarding the use of SIGS to control a plant pathogen.

SELECTING THE TARGET(S) FOR RNAi SILENCING

Once the core components of the RNAi pathway have been identified and the susceptibility to silencing evaluated, the following step is to decide what gene(s) to target: (i) essential or pathogenicity ones; (ii) rare or highly abundant mRNA targets; and (iii) single-gene-target or multiple-gene-target dsRNAs, while taking into account the off-target gene silencing possibilities.

Essential or pathogenicity genes?

Targeting genes relevant to fungal development (hereafter essential genes) can reduce fungal proliferation through the entire disease cycle, from the arrival of the fungi to its host until late in the disease. Examples of such genes (Table 1) include the Cytochrome P450 sterol 14α-demethylases (*CYP51*) genes, which are the targets of many fungicides, and DCL genes (Wang et al., 2016; Zhang et al., 2019). Silencing *CYP51* in *F. graminearum* (Wang et al., 2016) and *DCL1* in *B. cinerea* (Koch et al., 2016) reduced fungal proliferation in the host, leading to a delay in symptom development. Similarly, silencing genes in the vesicle trafficking pathway such as the protein sorting 51 (*VPS51*), dynactin (*DCTN1*), and suppressor of actin (*SAC1*) genes

Table 1. Essential and pathogenicity genes that have been used as targets during SIGS to control plant pathogenic fungi

Target gene(s)	Pathogen	Function	References
Essential genes			
CYP51 genes	*Fusarium graminearum*, *Fusarium culmorum*, *Erysiphe quercicola*, *Golovinomyces orontii*, *Erysiphe necator*	Sterol 14α-demethylase genes	Koch et al. (2018, 2019); Höfle et al., 2020; McRae et al., 2023; Cao et al., 2024)
AGO and *DCL* genes	*Botrytis cinerea*, *Plasmopara viticola*, *Fusarium graminearum*, *Fusarium culmorum*, *Colletotrichum gloeosporioides*, *Verticillium dahliae*, and *Sclerotinia sclerotiorum*	RNAi pathway	(Wang et al., 2016; Werner et al., 2020; Qiao et al., 2021; Zm et al., 2021; Mukherjee et al., 2024)
VPS51, *DCTN1*, and *SAC1*	*Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Aspergillus niger*, *Colletotrichum gloeosporioides*, *V. dahliae*, and *Rhizoctonia solani*	Vesicle trafficking genes	(Qiao et al., 2021)
βTUB	*Fusarium asiaticum*, *Magnaporthe oryzae*, *Botrytis cinerea*, *Colletotrichum truncatum*, *Austropuccinia psidii*, *Chlamydia plumeriae* and *Erysiphe quercicola*	Cytoskeleton major component	(Gu et al., 2019; Degnan et al., 2023; Cao et al., 2024)
ERG genes	*Botrytis cinerea*	Ergosterol biosynthesis pathway	(Duanis-Assaf et al., 2022)
MAPK	*Botrytis cinerea*, *Aspergillus psidii*, and *Colletotrichum plumeriae*	Mitogen-activated protein kinases	(Degnan et al., 2023; Spada et al., 2023)
CHS genes	*Phakosporta pachyrizi*, *V. dahliae* and *Erysiphe quercicola*	Chitin synthase	(Xu et al., 2016; Saito et al., 2022; Cao et al., 2024)
ATC	*Plasmopar pachyriz*	Acetyl-CoA acyltransferase	(Hu et al., 2020)
GCS_H	*Plasmopar pachyriz*	Glycine cleavage system H protein	(Hu et al., 2020)
RP_S16	*Plasmopar pachyriz*	40S ribosomal protein S16	(Hu et al., 2020)
28S rRNA	*Aspergillus psidii* and *Colletotrichum plumeriae*	28S ribosomal RNA gene	(Degnan et al., 2023)
EF1-α	*Aspergillus psidii* and *Colletotrichum plumeriae*	Translation elongation factor 1α	(Degnan et al., 2023)
LIP1, *LIPA* and *ACX*	*Golovinomyces orontii*, *and Erysiphe necator* (*LIP1*, *LIPA only*)	Lipid catabolism	(McRae et al., 2023)
Pathogenicity genes			
SAS1	*Botrytis cinerea*	Virulence factor	(Niño-Sánchez et al., 2021)
PLS1	*Botrytis cinerea*	Tetraspanin involve in appressoria-mediated penetration of the host tissue	(Spada et al., 2023)
TRI5	*Fusarium culmorum*	Virulence factor	(Tretiakova et al., 2022)
DES1	*Magnaporthe oryzae*	Virulence factor	(Sarkar and Roy-Barman, 2021)
PG	*Rhizoctonia solani*	Polygalacturonase (PG)	(Qiao et al., 2021)
PGXB	*Aspergillus niger*	Exo-polygalacturonase b	(Qiao et al., 2021)
NCED, *ABA2*, and *ABAr*	*Golovinomyces orontii*, *Erysiphe necator (NCED only)*	Manipulation of plant host ABA metabolism	(McRae et al., 2023)
EC2	*Golovinomyces orontii*, *Erysiphe necator*	Effector protein	(McRae et al., 2023)
CIN1, *CE5*, *VICE12*, *and VICE16*	*Venturia inaequalis*	Pathogenicity genes	(Bhagta et al., 2023)

delayed fungal and symptoms development *B. cinerea*, *S. sclerotiorum*, *A. niger*, *R. solani*, and *V. dahliae* in their respective hosts (Qiao et al., 2021) and *F. circinatum* in pines (Bocos-Asenjo et al., 2024). A drawback of targeting essential genes is that it might increase off-target risks (discussed in "Off-target gene silencing") as they are widely conserved across the fungal kingdom as they participate in common and conserved metabolic pathways.

Alternatively, pathogenicity genes specific to the pathosystems of interest can be targeted (Table 1). For example, the *F. culmorum TRI5* gene is involved in trichothecene metabolism, a mycotoxin relevant to the virulence in *Fusarium*-plant pathogens (Maier et al., 2006). Double-stranded RNAs targeting this gene reduced trichothecene production and, in consequence, limited symptom development and fungal proliferation in wheat leaves inoculated with the pathogen (Tretiakova et al., 2022). Another example of pathogenicity genes constituting potential targets for SIGS are genes encoding fungal effectors. Fungal effectors are secreted proteins suppressing immune responses or manipulating cellular physiology in the plant host, thereby facilitating disease establishment (Lo Presti et al., 2015). Various fungal effectors have been described. For instance, in *V. dahliae*, the accumulation of miRNA relevant to the host RNAi-dependent immunity response is suppressed by SSR1, a secretory protein that translocates into the host cell and into the nucleus where it interacts with proteins in the RNAi pathway (Zhu et al., 2022). The PSR1 effector of oomycetes of the *Phytophthora* genus interacts with the plant pre-mRNA splicing factor PINP1 and affects the normal splicing of mRNA of genes in the pathogen-related immune response pathway (Gui et al., 2022).

Targeting such genes can increase the silencing specificity during SIGS. However, the genes responsible for pathogenicity are not necessarily known. Important plant pathogens still have incomplete and inconsistent gene sets. The absence of comprehensive gene annotation represents a significant challenge in the identification of suitable target genes for SIGS technology and increases the risk of OTEs. Therefore, an accurate and complete annotation of plant pathogens' genomes would facilitate advances in plant disease management.

Plants can deliver dsRNA to pathogenic fungi to reduce their virulence (Cai et al., 2018; Ma et al., 2020). Although the mechanisms of RNA-mediated communication between plants and fungi are not yet fully understood (Šečić and Kogel, 2021; Cai et al., 2023), several naturally occurring plant dsRNAs and their fungal mRNA targets have been described. For example, Zhang et al. (2016) identified two cotton dsRNAs (miR166 and miRN159) in *V. dahliae* cells upon infection that reduced the expression of two virulence genes (calpain clp-1 and C-15 hydroxylase genes). Similarly, upon infection, dsRNAs predicted to target vesicle trafficking pathways genes are transferred from *Arabidopsis* exosomes into *B. cinerea* cells (Cai et al., 2018). Identifying genes targeted by the host's defense mechanism can effectively find targets for SIGS. Therefore, researchers could use high-throughput sequencing to identify plant siRNAs overly expressed during infection and sequence alignment to find possible targets in the pathogen transcriptome. Likewise, mycoparasitic fungi might use RNAi interferences to disrupt the metabolism of their hosts (Piombo et al., 2022). Suitable target genes for SIGS can thus be identified by predicting potential targets of sRNAs and microRNAs-like delivered by parasitic fungi into their plant pathogenic hosts (Piombo et al., 2022).

Rare or highly abundant mRNA targets?

Some gene intrinsic features might impact the gene suitability as a target for SIGS (Riolo et al., 2020). For example, in mammalian cells, the abundance and turnover ratio of the targeted gene affect the silencing, with short- and medium-half-life mRNAs being less susceptible to gene silencing than long-lived transcripts (Larsson et al., 2010). Moreover, in mammalian cells and insects, gene expression level and silencing are strongly associated (Hong et al., 2014; Chen et al., 2021a). This suggests that abundant and highly transcribed genes with stable mRNAs make good targets, but whether this is also valid for fungi is yet to be confirmed. Nevertheless, it could explain the success of targeting abundant and highly expressed genes for controlling fungal pathogens, such as *EF2* (Nerva et al., 2020), *β-TUB* (Gu et al., 2019; Degnan et al., 2023; Cao et al., 2024), *EF1-α*, 28S ribosomal RNA (Degnan et al., 2023), or EC2 (McRae et al., 2023).

An important consideration related to this is that gene expression levels can change throughout fungi developmental stages or even vary across tissues. For instance, the EC2 effector gene of *G. orontii* is highly abundant only during early or late infection (McRae et al., 2023), and *F. graminearum Fg*AGO2 and *Fg*DICER1 are highly abundant in conidia but not in mycelia (Chen et al., 2015). Therefore, one should check that the considered target genes are highly transcribed at a time relevant to disease control.

Single-gene-target or multiple-gene-target dsRNAs?

In therapeutic applications, dsRNAs usually target a single gene (Aigner, 2007; Davidson and McCray, 2011). Double-stranded RNAs targeting only one gene have been used successfully in various SIGS studies targeting fungi. For instance, Spada et al. (2021) used a dsRNA targeting the Slt2-type MAP kinase *Bmp3* gene in *B. cinerea* and observed delayed symptom development in lettuce leaves. Similarly, dsRNAs targeting single polygalacturonase genes were effective against *A. niger* and *R. solani* (Qiao et al., 2021). Targeting a single gene presents the advantages of simplifying the dsRNA design, potentially facilitating uptake due to the smaller molecule size, and lowering the risk of OTEs (see "Off-target gene silencing").

However, it is also possible to target multiple genes, either using dsRNA chimeras with segments targeting different genes (Koch et al., 2019; Qiao et al., 2021; Zm et al., 2021) or by mixing dsRNAs each targeting a specific gene (Yang et al., 2022). In nature, such multiple gene targeting is a widespread phenomenon and plants commonly target multiple genes in their pathogen, as illustrated by *Arabidopsis* delivery of dsRNAs targeting various genes into *B. cinerea upon*

infection (Cai et al., 2018). We believe there are cases when simultaneously silencing more than one gene might be also advantageous for SIGS.

First, if the target gene has a paralog (or more), this latter might compensate for the silencing, as observed with *F. graminearum CYP51* genes (Koch et al., 2019). Another classic example of a potential target with paralogs is the DCL genes, and thus studies assessing control based on this target often aim at silencing all DCL genes present (Wang et al., 2016; Qiao et al., 2021; Zm et al., 2021).

Second, the expression of genes in complex pathways might be highly regulated by feedback and feedforward loops that have evolved to help the cells maintain homeostasis (Tang et al., 2010; Tsang et al., 2007). Consequently, when the aim is to disrupt such finely tuned pathways, targeting more than one gene, preferably in different steps of the pathway, could help surpass the effects of this tight regulation, thus increasing the chances of strong silencing.

Third, targeting multiple, non-redundant, genes might reduce the risk of resistance arising, especially when targeting genes with a history of resistance development to other antifungals due to target site modifications or genes whose high mutation rates can lead to low-cost resistance mutations. Indeed, even in cases of mutation in one of the target genes, the control exerted by SIGS will still be ensured through silencing of the others.

To date, surprisingly few studies have compared the effect of multiple-gene-target dsRNAs with that of their corresponding single-gene-target dsRNAs in pathogenic fungi. Regarding paralog genes, in *F. graminearum*, targeting all three *CYP51* genes was more effective in reducing the fungal proliferation and symptom development in barley than dsRNAs targeting these genes individually or pairwise (Koch et al., 2019), highlighting the importance of silencing all paralogs to avoid compensation. Conversely, Werner et al. (2020) did not observe a higher reduction in infection when targeting both DCL genes of *F. graminearum* compared with targeting various pairs consisting of one AGO and one DCL gene.

A dsRNA targeting multiple genes in distinct pathways of the plant pathogenic oomycete *Phytophthora infestans* yielded a greater reduction in lesion area and sporulation than dsRNAs targeting any of the genes alone (Siddappa et al., 2022). Pant and Kaur (2024) used dsRNAs targeting pairs of genes in *S. sclerotiorum* and reported a non-significant reduction of lesion area for all three dsRNAs tested in comparison with dsRNAs targeting each single gene. In contrast, dsRNAs targeting genes in different pathways were equally effective in mixtures and by themselves in reducing symptom development in *F. graminearum*-infected wheat (Yang et al., 2021); however, in this study mixtures of single gene targeting dsRNAs were used, rather than dsRNAs targeting multiple genes.

Overall, it seems that using dsRNAs targeting multiple genes could be beneficial for the control plant pathogenic fungi via SIGS. Nevertheless, studies regarding this are still lacking and it is therefore important to compare the effect of multiple-gene-target dsRNAs with their corresponding single-gene-target dsRNAs to ensure the best results.

Off-target gene silencing

Although a commonly mentioned advantage of RNAi-based pest and disease control is its high specificity, OTEs have been identified as a potential risk on which current knowledge is very limited (Casacuberta et al., 2015; Christiaens et al., 2018). Therefore, the risk and consequences of off-target silencing must be seriously evaluated while designing a SIGS strategy to control fungal pathogens in the field.

To the best of our knowledge, gene silencing in non-target species caused by dsRNA targeting fungal genes has so far only been reported between the two closely related fungi: *B. cinerea* and *S. sclerotiorum* (Spada et al., 2021). However, several such instances of OTE in closely related species, with highly similar gene sequences, have been documented in insects (Baum et al., 2007; Bachman et al., 2013). For example, a dsRNA targeting a western corn rootworm gene affected insects of the same subfamily but not insects from either other orders or Coleoptera from other families (Bachman et al., 2013). Adverse activity against closely related insects required a sequence homology over 90% and/or a sequence with at ≥21 nt continuous matches to the target gene sequence (even if just a few matches were present over the length of the dsRNA), although it was unclear which of the two criteria was the determining factor (Bachman et al., 2013).

A more recent study by Chen et al. (2021a, 2021b) showed that, in insects, OTEs are possible even in non-closely related species and defined several thresholds for triggering RNAi:

- a sequence identity above 80%,
- a sequence homology ≥53% and ≥16 bp fragments of a perfectly matched sequence,
- a sequence homology ≥53% and >26 bp fragments of almost perfectly matched sequence with single mismatches separated by ≥5 bp matching segments or mismatched couplets with ≥8 bp matching segments in between). Importantly, they considered such segments to already represent a warning zone above 19 bp.

Wang et al. (2023a) demonstrated that careful selection of the target region of a given gene based on these criteria can minimize OTE. Indeed, they predicted the potential non-target effects against *Propylea japonica* of dsRNA fragments targeting various regions of the actinin gene in pea aphids and found that the dsRNA predicted to have a low off-target potential caused no adverse effect in *P. japonica*, whereas the dsRNAs with intermediate and high risk were detrimental.

Various bioinformatics tools can be used to predict off-target risks (see "Bioinformatic tools for target selection and dsRNA design"). During this step, it is very important to analyze both strands of the dsRNAs as both can cause OTE. If

the risk of off-target is high, other dsRNAs should be designed. Alternatively, it might be possible to chemically modify dsRNAs to reduce their unintended toxicity (Bartoszewski and Sikorski, 2019). In HeLa cells that paired 2′-*O*-methyl ribosyl substitution at position 2 in the guide strand, which modulates the strength with which the seed region binds to the target RNA, led to a lower number of unintended transcripts being targeted, without affecting the silencing of the intended target (Jackson et al., 2006) and it would be of great interest to check whether this method is also effective for reducing OTE in fungi.

Nevertheless, there are still major limitations in the prediction of off-target risk. First of all, predicted OTE might actually not materialize biologically due to, for instance, lack of uptake, no physiological effect of the silencing, insufficiently efficient silencing, or other unknown reasons (Fletcher et al., 2020; Svoboda, 2020). For instance, Taning et al. (2021) identified a large number of hits when conducting silico-based predictions of potential off-target genes in the bumblebee of dsRNA targeting the pest pollen beetle. However, when trying a few of these candidates *in vivo*, they observed no negative effects on bumblebees. Overestimation of OTE might complicate the identification of novel, efficient dsRNA, but pose little threat to the environment. In contrast, failure to correctly predict OTE might have severe consequences. First, off-target predictions are sometimes based on BLAST searches (e.g., Ulrich et al., 2015) and thus might only yield the most obvious off-target genes, omitting many potential candidates (Birmingham et al., 2006). Second, studies on silencing in unintended species are still scarce and little information is known with certainty about how much sequence homology is required to trigger RNAi. Last, and most importantly, despite tremendous advances in sequencing technology, the genomes of many species have not yet been sequenced and, even when the genome is available, intraspecific genomic variations rendering a population sensitive to the prospective dsRNA cannot be excluded (Fletcher et al., 2020; Majumdar et al., 2017).

To complicate matters, exogenous dsRNAs might have silencing-independent effects on both target and non-target organisms. Intriguingly, exogenous dsRNAs with no predicted targets in *Magnaporthe oryzae* were observed to induce germ tube elongation in the fungus during *in vitro* assays (Ladera-Carmona et al., 2024). Exogenous dsRNAs have been associated with the natural phenomenon of root extracellular traps (RET). In RET, plants release organic molecules, including proteins, DNA, and RNA, forming a protective matrix around roots (Meitha et al., 2021). The antimicrobial activity in these matrices has been primarily attributed to extracellular self-DNAs (Mazzoleni et al., 2015a, 2015b; Tran et al., 2016). However, dsRNA participation in this activity has not been ruled out. Furthermore, when applied above a certain concentration, such dsRNAs reduced necrotic lesions on leaves, albeit much less than dsRNAs targeting a MAP kinase, and induced the high-osmolarity glycerol stress pathway and the production of reactive oxygen species in germ tubes (Ladera-Carmona et al., 2024). If confirmed, these results would imply that non-pathogenic fungi might be at risk of sequence-nonspecific adverse effects, especially if high dsRNA concentrations are used. Furthermore, dsRNAs can act as elicitors in plants, with *in vitro*-generated dsRNAs and viral dsRNAs inducing a pattern-triggered immunity response in *Arabidopsis thaliana* (Niehl et al., 2016). While this might reinforce the protection conferred by SIGS, the possibility of undesirable plant responses, such as stress responses, cannot be excluded.

In conclusion, OTE could be a serious concern for field applications of RNAi-based plant disease management. There is an evident lack of research on OTE on fungal species, a major concern as beneficial fungi are an integral part of many ecosystems. To date, studies aiming at determining minimum sequence homology requirements have been aimed at either mammalian cells or insects (Jackson et al., 2006; Bachman et al., 2013; Chen et al., 2021b). Furthermore, studies on plant pathogen control rarely describe attempts to minimize the risks posed to other organisms. Finally, sequence-nonspecific effects of dsRNAs have received little attention so far. Therefore, more research on the potential ecotoxicology of RNAi-based plant disease management is urgently needed.

Until more information is available on this topic, we recommend avoiding dsRNAs with high complementarity or long continuous stretches with nearly matching sequences to unintended targets and assessing the effects on the host plant and other organisms at risk of exposure, using various dsRNA concentrations, both *in vitro* and in greenhouse settings.

Another argument used to warrant the safety of dsRNA is its short persistence in the environment, which limits the exposition of other organisms. However, this lack of stability also represents a major setback for RNAi-fungicide efficiency; to circumvent this, efforts are made to develop formulations increasing dsRNA lifetime (see “Nanoparticle formulations”). Such formulation might also improve dsRNA uptake. Increased stability and easier uptake go hand in hand with higher off-target potential. We thus encourage researchers to take not only the naked molecules into consideration but also the encapsulated complexes when evaluating the off-target potential of dsRNAs.

Concluding remarks and other considerations for target gene selection

There are multiple strategies for selecting target genes: (i) choosing targets already validated for SIGS in other pathogens, (ii) testing new target genes among genes known to be essential for survival and growth, or affecting the fungus pathogenicity, or (iii) actively seek target genes based on observations of host–pathogen communication, with an emphasis on host defense mechanisms in nature.

Factors that can help to increase the chances of SIGS success are summarized in this review. Abundant genes with

stable mRNAs make good targets (Larsson et al., 2010; Chen et al., 2021b). Both essential or pathogenicity genes can be targeted (McRae et al., 2023). Spray-induced gene silencing can target single or multiple genes (Koch et al., 2019), the latter is possibly particularly beneficial when dealing with paralog genes, genes in complex pathways under expression-regulatory mechanisms, or genes with a history of resistance development or a high rate of mutation. In contrast, highly conserved genes with high homologies to orthologous genes in other organisms should be avoided to reduce the risk of off-targets (Bachman et al., 2013; Chen et al., 2021a).

Information about the pathogen is crucial for appropriate target selection. Indeed, an annotated genome and transcriptome are required to identify the potential target gene function, paralogous genes, transcript variants, and so forth. Data on closely related organisms might be used, although we do not recommend this practice if it can be avoided. Furthermore, an expression profile of the prospect target(s) during fungal development on the host is necessary, not only to check whether they are really expressed (and thus susceptible to silencing), but also to determine when they are most expressed (and thus must be targeted) (McRae et al., 2023). This is especially relevant when targeting pathogenicity genes as their expression is dependent on the disease development stages.

As research gaps on RNAi are filled, procedures for target selection will certainly become more straightforward, allowing for more precise guidelines. Until then, we recommend not giving up if a successful SIGS strategy against one pathogen turns out ineffective against another. It is likely that differences between pathosystems explain the failure and a different SIGS approach might yield the desired results.

DESIGNING THE dsRNA

Defining the target region within the gene, the accessibility to the target region

Choosing a suitable target gene, or suitable target genes, is not enough, the target site selection within the gene is also critical. dsRNAs and siRNAs targeting different regions within the *CYP51* gene of *Golovinomyces orontii* can yield different results in terms of fungal development reductions in SIGS experiments with *A. thaliana* plants, illustrating the importance of the target region (McRae et al., 2023).

During silencing, RISC interacts with a region within the targeted mRNA complementary to the siRNA guide strand (Wilson and Doudna, 2013). This region must be accessible to facilitate siRNA/RNA duplex formation (Bohula et al., 2003; Vickers et al., 2003; Lu and Mathews, 2007; Westerhout and Berkhout, 2007). Therefore, dsRNAs must be designed to target accessible sites in the mRNA of the targeted gene(s). Assessing the efficiency of RNAi against RNA hairpin mutants with decreasing stability levels showed that the hairpins with more accessible target sites were also more susceptible to silencing (Westerhout and Berkhout, 2007). This might indicate that thermodynamically unstable genes lacking complex secondary structures make good targets (Vickers et al., 2003; Westerhout and Berkhout, 2007).

Gene GC content influences their thermodynamic stability and thus might influence silencing (Chan et al., 2009). However, while some authors have shown negative associations between GC content and silencing (Pascut et al., 2015), others have found that GC content is a poor silencing predictor (Tafer et al., 2008).

Importantly, the target region should be within exonic regions of the gene as targeting siRNA to introns has led to no silencing (Vickers et al., 2003; Westerhout and Berkhout, 2007). Additionally, targeting should be located toward the 3′-end of the mRNA, that is the end most susceptible to RNAi and initially recognized and bound by the RISC/siRNA complex (Haley and Zamore, 2004; Westerhout and Berkhout, 2007).

The length of the dsRNA

The length of the dsRNA molecules is an important parameter as it might affect the fungus's ability to process them and determine the number of siRNAs generated by DCL protein cleavage. Yet, to date, very few studies have focused on dsRNA length optimization for SIGS, and results obtained in HIGS do not seem applicable (Höfle et al., 2020).

Intuitively, one might be tempted to think that longer dsRNAs imply stronger silencing, because a wider variety of siRNAs would be generated. Indeed, *in silico* analyses predict that substantially more efficient siRNAs are generated from longer dsRNA constructs (Höfle et al., 2020; Werner et al., 2020). However, this does not necessarily translate into stronger disease control. When applying dsRNAs on barley leaves and measuring *F. graminearum* infection, one study found that long (1,528–1,783 nt) and short (355–374 nt) dsRNAs had comparable efficiencies (Werner et al., 2020) while, in the other study, the efficiency of dsRNAs decreased as the length was increased from 220 nt to 1,500 nt (Höfle et al., 2020). Moreover, a 340 nt-long dsRNA was more effective for silencing the *Austropuccinia psidii* 28S rRNA gene than the 685 nt-long dsRNA (Degnan et al., 2023).

It is possible that the efficacy of longer dsRNAs is constrained by the uptake capacity of the pathogen. For instance, *F. graminearum* seems unable to take up dsRNAs of approximately 1,500 nt, as these molecules yield no silencing when applied to liquid cultures (Höfle et al., 2020). Despite this, spraying barley leaves with ≥1,500 nt dsRNAs still reduced *F. graminearum* infection (Höfle et al., 2020; Werner et al., 2020), possibly because *in vitro* conditions differed from those of SIGS (Höfle et al., 2020), or because the plant pre-processed the molecules into smaller dsRNAs or siRNAs. Interestingly, the pathogenic oomycetes of the *Phytophthora* genus appeared not to take up dsRNA from the environment, independently of the length (151–766 nt) (Cheng et al., 2022; Qiao et al., 2021; Wang et al., 2023b). Nevertheless, SIGS against these pathogens is still feasible using short siRNAs (Cheng et al., 2022). Therefore, potential limitations due to

long dsRNA uptake might be circumvented with the use of smaller molecules (dsRNAs or siRNAs). In addition, the optimal length might be dictated by DCL proteins substrate preference as some DCLs cleave dsRNA more efficiently within a given size range (Tabara et al., 2021), however next to no information is available on this topic.

In summary, optimal dsRNA lengths appear pathogen specific as they are dictated by both DCL protein properties and pathogen uptake capacities. Furthermore, even for a given pathogen the best length might be variable, as shown by the fact that different relationships between dsRNA length and *F. graminearum* development were observed when targeting the CYP51 genes (Höfle et al., 2020) and when targeting the AGO and DCL genes (Werner et al., 2020). In addition, if dsRNAs are taken up first by the host plant and then transferred into their pathogen, then plant uptake and DCL preferences might also affect the optimal length. Currently, the lack of knowledge of these mechanisms prevents the development of easy prediction methods. Therefore, we recommend testing different dsRNA lengths when developing a new SIGS strategy. In the literature, dsRNA lengths between 118 and 1,403 nt have been successfully tested for SIGS (Wang et al., 2016; Koch et al., 2018; Sarkar and Roy-Barman, 2021; Zm et al., 2021; Bocos-Asenjo et al., 2022; Tretiakova et al., 2022; Degnan et al., 2023; McRae et al., 2023; Cao et al., 2024; Mukherjee et al., 2024; Pant and Kaur, 2024), with dsRNA sizes within the ranges 150–550 nt being the most prevalent. Therefore, the use of lengths between this range (150–550 nt) constitutes a good starting point, but longer dsRNAs are also worth investigating as they have sometimes led to satisfactory silencing.

Understanding how DCL proteins affect siRNA generation

As silencing depends on the siRNA sequence and as the siRNAs depend on the cutting length and cutting mechanism of DCL proteins, the design of dsRNA for a specific purpose requires some knowledge about DCL cleavage functioning.

DCL proteins are enzymes with ribonuclease activity highly conserved across eukaryotes (Paturi and Deshmukh, 2021). They have a basic domain architecture that includes an RNase III cleavage domain, responsible for cleaving dsRNAs into siRNAs, and an RNA-binding domain that determines the enzyme–substrate preference and siRNA-product length (Nykänen et al., 2001; Zhang et al., 2004; MacRae et al., 2006; 2007; Takeshita et al., 2007). The multiple duplication events of DCL genes throughout evolution have allowed their diversification (Raman et al., 2017; Jeseničnik et al., 2019; Mann et al., 2023). In turn, this diversification has led to proteins with varying substrate lengths and feature preferences, as well as different product lengths (Figure 3), although most DCLs have retained their core function in the RNAi pathway (Mann et al., 2023).

The DCL proteins of the genetic plant model *A. thaliana* are well characterized and provide a good example of DCL specificities. *At*DCL3 prefers short dsRNAs (~30 nt) with 1 nt or 2 nt 3′ overhangs or 5′-adenosine or uridine, while *At*DCL4 prefers long dsRNAs (preferably over 50 nt) with blunt ends or with a 1 nt or 2 nt 3′ overhang (Nagano et al., 2014). The products of *At*DCL1 and *At*DCL4 are 21 nt miRNAs and siRNAs, respectively, while AtDCL2 and *At*DCL3 produce 22 and 24 nt siRNAs, respectively (Xie et al., 2004; Nagano et al., 2014; Paturi and Deshmukh, 2021).

Many fungi also have several DCL genes although exceptions exist, such as *S. pombe*, which possesses a single DCL copy (Sigova et al., 2004). Determining the role of each DCL in RNAi is a first step toward understanding the fate of dsRNAs following fungal uptake. Indeed, DCL copies often have distinct functions, with only partial redundancy. For example, in *M. oryzae* (Kadotani et al., 2003; Raman et al., 2017), *F. graminearum* (Chen et al., 2015), *N. crassa* (Catalanotto et al., 2004; Tabara et al., 2021), and the entomopathogenic fungus *Metarhizium robertsii* (Meng et al., 2017), DCL2 seems to be the major component of the RNAi machinery.

However, understanding the exact role of DCL proteins is not straightforward. For instance, *N. crassa* DCL1 and DCL2 are usually considered to be at least partially redundant, with the deletion of both required to abolish silencing (Catalanotto et al., 2004; ReFalo and Sachs, 2004; Chang et al., 2012); yet, Tabara et al. (2021) observed no dsRNA-cleaving activity in the absence of DCL2. Discrepancies between studies might be due to several factors. First, the length of the substrate used can affect the results. For instance, *N. crassa* DCL2 is able to efficiently cut 50 and 130 nt dsRNA but efficacy is reduced for shorter molecules (30 and 37 nt); longer dsRNAs were not tested (Tabara et al., 2021). Second, DCLs activity might depend on the development stage of the fungus. In *M. robertsii*, DCL1 was only expressed in spores, while DCL2 was expressed during all stages but the expression was highest during late conidiogenesis (Meng et al., 2017). Third, DCLs might be active in different tissues. In *F. graminearum*, DCL1 gene expression was much higher in conidia than in mycelia (Chen et al., 2015).

The next question of relevance for SIGS regards the characteristics of the siRNAs generated. Even in a well characterized fungus, such as the model fungus *N. crassa*, knowledge on siRNAs generation is imprecise: siRNAs lengths of 21–26 nt were reported by ReFalo and Sachs (2004), while Tabara et al. (2021) found a wider siRNA range of 18–28 nt. Both studies found that 23 nt was the predominant length, whereas a length of approximately 25 nt was reported elsewhere (Catalanotto et al., 2004). In *M. oryzae* (Raman et al., 2017) and *F. graminearum* (Chen et al., 2015), siRNA populations with lengths of 21–26 nt (peak at 21 nt) and 17–32 nt (peak at 27 nt) were reported, respectively. The size range of siRNAs is thus wider in fungi than in *A. thaliana*. As the length of the siRNA can influence the efficacy of the silencing (Kim et al., 2005; Chang et al., 2007), knowing ahead the lengths of the siRNAs that are likely to be generated from the dsRNA molecule could help during dsRNAs design.

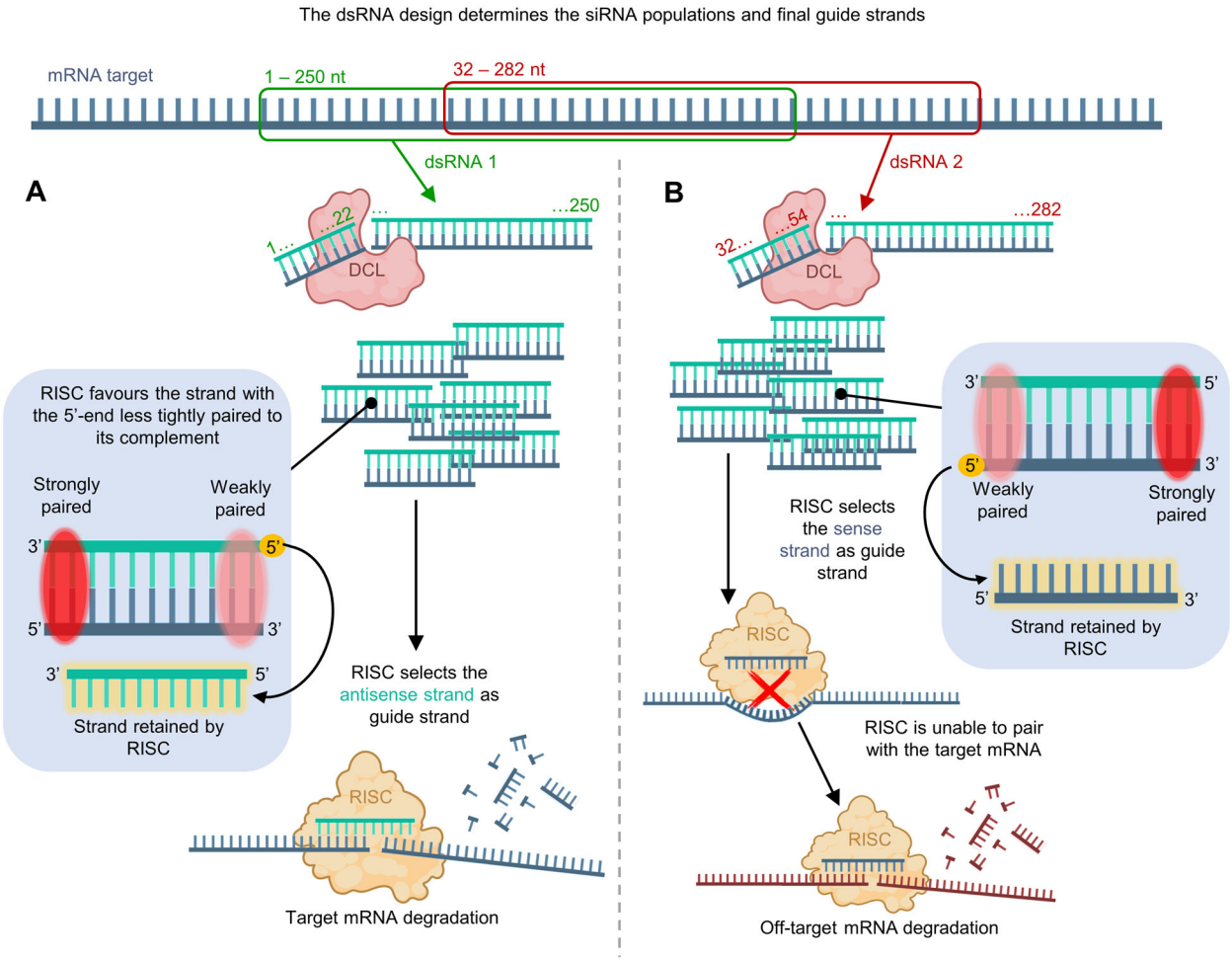

Figure 3. Considerations for designing the double-stranded RNAs (dsRNA)
Double-stranded RNA features influence silencing as they determine siRNAs resulting from DCL proteins cut and which strand is retained by RISC as the guide strand. The guide strand must be complementary to the target mRNA for successful silencing **(A)**, otherwise, it can result in the degradation of off-target mRNA degradation **(B)**.

The abundance of ions in the environment also influences the activity of DCL enzymes. Studies in genetic model organisms, such as *Drosophila melanogaster*, *N. crassa*, and *A. thaliana*, have shown that some salts can enhance or inhibit the activity of DCL proteins. Magnesium chloride promotes the activity of *Dm*DCL2, *Nc*DCL2, *At*DCL3, and *At*DCL4 (Tabara et al., 2021). High concentrations of sodium chloride promote the activity of *At*DCL3 proteins but suppress the activity of *At*DCL2, *At*DCL4, and *Nc*DCL2 proteins (Nagano et al., 2014; Tabara et al., 2021). Potassium chloride, some phosphates, and some sulfates were also found to affect the activity of other DCLs (Fukunaga et al., 2014; Nagano et al., 2014; Seta et al., 2017). The link between salts and the activity of DCL proteins is interesting as it suggests that we can modulate the activity of these enzymes using salts. However, salts appear to have different effects on distinct DCLs, enhancing or promoting their activity. Therefore, what salt could be beneficial would need to be determined for each pathosystem.

To summarize, DCL proteins have varying substrate affinities in terms of dsRNA length and can produce siRNAs of different lengths. Knowing how DCL proteins process dsRNA substrates into siRNA products can help to design dsRNA molecules that will be efficiently processed into siRNA by DCL proteins of the fungal pathogen. For example, the dsRNAs could be designed in such a manner that they would be processed by a specific DCL protein to produce siRNAs with lengths between 21 and 25 nt, which are the common lengths reported for fungal siRNAs (Chang et al., 2012; Chen et al., 2015). Furthermore, as some salts are known to potentiate DCL activity, it is possible that adding salts as components of dsRNA formulations could enhance the silencing of the genes targeted in the fungal pathogen during SIGS.

Understanding the guide strand selection to improve SIGS efficacy

Following DCL cleavage, the resulting siRNAs are loaded onto AGO proteins, forming the RISC complex. Here, one of the two siRNA strands, referred to as passenger strands, dissociates from the AGO protein, while the other, the guide strand, remains bound to the RISC and guides it to the

complementary mRNA. Then, the dissociated strand is degraded (Nykänen et al., 2001; Wilson and Doudna, 2013) (Figure 3). dsRNA features influence silencing as they determine siRNAs resulting from DCL proteins cut and which strand is retained by RISC as the guide strand. The guide strand must be complementary to the target mRNA for successful silencing.

As only one strand will be complementary to the mRNA target, but any of the two siRNA strands can be degraded or serve as a guide strand (Schwarz et al., 2003), it is crucial that the RISC favors the load of the strand that will result in silencing of the desired gene (Figure 3A). Loading of the other strand as a guide strand could cause degradation of unintended mRNA molecules, potentially triggering OTEs (Figure 3B).

To date, the mechanism behind the guide strand selections is not fully known (Liu et al., 2022). In animal systems, it is governed by a sequence asymmetry-sensing mechanism based on the thermodynamic stability along the siRNA duplex (Hutvagner, 2005). The RISC favors the strand with the 5′-end less tightly paired to its complement: strands having U or nucleotides resulting in a mismatch at the 5′-end will be more likely to be selected as guide strands than those with nucleotides leading to a stronger base pairing (G:C) (Khvorova et al., 2003; Schwarz et al., 2003).

siRNA and miRNA duplexes with 5′-U ends have also been reported in several fungi, including *N. crassa* (Lee et al., 2010), *M. circinelloides* (Nicolas et al., 2010), *M. oryzae* (Raman et al., 2017), and some yeasts (Bühler et al., 2008). In these organisms the guide strand selection might thus follow a mechanism similar to the asymmetry-sensing mechanism observed in animal systems (Hutvagner, 2005). Interestingly, in *A. thaliana*, while AGO1 is also associated with the siRNA and miRNA duplex with 5′-U ends, AGO2 and AGO5 proteins seem to favor siRNAs with a 5′ terminal A and C, respectively, suggesting that the guide strand selection is more complex than previously thought and can depend on the AGO protein (Takeda et al., 2008). Improved knowledge of the guide strand selection mechanisms in fungi would be greatly beneficial for the development of SIGS as it would enable the design of dsRNAs in a way that ensures the selection of the strand complementary to the target gene as the guide strand.

Bioinformatic tools for target selection and dsRNA design

Many factors must be considered when selecting a target gene and designing the dsRNA molecule. Fortunately, various bioinformatics tools can be of assistance (Riolo et al., 2020). Table 2 provides a list of tools that are helpful for dsRNA design, the prediction of off-targets, and gene-target selection. Many of these tools are relatively simple and are specific for designing siRNA candidates for a target gene. For instance, the web servers Oligowalk (Lu and Mathews, 2008) and RNAxs (Tafer et al., 2008) provide a list of candidate siRNAs for a mRNA-target sequence given by the user.

Other tools are more complex and can provide additional information. For example, the psRNATarget web server can be used to look for target genes in a given transcriptome once the dsRNA is provided. Also, it can be used to look for candidate miRNAs for a given mRNA in miRNA databases. In this case, the user provides the miRNA or mRNA sequences and selects the transcriptome or miRNA database from drop-down menus (Dai et al., 2018). Furthermore, the psRNA-Target can help to select between siRNAs and can be particularly helpful in assessing the off-target risk of a dsRNA against a wide range of plant and fungal transcriptomes. Another useful tool for predicting the off-target risk is the publicly available software si-Fi, which enables checking the potential OTEs of a long dsRNA sequence within a local sequence database by computing all the possible siRNA sequences of a selectable length that can be derived from the dsRNA and checking for matches in the database (Lück et al., 2019). For instance, Spada et al. (2021, 2023) used si-Fi to check the potential OTEs of *B. cinerea*-specific dsRNA in closely related fungi and in lettuce, the host plant.

There are no bioinformatic tools specific for gene-target selection. However, some tools, such as the sfold web server, intended to find secondary structures in mRNAs, can help to determine whether the target gene has accessible sites (Shao et al., 2007). Most of these tools are freely available as web services or desktop software with a user-friendly interface, making them easy to use. It is worth noting that, being based on a restricted number of dsRNA and target features, most tools are not faultless. Therefore, results must be experimentally validated (Riolo et al., 2020; Ghanbarian et al., 2022).

Unfortunately, most of these tools are limited to siRNAs or miRNAs. Nevertheless, they can still be useful in identifying open regions in the mRNA targets or checking for off-targets by manually assessing all the siRNAs possibly derived from the dsRNA molecule. To the best of our knowledge, si-Fi is the only software considering dsRNAs (Lück et al., 2019). This software can not only help the design of longer dsRNAs but is also particularly useful when checking the off-target risk as it checks all possible siRNA sequences that can be derived from long dsRNAs for potential OTEs.

Concluding remarks and other considerations for designing the dsRNA

In conclusion, the design of new dsRNA molecules is a complex process. Our main recommendations, based on current knowledge, are summarized below.

As a general rule, dsRNAs must be designed to target sites in the mRNA of the targeted gene that are accessible and lack of complex secondary structures (Vickers et al., 2003; Westerhout and Berkhout, 2007), which can be identified using some of the bioinformatics tools listed in Table 2. As optimal dsRNA length cannot yet be predicted, we recommend testing various sizes, preferably within the 150–550 nt range, as such lengths have resulted in satisfactory silencing in a variety of pathogens. Larger molecules, despite being more likely to be cut by the DCL protein into a wide variety of effective siRNA, might compromise the

Table 2. Useful bioinformatic tools for the development of SIGS strategies to control plant pathogenic fungi

Bioinformatic tools	Input	Output	References
dsRNA design			
Oligowalk rna.urmc.rochester.edu/servers/oligowalk	Target sequence	List of siRNA	(Lu and Mathews, 2008)
RNAxs http://rna.tbi.univie.ac.at/cgi-bin/RNAxs/RNAxs.cgi	Target sequence	List of siRNA	(Tafer et al., 2008)
siDirect 2.0 http://siDirect2.RNAi.jp/	Target sequence	List of siRNA	(Naito et al., 2009)
BLOCK-iT™ RNAi Designer https://rnaidesigner.thermofisher.com/rnaiexpress/setOption.do?designOption=stealth&pid=-7982084108525566116	Target sequence	List of siRNA	Thermo Fisher
Off-target prediction			
psRNATarget https://www.zhaolab.org/psRNATarget/home	Target or dsRNA sequence, transcriptome or miRNA database (available as drop-down menu); a custom transcriptome or databases can be provided	List of miRNA or genes	(Dai et al., 2018)
si-Fi https://labtools.ipk-gatersleben.de/	Target sequence; custom transcriptome	Accessible regions in the target mRNA for dsRNA, list of possible off-targets in the provided database	(Lück et al., 2019)
dsCheck http://dscheck.rnai.jp/	dsRNA sequence and transcriptome of interest (restricted to limited previously uploaded transcriptome options)	List of mRNA with sequence similarity	(Naito et al., 2009)
online NCBI nblast https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome	dsRNA sequence and transcriptome of interest	List of mRNA with sequence similarity	(Sayers et al., 2022)
Gene-target selection			
RNAfold WebServer http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cg	Target sequence	Target secondary and accessible regions	Institute for Theoretical Chemistry (University of Vienna)
sfold web server https://sfold.wadsworth.org/cgi-bin/index.pl	Target sequence	Target secondary and accessible regions	(Shao et al., 2007)

fungal uptake or affinity with DCL proteins, all key factors for SIGS success.

Unfortunately, important knowledge gaps regarding mechanisms behind RNAi silencing in fungi still prevent the establishment of a more precise protocol for the design of dsRNA molecules. To hasten the development of SIGS, further research should focus on: (i) fungal DCL proteins substrate affinities, cutting mechanisms, and siRNA-product characteristics, (ii) fungal AGO proteins guide strand selection, and (iii) the steps downstream of these processes that lead to silencing, with a view to enhancing the dsRNA design.

MAKING ENGINEERED dsRNA MOLECULES EFFECTIVE

Once the dsRNA molecules have been designed, it is necessary to verify that they silence the targeted gene(s) in the fungal pathogen and reduce symptom development in the plant. This can be done by challenging the fungus with the molecule under *in vitro* conditions and assessing changes in fungal development, phenotype, or expression of the targeted gene (Yang et al., 2021; Bhagta et al., 2023).

The efficacy of the dsRNA can then be tested *in planta*. A critical determinant of SIGS success is the number of RNAi-inducing molecules reaching the RNAi machinery (Nami et al., 2017; Schwartz et al., 2020; Qiao et al., 2021). However, once dsRNAs are applied onto the plant surface, they might face stability issues or not be taken properly by fungal cells.

Double-stranded RNA stability

Spray-induced gene silencing success depends on retaining an effective dsRNA concentration before and after application to the plant (Nami et al., 2017; Bachman et al., 2020). To date, little information is known about dsRNA shelf life, although they seem to be relatively stable when stored as buffer suspensions at 4°C, −20°C, and −80°C (Bai et al., 2023).

However, on the plant surface, dsRNAs must endure harsh environmental conditions compromising their stability (Bachman et al., 2020; Qiao et al., 2021). Several SIGS studies have found that the control provided by dsRNAs is lost within 1 week following their application on detached fruits or leaves (Islam et al., 2021; Niño-Sánchez et al., 2022; Qiao et al., 2023). This is probably due to a decline in dsRNA concentration. Indeed, once applied, dsRNAs can be degraded by environmental nucleases and sunlight UV radiation (San Miguel and Scott, 2016; Dhandapani et al., 2019; Bennett et al., 2020) or washed out by rain and irrigation (Mitter et al., 2017; Qiao et al., 2023). For instance, dsRNAs applied to detached tobacco leaves become undetectable 20 d following application (Mitter et al., 2017).

In some cases, some measures must be taken to protect the dsRNAs during storage and once in the plant to avoid this decline in dsRNA concentration. Some of these measurements include stabilizing dsRNAs with nanoformulations, which will be discussed in a later section (see "Nanoparticle formulations") (Rank and Koch, 2021).

Fungal dsRNA uptake

Spray-induced gene silencing success also depends on the dsRNA molecules finding their way into the fungal cells (Qiao et al., 2021). Two pathways for uptake are possible. Fungi can take up dsRNAs either directly from the plant surface (direct uptake) or after they have been taken up and processed by the plant host (indirect uptake).

Direct uptake

In vitro experiments using fluorescently labeled dsRNAs have shown that fungal cells can take up dsRNAs without plant intervention. However, not all fungi are equally capable of direct uptake. For instance, the fungal pathogens *B. cinerea* (Wang et al., 2016), *S. sclerotiorum*, *R. solani*, *A. niger*, *V. dahliae* (Qiao et al., 2021), and *A. psidii* (Degnan et al., 2023) have a better uptake capacity than *Zymoseptoria tritici* (Kettles et al., 2019), *Trichoderma virens*, and *C. gloeosporioides* (Qiao et al., 2021), which show poor to no uptake. The developmental stage of the fungus can also affect uptake. For example, *A. psidii* germinating urediniospores can take up dsRNAs but not its teliospores (Degnan et al., 2023).

Knowing whether the fungus is capable of dsRNA uptake is recommended as it will increase the chances of silencing in the field (Qiao et al., 2021). Therefore, we suggest evaluating the fungal uptake *in vitro* before moving to *in planta* evaluations. This can be done by exposing the fungus to fluorescently labeled dsRNAs in culture medium (Hamby et al., 2020).

Indirect uptake

Plants can take up dsRNAs, possibly process them into siRNAs, and transfer them to fungal cells (Koch et al., 2016; Wang et al., 2016; Cai et al., 2018). Interestingly, before being transferred into fungal cells, dsRNAs, or the resulting siRNAs, can travel systematically through the plant (Figure 4) (Koch et al., 2016; Biedenkopf et al., 2020; Delgado-Martín et al., 2022). Therefore, even untreated plant tissues might be protected by SIGS.

Even though the discovery of RNAi-signaling molecule movements between plants and fungi is relatively new, such exchanges are actually widespread and are key mediators of plant–pathogenic fungi interactions (Weiberg et al., 2013; Wang et al., 2016, 2017). These molecules travel between plants and fungi inside extracellular vesicles (Figure 4) (Cai et al., 2018).

Constraints for dsRNA uptake

Before entering plant or fungal cells, dsRNAs have to cross the cell wall, cell membrane and, in the case of plant uptake, the plant cuticle (Garcia-Rubio et al., 2020; Qiao et al., 2021). These biological barriers have specific permeability properties and control the trafficking of nutrients and other molecules inside the cell. Therefore, they might limit RNAi-signaling molecules moving inside fungal and plant cells (Bennett et al., 2020; Šečić and Kogel, 2021).

Constraints in the direct uptake

The complex polysaccharide matrices of the fungal cell wall and the lipid bilayers in the fungal cell membrane are the main biological barriers to the direct uptake of dsRNA by fungi. Cell walls are perhaps the least restrictive obstacle as they have a porous nature (Lipke and Ovalle, 1998; Garcia-Rubio et al., 2020). They allow the movement of small molecules into the cell, but can limit the movement of long dsRNAs (Casadevall et al., 2009; Walker et al., 2018). However, fungal cell walls are not rigid barriers but dynamic structures, with viscoelastic properties fluctuating and interacting with molecules. Therefore, the movement of molecules across cell walls does not depend exclusively on the molecule's size. For instance, Walker et al. (2018) showed that gold nanoparticles crossed *Candida albicans* and *Cryptococcus neoformans* cell walls only when encapsulated in liposomes containing amphotericin B, despite encapsulation not affecting the particles' size.

Fungal cell membranes, conversely, are less permeable. While they allow the movement of small and relatively hydrophobic molecules through passive diffusion, other molecules require active mechanisms (Barata-Antunes et al., 2021). This is the case for dsRNAs as they have a cationic and hydrophilic nature (Shin et al., 2018). In insects, dsRNAs enter the cell using transmembrane channel proteins (Systemic RNAi Defective (SID) Proteins and SID-like proteins) and endocytic pathways (Wytinck et al., 2020). SID-like proteins seem absent in fungi (Cai et al., 2019; Wytinck et al., 2020). Therefore, it is believed that the movement of dsRNAs across fungal cell membranes relies mostly on endocytosis. Supporting this hypothesis, Wytinck et al. (2020) have shown that the inhibition of the clathrin-mediated endocytosis disrupts dsRNA uptake in *S. sclerotium*.

Constraints in the indirect uptake

During indirect uptake, the cuticle, the plant cell wall and the cell membrane represent additional biological barriers for fungal uptake as dsRNAs must first make their way in and out of the plant cell.

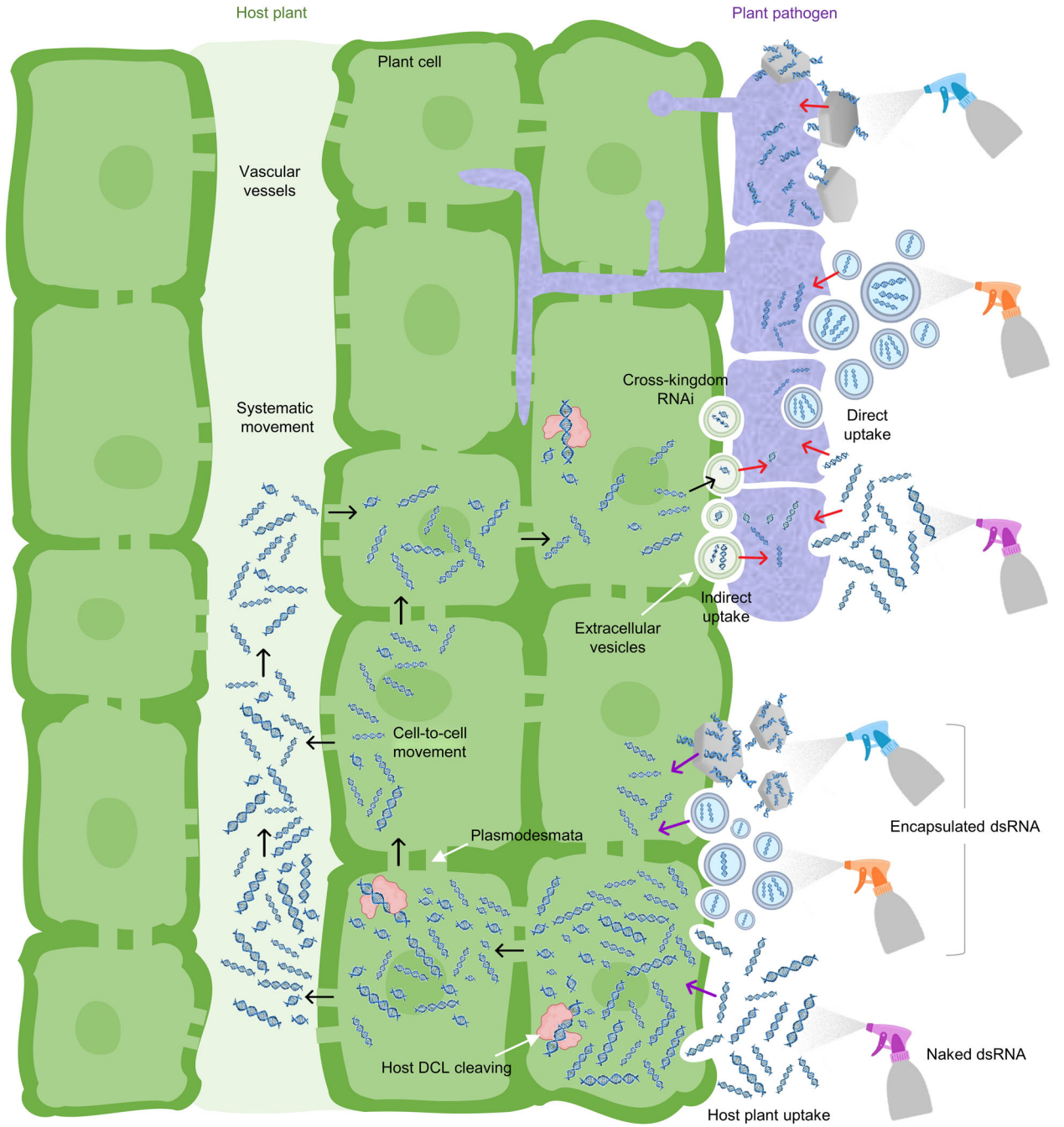

Figure 4. Fungal uptake of double-stranded RNAs (dsRNAs)
Double-stranded RNAs are applied to the plant as naked molecules or within nanoformulations to protect the molecules from the environment. Fungi can take up the dsRNAs from the environment or the host plant (red arrows). The plant can take up dsRNAs from the environment (purple arrows), and these molecules can be processed by DCL plant enzymes into siRNAs and both (dsRNAs and siRNAs) move from cell to cell through the plasmodesmata and systemically through the vascular system (black arrows) and, between plant and fungi, inside extracellular vesicles.

Plant cell walls and cell membranes behave similarly to fungal ones, the former permitting the passage of small dsRNAs molecules (Li et al., 2019; Bennett et al., 2020), and the latter being more restrictive to their crossing (Shin et al., 2018; Bennett et al., 2020). Bennett et al. (2020) have shown that dsRNAs of less than 50 bp easily cross tobacco cell walls, but not longer dsRNAs. However, one study in plant cells found that 70 kDa dextran molecules (~6 nm hydrodynamic radius) can cross plant cell walls, but not the smaller α-amylase molecules (~4 nm hydrodynamic radius) (Li et al., 2021). Therefore, as in fungi, molecule size is not the only factor defining the movement of molecules across plant cell walls.

Like fungi, plants do not have SID-like proteins and seem to rely on endocytosis for the dsRNA movement across cell membranes. For instance, Bennett et al. (2020) have shown

that flg22 (a 22-amino acid flagellin fragment), which is known to induce ligand-induced endocytosis in plants, enhances the uptake of small dsDNA molecules in tobacco plant cells.

The plant cuticle is relatively impermeable to nucleic acids, including dsRNAs. At laboratory scale, pressure, abrasion, and biolistic transfection can be used to transfect plant cells with nucleic acids and dsRNAs (Bennett et al., 2020). However, these methods are not feasible at field scale. Alternatively, dsRNAs can be applied in formulations with surfactants. Indeed, dsRNA formulations with organosilicon surfactants (i.e., Silwet L-77 and Pulse) facilitate the delivery of dsRNAs through the plant stomata (Bennett et al., 2020; Degnan et al., 2023).

In addition to surfactants, nanocarrier formulations have shown some promising results for the delivery of dsRNAs into plant and fungal cells and for SIGS. In the following section, some nanocarriers are discussed, with an emphasis on those tested for SIGS applications.

Nanoparticle formulations

Nanocarriers can be divided into three broad categories: organic, inorganic, and carbon-based nanocarriers, depending on their chemistries (Jat et al., 2020) (Figure 5). They have been used intensively in the medical field for RNA-based therapeutics (Blanco et al., 2015; Shin et al., 2018). *In vitro* and in greenhouse studies with plant pathogenic fungi have yielded promising results, showing that nanocarriers improve dsRNA stability and cellular uptake (Qiao et al., 2023) and promote dsRNA systemic movement inside the plant (Chen et al., 2022; Molesini et al., 2022), leading to more sustained protection of the plant host (Mosa and Youssef, 2021; Niño-Sánchez et al., 2022). Some nanocarriers can even double the protection time of the dsRNA. For example, Niño-Sánchez et al. (2022) have shown that the protection of tomato fruits and detached leaves against *B. cinerea* was increased from 1 week to up to 2 weeks when using a formulation with layered double hydroxide (LDH) nanoparticles compared with naked dsRNAs. Similar results have been reported for other nanoparticles and pathosystems, including LDH nanoparticles with *F. oxysporum* in tomato plants (Mosa and Youssef, 2021), lipid vesicles with *B. cinerea* in tomato fruits, grape berries, and *Vitis vinifera* leaves (Qiao et al., 2023), and chitosan/star polymer (SPc) nanoparticles complex with *R. solani* in detached-rice leaves (Wang et al., 2022). The number of studies regarding the use of dsRNAs nanoformulations for protecting plants against plant pathogenic fungi is still low, but it will certainly increase considering the positive results already obtained.

Organic nanocarriers

Various organic nanocarriers, namely lipid vesicles, and protein-based or organic polymer-based nanocarriers, have been tested for delivering dsRNAs to plant pathogenic fungi (Figure 5).

Lipid-based nanocarriers

Lipid vesicles are non-toxic self-contained structures enclosed by a lipid bi-layer composed mainly of phospholipids and sterols that can encapsulate different cargoes, including dsRNAs (Podesta and Kostarelos, 2009; Qiao et al., 2023). Lipid vesicles, in particular liposomes, have been used

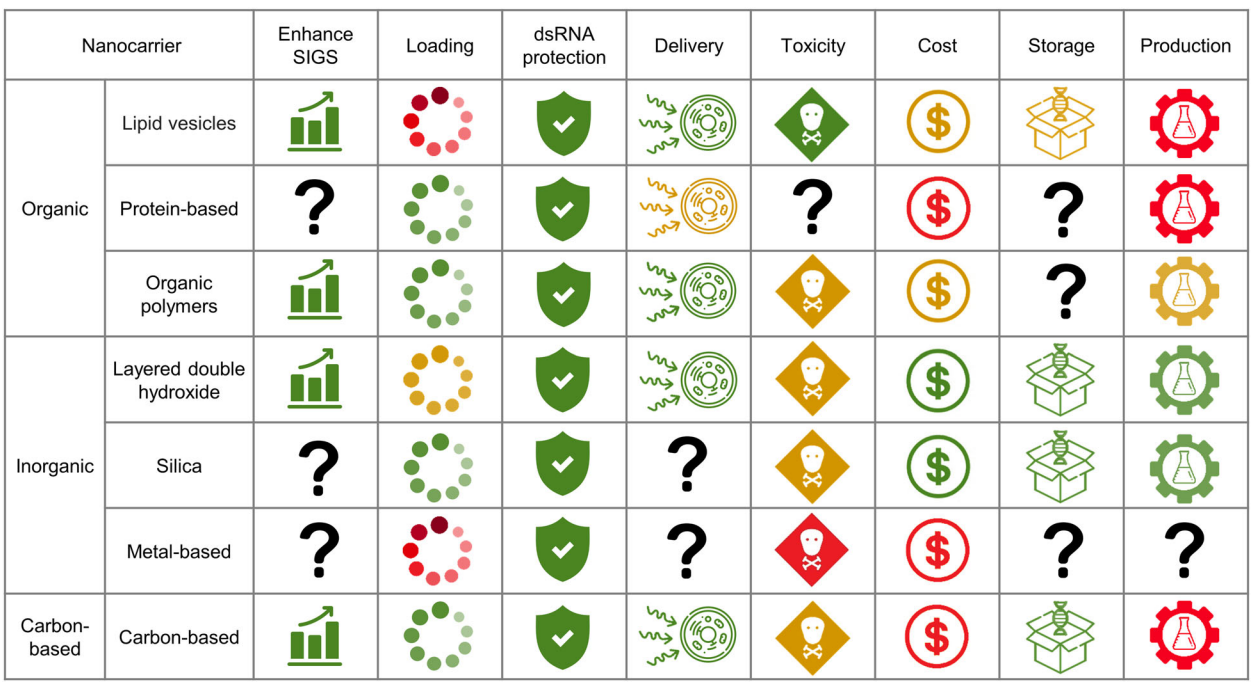

Figure 5. Summary of the relevant features of the main nanocarriers used for spray-induced gene silencing (SIGS)
The figure shows the performance of nanocarriers across categories, with green, orange, and red symbols representing good, average, and poor performance, respectively. A black question mark indicates missing information.

extensively for delivering dsRNAs and other cargoes in the medical field (Shim et al., 2011; Shin et al., 2018; Giordani et al., 2023), and their success has motivated their use in other fields, including plant protection (Sanitt et al., 2016; Tayler et al., 2019; Qiao et al., 2023). Only one study has tested liposomes for SIGS against fungal pathogens (Qiao et al., 2023). This study, as well as other SIGS studies for pest control, have shown that liposomes can protect dsRNAs from the environment, and enhance cellular uptake, silencing, and plant protection (Zhang et al., 2018; Tayler et al., 2019; Qiao et al., 2023). Liposomes also provide a regulated release of the dsRNAs and aid their attachment to the plant surface, preventing rain wash (Qiao et al., 2023; Su et al., 2023).

A downside of liposomes is that they can have a short shelf life. Depending on the lipidic composition and storage conditions, liposomes fuse during long-term storage, leading to enlarged particle sizes and unwanted cargo release (Lehman et al., 2023; Budavári et al., 2024). They also have relatively low loading capacities and are hard to mass produce as they require laborious and low-efficient isolations and cargo-loading methodologies, most of which have low efficiencies and are limited to the laboratory scale (Shin et al., 2018; Schlemmer et al., 2021; Giordani et al., 2023). The cost derived from the liposome complex production process and raw materials might be a limitation for some applications. Therefore, developing simple and easy-to-scale production processes using less expensive lipid constituents is necessary before using liposomes for SIGS in the field (Qiao et al., 2023).

Lipid vesicles produced by living cells (i.e., extracellular vesicles and minicells) can be an interesting alternative to liposomes (Shin et al., 2018; Islam et al., 2021). To our knowledge, only one study has tested such vesicles for SIGS, showing that *E. coli* minicells can be used to deliver dsRNAs targeting the CHS3b2, DCL1, and DCL2 genes of *B. fuckeliana*, reducing fungal growth and symptom development in strawberries (Islam et al., 2021). Furthermore, living bacteria or the cell-free lysate of an *E. coli* strain producing dsRNAs targeting the virulence *SAS1* gene of *B. cinerea* reduced fungal proliferation, symptom development, and the expression of the targeted gene when applied on *N. benthamiana* detached leaves (Niño-Sánchez et al., 2021). Whether the dsRNAs produced were subsequently encapsulated in bacterial vesicles was not evaluated. However, it is a possibility as it has been shown that bacteria genetically modified to express different cargoes can encapsulate these cargoes in extracellular vesicles (Wang et al., 2018).

Protein-based nanocarriers

Protein-based nanoparticles are based on polypeptides with various cationic domains that can interact with the negatively charged dsRNAs through electrostatic interactions, forming three-dimensional structures that protect dsRNAs and facilitate their delivery into cells (Unnamalai et al., 2004; Elzoghby et al., 2012; de Schutter et al., 2021). These nanoparticles have also been used extensively in the medical field. However, they are beginning to be replaced by liposomes, following the approval of various lipid-based therapeutic products (Giordani et al., 2023).

Among protein-based nanocarriers, polycationic and amphipathic cell-penetrating peptides (CPP)-based and lectin-based nanocarriers have been the most explored for delivering dsRNAs in agricultural applications, but mostly into plant and insect cells (Fitches et al., 2002; Down et al., 2006; Yang et al., 2017; Numata et al., 2014, 2018; Martinez et al., 2021). To the best of our knowledge, the only protein-based nanocarrier tested for SIGS against a fungal pathogen was protamine nanoparticles, which had lower loading, delivery, nucleases-protection, and disease-protection capacities than other nanoparticles (i.e., chitosan, polyethyleneimine, carbon quantum dots, polyamidoamine, and chitosan/star polymer (SPc) complex) (Wang et al., 2023c). The main disadvantage of protein-based nanocarriers is their complex production processes (Yang et al., 2017), which can translate into high production costs and market prices. In addition, some protein-based nanocarriers have shown low dsRNA-releasing capabilities due to strong polypeptide–dsRNA interactions, which can interfere with the delivery (Yang et al., 2017).

Organic polymer-based nanocarriers

Among organic polymer-based nanocarriers are polyethyleneimine, polyamidoamine (PAMAM), polylactic-co-glycolic acid, and chitosan, which are commonly used as nanocarriers because their amine/imine groups confer to them a high loading capacity by binding dsRNAs through electrostatic interaction (Quilez-Molina et al., 2024). Also, they can be functionalized with additional amine groups increasing their loading capacities (Shin et al., 2018; Wang et al., 2023c). Most of these polymers protect the dsRNAs from the environment (Mudo et al., 2022) and facilitate their delivery into fungal (De Angelis et al., 2022; Wang et al., 2023c) and plant cells (Silva et al., 2010). However, they can differ in these and other capacities and some of these polymer nanocarriers can have high production costs (Saharan et al., 2013; Sathiyabama and Parthasarathy, 2016). Therefore, polymer selection requires careful consideration.

For example, a study in *R. solani* showed that chitosan, polyethyleneimine, and chitosan/SPc complex nanoparticles were better at delivering dsRNAs into fungal cells than PAMAM, and that the chitosan/SPc complex was the best in protecting the dsRNA from nuclease degradation and reducing symptom development in rice (Wang et al., 2023c). Polyethyleneimine nanoparticles can be used to deliver dsRNAs into tobacco protoplast, but are less effective in complete cells, suggesting that plant cell walls limit the movement of these nanoparticles (Silva et al., 2010). Chitosan nanoparticles can protect dsRNAs from degradation when subjected to 80°C and ultraviolet light (Mudo et al., 2022; Scarpin et al., 2023). They also improved the control of *B. cinerea* in *N. benthamiana* leaves with dsRNAs targeting *BcCYP51*, *Bcchs1*, and *BcEF2* genes (Scarpin et al., 2023). Furthermore, chitosan nanoparticles have been extensively

used to boost a defense response and promote plant growth in several crops. Finally, another advantage of chitosan is its low cost (Sathiyabama and Parthasarathy, 2016; Kumaraswamy et al., 2018).

Inorganic nanocarriers

Inorganic nanocarriers, such as synthetic anionic clay, metal-based, and silica nanoparticles, have been commonly used to deliver dsRNAs and other cargoes in agriculture applications.

Layered double hydroxide

Layered double hydroxide nanoparticles are part of the synthetic anionic clay nanoparticles and are probably the most explored option for the delivery of dsRNAs into plant and fungal cells (Mosa and Youssef, 2021; Chen et al., 2022; Molesini et al., 2022; Niño-Sánchez et al., 2022; Yong et al., 2022; Mukherjee et al., 2024). Due to their anion exchange capacity, they interact with dsRNA providing them protection against nucleases (Mosa and Youssef, 2021), facilitating their cellular uptake (Chen et al., 2022), promoting their systemic move across the plant (Chen et al., 2022; Yong et al., 2022), and improving their adhesion to the plant surface (Chen et al., 2022). Layered double hydroxide nanoparticles release dsRNA molecules in a controlled manner as the clay degrades (Jain et al., 2022), which results in a prolonged control of the host (Niño-Sánchez et al., 2022). dsRNA–LDH nanoformulations have proven effective to retard symptom development in multiple pathosystems, including tomato–*F. oxysporum* (Mosa and Youssef, 2021), chickpea–*B. cinerea* (Niño-Sánchez et al., 2022), and maize–*R. solani* (Chen et al., 2022).

Layered double hydroxide nanoparticles are low-cost nanocarriers. However, their low number of adsorption active sites limits their loading capacity (Dou et al., 2020; Li et al., 2021). The additional processes to functionalize their surfaces and increase their loadings might increase their costs (Tang et al., 2020). Furthermore, some plant tissues can take up LDH more readily than others (Mosa and Youssef, 2021; Chen et al., 2022). To our knowledge, no studies have evaluated the differential uptake in fungal structures. Also, it should be noted that this might not be unique to LDH nanoparticles, as it has not been tested for other nanocarriers. This differential uptake observed between plant tissues must be considered, as it will determine how the nanocarrier will be applied to the plant.

Silica-based and metal-based nanoparticles

Silica and metal nanoparticles have been used for intracellular delivery of several cargoes, including dsRNAs, into mammalian cells but not into fungal or plant cells (Giljohann et al., 2009; Meng et al., 2010; Shin et al., 2018). A recent publication by Xu et al. (2023) showed that spraying *N. benthamiana* with amine-functionalized silica nanoparticles carrying dsRNAs targeting the Potato virus Y reduced, to a greater extent, virus titers in infected plants when compared with the naked molecules, especially during late infections. However, the feasibility of using silica and metal nanoparticles as carriers for delivering dsRNAs into the plant and fungal cells remains mostly unexplored.

While both metal and silica nanoparticles protect dsRNAs from nuclease degradation and facilitate cellular uptake in animal cells (Paul et al., 2014; Shin et al., 2018), the former underperforms the latter in some other aspects. Indeed, metal nanoparticles have low loading capacities and thus require surface functionalization or complexation with other nanocarriers to increase their loading (Giljohann et al., 2009; Meng et al., 2010; Shin et al., 2018), are expensive, and can be toxic (Li et al., 2021). In contrast, silica nanoparticles are innocuous, stable, and have simple and inexpensive production processes. They have high loading capacities, due to their porous structure and large surface area, but can also be functionalized to improve their cargo loading or to target specific sites in the targeted organism (Li et al., 2021). Thus, silicate nanoparticles might be interesting to further assess for SIGS purposes.

Carbon-based nanocarriers

Carbon-based nanocarriers include carbon dots (CD), nanotubes, nanohorns, fullerenes, nanoparticles, nanobeads, nanofibers, and nanodiamonds (Verma et al., 2019), some of which have already been evaluated for dsRNAs delivery into plant cells (Verma et al., 2019; Demirer et al., 2020). Overall, carbon-based nanocarriers are characterized by their stable molecular architecture, high chemical reactivity, and high surface area (Verma et al., 2019; Demirer et al., 2020; Jat et al., 2020). However, they have distinctive properties depending on their agglomeration patterns, surface structure, and sizes (Verma et al., 2019). For example, the CD can cross biological barriers, are water soluble, and exhibit fluorescence, making them a convenient tool for tracking nanoparticles inside plant tissues. However, they can be toxic to plants at high concentrations (Shin et al., 2018; Verma et al., 2019). Graphenes can also be toxic to plants at high concentrations, but they are not toxic when functionalized with oxygen groups (Husen and Siddiqi, 2014). Nanotubes have high cargo-loading capacities due to their large surface areas (Verma et al., 2019; Demirer et al., 2020; Jat et al., 2020).

Carbon dots and carbon nanotubes have been evaluated for SIGS or to deliver dsRNA with promising results (Kostov et al., 2022; Su et al., 2023; Wang et al., 2023c). For instance, these nanoparticles protect the dsRNAs from the environment and facilitate the delivery inside the cell (Demirer et al., 2020; Schwartz et al., 2020). Furthermore, dsRNA–CD nanoformulations targeting *R. solani* pathogenicity genes reduced symptom development in rice-detached leaves (Wang et al., 2023c), and leaf applications of dsRNA–CD and dsRNA–carbon nanotubes silence the targeted genes in tobacco and tomato (Demirer et al., 2020; Kostov et al., 2022). These results show that CD and carbon nanotubes can be a good alternative for SIGS. However, due to the toxicity demonstrated for other carbon-based nanocarriers

(Husen and Siddiqi, 2014; Verma et al., 2019), their toxicity should not be overlooked. Therefore, SIGS evaluations using CD and carbon nanotube nanoformulations should include experiments assessing the phytotoxicity of these nanocarriers at different concentrations.

Concluding remarks and other considerations for dsRNA molecules effective

Despite SIGS' multiple advantages (versatility and adaptability, precise and sequence-specific control, low environmental impact, reduced risk for resistance development, etc.), this technology is constrained by the instability and easy degradation of the RNA molecule, rendering the protection of the plant transient. Also, there are concerns regarding the uptake of dsRNA molecules by plants and pathogens, which may also affect the efficacy of the silencing. Consequently, significant efforts are being devoted to developing nanocarriers that can shield dsRNA from environmental conditions and, in turn, enhance its uptake.

So far, studies on nanocarriers for SIGS have mainly focused on LDH and chitosan, which appear to be good options as they perform well at a relatively low cost. However, the lack of comparison with other nanocarriers for SIGS does not allow us to assert whether they really are the best alternative and perhaps, there is not a single nanocarrier that can guarantee protection for the several application methods required depending on the infection mode of the fungal pathogen (foliar, vascular, soil-borne, post-harvest). In addition, price-wise comparisons of nanocarriers are limited as no cost evaluation study has been performed to date.

PERSPECTIVE OF SIGS CONTROL STRATEGY

It is well recognized that SIGS is a promising strategy for the control and management of plant diseases. However, there are some aspects of this technology that require more extensive research and open up possibilities for further investigation. Along this review, we have pointed out the knowledge gaps that we found regarding the mechanisms behind fungal RNAi silencing. Little information is known about fungal DCL processing of dsRNAs, AGO guide strand selection, and downstream processes leading to silencing. Also, we have stressed the need for more robust studies evaluating the potential OTE effect of dsRNAs on varying organisms and the ecotoxicology of RNAi-based plant disease management. In the last section of this review, we have focused on the dsRNA instability and low pathogen uptake, as we consider them to be the most limiting factors for SIGS success in the field. We have discussed how they can reduce SIGS efficacy and some efforts that are being taken to overcome these limitations. The work on the encapsulation of the dsRNAs using nanocarriers is relatively new, and has shown promising results. However, most studies have been focused on limited combinations of pathogens and nanocarriers. It is necessary to expand these studies to other pathogens and nanocarriers, comparing the efficacy of the different nanocarriers.

To conclude, we would like to stress that other factors can also influence the implementation of SIGS in the field. Thus, despite being out of the scope of this review, they should not be overlooked. One of them relates to the cost and production of dsRNAs. Spray-induced gene silencing technology must be cost-effective in order to be implemented on a large scale. Since the advent of RNAi, the industry has addressed these challenges due to growing interest in these technologies. The growing interest of companies in SIGS technology has led to remarkable advances in production and encapsulation methods, resulting in significantly reduced costs (Rank and Koch, 2021; Bocos-Asenjo et al., 2022). Another aspect that cannot be overlooked is the issues pertaining to the development of new fungicide products, including their biosafety and the legislative framework governing their use in the field (Rank and Koch, 2021). Currently, these products are considered safe, given their minimal risk to human and animal health. However, a robust regulatory framework has yet to be established for these new biopesticides (Rank and Koch, 2021). Last, a comprehensive effort is necessary to establish crop application guidelines to facilitate the implementation of SIGS by farmers. We need to study optimal timing and dsRNA concentration for field applications, develop effective application methods, and assess potential synergies with other control methods.

CONCLUSIONS

SIGS is a promising strategy for the control of plant pathogenic fungi in agriculture. As an alternative to fungicides, it is a cheaper, more convenient, and more versatile option than other RNAi-based technologies, such as HIGS. For the SIGS technology to progress, more fundamental studies are needed to fully elucidate the biological mechanisms behind it, but even more applied studies. Therefore, a step-by-step description of how to develop a SIGS strategy, highlighting relevant considerations, can come in handy.

The first step in designing a SIGS strategy is to ensure that the target fungus is susceptible to SIGS. Here, the researcher can start by checking whether the fungus has the well characterized RNAi machinery. Indeed, researchers can use this information to look for homologies in the target fungus. However, it is important to keep in mind that having RNAi machinery is not a guarantee of SIGS susceptibility but only a good starting point.

The second step is the selection of the gene(s) to target. Targeted genes must play central roles in the fungal pathogenicity, be it because they are essential genes participating in fungal proliferation or because they are directly responsible for virulence. Besides, the literature suggests that non-conserved, abundant, and highly transcribed genes with accessible regions make good targets.

The third step is to design *in silico* dsRNA sequences targeting those genes, which is facilitated by a variety of bioinformatic tools. The dsRNA length must be carefully chosen. In addition, it is important to remember that only one of the dsRNA strands is complementary to the target mRNA and thus can yield functional siRNA guide strands. Which strand is turned into guide strands depends on the DCL and AGO proteins cut and recognition affinity. Unfortunately, knowledge directly applicable to dsRNA design is scarce as the mechanisms behind these phenomena are still being researched. To reduce the risk of adverse outcomes on biodiversity, the potential of the designed molecules to trigger silencing in non-target organisms should be assessed. At this point, the efficacy of the dsRNAs can be checked through preliminary *in vitro* and *in planta* experiments assessing the dsRNAs uptake, gene silencing, and disease control to select a couple of functional dsRNAs.

The final step is to address the major SIGS limitations, dsRNA stability and cellular uptake. This can be done effectively by selecting a nanocarrier from a variety of nanoparticles with different characteristics. Their capacity of improving dsRNA uptake, gene silencing, and disease control can be evaluated in preliminary evaluations as for the naked dsRNA molecules. Once the best combinations of dsRNAs and nanoparticles have been selected, researchers can move to greenhouse and field evaluations to test them for disease control. It is imperative to investigate the optimal timing and frequency of RNAi application to determine the most effective spraying intervals and timing for achieving maximum efficacy and outcomes.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

S.M. and M.G. wrote most parts of the review. I.T.B.-A. contributed to writing and drawing the figures. H.A. assisted in writing the bioinformatics-related sections. S.D.-H. contributed to writing, drawing Figure 1, and organizing the material. J.J.D. revised the manuscript. J.N.S. conceived the idea, structured the review, contributed to writing, and revised the manuscript. All authors read and approved its content.

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