

Spray-induced gene silencing: an innovative strategy for plant trait improvement and disease control

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Abstract: Modern plant breeding is still a time-consuming and costly process, even with the most advanced technologies such as gene editing. Hence, there is an urgent need to develop alternative means for plant trait manipulation and plant protection. RNA interference (RNAi) is a conserved cellular mechanism mediated by naturally occurring double-stranded RNA (dsRNA) and small RNAs (sRNAs) that can target mRNAs for destruction or transcript reduction. Here, we review the potential of technology based on RNAi, called spray-induced gene silencing (SIGS), as an alternative or adjunct to breeding for manipulation of endogenous gene expression in plants or pathogen control. SIGS based on exogenous application of RNA molecules in plants may be especially useful in reducing pest or pathogen impacts, thereby ameliorating biotic stresses and increasing the agronomic performance of crops.

Keywords: RNA interference, small RNAs, SIGS, dsRNAs

INTRODUCTION

Over the last century, global food demand has increased due to the quadrupling of the human population from 1.8 billion people in 1915 to 7.9 billion at present and is expected to reach 9.7 billion by 2050 (UN 2019). To meet the growing population demands, food production needs to increase by 98% by 2050 despite challenges such as pests, pathogens, climate change and urbanization (Valin et al. 2014). Global food production demands can only be met by integrating modern breeding and plant biotechnological tools for precision farming to maximize edible yields and minimize losses to both biotic and abiotic stresses. Modern precision breeding technologies of introducing genes into elite germplasm using the most advanced technologies such as gene editing and speed breeding will still require years to deliver individual improved cultivars. Furthermore, not all crop plants are amenable to genetic modification with tools such as gene editing, or they have breeding cycles that require years between crosses and subsequent phenotyping (e.g. trees).

Today, bringing a transgenic crop from development to commercialization costs US\$ 140 million (Rosa et al. 2018). There is also public opposition and legislative limitations on cultivation of genetically modified (GM) crops in many countries (particularly in Africa, most of Asia, and Europe). Similarly, to bring a new synthetic crop protection chemical to market costs over US\$ 250 million and takes over 10 years (Sparks and Lorschbach 2017). Thus there is a need to develop

Crop Breeding and Applied Biotechnology
21(S): e387921S11, 2021
Brazilian Society of Plant Breeding.
Printed in Brazil
<http://dx.doi.org/10.1590/1984-70332021v21S11>



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Received: 25 June 2021

Accepted: 27 June 2021

Published: 06 July 2021

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new sustainable and GMO-free approaches to improve plant traits and agronomic performance, that support and work in parallel with modern breeding. Research elsewhere has shown that gene silencing approaches can aid precision breeding and plant protection via manipulation of plant traits involved in growth and different stress tolerances (biotic and abiotic) (Younis et al. 2014). In particular, the strategy of spray induced gene silencing (SIGS), which uses topical application of double stranded RNA (dsRNA) molecules, has an advantage of being more environmentally acceptable and GMO-free. In this review, RNAi approaches involving topical application of RNA molecules directly on plants (SIGS) are discussed (Figure 1).

REGULATION OF PLANT TRAITS VIA TOPICAL APPLICATION OF RNAS

Small non-coding RNAs (sRNAs) of 20–40 nucleotides (nt) have emerged as key players in transcriptional and post-transcriptional gene regulation, also known as RNA interference (RNAi), in an array of organisms (Ghildiyal and Zamore 2009, Holoch and Moazed 2015, Dalakouras et al. 2020). RNA silencing or RNAi is a conserved eukaryotic process in which transcript accumulation is either modulated or reduced in a sequence-specific manner. sRNAs, through RNAi, play important roles in gene regulation, genome stability, chromatin organization, and pathogen/pest resistance (Ding 2010, Ketting 2011, Castel and Martienssen 2013). The biogenesis and function of sRNAs typically involves the processing of dsRNAs by a core set of proteins, namely Dicer or Dicer-like (DCR or DCL; double-stranded (ds)RNA ribonuclease), Argonaute (AGO; RNA binding, slicer ribonuclease), and RNA-dependent RNA polymerase (RdR; secondary sRNA production) (Holoch and Moazed 2015). The sRNAs move from their site of biogenesis to neighboring cells across plasmodesmata, the nano-channels that allow cell continuity in plants, or apoplastically through intercellular spaces (Mermigka et al. 2016). The sRNAs can also be transported long-distance to distal parts of the plant through the vasculature, a process termed systemic RNAi (Melnik et al. 2011). As a result of their role in regulating the developmental pathways of eukaryotic organisms, there has been an increasing interest in exploiting sRNAs to modulate plant gene expression towards improving crop productivity and enhancing stress tolerance.

RNA-based applications have been reported to effectively target plant transgenes and endogenous genes in only a few plant systems such as *Nicotiana benthamiana*, *Arabidopsis* and rice through various methods, including formulations with adjuvants to enhance dsRNA delivery, syringe infiltrations, foliar spraying, and irrigation methods. In one of the first reports utilizing SIGS technology, *N. benthamiana* leaves were treated with 200 bp and 700 bp dsRNA, 21-nt sRNA, or 21-mer anti-sense ssDNA targeting an endogenous *PHYTOENE DESATURASE (PDS)* gene, resulting in bleaching of leaves within ten days (Sammons et al. 2011). In this study, leaves were treated either with carborundum or Silwet L-77 surfactant as an adjuvant before dsRNA application.

Syringe infiltration is considered as one of the simplest methods for introducing transgenes in model plants. Surprisingly though, leaf infiltration with dsRNA targeting a YFP transgene in *Arabidopsis* did not result in RNAi. However, delivery of dsRNA through a carrier complex containing a copolymer of histidine and lysine along with a cell-penetrating peptide resulted in efficient delivery of dsRNA targeting the YFP transgene and an endogenous *CHALCONE SYNTHASE (CHS)* gene, followed by decreased mRNA accumulation for the respective genes (Numata et al. 2014). The Cy3-labelled dsRNA was observed in the cytosol, indicating that symplastic delivery of dsRNA is important for effective dsRNA-mediated target gene silencing. Similarly, Jiang et al. (2014) reported that cationic fluorescent nanoparticle-based formulation of dsRNA (G2 nanoparticle – dsRNA at 2:1 ratio) effectively delivered the dsRNA into plant cells. Application of G2-dsRNA targeting

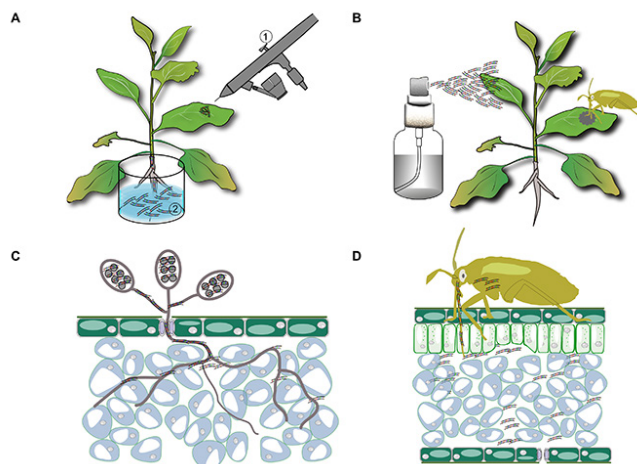


Figure 1. Illustration of means of delivery of dsRNA for modulation of plant traits and plant protection. A. High pressure spraying of dsRNA using an airbrush (1) and delivery of dsRNA through irrigation (2) for plant trait modulation to achieve intracellular delivery. B. Automizer assisted low-pressure spray results in apoplastic delivery of dsRNA allowing fungal, oomycete pathogens, pests/insects to take up dsRNA (C, D).

SHOOT MERISTEMLESS (STM), a gene required for formation and maintenance of shoot apical meristem, and *WEREWOLF (WER)*, a transcription factor (TF) involved in specifying non-hair identity in the root epidermis, were applied for three subsequent days at the root tip, resulting in reduction in transcription levels by 84% and 87%, respectively, compared to mock or water treated controls (Jiang et al. 2014). These reports demonstrate that accumulation of specific mRNAs can be targeted by exogenously applied dsRNAs in both above and below ground plant parts, but that the uptake of the dsRNA can be strongly influenced by the formulation.

In addition to carrier-based formulations, mechanical inoculation through spreading of dsRNA with soft sterile brushes and gentle rub inoculation also has been shown to aid dsRNA delivery to induce RNAi. Application of dsRNA targeting *eGFP* and *NEOMYCIN PHOSPHOTRANSFERASE II (NPTII)* in transgenic *Arabidopsis* plants using soft brushes resulted in significant reduction in the target transcript and protein levels (Dubrovina et al. 2019). Gentle rub inoculation of dsRNA targeting *MYB1*, a TF involved in flower morphogenesis, onto the floral buds of the orchid plant *Dendrobium hybrida* resulted in formation of flattened epidermal cells compared to conical shaped cells (Lau et al. 2015). The authors of this study used crude bacterial extracts containing a 430 bp dsRNA targeting *MYB1*, expanding the scope of dsRNA-based strategies in manipulating plant traits, by potentially enabling large scale application. However, application of dsRNAs through rub inoculation, or infiltration-based methods are not suitable at a large greenhouse or field scale. Such limitations can be overcome through irrigation or spray-based technologies.

Promising research results were obtained after roots of two-week old *Arabidopsis* seedlings soaked in 1 mL of a ~ 500 bp dsRNA targeting *MOB KINASE ACTIVATOR-LIKE 1A (MOB1A)*, gene involved in tissue patterning and organ growth, and *WRKY23*, a TF involved in auxin mediated root development resulted in absorption of dsRNA. As expected, dsRNA^{Mob1A} caused suppression of flowering, while dsRNA^{WRKY23} resulted in impaired root growth (Li et al. 2015). To further examine the possibility of uptake of dsRNA through the root system, Li et al. (2015) submerged the roots of three-leaf rice plants in fluorescently-labelled dsRNA^{eYFP} and dsRNA^{Actin}. In line with their previous observation, fluorescence was observed in the rice sheath and growth was severely repressed in dsRNA^{Actin} treated plants. The expression levels of *Ago* and *Dicer* were also upregulated, suggesting the activation of the RNAi pathway (Li et al. 2015). Taken together, the authors provide evidence for successful absorption of dsRNA through irrigation systems, a step towards modulating endogenous traits. That this research led to clear phenotypic changes indicates the scope for RNAi, initiated by exogenously applied dsRNA, for regulating plant endogenous genes. However, there is a pressing need for more research to corroborate these findings in other plant systems and using a large-scale approach. Delivery of dsRNAs via irrigation would be particularly well suited to crops grown under hydroponic conditions.

Following patent WO 2011/112570 describing that dsRNAs, small interfering RNAs (siRNAs) and single stranded DNA oligonucleotides can trigger efficient local and systemic silencing of *N. benthamiana* endogenes, foliar application of dsRNA for targeting endogenous genes has attracted interest in the agricultural community (Sammons et al. 2011). However, low-pressure spraying of siRNA^{GFP} on transgenic *N. benthamiana* 16C plants did not result in GFP silencing, probably owing to failure in achieving symplastic entry (Dalakouras et al. 2016). This problem was overcome by using high-pressure spraying of siRNAs targeting GFP, although silencing was only achieved in a few cells. Further research is necessary to establish whether high-pressure spraying is functional in targeting plant endogenous genes, or if dsRNA uptake from spray application can be improved through addition of adjuvants to the dsRNA formulation.

CROP TRAITS FOR MODULATION BY SIGS

Since RNAi functions to remove the target mRNA from the transcriptome, it is particularly well suited to modulation of plant traits where negative regulators have a significant impact on plant phenotype. That is, plant genes that, when the mRNAs are knocked down, lead to an advantageous phenotype. Examples of traits where SIGS targeting negative regulators could improve crop performance include: drought and salinity tolerance (Feng et al. 2021, Li et al. 2021, Min et al. 2021, Myo et al. 2021), flowering time (Hong et al. 2021), and host plant resistance to pathogens (He et al. 2020). As an example, for successful infection, pathogens deploy effector proteins into host plant tissue to target proteins and thereby suppress the host immune response. Some effectors target host proteins that are crucial for successful host-pathogen interactions; these plant proteins are often called susceptibility factors (Van Schie and Takken 2014).

In transgenic potato, silencing of five susceptibility factors led to reduced late blight disease, caused by *Phytophthora infestans* (Sun et al. 2016). Similarly, knockout of DOWNY MILDEW RESISTANT 6 (DMR6) in sweet basil (*Ocimum basilicum*) resulted in up to 93% reduction in downy mildew pathogen biomass (Hasley et al. 2021). SIGS of these susceptibility factors would be a good non-GM alternative for pest control. Furthermore, there is also the possibility that a GM crop plant where a gene encoding a negative regulator has been knocked out will also have an undesirable impact on plant performance. More precise timing of knock down of the target gene mRNA may avoid the undesirable effects, while delivering the desired trait at the time when it is required. In transgenic plants, this could be achieved by inducible gene silencing (Chen et al. 2003, Masclaux et al. 2004, Liu and Yoder 2016). However, this is also a situation where SIGS may be useful, especially in crops that cannot be easily genetically modified through transgenes or gene editing.

SIGS IN CROP PROTECTION

In addition to mediating a broad range of cellular processes related to growth and development, roles for sRNA-mediated RNAi in host-pathogen interactions have been described (Chaloner et al. 2016, Qiao et al. 2021). While most sRNAs function endogenously, some sRNAs move bidirectionally from one species to another and modulate cellular functions of recipient cells by regulating their gene expression through targeted gene silencing (Weiberg et al. 2013, Chaloner et al. 2016, Cai et al. 2018). This cross-kingdom RNAi can be exploited in crop-protection by generating transgenic plants expressing dsRNAs against selected gene targets of pathogens and pests, a phenomenon called host-induced gene silencing (HIGS; Baulcombe 2015). However, the use of HIGS requires transgenes and genetically modified organisms, which may raise concerns among some consumers, particularly in Europe.

This limitation can be overcome by the application of SIGS (Wang and Jin 2017, Koch et al. 2019). SIGS for crop protection is based on recent findings demonstrating that certain plant pathogens and pests can take up externally applied dsRNA/sRNA (Chaloner et al. 2016, Wang et al. 2016), which can then silence target pathogen genes essential for disease development (environmental RNAi). These findings raise the possibility of applying a SIGS-based strategy to improve crop performance. SIGS against pests and pathogens is an innovative and ecofriendly strategy for plant disease control at preharvest and postharvest stages. Moreover, compared to synthetic fungicide-based crop protection strategies, an RNA-based approach promises lower off-target effects, because it is possible to design short nucleotide sequences that are specific to the target genes. Further, with the ongoing rapid expansion of sequence databases, gene sequences that are specific to pests and pathogens can readily be identified.

SIGS to control fungal and oomycete plant pathogens

In recent years there has been an increasing number of publications that report on exogenous application of dsRNAs on crop plants to decrease disease caused by plant pathogenic fungi, oomycetes, viruses, and insect pests (Dalakouras et al. 2020). These strategies rely on direct uptake of the applied dsRNA by the pest or pathogen, leading to RNAi of the targeted genes. Demonstrations that SIGS can be used to control fungal and oomycete pathogens have been reported for several years. Spray application of detached barley leaves with dsRNAs targeting *Fusarium graminearum* ergosterol biosynthesis genes (*CYP51A*, *CYP51B*, and *CYP51C*) strongly inhibited fungal growth. Intriguingly, sprayed dsRNAs moved from site of application to different parts of the plant and could inhibit fungal growth at non-sprayed parts of detached leaves (systemic RNAi) (Koch et al. 2016). In another study, dsRNAs targeting ARGONAUTE and DICER genes in *F. graminearum* protected barley leaves from infection (Werner et al. 2020).

Similarly, SIGS against *Sclerotinia sclerotium* and *Botrytis cinerea* on oilseed rape and *Arabidopsis thaliana*, and against *F. asiaticum* on wheat resulted in reduced fungal disease (McLoughlin et al. 2018, Song et al. 2018). A recent study also showed that SIGS can also be applied against the basidiomycete fungus *Rhizoctonia solani* to control sheath blight of rice (Qiao et al. 2021). In this study, topical application of dsRNA targeting vesicle trafficking pathway genes *DYNACTIN 1* (*DCN1*) and *SUPPRESSOR OF ACTIN 1* (*SAC1*), or a gene coding for polygalacturonase (a known virulence factor gene of *R. solani*) resulted in a significant reduction of fungal biomass and disease symptoms on rice plants (Qiao et al. 2021). SIGS has also been used to control post-harvest diseases in fruit and berry crops, as well as in vegetables. Exogenous application of dsRNAs or sRNAs targeting *B. cinerea* dicer-like genes (*Bc-DCL1* or *Bc-DCL2*) significantly reduced gray mold disease on tomato, grape, strawberry, lettuce, onion, and rose (Wang et al. 2016). In the downy mildew pathogen, *Hyaloperonospora arabidopsidis*, mixing dsRNA targeting CELLULOSE SYNTHASE (*CESA3*), suppressed infection on *Arabidopsis*, thereby suggesting that SIGS may also be effective against oomycetes (Bilir et al. 2019).

Using a similar approach, exogenously applied dsRNAs did not lead to RNAi of target genes in the potato late blight pathogen, *Phytophthora infestans* (Qiao et al. 2021). However, by spray application of dsRNAs onto potato leaves, another study demonstrated that dsRNAs targeting developmentally important pathogen genes coding for guanine-nucleotide binding (G) protein β -subunit (PiGPB1), haustorial membrane protein (PiHMP1), cutinase (PiCUT3), or endo-1,3(4)- β -glucanase (PiENDO3) in *P. infestans* can potentially be used as a tool in controlling potato late blight disease (Kalyandurg et al. 2021). These results suggest that the optimal outcomes for SIGS against oomycete pathogens may be strongly influenced by the manner in which the dsRNA is applied. Although dsRNA has high target specificity, it is possible to design dsRNAs that can target multiple groups of pathogens. For instance, dsRNAs derived from the *F. asiaticum* β 2-tubulin gene are shown to be effective against *F. asiaticum*, *B. cinerea*, *Magnaporthe oryzae*, and *Colletotrichum truncatum* in wheat, cucumber, barley, and soybean, respectively (Gu et al. 2019).

SIGS against insect pests

The application of RNAi for controlling insect pests has been proposed for over a decade (Baum et al. 2007, Mao et al. 2007). However, the first demonstration of exogenous application of dsRNA against insect pests came from a study where dsRNA targeting an arginine kinase gene was applied on citrus trees and grapevine to control psyllids and sharpshooter pests (Hunter et al. 2012). After this breakthrough, the potential for dsRNAs in insect pest management were demonstrated elsewhere (e.g. Cagliari et al. 2019, Dalakouras et al. 2020). For instance, spray application of *Brassica oleracea* and *Brassica alboglabra* leaves with siRNAs targeting the acetylcholine esterase genes AchE1 led to significant mortality of diamondback moth, *Plutella xylostella*, under both laboratory conditions and in the field (Gong et al. 2013). Similarly, foliar application of dsRNA targeting the *Leptinotarsa decemlineata actin* gene resulted in significant control of Colorado potato beetle on potato plants (San Miguel and Scott 2016). The result showed that dsRNA applied on potato leaves was stable for at least four weeks under greenhouse conditions (San Miguel and Scott 2016).

While a considerable amount of data have shown that SIGS can control insect pests on crop plants (Zotti and Smaghe 2015, Cagliari et al. 2019, Dalakouras et al. 2020), considerable variation in efficacy was reported. The variation in efficacy is plausibly due to the differences in the feeding behaviour of insects. For instance, pests belonging to orders Coleoptera and Lepidoptera are relatively less susceptible to external dsRNA, possibly because lepidopterans confine the ingested dsRNA to endocytic compartments and hemipterans release nucleases into the plant tissue before sucking (Lomate and Bonning 2016, Shukla et al. 2016). Finding suitable delivery methods will be therefore crucial to improve the efficacy of SIGS, as after ingestion dsRNA must survive the degradation by salivary nucleases in the midgut or hemolymph of insect pests. In recent years, various options such as chitosan nanoparticles, cationic core-shell nanoparticles, and guanlylated polymers have been explored to develop stable insecticidal dsRNA formulations (Joga et al. 2016, Vélez and Fishilevich 2018). In addition, understanding the mechanisms underlying the systemic spread of ingested dsRNAs to distant cells will further enhance the application of SIGS for insect pest control.

SIGS to control viral pathogens

Topical application of virus-specific dsRNAs to protect plants against plant viruses has been demonstrated (Mitter et al. 2017a, Dalakouras et al. 2020, Das and Sherif 2020). This was first shown against *Pepper mild mottle virus* (PMMoV), *Alfalfa mosaic virus* (AMV) and *Tobacco etch virus* (TEV) where mechanical application of dsRNA derived from viral sequences targeting replicase genes attenuated viral infections of the respective host plants (Tenllado and Díaz-Ruiz 2001). Since then, more investigations have reported external application of dsRNA to confer viral resistance in various host plants and against a plethora of viruses (Mitter et al. 2017a, Dubrovina and Kiselev 2019, Dalakouras et al. 2020, Das and Sherif 2020).

dsRNA persistence and uptake are major considerations

Despite the success of SIGS in controlling fungal and oomycete plant diseases, its application is limited against certain phytopathogenic fungi. Furthermore, the determining factors for effective SIGS against a broad range of plant pathogens still remain to be determined, such as efficacy of exogenous RNA uptake by these organisms. For example, a recent study by Qiao et al. (2021) reported a varied level of dsRNA uptake among the pathogenic fungi such as *Aspergillus niger*, *B. cinera*, *S. sclerotiorum*, *Verticillium dahliae* (Ascomycota), *R. solani* (Basidiomycota), and the mycoparasitic fungus

Trichoderma virens (Qiao et al. 2021). Moreover, a study on *Zymoseptoria tritici* (a major fungal pathogen of wheat) and *Colletotrichum gloeosporioides* (causal agent of anthracnose), showed that these pathogens were incapable of taking up exogenous dsRNA (Kettles et al. 2019, Qiao et al. 2021). The underlying mechanisms associated with variations in RNA uptake remain unknown, but inherent differences including variation in cell wall and plasma membrane composition between different fungal species are hypothesized to be major factors. As efficient dsRNA uptake is vital for successful application of SIGS in plant disease control, investigating dsRNA uptake across a wide range of fungal pathogens is a critical next step in the development of this technology (Qiao et al. 2021).

Despite the potential use of dsRNA in crop protection and improvement, the short-term stability of this type of molecule in the environment is one of the main constraints to practical application. The stability of dsRNA can be increased by developing RNA formulations with nanoparticles that protect the dsRNA/sRNA from degradation (Mitter et al. 2017b). Mixing of a double-layered hydroxide (LDH) nanoparticle with dsRNA molecules (BioClay) resulted in protection of tobacco plants from virus infection for up to 20 days compared with five to seven days using naked dsRNA (Mitter et al. 2017b, Mitter et al. 2017a). Similarly, a preceding application (five days before exposure to aphids) of tobacco and cowpea plants with BioClay formulated dsRNA targeting the coat protein from *Bean common mosaic virus* inhibited BCMV infection due to aphid-mediated virus transmission (Worrall et al. 2019). Chitosan and carbon dot based nanoparticles have also been used to enhance self-life and uptake of dsRNA (Zhang et al. 2010, Schwartz et al. 2020, Wang et al. 2020). Nanoparticle based formulations promise enhanced delivery of exogenous dsRNAs by increasing their stability and uptake. However, the main concern associated with nanoparticle-based formulation is that they are quite expensive or difficult to synthesize. The efficacy of topically applied dsRNAs can also be affected by the concentration and length of dsRNAs (Dubrovina et al. 2019). Further research in these areas could be helpful for the application and delivery of dsRNAs for RNAi-based plant disease control and improved crop performance.

OUTLOOK

The growing world population is an important driver for increased food demand that is threatened by production constraints including low yield and diseases. However plant breeding is expensive and time consuming, and also a limited number of traits are accessible to genetic transformation. New traits are sometimes required in a short timeframe to deal with threats such as environmental change, new applications, and emerging pests and pathogens, so urgent solutions are needed. The tools for these solutions need to deliver increased food production, but in a more sustainable manner, with less detrimental environmental impact, and consumer acceptance. SIGS has potential to be used for manipulating plant traits to increase agronomic performance and control plant disease development, but more research is needed for development of optimum methods for dsRNA stability, delivery, and reducing the cost for preparation of dsRNA formulation. Furthermore, risk assessments for potential impacts of SIGS on plant health, human health, and the environment need to be undertaken. SIGS has potential to be integrated into integrated pest management (IPM) strategies aiming to both increase crop yields and pest control. Topical application of dsRNAs to modulate crop traits also suggests that dsRNAs may be applied singly or in combinations, allowing 'tuning' of crop traits to best fit the growing conditions. SIGS may be a valuable addition to the precision farming toolbox in the near future.

ACKNOWLEDGEMENTS

This work has been supported by FORMAS (2019-01316), The Swedish Research Council (2019-04270), NKJ-SNS – Dialogue Biocontrol network (NKJ-SNS 06), Carl Tryggers Stiftelse för Vetenskaplig Forskning (CTS 20:464), The Crafoord foundation (20200818), Partnerskap Alnarp (1317/Trg,VO/2020, 1353/VO/2021) and Alnarp stipendiekommitténs. MD was supported by FORMAS (2018-01420) and Carl Tryggers foundation (19:82). SCW acknowledges financial support from the Scottish Government Rural and Environment Science and Analytical Services Division (RESAS). The authors have no conflicts of interest to declare.

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