ADVANCES AND CHALLENGES OF RNAi BASED TECHNOLOGIES FOR PLANTS

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ADVANCES AND CHALLENGES OF RNAi BASED TECHNOLOGIES FOR PLANTS

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EDITORIAL
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Editorial: Advances and Challenges of RNAi Based Technologies for Plants

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Keywords: gene silencing (siRNA), cross kingdom, pathogen control, biosafety, sustainable agriculture

Editorial on the Research Topic

Advances and Challenges of RNAi Based Technologies for Plants

In this Research Topic, the focus is on recent research and developments in the use of RNAi techniques to protect plants. Cross kingdom effects of RNA expressed in plants primarily for pathogen control are discussed by Schaefer et al. in *Cross-Kingdom RNAi of Pathogen Effectors Leads to Quantitative Adult Plant Resistance in Wheat*.

Pest as well as pathogen control is considered in *Plant miRNA Cross-Kingdom Transfer Targeting Parasitic and Mutualistic Organisms as a Tool to Advance Modern Agriculture* by Gualtieri et al..

Bachman et al. consider aspects of their research on dsRNA expressed in maize/corn for root worm beetle control in *Sequence–Activity Relationships for the Snf7 Insecticidal dsRNA in Chrysomelidae.*

The efficiency and efficacy of a system for lepidopteran control is discussed in *Comparative Analysis of Chitin SynthaseA dsRNA Mediated RNA Interference for Management of Crop Pests of Different Families of Lepidoptera* by Rana et al..

The potential for plants to express RNA viruses which can infect both plants and insects but which are pathogenic in their insect vectors is considered as a strategy for vector insect control in *The Use of Engineered Plant Viruses in a Trans-Kingdom Silencing Strategy Against Their Insect Vectors* by Kolliopoulou et al..

The biosafety assessments of these and other host induced gene silencing (HIGS) systems are discuss in *Biosafety of GM Crop Plants Expressing dsRNA: Data Requirements and EU Regulatory Considerations* by Arpaia et al..

Exogenous application of dsRNA (SIGS) for pest and pathogen control is an attractive alternative but is confronted with several problems related to application, uptake, persistence, and efficacy. In *Barriers to Efficient Foliar Uptake of dsRNA and Molecular Barriers to dsRNA Activity in Plant Cells*, these are discussed by Bennett et al..

Uslu et al. describe studies of high pressure spraying to improve efficacy in *High-Pressure-Sprayed Double Stranded RNA Does Not Induce RNA Interference of a Reporter Gene.*

In relation to virus control, Tabein et al. discuss *The Induction of an Effective dsRNA-Mediated Resistance Against Tomato Spotted Wilt Virus by Exogenous Application of Double-Stranded RNA Largely Depends on the Selection of the Viral RNA Target Region.*

Research on control of a major insect pest is reviewed in *Validating the Potential of Double-Stranded RNA Targeting Colorado Potato Beetle Mesh Gene in Laboratory and Field Trials* by Petek et al..

These papers give an insight into the many areas of research on the potential applications for RNAi for crop protection. Some applications are being commercialized while others are

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at earlier stages of research (Taning et al., 2020a). The Editors believe that applications of HIGS and SIGS can make major contributions to sustainable and integrated crop protection and support "Green" systems for intensification of agricultural production as advocated in UN Sustainability Development Goals and by FAO, EU, and many national governments (Mezzetti et al., 2020; Taning et al., 2020b). The editors would like to acknowledge the opportunity provided by Frontiers for publishing these papers and thank the authors for their contributions. The editors and many of the authors are members of the iPlanta COST action CA 15223 and further information on iPlanta and publications produced by iPlanta are available at https://iplanta.univpm.it/.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Cross-Kingdom RNAi of Pathogen Effectors Leads to Quantitative Adult Plant Resistance in Wheat

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Cross-kingdom RNA interference (RNAi) is a biological process allowing plants to transfer small regulatory RNAs to invading pathogens to trigger the silencing of target virulence genes. Transient assays in cereal powdery mildews suggest that silencing of one or two effectors could lead to near loss of virulence, but evidence from stable RNAi lines is lacking. We established transient host-induced gene silencing (HIGS) in wheat, and demonstrate that targeting an essential housekeeping gene in the wheat powdery mildew pathogen (*Blumeria graminis* f. sp. *tritici*) results in significant reduction of virulence at an early stage of infection. We generated stable transgenic RNAi wheat lines encoding a HIGS construct simultaneously silencing three *B.g. tritici* effectors including *SvrPm3*$^{a1/f1}$, a virulence factor involved in the suppression of the *Pm3* powdery mildew resistance gene. We show that all targeted effectors are effectively downregulated by HIGS, resulting in reduced fungal virulence on adult wheat plants. Our findings demonstrate that stable HIGS of effector genes can lead to quantitative gain of resistance without major pleiotropic effects in wheat.

Keywords: cross-kingdom RNAi, ck-RNAi, host-induced gene silencing, HIGS, ribonuclease-like effectors, effectors, *Blumeria graminis*, wheat

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INTRODUCTION

RNA interference (RNAi) is a biological process in which small non-coding RNAs (sRNAs) are employed to selectively downregulate gene expression at the transcriptional or post-transcriptional level. Post-transcriptional gene silencing is a highly regulated mechanism relying on a cohort of proteins that direct gene silencing based on sequence complementarity between the sRNA and a target mRNA (Gheysen and Vanholme, 2007; Shabalina and Koonin, 2008). Plants encode functionally diverse populations of regulatory sRNAs which include microRNAs (miRNAs) and short interfering RNAs (siRNAs). While miRNAs correspond to 20–22-nt sequences typically derived from imperfect RNA hairpin structures, siRNAs refer to 20–24-nt sequences typically processed from long double-stranded RNA (dsRNA) precursors (Borges and Martienssen, 2015; D'Ario et al., 2017). Both siRNAs and miRNAs are processed from dsRNA precursors by the ribonuclease Dicer, and both can regulate gene expression. RNAi participates in the regulation of diverse biological processes including plant immunity (Brant and Budak, 2018; Deng et al., 2018), and several siRNAs and miRNAs have been described as important players in plant defense against viruses, bacteria, and fungi (Hua et al., 2018; Rosa et al., 2018; Muhammad et al., 2019).

Small non-coding RNAs can be expressed in a tissue-specific or stage-specific manner (Mohorianu et al., 2011; Mao et al., 2012) and correspond to highly mobile molecules that can travel from cell-to-cell (short-range) or systemically (long-range) in the plant (Dunoyer et al., 2013). There is increasing evidence demonstrating that sRNAs are also mobilized in bi-directional exchanges between plants and their parasites, thus providing a basis for cross-kingdom RNAi (ck-RNAi) as a plant defense mechanism (Knip et al., 2014; Hua et al., 2018). Prominent examples include miRNAs from the parasitic plant *Cuscuta campestris*, and siRNA *Bc-siR37* from the fungal pathogen *Botrytis cinerea*, which act as virulence factors downregulating several *Arabidopsis thaliana* genes involved in immunity (Weiberg et al., 2013; Wang et al., 2017; Shahid et al., 2018). This phenomenon can be partially explained by the fact that the three proteins that are important for RNAi, an argonaute protein, a dicer-like (DCL) protein, and a RNA-dependent RNA polymerase, are commonly found across eukaryotes (Shabalina and Koonin, 2008). While experimental evidence for the transfer of sRNAs from fungi to their host has been provided recently (Weiberg et al., 2013; Wang et al., 2017), sRNA mobility from plant to fungi has been used for host-induced gene silencing (HIGS) for almost a decade (Nowara et al., 2010; Qi et al., 2019). In plant-pathogen interactions, HIGS can be based on the uptake of exosome-like vesicles, containing transgene-derived siRNAs from the host (Cai et al., 2018).

In the obligate biotrophic cereal powdery mildew *formae speciales* (*Blumeria graminis* ff. spp.), HIGS is an important tool for functional genomics (Nowara et al., 2010; Pliego et al., 2013). In this system, successful infection is typically characterized by the formation of a highly specialized feeding structure called the haustorium, shortly after an appressorium-mediated penetration of the plant cell wall (Praz et al., 2018). The haustorium is a poorly understood membrane invagination that develops inside the host epidermal cell, and serves as a basis for the emergence of a network of strictly epiphytic hyphae which can form secondary appressoria (Kwaaitaal et al., 2017). The latter will then infect the same cell or the surrounding cells and form additional haustoria. These invasive structures are the sites of molecular exchange between *B. graminis* and its hosts. In particular, it was demonstrated that haustoria are the main interface for the delivery of candidate small secreted effector proteins (CSEPs) which promote pathogen virulence (Bourras et al., 2018).

The *B. graminis* genomes encode one of the largest repertoires of effectors in fungi, consisting of over 800 annotated proteins (Bourras et al., 2018; Frantzeskakis et al., 2018; Müller et al., 2019). Transient HIGS has been successfully used to functionally validate such candidate virulence factors in barley powdery mildew (*B. graminis* f. sp. *hordei*), leading to the identification of 21 *bona fide* effectors involved in host penetration or promoting haustorium formation (Zhang et al., 2012; Pliego et al., 2013; Ahmed et al., 2015, 2016). Of these, HIGS of the *B.g. hordei* effectors BEC1054 and BEC1011 resulted in a 60–70% reduction of haustorium formation, suggesting that some *B. graminis* CSEPs are probably essential for virulence (Pliego et al., 2013). Further molecular and biochemical characterization of these HIGS-assayed effectors revealed that they are interacting with host proteins involved in plant immunity (reviewed in Bourras et al., 2018). Relevant examples include CSEP0055 which interacts with the barley pathogenesis-related protein PR17c involved in resistance to penetration (Zhang et al., 2012), and the ribonuclease-like CSEP0064/BEC1054 which targets several barley proteins implicated in defense responses (Pennington et al., 2016, 2019). Based on these results, it has been suggested that HIGS of a few essential *B. graminis* effectors via stable host transformation could lead to a significant to permanent loss of pathogen virulence.

In the recent decade, ck-RNAi has emerged as a possible approach to control diseases in crops (Koch and Kogel, 2014; Hua et al., 2018). Several studies have described the use of stable HIGS in plants to confer resistance to fungal pathogens (Nowara et al., 2010; Koch et al., 2013; Chen et al., 2016; Panwar et al., 2017; Guo et al., 2019). Based on experimental evidence from transient assays in barley, a preliminary study using stable transgenics showed that HIGS of the *B.g. hordei 1,3-β-glucanosyl-transferase (GTF1)* gene, resulted in a decrease of fungal virulence on barley T1 seedlings (Nowara et al., 2010). So far, fungal house-keeping genes and pathogenesis-related genes were the primary gene targets for stable HIGS. In *B. graminis*, the discovery of *bona fide* effectors such as BEC1054 and BEC1011 raised the question whether stable silencing of such CSEPs would be equivalent to silencing an essential gene. In the BEC1054 and BEC1011 HIGS assays, single barley epidermal cells were biolistically transformed with a HIGS construct encoding a long dsRNA sequence, perfectly complementary to a segment of the effector mRNA sequence (Pliego et al., 2013). *B.g. hordei* virulence was scored microscopically at the haustorial stage, based on the number of formed haustoria, normalized to the number of penetration attempts (Pliego et al., 2013). Because of the technical limitation of this strategy, it was not possible to quantify target gene expression or to assess macroscopic phenotypes. In this context, HIGS of effectors using stable transgenic lines would be an opportunity to understand the impact of ck-RNAi on pathogen virulence, based on accurate, whole tissue assessment of target gene silencing in relation to plant resistance.

Here, we demonstrate the applicability of HIGS in *B.g. tritici* based on the silencing of the *β2-tubulin (β2-tub)* housekeeping gene. We also present the generation, selection, and characterization of stable wheat lines expressing a HIGS construct targeting the suppressor of avirulence gene $SvrPm3^{a1/f1}$. This wheat powdery mildew effector is involved in the suppression of several allelic *Pm3* resistance gene variants in wheat (Bourras et al., 2015, 2019; Parlange et al., 2015). We also show that $SvrPm3^{a1/f1}$ is expressed at significantly lower levels in *B.g. tritici* colonies growing on the HIGS lines as compared to the non-transgenic control. Consistent with *in silico* prediction of possible targets in the pathogen, two additional members of the $SvrPm3^{a1/f1}$ effector gene family sharing sequence homology are also downregulated by HIGS. Finally, based on extensive phenotypic characterization of infected wheat material from laboratory and semi-field experiments, we show that HIGS of $SvrPm3^{a1/f1}$ results in a quantitative gain of resistance against *B.g. tritici*.

RESULTS

Establishing HIGS of *B.g. tritici* Genes in Wheat

Host-induced gene silencing of *B. graminis* genes was originally established in barley (Nowara et al., 2010). Here, we aimed at establishing HIGS in wheat for targeting *B.g. tritici* genes and at comparing HIGS efficacy in wheat and barley powdery mildew. The *B. graminis β2-tub* gene, an essential housekeeping gene and a canonical fungicide target (Zhou et al., 2016; Vela-Corcía et al., 2018), was selected as target gene for this assay. There is no *β1-tub* gene in *B. graminis*, indicating that transcriptional knockdown of the single-copy *β2-tub* gene is highly relevant for assessing the impact of HIGS on fungal virulence. Due to high sequence similarity between *β2-tub* genes across *B. graminis* ff. spp., the sequence from *B.g. hordei* was used as a template to design a HIGS construct capable of silencing this gene in *B.g. tritici* and *B.g. hordei* (**Figure 1A**). The silencing construct is based on a 140-nt segment of exon 6 from the *B.g. hordei β2-tub* gene (**Supplementary File 1**). The *β2-tub*-RNAi sequence used in this study was analyzed for possible off-targets in both *formae speciales* and their host species wheat and barley using the si-Fi software, which predicted no off-targets in either of the analyzed *B. graminis* and host genomes (Lück et al., 2019).

Wheat and barley leaves were biolistically co-transfected with a GUS reporter plasmid and the *β2-tub*-RNAi plasmid and infected with *B.g. tritici* and *B.g. hordei*, respectively. Fungal virulence was assessed microscopically 2 days post infection (dpi) based on successful haustoria formation (haustorium index, see section "Materials and Methods"). The empty pIPKTA30 vector was used as negative control. Results from three independent biological replicates showed statistically significant reduction of fungal virulence, corresponding to a decrease of the haustorium index by −42% in *B.g. tritici* and −43% in *B.g. hordei* as compared to the negative control (**Figure 1B**). We observed no effect on hyphae formation in the successful colonies. We conclude that transient HIGS of an essential housekeeping gene in *B.g. tritici* and *B.g. hordei* leads to very similar, quantitative loss of virulence in both *formae speciales*. Thus, HIGS studies are feasible in wheat using the same vector design and phenotyping strategy. We suggest that targeting of a housekeeping gene, such as *β2-tub*, can be used as a technical reference for assessing the relative efficiency of HIGS in future assays.

Generation of Stable *SvrPm3$^{a1/f1}$*-RNAi Wheat Lines

We designed a new HIGS construct targeting the *B.g. tritici* effector *SvrPm3$^{a1/f1}$*. We selected a 500-nt fragment of the *SvrPm3$^{a1/f1}$* mRNA, which includes a segment of the 5′UTR and both exons excluding the last two codons (**Figure 2A**). Off-target analysis using the si-Fi software (Lück et al., 2019) predicted four putative siRNAs that map to a single 30-nt off-target locus in a putative pseudogene on the wheat chromosome 6A. However, these siRNAs are predicted to be inefficient in directing silencing (**Supplementary Note 1** and **Supplementary File 2**). Off-target analysis using the *B.g. tritici* genome identified two additional target genes, *Bgt_Bcg-6* and *Bgt_Bcg-7* (**Figure 2A** and **Supplementary Table 1**). Consistent with the off-target predictions, both effector genes share nucleotide homology with *SvrPm3$^{a1/f1}$*, with *Bgt_Bcg-6* being identical in the 5′ first 120-nt of the HIGS construct (**Supplementary Figure 1**). *Bgt_Bcg-6* and *Bgt_Bcg-7* both belong to the *SvrPm3$^{a1/f1}$* effector gene family and are the closest homologs of the target effector (Parlange et al., 2015; Müller et al., 2019). Based on these results, we conclude that *SvrPm3$^{a1/f1}$*, *Bgt_Bcg-6*, and *Bgt_Bcg-7* are potent targets of the HIGS construct designed for the generation of stable transgenics.

We cloned the selected *SvrPm3$^{a1/f1}$*-RNAi sequence into the pIPKb007 vector, in which sense and antisense sequences are separated by a wheat *RGA2* intron allowing hairpin formation (Himmelbach et al., 2007). Transgene expression is controlled by the maize ubiquitin promoter and the CaMV 35S terminator (**Supplementary Figure 2**). The spring wheat cultivar Bobwhite SH 98 26 (BW) was co-transformed with the linearized HIGS construct and the *phosphomannose isomerase* gene (*Pmi*) as a selection marker by particle bombardment. We obtained 138 independent T0 transformants (events), which were propagated to the T1 generation. Macroscopic scoring of leaf coverage by *B.g. tritici* colonies in T1 seedling leaves, did not allow us to discriminate between the segregating transgenic and non-transgenic T1 progeny. We hypothesized that our assay was not sensitive enough for scoring quantitative effects. We therefore aimed at selecting four events with the following criteria (i) 1–2 copies of the transgene can be detected by southern blot (**Supplementary Figure 3**), (ii) both the sense and antisense copy of the HIGS construct can be amplified by PCR indicating that the complete HIGS transgene is integrated into the genome, (iii) events are homozygous, (iv) transgene expression can be detected by RT-PCR, and (v) no major pleiotropic effects on plant development resulting from tissue culture can be observed. Based on this combination of criteria, we could confidently select four events, #51, #59, #67, and #68. All are single copy events, except event #67 which contains two genetically linked transgene copies.

We conclude that the generation of stable transgenic HIGS wheat plants can be restricted by the following challenges: First, T1 plants are difficult to phenotype for quantitative, small effects in which replication and scalability are key for inferring statistically significant differences. Second, the hairpin secondary structure inherent to the design of the HIGS construct complicates the molecular detection of transgene copies, both at the DNA and cDNA levels, using PCR-based methods. Considering these technical limitations and our selection process in which only events that pass all selection criteria are further characterized, we suggest that we are probably underestimating the proportion of successful transformation events.

The *SvrPm3$^{a1/f1}$*-RNAi Transgene Is Constitutively Expressed

In recent work by Wang et al. (2018), it was demonstrated that standard RT-qPCR assays are not suitable for the quantification of dsRNA transcripts produced by RNAi transgenes. Standard protocols for mRNA quantification by RT-qPCR primarily

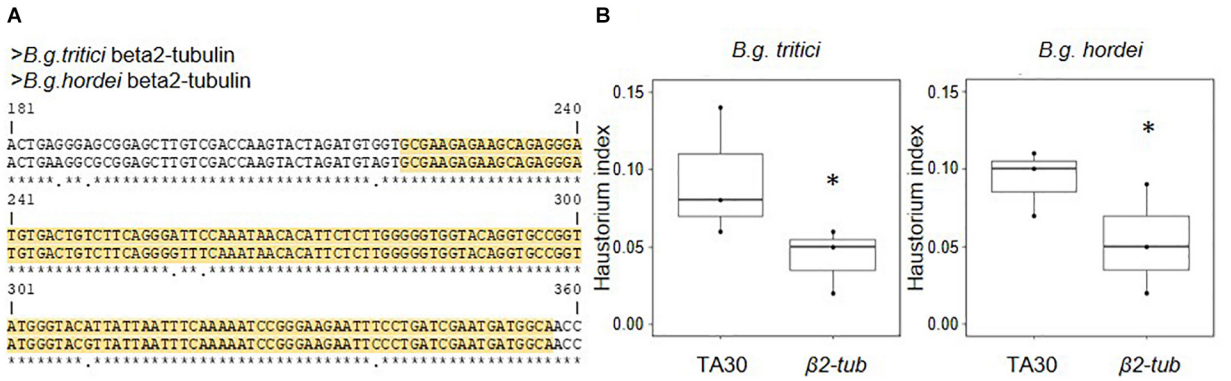

FIGURE 1 | Transient HIGS of fungal *β2-tubulin* (*β2-tub*) reduces virulence in *B.g. hordei* and *B.g. tritici*. The *β2-tub*-RNAi construct was transiently expressed in barley and wheat epidermal cells, which were infected with *B.g. hordei* and *B.g. tritici*, respectively. **(A)** Alignment of the *B.g. tritici* and *B.g. hordei β2-tub* gene sequences. Only the fragment including the donor sequence for the *β2-tub*-RNAi construct (yellow) is depicted. The *B.g. hordei* sequence was used to construct the *β2-tub*-RNAi plasmid. **(B)** Effect of the *β2-tub*-RNAi construct on virulence in both *formae speciales*. The effect on fungal virulence was measured 2 days post infection, by scoring the ratio of successful over total infection attempts (haustorium index). Results were compared to the empty vector control pIPKTA30 (TA30) using a proportion test. *$p < 0.05$.

quantify aberrant single-stranded RNA (ssRNA) transcripts, which are inefficient in directing gene silencing (Wang et al., 2018). To further characterize the four selected events, we adapted the protocol described by Wang et al. (2018) and quantified dsRNA and ssRNA transcripts in non-infected seedlings. Consistent with previous findings by Wang et al. (2018), our assays show that expression of dsRNA and ssRNA transcripts are not correlated, thus further corroborating the importance of validating dsRNA expression from RNAi transgenes. But most importantly, we found that dsRNA transcripts are generally more abundant than ssRNA transcripts thus demonstrating that the HIGS transgene is giving rise to a proper template for siRNA biogenesis (**Figure 2B**). Together this data demonstrate that all four events contain a full, transcriptionally active copy of the *SvrPm3$^{a1/f1}$*-RNAi transgene, which is constitutively expressed in absence of *B.g. tritici* infection.

We conclude that our selection procedure, based on careful molecular validation of transgene integrity at the DNA and RNA levels, led to the identification of relevant events, constitutively expressing the HIGS transgene. In this context, we propose that this material is ideal for assessing the effect of stable HIGS on mildew virulence at different stages of wheat development.

The *SvrPm3$^{a1/f1}$*-RNAi Transgene Reduces Target Effector Gene Expression

The *B.g. tritici* effectors *SvrPm3$^{a1/f1}$*, *Bgt_Bcg-6*, and *Bgt_Bcg-7* are equally valid potential targets of the *SvrPm3$^{a1/f1}$*-RNAi transgene (**Figure 2A**). To test whether the three targets are effectively silenced, we quantified mRNA expression from *B.g. tritici* infected seedlings at three dpi, a time point corresponding to the haustorial stage. Previous studies have shown that gene expression levels of *SvrPm3$^{a1/f1}$* are the highest during haustorium formation (Bourras et al., 2015, 2019; Praz et al., 2018). Similarly, several members of the *SvrPm3$^{a1/f1}$* effector gene family, including *Bgt_Bcg-6* and *Bgt_Bcg-7*, are induced at that same stage (**Supplementary Figure 4**) (Praz et al., 2018; Bourras et al., 2019), suggesting that this time point is particularly appropriate for the quantification of mRNA levels of all three effectors, and to assess HIGS efficiency. Transgenic events and wildtype BW, were infected with the *B.g. tritici* isolate Bgt_JIW2, which shows intermediate levels of expression of *SvrPm3$^{a1/f1}$* (Bourras et al., 2015, 2019). Primer design for specific amplification of the respective fungal *SvrPm3$^{a1/f1}$*, *Bgt_Bcg-6*, and *Bgt_Bcg-7* mRNAs was challenging, as the sequences of the three effectors are rather similar, and since the transgene corresponded to almost the complete *SvrPm3$^{a1/f1}$* coding sequence. Therefore, RT-qPCR primers could only be designed on the 3′UTR to discriminate between the transgene, *SvrPm3$^{a1/f1}$* and *Bgt_Bcg-6* in particular. Specificity was verified by Sanger sequencing of amplicons from infected leaf material and non-infected controls.

The mRNA levels of the target genes *SvrPm3$^{a1/f1}$* and *Bgt_Bcg-6* were significantly reduced as compared to the wildtype BW control in all events except #67 (**Figures 2C,D**). Interestingly, silencing of *Bgt_Bcg-6* was consistently similar and sometimes even more effective than silencing of *SvrPm3$^{a1/f1}$* (**Figures 2C,D**). For *Bgt_Bcg-7*, we observed a less pronounced but yet significant reduction of mRNA levels in events #51 and #59, but not in event #68 (**Figure 2E**). Here, differences in target gene silencing are in agreement with the differences in sequence similarity between the three effectors. In particular, we propose that the 120-nt segment that is identical between *SvrPm3$^{a1/f1}$* and *Bgt_Bcg-6* is possibly responsible for the silencing of both genes, while lower sequence identity between these two effectors and *Bgt_Bcg-7* in that same segment is likely responsible for reduced HIGS efficiency on the latter. All effector targets considered, target gene downregulation ranged from −16% (*Bgt_Bcg-7* on #51) to −58% (*Bgt_Bcg-6* on #51), thus

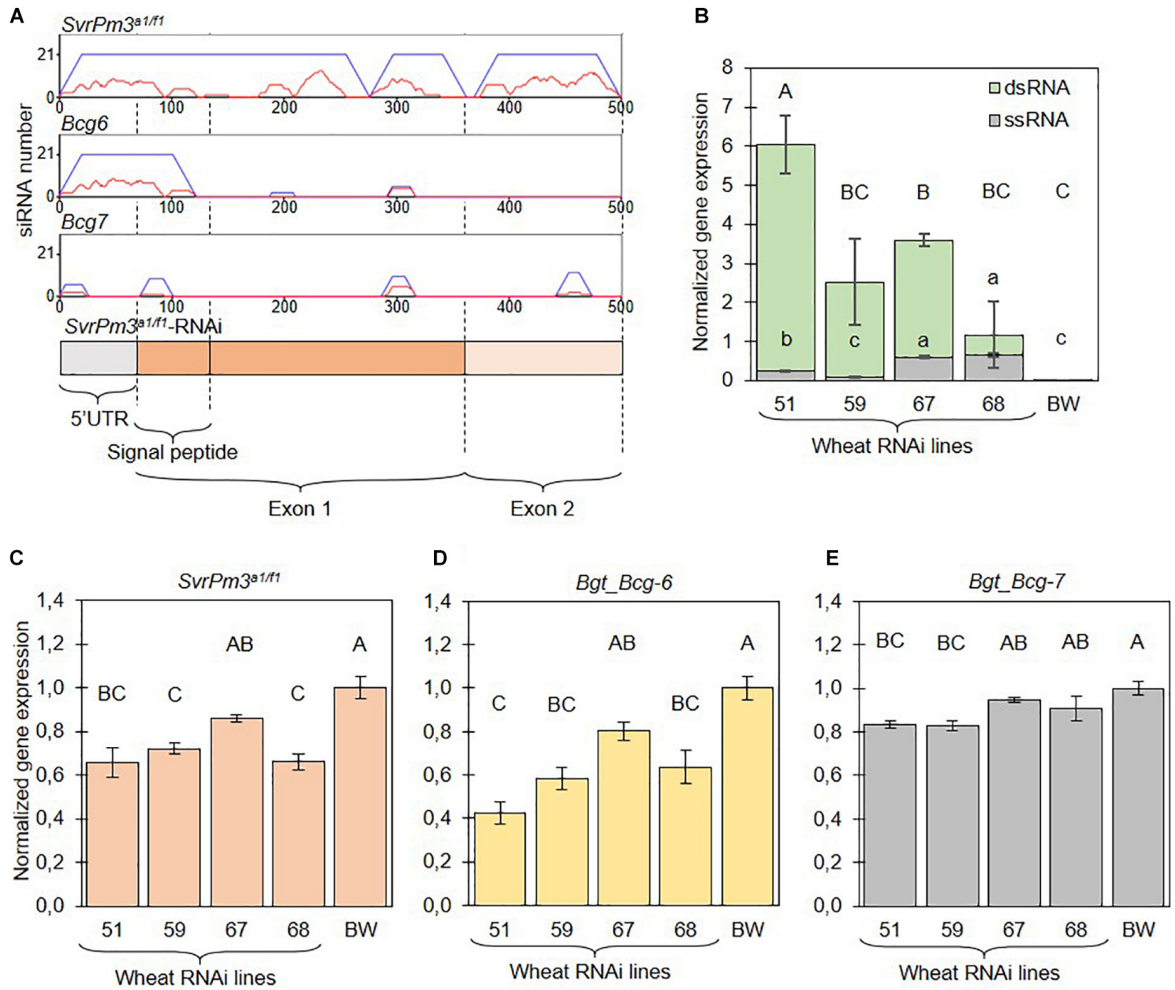

FIGURE 2 | The *SvrPm3*$^{a1/f1}$-RNAi transgene is constitutively expressed in transgenic wheat lines and silences three *B.g. tritici* effectors. **(A)** si-Fi software predictions of *SvrPm3*$^{a1/f1}$-RNAi transgene-derived siRNAs that are efficient (red lines) or inefficient (blue lines) in directing silencing of the three putative target effectors *SvrPm3*$^{a1/f1}$, *Bgt_Bcg-6*, and *Bgt_Bcg-7*. On the y-axis is the number of siRNAs that bind to a given locus on the x-axis. **(B)** Quantification of transgene-derived ssRNA and dsRNA transcripts from non-infected samples. **(C–E)** Quantification of target gene transcripts from infected samples. Samples were collected 3 days post infection. Error bars represent the standard error. Statistical difference was tested using ANOVA **(B:**dsRNA,**D,E)** or Welch ANOVA **(B:**ssRNA,**C)**, according to the data distribution and variance. Different letters indicate statistical significance.

molecularly demonstrating that the HIGS transgene is functional in the events #51, #59 and #68, and does mediate ck-RNAi.

We conclude that stable wheat lines constitutively expressing a HIGS transgene can effectively mediate target gene silencing in *B.g. tritici*. We also conclude that the 5′ 120nt of the HIGS construct are probably sufficient for achieving high levels of target silencing, which could also be combined with other RNAi sequences for simultaneous silencing of entire gene families to potentially strengthen the impact of HIGS.

Effector Silencing Results in an Event-Specific Quantitative Loss of Virulence

Based on the functional validation of target gene silencing, the impact of simultaneous silencing of three effectors, *SvrPm3*$^{a1/f1}$, *Bgt_Bcg-6*, and *Bgt_Bcg-7*, on *B.g. tritici* virulence was assessed at different developmental stages (**Figures 3A–D**). First, we infected leaf segments from seedlings of the transgenic events and the wildtype control with the *B.g. tritici* isolate Bgt_JIW2 using a low-density inoculum, and quantitatively scored virulence based on the infected leaf area at six dpi. In this assay, we observed no reduction of virulence of *B.g. tritici* on seedlings (**Supplementary Figure 5**). We hypothesized that the effect of HIGS is best observed at the haustorial stage when the target effectors, in particular *SvrPm3*$^{a1/f1}$ are transcriptionally active (Bourras et al., 2015, 2019; Praz et al., 2018). So, we prepared infected leaf segments for microscopy to score virulence at two dpi. Interestingly, we found a significant reduction in the haustorium index of −46% in event #68 compared to the wildtype (**Figure 3E**). The effect was stronger on the subgroup of immature haustoria, which are not yet showing the fully

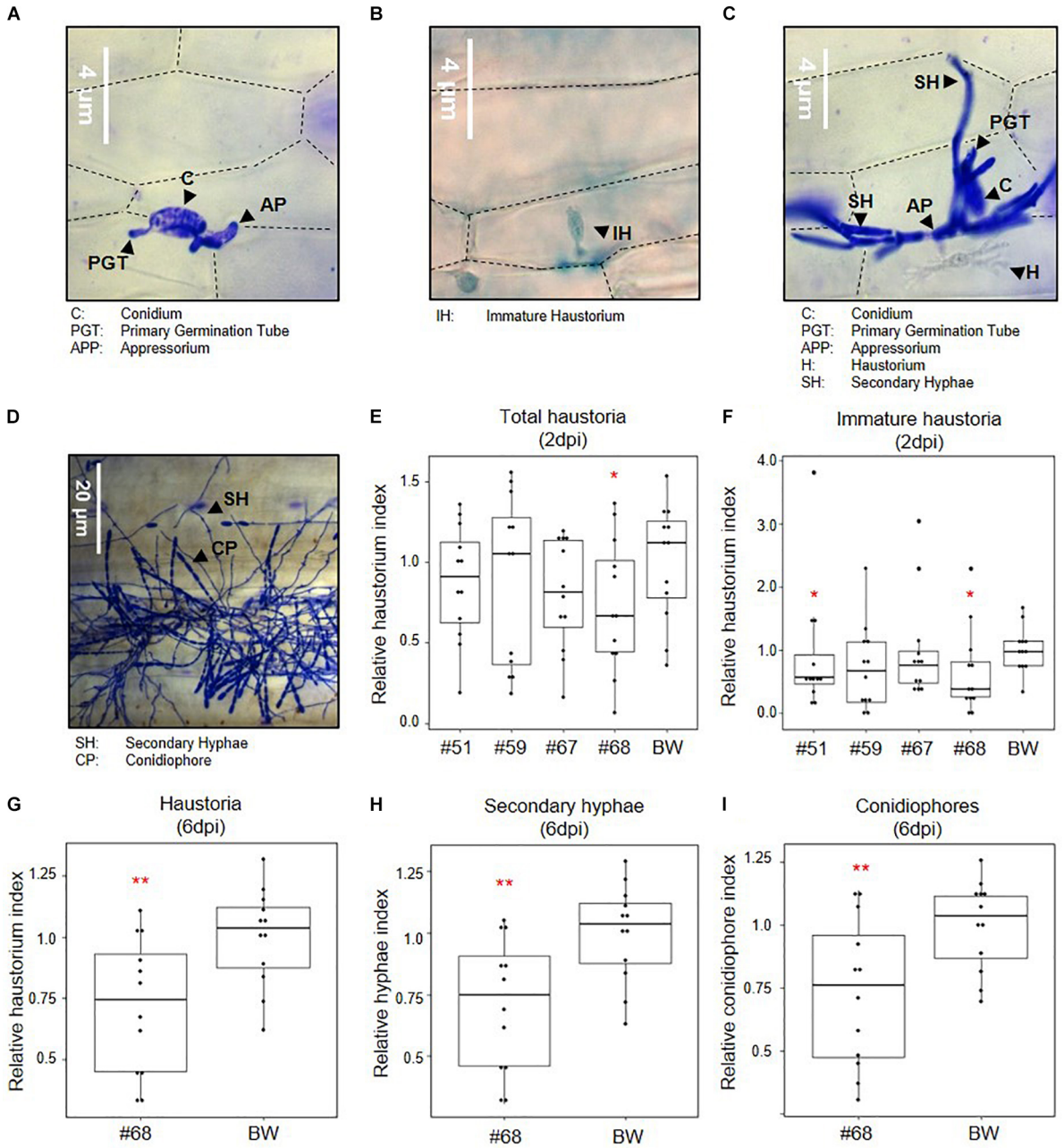

FIGURE 3 | Effect of effector gene silencing on infection success. Seedlings of *SvrPm3*$^{a1/f1}$-RNAi wheat lines were infected with *B.g. tritici*, and virulence was scored at two- and six-days post infection (dpi). All data is normalized to the wildtype control Bobwhite (BW). **(A–D)** Representative pictures of *B.g. tritici* developmental stages. **(A)** A spore has formed an appressorium, but fails at infection. **(B)** An immature haustorium. **(D)** A successful colonization at ca. two dpi with a mature haustorium and secondary hyphae. **(D)** A successful *B.g. tritici* colony at six dpi with mature conidiophores. **(E)** Total haustorium index at two dpi. **(F)** Immature haustorium index at two dpi. **(G)** Haustorium index at six dpi. **(H)** Hyphae index at six dpi. **(I)** Conidiophore index at six dpi. Pairwise comparison of transgenic events to the wildtype BW was carried out using a one-sided *t*-test **(E,G–I)** or a Wilcoxon rank sum test **(F)**, according to the data distribution and variance. $^{*}p < 0.05$; $^{**}p < 0.01$.

branched structure typical of mature haustoria (**Figures 3A–C**). For these, we found a significant reduction in the haustorium index of −41% in event #51 and −60% in event #68 compared to the wildtype (**Figure 3F**). The reduction of haustorium indices in the events #59 and #67 were not significant (**Figures 3E,F**). These results suggest that silencing of the three target effectors impairs *B.g. tritici* virulence at the haustorial stage in an event-specific, quantitative manner. Next, we microscopically assessed *B.g. tritici* development at four and six dpi on line #68, as it shows the strongest effect at the haustorial stage. Here, we

scored *B.g. tritici* virulence by assessing the presence of haustoria, hyphae, and conidiophores. We found no significant differences at four dpi (**Supplementary Figures 6C–H**). However, we observed a significant reduction of the haustorium index of −30% in event #68 at six dpi compared to wildtype (**Figure 3G**), although no macroscopic differences in infected leaf area were observed in the initial seedling infection assays at this time point. Congruently, no additive effect was observed on hyphae and conidiophore formation (**Figures 3D,H,I** and **Supplementary Figures 6A,B,I**). Altogether, these results indicate that there is a consistent quantitative effect from HIGS of the three target effectors specifically impairing primary haustoria formation.

We conclude that the observed effects of HIGS on *B.g. tritici* development on seedlings, is in agreement with previous evidence suggesting that the effectors *SvrPm3*$^{a1/f1}$, *Bgt_Bcg-6*, and *Bgt_Bcg-7* are important virulence factors particularly active at the haustorial stage. We also conclude that the observed effect is quantitative and event-specific, which could be due to genomic location of the transgene and subsequent tissue-specific variations of transgene expression.

SvrPm3$^{a1/f1}$-RNAi Transgenic Wheat Shows Quantitative Adult Plant Resistance to *B.g. tritici* in Semi-Field Conditions

In a final phenotypic analysis, we characterized all events for susceptibility to *B.g. tritici* at the adult stage in a semi-field setup. Here, the plants were exposed to local climate, and the local *B.g. tritici* population, We monitored *B.g. tritici* disease development over time according to Brunner et al. (2011) and calculated the area under the disease progression curve (AUDPC). We additionally collected representative samples for each test plot of flag leaves (F0), F-1 and F-2 leaves for precise quantification of infected leaf area. F0 leaves were sampled at the beginning of flag leaf infection at 70 days post sowing (dps), concurrent with spike emergence, and a second time at the end of the *B.g. tritici* disease progression at 92 dps, concurrent with seed ripening. F-1 and F-2 leaves were sampled at 78 dps.

Area under the disease progression curves based on all plant development stages did not indicate significant differences between the transgenic events and the wildtype control (**Supplementary Figure 7A**). However, AUDPCs based on flag leaves indicated significantly lower *B.g. tritici* infection than wildtype in the three events that also show target gene silencing (#51, #59, and #68) (**Figure 4A**). AUDPC is designed to score disease development on whole plants, but in our assay, it was only informative when considering the flag leaves. Therefore, we used image-based precise quantification of infected leaf area in the representative leaf samples to further support our results. In line with the AUDPC results, we found a significant reduction of infected leaf area in the flag leaf at 70 dps compared to wildtype in the same three events (−71% for #51, −50% for #59, and −70% for #68) (**Figures 4B,D**). Consistent with the results from seedling assays, this effect decreased over time to no significant difference at 92 dps with the exception of event #68, in which a significant reduction in infected leaf area (−69%) could still be observed (**Figures 4C,E**). The effect also decreased with the age of the leaves. While the F-1 leaves show a significant reduction in infected leaf area at 78 dps for events #59 (−36%) and #68 (−60%) (**Figure 4F** and **Supplementary Figure 7B**), there was no significant effect in the F-2 leaves (**Supplementary Figures 7C,D**).

We also compared leaf area from adult leaves and took measurements of physiological traits including spike emergence and flowering time during the semi-field experiment. Additionally, we scored plant height, seed set, and seed weight per area both in the semi-field experiment and under controlled greenhouse conditions. While we acknowledge the small scale of these experiments, our results indicate that while there are no significant differences in seed weight per area in the semi-field, there is a trend toward higher seed weight per area in lines #59 and #68 which correlates with lower infected leaf area in F-1 leaves (**Figure 4G**). Under controlled greenhouse conditions, the seed weight per area was significantly higher in all three lines that showed target gene silencing and reduced infected leaf area in the semi-field (#51, #59, and #68) (**Supplementary Figure 8A**). Higher seed weight per area partially correlates with significantly higher spike number per plant in event #51 and #68 (**Supplementary Figure 8B**). We also observed that the pooled F0, F-1, and F-2 leaves of the three events showing increased adult plant resistance have significantly larger leaf area than wildtype, which could explain the increase in seed weight per area (**Supplementary Figure 8C**). We observed no meaningful differences in flowering time, plant height, or seed set neither in the semi-field nor in the greenhouse (**Supplementary Figures 8D–H,J**). But we observed a trend toward a 2- to 3-day delay in median spike emergence in all four transgenic lines, which was significant for events #51 and #67 (**Supplementary Figure 8I**). Overall, these results indicate that the virulence reducing effect of the *SvrPm3*$^{a1/f1}$-RNAi transgene on the *B.g. tritici* pathogen is not accompanied by a major effect on host performance.

We conclude that stable HIGS of the three *B.g. tritici* effectors resulted in a quantitative gain of powdery mildew resistance in wheat. Resistant events can impair haustorium formation on seedlings, and restrict *B.g. tritici* growth on adult leaves in a quantitative manner.

DISCUSSION

Transient HIGS has been an important tool for functional genomics in *B. graminis*. In this study, we showed that HIGS can be used in stable transgenic wheat plants to effectively target three *B.g. tritici* effectors. Most importantly, we show that the use of this strategy to impair pathogen virulence results in a quantitative gain of resistance to wheat powdery mildew in adult plants.

Evidence for Stable Cross-Kingdom RNAi in Wheat Powdery Mildew

The stable transgenic events generated in this study constitutively express the *SvrPm3*$^{a1/f1}$-RNAi transgene. Therefore, we could confidently assess and correlate the observed phenotypes with

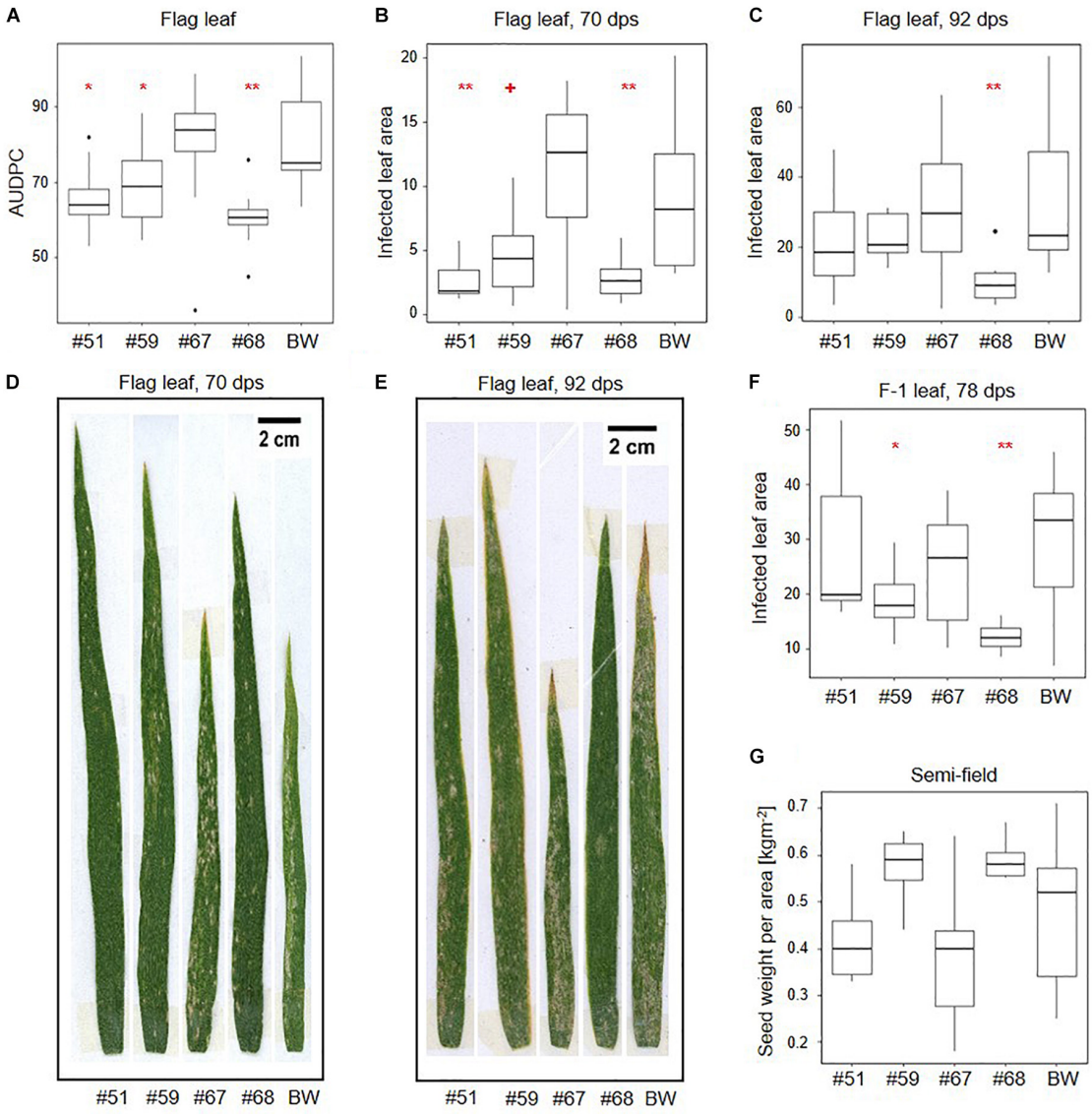

FIGURE 4 | *SvrPm3*$^{a1/f1}$-RNAi wheat lines show quantitative adult plant resistance in semi-field conditions. Transgenic events and wild-type Bobwhite (BW) were exposed to natural *B.g. tritici* infection in a semi-field trial. **(A)** Disease was monitored over time on the flag leaves and the area under the disease progression curve (AUDPC) was calculated. **(B–F)** Image-based quantification of the infected leaf area in flag and F-1 leaves. **(B,D)** At the beginning of flag leaf infection, 70 days post sowing (dps). **(C,E)** At the end of the *B.g. tritici* disease progression, 92 dps. **(F)** F-1 leaves at 78 dps. **(G)** Quantification of seed weight per area. Pairwise comparison of transgenic events with wildtype BW were carried out using one-sided *t*-test **(A)**, Welch *t*-test **(C,F,G)** or Wilcoxon rank sum test **(B)** depending on the distribution and variance of the data. + < 0.06; *p < 0.05; **p < 0.01.

the actual downregulation of the target genes, which is a major improvement to single cell transient HIGS assays. In this study, three effector genes were targeted (*SvrPm3*$^{a1/f1}$, *Bgt_Bcg-6*, and *Bgt_Bcg-7*) and RT-qPCR assays showed that all three are downregulated when the pathogen is growing on transgenic HIGS events as compared to the non-transgenic control. The level of target gene silencing ranged from −16 to −58%, which is similar to the levels of HIGS reported in other fungal pathogens such as *B. cinerea* on Arabidopsis and *Puccinia triticina* on wheat (Wang et al., 2016; Panwar et al., 2017). In *Fusarium graminearum*, an even higher level of target gene silencing was reported (−77 to −92%) on Arabidopsis, suggesting that HIGS efficiency is intrinsically regulated by the two organisms involved in ck-RNAi (Koch et al., 2013).

The biology behind sRNA transport from the host to the fungal cell through the haustorial interface is not well understood. In principle, the transgene-derived hairpin transcripts are processed by host DCL enzymes into siRNAs. These siRNAs are then secreted in exosome-like vesicles that can be taken up by the fungus via the haustorial interface (Cai et al., 2018). Once the siRNAs are taken up, they guide the RNA-induced silencing

complex (RISC) to cleave homologous, fungal mRNA transcripts. In plants and worms, mRNA-cleavage by a siRNA-primed RISC leads to the accumulation of secondary siRNAs through a specific RNA-dependent RNA polymerase (RdRP), which uses the diced mRNA molecules as a template to synthesize secondary dsRNA precursors or directly siRNAs. The accumulation of secondary siRNAs is known as transitivity, and is thought to result in a more persistent and stable target gene silencing (Calo et al., 2012).

In fungi, transitivity has been described in the zygomycete *Mucor circinelloides* and relies on its RdRP homolog *McRdRP-2* (Calo et al., 2012). In ascomcyetes such as *Neurospora crassa* and *Aspergillus nidulans*, whole genome sequencing data indicates that the orthologs of the *McRdRP-2* gene have been lost (Torres-Martínez and Ruiz-Vázquez, 2017). Instead, ascomycetes have orthologs of *McRdRP-1*, e.g., *NcQde-1*, which are necessary for sense but not for inverted repeat transgene-triggered RNAi. It is unclear whether the ascomycete orthologs of *McRdRP-1* are capable of transitivity. In the case of the *Blumeria* clade, Kusch et al. (2018) found that this group of fungi has lost all RdRP paralogs except one single copy. It is unknown which specific RNA processing properties were retained by this single representative of the RdRP family in *Blumeria*, and how this protein is contributing to ck-RNAi. Based on our results, we hypothesize that ck-RNAi in *Blumeria* is possibly restricted by the absence of a transitivity-capable RdRP. This hypothesis provides an explanation for the relatively moderate silencing of target gene expression achieved by HIGS in this study. We propose that further characterization of the fungal RNAi machinery can provide meaningful information about the limitations and possible improvements of HIGS in *Blumeria*.

The Molecular Basis for Effective Cross-Kingdom RNAi in Wheat Powdery Mildew

Efficient transgene-derived dsRNA accumulation *in planta* is thought to be decisive for efficient RNAi-directed control of insect pests (Wang et al., 2018). In fact, both dsRNA and ssRNA transcripts can be derived from a hairpin transgene, however, since only dsRNA is an efficient template for RNAi, it is anticipated that higher accumulation of dsRNA molecules should correlate with a higher accumulation of siRNAs, and thus higher target gene silencing efficiency. Here, we had a unique opportunity to test this hypothesis in a stable system in which we had clear evidence for HIGS. Interestingly, our results showed that the dsRNA proxy performed poorly in predicting the efficacy of ck-RNAi. While we could show that all events express a detectable dsRNA molecule, we found little correlation between ssRNA/dsRNA expression levels and target gene silencing. This could be explained by differences in transgene expression at the tissue level, which cannot be detected in our RT-qPCR assays based on RNA extraction from entire leaf segments. Here, higher levels of target gene silencing can possibly be associated with higher dsRNA expression in epidermal cells which are the only cells infected by mildew, and the primary target tissue of transient HIGS. It is also possible that dsRNA accumulation is not the only factor relevant for efficient ck-RNAi in *Blumeria*. In fungi, evidence suggests that dsRNA-derived siRNAs are the bioactive molecule driving HIGS (Cai et al., 2018), thus additional factors influencing dsRNA processing by the host DCL enzymes, siRNA packaging into exosome-like vesicles, and vesicle uptake by the fungus can have an impact on silencing efficiency, either acting as bottlenecks or enhancers. In humans, the discovery of sequence motifs that are over-represented in exosome-associated miRNAs led to the identification of specific proteins that control miRNA sorting into exosomes (Villarroya-Beltri et al., 2013). We therefore suggest that the identification of the genetic factors associated with RNA mobility from its hosts to *Blumeria* can provide an important leverage for improving HIGS efficiency. We speculate that favorable HIGS traits can be identified from the natural diversity of wheat cultivars, introduced through breeding or genome editing, and inform better HIGS transgene design.

Evidence for a Conserved Role of Ribonuclease-Like Effectors in Wheat Powdery Mildew Virulence

Small secreted proteins with a predicted ribonuclease fold constitute the largest class of candidate effectors in the *Blumeria* genomes (Pedersen et al., 2012; Bourras et al., 2018). This RNase-like class of effectors also includes the $SvrPm3^{a1/f1}$ family which encodes all three effectors targeted by HIGS in this study. Evidence from previous transient HIGS experiments indicated that this class of effectors is highly important for *Blumeria* virulence at the early stages of infection (Pliego et al., 2013). In this study, we extensively scored several attributes of *B.g. tritici* virulence on stable $SvrPm3^{a1/f1}$-RNAi wheat lines at the macroscopic and microscopic level. We observed that HIGS of the three RNase-like effectors, $SvrPm3^{a1/f1}$, *Bgt_Bcg-6*, and *Bgt_Bcg-7*, leads to a consistent loss of virulence at the haustorial stage, in the form of a reduced ability of the fungus to penetrate and form such feeding structures, and to a significant reduction in infected leaf area on adult leaves. Our results thereby further substantiate the importance of this class of proteins for *Blumeria* virulence. Previous transient HIGS experiments in *B.g. hordei* also described an effect primarily on haustorial development upon targeting of effectors (Nowara et al., 2010; Zhang et al., 2012; Pliego et al., 2013; Ahmed et al., 2015; Aguilar et al., 2016).

Several families of *Blumeria* effectors, including the effector targets in this study, are specifically induced at the haustorial stage (Praz et al., 2018), suggesting that temporal expression patterns of the target gene could explain the limitation of loss of virulence to this developmental stage. However, we observed the same limited impact on virulence to the haustorial stage when we used transient HIGS to target *β2-tub*. Thus, demonstrating that this stage of development is highly relevant to control the pathogen via HIGS, independently of temporal target expression patterns. Similarly, it was speculated previously that stable HIGS lines could cause complete loss of virulence, instead our

results suggest that the effect of HIGS on fungal virulence is quantitative by nature.

The primary target of this study *SvrPm3*$^{a1/f1}$ has first been characterized as a suppressor of the cell death triggered by the *Pm3* wheat resistance gene alleles (Bourras et al., 2015, 2019). By interfering with the recognition of cognate *AvrPm3* genes, *SvrPm3*$^{a1/f1}$ reduces the resistance spectrum of *Pm3* alleles. McNally et al. (2018) showed that the suppressor variant of *SvrPm3*$^{a1/f1}$ is present in wheat powdery mildew populations globally. We therefore conclude that combining HIGS of *SvrPm3*$^{a1/f1}$ with *Pm3* resistance alleles has the potential to increase the resistance spectrum of such wheat lines.

To our knowledge, this is the first comprehensive study of ck-RNAi in *Blumeria* based on stable transgenic HIGS lines. We conclude that ck-RNAi has great potential to complement current pest control strategies by e.g. broadening the resistance spectra of host resistance genes. We suggest that using ck-RNAi to tap naturally occurring RNA exchanges can provide new routes for crop improvement through genetic engineering as well as classical breeding.

MATERIALS AND METHODS

Infection Tests

The United Kingdom *B.g. tritici* isolate Bgt_JIW2 (Wicker et al., 2013) and the Swiss *B.g. hordei* isolate K1 were maintained as described by Parlange et al. (2011) and Jordan et al. (2011), respectively. For high-density infection tests, inoculum for a 12 cm square plate was derived from three heavily infected 3 cm wheat leaves 10 dpi and applied using an infection tower of 25 cm height. Infected wheat leaves were scored macroscopically at 10 dpi. For low-density infection tests, inoculum was harvested at eight dpi from two mildly infected 2 cm wheat leaf segments, when the first mildew colonies start to sporulate, then dusted on leaf material in an infection tower. Infected wheat leaves were scanned six dpi using an HD-Scanner and "leaf area" and area covered by powdery mildew were estimated using Fiji[1] based on color thresholding. The "infected leaf area" was calculated for each leaf by normalizing the infected area to the "leaf area". The "relative infected leaf area" was further calculated by normalizing the "infected leaf area" to the mean infected leaf area of the non-transformed BW control from the same infection plate. For microscopy, high-density infection was used for two dpi samples and low-density infection for four and six dpi samples. Leave samples were destained (in 8% lactic acid, 16% glycerol, and 66% ethanol) and fungal structures were stained using coomassie blue. Aniline blue was used for papilla staining of a subset of two dpi samples, but since there were no observed effects on papilla formation it was omitted for the remaining samples. For each leaf, a minimum of 50 powdery mildew-wheat interactions, corresponding to an appressorium attacking an A- or B-type epidermal cell (Rubiales and Carver, 2000), were assessed for haustorium formation and shape, hyphae formation and number, and conidiophore formation and number.

[1]https://imagej.net/Fiji

Construct Design and Cloning

The si-Fi software was used to select the donor sequences for the HIGS constructs (Lück et al., 2019). The candidate sequences were analyzed for putative off-targets in the respective donor genomes, *B.g. tritici* and *B.g. hordei*, and in the respective host genomes, wheat and barley (Mascher et al., 2017; Frantzeskakis et al., 2018; International Wheat Genome Sequencing Consortium, 2018; Müller et al., 2019). Additionally, CDS files of manually annotated *SvrPm3*$^{a1/f1}$ family members were used for target prediction in the isolate Bgt_JIW2. The *SvrPm3*$^{a1/f1}$-RNAi sequence was amplified from genomic DNA of the Swiss *B.g. tritici* isolate Bgt_96224 (Stirnweis et al., 2014) using the primers BgtSvr-RNAi-F/R (**Supplementary Table 2**), cloned in reverse into the pIPKTA38 entry vector according to Douchkov et al. (2005) and transferred via Gateway cloning to the pIPKb007-RNAi vector (Himmelbach et al., 2007). The *β2-tub*-RNAi sequence was cloned into the pIPKTA30-RNAi vector from genomic DNA of isolate K1 using the primers Bgtβ2tub-RNAi-F/R (**Supplementary Table 2**).

Transient HIGS

Transient HIGS was performed as described by Nowara et al. (2010). Here, the barley (*Hordeum vulgare*) cultivar "Golden Promise" and the wheat (*Triticum aestivum*) cultivar "Chancellor" were used. Three days after bombardment, leaves were infected with the barley powdery mildew isolate K1 and the wheat powdery mildew isolate Bgt_JIW2, respectively. The "haustorium index" represents the ratio of appressorium-forming spores that established a haustorium. Additionally, the presence of secondary hyphae was recorded. Results are derived from three independent experiments.

Generation and Selection of Transgenic Events

Wheat transformation and transformant selection is described in Brunner et al. (2011). The *ubi*:*SvrPm3*$^{a1/f1}$-RNAi transgene was excised from the pIPKb007 plasmid using *SfiI*. *SvrPm3*$^{a1/f1}$-RNAi transgene presence was confirmed by PCR using the primers BgtSvr-RNAi-F/R (**Supplementary Table 2**). The selected T0 seedlings were transferred to the greenhouse and allowed to self. To select transgenic events for further analysis presence of both repeat sequences, copy number and transgene expression was assessed. Genomic DNA extraction is described in Brunner et al. (2011). Antisense repeat-specific primers pIPKb007-4/BgtSvr-RNAi-F and the sense repeat-specific primers pIPKb007-5/BgtSvr-RNAi-R were used to confirm presence of both repeats by PCR (**Supplementary Table 2**). Copy number was assessed by southern blotting 20μg *HindIII*-digested genomic DNA as described in Graner et al. (1990). For the probe, the repeat sequence was amplified using the BgtSvr-RNAi-F/R primers. Transgene expression was assessed by RT-PCR on cDNA. RNA was isolated as described in Brunner et al. (2011) with the alteration that leaves were collected in tubes and ground using glass beads. cDNA was synthesized from 500 ng RNA using the iScript^{TM} Advanced cDNA Synthesis

Kit. The qRT-BgtBcg1F primer from Bourras et al. (2015) and BgtSvr-RNAi-R were used for amplification.

RT-qPCR Assays

Biological replicates consist of first leaves of three 10-days-old seedlings which were pooled, immediately frozen in liquid nitrogen and stored at −80°C. RNA was extracted as described above with the alteration that infected samples were ground using metal beads. For target gene expression analysis, cDNA was synthesized as described above. Primers were designed to be specific to the fungal *SvrPm3*$^{a1/f1}$, *Bgt_Bcg-6*, and *Bgt_Bcg-7* mRNAs (**Supplementary Table 2**). They were checked for specificity on plasmid DNA containing the transgene or either fungal target gene and on cDNA from infected non-transgenic and non-infected transgenic leaf material. Amplicons were verified by Sanger sequencing. *B.g. tritici gapdh* was used to normalize expression (Bourras et al., 2015). To further increase specificity, primer annealing was performed at 63°C and cDNA was quantified at 72°C. For consistency, these settings were used in all RT-qPCR analysis performed in this study. For transgene expression analysis, dsRNA and ssRNA were quantified according to Wang et al. (2018) with the following modifications: (1) RNA digested with *RNase If* was purified using the Promega SV Total RNA Isolation System, following the instructions in Promega Notes No. 86 2004 17, but using EconoSpin Micro Volume DNA/RNA Spin Columns. And (2) iScript^{TM} Advanced cDNA Synthesis Kit was used after hexamer incubation. qRT-BgtBcg1F/R primers from Bourras et al. (2015) were used to quantify transgene-derived transcripts. The wheat reference genes *Ta2291*, *Ta.6863*, and *Ta.25640* were used to normalize transgene expression (**Supplementary Table 2**; Giménez et al., 2011; Hurni et al., 2013). Results represent 3–4 biological replicates. The Kapa Sybr Fast qPCR Kit and the CFX384 Real-Time PCR Detection System were used.

Semi-Field and Greenhouse Experiments

For the semi-field experiment we used the convertible glasshouse previously described in Romeis et al. (2007) and Boni et al. (2017) with a convertible roof programed to close in case of wind, rain, or cold. In this experimental setup the transgenic plants are retained from spreading to the environment in compliance with Swiss federal regulation. We planted a randomized setup of eight irrigated plots per event and wildtype BW, containing 10 plants each. Pre-germinated seeds were sown on March 29, 2018 into the 0.055 m^2 central cylinders. More BW was sown as buffering plants around the central cylinders to reduce border effects. The soil was fertilized with 50 kg/ha P_2O_5, 85 kg/ha K_2O, 40 kg/ha N, 11.7 kg/ha S, and 8.3 kg/ha Mg before sowing. At the start of stem elongation, test plants were reduced to 10 plants per cylinder and fertilized with 40 kg/ha N. The last fertilization with 40 kg/ha N was carried out before booting. Insecticides were applied as needed; weeding was done by hand. *B.g. tritici* infection was provided by natural inoculum. For AUDPC calculations, *B.g. tritici* disease was scored twice a week from beginning of May to end of June as described in Brunner et al. (2011). Representative leaves of adult plants were sampled per plot and infected leaf area was quantified as described above. Agronomical data was recorded per plot. 10 plants of each transgenic event and BW were also grown under greenhouse conditions. Data was recorded per plant. Plants were hand-harvested and threshed.

Statistical Analyses

The statistical "exact and approximate test for proportions" in R was used to compare the *β2-tub*-RNAi construct results to the empty vector control. RT-qPCR experiment results were assessed for normal distribution and homogenous variance using the Shapiro–Wilk test and the Levene test, respectively. Based on this, one-way ANOVA or Welch ANOVA followed by the Tukey multiple pairwise comparison or the Kruskal–Wallis rank sum test followed by the Dunn's *post hoc* test was used to test for statistical significance. Phenotyping results were assessed for normal distribution and homogenous variance using the Shapiro–Wilk test and the F-test, respectively. Based on this, the Wilcoxon rank sum, Welch *t*, or *t*-test was used to compare independent transgenic events to BW. All analyses were carried out using R software[2].

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

LS, BK, and SB wrote the manuscript. LS, BK, SB, FP, and MM designed the experiments. LS, FP, GB, and GH performed the lab experiments. LS, EJ, JS, and RS performed the field experiments. LS, FP, TW, and SB performed the bioinformatics analyses. LS and AW performed the statistical analyses.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00253/full#supplementary-material

[2]https://www.r-project.org/

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frontiers in Plant Science

ORIGINAL RESEARCH
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Comparative Analysis of Chitin SynthaseA dsRNA Mediated RNA Interference for Management of Crop Pests of Different Families of Lepidoptera

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RNA interference (RNAi) is a sequence-specific down-regulation in the expression of a particular gene, induced by double-stranded RNA (dsRNA). Feeding of dsRNA either directly or through transgenic plants expressing dsRNA of insect genes has been proven successful against lepidopteran and coleopteran pests, establishing an additional alternative to control insect pests. Lepidopteran crop pests including *Spodoptera litura* (Fabricius) (Noctuidae), *Chilo partellus* (Swinhoe) (Crambidae), *Plutella xylostella* (Linnaeus) (Plutellidae), and *Maruca vitrata* (Fabricius) (Pyralidae) are the devastating pests of a variety of crops. To tap the potential of RNAi against insect pests, a gene coding for the key enzyme in chitin biosynthesis in arthropods, the *chitin synthaseA* (*CHSA*), has been targeted through an exogenous delivery of dsRNA and plant-mediated RNAi. The introduction of *dsCHSA* caused "Half ecdysis" and "Black body" type lethal phenotypes and a significant reduction in larval body weight. Subsequent RT-qPCR analysis demonstrated the down-regulation of *CHSA* gene transcripts from 1.38- to 8.33-fold in the four target species. Meanwhile, when *S. litura* larvae fed with leaves of transgenic tobacco plants expressing *dsSlCHSA*, the mRNA abundance of *CHSA* gene was significantly decreased resulting in lethal phenotypes like "Double head formation," "Half ecdysis," and "Black body." In addition, abnormalities in pupal–adult and adult stage were also documented, strongly suggesting the RNAi effect of *CHSA* gene at late developmental stages. Overall, the results demonstrated that *CHSA* gene expression in Lepidopteran crop pests could be suppressed by application of dsRNA either as feeding or through transgenic crop plants.

Keywords: ***Spodoptera litura***, ***Chilo partellus***, ***Plutella xylostella***, ***Maruca vitrata***, **RNAi**, ***CHSA***

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INTRODUCTION

Lepidopteran crop pests including tobacco cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae); maize/sorghum stem borer, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae); diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae), and legume pod borer, *Maruca vitrata* (Fabricius) (Lepidoptera: Pyralidae) are considered as most destructive pests of several economically important agricultural and horticultural crops worldwide. Significant

amount of yield losses caused by those insect pests has been reported in several regions of world (Ahmad et al., 2008; Sharma et al., 2010; Zalucki et al., 2012). The most common method for the management of those insect pest is the use of insecticides or bio-control agents particularly *Bacillus thuringiensis* (Bt) toxins. However, the management of those insect pests is a daunting task because of the development of resistance against insecticides and Bt toxins (Storer et al., 2010). These problems necessitate finding an alternative pest control strategy to supplement the present pest management methods.

RNA interference (RNAi)-based strategy through the expression of double-stranded RNA (dsRNA) targeting potential genes in crop pests has paved the way for new generation of integrated pest management (IPM). RNAi is a conserved phenomenon in which a dsRNA knocks down the expression of a target gene. However, its efficiency varies with the target insect species (Terenius et al., 2011), the method of RNAi administration (Scott et al., 2013), and the candidate gene targeted by RNAi (Kola et al., 2015). In the preceding years, several successful RNAi experiments in lepidopterans have been reported and published. However, the comparative analysis to study the effect of RNAi among crop pests belonging to different families in lepidopterans has not been appeared so far.

The basic methods of dsRNA administration in insects are injection and feeding. Most of the RNAi experiments in different insect orders including lepidopterans were conducted through droplet feeding or microinjection, particularly in *Drosophila melanogaster* (Meigen) (Miller et al., 2008), *Tribolium castaneum* (Herbst) (Tomoyasu and Denell, 2004; Bai et al., 2011), *Acyrthosiphon pisum* (Harris) (Ye et al., 2019), and *Bombyx mori* (Linnaeus) (Hossain et al., 2008), *Spodoptera exigua* (Hubner) (Kim et al., 2015). Initially, the RNAi aimed as a functional genomic tool to elucidate the functions of genes. For example, Bettencourt et al. (2002) and Quan et al. (2002) observed phenotypic variations in embryos in *Hyalophora cecropia* (Linnaeus) and *B. mori*, after injection of dsRNA into the pupa demonstrating systemic RNAi. Chen et al. (2008) also observed abnormal larval growth and development in *S. exigua* after injection of dsRNA of *chitin synthaseA* (*CHSA*). Though the injection method showed phenotypic variations, the delivery of dsRNA through injection has low survival rate and is not feasible in the field conditions to control crop pests. Furthermore, Wang et al. (2011) demonstrated that a direct spray of dsRNA in *Ostrinia furnalalis* (Linnaeus) larvae, resulting in down regulation in the expression of target genes, delayed growth and development, and mortality. However, high amount of dsRNA is required to reach the threshold and produce the desired results, raising the question of specificity and cost effectiveness. Therefore, through the expression of dsRNA in plants involving transgenic plant-mediated RNAi is quite economical, its administration is easy and practicable in field conditions. Moreover, the transgenic plants expressing dsRNA of insect genes, to protect the plants against insect feeding damage have been proven successful against coleopteran and lepidopteran insect pests (Baum et al., 2007; Mao et al., 2007; Zha et al., 2011). However, to our knowledge, no studies have been documented deploying two different methods, both the direct application of dsRNA and plant-mediated RNAi by feeding to control crop pests of different families of lepidoptera.

Another important factor for the success of RNAi is the selection of potential target genes. The simplest and effective way is to select known ideal genes based on the literature. In the present study, we have selected *CHSA* as a target gene for dsRNA-transgenic plant-mediated RNAi. Chitin synthesis is essential for insect growth and development. Chitin, a polysaccharide of *N*-acetyl-B-D-glucosamine is an important component of insect cuticle which forms an exoskeleton (exo and endocuticle) and plays important role in protecting insects from environmental stresses and pathogenic microbes (Kramer and Muthukrishnan, 2004). The insect *chitin synthases* (*CHS*) are encoded by two genes, *CHSA*/*CHS1* and *CHSB*/*CHS2* (Hogenkamp et al., 2005). *CHSA* function exclusively in the formation of chitin found in the insect cuticle (epidermal and ectodermal cells), while *CHSB* function mainly in the formation of chitin in the peritrophic membrane of epithelial cells (Arakane et al., 2005; Wang et al., 2012). Moreover, chitin is mainly present in arthropods and absent in vertebrates and plants, could address an important concern of RNAi, i.e., the specificity of dsRNA (Tian et al., 2009). Significant promising results documenting various phenotypic abnormalities and lethality in *S. exigua* through disruption of *SeCHSA* by injection and bacterial expressed dsRNA of *SeCHSA* have been shown (Chen et al., 2008; Tian et al., 2009). However, the present comparative study using dsRNA-transgenic plant-mediated RNAi of *CHSA* in lepidopteran crop pests has proven new insights for designing futuristic pest management strategies.

In this study, *CHSA* gene was isolated, cloned, and sequences were compared from *S. litura*, *C. partellus*, *P. xylostella*, and *M. vitrata*. dsRNA of *CHSA* gene was synthesized and insect feeding study demonstrated its effect against the target species. We also developed hairpin RNAi construct targeting *CHSA* gene and transformed into tobacco plants. Both the direct feeding of dsRNA of *CHSA* gene and plant mediated RNAi demonstrated the significant reduction in larval body weight, higher lethality rate, and down-regulation in mRNA abundance of *CHSA* gene in all four lepidopteran insects studied.

MATERIALS AND METHODS

Insects Studied

The larvae of *S. litura*, *P. xylostella*, and *M. vitrata* were collected from the research fields of Tamil Nadu Agricultural University, Coimbatore, and the eggs of *C. partellus* were obtained from the National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, Karnataka, India. They were reared on castor leaves (*Ricinus communis*) (Euphorbiaceae), cauliflower leaves (*Brassica oleracea* L.) (Brassicaceae), lablab pods (*Lablab purpureus* L.) (Fabaceae), and baby corn (*Zea mays*) (Poaceae), respectively, at the Molecular Ecology Laboratory, Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore, India.

Mass Culturing of Insects

Mass culturing was done with slight modification to the methodologies of Britto (1980) and described briefly here. The

TABLE 1 | Primers used in this study.

Sl. No.	Primer name	Sequence (5′–3′)	Amplicon size (bp)
1	CHSA	F-GTGATGATGATTCGCAAGTGA	518
		R-AGGATGAATACGACCGCAAG	
2	dsCHSA	F-taatacgactcactatagggGTGATGATGATTCGCAAGTGA	616
		R-taatacgactcactatagggAGGATGAATACGACCGCAAG	
3	S SlCHSA	F-CTCGAGGTGATGATGATTCGCAAGTGA	530
		R-GGTACCAGGATGAATACGACCGCAAG	
4	A SlCHSA	F-TCTAGAGTGATGATGATTCGCAAGTGA	530
		R-AAGCTTAGGATGAATACGACCGCAAG	
5	qRT CHSA	F-GACTCTGGACGGAGACAT	112
		R-GCCTACAGGATGAATACGAC	
6	qRT Actin	F-AATCGTGCGTGACATCAA	218
		R-TGTAAGTGGTCTCGTGGAT	

larvae hatched out from the eggs were reared on respective feed till pupation in plastic buckets (22.5 cm dia. and 25 cm height). The feed was changed once in 2 days during earlier stages and daily in later stages. The pupae were collected, surface sterilized with 0.5% sodium hypochlorite, rinsed with distilled water, and kept in an adult emergence cage. The newly emerged adults were transferred to plastic buckets for mating and oviposition and were fed with 10% sugar solution enriched with vitamin. Folded wax paper was placed inside the plastic buckets to lay the eggs. The temperature and relative humidity were maintained at 28 ± 3°C and 70–75%, respectively, inside the culture room.

Cloning of *CHSA* Gene in *S. litura*, *C. partellus*, *P. xylostella*, and *M. vitrata*

RNA Isolation and cDNA Synthesis

Total RNA was extracted by homogenizing single third instar larvae of *S. litura*, *C. partellus*, *P. xylostella*, and *M. vitrata* individually by employing Trizol method (Chomczynski and Mackey, 1995). The isolated RNA was reverse transcribed using cDNA Synthesis Kit (Thermo Scientific, United States) after treating with RNase-free DNase I (Thermo Scientific, United States). The partial *CHSA* gene from *S. litura*, *C. partellus*, *P. xylostella*, and *M. vitrata* was amplified using a set of *CHSA* specific primers (**Table 1**). The PCR product was column purified as per the manufacture's instruction provided by purification spin kit (BIOBASIC). The purified DNA fragments were used for cloning (pTZ57R/T vector, Thermo Scientific, United States) and bacterial transformation. Further validation of recombinant colonies was done by restriction digestion analysis and sequenced at SciGenom Labs Pvt. Ltd., Cochin, Kerala, India.

Sequencing of Cloned Fragment and Analysis

The samples were sequenced through single pass analysis from forward and reverse direction. DNA sequence data was compared with available *CHSA* gene sequences in National Center for Biotechnology Information (NCBI) data bank[1] by using BLASTn analysis tool. The sequences in different species were edited and aligned with reference sequences of *CHSA* (Accession No: JN003621.1) by ClustalW v2.0 online tool[2]. The *CHSA* nucleotide sequences resulted through cloning were deduced into amino acid sequences via EMBOSS Transeq[3]. Simultaneously, the amino acid sequences of *CHSA* gene from different insect species in lepidopteran order were collected from NCBI database and multiple sequence alignment was carried out in BioEdit software using ClustalW option.

To know the relatedness of *CHSA* gene among the lepidopteran insects, the phylogenetic analysis was conducted using MEGA v5.05 software (Tamura et al., 2013). A bootstrap analysis was done, and robustness of each cluster was verified in 1000 replicates. The nucleotide sequences of *CHSA* gene were used from different insects of lepidopteran order, viz., *B. mori* (Accession No: JQ320074), *Choristoneura fumiferana* (Clemens) (Accession No: EU561238), *Cnaphalocrocis medinalis* (Guenée) (Accession No: KP000843), *Earias vitella* (Fabricius) (Accession No: JX444555), *Ectropis obliqua* (Prout) (Accession No: EU482034), *Helicoverpa armigera* (Hubner) (Accession No: KP939100), *Helicoverpa zea* (Boddie) (Accession No: AF229127), *Hyblaea puera* (Cramer) (Accession No: JQ289043), *Leucinodes orbonalis* (Guenée) (Accession No: JX461234), *Mamestra brassicae* (Linnaeus) (Accession No: GQ281761), *Manduca sexta* (Linnaeus) (Accession No: AY062175), *Mythimna separata* (Walker) (Accession No: KT948989), Ostrinia *furnacalis* (Accession No: EU376026), *Phthorimaea operculella* (Zeller) (Accession No: KU720384), *P. xylostella* (Accession No: AB271784), and *S. exigua* (Accession No: KT932387).

Double-Stranded RNA (dsRNA) Synthesis

The dsRNA was synthesized from the particular region of *CHSA* gene (Accession No: JN003621.1) which did not show off-target effects using dsCheck online software[4] (Naito et al., 2005). To this region, primers were designed and T7 promoter sequence (TAATACGACTCACTATAGGGAGA) was incorporated at the 5'ends (**Table 1**). The purified PCR products were used for

[1]https://www.ncbi.nlm.nih.gov/

[2]www.ebi.ac.uk/Tools/msa/clustalw2/

[3]http://www.ebi.ac.uk/Tools/st/emboss_transeq

[4]http://dsCheck.RNAi.jp/

in vitro transcription with MEGAscript RNAi kit (Ambion Life Technologies, United States). The dsRNAs were annealed by incubating at 37°C for 3.5 h, followed by slow cooling to room temperature. The annealed dsRNAs were treated with DNase I and RNase at 37°C for 1 h, purified, and stored at −20°C.

Feeding Bioassays Against Target Insects

Bioassay Study With *SlCHSA*, *CpCHSA*, *PxCHSA*, *MvCHSA dsRNA*

The dose effect was determined by diluting *SlCHSA* dsRNA with diethyl pyrocarbonate (DEPC) treated water to give a final concentration of 1, 3, 5, 7, and 9 μg/μl and further standardized dose of 3 μg/larvae was used for bioassay studies considering the production cost and efficacy. Similarly, the time-course expression analysis of the target gene was performed from day 1 onward until the end of the experiment. The bioassay was performed against *S. litura, C. partellus*, *P. xylostella*, *M. vitrata*, single second instar larvae were treated individually with 3 μg *SlCHSA*, *CpCHSA*, *PxCHSA*, *MvCHSA* dsRNA, respectively. The dsRNA was overlaid on castor leaf, baby corn, cauliflower leaf, and lablab pod, respectively, and were maintained in 80 mm sterile plastic cups with agar base for providing moisture till the end of the experiment. For control, the same number of larvae fed on respective feed overlaid with DEPC treated water (control) and Novaluron (Rimon—1.25 ml/l, benzoylphenyl urea, inhibits chitin formation) used as positive control in the experiment. There were 20 biological replicates in each group and the observations, viz., larval body weight, lethality rate, were observed. The data were analyzed statistically by one-way analysis of variance (ANOVA) at significance level (0.05) using the STAR software (International Rice research Institute [IRRI], 2013).

Transgenic Tobacco Plants Expressing Hairpin *dsSlCHSA*

The RNAi intermediate vector, pHANNIBAL (Source: CSIRO plant industry, Australia) contains RE site for directional insertion of PCR products on either side of the PDK intron. The 530 bp *SlCHSA* fragments cloned into pTZ57R/T vector were digested with *Hin*dIII and *Xba*I (*CHSA* antisense strand) and *Xho*I and *Kpn*I (*CHSA* sense strand), and subsequently inserted on either side of intron present in pHANNIBAL vector. The 3.0 kb RNAi-cassette was released from the cloned pHANNIBAL vector by *Not*I digestion, which was then cloned into pART27, plant transformation vector. The constructed pART27 expressing hairpin *SlCHSA* dsRNA (pRNAi-CHSA) contains a CaMV35S promoter, a sense strand of *SlCHSA*, a 741 bp intron, an antisense strand of *SlCHSA*, and an NOS terminator (**Figure 1** and **Supplementary Figure 1**). *Agrobacterium tumefaciens* strain LBA4404 containing the binary plasmid pRNAi-CHSA was used for tobacco transformation. The plant transformation was done using standardized protocol and transformants were selected using 100 mg/l Kanamycin on MS medium (Horsch et al., 1985). After 1 month, rooted tobacco plants were transferred to greenhouse. Genomic DNA was isolated from the putative tobacco transformants and wild-type (WT) and checked by PCR using gene specific primers.

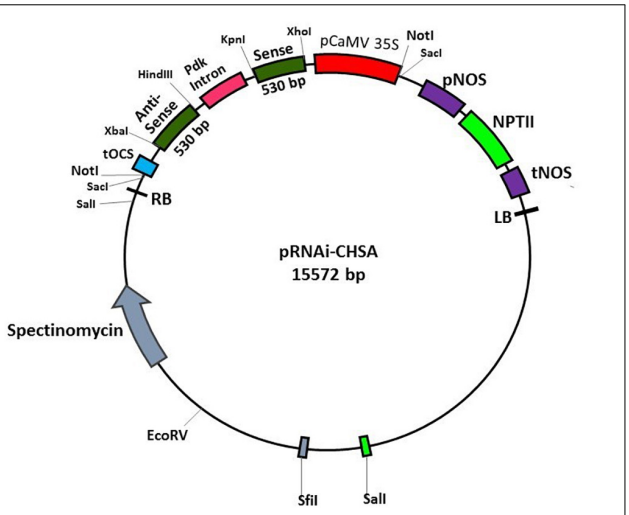

FIGURE 1 | Physical map of pRNAi-CHSA vector.

Bioassay Study With Transgenic Tobacco Plants Expressing Hairpin *dsSlCHSA*

To assess the effects of transgenic tobacco plants expressing *dsSlCHSA*, on *S. litura* growth, second instar larvae (ten biological replicates) were reared on detached mature leaves (∼45 days old) of the control-WT, and five transgenic tobacco plants (dsSlCHSA E4, E5, E6, E9, and E11) and were weighed, respectively, for 7 days. Small leaf discs with 3 cm diameter were maintained in an 80 mm sterile plastic cup with agar base for providing moisture, and the observations were carried out.

Assessment of Effect of dsRNA on the Target Gene Expression

RT-qPCR Analysis

Total RNA was extracted and pooled from three to five treated and untreated larvae in three groups independently and considered as three biological replicates. The reverse transcription product (100 ng) was used to perform the RT-qPCR amplification on CFX Connect^{TM} Real-Time PCR Systems (Bio-Rad, United States) using primers specific to *CHSA* gene (**Table 1**); 20 μl RT-qPCR reaction mixture included 1 μl of cDNA, 10 μl of 2X iQ^{TM} SYBR Green supermix (Bio-Rad, United States), 100 nM of primers, and nuclease free water to make up the total volume. The relative quantification was performed on CFX Connect^{TM} systems (Bio-Rad, United States) with the initial denaturation for 3 min at 95°C followed by 40 cycles at 95°C for 15 s and 55°C for 1 min. Each of the reactions was performed with three biological replicates, and the results were normalized with constitutively expressing *Actin* gene. RT-qPCR data were analyzed by using CFX Manager 2.1 (Bio-Rad, United States) software and was further verified using the standard delta-delta-Ct (ddCt) method.

RESULTS

Sequencing and Phylogenetic Analysis of *CHSA* Gene

The multiple sequence alignment of the cloned partial sequence of *CHSA* gene of *S. litura*, *C. partellus*, *P. xylostella*, and *M. vitrata* with the reference sequence of *S. litura CHSA* (Accession No: JN003621.1) showed match of 518 bp, as of expected size (**Supplementary Figure S2**). There were nucleotide variation at 143, 338, 355, 383, and 415 bp positions among the amplified sequence of *CHSA* from *S. litura*, *C. partellus*, *P. xylostella*, and *M. vitrata* and rest other sequences were similar to the reference sequence of *S. litura CHSA* (**Supplementary Figure S3**). The multiple sequence alignment of the deduced amino acid sequences of amplified *CHSA* from the four species with the other insects of lepidopteran order showed that most of the residues were conserved with 72.86% identity (**Supplementary Figure S4**). The phylogenetic tree formed two principle clusters, viz., A and B. Principle cluster A comprises of 13 genus of different families of Lepidoptera, viz., *B. mori*, *C. fumiferana*, *C. medinalis*, *Helicoverpa* sp., *H. puera*, *L. orbonalis*, *M. sexta*, *M. brassicae*, *M. separata*, *O. furnacalis*, *P. operculella*, *P. xylostella*, *Spodoptera* sp. Species from Noctuidae formed a separate subcluster A1 while *B. mori* (Bombycidae) and *H. puera* (Hyblacidae) formed subcluster A2 and showed the relatedness among them. *C. fumiferana* (Tortricidae) and *M. sexta* (Sphingidae) formed subcluster A3 while *L. orbonalis*, *C. medinalis*, *O. furnacalis* (Crambidae) formed subcluster A4. Notably, *P. operculella* (Gelechiidae) and *P. xylostella* (Plutellidae) falls in a separate subcluster A5 and A6 individually. Principle cluster B comprises two species *E. obliqua* (Geometridae) and *E. vitella* (Nolidae). All lepidopteran *CHSA* have a common lineage as high bootstrap value of 99 confirmed its phylogeny (**Figure 2**).

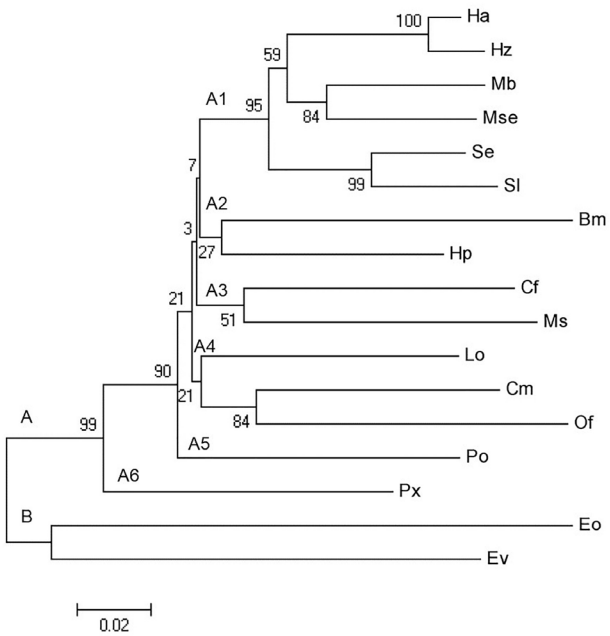

FIGURE 2 | Phylogenetic tree for *chitin synthaseA* gene in different families of lepidopterans using MEGA v5.05 software. The scale on the *x*-axis represents the branch length and the numbers indicate the bootstrap values. *Chitin synthaseA* were from *Bombyx mori* (Bm), *Choristoneura fumiferana* (Cf), *Cnaphalocrocis medinalis* (Cm), *Earias vitella* (Ev), *Ectropis obliqua* (Eo), *Helicoverpa armigera* (Ha), *Helicoverpa zea* (Hz), *Hyblaea puera* (Hp), *Leucinodes orbonalis* (Lo), *Mamestra brassicae* (Mb), *Manduca sexta* (Ms), *Mythimna separata* (Mse), *O. furnacalis* (Of), *Phthorimaea operculella* (Po), *Plutella xylostella* (Px), *Spodoptera exigua* (Se), and *Spodoptera litura* (Sl). The accession numbers for various *chitin synthaseA* used in the phylogenetic analysis are provided in Section "Materials and Methods."

Synthesis of dsRNA, Dose Effect, and Persistence of dsRNA on Target Gene

The dsRNA was synthesized from 518 bp of *SlCHSA* and "dsCheck" showed no off target gene candidate from selected region (**Supplementary Figures S5, S6**). NCBI-BLAST analysis of the nucleotide sequence of dsRNA also showed high level of sequence homology (99.00% identity) to *S. litura CHSA* gene and no significant homology to other genes of *S. litura* and other species.

The assessment of dose effect of dsRNA demonstrated that the concentration of 3–9 μg dsRNA is ideal for further bioassay (**Supplementary Figure S7A**). Bioassay results performed with standardized 3 μg/larvae dsRNA are presented and discussed. Time course expression analysis showed the maximum down-regulation at 48–72 h after treatment than 24 and 96 h after treatment (**Supplementary Figure S7B**).

Bioassay Study With *SlCHSA* dsRNA

The *SlCHSA* dsRNA exhibited the lethal phenotypes like "Half-ecdysis" and "Black body." The phenotype terminologies were used as per reported in *S. exigua* (Tian et al., 2009). Arrest in molting process also called as "Half- ecdysis" in which insects were not able to molt to next instar, was recorded in more than 80% of the larvae at 24–48 h after treatment. Notably, molting process was delayed by 24 h in dsRNA treated larvae as compared to control (DEPC treated H_2O). About 25% larvae turned black at 48 h after treatment and designated as "Black body" phenotype or hyper pigmented phenotype (**Figure 3A**).

The effect of *dsSlCHSA* on the growth and development of *S. litura* larvae was assessed by recording the larval body weight and lethality. The larval body weight reduction of 8.0% was observed at 24 h in *S. litura* fed with *dsSlCHSA* as compared to control. On the contrary, positive control (Novaluron) showed a significant reduction in larval body weight until pupation (**Figure 4A**). Similarly, lethality in *dsSlCHSA*-treated groups was calculated at different developmental stages. Lethality in dsRNA-treated groups continuously increased with time and lies from 12.5 to 57.5% while 40.0 to 87.5% in the positive control. Significant lethality was observed in fourth and fifth instar larvae of *S. litura* and its pupal stage. There were no lethal effects on growth and development of *S. litura* larvae fed with control (**Figure 4A**). The relative expression level using RT-qPCR analysis also showed the abundance of *SlCHSA* on treated larvae with 2.32-fold lesser expression than the control (**Figure 4A**).

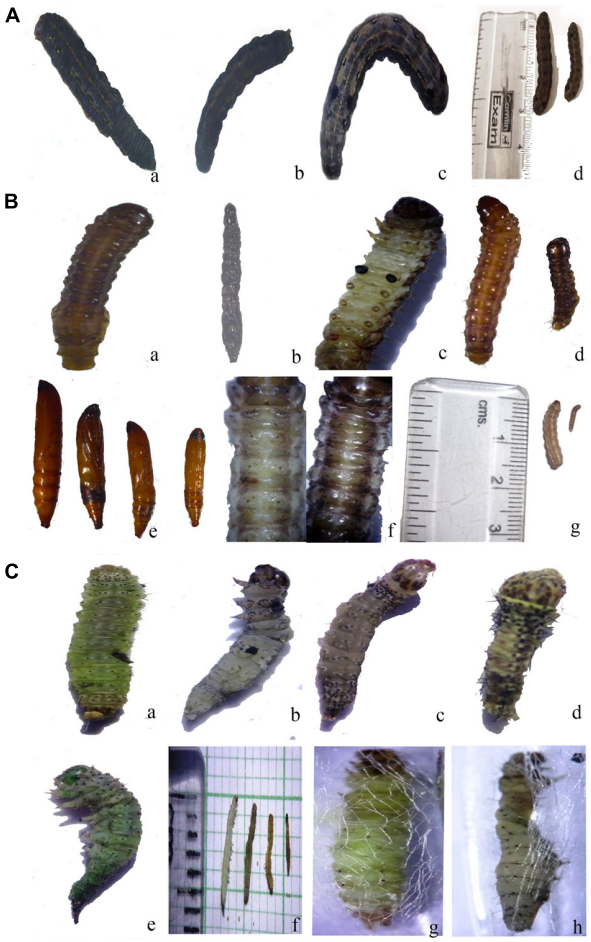

FIGURE 3 | (A) Phenotype of *S. litura* after ingestion of *SlCHSA* dsRNA **(a)** "half ecdysis" phenotype of fourth instar larvae; **(b)** "black body" phenotype of fourth instar larvae; **(c)** normal phenotype of fourth instar larvae; **(d)** larvae of *S. litura*; control and treatment. **(B)** Phenotype of *C. partellus* after ingestion of *CpCHSA* dsRNA **(a)** "half ecdysis" phenotype of fourth instar larvae; **(b)** "black body" phenotype of fourth instar larvae; **(c)** pigmented spiracle; **(e)** abnormal phenotype at pupal stage; control and treatment; **(d,f,g)** larvae of *C. partellus*; control and treatment. **(C)** Phenotype of *P. xylostella* after ingestion of *PxCHSA* dsRNA **(a)** Control; **(b,c)** "black body" and melanized phenotype; **(d,e)** abnormal phenotype cramped, shrinked; **(f)** control and treated larvae; **(g)** control pupal stage and **(h)** abnormal pupal stage.

Bioassay Study With *CpCHSA* dsRNA

Similar, "half-ecdysis" and "black body" phenotypes were observed in *C. partellus* larvae at 48 h after treatment. In addition, the melanized spiracle, shrinked larvae, and abnormal pupae were also observed in the larvae fed with *CpCHSA* dsRNA and no lethal phenotypes in control (**Figure 3B**). The larval body weight was reduced by 26.00% at 24 and 48 h after treatment compared to control (**Figure 4B**). dsRNA-treated groups shown lethality from 10.0 to 67.5%, affecting significantly the later larval (fifth, sixth instar) and pupal stages while positive control showed obvious lethality of 52.0–85.0% (**Figure 4B**). Further, RT-qPCR analysis showed that the abundance of *CpCHSA* was 1.49-fold lower than the control (**Figure 4B**).

Bioassay Study With *PxCHSA* dsRNA

The *PxCHSA* dsRNA resulted in "black body" phenotype in larval as well as in the pupal stage at 48–72 h after treatment. Similar, cramped, shrinked, melanized, hyperpigmented cuticle abnormalities were observed. Positive control also showed similar phenotypes (**Figure 3C** and **Supplementary Figure S8**). Notably, highest significant reduction of 64.00% in larval body weight was observed at 48 and 72 h after treatment (**Figure 4C**). Similarly, *dsPxCHSA*-treated groups showed increased lethality from 26.66 to 70.00 with significant lethal effects at fourth instar and pupal stage (**Figure 4C**). The results also correlate with the 8.33-fold decreased expression of *PxCHSA* in *dsPxCHSA*-treated group than the control (**Figure 4C**).

Bioassay Study With *MvCHSA* dsRNA

In *M. vitrata*, about 50% of larvae turned black at 48 h after treatment and mortality was observed at 96 h after treatment while no lethal phenotypes were observed in control group. Significant differences in larval body weight was observed at 48, 72, and 96 h after treatment with 56% reduction in larval body weight compared to control (**Figure 4D**). Similarly, lethality in *dsMvCHSA*-treated groups was substantially increased from 5.0 to 50.0 and 52.5 to 85.0% in the positive control, at different developmental stages. Statistical analysis showed significant lethal effects at fifth instar and pupal stage of *M. vitrata* and the relative expression level of *MvCHSA* gene showed 1.38-fold lower in treated and control groups (**Figure 4D**).

Molecular Analysis of Tobacco Transformants

A total of 45 independent putative transgenic tobacco shoots were recovered from 80 leaf explants after 4 weeks of co-cultivation with *A. tumefaciens* LBA4404 harboring pRNAi-CHSA construct. Fourteen of the 45 shoots were regenerated into whole plant and established in greenhouse. These established putative plants were used in further molecular analysis. Twelve of the 14 plants regenerated with pRNAi-CHSA construct found to be positive for the amplification of ∼296 and ∼530 bp partial sequences of *nptII* and *SlCHSA* genes. A similar band was observed in their respective positive control, pRNAi-CHSA, whereas untransformed control tobacco plants did not show any amplification (**Supplementary Figure S9**). The PCR positive tobacco plants were further validated using RT-qPCR which showed substantial amount of the *SlCHSA* expression level with highest in *dsSlCHSA* E4 plants (>4-fold change). The plants showing > 2-fold change (*dsSlCHSA* E4, E5, E6, E9, and E11) were selected for the bioassay studies (**Supplementary Figure S10**).

Feeding Bioassay With Transgenic Tobacco Plants Expressing *dsSlCHSA*

Insect feeding trials with detached mature transgenic tobacco leaves expressing *dsSlCHSA* dsRNA showed lethal phenotypes such as "Half ecdysis," "Black body," and "Double head" at larval stage. Some abnormal phenotypes were also observed at pupal, pupal–adult intermediates, and adult stage (**Figure 5**). Significant

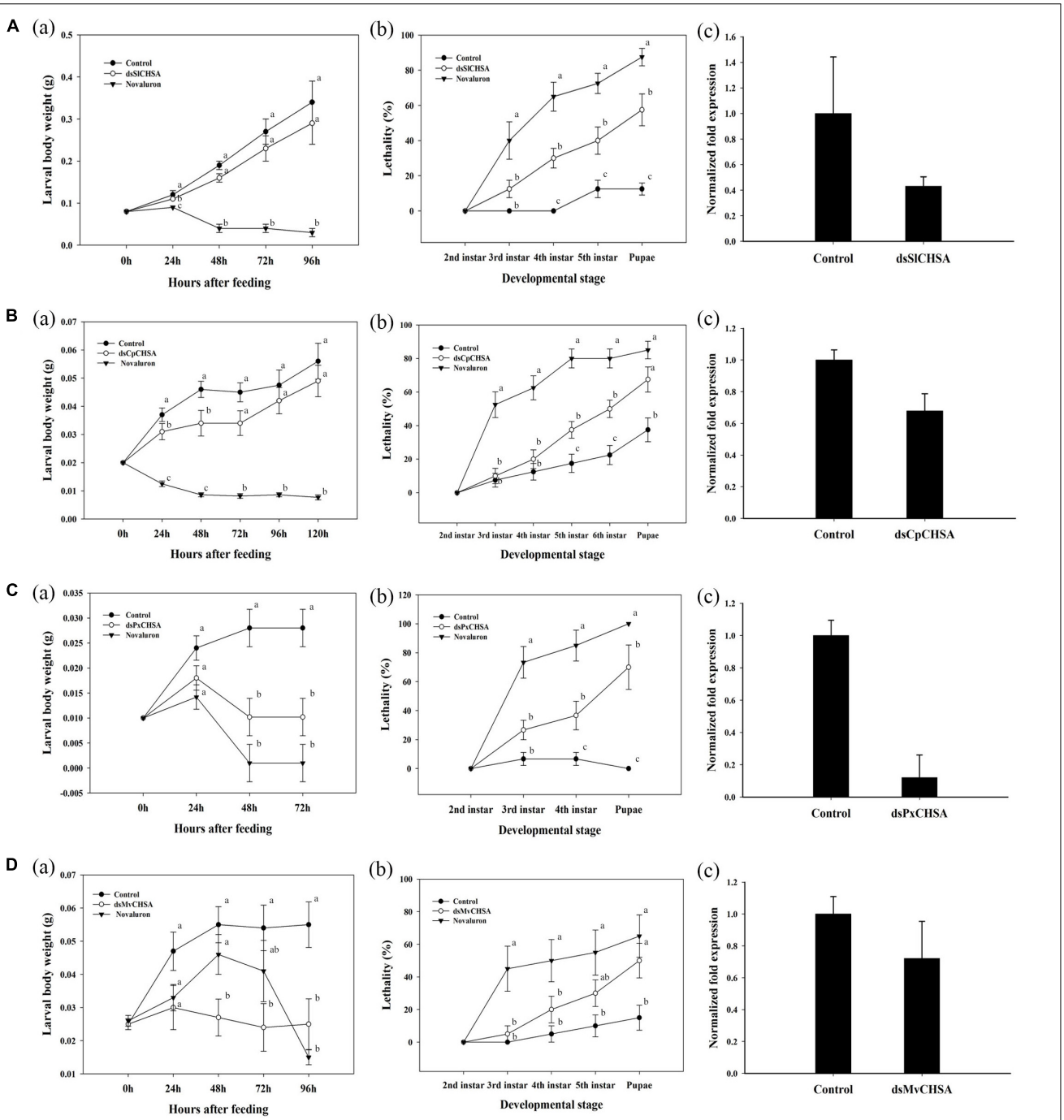

FIGURE 4 | (A) Effect of ingested *dsSlCHSA* on *S. litura* larval body weight, lethality, and expression level. **(a)** The growth of *S. litura* after feeding *dsSlCHSA* was significantly delayed and larval weight reduced. **(b)** Ingestion of *dsSlCHSA* caused significantly higher lethality than in control. **(c)** *SlCHSA* mRNA level in *S. litura* was decreased after 96 h of feeding. **(B)** Effect of ingested *dsCpCHSA* on *C. partellus* larval body weight, lethality, and expression level. **(a)** The growth of *C. partellus* after feeding *dsCpCHSA* was significantly delayed and larval weight reduced. **(b)** Ingestion of *dsCpCHSA* caused significantly higher lethality than in control. **(c)** *CpCHSA* mRNA level in *C. partellus* was decreased after 120 h of feeding. **(C)** Effect of ingested *dsPxCHSA* on *P. xylostella* larval body weight, lethality, and expression level. **(a)** The growth of *P. xylostella* after feeding *dsPxCHSA* was significantly delayed and larval weight reduced. **(b)** Ingestion of *dsPxCHSA* caused significantly higher lethality than in control. **(c)** *PxCHSA* mRNA level in *P. xylostella* was decreased after 72 h of feeding. **(D)** Effects of ingested *dsMvCHSA* on *M. vitrata* larval body weight, lethality, and expression level. **(a)** The growth of *M. vitrata* after feeding *dsMvCHSA* was significantly delayed and larval weight reduced. **(b)** Ingestion of *dsMvCHSA* caused significantly higher lethality than in control. **(c)** *MvCHSA* mRNA level in *M. vitrata* were decreased after 96 h of feeding. Three biological replicates, each consist of pooled RNA from three to five larvae were used for analysis. The RT-qPCR data were analyzed using the delta–delta Ct method. Housekeeping gene, *Actin* used as internal control. The mRNA level in the treated group was relative to control group at the same time point. Error bars indicate standard error of mean. Statistical significance of difference was analyzed with ANOVA ($P = 0.05$). Means with different letters are significantly different (Tukey's test).

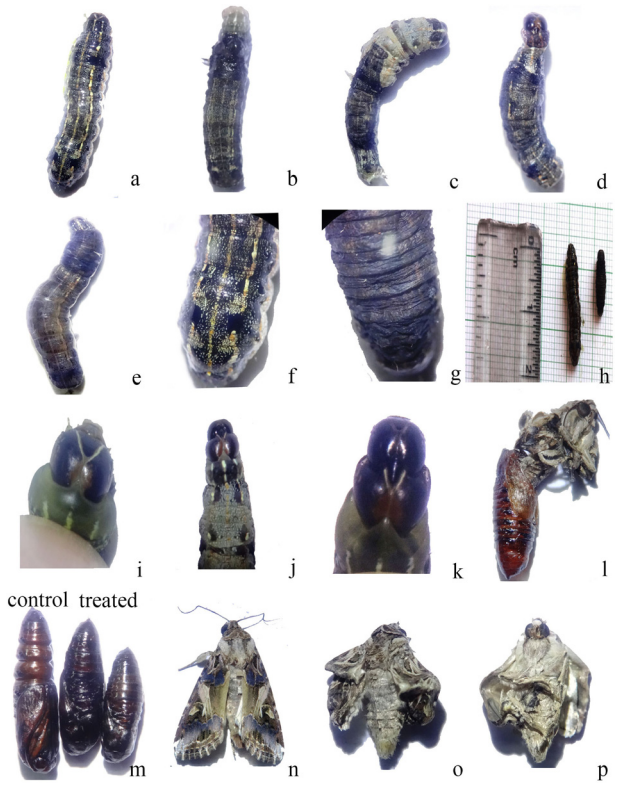

FIGURE 5 | Phenotype of *S. litura* larvae after feeding with transgenic tobacco plants expressing *dsSlCHSA* **(a,f)** Control; **(b,e,g)** "black body" and melanized phenotype; **(c,d)** "half ecdysis" phenotype of fourth instar larvae; **(h)** larvae of *S. litura*; control and treatment; **(i)** Control larvae with normal head; **(j,k)** "double head" phenotype of fourth instar larvae; **(l)** lethal phenotype at pupal–adult intermediates; **(m)** abnormal phenotype at pupal stage; control and treatment; **(n)** normal phenotype at adult stage; **(o,p)** lethal phenotype (crimpled, weak cuticle with not spreading wings) at adult stage.

reduction in larval body weight was observed at 120, 144, and 168 h after feeding of transgenic tobacco plants (*dsSlCHSA* E4, E5, E6, E9, and E11) as compared to WT (**Figure 6A**). Moreover, lethality was found more in larvae fed with *dsSlCHSA* E4 plants (25–70%) relative to WT (5.0%) (**Figure 6B**). RT-qPCR analysis had shown the varied amount of down-regulation of *SlCHSA* gene in insects at 24, 72, 120, and 168 h after feeding with an evident reduction in larvae fed with *dsSlCHSA* E4 (3–100-fold change) and *dsSlCHSA* E11 (1.5–33-fold change) transgenic tobacco plants (**Figure 6C**).

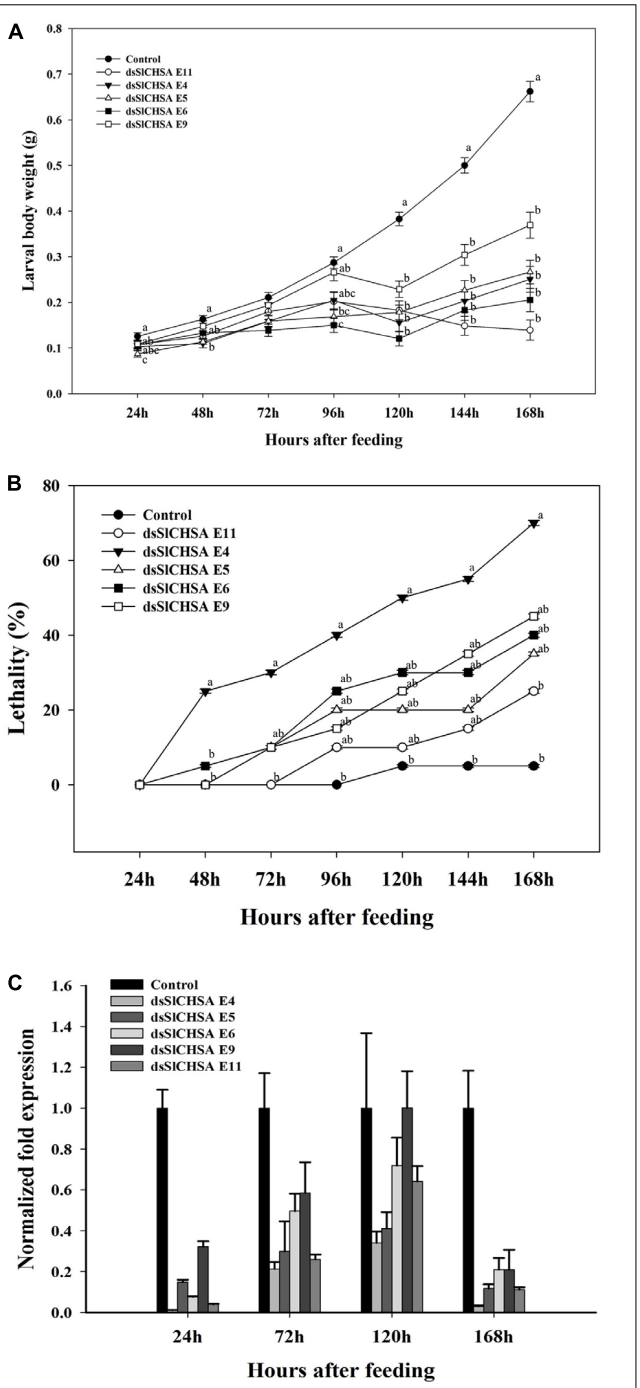

FIGURE 6 | Effect of ingestion of transgenic tobacco plant tissues expressing *dsSlCHSA* on *S. litura* larval body weight, lethality, and expression level. **(A)** The growth of *S. litura* after feeding detached mature tobacco leaves expressing *dsSlCHSA* (E4, E5 E6, E9, and E11). **(B)** Feeding caused significantly higher lethality than in control. **(C)** *SlCHSA* mRNA level in *S. litura* at 24, 72, 120, and 168 h after feeding. Three biological replicates, each consist of pooled RNA from three to five larvae were used for analysis. The RT-qPCR data were analyzed using the delta–delta Ct method. Housekeeping gene, *Actin* used as internal control. The mRNA level in the treated group was relative to control group at the same time point. Error bars indicate standard error of mean. Statistical significance of difference was analyzed with ANOVA ($P = 0.05$). Means with different letters are significantly different (Tukey's test).

DISCUSSION

RNAi technologies hold great promise for the management of insect pests. Ingestible dsRNAs/siRNAs targeting key insect genes triggered through RNAi lead to growth inhibition, developmental aberrations, reduced fecundity, and mortality. However, previous reports have shown great variability and inconsistency with the targeted insect species, targeted gene, and mode of administration of RNAi in lepidopterans from time

to time (Yang and Han, 2014). This study offers new insights to explore the potential of dsRNA-transgenic plant-mediated RNAi and design strategy for management of four key lepidopteran crop pests, viz., *S. litura*, *C. partellus*, *P. xylostella*, and *M. vitrata.*

In the present study, sequencing of partial *CHSA* gene from *S. litura*, *C. partellus*, *P. xylostella*, and *M. vitrata* has demonstrated that it forms an integral role in almost all crop pest, they are highly conserved among different species at genus level and with minor degree of variation at species level. Also, phylogenetic tree constructed using protein sequences of *CHSA* gene from different lepidopteran order is consistent with the inferred phylogeny of these larvae based on DNA sequences. Further, multiple sequence alignment and multi-protein alignment validated the sequence similarity of 72.86% for *CHSA* gene among the different lepidopteran species. Together signifying that, *CHSA* gene which is conserved in all insects of lepidopteran order is also one of the ideal targets for RNAi suppression.

Dose Effect and Persistence of dsRNA

RNAi efficiency depends on the concentration of dsRNA (Chen et al., 2008). It differs in different order of insects (Tian et al., 2009). In earlier studies, the amount of dsRNA injected into lepidopteran insect ranged from 0.1 to 6 μg/larva (Rajagopal et al., 2002; Turner et al., 2006; Chen et al., 2008). As the earlier studies were based on injection method, different from the direct dsRNA feeding method, the dose effect of 1, 3, 5, 7, 9 μg/larvae dsRNA concentration on second instar *S. litura* was studied. RT-qPCR analysis exhibited the detectable amount of down-regulation at all the concentrations, but it was more significant at 3, 5, 7, 9 μg/larvae concentrations. Furthermore, the higher concentration did not make any difference; it was constant for 3–9 μg/larvae concentrations, revealing dose saturation effect. However, considering the cost of *in vitro* dsRNA synthesis and to avoid off-target effects with excessive dsRNA, we had employed 3 μg/larvae. These results were different from an earlier study on *H. armigera* where a linear increase in down-regulation was observed from 0.4 to 10 μg dsRNA concentrations and showed dose saturation effect at 53 μg/day (Yang and Han, 2014).

Simultaneously, we demonstrated the higher effect of dsRNA on the persistence of RNAi at time intervals of 48 and 72 h in comparison to 24 and 96 h after treatment. This suggested that single application of dsRNA triggers RNAi at 48 h and sustained till 72 h after feeding. Asokan et al. (2013) also observed single application of dsRNA resulted in delayed and transient silencing, while multiple applications led to an early onset and sustained silencing in case of chymotrypsin and jhamt.

dsRNA-Transgenic Plant-Mediated RNAi Resulted in Lethal Phenotypes

The knockdown of *SlCHSA*, *CpCHSA*, *PxCHSA*, and *MvCHSA* through dsRNA in *S. litura, C. partellus*, *P. xylostella*, and *M. vitrata* respectively, documented lethal phenotypes like "Half ecdysis" and "Black body" at larval stage, which is consistent with the earlier study done in *S. exigua* (Tian et al., 2009; Li et al., 2013). On the contrary, we have also demonstrated significant change in pupal phenotype in *C. partellus* and *P. xylostella* though they did not complete the pupation stage and were not able to emerge out as adults. Similar observation was reported by Arakane et al. (2005) in *T. castaneum* larvae injected with dsRNA in the penultimate larvae which failed to pupate and did not complete pupal development. However, the insect pests targeted in our study are destructive at larval stage and control of them at early larval stage is important. The positive control Novaluron belonging to benzoylphenyl urea group and acts as an insect growth regulator by inhibiting the biosynthesis of chitin, also showed similar phenotypes suggesting dsRNA might have alike mode of action to that Novaluron. Retnakaran and Wright (1987) also observed similar phenotypes in insect treated with acrylureas which also belongs to benzoylphenyl urea group and acts as a selective disrupter of chitin synthesis in insects. Apparently, no lethal phenotypes were observed in the control group, so it was unlikely that these phenotypes were caused by injury or infection. Besides that, molting was delayed ∼24 h in the RNAi-treated group as compared to control larvae which molted normally into next larval instar.

Nevertheless, *in vitro* synthesized dsRNA is not applicable for the management of insect pest in the fields because of high concentration of dsRNA is required to cause severe RNAi effects as they are degraded in the digestive system (Zhu et al., 2012). Therefore, it is important to develop an efficient method of delivery for large scale pest control in the fields. Transgenic technology has generated insect resistant plants to reduce yield loss and utilization of pesticides (Kos et al., 2009). However, there is continuous development of insect resistance and outbreak of non-target pests (Lu et al., 2010; Jin et al., 2015). Thus, transgenic plants expressing suitable insect dsRNA can be exploited to control insect pests. To determine its potential over the direct dsRNA feeding, we constructed pRNAi-CHSA vector to express *dsSlCHSA* genes and validated in tobacco for mRNA abundance of the transgene, and *S. litura* larvae were evaluated for down-regulation of *CHSA* gene expression. Tobacco and *S. litura* were used as plant and insect model system, respectively, for the preliminary studies and the generation of transgenic plants specific for other insects is future line of work. Our results suggested that *S. litura* larvae fed with leaves of transgenic tobacco plants expressing *dsSlCHSA* manifested same lethal phenotypes such as "Half ecdysis," "Black body," and "Double head phenotype" leading to mortality. These phenotypes were like those reported in *S. exigua* after feeding bacterially expressed dsRNA of *SeCHSA* gene (Tian et al., 2009) but they did not observe any abnormality at later developmental stages which was different from our observations. We also observed abnormal phenotypes (crimpled) in pupal, pupal–adult intermediates, and adult stage as reported in *H. armigera* (Zhu et al., 2012) and partly in agreement with insects of other orders, viz., *T. castaneum* (Arakane et al., 2005, 2008), *L. migratoria* (Zhang et al., 2010; Liu et al., 2012) and *N. lugens* (Wang et al., 2012). Importantly, the lethal phenotypes observed in larva–pupa stage and pupa–adult stage were in agreement with the earlier report in *S. exigua* targeting trehalase gene (*SeTre-1*) which inhibits the expression of *CHSA* gene (Chen et al., 2010). These lethal phenotypes were

more pronounced compared to phenotypes observed with direct application of dsRNA.

dsRNA-Transgenic Plant-Mediated RNAi Affecting Growth and Development

Reduction in larval body weight at different times period among the four lepidopteran species suggested the time sensitivity of dsRNA molecules, which may reach at threshold level (required to inhibit the target) at different time depending on the species. The *S. litura* larvae showed less reduction in larval body weight compared to *C. partellus*, *M. vitrata*, and *P. xylostella*. One possible reason could be the variation in the physiological conditions of the gut fluids or the presence of dsRNAase in the digestive tract of insects. Still the mechanism underlying the variability is not well understood in lepidopterans and it is likely that a lot remains to be discovered. Moreover, the growth pattern of the RNAi-treated group was more like Novaluron treated group compared to control in *P. xylostella* and *M. vitrata*, with a drastic reduction at the later developmental stages. This presents an opportunity for molecular biologists in developing insect-specific molecular biopesticides using dsRNA. To our knowledge, this is only study where commercially available product for controlling insect pests has been used for the comparative studies. Another factor measured lethality demonstrated higher effects during late developmental stages in all four lepidopteran spp. A previous study in lepidopteran *M. separate* also reported effects at later developmental stages with only 10–16% mortality using bacterially expressing chitinase gene (Ganbaatar et al., 2017). We showed higher lethality in *dsPxCHSA* treated group and *S. litura* fed with leaves of transgenic tobacco plants expressing *dsSlCHSA* (dsSlCHSA E4) (∼70%). The down-regulation of *CHSA* gene did not yield cent percent lethality or phenotypic variation in all the four insects, which may be due to the incomplete down-regulation of *CHSA* gene and some amount of *CHSA* transcript might have translated to produce chitin.

dsRNA-Transgenic Plant-Mediated RNAi Reduces *CHSA* mRNA Level

Expression analysis through RT-qPCR showed decreased *CHSA* mRNA level in all the four lepidopterans. Higher down-regulation of *CHSA* was observed in *dsPxCHSA* and transgenic plant-mediated RNAi, affecting all molting stages (larval–larval, larval–pupal, and pupal–adult) and cuticular chitin synthesis. A previous study in *S. litura* using injection of dsRNA did not cause lethal phenotypes and lethality, in spite of reduction in expression level (Rajagopal et al., 2002). We also demonstrated that transgenic tobacco plants expressing *dsSlCHSA* showed positive correlation between the mRNA abundance of *SlCHSA* and percent lethality, which was further validated by RT-qPCR analysis that showed higher down-regulation of *SlCHSA* gene in insects fed on leaves of dsSlCHSA E4 plant. Such positive correlation between expression level of Cry protein and insect lethality have been also reported in many studies (Bhattacharya et al., 2002; Estrada et al., 2007; Ramu et al., 2012). Furthermore, the bell-shaped trend of normal *CHSA* gene expression remains the same after down-regulation of *CHSA*, which increases continually from first to the last instar and decreases from pupal to the adult stage. This exhibited stage specific and target specific manner of RNAi efficacy. Therefore, stage of insect to be targeted based on target gene expression and selection of target genes are the critical considerations before commencing RNAi experiments.

CONCLUSION

RNAi is a sequence-specific gene silencing mechanism mediated by dsRNA, which has been harnessed as a useful tool in devising novel insect pest management strategies. Our study demonstrates efficacy of dsRNA-transgenic plant-mediated RNAi in reducing mRNA transcript level of specific targeted gene causing lethal phenotypes, reduction in larval body weight, and eventually mortality. Although the inconsistencies lie between the insects belonging to same order, targeting same gene nevertheless the technology has proved its potentiality. *CHSA* targeted in present study is as an ideal target gene and presents an effective alternative opportunity for designing broad spectrum biopesticide for the management of insect pests.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SR conducted the experiments and wrote the manuscript. SR and AR analyzed the data. SM and KK conceptualized and supervised the project. AR, SM, and KK edited the manuscript. All authors read and approved the final document.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00427/full#supplementary-material

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Barriers to Efficient Foliar Uptake of dsRNA and Molecular Barriers to dsRNA Activity in Plant Cells

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Foliar application of dsRNA to elicit an RNA interference (RNAi) response is currently under consideration as a crop protection strategy. To access the RNAi machinery of a plant, foliarly applied dsRNAs must traverse the plant cuticle, avoid nuclease degradation, and penetrate the cell wall and plasma membrane. Application methods and co-formulants have been identified by Bayer Crop Science researchers and others that can help bypass barriers to dsRNA uptake in plants leading to an RNAi response in greenhouse grown, young plants and cell cultures. However, these advances in dsRNA delivery have yet to yield systemic RNAi silencing of an endogenous gene target required for product concepts such as weed control. Systemic RNAi silencing in plants has only been observed with the *GFP* transgene in *Nicotiana benthamiana*. Because biologically meaningful whole plant RNAi has not been observed for endogenous gene products in *N. benthamiana* or in other plant species tested, under growing conditions including field production, the regulatory risk assessment of foliarly applied dsRNA-based products should not consider exposure scenarios that include systemic response to small RNAs in treated plants.

Keywords: RNAi, dsRNA, barriers in plants, systemic silencing, local silencing

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INTRODUCTION

Weed control in crops using herbicides is one of the most important agronomic practices in modern agriculture. In order to work toward the development of biologically based herbicides, foliar application of double-stranded RNA (dsRNA) to plants was evaluated as a method to control weeds (Sammons et al., 2011) as well as plant pests and pathogens though a mechanism known as RNA interference (RNAi). To achieve the desired RNAi response and subsequent phenotype for weed control (including those with an herbicide-resistant phenotype), the foliarly applied dsRNA must ultimately be delivered into a sufficient number of responsive cells in the target organism. This dsRNA must travel from the surface of a leaf through the waxy cuticle, and then traverse the apoplast, cell wall, and plasma membrane to gain access to the plant cell's RNAi machinery. Once inside the cell, the applied dsRNA can move to adjacent cells through plasmodesmata and subsequently to distal cells through the phloem vasculature. Alternatively, small dsRNAs (aka secondary or transitive siRNAs) generated through the cell's RNAi machinery can also travel symplastically to the phloem vasculature and to distal cells. Distal movement of applied or generated dsRNA can result in distal or systemic RNAi thus facilitating efforts to employ dsRNA-based products to manage weeds. This differs from the proposed use of dsRNA as an agriculture insecticide where

access to the plant cell's RNAi machinery isn't required to get the desired phenotype (Vogel et al., 2019). This document will summarize the current understanding of the barriers to efficient cellular delivery of nucleic acids (dsRNA and dsDNA) after foliar application to plants and some of the efforts to identify formulations that overcome these barriers. Specifically, this document will focus on the cuticle, nucleases, and cellular uptake as barriers, and will also discuss the requirements of the dsRNA structure to complete successful RNAi.

BARRIERS TO EFFICIENT FOLIAR UPTAKE OF dsRNA

Cuticle as a Barrier

The plant cuticle is a lipophilic film generated by and covering the epidermis of leaves, young shoots, and fruit. The primary purpose of the cuticle is to prevent dehydration of plant surfaces. The cuticle is also known to impede the absorption of exogenous water and solutes (Schreiber, 2005). We hypothesize that it is these properties of the cuticle that make it resistant to dsRNA absorption, given the water solubility of dsRNA. To test this hypothesis, a fluorescently labeled (Cy3) 21 base pair (bp) siRNA was applied to the adaxial surface of Palmer Amaranth (*Amaranthus palmeri*) with 0.5% of the spreading surfactant Silwet L-77. Treated leaf cross-sections were collected at 4 h post application and assessed for the presence of Cy3-siRNA using fluorescence microscopy. A representative image obtained from this study is provided in the **Supplementary Figure S1**. Note that most of the applied Cy3-siRNA was found on the surface of the leaf. The fact that the Cy3-siRNA was mostly on the leaf's surface was not surprising due to its size (MW > 14,000 Da for a 21-mer) and that it is a relatively water-soluble molecule.

To enhance cuticle penetration of siRNA and achieve robust visual RNAi phenotypes in this and other research (Dalakouras et al., 2016; Huang et al., 2018), abrasion, high pressure spraying, and abaxial stomatal flooding have been utilized. We have observed that spraying particles (celite, alumina, etc.) of sizes >2 microns with or after siRNA application at pressures <700 kPa resulted in improved cuticle penetration of the siRNA (**Figure 1**) and resulted in robust visual RNAi phenotypes (Huang et al., 2018). In addition to sprayed particles, other abrasive methods, such the use of sandpaper, resulted in improved siRNA penetration and RNAi silencing after adaxial foliar siRNA application. Consistent with these results, Dalakouras et al. (2016) did not observe RNAi silencing of green fluorescent protein (GFP) phenotypes in *Nicotiana benthamiana* in the absence of tissue wounding. They accomplished this either biolistically with gold particles (1 μm) and a gene gun or by spraying an aqueous siRNA solution at high pressure (7–8 bar) and close (2–4 cm) to the plant surface. Dalakouras et al. (2016) reported that they did not observe GFP silencing by either pipetting the solution to the adaxial surface of the plant or by infiltration. This lack of dsRNA delivery with infiltration suggests that abrasion and high-powered spray must be impacting other aspects of siRNA delivery in addition to cuticle penetration.

Successful siRNA delivery resulting in robust visual RNAi endogenous gene and transgene phenotypes in several plant species has been demonstrated using either pipet application (**Supplementary Figure S2**) or relatively low pressure (70–140 kPa) spray (not shown) to the abaxial leaf surface. This was accomplished by including ≥0.3% of a super spreading surfactant, such as Silwet L-77[1], in the siRNA solution (0.1–1.0 mg/mL) at the time of application, which drives uptake of the dsRNA by stomatal flooding. An example of stomatal flooding siRNA delivery and resulting GFP silencing are featured in **Supplementary Figure S2**. It should be noted that the application conditions, e.g., application to the abaxial surface and surfactant concentrations, utilized in these experiments are not employed in commercial agriculture.

Nuclease Stability as a Barrier

In vivo nuclease degradation is known to impact the efficiency of applied siRNA to elicit an RNAi response in mammalian systems (Behlke, 2008). Not much is known about the impact of nucleases on the delivery efficiency of foliarly applied siRNAs in plants; however, the well documented presence of nucleases in plants suggests that nuclease degradation could impact the ability of a foliarly applied siRNA to gain access to the plant cell (Pérez-Amador et al., 2000). To investigate this, we applied 22 bp siRNA by syringe infiltration to an expanded leaf of *N. benthamiana*. Tissue samples from the infiltration site were collected at 0, 1, 2, 4, 6, and 8 h post application. Analysis of RNA extracts obtained from the collected tissues by anion exchange HPLC revealed that applied siRNA was not detected at 6 h post application (**Supplementary Figure S3**).

In a similar experiment, 22 bp siRNA was infiltrated in the presence or absence of a nuclease inhibitor or the cationic polymer polybrene. Tissue samples were collected from the infiltration site at 0, 1, 2, 4, 6, 8, and 24 h post application and analyzed using anionic exchange HPLC. As with the experiment mentioned above, siRNA applied in the absence of a nuclease inhibitor or polybrene was not detectable at 6 h post application (**Figure 2**). siRNA applied with a nuclease inhibitor or polybrene which binds dsRNA was still detectable at 24 h. Both sets of experimental results strongly suggest that nucleases act as a barrier to efficient dsRNA delivery. This assertion is further supported by recent publications demonstrating that agents such as carbon dots, single-walled carbon nanotubes, and clay nanosheets enhance nuclease stability and delivery efficiency of applied nucleic acids in plants (Mitter et al., 2017; Demirer et al., 2019; Schwartz et al., 2019).

Cellular Uptake as a Barrier

Plant cells have both a cell wall and plasma membrane that can pose as barriers to efficient nucleic acid delivery. The plant cell wall is a matrix comprised of cellulose, hemicelluloses, pectin, and other biopolymers. It provides support and protection, and acts as a filtering mechanism for plant cells. The cell wall pore size is primarily dependent on environmental factors, plant

[1]Note this level of Silwet L-77 is at least a level of magnitude higher than normally used in agriculture.

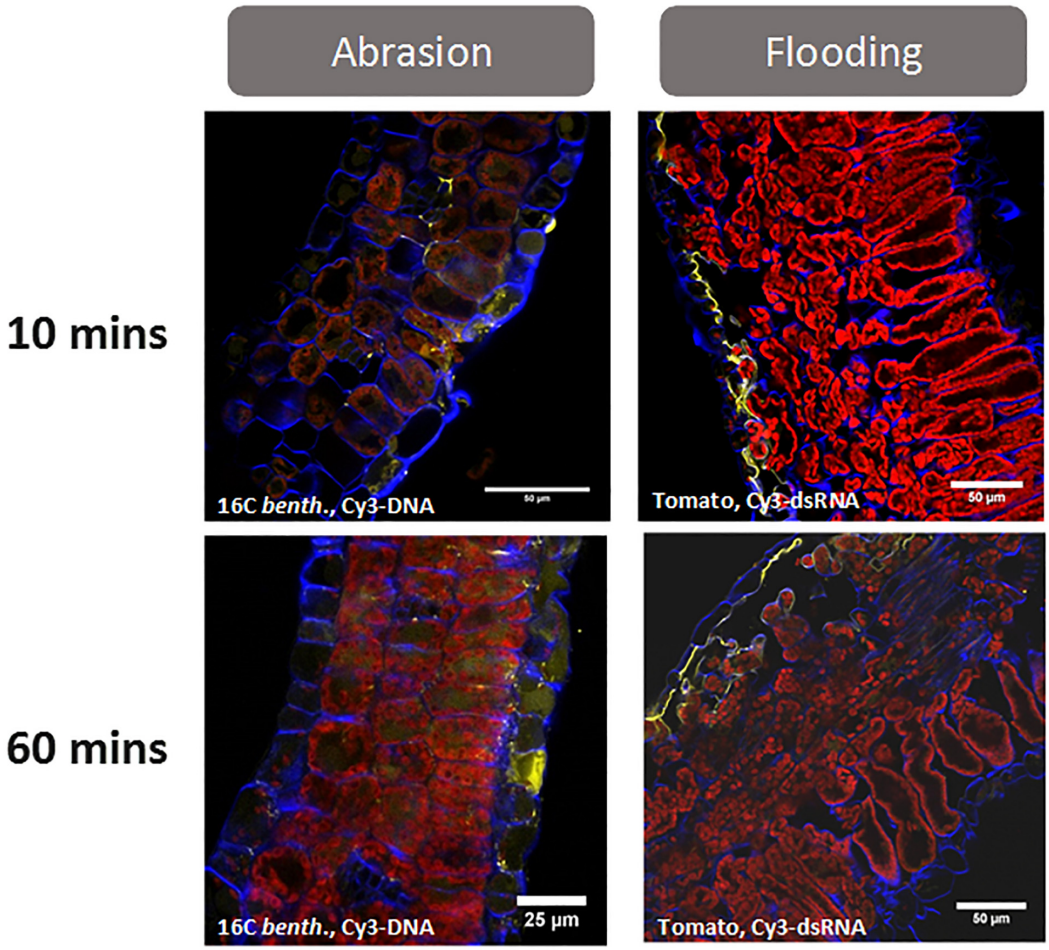

FIGURE 1 | Uptake of fluorescently labeled (Cy3) nucleic acids (dsDNA-1 and siRNA-1 are GFP sequences, see **Supplementary Table S1** for sequence information) after foliar application with either abrasion (*N. benthamiana*) or abaxial stomatal flooding (tomato). Leaf cross sections were collected at 10- and 60-min post application and visualized by confocal microscopy. The images show that both abrasion and abaxial stomatal flooding facilitate transcuticular movement of the applied nucleic acids within 10 min of treatment. Most of the Cy 3-labeled nucleic acids (yellow colored) can be found at the cells adjacent to the site of application and in the apoplast. While the difference between Cy3 and Cy3-labeled nucleic acid cannot be determined using a traditional confocal microscope, hyperspectral confocal fluorescence microscopy (Pedroso et al., 2009) was used in previous validation studies to confirm the yellow signal was due to Cy3 -labeled nucleic acid (unpublished Bayer Crop Science research). Abrasion was conducted using 0.5 mg.ml^{-1} Cy3-dsDNA^{-1} in water, 10 µl.leaf^{-1} and abraded with a 600-grit sandpaper. The stomatal flooding method is described in **Supplementary Figure S2**. For microscopy, a 5 mm punch from treated leaves was infiltrated in 4% paraformaldehyde/l×PBS followed by sucrose equilibration (10, 20, and 30% in l×PBS for 2–3 h each). Blue fluorescence represents cell wall, and red fluorescence is from chlorophyll.

species, and cell type (Carpita et al., 1979). Macromolecules such as globular proteins (MW≈17 kDa) and extracellular polysaccharides (MW > 100 kDa) have been shown to pass through plant cell walls (Bauer et al., 1973; Carpita et al., 1979). To characterize the nucleic acid exclusion limit for plant cells, we incubated GFP-expressing BY-2 suspension cells with and without flg22 (a 22-amino acid flagellin fragment) and either a 21, 50, or 90 bp (MW = 12.9 kDa, 32.8 kDa, and 55.5 kDa, respectively) fluorescently labeled (pHRodo) double-stranded DNA (dsDNA). DNA was used as a surrogate for double-stranded RNA in this study because of challenges getting synthesized RNAs >48 bp from commercial sources. DNA has similar physiochemical attributes (charge density, hydrophilicity, etc.) as RNA that make it a suitable replacement in delivery studies. Flg22 is known to stimulate ligand-induced endocytosis in plants. Uptake of the applied DNA by formed endosomes would be an indication that it was able to successfully pass through the cell wall thus gaining access to the plasma membrane. Fluorescent imaging of the treated BY-2 cells indicated that the 21 and 50 bp DNAs were more readily taken up by flg22-stimulated endosomes than the 90 bp DNA (**Supplementary Figure S4**). This result indicates an exclusion limit of between 32.8 and 55.5 kDa for nucleic acids in BY-2 cells. It should be noted that none of the DNAs screened were internalized by the cells without flg22 stimulation, suggesting that the plasma membrane is also a barrier to efficient cellular uptake and that transfection agents are required for nucleic acid delivery to plant cells. Transfection agents have been shown to improve both DNA and RNA delivery

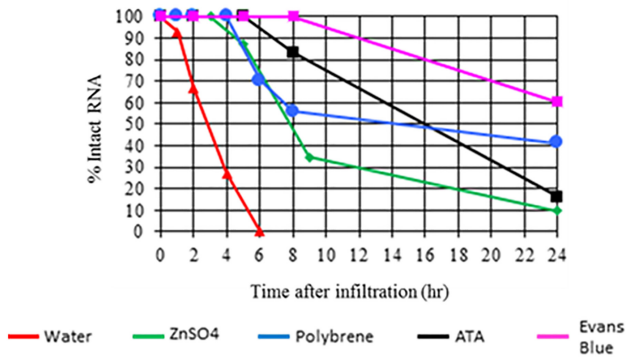

FIGURE 2 | Impact of nuclease inhibitors and polybrene on *in planta* persistence of applied 22 bp siRNA (siRNA-2 from GFP, see **Supplementary Table S1** for sequence information and additional experiment information). Aqueous solutions of siRNA-2 either alone or with a nuclease inhibitor or polybrene were applied to expanded leaves of *N. benthamiana* by syringe infiltration. Tissue samples were collected from the infiltration site at 0, 1, 2, 4, 6, 8, and 24 h after application. The applied siRNA was extracted from the samples and analyzed by anionic exchange HPLC. Results indicate that siRNA applied without a nuclease inhibitor or polybrene was completely degraded by 6 h. siRNA applied with nuclease inhibitors or polybrene was still detectable at 24 h.

efficiency in plant cells (Bennett et al., 2017; Demirer et al., 2019; Schwartz et al., 2019).

To achieve the robust and reproducible whole plant RNAi phenotypes necessary for weed and plant virus control product concepts, the applied dsRNA or the resultant secondary/transitive sRNAs must be able to assert their activity throughout the plant, including tissues not accessible to topical application. Identification of co-formulants that enable foliar applied dsRNA to overcome the barriers listed above have indeed yielded improvements in both dsRNA delivery efficiency and RNAi triggered endogenous gene and transgene phenotypes in plants (Huang et al., 2018). Literature examples of robust and reproducible whole plant RNAi after foliar application of dsRNA have been limited to transgene silencing in plant species such as *N. benthamiana* (**Supplementary Figure S5**; Dalakouras et al., 2016; Huang et al., 2018). Other examples in the literature of whole plant endogenous gene silencing phenotypes through foliar application of exogenous nucleic acid application involved the use of biological vectors such as viral induced gene silencing (VIGS) (Burch-Smith et al., 2006). Application of such vectors would likely not be suitable for commercial applications, due to the additional regulatory burden and region-specific acceptance of crops with biotechnology derived traits.

We have made progress toward achieving robust and reproducible gene silencing in treated leaves of plants by identifying methods and co-formulants that overcome the barriers to efficient delivery of foliarly applied dsRNA delivery. However, these methods, which result in both local (where applied to leaf) and systemic (untreated leaf) silencing of a GFP transgene in 16C *N. benthamiana*, result only in local silencing of the GFP transgene in other plant species such as *Arabidopsis thaliana* and for endogenous genes such as magnesium chelatase in *Amaranthus cruentus* (**Supplementary Figure S5**). Attempts by others to improve the movement of applied dsRNA through the plant's vasculature and achieve systemic distribution and RNAi in tissues not readily accessible to a spray such as the apical meristem have not been successful (Dalakouras et al., 2018). In research published by Dalakouras et al. (2018) exogenous dsRNA was applied directly to the vasculature by petiole absorption of 16C *N. benthamiana* and other plants. This approach was successful in achieving systemic distribution of exogenous dsRNA, however, it did not result in down regulating the transgene product levels since the observed movement was through the apoplast and xylem. Cellular uptake and movement through the phloem was not observed so the applied dsRNA did not have access to the dicer-like endonucleases or other RNAi pathway enzymes required for initiating RNAi (Dalakouras et al., 2018). Distribution such as this through the apoplast and xylem might be applicable for pest management by targeting RNAi in insects and fungi (Koch et al., 2016; San Miguel and Scott, 2016). It is not useful, however, to achieve the whole plant silencing phenotypes desired for weed and plant virus control product concepts.

dsRNA Properties Constrain RNAi Activity in Planta

In addition to containing sequences that match the target gene, the specific dsRNA size and structure is important for successful local or systemic down-regulation of a plant gene product from topically applied dsRNA.

Down-regulation of targeted genes using topically applied RNAs has been observed with dsRNAs from 21 to ∼150 bp, but 22 bp siRNAs produced the strongest silencing phenotypes (Dalakouras et al., 2018; Hendrix et al., 2020; unpublished Bayer Crop Science data). It is hypothesized that 22 bp siRNAs provide the greatest silencing efficiency, compared to the other dsRNAs tested, because they can induce production of secondary (transitive) siRNAs for the targeted mRNAs, which could increase the number of active silencing siRNAs for that gene target (Chen et al., 2010; Cuperus et al., 2010). Topically applied 22 bp siRNAs induced production of secondary siRNAs homologous to the target mRNA for all targeted genes examined, although the amount of secondary siRNAs varied by gene (Hendrix et al., 2020). These 22 bp siRNAs also generated a greater silencing phenotype in the leaf to which they were applied compared to 21 bp siRNAs for a variety of targeted genes. Importantly, 22 bp siRNAs, but not 21 bp siRNAs, were also able to trigger systemic silencing for the GFP transgene in *N. benthamiana* (Dalakouras et al., 2016; Hendrix et al., 2020), which is discussed further below.

In our research we observed that 2 bp 3′ overhangs are required for strong silencing activity of topically applied small to mid-sized dsRNAs (21 – 60 bp) in plant cells (unpublished Bayer Crop Science research). These overhangs are particularly sensitive to degradation by RNAses found in plant tissues

(unpublished Bayer Crop Science research), and so these structures may not be very long-lived in the environment.

Work in Bayer laboratories has shown that not every target gene can be successfully down-regulated using topically applied siRNAs or dsRNAs. Reduced mRNA concentration was observed for only 40% of gene products targeted, after testing at least 4 homologous 22 bp siRNAs per gene (unpublished Bayer Crop Science research). A weak correlation between target gene expression level and ability to down-regulate that gene with topically applied siRNA suggested that highly expressed genes may be more readily silenced. However, reliable predictors of targets that can be silenced using topical RNA have not yet been identified. Other hypotheses for the limitation on silencing of targets is that secondary structure of the mRNA *in vivo* (Ding et al., 2013), or protein interactions with an mRNA may limit accessibility of the sequence to a dsRNA (Liu et al., 2014).

Further, not all 22 bp siRNAs against responsive targets are efficacious. Rules for efficacious siRNAs have been described in the literature based on research with mammalian cells (Reynolds et al., 2004; Jagla et al., 2005), and some of these are expected to be important for plant cells also. However, only approximately half of siRNAs that were selected based on such guidelines were efficacious in reducing mRNA concentration for a targeted gene that could be silenced (unpublished Bayer Crop Science research). More recently, an *in vitro* assay was developed to identify efficacious siRNAs (esiRNAs) targeting a plant virus gene (Gago-Zachert et al., 2019). The two critical features for the esiRNA were ability to bind Argonaute proteins and the ability to access the target RNA. The *in vitro* assay correlated well with *in planta* anti-viral activity. However, further refinement of rules for selecting *in silico* which siRNAs will be efficacious for silencing genes would be useful.

Systemic Silencing Using Topical dsRNA Has Only Been Documented for the GFP Transgene in *N. benthamiana*

Systemic RNAi has been observed for the GFP transgene in *N. benthamiana* line 16C (Ruiz et al., 1998) using topical RNA with dsRNAs that are 124 bp or 22 bp, but not 21 bp (Dalakouras et al., 2016; Schwartz et al., 2019; Hendrix et al., 2020; unpublished Bayer Crop Science research). The ability of 22 bp siRNAs to cause systemic silencing is likely related to their ability to induce production of secondary sRNAs (Chen et al., 2010; Cuperus et al., 2010; Dalakouras et al., 2020; Hendrix et al., 2020). Activity by longer dsRNAs to induce systemic silencing may result from generation of 22 bp siRNAs by dicer-like enzymes in plant cells.

Strongly expressed GFP transgenes have been targeted in tomato and *Arabidopsis* by topical application of 22 bp siRNAs, and while strong local silencing was achieved, no systemic silencing was ever observed those species (**Supplementary Figure S5** and unpublished Bayer Crop Science research).

A number of endogenous genes were down-regulated with topically applied siRNAs in *N. benthamiana*, but systemic silencing was not observed (unpublished Bayer Crop Science research). Endogenous genes that have been targeted in *N. benthamiana* included magnesium cheletase subunit H (CHL-H), magnesium cheletase subunit I, GUN4, and phosphoribosylanthranilate transferase (Hendrix et al., 2020; and unpublished Bayer Crop Science research). Visible local silencing and/or reduced mRNA levels were observed after targeting each of these gene products in *N. benthamiana*, but no systemic phenotypes were detected. Endogenous genes including CHL-H, HSP70, Ubiquitin B and others were down-regulated in Amaranth species and strong local phenotypes were evident, but no systemic RNAi response was observed (**Supplementary Figure S5** and unpublished Bayer Crop Science research). In **Supplementary Figure S5C**, magnesium chelatase silencing can be observed as yellow spots in several leaves after treatment of young plants with *CHL-H* siRNA delivered with the particle spray method. All of the leaves showing silencing of magnesium chelatase were present at the time of treatment, and all observed silencing phenotypes can be explained by direct delivery of siRNA. In canola, the strongly expressed CP4 gene that conveys tolerance to the herbicide glyphosate was silenced locally but no systemic silencing was observed (unpublished Bayer Crop Science research).

The presence of introns in most endogenous genes may limit their susceptibility to systemic gene silencing (Christie et al., 2011; Dadami et al., 2014; Dalakouras et al., 2020). However, McHale et al. (2013) showed systemic silencing of the endogenous chalcone synthase gene in *Arabidopsis* using a 22-nt artificial miRNA. Further work should be done to better understand the role of introns in systemic gene silencing.

Currently, the underlying reason(s) for the limitation of systemic RNAi response by topical dsRNA application to only the GFP transgene and only in 16C *N. benthamiana* is unclear.

CONCLUSION

Plant tissues have many barriers to entry of foreign and topically applied nucleic acids. Even if a sprayed dsRNA is formulated or applied in a manner that helps to bypass the plant's physical (e.g., cuticle, cell wall) and biochemical barriers (e.g., nucleases) and does enter a plant cell, many molecular barriers exist that must be overcome to generate a biologically meaningful RNAi response from the plant. As with all RNAi, the first requirement is that the nucleic acid sequence is complementary to the sequence of a gene product that is expressed in the cell that received the dsRNA; however, our research demonstrated that sequence match alone does not guarantee a biologically meaningful RNAi response. The structure of the dsRNA needs to be efficacious for an RNAi response, including presence of 2 nt 3′ overhangs - which tend to be degraded by plant or microbial nucleases. Finally, even if local down-regulation of a gene product occurs, evidence from this research as well as the literature suggests that a systemic RNAi response would be very unlikely to occur. Therefore because biologically meaningful whole plant RNAi has not been

observed for endogenous gene products in *N. benthamiana* or in other plant species tested, under growing conditions including field testing, the regulatory risk assessment of foliarly applied dsRNA-based products should not consider exposure scenarios that include systemic response to small RNAs in treated plants.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made publicly available, the authors are willing to accept requests for data sets referenced in this article. However, since legal intellectual property review may be necessary to release the data sets, the authors cannot commit to release the data sets at this time.

AUTHOR CONTRIBUTIONS

MB and JD contributed equally to the research reported and wrote the manuscript. BH and AI contributed to the plant uptake and siRNA testing work reported in this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00816/full#supplementary-material

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MINI REVIEW
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Plant miRNA Cross-Kingdom Transfer Targeting Parasitic and Mutualistic Organisms as a Tool to Advance Modern Agriculture

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MicroRNAs (miRNAs), defined as small non-coding RNA molecules, are fine regulators of gene expression. In plants, miRNAs are well-known for regulating processes spanning from cell development to biotic and abiotic stress responses. Recently, miRNAs have been investigated for their potential transfer to distantly related organisms where they may exert regulatory functions in a cross-kingdom fashion. Cross-kingdom miRNA transfer has been observed in host-pathogen relations as well as symbiotic or mutualistic relations. All these can have important implications as plant miRNAs can be exploited to inhibit pathogen development or aid mutualistic relations. Similarly, miRNAs from eukaryotic organisms can be transferred to plants, thus suppressing host immunity. This two-way lane could have a significant impact on understanding inter-species relations and, more importantly, could leverage miRNA-based technologies for agricultural practices. Additionally, artificial miRNAs (amiRNAs) produced by engineered plants can be transferred to plant-feeding organisms in order to specifically regulate their cross-kingdom target genes. This minireview provides a brief overview of cross-kingdom plant miRNA transfer, focusing on parasitic and mutualistic relations that can have an impact on agricultural practices and discusses some opportunities related to miRNA-based technologies. Although promising, miRNA cross-kingdom transfer remains a debated argument. Several mechanistic aspects, such as the availability, transfer, and uptake of miRNAs, as well as their potential to alter gene expression in a cross-kingdom manner, remain to be addressed.

Keywords: agriculture, cross-kingdom, microRNAs, mutualism, pathogen, plant

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INTRODUCTION

Plants have evolved sophisticated mechanisms to adapt to environmental changes and to interact with different organisms. Many of these strategies are based on the activation and repression of large sets of genes, and miRNAs are important regulator molecules in this scenario. They may be induced or repressed to subsequently regulate the expression of target genes through post-transcriptional silencing or translational inhibition of their mRNA targets. MicroRNAs are defined as small,

non-coding, single-stranded RNAs acting as regulators of multiple biological and physiological processes. In plants, these small molecules derive from stem-loop precursors that are processed through a Dicer-like (DCL) enzyme and loaded, in association to Argonaute (AGO) proteins, into the RNA-induced silencing complex (RISC) that serve to direct them to their target site where cleavage of mRNAs or inhibition of translation happens (Jones-Rhoades et al., 2006).

Nowadays, miRNAs are starting to be envisioned for their ability to move not only within an organism, but also across kingdoms and influence gene expression in evolutionary distant organisms (LaMonte et al., 2012; Cheng et al., 2013; Shahid et al., 2018; Zhang et al., 2019a). The presence of a methyl group on the ribose of the last nucleotide together with the association with RNA binding proteins and packing into exosomes may contribute to the stability and transfer of plant miRNAs across kingdoms (Valadi et al., 2007; Zhao et al., 2012). The miRNA cross-kingdom transfer may be favored by the conserved features of the RNA silencing machinery among eukaryotes, though taxon-specific variations exist. Such differences are mainly related to the ability of organisms to incorporate RNA molecules, systematically transmit the RNA signals to other tissues and to the magnitude and duration of the RNA silencing response (Winston et al., 2007; Shannon et al., 2008; Wang et al., 2015; Wang et al., 2016). Most examples of miRNA cross-kingdom transfer come from plant-pathogen/parasite interactions (Zhang et al., 2016; Wang et al., 2017a; Zhang et al., 2019a). The cross-kingdom transfer of endogenous plant miRNAs to pathogens or parasites may inhibit their invasive powers while the miRNA transfer from parasitic eukaryotes to plants may suppress the immunity of the host plants. In the case of symbiotic/mutualistic relations, the miRNA transfer from plants may influence essential processes such as growth and development (Zhu et al., 2017).

Understanding the complex network of interactions between plants and eukaryotic organisms and the translation of these information from the bench to the field can pave the way for the development of new technologies. In view of this, miRNA-based strategies exploiting the potential of plant miRNAs to move across kingdoms and silence specific genes in distantly related organisms, are gaining ground. The use of artificial miRNAs (amiRNAs) can be regarded as valuable tools that can complement the already existing technologies to face the global climate changes and associated agricultural challenges (Chen et al., 2013; Mitter et al., 2016).

The current minireview focuses on the latest information related to cross-kingdom miRNA addressing plant-parasite/mutualistic relations. Specific examples of cross-kingdom transferring plant miRNAs and potential gene targeting are provided and their potential implication in improving agricultural practices are discussed. Since this is still a highly debated topic, where both positive and negative results are available with regard to plant miRNA stability, abundance, and especially cross-kingdom targeting ability, several open questions are being proposed relative to methodological and mechanistic issues.

PLANT-PARASITE MIRNAS CROSS-KINGDOM TRANSFER: ALTERNATIVE TOOLS TO FIGHT PLANT PESTS AND DISEASES

Among plant diseases, agricultural crop infection by fungal pathogens annually cause multimillion dollars losses. While the most used methods to combat fungal-borne diseases are fungicides and chemical sprays, these have negative impacts on human health and surrounding environment (Almeida et al., 2019). The cross-kingdom miRNAs delivery between plants and fungi may represent alternative, environmental-friendly approaches to fight fungal diseases and confer crop protection (Wang et al., 2016). To date, miRNAs have been observed to move in a cross-kingdom manner from plants to fungi and vice versa (**Table 1**). An example of plant miRNA transfer to pathogenic fungi is constituted by miR159 and miR166 from cotton (*Gossypium hirsutum*), shown to confer resistance to *Verticillium dahlia* (Zhang et al., 2016). These miRNAs, found in fungal hyphae isolated from infected cotton tissues, were predicted to hit the virulence-related proteins HiC-15 (isotrichodermin C-15 hydroxylase) and Clp-1 (Ca^{2+}-dependent cysteine protease). The targets were validated by transiently expressing miRNA-resistant HiC-15 and Clp-1 in tobacco and *V. dahliae*. Subsequent analysis of *V. dahliae* mutants confirmed that the targeted fungal genes had an important role to play during fungal virulence and that they were specifically targeted by the miRNAs exported from the infected cotton plants to achieve silencing, hence conferring resistance to the fungal pathogen (Zhang et al., 2016). An example of fungal miRNA delivery to host plants is the case of a novel miRNA-like RNA from *Puccinia striiformis* f. sp. *tritici* (*Pst*), the agent causing the wheat stripe rust disease, able to act as a pathogen effector and suppress wheat innate immunity (Wang et al., 2017a). Pst-milR1, identified by high-throughput analysis of *Pst* sRNA library, was predicted to target the β-1,3-glucanase *SM638* (pathogenesis-related 2) gene in wheat. Co-transformation analyses and RACE (rapid amplification of the

TABLE 1 | Examples of cross-kingdom miRNA transfer related to plant parasitic and mutualistic relations.

Donor	Receiver	Relation	miRNA	Target	Function	Reference
G. hirsutum	*V. dahlia*	Parasitic	miR159 miR166	*HiC-15* *Clp-1*	Fungal virulence	Zhang et al., 2016
P. striiformis	*T. aestivum*	Parasitic	pst-milR1	*SM638*	Innate immunity	Wang et al., 2017a
A. thaliana	*P. xylostella*	Parasitic	miR159c novel-7703-5p	*BJHSP1* *PPO2*	Pupae development	Zhang et al., 2019a
B. campestris	*A. mellifera*	Mutualistic	miR162a	*TOR*	Caste development	Zhu et al., 2017

cDNA ends) validation in tobacco leaves confirmed that *SM638* was targeted by Pst-milR1.

When considering insect pests, the cross-kingdom transfer of miRNAs has been investigated for its communication role between plants and plant-feeding insects, such as *Plutella xylostella* (diamondback or cabbage moth) (Zhang et al., 2019a). RNA sequencing analysis has evidenced the presence of 39 plant miRNAs in the moth hemolymph. The plant-derived miR159a, miR166a-3p, and the novel-7703-5p were predicted to influence cellular and metabolic processes in *P. xylostella* through binding and suppressing *BJHSP1, BJHSP2* (basic juvenile hormone-suppressible protein 1 and 2), and *PPO2* (polyphenol oxidase subunit 2) genes. QRT-PCR analyses carried out following treatment with the specific miRNA agomir sequences, demonstrated the downregulation of the predicted targets whereas a luciferase assay proved the binding to their respective targets. Further insect development studies revealed that treatments with agomir-7703-5p resulted in the development of abnormal pupae and decreased adult emergence rates (Zhang et al., 2019a).

Other examples focused on showing the presence of plant-derived miRNAs in insect pests. For instance, Zhang et al. (2012) investigated this aspect in several *Lepidoptera* and *Coleoptera* species subjected to controlled feeding experiments. This study focused on determining the presence of conserved miR168 sequences in insects by means of northern blot and deep sequencing; while northern blot analyses were negative, the deep sequencing data revealed the presence of miR168 in moderate quantities. Hence, the authors discuss the possibility of sample contamination evidencing the existence of some artefacts during sequencing data analysis (Zhang et al., 2012). Deep sequencing was used to reveal plant miRNAs in cereal aphids (*Schizaphis graminum*, *Sipha flava*) causing serious losses in sorghum (*Sorghum bicolor*) and switchgrass (*Panicum virgatum*) crops (Wang et al., 2017b). Thirteen sorghum miRNAs and three barley miRNAs were detected and predicted to target aphid genes playing important roles in detoxification, starch and sucrose metabolism.

MiRNA cross-kingdom transfer probably occurs also during the interplay between plants and parasitic nematodes (phytonematodes) (Jaubert-Possamai et al., 2019). Plant-parasitic nematodes are responsible for considerable crop losses worldwide. Understanding how plants respond to these organisms is necessary to bridge the gap between agricultural production and the growing food demand. Most of the scientific literature on gene silencing mechanisms comes from nematodes, specifically from *Caenorabditis elegans*. However, these studies mostly focus on the ability to uptake double strand RNAs (dsRNAs) from the environment (Huang et al., 2006; Tian et al., 2019) rather than on the cross-kingdom transfer of plant miRNAs. Many studies have investigated the involvement of plant miRNAs and their corresponded gene targets in response to phytonematodes infection (Hewezi et al., 2008; Li et al., 2012; Lei et al., 2019; Pan et al., 2019). Transcriptomic analyses evidenced extensive reprogramming of gene expression at the nematode feeding sites, modulated by plant miRNAs; also, some conserved miRNAs were shown to have analogous roles in feeding site formation in different plant species (Jaubert-Possamai et al., 2019).

The cited examples depict a promising research area. Understanding the complex interactions between host plants and parasitic organisms would pave the way for the development of new technologies for a more sustainable control of plant pests and diseases.

MIRNAS CROSS-KINGDOM TRANSFER IN PLANT MUTUALISTIC INTERACTIONS

Several studies on mutualistic relations have regarded many miRNAs target processes related to hormone-responsive pathways and innate immune function (Formey et al., 2014; Wu et al., 2016). The majority of these processes correspond to turning off defense pathways that would otherwise block fungal or bacterial proliferation within plant tissues (Plett and Martin, 2018). In a recent study, Silvestri et al. (2019) have looked into the symbiosis between the arbuscular mycorrhiza (AM) *Rhizophagus irregularis* and the model legume *Medicago truncatula*, showing the presence of fungal microRNA-like sequences potentially able to target plant transcripts. The *in silico* analysis, verified through a degradome analysis, predicted more than 200 plant genes as putative targets of specific fungal sRNAs and miRNAs, many of which had specific roles in AM symbiosis. For instance, three miRNA-like sequences (*Rir*-miRNA-like 341, 342, and 828) shown to be up-regulated in the intraradical phase were suggested to be responsible for the regulation of AMF genes required to manipulate fungal or host plant gene expression. The predicted target genes encode for a DHHC-type zinc finger protein (AES89412), integral membrane family protein (AES91391), and carboxy-terminal region remorin (AES81367).

In recent years, evidence that plant miRNAs target genes in a trans-kingdom fashion in pollinator insects is steadily accumulating. Currently available studies report pre-eminently on dietary intake of plant miRNAs by honey bees (*Apis mellifera*) (Ashby et al., 2016; Gismondi et al., 2017; Zhu et al., 2017). The plant-pollinator relationship is partly mutualistic considering the nutrients intake in exchange for the pollination service that enables plant reproduction. The presence of plant miRNAs in honey was reported by Gismondi and collegues (2017) who detected and quantified several miRNAs belonging to conserved families (miR482b, miR156a, miR396c, miR171a, miR858, miR162a, miR159c, miR395a, miR2118a) in different types of honey. The authors found that the most enriched in plant-miRNAs was the honey obtained from sweet chestnut (*Castanea sativa*) flowers. In bees, the dietary intake of pollen-derived miR162a was proven to regulate caste development at larval stage (Zhu et al., 2017). It was shown that miR162a targets *TOR* (target of rapamycin) mRNA downregulating its expression at the post-transcriptional level. Interestingly, this mechanism was found to be conserved in *Drosophila melanogaster* (common fruit fly), a non-social type of insect (Zhu et al., 2017).

Nonetheless, contrasting results are also reported. Although Masood et al. (2016) observed accumulation of plant miRNAs after pollen ingestion in adult bees, they did not find any evidence of biologically relevant roles of these plant miRNAs in bees. Likewise, expression analysis of pollen-derived miRNAs ingested by bees, revealed the absence of substantial uptake and systemic delivery of miR156a, highly expressed in bee-bread and honey (Snow et al., 2013). In a different system, silkworm (*Bombyx mori*) and mulberry (*Morus* spp.) was used as model to study the proposed miRNA-mediated crosstalk between plants and insects (Jia et al., 2015). Sanger sequencing and digital PCR demonstrated the presence of mulberry-derived miRNAs in silkworm tissues while the administration of synthetic miR166b did not influence silkworm physiological progress.

CROSS-KINGDOM TRANSFER OF AMIRNAS FOR AGRICULTURAL PURPOSES

The knowledge acquired on endogenous miRNAs as regulators of gene functions within and among organisms led researchers to develop increasingly sophisticated agricultural technologies based on miRNAs. Among these, the amiRNA (artificial miRNA) strategy was developed to produce specific miRNAs that can effectively silence designated genes (Zhang et al., 2018). One of the main characteristics of amiRNAs is the conserved secondary foldback structure that has to be similar to that of a typical pre-miRNA. In this case, the original structure of the miRNA-5p:miRNA-3p sequence will be replaced by an engineered miRNA targeting a designated mRNA, and the most preferred structures are those existing in conserved miRNA families. In this way, amiRNAs can be engineered to target any mRNA with higher specificity compared to other strategies like dsRNA overexpression or siRNA accumulation. Since pre-amiRNA processing results in a single amiRNA targeting a designated sequence, this eliminates the off-target effects and the production of secondary siRNAs is quite limited (Manavella et al., 2012). A highly relevant attribute for agricultural purposes is the fact that amiRNAs are stable and inheritable. Moreover, the amiRNA-mediated silencing is believed to pose less problems regarding bio-safety and environmental security with respect to other strategies (Liu and Chen, 2010; Toppino et al., 2011), due to the small size of the inserts and reduced probabilities for horizontal transfer. Aside the study of gene functionality (Schwab et al., 2006; Warthmann et al., 2008), amiRNA technology has been applied to knock out genes from insect pests, nematodes, viruses, and other phytopathogens (Niu et al., 2006; Fahim et al., 2012; Guo et al., 2014; Kis et al., 2016; Wagaba et al., 2016).

Several pre-miRNAs have been used as backbones to synthesize artificial miRNAs in engineered plants with the aim to control agricultural pests (**Table 2**). This strategy is based on the possibility of miRNAs to be transferred through diet across kingdoms and the ability of these small molecules to exercise their biological activity in recipient organisms. Indeed, the miRNAs in the transgenic plant may be taken up by plant feeding organisms and then suppress selected genes such as those related to metabolism, development but also to pathogenesis/parasitism by exploiting the endogenous silencing machinery of the plant feeding organism. Essential genes either involved in pathogen metabolism, or causing resistance to plant toxins, or encoding effectors involved in pathogenicity, have been considered as potential targets. For instance, enhanced resistance to the aphid *Myzus persicae* was reported in transgenic plants expressing amiRNAs targeting the *MpAChE2* (aphid acetylcholinesterase 2) gene (Guo et al., 2014). The *AChE* gene encodes for hydrolase enzyme that hydrolyses the neurotransmitter acetylcholine and plays vital roles in insect growth and development (Kumar et al., 2009). In a recent investigation, amiRNA-based technology targeting AChE was also applied by Saini and co-workers (2018) to defeat *Helicoverpa armigera*. They demonstrated that the silencing of *HaAce1* gene by host-delivered amiRNAs disrupted growth and development in the polyphagous insect. Another example relates to the use of amiR-24 targeting the 3′-UTR of the *chitinase* gene. Transgenic tobacco plants producing amiR-24 were fed *H. armigera* caterpillars, resulting in delayed molting and enhanced lethality (Agrawal et al., 2015). In a different study, amiR15 was used to design transgenic rice plants resistant to the striped stem borer, *Chilo suppressalis* (Jiang et al., 2016). The amiR15, design staring from the insect specific miRNA, *Csu*-miR-15, targets the *CsSpo* (Cytochrome P450 307a1) and *CsEcR* (Ecdysone receptor) genes involved in the ecdysone signaling pathway. Feeding trials carried out using the transgenic miR-15 rice resulted into increased mortality and developmental defects in the targeted insect pest. The effect of amiRNAs was studied also on the *Avr3a* gene, the target transcript of *Phytophtora infestans.* AmiRNAs targeting different regions of the *Avr3a* gene imparted moderate type of late blight resistance into two transformed Indian potato cultivars (Thakur et al., 2015).

AmiRNA delivery may be considered as a species-specific pesticide and as a potential and powerful alternative to the chemical strategies used so far. This miRNA-based technology may be considered as an alternative method for intragenic crop

TABLE 2 | Examples of cross-kingdom transfer of artificial microRNAs (amiRNAs) from transgenic plants to their respective pathogens/parasites.

Modified plant	Pathogen/parasite	Target	Function	Reference
N. tabacum	*M. persicae*	*MpAChE2*	Synaptic transmission	Guo et al., 2014
A. thaliana	*H. armigera*	*HaAce1*	Synaptic transmission	Saini et al., 2018
N. tabacum	*H. armigera*	*Chitinase*	Chitin synthesis	Agrawal et al., 2015
O. sativa	*C. suppressalis*	*CsSpo CsEcR*	Embryonic development	Jiang et al., 2016
S. tuberosum	*P. infestans*	*Avr3a*	Fungal virulence	Thakur et al., 2015

engineering causing less public concern. For beneficial insects, such as honey bees, amiRNA-based technology may be used to counteract virus infections by feeding them in large field treatment with amiRNAs able to reduce the expression of viral genes. Apart from transgenic plants permanently expressing amiRNAs, amiRNAs sprayed onto leaves in conjunction with miRNAs enriched soil can minimize pest damages (Cagliari et al., 2019).

CONCLUSIONS AND FUTURE PERSPECTIVES

Plant pathogens place a global burden on major crops being responsible for reduced crop yields with great repercussions on food production and food security (Savary et al., 2019). On the other hand, promoting the investigation of mutualistic relations has the potential to better assist sustainable agricultural practices (Duhamel and Vandenkoornhuyse, 2013).

As shown in the presented examples, understanding the miRNA cross-kingdom transfer and mode of action could contribute to decrease the pathogenicity of fungi and pests, hence promoting better plant productivity. In the case of insects (pests or pollinators), administration of plant miRNAs (through genetic engineering, nanoparticles, or spraying) may actively contribute to population control, reducing the prevalence of pests while enhancing the preponderance of pollinators. In this context, researches could be envisioned to grasp on how plant miRNA trans-kingdom regulation could be used to avoid the extinction of bees, as exemplified in the studies demonstrating their involvement in cast development (Zhu et al., 2017). But, to progress this debated field, many questions still need to be addressed and many additional steps must be taken to elucidate plant miRNAs uptake and potential cross-kingdom gene targeting. In this highly-technological era, the rapid progress of bioinformatics studies and tools to predict cross-kingdom miRNA targets (Mal et al., 2018; Bellato et al., 2019) sets the stage to advance new hypothesis to be subsequently experimentally tested. Nonetheless, many of the existing questions demand solid proofs from wet lab analyses. From the point of view of experimental design, questions related to the most appropriate techniques (deep sequencing, digital PCR, qRT-PCR) and references (samples and/or genes) to be used for cross-kingdom miRNA studies still need to be addressed and uniformized accordingly (Chan and Snow, 2016; Zhang et al., 2019b). Once these issues are settled, we can then proceed to investigate other challenges; for instance, why some plant miRNAs seem to be more stable and abundant than others? Are the levels of host plant miRNAs found in pathogen species high enough to exert a physiological impact? How do plant miRNAs reach their targets in the receiving organism? Considering the miRNAs mode of action (targeting mRNAs based on sequence complementarity), their impact on the receiving organism can variate depending on the targeted genes; hence, studies covering both favorable and unfavorable effects need to be encouraged to promote best-informed scientific solutions.

AUTHOR CONTRIBUTIONS

AM conceptualized the minireview. CG, PL, and AM wrote the manuscript.

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REVIEW
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Biosafety of GM Crop Plants Expressing dsRNA: Data Requirements and EU Regulatory Considerations

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The use of RNA interference (RNAi) enables the silencing of target genes in plants or plant-dwelling organisms, through the production of double stranded RNA (dsRNA) resulting in altered plant characteristics. Expression of properly synthesized dsRNAs in plants can lead to improved crop quality characteristics or exploit new mechanisms with activity against plant pests and pathogens. Genetically modified (GM) crops exhibiting resistance to viruses or insects *via* expression of dsRNA have received authorization for cultivation outside Europe. Some products derived from RNAi plants have received a favourable opinion from the European Food Safety Authority (EFSA) for import and processing in the European Union (EU). The authorization process in the EU requires applicants to produce a risk assessment considering food/feed and environmental safety aspects of living organisms or their derived food and feed products. The present paper discusses the main aspects of the safety assessment (comparative assessment, molecular characterization, toxicological assessment, nutritional assessment, gene transfer, interaction with target and non-target organisms) for GM plants expressing dsRNA, according to the guidelines of EFSA. Food/feed safety assessment of products from RNAi plants is expected to be simplified, in the light of the consideration that no novel proteins are produced. Therefore, some of the data requirements for risk assessment do not apply to these cases, and the comparative compositional analysis becomes the main source of evidence for food/feed safety of RNAi plants. During environmental risk assessment, the analysis of dsRNA expression levels of the GM trait, and the data concerning the observable effects on non-target organisms (NTO) will provide the necessary evidence for ensuring safety of species exposed to RNAi plants. Bioinformatics may provide support to risk assessment by selecting target gene sequences with low similarity to

the genome of NTOs possibly exposed to dsRNA. The analysis of these topics in risk assessment indicates that the science-based regulatory process in Europe is considered to be applicable to GM RNAi plants, therefore the evaluation of their safety can be effectively conducted without further modifications. Outcomes from the present paper offer suggestions for consideration in future updates of the EFSA Guidance documents on risk assessment of GM organisms.

Keywords: RNA interference, biosafety, food safety, genetically modified plants, bioinformatics, non-target organisms, GMO regulation

INTRODUCTION

In the last decade, a variety of new biotech methods have been developed, offering great technical potential for use in the agricultural sector. The European Union has established a legal framework to ensure that the application of modern biotechnology, and more specifically of genetically modified organisms (GMO), is developed under safe conditions. According to the EU legislation on GMO, in particular Regulation (EC) No 1829/2003 on genetically modified (GM) food & feed and Directive 2001/18/EC on deliberate release into the environment of GMO, every application for commercial use of a GMO has to be approved by the European Commission after a safety assessment. This assessment is conducted by the applicants according to the requirements of either of the two laws. For the preparation of dossiers, applicants need to conduct appropriate experimental studies and literature searches for collecting information regarding human and animal health, as well as environmental safety. The data requirements according to EU legislation on GMOs are laid down in the implementing Regulation No 503/2013 and Commission Directive (EU) 2018/350 for food/feed and environmental safety respectively. Support for applicants in preparing dossier for the commercialization of GM products is guaranteed by specific guidance documents (GDs) issued by the European Food Safety Authority (EFSA Panel on GMO, 2010; EFSA Panel on GMO, 2011) and periodically updated or supplemented by specific statements following the latest scientific developments in the field. As new biotech products are being developed, it is important that the rationale for the safety assessment is built on a scientific consideration of the characteristics of the new products in accordance with the principles of the EFSA GDs.

The RNA-interference (RNAi) technique exploits a natural mechanism present in almost all eukaryotic organisms, which leads to the loss of functionality of a gene by blocking the messenger RNA (mRNA) molecules essential for the formation of a protein. The RNA expression constructs are normally delivered as transgenes, *via* plant transformation or as a part of virus-vectors (Yin et al., 2005), and therefore, they are required to undergo GMO regulatory procedures to enable authorization for commercial use. However, unlike classical GM plants that are generally modified to express a specific protein, GM plants expressing dsRNA (hereafter, RNAi plants) do not need to express novel proteins to produce a new phenotype. RNAi plants have been modified to express double stranded RNA (dsRNA) molecules that enable specific post-transcriptional partial or complete silencing of plant target genes or target genes from plant pathogens or pests.

RNAi can be used in a "within species" mode to improve plant composition by removing or reducing anti-nutrients, allergens and toxins while enhancing levels of beneficial nutrients, and to improve plant growth and productivity by suppressing undesirable traits. The same mechanism can be exploited by expressing dsRNA in plants that silence genes in other organisms exposed to the plants (Zotti et al., 2018). Virus resistant and insect resistant plant varieties obtained using RNAi mechanisms have already been approved for cultivation outside Europe (e.g. papaya resistant to Papaya Ringspot Virus in USA, Canada and Japan; plum tree resistant to plum pox virus (PPV) approved in the USA; common bean resistant to Bean Golden Mosaic Virus (BGMV) in Brazil; SmartStax™ maize with multiple resistance traits, including dsRNA against *Diabrotica virgifera virgifera* approved in the USA and Canada), cassava resistant to Brown Streak Virus and Ugandan Cassava Brown Stick Virus in Nigeria. In the EU, several RNAi plants with enhanced nutritional characteristics including Soybean DP305423 and soybean MON87705 with increased oleic acid acid have been authorised for placing on the market with the exception of cultivation. More recently, EFSA has given a positive opinion for import and processing of products derived from corn rootworm resistant GM maize MON87411 (EFSA Panel on GMOs, 2018). No applications for cultivation of plants producing dsRNA have been submitted so far to European authorities. However, EFSA has published an opinion on a GM potato event with antisense-mediated gene silencing (EFSA, 2006).

As knowledge in this field is rapidly accumulating, EFSA has supported the publication of 3 systematic literature searches and reviews, summarizing the available information on RNAi technology to support the risk assessment of RNAi plants (Paces et al., 2017; Christiaens et al., 2018; Dàvalos et al., 2019). While these studies did not directly offer any recommendations for risk assessment, they offer valuable baseline information supporting any future risk assessment framework. Previously, EFSA had also organized an international workshop on "Risk assessment considerations for RNAi-based GM plants" (European Food Safety Authority, 2014) which also led to a number of external peer-reviewed publications discussing the risk assessment considerations of these plants (Ramon et al., 2014; Casacuberta et al., 2015).

Together also with EPA's FIFRA Scientific Advisory Panel meeting publication (USEPA, 2014), these publications form the first wave of risk assessment considerations.

In the field of pest management, RNAi applications are exploring completely novel mechanisms of actions, as has been shown from work on RNAi plants as well as from direct applications of dsRNA as pesticide product. Effective examples are: silencing of the *DvSnf7* gene, which results in suppression of mRNA encoding the class E vacuolar sorting protein in *D. v. virgifera* (Bachman et al., 2013); targeting of vacuolar-ATPase subunit A (v-ATPase A) in *D. v. virgifera* (Baum et al., 2007) and in *Bemisia tabaci* (Thakur et al., 2014) by the expression of dsRNA. Furthermore, examples of RNAi-based control to manage filamentous fungal plant pathogens are steadily increasing (Machado et al., 2018). Progress in the development of RNAi plants and also new RNA-based biopesticides is expected to bring to the market new plant varieties or plant protection products within a few years (Taning et al., 2020).

The COST Action iPlanta (iplanta.univpm.it) is the largest network of European scientists actively engaged in research on RNAi systems and applications, including host-induced gene silencing (HIGS) and spray induced gene silencing (SIGS). Among the goals of iPlanta are the identification of the specific biosafety data requirements for the risk assessment and risk management of RNAi plants and their products (i.e. food and feed) and the elucidation of knowledge gaps arising in the area of potential food and feed and/or environmental risks specific to RNAi applications.

The present paper is the result of an iPlanta working group activity and aims to discuss the relevance and applicability of the existing EFSA GMO Guidelines for environmental and food/feed risk assessment for RNAi plants. Starting from some key elements of the EFSA GDs, the paper discusses the applicability of the principles of the GDs to RNAi plants and, based on the considerations of their scientific aspects involved, suggests the data requirements considered significant for preparation of dossiers.

COMPARATIVE ASSESSMENT

In the EFSA GDs, comparative safety assessment is indicated as a general principle for the risk assessment of GM plants. The GDs therefore suggest optimal methodologies for data collection on relevant assessment endpoints, to compare GM plants and derived food and feed, with their respective comparators (e.g. the near isogenic control line). The comparative safety assessment of GM plants is considered effective to identify differences to their non-GM counterparts and assess the consequences of these differences.

Comparative safety assessment is based on data collected for molecular characterization, the agronomic and phenotypic characteristics of the GM plant, as well as its compositional analysis. In addition, the comparative safety assessment within environmental risk assessment (ERA) requires information on the interactions of the GM plant with other biota and its receiving environment(s).

The main requirements and questions raised by the EFSA GD are the following:

- Describe how the tested GM line was produced (breeding tree);
- Document that the selected conventional counterpart is genetically as close as possible to the GM line and has a history of safe use;
- Select (a minimum of 8) experimental sites for conducting field experiments concerning compositional, agronomic and phenotypic analysis, where the meteorological and agronomic conditions reflect the ones under which the crop is to be grown;
- Select appropriate plant genotypes, e.g. the GM line genetic background to ensure the quality and stability of the selected test material;
- Include in the field experiments the GM line(s), the near isogenic control and 3 reference commercial varieties at each site (minimum 6 different varieties in total), on the basis of having adequate statistical power to detect differences or equivalence;
- Indicate the measurement endpoints to be used;

These power calculations depend respectively on the typical variability found between small agronomic plots used for food-feed trials and between the larger plots typically employed for environmental trials (see EFSA Panel on Genetically Modified Organisms, 2010; EFSA Panel on Genetically Modified Organisms, 2011).

In neither case is the methodology of the field trial assessment related to the technology adopted to produce the novel plant or food under assessment. For the above-mentioned points, it is considered that the implementation of field experiments for RNAi plants will not be different from all currently grown GM events.

It could be problematic to field test RNAi plants with tolerance to extremes of environmental conditions such as temperature, salinity and soil moisture, but this is also the case for other GM plants. In addition, comparative field testing in the presence of a target pathogen or pest may not be easily realized. For instance, in the case of virus resistant RNAi plants interactions between GM plants and the targeted viruses have to be assessed in preliminary tests. It is also important to consider that the composition of an RNAi plant may be altered after the attack by a specific pest or pathogen due to general and dsRNA- specific defence responses, which may change plant physiology/metabolism (Eschen-Lippold et al., 2012). Consequently, specially designed field studies located in regions with high pathogen or pest pressure are useful to assess the safety of newly introduced plant or pest resistance mechanisms under typical cultivation conditions and to detect any possible unintended effects.

The current EFSA GD have been used mostly to consider applications for herbaceous GM plants. However perennial RNAi plants such as virus resistant RNAi GM trees (e.g. Rainbow papaya, Ferreira et al., 2002, Honey sweet plum, Scorza et al., 2013), may require other measurement endpoints

relating to their development and seasonal growth in comparative field studies for safety evaluations (Aguilera et al., 2013).

In some applications (e.g. virus resistance in GM trees) dsRNA can be used for transforming rootstocks so that the effects occur in the whole plant, because of the translocation of siRNA into scions grafted to the transformed rootstocks (Limera et al., 2017; De Francesco et al., 2020). Several studies demonstrated the transfer of siRNAs from the transgenic rootstock to the nontransgenic scion conferring resistance to virus in fruit trees (Zhao and Song, 2014) and grafted cucumber (Bai et al., 2016). This technology is now studied also for conferring resistance to pests (Taning et al., 2020) and diseases (Sabbadini et al., 2019). Such systemic silencing probably depends on amplification of siRNAs through secondary siRNA production. However, in contrast to highly expressed or aberrant RNAs (such as viral RNAs or RNA from transgenes), endogenous plant mRNAs are usually not prone to secondary siRNA production (Luo and Chen, 2007; Baeg et al., 2017) and to systemic silencing (Frizzi and Huang, 2010; Dadami et al., 2014). Therefore, translocation of siRNAs to scions and fruits and the usage of grafting technologies will be available only for specific applications.

MOLECULAR CHARACTERIZATION

According to the strategy of the EFSA GDs, following a case-specific problem formulation, the risk assessment starts with the comprehensive molecular characterization of the GM plant under scrutiny.

The main areas on which information is required during the molecular characterization are:

- Information on the intended genetic modification which includes: the transformation process (method of transformation; recipient plant tissue; details of *Agrobacterium* strain used, helper plasmids etc.); the source of DNA and design of vector constructs (sequence of DNA to be inserted, codon optimisation, promoters etc.); the role of each functional element
- Information on the GM plant, including: the trait(s) modified; the transgene constructs actually present in the GM plant; protein characterization and equivalence; expression and stability of the insert.
- Bioinformatics data/open reading frame analysis to identify potential for newly created toxins/allergens.

These data also inform the risk analysis of horizontal gene transfer (see below).

Data concerning the transformation process and the sequence and function of the inserted DNA for RNAi plants are not different from other transgenic events. Also, where the RNAi event possesses additional conventional transgenes for selection etc., the normal data requirements still apply to those parts.

However, for the inserted dsRNA cassette specifically, some of the data normally required for GM plants simply cannot be obtained. For instance, data related to newly expressed proteins, protein equivalence and the codon optimization are irrelevant for this inserted DNA as long as no part is translated into protein. Data on levels of dsRNA expression over time and plant development stages in different tissues and environmental conditions relevant for the crop in question will be necessary to estimate exposure of humans and other animals to the dsRNA in food or feed. dsRNA levels in plant tissues are also dependent on the type of transformation. In nuclear transformants dsRNA is to a large part processed in plant cells into siRNAs (Frizzi and Huang, 2010) which are not efficiently taken up by insects. In contrast, transplastomic plants accumulate dsRNA within the chloroplasts (Bally et al., 2016), resulting in high amounts of unspliced dsRNA in green plant parts (Zhang et al., 2015) and may therefore be used particularly for targeting leaf-feeding insect pests. Exposure of organisms to dsRNA through roots, tubers and pollen, however, is low in transplastomic RNAi plants, which implies that nuclear transformants and transplastomic plants are likely to have different risks for non-target organisms (Schiemann et al., 2019). Bioinformatics analyses offer the availability of additional measurement endpoints, which can be potentially more specific for events transformed with dsRNA. According to EU implementing regulation 503/2013, bioinformatics analyses are requested for the recipient plant genome to detect potential off-target plant genes that might be suppressed unintentionally. Considerations including a set of parameters that allow the prediction of possible off-target transcripts in plants have been published by the EFSA GMO Panel (EFSA GMO Panel (2017) Annex II of the minutes of the 118th GMO Plenary meeting: Internal note on the strategy and technical aspects for small RNA plant off-target bioinformatics studies. Available at: https://www.efsa.europa.eu/sites/default/files/event/171025-m.pdf). On a case-by-case basis and depending on the function of the potential off-targets, additional data may be required for safety assessment.

Such information could also be considered, although with a more limited value, for estimating possible off-target effects in outcrossing plant species or non-target organisms in the receiving environment, however this is feasible only when relevant genomic information is available on organisms exposed to the GM plant or its dsRNA.

A key benefit of the sequence-based mechanism of action for RNAi plants for pest control is the possibility to achieve a high degree of specificity to the target organism while not harming exposed valued NTOs in the agroecosystem (Bramlett et al., 2020). Bioinformatics are informative for the selection of regions within target genes that possess high divergence across species, thus allowing for the selection of gene sequences specific to the target pest and minimizing the potential for homology to NTOs that are potentially exposed to the GM plants or its products. In order to achieve this, the search for 21bp homologies between the dsRNA and possible target sequences in NTOs is a good starting point, but the exact length and sequence of the construct that may produce gene suppression in different invertebrate species are not precisely known. Paces et al. (2017) indicates that while siRNA-target base pairing is highly specific, mismatches do not

necessarily prevent RNAi silencing, depending on position and type of mismatch. Furthermore, available literature is in disagreement regarding the minimum effective base pairs length, number and location of allowed mismatches (Christiaens et al., 2018). SiRNAs also appear to vary in length in different insect species. A recent research paper looking at siRNA populations after viral infections showed two tested lepidopteran species having predominantly 20nt siRNAs while siRNAs in the examined orthopteran and hymenopteran species were mostly 22nt long (Santos et al., 2019). This could imply that the necessary homology for successful RNAi silencing could differ between species as well. Coleoptera typically have 21nt long siRNAs and research experience supporting the development of the MON87411 maize with dsRNA targeting the *DvSnf7* gene, indicates that shorter than 21 nt sequence length shared between the dsRNA construct and the target gene (19, 20 bp) did not result in an efficient silencing effect in the target insect (Bachman et al., 2013).

Finally, it must be noted that little sequence information is available for many NTOs and factors other than sequence homology (e.g. successful uptake and stability of the dsRNA molecules into the insect, accessibility of the mRNA site of action) may affect the efficacy of siRNA. Therefore, it may be necessary to generate data on the effects of the dsRNA on exposed NTOs during ERA. This is discussed below.

FOOD/FEED SAFETY ASSESSMENT

The main pillars on which is based the safety assessment of food/feed containing or derived from GMOs according to the EFSA GD (EFSA Panel on GMO, 2011) are:

- Toxicological assessment of newly expressed proteins and/or new constituents other than proteins;
- Assessment of allergenicity of the newly expressed proteins and the new plant;
- Safety assessment of altered levels of food and feed constituents;
- Safety assessment of the whole food and/or feed derived from GM plants;
- Nutritional assessment of food/feed derived from GM plants.

No new proteins are intended to be produced by the dsRNA cassette in RNAi plants, therefore the toxicological assessment of newly expressed proteins is not relevant for food/feed products derived from them. Consequently, since all known food allergens are proteins, the allergenicity can also generally be considered not a relevant concern for RNAi plants, unless expression of genes coding for enzymes involved in the metabolism of existing plant allergens are silenced in the GM plant.

Compositional analyses can then constitute the key requirement for analysing the effects of identified differences, as well as for nutritional evaluation of food and feed derived from RNAi plants. Indeed, major metabolic changes in comparison with the near-isogenic control plants can be detected with compositional analyses, for which international standards are available and commonly applied for food safety assessment. For instance, the Organisation for Economic Co-operation and Development (OECD) consensus documents on the safety assessment of transgenic organisms (http://www.oecd.org/env/ehs/biotrack/safetyassessmentoftransgenicorganismsoecdconsensusdocuments.htm, accessed on 12 December 2019) include proximates (comprising moisture and total ash), key macro- and micro-nutrients, anti-nutritional compounds, natural toxins, and allergens, as well as other plant metabolites characteristic for the plant species.

For the safety assessment of whole food/feed, animal feeding trials are recommended by EFSA only on a case-by-case basis, specifically those cases for which the quality of available analytical data does not allow excluding possible safety issues for the specific product or fail to demonstrate nutritional equivalence with its comparator. While 90-days feeding studies are deemed effective methods to detect toxic effects of single substances, there has been considerable discussion over their relevance and sensitivity for the detection of potential unintended effects of whole food and feed. It is considered unlikely that substances present in small amounts and with a low toxic potential will result in any observable unintended effects in a 90-day rodent feeding study (EFSA, 2008). However, following the adoption of the Implementing Regulation (EU) 503/2013, a 90-day study in rodents on whole food/feed is required for all GM plant products in the EU. This new legal requirement changes the assessment from a hypothesis-driven case-by-case exercise, as originally indicated by the EFSA GD.

Exposure to dsRNA and siRNA through GM RNAi plant-derived food/feed by humans and farm animals is estimated to be low, due to some considerations regarding the metabolism of organisms ingesting exogenous RNA (Dàvalos et al., 2019). First of all, the uptake of ingested exogenous nucleic acids is limited by biological barriers in the gastro-intestinal (GI) tract, such as degradation by nucleases or an impaired cellular uptake (e.g. O'Neill et al., 2011; Petrick et al., 2013). Moreover, RNA absorption from the GI tract remains questionable (Jain, 2008; Thompson et al., 2012). Even if ingested siRNA is absorbed from the GI tract, it is normally rapidly degraded within the cardiovascular system and cleared through liver and kidneys (Christensen et al., 2013). These barriers represent the main difficulty for the development of targeted human RNAi drugs (e.g. Vaishnaw et al., 2010) which require specific formulations to deliver siRNA into the target cells. Consequently, it is unlikely that siRNA concentrations from GM RNAi plants will be sufficient to exert biologically relevant effects in mammals. In order to lead to harmful effects, the uptake of dsRNA should be followed by the delivery in sufficient quantity and in an active form to trigger RNAi and there would also need to be sufficient sequence complementarity with an mRNA transcript in the targeted cells (Roberts et al., 2015). Therefore, the risk of unwanted gene silencing in humans and animals upon ingestion of food/feed derived from RNAi plants can be considered negligible.

ENVIRONMENTAL SAFETY ASSESSMENT

The EFSA GD on Environmental Risk Assessment (EFSA Panel on GMO, 2010) requires a safety evaluation regarding different specific areas of concern.

- **Persistence and Invasiveness**

  Cultivated plants may persist in the environment even after harvest and some crop species have wild relatives with which they can hybridize allowing genes to flow from crops into other species. However, gene flow frequency and its consequences are very variable. For instance, gene flow is already an important issue in open pollinating plants with common wild relatives e.g., rice and beet but not in predominantly self-pollinating species such as wheat or beans. The focus of the ERA is on the expected consequences of gene flow, once it happens.

  The information needed for assessing consequences of plant transformation on persistence and invasiveness is based on the following points in order to analyse the possibility of environmental harm:
  - Understand the biological features of the plant that has been genetically modified (e.g. life cycle, dispersal, gene flow, persistence, invasiveness, etc.);
  - Analyse how the transgene affects the phenotype, behaviour, and interaction of the GM plant with the hybridizing wild relatives present in the receiving environment.

  The likelihood of increased persistence of the RNAi plant and recipient wild relatives in the environment is linked to the expressed traits of the GM plant, therefore the assessment for RNAi plants is similar to that of other GM plants and is case by case.

  The possibility of off-target effects in wild relatives of the GM plant could be estimated starting from the provided bioinformatics results and the acquired characteristics of the transformed plant species. Bioinformatics could be helpful as a predictive tool to detect possible off-target sequence alignment considering the similarities between the genome of the GM donor crop and the recipient plant. However, information on the genome of the wild relatives might be only partially available or completely lacking in the scientific literature.

  If transgene transfer to a wild relative occurs, then NTOs associated with the wild plant may be exposed to the dsRNA and should be considered in the safety assessment. The expression and environmental persistence of dsRNA in different tissues over time needs attention, as their environmental stability is expected to be different (Christiaens et al., 2018). However, this exposure pathway is not specific to RNAi plants as similar data may be required for GM plants expressing other biologically active compounds such as Cry proteins.

- **Horizontal Gene Transfer**

  The main issues to be considered in this case are:
  - Molecular characterization of the DNA sequences inserted in the plant, including information on the potential of the promoter elements that could drive expression in microorganisms;
  - presence of antibiotic resistance marker (ARM) genes;
  - presence of recipient microorganisms for transgenic DNA in the receiving environments;
  - presence of inserted DNA sequences showing homologies with DNA sequences from relevant microbial recipients, enhancing the probability of recombination, or mobile elements in the vicinity of the insertion site which could enhance the potential for gene transfer;
  - selective conditions enhancing the probability of dissemination and maintenance of the genetic material from GM plants in natural microbial communities (e. g. the presence of antibiotics in the receiving environment(s);
  - environmental persistence of GM plant material after harvesting;
  - potential for long-term establishment of the genetic materials from GM plants in microbial communities;
  - ecological or human/animal health consequences of a potential HGT from a GM plant to microorganisms (e.g. potential spread of antibiotic resistance genes and probability of reduced efficiency of antibiotic treatments in humans);
  - information on the prevalence and distribution of genes identical or similar to the transgene in microorganisms in natural environments.

  The likelihood of DNA transfer to bacteria is generally independent of the function of the DNA sequence, therefore the probability is the same as for other transgenes. However, integration of transferred DNA into the bacterial genome by homologous recombination depends on sequence homologies. For sequences that encode dsRNA targeting genes of plants or plant pests the likelihood for sufficient sequence homologies to a bacterial genome is rather low. As for other GM plants, if regulatory sequences from bacteria are present in the transgene construct (like the nopaline synthase promoter and terminator from *Agrobacterium tumefaciens*), they may provide homologies necessary for integration into the genome of microorganisms. Maintenance of transferred DNA in bacteria is dependent on the encoded trait and on possible selective conditions (i.e. transfer of an antibiotic resistance marker gene can allow a selective advantage in the presence of the respective antibiotic). One of the differences between RNAi plants and a conventional GM plant is that the newly inserted DNA does not code for a protein. This implies that in case of horizontal DNA transfer from RNAi plants to bacteria no new functions will be acquired through expression of a novel protein. In addition, bacteria do not possess the RNAi machinery that is homologous to eukaryote cells so targeted modification of gene expression is not easily possible.

  Gene transfer is a rare event, but frequency may increase if there is positive selection of the transferred sequences to levels where it may be relevant. Potential ecological consequences due to the transfer to microorganism of a gene or trait that is already widespread in the environment (like many of the ARM genes) need to be estimated case by case; however, also this aspect is not specifically related to RNAi plants.

For some of the GM events transformed with dsRNA currently authorized in non-EU countries (e.g Rainbow papaya 55-1, Arctic™ apple), antibiotic resistance marker genes are included in the cassette (http://www.isaaa.org/gmapprovaldatabase/default.asp); however, there are no reports concerning negative effects on the spread of ARM genes in the environments where these crops are being cultivated. Other RNAi events were obtained without insertion of ARM genes in the cassette. This prevents concerns for possible spreading of ARM genes and can be confirmed with molecular characterization of every newly developed event.

Exposure characterization steps are similar for all GM plants, no specific additional requirements are deemed necessary and the existing requirements are considered equally applicable.

- **Target and Non-Target Organisms**

The main questions raised by the EFSA ERA GD are the following:

Target Organisms

- Data on the exposure of target organisms to the GM plant;
- Data on the potential for resistance development in the target organisms.

Exposure data describing dsRNA expression in different plant parts are derived from the compositional and molecular characterisation of GM RNAi plants as previously discussed. This will show possible exposure levels of targeted plant pests and pathogens throughout the growing season and post-harvest in seeds, plant biomass and plant debris. dsRNA may be designed to have sub-lethal effects on target species, for example by preventing mature development or inducing sterility. Thus *in vivo* studies involving *in planta* exposure are required to determine effects at both individual and population level.

Continuous or repeated exposure exerts a selection pressure which can promote onset of resistance in target pest populations. RNAi mechanisms are not an exception to this general rule. Khajuria et al. (2018) reported the first known case of a pest resistant to a mechanism of gene silencing. The authors selected in laboratory conditions a strain of the western corn rootworm, *D. v. virgifera* resistant to dsRNA targeting *DvSnf7* gene, through exposure to GM maize MON 87411. The study demonstrated that the resistance mechanism is based on an altered uptake of RNA molecules and individuals of this colony showed cross-resistance also to other dsRNA tested in their experiments.

Though the insurgence of resistance in target organisms represents mainly an agronomic problem, a possible drawback for the environment is the fact that the spread of a resistant strain could damage crop species in the area and then require additional use of pesticides. Data necessary for estimating the potential for resistance development in target organisms refer to the biology of target organisms (e.g. allele frequency, fitness, mobility). Data requirements for estimating the exposure of target organisms pertain to the ecology of the pest species and the levels of exposure to dsRNA in plant parts. In cases of RNAi plants where a dsRNA targets an insect pest gene and the plant also expresses a Cry toxin targeting the pest (e.g. as in maize MON 87411), the insects ability to develop resistance to the GM plant will be influenced. The resistance management strategies of EFSA (EFSA, 2015) apply here, and post release monitoring plans should therefore consider the likelihood of resistance development to such pyramided or stacked traits.

In planta tests are applicable for the study of pathogen resistance induced *via* RNAi (Rosa et al., 2018), for instance, by modulating the expression of membrane surface proteins. This type of response has to be tested in actively growing plants as surface protein responses may vary with plant development, and this may have different impacts on the infectivity of pathogens.

Non-Target Organisms

The steps needed to conduct an environmental safety assessment according to the EFSA ERA GD are the following:

- Collection of available knowledge of the environments and ecosystems likely to be exposed to the RNAi plants or plant parts or to hybridising relatives;
- Selection of NTO focal species to be tested based on the presence and ecological relevance of the species occurring in receiving environments of the GM plant, their sensitivity to the potential stressor and likely to be exposed to the dsRNA either through the GM plant or food chains;
- selection of measurement endpoints representative for protection goals;
- setup experimental protocols for bioassays to assess direct and indirect effects on selected NTO;
- data collection for estimating the exposure of NTOs to the dsRNA in GM plant and food chains.

The criteria for the selection of non-target focal species are based on the ecology of the NTOs, therefore no different assessment is needed compared to any other GM events, however bioinformatics can provide support to the selection procedure. Information on the presence of the same gene sequences targeted by the dsRNA in the genome of NTO can be used to select species potentially sensitive to the dsRNA expressed in GM plants. However, sequence match does not necessarily mean risk as the organism possess digestive and other physiological barriers that degrade/exclude the dsRNA from being taken up. Given the large variability in RNAi efficacy in invertebrates, even between closely related species sometimes, the species' sensitivity to RNAi could also be taken into account during the selection procedure. Species known as being barely sensitive to dsRNA after oral uptake are less valuable as NTOs in risk assessment.

In order to estimate the exposure potential of NTOs, in addition to some pheno-ecological characteristics of the NTO and its host plants (e.g. host spatial distribution and life cycle, overlap of NTO and plant life cycle) it is necessary that the presence of dsRNA/siRNA in plant tissues over time is estimated. While the full-length dsRNA is expressed in plants and can be reliably measured across tissues, the endemic dicer in plants will result in pools of siRNAs. siRNA estimates may not be relevant, since they are not expressed, but become biologically available

upon metabolic activation of the RNA machinery. Specifically, levels of siRNAs in plant tissues would likely be pools and therefore of limited biological impact, which can be better assessed with bioassays. Measuring the dsRNA instead, can provide a better estimate for exposure, based on the assumption that no greater number of siRNAs would be present than starting dsRNA. The important information is the minimum amounts of dsRNA required to initiate siRNA silencing activity, once that threshold is reached there may not be a direct correlation between quantities of dsRNA and activity levels. Therefore, quantification of full-length dsRNA provides the most appropriate exposure value for NTOs.

Organisms at the third trophic level (e.g. parasitoids, predators), can also be exposed indirectly to dsRNA by feeding on herbivore hosts/preys. The movement and the residual activity of dsRNA at the third trophic level was detected by Garbian et al. (2012) in *Varroa destructor* individuals feeding on *Apis mellifera*. It is known that some predator species show sensitivity to dietary RNAi upon ingestion (Haller et al., 2019), however the occurrence of such effects if exposed to a natural feeding regime (i.e. ingestion of prey feeding on dsRNA containing diet and pollen) in controlled conditions needs to be confirmed. To date the tritrophic bioassay with *Varroa* mites represents the only studied case in which exposure at the third trophic level was experimentally demonstrated and therefore specific bioassays will be needed to prove actual exposure of non-target carnivores.

Experimental protocols for evaluating effects on NTOs may need to be adapted to the case of RNAi plants. The most relevant route of exposure for NTOs in nature is likely to be oral exposure. A different effect can be obtained in some cases, when dsRNA is directly injected into the body of specimen toxic or supplied *via* ingestion (Powell et al., 2017). Therefore, toxicity tests should incorporate a dietary exposure to the test substance to ensure physiological exposure of specimen to dsRNA. A designed NTO study of sufficient length should allow for the elucidation of sublethal and lethal effects. Apical endpoints such as growth, development, reproduction/fecundity, and mortality are clearly linked to population level effects in NTOs and thus can be related back to protection goals.

Sub-lethal effects need to be duly considered, as gene silencing is expected in many cases not to induce acute lethal effects, but alteration of the physiology of target organisms may lead to a delayed effect and/or induce transient effects (e.g. Vargas et al., 2008; Kumar et al., 2012). In addition, studies of gene expression in the tested species where a matching sequence with the dsRNA has been observed in genomic analyses can ensure that the possible effect is indeed caused by silencing and not by environmental conditions during testing, which may affect gene expression in the target/non-target species.

In planta tests may also be necessary to ensure that safety for NTOs is demonstrated under environmental conditions that allow optimal gene expression in NTOs exposed to (near isogenic) control plants as well as test plants (Arpaia et al., 2017). Information regarding susceptibility of 'control' specimen in different conditions is then to be provided.

Another case in which *in planta* tests are applicable for ERA, is the study of pathogen resistance induced *via* RNAi, for instance, by modulating the expression of membrane surface proteins. This type of proteins cannot be tested in isolation, therefore *in planta* tests can be effectively used to assess impacts on NTOs (e.g. endophytic and mycorrhizal fungi) exposed directly or indirectly to dsRNA in GM plants.

Also in this area of ERA of RNAi plants, an additional source of available information in support to ERA is the availability of bioinformatics tools. However, due to the limited availability of genome sequencing for non-target organisms, and the restricted predictability of RNAi off-target genes in animals, bioinformatics cannot reliably determine the possibility of unintended silencing effects on those species but may represent a first screening, which may need further confirmation with the support of more traditional toxicity bioassays. Routinely screening the genomes of non-target organisms to identify genes that might be silenced, may not be practical due to the absence of sequence information on many NTOs. It may be possible to collect relevant information from the observation of off target silencing in the intended target species in order to estimate potential effects on NTOs, especially if they are taxonomically close to the target species.

STACKED EVENTS

In some cases, GM RNAi plants may be transformed to express more than one dsRNA, for example to silence a plant gene to confer a quality change as well as pest resistance. In these cases, the risk assessment should follow the EFSA guidance approach (EFSA, 2007) and carefully consider whether any interactions occur between the transgenes and/or the traits that may alter their expression or effects on targets and non-targets. For example, silencing a plant gene which has antifeedant activity may render the plant more susceptible to pests and thus affect pest plant relationship.

In addition, GM RNAi plants may contain transgenes conferring activity against the same or other pests and pathogens and providing other characteristics such as herbicide tolerance. For example maize MON 87411 expresses dsRNA targeting *DvSnf7* gene in *Diabrotica* spp., is also transformed to express a Cry3 toxin lethal to the same species. Other GM RNAi plants may contain transgenes stacked by hybridisation of lines with single and numbers of events such as other pest resistance genes, herbicide tolerance or other complimentary traits (e.g. maize MON87427×MON89034×MIR162×MON87411). In these cases the non RNAi GM events require the normal risk assessment procedures and so no changes in data requirements are envisaged for plants containing stacked RNAi and non-RNAi events for both ERA and food/feed safety evaluation. However, the risk assessments should carefully consider whether any interactions occur between the transgenes and/or traits, which may alter their expression or effects on target and non-target organisms. In the case of maize MON 87411 the expression of both the dsRNA and Cry3 toxin will result in different effects in

the target organism. The acute toxicity of Cry3 may limit the exposure of *Diabrotica* individuals to dsRNA through feeding, whereas *Diabrotica* with some resistance to Cry 3 will feed for longer and receive higher doses of dsRNA. Studies of non-target organisms should consider the effects of both traits together where they are combined in single events.

In GM plants with events stacked by hybridisation it is also important to note that there can be segregation of events in subsequent generations. As advised in the EFSA Guidance note on stacks (EFSA, 2007), all potential novel combinations of events should be risk assessed as well as the stack and the single events.

DISCUSSION

Genetically modified plants expressing interfering RNAs represent a new generation of GM plants for which more applications for commercialization are expected in the near future. Some aspects of their physiology make them quite different from the "first generation" of currently marketed GM plants expressing a few phenotypic traits (mostly insect resistance *via* expression of Cry toxins or herbicide tolerance). For instance, RNAi plants for insect resistance exploit completely new mechanisms of action (e.g. targeting the endosomal sorting complexes required for insect cellular transport in the SmartStaxPro® maize).

Therefore, in species with significant sequence similarity, underlying physiological mechanisms need to be considered in the context of the overall mechanism of action and previous history of use for products targeting some physiological functions in plant-dwelling organisms. Knowledge gaps (for example possible effects of the dsRNA in non-target organisms as well as off-target effects in the plant genome) need to be specifically tackled by applicants during risk assessment.

In their evaluation of Monsanto's GM maize MON 87411 and the stack, MON 89034 x TC1507 x MON 87411 x DAS-59122-7 combined trait maize (SmartStax® PRO) expressing a dsRNA targeting the Western Corn Rootworm *Snf7* gene, USEPA reviewed the extensive data set provided by the applicant on the effects of dsRNA in the context of its application in agriculture.

In considering potential human health risks from the dsRNA, USEPA's risk assessment was based on the evidence provided, including a 28-day toxicology study on the dsRNA, supporting the findings from a USEPA Scientific Advisory Panel (USEPA, 2014; USEPA, 2016a). The report indicated that "no reliable evidence that exogenous dsRNAs are taken up from the gut" existed. The Panel concluded that the combination of RNAses and acids founds in the human digestive system ensure that all forms of RNAs expressed in plant material and consumed by humans are likely to be degraded.

When considering the ecological risk assessment of DvSnf7 RNA in the GM maize events, USEPA analyzed the battery of laboratory tests on non-target organisms including invertebrate predators, parasitoids, pollinators, soil biota, and aquatic and terrestrial vertebrate species. Data presented were considered a reasonable framework for future environmental assessments of pesticide products based on environmental dsRNA (USEPA, 2017). As a new mode of action was involved, the DvSnf7 RNA ecological risk assessment was also reviewed by a FIFRA Science Advisory Panel (SAP). Several aspects of the risk assessment approach, including exposure assumptions, environmental fate and non-target effects data, on toxicity and possible synergism with stacked *Bt* traits were considered. Furthermore, the risk assessment went on to state that "*in silico* evaluations are not considered to be predictive of adverse effects" (USEPA, 2017), and that such evaluations are currently only considered as supplemental information to provide additional evidence for risk determination.

Taken together, the data in support of the approval of GM maize events were considered adequate by EPA and they concluded that the application of RNAi-based mode of action for pest control in agriculture presents minimal hazard and risk to non-target organisms with protection goals (USEPA, 2016b; USEPA, 2017).

Likewise, Food Standard Australia and New Zealand applies a case-by-case approach to GM food safety assessment, which is considered sufficiently broad and flexible to address the safety of GM foods developed using gene silencing techniques such as RNAi technology (FSANZ, 2013).

The EFSA GDs are the technical support for applicants conducting risk assessment of GM plants according to the European legislative framework. The GDs indicate the general principles for conducting risk assessment, purposefully leaving room for selecting the necessary information for preparing dossiers case by case.

In this paper, we considered the main principles described in the current EFSA GDs for risk assessment of GM plants, to determine which areas of the existing risk assessment approaches for GM plants are appropriate or could be refined, and if complementary or alternative risk assessment strategies need to be developed for RNAi plants in the EU. We aimed at highlighting the rationale for defining specific biosafety data requirements for the risk assessment and risk management of RNAi plants and their derived products (i.e. food and feed).

The outcomes of our analyses, suggest that data requirements for the risk assessment of RNAi plants will be similar to other GM plants and therefore the risk assessment framework used so far for other GM plants is still valid. Likewise, the case by case approach depending on plant species, event and trait also applies for case specific post market monitoring. Guidelines for risk assessment cannot be "cookbooks" and some flexibility should be left for risk assessors to adapt and justify the details of their assessment. It is up to regulatory agencies to judge the validity of risk assessment approaches and support applicants in delivering estimates of risk at the highest safety standard, considering the severity and the likelihood of possible impacts on human, animal and environmental safety.

The approach outlined in this paper could provide support to future updates of the EFSA GDs, since there is not yet much experience, especially for environmental risk assessment, of RNAi plants. In particular, we wish to highlight a few characteristics of RNAi mechanisms, which need consideration during specific steps of the risk assessment (e.g. molecular characterization, NTO species selection).

A Simplified Risk Assessment for Food and Feed Derived From RNAi Plants

Risk assessment of RNAi plants may need less data in some steps of the process. For instance, the molecular analysis and comparative compositional analyses between the GM plants and their comparators might show that no new proteins are produced and endogenous protein levels are unchanged. In this case, assessment of toxicity and allergenicity of new plant products are unnecessary. However according to the implementing regulation (EU 503/2013), 90-days feeding studies on rodents are currently mandatory regardless of the additional data available from chemical analyses and their interpretation.

The Role of Bioinformatics

Bioinformatics may have an important role in supporting the risk assessment of RNAi plants, e.g. through opportune comparisons of the genomes (even if only partially known) of target and relevant non-target organisms which might be exposed to the interfering RNA in the field. In fact, a thoughtful design of dsRNA at the beginning of the process of development of new RNAi plants, can limit the possibility of non-target effects due to sequence similarity (USEPA, Scientific Advisory Panel, 2014). It is known that the taxonomic and genomic proximity of target and non-target species renders silencing effects more likely (Christiaens et al., 2018), however the extent of these effects may be variable between species in the same family (Haller et al., 2019). The limited availability of insect genomes in currently accessible databases further limits the predictive ability of bioinformatics, so that supporting data for an absence of non-target effects needs to be obtained through bioassays. However, results from bioinformatics analyses may contribute to build a weight of evidence on the safety assessment of RNAi plants.

Specifically Tailored Bioassays

Experimental protocols for bioassays may need to be adapted in order to achieve the required sensitivity in detecting possible effects. While exposing test specimens in laboratory conditions to dsRNA *via* injection is a useful tool for elucidating silencing mechanisms, this pathway cannot be considered biologically relevant for estimating *in vivo* exposure of TOs and NTOs in nature; therefore ingestion of RNAi plant tissues or diet-incorporated dsRNA should be adopted for testing NTOs. Experimental conditions should ensure that the expression of the target gene is optimal in the specimen not exposed to dsRNA containing diet for the whole duration of the test. Enzymatic barriers in some species may degrade dsRNA, so the silencing effect in a given species might not occur in other taxa. Additional measurement endpoints (e.g. measurement of expression of the target gene) can increase confidence on the results of the bioassays. For instance, an analysis of silencing effects on the target gene (and possibly on a few essential off-target genes with sequence similarities) can clarify the physiological mechanisms determining phenotypical characters (i.e. mortality and sub-lethal effects).

Observations of the presence of dsRNA in different tissues and over the growth period of plants will be necessary to estimate exposure of target and some selected non-target organisms. In addition, data will be required on expression levels of dsRNA in TOs to enable the selection of significant pathways necessary to estimate exposure of their natural enemies to the dsRNA.

A special remark concerns the risk assessment of GM RNAi trees, some of which have already been authorized for commercialization outside the EU. Though it is not unique for RNAi-based events, the possible applications for commercial release of GM rootstocks will need a reflection on what kind of information will be needed regarding scions, and consequently on the fruits produced by such varieties. Current European regulation states that if part of a plant is GM then the whole plant is designated as GM. Consequently, a product of a GM plant, even if does not contain transgenic DNA, is classified as GM. In the European system, approval is given for a GM event. Once the event is approved it can be put into any genetic background through hybridization. For instance, if the same GM *Prunus* rootstock is used for plum, cherry, peach, apricot scions, then no new application is required.

Finally, reference is to be made to the recent developments of RNAi-based pesticide products for external application. While these products are subject to pesticide regulation, risk assessment requirements and protocols are likely to be derived from experiences with GM RNAi plants. However, there are some unique aspects, which will have to be considered for externally applied RNAi products, such as issues of plant uptake, effect of chemical modifications, carriers or formulations on dsRNA stability, the effects of stabilizing measures on the exposure and impacts of non-target species (Taning et al., 2020).

CONCLUSIONS

A working group of the COST Action iPlanta discussed the main aspects, relevance and applicability of the principles of the existing EFSA guidelines to environmental and food/feed risk assessment for RNAi plants. The authors consider that the current science-based regulatory process in Europe is still applicable to RNAi plants; nevertheless, the assessment process should permit some flexibility for risk assessors to adapt and justify the case-by-case assessment of their RNAi plants.

We highlight the following considerations linked to the peculiarity of RNAi GM plants that could be also considered for further updates of the existing EFSA Guidance Documents on risk assessment for GMOs:

- The data related to newly expressed proteins, protein equivalence and the codon optimization are irrelevant for the inserted DNA as long as no part is translated to protein. Consequently, the food and feed safety assessment could be simplified with regard to novel proteins and their potential allergenicity if no novel proteins are produced. Intended as well as unintended effects triggered by siRNA in the plant can be detected by compositional analyses and, where applicable, nutritional evaluation of food and feed derived from RNAi

plants. Potential siRNA effects in humans and farm animals through dietary dsRNA/siRNA are highly unlikely because of rapid degradation in the GI tract and several barriers to cellular uptake in mammals. The data on levels of dsRNA expression over time, in different plant tissues and related to environmental conditions during the experiments are necessary for estimating the possible exposure to the dsRNA in plants or derived food or feed for consumers and non-target organisms in the receiving environment;

- Bioinformatics can offer a good support to risk assessment, especially when designing dsRNA sequences specific to the target gene and minimizing the potential for off-target binding sites. Due to the limitations in genome sequences for NTOs, for ERA bioinformatics analyses should be complemented with specifically developed bioassays and measurement of gene suppression.

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The Use of Engineered Plant Viruses in a *Trans*-Kingdom Silencing Strategy Against Their Insect Vectors

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Plants, plant viruses, and their vectors are co-evolving actors that co-exist and interact in nature. Insects are the most important vectors of plant viruses, serving as both carriers and hosts for the virus. This *trans*-kingdom interaction can be harnessed for the production of recombinant plant viruses designed to target insect genes via the RNAi machinery. The selection of the adequate viruses is important since they must infect and preferentially replicate in both the host plant and the insect vector. The routes of transmission that determine the extent of the infection inside the insect vary among different plant viruses. In the context of the proposed strategy, plant viruses that are capable of transversing the insect gut-hemocoel barrier and replicating in insect tissues are attractive candidates. Thus, the transmission of such viruses in a persistent and propagative manner is considered as a prerequisite for this strategy to be feasible, a characteristic that is found in viruses from the families *Bunyaviridae*, *Reoviridae,* and *Rhabdoviridae*. In addition, several RNA viruses are known that replicate in both plant and insect tissues via a yet unclarified transmission route. In this review, advances in knowledge of *trans*-kingdom transmission of plant viruses and future perspectives for their engineering as silencing vectors are thoroughly discussed.

Keywords: VIGS, VDPS, plant virus, insect virus, insect vector, *trans*-kingdom

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INTRODUCTION

Studies on plant viruses' biology have shown their dependence on a plethora of vector organisms for their transmission to a new host. This vector repertoire involves insects, mites, nematodes, plasmodiophorids, and fungi (Bragard et al., 2013; Blanc et al., 2014). Although differences exist between vectors, the plant virus transmission cycle includes certain standard steps that seem to apply to almost all occasions. In the case of an insect vector, for example, (1) an infected plant is first detected as a source of food, (2) the insect feeds from the plant, (3) it acquires the virus, (4) as a carrier of the virus it can transport it, and (5) in the search for a new food source, the virus is transmitted to the next plant that the insect vector selects to feed from (Whitfield and Rotenberg, 2016).

However, different strategies (**Table 1**) are employed during plant virus transmission by insect vectors that are distinguished by the acquisition time and retention period of the virus by the vector (Nault, 1997). Non-persistently or semi-persistently transmitted viruses have a half-life of minutes to hours and typically involve temporary attachment to the stylet or the foregut in hemipteran

insects (Blanc et al., 2014). By contrast, viruses that are persistently transmitted cross the midgut barrier and accumulate in the salivary gland, while their half-life of retention by the vectors can last from days to months (Nault, 1997; Blanc et al., 2014). Among persistently infecting viruses, plant viruses exist that also replicate in their insect vectors, a strategy called "propagative." While the distribution of propagative viruses among insect vectors may be restricted (Hogenhout et al., 2008; Bragard et al., 2013), they raise particular interest from the biotechnological viewpoint. More specifically, genomes of propagative viruses have the potential to be engineered into agents that cause gene silencing in the insect vectors by the RNAi mechanism, which could be developed into an environmentally safe strategy to control the vectors.

When viruses are used as viral dsRNA-producing systems (VDPS; see also further below), two main strategies can be followed regarding the introduction of the "silencing" fragment into the viral genome, as described for plant closteroviruses (Qiao and Falk, 2018): (1) An "add-a-gene" strategy that, in simple words, involves the insertion of a foreign sequence in a particular position so that it minimally interferes with the existing viral sequences. As it has been suggested, insertion near the 3′ terminus of an ORF may enhance the production of the new fragment during transcription (Navas-Castillo et al., 1997). (2) A "gene replacement" strategy of a viral genomic part encoding a non-essential protein. This second strategy is often used; however, it has been criticized for reduced efficiency compared to the previous one (Kiss et al., 2013). Because of ease of manipulation, VDPS employing plant viral vectors can prove useful as a fast-track approach to identify suitable target genes to cause toxic effects in target insects following silencing by RNAi (Kolliopoulou et al., 2017). Furthermore, similar methods of engineering of viral genomes can be used to create recombinant plant viruses that can trigger RNAi effects in insect vectors by a strategy named as "*Trans*-kingdom virus-induced gene silencing" (TK-VIGS) (**Figure 1**), as outlined further below.

ROUTES OF TRANSMISSION

Plant viruses that are known to be transmitted via particular insect families have been observed to follow diverse routes in their vectors' bodies (Hogenhout et al., 2008; Blanc et al., 2014). This diversification in the mode of transmission can be attributed to differences in the virus genomic and structural properties, as well as to physiological and anatomical variations among insect vectors belonging to distinct genera and species (Bragard et al., 2013). A general classification of these modes of transmission has led to the establishment of three main categories that differentiate one from another regarding the time window that they can be transmissible by the insect vector to a new plant. This classification includes the non-persistent, the semi-persistent and the persistent types (Fereres and Raccah, 2015; Dietzgen et al., 2016), as discussed below. Examples of the different transmission strategies are displayed in **Table 1**.

In all the cases, for vectors with piercing-probing mouthparts (Hemiptera and Thysanoptera), the virus is acquired during the probing and feeding activity. In the case of the non-persistent way of transmission, plant virus acquisition by stylet piercing of plant tissue is followed by transmission to the next plant by piercing within a period of seconds to minutes. This non-persistent strategy is mainly used when aphids repetitively puncture cells during the probing of plant tissue en route to feeding with phloem sap (Stafford et al., 2012; Nalam et al., 2019). For plant virus transmission in a semi-persistent manner, the virus is known to be retained in the more proximal part of the feeding apparatus, the acrostyle, or to become bound to the chitin lining of the foregut (Killiny et al., 2016; Webster et al., 2018). The involvement of specific viral capsid proteins aiding the transmission process has been reported for the whitefly vector *Bemisia tabaci* and the *Lettuce infectious yellow virus* (LIYV) (Stewart et al., 2010; Chen et al., 2011). In that case, the virus can be transmitted over a period from several hours to days to the next target plant. Both cases of non-persistent and semi-persistent transmission are also described as non-circulative, since upon entry the plant virus is localized only at specific spots on the vector's body (i.e., stylet, gut chitin lining) and does not cross the insect gut barrier in order to circulate to other tissues.

However, the most interesting category for biotechnological applications is that of plant viruses that are transmitted in a persistent manner, with considerable variation in terms of transmission window. This transmission route is also characterized as circulative, because the acquired virus must transverse the gut epithelium from the alimentary canal to other tissues and organs, so that it can finally reach the salivary glands. Depending on the particular plant virus–insect interaction, the transmission process may rely on specific viral proteins. Extended duration of feeding on infected plant phloem (hours), as well as prolonged retention time and a latent period, is required in order to obtain adequate transmission efficiency of the virus. Circulative plant viruses that do not replicate in the insect vectors include luteovirids, geminiviruses, and nanoviruses and are transmitted by aphids, whiteflies, and leafhoppers (Nault, 1997; Hogenhout et al., 2008). The capacity of luteoviruses to transverse the midgut epithelium has raised interest for biotechnological applications such as a new strategy to deliver peptide toxins into the hemocoel of aphids after feeding (Bonning et al., 2014).

On the other hand, viruses exist that are able to replicate and systemically invade insect tissues before reaching the salivary gland (thus taking up a propagative strategy), such as those belonging to *Reoviridae*, *Bunyaviridae,* and *Rhabdoviridae* families, as well as the *Bunyaviridae*-related genus *Tenuivirus* (Whitfield et al., 2015; Dietzgen et al., 2016). In addition to their circulation between plant and insect hosts, many propagative viruses can also be transmitted transovarially (vertically) to the insect's offspring (Nault, 1997; Hogenhout et al., 2008). Plant viruses with a propagative transmission strategy in their insect vectors are discussed in more detail below.

WHICH INSECT VECTORS?

By definition, plants lack any ability to move and they depend on their root system that provides connection with the soil in order

TABLE 1 | Examples of plant viruses and their insect vectors with different transmission strategies.

Transmission Strategy	Virus	Family (Genus)	Insect vector(s)	References
Non-persistent Non-circulative	*Potato virus Y* (PVY)	*Potyviridae (Potyvirus)*	Aphids e.g., *Myzus persicae*	Mondal and Gray, 2017
	Cucumber mosaic virus (CMV)	*Bromoviridae (Cucumovirus)*	Aphids e.g., *Myzus persicae*	Moreno et al., 2005
Semi-persistent Non-circulative	*Cauliflower mosaic virus* (CaMV)	*Caulimoviridae (Caulimovirus)*	Aphids e.g., *Acyrthosiphon pisum*	Webster et al., 2018
	Lettuce infectious yellows virus (LIYV)	*Closteroviridae (Crinivirus)*	Sweet potato whitefly *Bemisia tabaci*	Stewart et al., 2010
Persistent Circulative Non-propagative	*Beet western yellows virus* (BWYV)	*Luteoviridae (Polerovirus)*	Green peach aphid *Myzus persicae*	Brault et al., 1995
	Tomato yellow leaf curl virus (TYLCV)	*Geminiviridae (Begomovirus)*	Sweet potato whitefly *Bemisia tabaci*	Medina et al., 2006
Persistent Circulative Propagative	*Tomato spotted wilt virus* (TSWV)	*Peribunyaviridae (Tospovirus)*	Thrips e.g., *Frankliniella occidentalis*	Whitfield et al., 2005
	Rice stripe virus (RSV)	*Phenuiviridae (Tenuivirus)*	Planthopper *Laodelphax striatellus*	Liang et al., 2005
	Fiji disease virus (FDV)	*Reoviridae (Fijivirus)*	Planthopper *Perkinsiella saccharicida*	Ridley et al., 2008
	Rice ragged stunt virus (RRSV)	*Reoviridae (Oryzavirus)*	Brown planthopper *Nilaparvata lugens*	Huang et al., 2015
	Rice dwarf virus (RDV)	*Reoviridae (Phytoreovirus)*	Leafhopper *Nephotettix cincticeps*	Honda et al., 2007
	Lettuce necrotic yellows virus (LNYV)	*Rhabdoviridae (Cytorhabdovirus)*	Aphid *Hyperomyzus lactucae*	Randles, 1983
	Rice yellow stunt virus (RYSV)	*Rhabdoviridae (Nucleorhabdovirus)*	Leafhopper *Nephotettix cincticeps*	Wang et al., 2019

for a plant to feed, grow, and remain alive. So, for any virus that inhabits a plant, it is normal to rely on mobile vectors so that it is transmitted to other plant hosts.

Among the 32 orders of Insecta, seven of them have been found to encompass vectors for plant virus transmission. Most of these vector insects belong to Hemiptera (300) and Thysanoptera (6), which are characterized by pierce-sucking mouthparts (Fereres and Raccah, 2015). It must be stressed that Hemiptera are by far the most important vector group, since they cause minimal damage while feeding and leaving cells alive for virus to replicate while probing. On the contrary, Thysanoptera tend to provoke extensive cell damage during ingestion probes, thus hampering viral replication (Stafford et al., 2012). The anatomy of the mouthparts of these insects plays an important role in the virus transmission from plant to plant, as piercing of the epidermis that is covered by an impermeable cuticle allows for the virus to be delivered intracellularly and maintains the integrity of the plant cell (Fereres and Raccah, 2015). Thrips is a characteristic type of thysanopteran insects, with 14 species being vectors of the plant-infecting *Tospovirus* genus (*Bunyaviridae*) (Riley et al., 2011; Badillo-Vargas et al., 2015).

More vectors have been observed in other orders too (i.e., Coleoptera, Orthoptera, Lepidoptera, Diptera, and Dermaptera) (Fereres and Raccah, 2015), in which however different ways of transmission are utilized that lie beyond the scope of this mini review.

WHICH VIRUSES?

An important feature that defines the suitability of plant viral vectors for the delivery of the dsRNA silencing signal to their target insect regards the ability of the virus to replicate or not in the insect cells. Most plant viruses are not able to replicate in their insect vectors, so they can only be considered as vehicles for transfer of dsRNA that has already been synthesized inside the plant to the insect. On the other hand, replicating viruses not only are able to transverse the gut-hemocoel barrier (as "persistent circulative" viruses mentioned earlier) but also adopt a propagative strategy, which means that dsRNA intermediates are produced during their life cycle.

The viruses adaptable for gene silencing strategy, i.e., circulative-propagative viruses that do replicate in the insect body before transmission, are members of the families of *Bunyaviridae* [segmented ambisense ssRNA genome, such as *Tomato spotted wilt virus* (TSWV) whose main vector is the western flower thrips *Frankliniella occidentalis*], *Reoviridae* [segmented dsRNA genome, such as *Rice dwarf virus* (RDV) transmitted by the leafhopper *Nephotettix cincticeps*], and *Rhabdoviridae* [(−)ssRNA genome, such as *Maize mosaic virus* (MMV) vectored by the planthopper *Peregrinus maidis*]. Also *Tenuivirus* genus [(−)ssRNA genome, such as *Rice stripe virus* (RSV) and its vector planthopper *Nilaparvata lugens*] belong to this type of viruses (Whitfield et al., 2015; Dietzgen et al.,

2016). Interestingly, as it will be explained later, *Tymoviridae* and *Nodaviridae* virus families that are known to have multiple kingdom hosts have already been proposed too as possible gene silencing vehicles via employment of reverse genetics techniques by taking also advantage of the existing interplay between plants and insects (Katsuma et al., 2005; Taning et al., 2018). Rhabdoviruses and bunyaviruses are the only enveloped plant viruses while reoviruses, tenuiviruses, tymoviruses, and nodaviruses do not contain a lipid envelope such as the other plant viruses that do not have a propagative transmission strategy (Hogenhout et al., 2008).

On the other hand, viruses that circulate in the insect body but in a non-propagative, thus not-replicative, manner includes members of the families of *Geminiviridae* [single or bipartite (+)ssDNA genome, such as *Tomato yellow leaf curl virus* (TYLCV) transmitted by different whitefly species], *Luteoviridae* [(+)ssRNA genome, such as *Pea enation mosaic virus* (PEMV) transmitted by the aphid *Acyrthosiphon pisum*], and *Nanoviridae* [multipartite (+)ssDNA genome, such as *Banana bunchy top virus* (BBTV) vectored by the aphid *Pentalonia nigronervosa*].

It needs to be stressed that a considerable amount of propagative plant viruses are strictly associated with particular insect vector families (Nault, 1997; Hogenhout et al., 2008). All tospoviruses are transmitted by thrips vectors; reoviruses of the *Phytoreovirus* genus and tymoviruses are transmitted by leafhoppers (Cicadellidae); and reoviruses of the genera *Fijivirus* and *Oryzavirus* as well as tenuiviruses are transmitted by planthoppers (Delphacidae). A notable exception, however, is the rhabdoviruses that can be transmitted either by aphids, leafhoppers and planthoppers (Nault, 1997; Hogenhout et al., 2008).

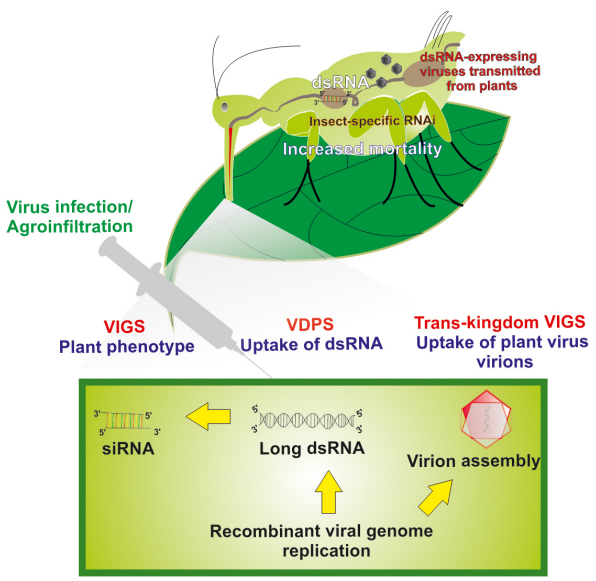

FIGURE 1 | Long dsRNA production and virus-mediated gene silencing in plant cells and insect cells. Transgenic viruses are being transmitted in plants via agroinfiltration and/or injection. After infection, replication of the viral genome's results in the production of long dsRNAs. Virus-based dsRNAs are being processed to siRNAs by the plant's core RNAi machinery causing gene-specific phenotypes in plants (virus-induced gene silencing, VIGS). Unprocessed insecticidal long dsRNAs produced in plant cells that are being taken up by insects also can cause insect-specific RNAi that leads to increased mortality rates (virus-dependent dsRNA production, VDPS). For particular instances, assembled virions in plant cells can also be taken up by insects and, during replication in insects, release insecticidal dsRNAs that cause insect-specific RNAi and increased mortality rates (*Trans*-kingdom VIGS, TK-VIGS).

PRODUCTION OF vsiRNAs AS A SIGN OF RNAi MECHANISM ACTIVATION

Double-stranded RNA molecules are expected to be produced by propagative viruses, during their replication or transcription steps inside the insect host's body after infection. Reoviruses possess a dsRNA multisegmented genome from which mRNA can be transcribed. For the production of new dsRNA molecules, (+)RNA strands are encapsidated in a subviral particle, where they are transcribed into (−)RNAs, so that dsRNA is then produced by base-pairing of the complementary (+) and (−) strands. In the case of single-stranded or ambisense RNA viruses, intermediate replicative steps normally may also lead to the temporary production of dsRNA molecules. Engineering of plant viruses that possess single-stranded RNA genomes with appropriately designed sequences in order to transcribe RNA molecules containing self-hybridizing regions would lead to the formation RNA hairpin structures. These RNA hairpins could then be processed in dsRNAs and siRNAs by the RNAi machinery of the insect vector. Also, regarding DNA viruses inverted repeats can be introduced in their sequence so that RNA hairpins with dsRNA structures can be formed upon transcription.

As the replication of propagative plant viruses is expected to lead to the production of viral short-interfering RNAs (vsiRNAs) in the insect vector, it can be assumed that the RNAi silencing machinery is turned on upon such an infection. Several examples of vsiRNA production in insects during plant virus infection have been reported in the literature, such as the infection of the small brown planthopper *Laodelphax striatellus* by the *Rice black-streaked dwarf virus* (RBSDV, *Reoviridae*) or RSV (*Tenuivirus*) (Xu et al., 2012; Li et al., 2013; Yang et al., 2018), of the white-backed planthopper *Sogatella furcifera* by RBSDV (Wang et al., 2016), in the zigzag leafhopper *Recilia dorsalis* by the *Rice gall dwarf virus* (RGDV, *Reoviridae*) (Lan et al., 2016a), and in the planthopper *Delphacodes kuscheli* by *Mal de Río Cuarto virus* (MRCV, *Reoviridae*) (De Haro et al., 2017). Furthermore, vsiRNA production was observed in the glassy-winged sharpshooter *Homalodisca vitripennis* (Cicadellidae) after infection with *H. vitripennis reovirus* (HoVRV) (*Phytoreovirus* genus) (Nandety et al., 2013) although a plant host for this virus was not identified (Stenger et al., 2009).

However, while deep sequencing efforts have detected vsiRNAs of propagative plant viruses in hemipteran vectors, their functionality (capacity of gene silencing) remains largely unexplored. Thus, a systematic effort needs to be initiated regarding the role of RNAi as an antiviral defense mechanism against replicating plant viruses in insect vectors, which will

include the effect of knockdown of the RNAi machinery on viral replication [of which limited data are available (Lan et al., 2016a,b)] and the possible existence of Viral Suppressors of RNAi (VSR) genes in viral genomes [which are known to act within plants (Cao et al., 2005; Xiong et al., 2009)]. In plants, on the other hand, RNAi has been used successfully to control plant viruses that are transmitted by insect vectors in a circulative propagative manner [e.g., RBSDV (Shimizu et al., 2011)].

EXAMPLES OF ENGINEERED PLANT VIRUSES TO TARGET THEIR INSECT VECTORS: FROM VDPS TO *TRANS*-KINGDOM VIGS

As a first straightforward application of engineered plant viruses causing gene silencing in insect vectors, the VDPS system (Kumar et al., 2012) was developed and its silencing efficiency was shown to compare favorably with conventional plant-mediated RNAi (PMRi; using transgenes to express dsRNA molecules). As will be explained in this section, the paradigm of VDPS was later expanded and further elaborated by the use of more sophisticated engineering concepts in several virus–vector combinations.

In the case of Hemiptera, an interesting example of efficient application of recombinant VIGS (here used as VDPS) technology to combat insect vectors was the construction of recombinant *Tobacco mosaic virus* (TMV, *Virgaviridae*) that produced RNAs in sense or antisense orientation targeting *actin*, *chitin synthase 1,* and *V-ATPase* genes of the hemipteran pest *Planococcus citri* that were initially inoculated to *Nicotiana benthamiana* plants through agroinfiltration. This method was successful in causing silencing of the above-mentioned genes of the insect vector (Khan et al., 2013) that is known to facilitate the transmission of *Grapevine leafroll associated virus 3* (GLRaV-3, *Closteroviridae*) among grapevine plants (Cabaleiro and Segura, 1997). Another hemipteran, the phloem-feeding *Bactricera cockerelli* was tested as a possible target by recombinant TMV viruses in which sequences against *BC-actin* and *BC-V-ATPase* genes were cloned, primarily infecting tomato (*Solanum lycopersicum*), tomatillo (*Physalis philadelphica*), and tobacco (*Nicotiana tabacum*) plants. In the same study, two other viruses (*Potato virus X*, PVX, *Alphaflexiviridae*; *Tobacco rattle virus*, TRV, *Virgaviridae*) were tested (Wuriyanghan and Falk, 2013). A few years later, however, PVX was repeatedly reported as an efficient and easy tool for *in planta* RNAi induction against the hemipteran phloem-feeding pest mealybug *Phenacoccus solenopsis* (by agroinfiltrating *N. tabacum* plants). In these studies, the *Bursicon*, *V-ATPase* and *Chitin synthase 1* genes of the insect vector were targeted, as proven by decrease in expression and increase in population mortality as well as other phenotypic effects (Khan et al., 2015, 2018). A virus of the same genus, *Alternanthera mosaic virus* (AltMV, *Alphaflexiviridae*), was used to create an RNAi vector for the silverleaf whitefly *Bemisia tabaci* where numerous cDNA clones could be then inserted and used as a screening method for finding the ideal target gene (Ko et al., 2015). Also *Citrus tristeza virus* (CTV, *Closteroviridae*) was effectively used to silence the *altered wing disc* (*Awd*) gene in CTV-based RNAi assays against the hemipteran phloem-sap sucking insect *Diaphorina citri*, although this particular virus is known to normally encode for three different silencing suppressors. Interestingly, these experiments involved a few additional steps, as CTV constructs were first agroinfiltrated into *N. benthamiana* plants, and virions produced there were then isolated and inoculated to *Citrus macrophylla* plants to trigger silencing in its target insect *D. citri* (Hajeri et al., 2014).

In the above examples, plant viruses were engineered as a VDPS to produce dsRNA in plant tissues at high levels to cause gene silencing and mortality in insects after feeding. The RNAi effects in the insect vectors were not caused by plant virus replication within the vectors but by the large amounts of dsRNA produced in the plant cells. VDPS is not considered a static system since it can be envisioned that the VDPS systems and the viruses that sustain them are spread through the plant populations by taking advantage of the virus transmissibility by insect vectors (Qiao and Falk, 2018). However, VDPS is not necessarily dependent on hemipteran vectors; for example, TRV is transmitted by nematode vectors (Ploeg et al., 1993) while PVX is only mechanically transmitted (Franc and Banttari, 2001). In such cases, spread in the field can be more limited and will depend on the transmission strategy of the virus that is used in the VDPS. By its nature, VDPS is also not limited to affect hemipteran insect vectors but can be employed to control agricultural pests belonging to different insect orders such as Lepidoptera (Kumar et al., 2012; Bao et al., 2016).

A more challenging category of viruses are those that infect and replicate in hosts belonging to different kingdoms, such as viruses of the *Tymoviridae* family that are known to infect plants but are also non-persistently transmitted by phloem-sucking insects (Martelli et al., 2002a,b). However, "tymovirus-like viruses" have the ability to replicate in insect tissues. *Culex Tymoviridae-like virus* (CuTLV) was isolated a few years ago from mosquitoes in China and found to present high sequence similarities with other plant-infecting *Tymoviridae* genera (Wang et al., 2012). Moreover, recently a new *Tymoviridae-like virus* was identified in *Culex quinquefasciatus* mosquitoes in Mexico (Charles et al., 2019). However, both these viruses have not been assigned officially to the *Tymoviridae* family, while it remains to be investigated whether they can be transmitted and possibly replicate in plants too (Wang et al., 2012; Charles et al., 2019). Similarly, sequencing has revealed the presence of *Bee macula-like virus* (BeeMLV) and *Bombyx mori macula-like virus* (BmMLV) in honeybee and silkworm samples, with the genus *Maculavirus* being one of the main genera of *Tymoviridae* family, thus meaning a possible interplay of these virus between hosts of plant and insect origin (Katsuma et al., 2005; De Miranda et al., 2015). Up to now, it remains unclear whether a particular "tymovirus-like virus" can replicate in both insect and plant tissues and whether host switching (from plant to insect and vice versa) occurs frequently in nature. Interestingly, *Flock house virus* (FHV) is an insect pathogenic virus that belongs to the *Nodaviridae* family, but can also replicate and produce functional virions

in plants (Selling et al., 1990). Engineered transencapsidated FHV has been efficiently packaged upon agroinfiltration in plant cells and used as vehicle for transfer as vaccine in mammalian cells (Zhou et al., 2015). Also, the modification of FHV genome for silencing specific genes was shown to lead to targeted gene suppression and mortality in insects *in vivo* and *in vitro* (Taning et al., 2018). Therefore, FHV could be engineered to carry purposely designed sequences against an insect host, so that it was ideally produced in a plant system and transmitted to insects in order to control a dangerous insect pest population. However, also in the case of nodaviruses the mode and efficiency of transmission between plants and insects needs much more investigation before the proposed strategy can be employed.

Sonchus yellow net rhabdovirus (SYNV) is another plant virus for which a reverse genetics system became available (Wang et al., 2015). Because SYNV presumably is transmitted by aphid vectors in a circulative-propagative manner, the potential exists for the usage of recombinant SYNV as a gene silencing inducing agent in aphids. For instance, recombinant SYNV viruses that target essential genes of the aphid vectors could be produced in plants and deployed to infect aphid vectors and cause toxic effects following replication and dsRNA production, an approach termed *Trans*-kingdom virus-induced gene silencing (TK-VIGS; **Figure 1**). However, reverse engineering of SYNV remains a major challenge because of its large size and possible pathological effects caused during infection of plant tissues. While this approach is very attractive conceptually, major engineering efforts are considered necessary to make it a viable tool.

CONCLUSION

The strategy of modifying plant viruses in order to produce novel tools that will offer the potential of virus-mediated gene silencing in insects is an attractive option for pest control. Because RNA molecules are considered safer than proteins (Petrick et al., 2013; Ramon et al., 2014; Casacuberta et al., 2015), the strategy of causing RNAi effects may be looked upon favorably by regulatory bodies. The engineering of plant viruses to silence genes in their insect vectors offers advantages such as the specificity of their target gene silencing, as nowadays RNAi has turned into a well-established and feasible control technique. For instance, VDPS is expected to accelerate the screening procedures for candidate genes, and thus for target sequences, in the frame of designing a well-orchestrated RNAi-mediated agricultural pest control strategy (Hajeri et al., 2014). Obviously, compared to other methods like the development of transgenic plants, VDPS is considered as an impressively rapid and cost-effective alternative technique (Bao et al., 2016). However, a relevant concern regarding the use of this technique is that recombinant plant viruses are considered to include a risk of being quite unstable, since the insertion of duplicated sequences increases the possibility of homologous recombination incidents, therefore stressing a tendency for them to return to the wild type (Donson et al., 1991; Qiao and Falk, 2018).

In this review, we propose a more radical strategy of using viral vectors to cause gene silencing in insects, which is called *Trans*-kingdom VIGS (**Figure 1**). The challenge will be to produce plant viruses that cause minimal damage in host plants but trigger cellular damage and mortality following infection and replication in insect vectors after transmission. Because the viruses replicate in both types of hosts, a high efficiency of transmission of RNAi triggers is expected. Furthermore, because propagative plant viruses can be transmitted vertically in insect vectors, the silencing effect may become amplified along the next generation.

In comparison to VDPS, however, *Trans*-kingdom VIGS has some restrictions. VDPS can be harnessed to control not only hemipteran vectors but also other agricultural pests, most notably lepidopteran larvae, because it is based on production of dsRNAs in plant tissues. In *Trans*-kingdom VIGS, dsRNAs are produced in insect (hemipteran) vectors during their transmission and its efficiency therefore is limited to particular insect species that are competent hosts for propagative transmission. *Trans*-kingdom VIGS is therefore more likely to be developed as a "tailor-made" application for the control of particular plant virus–insect vector combinations.

To achieve such goals, much more knowledge needs to be acquired for the establishment of reverse genetics systems for propagative plant viruses and the stimulation of the transmission between plant hosts and insect host/vectors. Furthermore, viruses (e.g., FHV) exist that have a very broad host range and that can replicate in both plants and insects also deserve renewed attention to see if they can be adapted to the strategy of *Trans*-kingdom VIGS.

AUTHOR CONTRIBUTIONS

AK, DK, and LS conceived the idea, designed the study, and wrote the manuscript. DK prepared the figures of the study. All authors read and approved the final version of the manuscript.

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Validating the Potential of Double-Stranded RNA Targeting Colorado Potato Beetle *Mesh* Gene in Laboratory and Field Trials

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Colorado potato beetle (CPB) is an agricultural pest of solanaceous crops, notorious for its rapid resistance development to chemical pesticides. Foliar spraying of dsRNA formulations is a promising innovative technology providing highly specific and environmentally acceptable option for CPB management. We designed dsRNA to silence CPB *mesh* gene (dsMESH) and performed laboratory feeding trials to assess impacts on beetle survival and development. We compared the effectiveness of *in vivo* and *in vitro* produced dsRNA in a series of laboratory experiments. We additionally performed a field trial in which the efficacy of dsRNA sprayed onto potato foliage was compared to a spinosad-based insecticide. We showed that dsMESH ingestion consistently and significantly impaired larval growth and decreased larval survival in laboratory feeding experiments. *In vivo* produced dsRNA performed similarly as *in vitro* synthesized dsRNA in laboratory settings. In the field trial, dsMESH was as effective in controlling CPB larvae as a commercial spinosad insecticide, its activity was however slower. We discuss limitations and benefits of a potential dsMESH-based CPB management strategy and list some important RNAi based CPB research topics, which will have to be addressed in future.

Keywords: RNA interference (RNAi) feeding, dsRNA, gene silencing, RNAi pest control, survival analysis, *Leptinotarsa decemlineata* (Say), field trial, *E coli* HT115 (DE3)

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INTRODUCTION

Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, is a serious pest of potato and other solanaceous crops. It is well known for its ability to rapidly evolve resistance to insecticides; it has already evolved resistance to all major insecticide classes (Alyokhin et al., 2008). Extensive use of conventional insecticides can have undesirable effects on the environment, non-target organisms and human health. Compared to chemical pesticides, double-stranded RNAs (dsRNAs) have the advantage of high selectivity towards the target organism and rapid environmental degradation into non-toxic compounds (Dubelman et al., 2014; Albright et al., 2017). Therefore, this novel pest management approach has the potential to decrease the extensive use of conventional insecticides.

When delivered into cells, dsRNAs activate the RNA interference (RNAi) mechanism that mediates a sequence-specific suppression of transcription, also called gene silencing (Joga et al., 2016). In CPB,

unlike some other insects, dsRNAs are not degraded by gut nucleases, are efficiently taken up by the gut epithelium cells, and can trigger local as well as systemic RNAi response (Cappelle et al., 2016). This makes CPB an excellent candidate for pest management using dsRNAs, which was first recognized in a study by Baum et al. (2007) that has also identified several RNAi targets. This was followed by studies that identified novel effective target genes in CPB (Zhu et al., 2011; Zhou et al., 2013; Wan et al., 2014; Fu et al., 2015; Lü et al., 2015; Meng et al., 2015; Zhu et al., 2015; Fu et al., 2016; Guo et al., 2016; Shi et al., 2016a; Shi et al., 2016b; Meng et al., 2018; Xu et al., 2018) and for some targets also validated in field trials (Guo et al., 2018). In western corn rootworm, Hu et al. (2016) identified another target gene—*mesh* (alternatively named dv*ssj2*) which encodes a smooth septate junction protein important for structural integrity of the midgut epithelium. They showed that silencing *mesh* impairs midgut barrier function which results in increased larval mortality (Hu et al., 2019).

In this study, we used *in vitro* and *in vivo* synthesized dsRNA designed to silence the *mesh* gene in CPB. We performed laboratory-based feeding assays with CPB at different stages of larval development as well as a small-scale field trial to validate the designed dsRNA's pesticidal potential in a commercial production system. Therefore, our study offers new data on dsMESH effectiveness in another coleopteran, CPB, which represents an important crop pest and the most probable first candidate for spray-induced gene silencing commercialization.

MATERIALS AND METHODS

Quantification of *Mesh* Gene Expression by qPCR

To quantify the expression levels of *mesh*, RNA was extracted from three to four individual larvae (three to four biological replicates), except for the study of *mesh* expression in CPB body parts where one pooled sample from 3–4 beetles for each body part was analyzed. RNA extraction was performed using TRIzol reagent (Invitrogen) and Direct-zol RNA Microprep kit (Zymo Research). DNase treatment and reverse transcription were performed as described previously (Petek et al., 2012). RNA concentration and integrity were validated using a NanoDrop ND-1000 spectrophotometer and agarose gel electrophoresis. The efficiency of DNase treatment was confirmed by qPCR with no RT samples.

The expression of *mesh* was assessed by quantitative PCR (qPCR). *Mesh* gene model from i5k genome version 0.5.3 (LDEC006484; Schoville et al., 2018) was corrected based on alternative models and mapped RNA-seq reads available in i5k's WebApollo instance (**Supplementary Figure 1** and **Supplementary Data 1.1**). The qPCR primers and probes were designed in Primer Express 2.3 (Applied Biosystems) using default parameters for TaqMan amplicons and were synthesized by IDT. Assay specificity was verified *in silico* using blastn queries against all transcripts predicted in the CPB genome (Schoville et al., 2018). The linear ranges and amplification efficiencies were determined across five 10-fold serial dilutions of cDNA. Target gene accumulation was normalized to three endogenous control genes: LdRP4 (Shi et al., 2013), 18S rRNA (Eukaryotic 18S rRNA Endogenous Control, Applied Biosystems) and LdSmt3 (Petek et al., 2014). Primer and probe sequences, qPCR chemistry and other assay metadata are available in **Supplementary Table 1**.

FastStart Universal Probe Master Rox mastermix (Roche) was used for TaqMan chemistry based assays and Power SYBR mastermix (Applied Biosystems) for SYBR Green chemistry based assays. Dilution of cDNA samples and pipetting of qPCR reagents onto 386-well plates was performed on a Microlab STARlet automated liquid handling system (Hamilton). Reactions were performed in 5 µl total volume on LightCycler 480 (Roche) as described previously (Petek et al., 2012). Melting curve analysis was applied for SYBR green chemistry based assays LdRP4 and LdSmt3 to control for primer dimer formation and amplification specificity in each reaction. Each sample was analyzed in two replicates of two dilutions to check for the presence of inhibitors in the sample. Cq values were calculated using instrument manufacturer software and exported as text files. Amplification quality control for each sample and relative quantification based on the standard curve method was performed in quantGenius software (Baebler et al., 2017). For every gene, the limit of quantification (LOQ) was determined from the standard curve. The normalized target copy numbers calculated by quantGenius were exported to an Excel file to calculate standard errors and Student's *t*-test statistics.

Design and *In Vitro* Synthesis of dsRNAs

To avoid sequence regions that might affect other species due to nucleotide conservation we used EMBOSS splitter (Rice et al., 2000) to generate all possible 21-mers for the CPB *mesh* transcript. These 21-nt sequences were queried using BLASTn against non-target organism transcriptomes including *Homo sapiens*, *Apis mellifera*, *Bombus terrestris*, *Danaus plexippus*, *Drosophila melanogaster*, *Megachile rotundata*, *Nasonia vitripennis*, and *Tribolium castaneum*. Regions of the transcripts with 20 or 21 nt BLASTn hits in non-target organisms were excluded from dsRNA design. Based on the above metrics, the longest CPB-specific region was selected as the input sequence to design a long dsRNA molecule using e-RNAi web service (Horn and Boutros, 2010) using default parameters. Such bioinformatics design however does not exclude the possibility of off-target effects. For example, due to crosstalk between siRNA and miRNA pathways, off-target silencing could be triggered by siRNAs with less sequence conservation. Also, due to limited genomics and transcriptomics sequence availability in Arthropods a comprehensive bioinformatics analysis is not possible (Christiaens et al., 2018). As non-specific dsRNA control, the dsEGFP with sequence corresponding to a fragment of enhanced green fluorescent protein (Guo et al., 2015) was used (sequences in **Supplementary Data 1.2**). *In vitro* synthesis of dsMESH and dsEGFP was performed by AgroRNA (South Korea). The quality and quantity of dsRNA was determined using agarose gel electrophoresis and NanoDrop.

In Vivo Synthesis of dsRNAs

To *in vivo* synthesize dsMESH, a 417 bp fragment of the gene was amplified by PCR from a pooled CPB midgut cDNA sample using Phusion DNA polymerase (Biolabs) and cloned into

L4440gtwy (Addgene) using pCR8/GW/TOPO TA Cloning Kit (Invitrogen) to obtain MESH::L4440. The correct fragment insertion was confirmed by Sanger sequencing (Eurofins Genomics). The GFP::L4440 plasmid (Addgene), containing a full-length (857 bp) green fluorescence protein sequence insert was used to produce dsGFP. Heat-shock induced competent *Escherichia coli* HT115 (DE3) bacteria were transformed with MESH::L4440 and GFP::L4440, respectively. Transformation was confirmed by colony PCR using KAPA2G Robust HotStart Polymerase (Kapa Biosystems).

To produce dsRNA, cultures of transformed bacteria were grown to OD600 0.5 in 250 ml of liquid LB media. Production of dsRNAs was induced with 400 μM IPTG (Thermo Scientific). After 4 h, cells were pelleted, re-suspended in 6 ml nuclease-free water (Sigma) and lysed by boiling followed by four freezing-thawing cycles and a 15 min treatment in ultrasonic bath SONIS 4 (Iskra PIO). Bacterial lysates were centrifuged at 9,000 g for 20 min and the supernatant was concentrated to 1/10 volume using GeneVac EZ-2plus (Genevac Ltd).

To estimate the quantity of dsRNA produced, total RNA was extracted from bacterial lysates using Direct-zol RNA MiniPrep Plus kit (Zymo Research), treated with DNase I (Zymo Research) and reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). *In vivo* synthesized dsMESH and dsGFP quantities were estimated from 1% agarose E-Gel EX (Thermo) RNA band intensities. Identity of dsRNA was confirmed by RNase I_f treatment (**Supplementary Data 1.3**, **Supplementary Figure 2**).

Laboratory Feeding Trials

CPBs were reared on potato plants cv. Désirée in conditions described previously (Petek et al., 2014). Larvae, which hatched on the same day, were reared on non-treated detached potato leaves until most larvae reached desirable treatment stage. For each feeding trial, larvae were randomly selected and assigned into treatment groups, enclosed into plastic or glass containers and reared on untreated potato foliage. DsRNA were either sprayed on detached leaves, potted whole plants, or CPB eggs, or pipetted onto freshly cut leaf disks (**Table 1**). To protect detached leaves from desiccation, the petioles were placed in sterile 2 ml microcentrifuge tubes filled with 0.5% agarose gel, whereas leaf disks were placed into flat bottom 24-well plates with bottom covered by 0.5% agarose gel. After consumption of leaf disk (trials three and four, **Table 1**), the larvae were moved back to plastic containers and daily supplied with untreated detached potato leaves.

Nuclease-free water (Sigma) was used for blank control treatment and dilution of all dsRNAs. In all, except trial four, *in vitro* synthesized dsEGFP (Guo et al., 2015) was used as non-specific dsRNA treatment control. In trial four, *in vivo* synthesized dsGFP sequence from GFP::L4440 plasmid (Addgene) was used instead. During the study, we adhered to national and institutional biosafety standards. More details on feeding trials are given in **Table 1** and **Supplementary Data 1.4-8**.

Analysis of right-censored survival data was performed using Cox proportional hazards regression model fit and statistical tests implemented in R survival package version 2.42 (Therneau and Grambsch, 2000). Data analysis execution calls are given in **Supplementary Data 1.9**.

Field Trial

A small-scale field trial was conducted in June and July 2019 on three locations near Ljubljana, Slovenia. Two trials were performed on adjacent potato fields in Šentjakob (46°05'13.4"N 14°34'06.8"E) and one in Iška vas (45°56'28.8"N 14°30'31.0"E). The experiment was designed following EPPO guidelines (EPPO, 2008). Cultural conditions were uniform for all plots of the trial at each location and conformed to local agricultural practice. To assess the efficacy of dsMESH the only difference between treatments was the method of CPB management. Three 25 m^2 plots were marked at each location. Each plot was divided into four replicate sub-plots, giving four replicates per treatment. On each sub-plot, an individual potato plant infested by at least 15 CPB larvae was randomly selected and marked, giving four plants per treatment at each location. Before treatment, foliage from potato plants and weeds surrounding the marked potato plants was removed to restrict larval movement between plants. Any unhatched CPB eggs from the marked potato plants were

TABLE 1 | Design of Colorado potato beetle dsRNA laboratory-based feeding trials.

Feeding trial number	Conducted	Negative controls	dsRNA production	CPBs per treatment	CPB stage at first treatment	Treatment regime (dsRNA spray concentration [serial dilution]; dose per larva)	Trial duration (d)
1	Jun–Jul 2016	water, dsEGFP	*In vitro*	40	2nd instar larvae	continuous feeding on sprayed detached leaves (conc. 0.4 μg/μl)	41
2	Jun–Jul 2016	water, dsEGFP	*In vitro*	30	4th instar larvae	continuous feeding on potted plants sprayed once (conc. 0.4 μg/μl)	24
3	Dec 2016	water, dsEGFP	*In vitro*	16	2nd instar larvae	discontinuous feeding on leaf disks (conc. 0.5 μg/μl; 0.75 μg/larva)	7
4	Apr 2018	water, dsGFP (*in vivo*)	*In vitro*, *In vivo*	24	2nd instar larvae	discontinuous feeding on leaf disks (*in vitro*: conc. 0.1 μg/μl [60x], 0.01 μg/μl [600x], 0.001 μg/μl [6000x]; dose 0.6 μg/larva, 0.06 μg/larva, 0.006 μg/larva, respectively) (*in vivo:* conc. 0.1 μg/μl; dose 0.6 μg/larva)	10
5	May 2018	water, dsEGFP	*In vitro*	20 egg masses	eggs	egg spraying (conc. 0.5 μg/μl)	7

removed. CPB larvae were counted and larval stages and plant defoliation percentages were determined for each plant separately. The leaf damage caused by the CPB larval herbivory was estimated visually by inspecting the first ten fully developed leaves from the topmost apical plant meristem downwards on each marked potato plant.

Marked plants were sprayed with *in-vitro* synthesized dsMESH in concentration 10 µg/ml. We used potato plants sprayed with the manufacturer recommended 0.5% diluted spinosad formulation (insecticide Laser 240 SC, Dow AgroSciences) as a positive control treatment and unsprayed plants as a negative control. Two days post treatment (dpt), CPB larvae on marked plants were recounted and stages determined, and 7 dpt larvae were counted again, their stages determined, and leaf damage estimated. From the relative change of leaf damage assessed before treatment and at 7 dpt, the parameter "leaf damage increase" was calculated.

Statistical differences in leaf damage, leaf damage increase, and larval mortality according to Henderson-Tilton were calculated using ANOVA and Bonferroni's Multiple Comparison Test in GraphPad Prism 5.00 (GraphPad Software). The dataset was also analyzed using a general linear model (GLM), where the effect of factors *treatment* (dsMESH, spinosad, and control), *experiment* (trials 1–3) and *replicate* (1–4) on previously mentioned parameters was assessed. Further, Fisher's least significance difference (LSD) procedure at 95% confidence level was used to discriminate between the treatments within the three-trial dataset. These analyses were performed with the statistical software Statgraphics Centurion XVI (StatPoint Technologies).

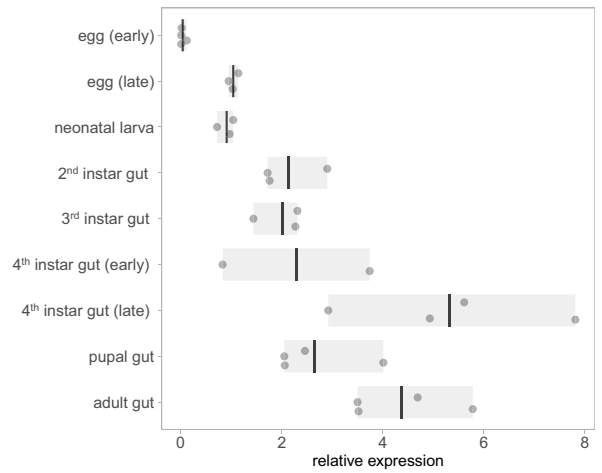

FIGURE 1 | Expression of *mesh* at different CPB developmental stages. Gene expression values are shown relative to early egg sample average. In the eggs and neonatal larval stages, entire organisms were sampled, whereas in later stages, only guts were sampled. Bars show the range of data and lines shown the mean.

RESULTS

The Target Gene *Mesh* Is Expressed Throughout All CPB Developmental Stages

To test whether an RNAi insecticide targeting *mesh* will work against all CPB's developmental stages, we profiled *mesh* expression through the stages. Constitutive expression of *mesh* was detected in all developmental stages. Expression in the gut is highest in fourth instar larvae preceding pupation and in adults (**Figure 1** and **Supplementary Dataset 1**). Constitutive expression of *mesh* in larval and adult stages is also evident from mapped RNA-Seq data available at i5k CPB genome browser (**Supplementary Figure 1A**). This expression pattern is suitable for RNAi insecticide targets as the gene is expressed in stages in which the beetles feed on plant leaves. We also qualitatively showed higher expression of *mesh* in samples of foregut, midgut and hindgut tissues compared to samples of legs, head and antennae (**Supplementary Figure 3** and **Supplementary Dataset 2**).

Laboratory Feeding Trials Confirm dsMESH Efficiency at Different CPB Life Stages

To test the efficiency of dsMESH in silencing the target gene and its potential as a bioinsecticide we performed three laboratory feeding trials in which we treated CPB at different life stages (**Table 1**). Firstly, we fed 2nd instar larvae continuously on *in vitro* synthesized dsRNA-sprayed potato foliage and left them to pupate and emerge as adults (trial one, **Table 1**). We confirmed silencing of *mesh* gene by dsMESH after 4 days of treatment. Compared to dsEGFP treatment, dsMESH reduced *mesh* expression by 71% ($p<0.001$, **Supplementary Dataset 3**) and larval survival at that point was 48, 80, and 95% for dsMESH, dsEGFP, and water treatment, respectively (**Supplementary Figure 4** and **Supplementary Dataset 4**).

We also tested the effectiveness of dsMESH on 4th instar larvae (trial two, **Table 1**), which is the final instar before pupation. We recorded adult emergence and inspected plant substrate for beetle carcasses at the end of the trial. Adult emergence rate was 11% in larvae exposed to dsMESH, which is significantly lower compared to more than 75% for dsEGFP and water treatments ($p<0.01$; **Figure 2B** and **Supplementary Dataset 5**). Additionally, in all three emerged adults from the dsMESH treated group we observed darkened deformed elytra (**Supplementary Figure 5**) and the beetles died within two days after emergence. In contrast, dsEGFP and water treated beetles exhibited normal phenotypes and no adult mortality. From the substrate of dsMESH treated plants, we recovered two larval and six adult carcasses (**Supplementary Figure 5**) whereas in substrates of dsEGFP and water treated plants we found no carcasses. Trial two thus shows that dsMESH is also effective against 4th instar larvae.

In trial five, we tested the effectiveness of dsMESH spraying on CPB eggs. We sprayed freshly laid CPB egg masses (**Supplementary Figure 6**) and transferred 1st instar larvae to untreated potato foliage. Most larvae hatched three days after egg treatment. We observed no difference in larval emergence between dsMESH and control treatments. Massive larval die-off in dsMESH treated group occurred in 6–7 days old larvae (9–10 days post egg treatment, **Figure 2C**). The survival of dsMESH treated 6 days old larvae was 61%, and a day later only 23%. In comparison, for both dsEGFP and

water treated groups, the survival at that time point was 100% (**Figure 2C** and **Supplementary Dataset 8**). Only 4% of dsMESH treated larvae survived until 13 dpt, whereas 97% and 95% larvae survived in dsEGFP and water treated groups, respectively ($p<0.001$; **Figure 2C** and **Supplementary Data 1.9**). This trial shows high insecticidal efficiency of dsMESH also when sprayed on CPB eggs.

The Treatment Regime Does Not Affect the Efficiency of dsRNA

We next compared the effect of the dsRNA administration approach. Contrary to the above-described feeding trials, where larvae were continuously fed with dsRNA-sprayed potato leaves, here we exposed each individual larva (2^{nd} instar) to the same dose of dsRNA by discontinuous administration *via* treated potato leaf disks (trial three and four, **Table 1**). We observed similar survival trends as the ones obtained with continuous treatment regime (trial one).

In the first leaf-disk feeding trial (trial three), we observed a substantial reduction in survival 4 dpt, reaching only 18% survival in the case of dsMESH treated larvae compared to more than 90% survival in dsEGFP and water treatments (**Figure 2A**, **Supplementary Dataset 6**). In trial four, most substantial survival reduction was observed 5 dpt, where dsMESH treated larval survival rate was 54% compared to 100% survival in both dsGFP and water treatments (**Figure 3**, **Supplementary Dataset 7**). In both trials, statistical analysis indicates highly significant survival reduction for dsMESH treatment ($p<0.001$; **Supplementary Data 1.9**).

Comparison of *In Vivo* and *In Vitro* Synthesized dsRNA Efficiency

Larval survival analysis and weight measurements in trial four (**Figure 3**; **Supplementary Datasets 7 and 12**) show that *in vivo* and *in vitro* synthesized dsMESH are similarly effective (**Supplementary Data 1.9**). In addition, by testing serial dilutions of *in vitro* synthesized dsMESH, we showed that ingestion of as little as 6 ng of dsMESH caused more than 90% larval mortality (**Figure 3B**). No significant effect of bacterially produced dsGFP treatment on larval weight or survival was observed (**Figure 3**).

The dsMESH Treatment Against CPB Is Also Efficient in the Field

In order to confirm the efficacy of dsMESH as potential insecticide also under environmental conditions we treated potato plants growing in three different fields with *in vitro* synthesized dsMESH. No formulation to increase dsRNA stability or uptake was used to make the results of the field trial more comparable to the laboratory-gained results. Mortality rates for dsMESH treatment after 7 days were significantly higher ($F_{2,\ 40} = 16$; $P<0.0001$) compared to untreated plants according to ANOVA and were 93, 84, and 95%, in the three locations, respectively. GLM analyses showed a significant effect of factor treatment on parameters leaf damage increase ($F_{2,\ 41} = 34, 8$; $P<0.0001$) and insect mortality rate ($F_{2,40} = 13.2$; $P<0.0001$; **Figure 4**). Factors experiment and replicate did not significantly affect the observed parameters. Spinosad acted more rapidly than dsMESH, causing on average 98% of larval mortality in just two days, whereas the average mortality rate of dsMESH treatment at that time point was 32% (**Supplementary Dataset 13**).

DISCUSSION

We performed a systematic evaluation of applicability of an RNAi-based insecticide targeting the *mesh* gene (dsMESH) and validated its insecticidal action in CPB. The incentive to use this target gene came from its high expression in CPB gut in most developmental stages and the lethal phenotypes observed in Drosophila knockout

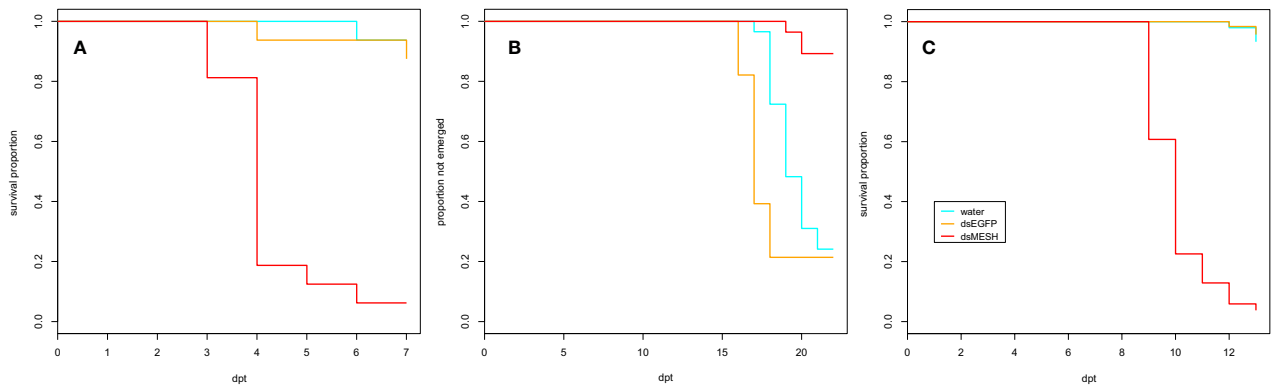

FIGURE 2 | Effectiveness of *in vitro* synthesized dsMESH treatment of different Colorado potato beetle developmental stages. **(A)** Kaplan-Meier survival curves of 2^{nd} instar larvae exposed to discontinuous administration of dsRNA (trial three). Survival was significantly reduced for dsMESH compared to water or dsEGFP treatment ($p<0.001$). **(B)** Proportion of beetles not emerged as adults after 4^{th} instar larval dsRNA treatment (trial two). Adult emergence was significantly lower for dsMESH treated larvae compared to water or dsEGFP treatment ($p<0.01$). **(C)** Kaplan-Meier survival curves of larvae hatched from dsRNA-treated eggs (trial five). Survival was significantly reduced for dsMESH compared to water or dsEGFP treatment ($p<0.001$). dpt – days post treatment.

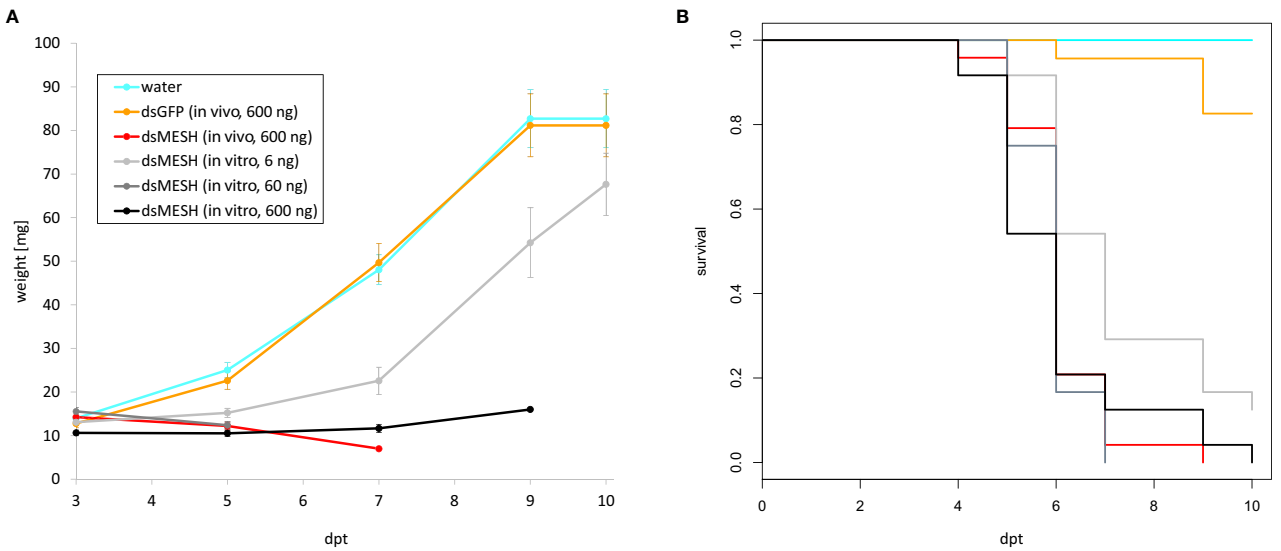

FIGURE 3 | Efficiency of *in vitro* and *in vivo* synthesized dsMESH (trial four). **(A)** Larval weight throughout the trial was significantly reduced when using either *in vitro* or *in vivo* synthesized dsMESH compared to water or dsEGFP treatment. Error bars show standard error of the mean. **(B)** Kaplan-Meier survival curves with survival proportions plotted. Survival was significantly reduced for dsMESH compared control treatments ($p<0.001$). dpt – days post treatment.

mutants (Gramates et al., 2017) and *Tribolium castaneum* RNAi screens (Ulrich et al., 2015). Mesh is a transmembrane protein important for proper organization of the insect midgut septate junctions and Drosophila *mesh* mutants show an impaired barrier function of the midgut (Izumi et al., 2012). Silencing *mesh* by RNAi in Drosophila adults, however, does not impair gut integrity but increases gut bacterial load by regulating dual oxidase expression (Xiao et al., 2017).

Mesh was first identified as an effective RNAi pesticide target in western corn rootworm, *Diabrotica virgifera virgifera*, another coleopteran pest closely related to CPB (Hu et al., 2016). Our CPB feeding trials with *in vitro* synthesized dsMESH consistently showed high mortality rates in larvae with effective dose in the ng range, similarly as reported for corn rootworm by Hu et al. (2016). In our first feeding trials we used 2nd instar larvae because the first two CPB instars were described as most susceptible to RNAi (Guo et al., 2015). In addition, we showed that dsMESH treatment is effective against 4th instar larvae and CPB eggs. Surprisingly, reports of insect egg treatment by spraying or soaking in dsRNA are rare and have different outcomes. Soaking Asian corn borer (*Ostrinia furnalalis*) eggs in pesticidal dsRNA solutions caused reduced hatching (Wang et al., 2011). On the contrary, in the corn earworm, *Helicoverpa zea*, soaking eggs in dsRNA as well as larval feeding delivery had no effect, whereas injecting eggs with same dsRNA induced RNAi and reduced egg hatching rate (Wang et al., 2018). In our trial, spraying eggs with dsMESH did not affect egg

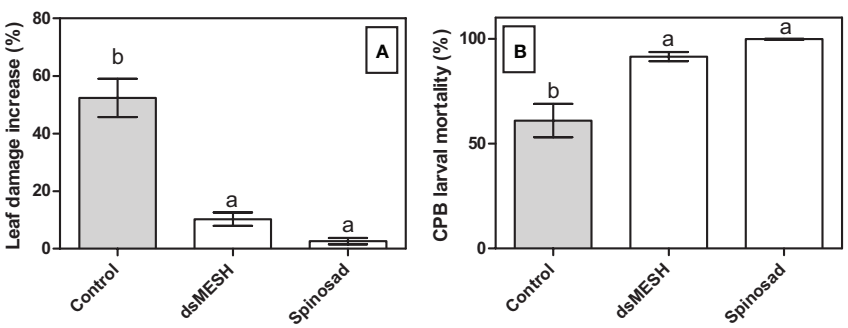

FIGURE 4 | Treatment with dsMESH reduced Colorado potato beetle potato infestation in the field. **(A)** Potato defoliation due to CPB herbivory expressed as leaf damage increase, which was calculated by subtracting data from initial and final leaf damage assessment after seven days. **(B)** CPB larval mortality in the field trial. The data from all three locations were used. Bars not sharing the same lowercase letter are significantly different.

hatching although we showed that *mesh* is expressed in eggs. The larval die-off six to seven days after emergence from dsMESH treated eggs leads to suggest that dsRNA was mostly taken up by neonatal larvae while feeding on eggshells.

The activity of dsRNA obtained in *in vitro* tests or laboratory feeding experiments might not reflect that on the field. Thus, we decided to validate our laboratory-based trial results in a field trial comparing dsMESH efficiency to that of spinosad. Spinosad was used as a positive control as a) it is highly effective against CPB, b) it is a bioinsecticide and can thus be used also in organic farming, c) it is an insecticide registered for control of CPB in Slovenia and d) it is an alternative to conventional chemical insecticides (e.g. thiacloprid, beta-cyfluthrin), which can be ecotoxicologically problematic, and for which we are trying to find alternatives for. The observed field mortality was slightly lower compared to laboratory trials, which is reasonable, as larval treatment on the field was not as controlled and uniform as in the laboratory. In addition, reduced dsRNA stability in the field is expected due to direct sunlight exposure and lack of formulation to improve dsRNA stability (Cagliari et al., 2019). Compared to the wide-spectrum insecticide spinosad (Kirst, 2010), dsMESH has an inherent lag phase in observed mortality, which can be attributed to its mode of action. The toxicity of dsRNA depends on target protein's half-life (Scott et al., 2013) therefore, we expected to observe lethal effects after a few days. Despite its slower activity, the final mortality and leaf damage caused by dsMESH treatment in the field trial were not statistically different to that of spinosad.

In our experiments we used *in vitro* synthesized dsRNA, however, for large scale field application applying crude extract of *E. coli* producing dsMESH might be a good option to reduce the costs. Our laboratory feeding trials showed that *in vivo* produced dsMESH and the dsMESH synthesized *in vitro* are similarly effective. Because the dsMESH amounts in bacterial extracts was approximated from the gel, a more accurate comparison is not possible. Despite the potential advantages of applying dsRNA as a crude bacterial extract, the approach has also additional risks such the presence of synthetic DNA elements and the possibility of having a GMO status assigned (Fletcher et al., 2020).

CONCLUSIONS

Although plant-incorporated protectants (transgenic plants) are the most cost-effective way of using RNAi-based pesticide technology, their public acceptance might prove challenging, at least in the European Union. Other possibilities were envisioned, such as transformed insect symbionts (Whitten et al., 2016) or viruses expressing pesticidal RNA molecules (Taning et al., 2018), albeit again using genetically modified organisms. As an alternative, non-transformative strategies of dsRNAs application, i.e. spray-induced gene silencing, are being investigated and CPB is the first agricultural pest for which this technology might be commercialized (Cagliari et al., 2019).

We have shown in laboratory trials as well as in the field that spraying with insecticidal dsRNA is a highly efficient strategy for managing CPB. We are planning to test dsMESH in a larger field trial using standard agricultural spraying equipment and against a range of other insecticides. For RNAi-recalcitrant agricultural pests, future research will have to focus on formulations to improve dsRNA stability and cellular uptake. Apart from efficiency, further research is needed on biosafety implications of this new pest management strategy. This includes investigating possible impact of dsRNA on human health and the environment (Rodrigues and Petrick, 2020). For sustainable use of this technology in agriculture, integrated pest management strategies will have to be employed to delay the development of pest resistance to dsRNAs (Khajuria et al., 2018).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

MP provided the initial concept and design of the study, performed gene expression analysis, laboratory-based feeding trials, contributed to execution and evaluation of the field trial and wrote the manuscript. AC and RF established the bacterial production of dsRNA. RF also helped with execution of the feeding trial four. JR designed and led the execution of the field trial. KG contributed to study design, data interpretation and manuscript drafting. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01250/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sequence–Activity Relationships for the Snf7 Insecticidal dsRNA in Chrysomelidae

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The responsiveness of insects to oral delivery of insecticidal dsRNA has been shown to be dependent on dsRNA length and sequence match. Previous work with the western corn rootworm (WCR, *Diabrotica virgifera virgifera*; Coleoptera: Chrysomelidae) demonstrated that at least one ≥21 nt match must be present in the DvSnf7 dsRNA of approximately ≥60 base-pairs (bp) for activity. Further data is needed on the activity of <21 nt matches along with characterization of relationship between activity and the number of ≥21 nt matches. To characterize the sequence–activity relationship for insecticidal dsRNA further, the activity of orthologous Snf7 dsRNAs with 19, 20, and 21 nt contiguous matches against WCR was compared. Neither 19 nor 20 nt sequence matches were active, supporting that a ≥21 nt sequence match is required for activity. The relationship between the number of 21 nt matches with activity of Snf7 dsRNA orthologs from several Chrysomelid species was characterized using WCR and Colorado potato beetle (CPB, *Leptinotarsa decemlineata;* Coleoptera Chrysomelidae). For WCR, there was a strong relationship between an increasing number of 21 nt matches and increased activity (*i.e.*, lower LC_{50} values). A similar relationship was observed for CPB with an exception for a single ortholog, which may be related to the exceptionally high rate of polymorphisms in CPB. Overall, these results demonstrate a general relationship between the number of 21 nt matches and activity, and this relationship could be used to inform a testing and assessment plan for an ecological risk assessment for an insecticidal dsRNA.

Keywords: RNAi, dsRNA, insecticidal, ortholog, Diabrotica, Leptinotarsa, non-target organism

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INTRODUCTION

The registration of genetically engineered (GE) plants that express double-stranded RNA (dsRNA) to control insect pests through RNA interference (RNAi) has brought a new mode of action (MOA) for pest control to the market. The efficacy and its potential for an improved environmental safety profile of such products have gained interest from academic, government, and private sector researchers. Several factors including exposure concentration, potency, sequence and length, time-to-effect, persistence of gene silencing, and the insect life-stage, have been identified as influencers

on efficacy against the target (Huvenne and Smagghe, 2010; Bolognesi et al., 2012; Bachman et al., 2013).

In general, long dsRNAs that incorporate a high degree of sequence match to mRNAs in the target insect have greater potential for potency as a result of the number of siRNAs that can be produced from the sequence of each long dsRNA (Baum et al., 2007; Miller et al., 2012; Haller et al., 2019). Bolognesi et al. (2012) and Miller et al. (2012) demonstrated that a dsRNA must be of sufficient length (*e.g.* ≥60 bp) to result in activity against western corn rootworm (WCR; *Diabrotica virgifera virgifera*) and *Tribolium castaneum*, respectively. Additionally, Bolognesi et al. (2012) demonstrated that a single 21 nucleotide (nt) contiguous sequence match in ≥60 bp was sufficient for southern corn rootworm (SCR, *Diabrotica undecimpunctata howardi*) activity. In comparison, Bachman et al. (2013) demonstrated that a single 19 nt contiguous match in a 240 bp Snf7 orthologous dsRNA did not have activity against WCR in diet bioassays. However, a gap exists with empirical data to address the potential activity of a single 20 nt contiguous match, and there was a need to further characterize the relationship between the number of 21 nt contiguous sequence matches and activity. Further, as demonstrated in Miller et al. (2012), the potency of a dsRNA is positively related to the number of potential 21 nt matches contained in the sequence, and therefore the number of 21 nt matches should be considered within the ecological risk assessment (ERA) and relevant environmental exposure necessary for activity under realistic exposure scenarios for non-target organism (NTOs) in the agroecosystem.

Here we provide additional bioassay data to address these gaps and further characterize the response of insects to environmental (exogenous) dsRNA. Efforts were focused on the beetles from the family Chrysomelidae (chrysomelids) because they have become well established models for these types of bioassays due to their responsiveness to oral delivery of dsRNA and are the target of the first registered insecticidal dsRNA product.

MATERIAL AND METHODS

A series of bioassays were performed using two chrysomelid beetles, WCR and the Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) with established methods for laboratory testing. Bioassays were conducted using diet-incorporation, and the test arthropods were fed dsRNA in diets *ad libitum*. Bioassays followed published methods described in Bachman et al. (2013) using 240 bp dsRNAs, DvSnf7_240 dsRNA targeting WCR and LdSnf7 dsRNA (240 bp) targeting CPB. Additional 240 bp Snf7 orthologous dsRNA sequences for testing were chosen from an internal library of insect sequences and selected for the number of 21 nt matches as compared back to the DvSnf7_240 and LdSnf7 dsRNAs as described in Bachman et al. (2013; **Supplementary Material S1**). dsRNAs were prepared as described in Bolognesi et al. (2012) and Bachman et al. (2013) with the MEGAscript kit (Ambion) following the manufacturer's protocol. Purified dsRNAs were quantified by spectroscopy and examined by agarose gel electrophoresis to ensure their integrity.

Examination of Minimum Sequence Necessary for Biological Activity

Single dietary concentrations of three dsRNA treatments were tested concurrently at limit concentrations in 12-day diet bioassays to determine if a dsRNA containing single 19 nt, 20 nt, or 21 nt contiguous matches to the DvSnf7_240 dsRNA have a biological effect on larval WCR. The single 19 nt match was identified in the Snf7 240 bp dsRNA ortholog from *Chrysolina quadrigemina* (CqSnf7) and was previously fed to WCR at 5,000 ng/ml diet with no significant impact on survival (Bachman et al., 2013) and served as the negative (no activity) control. A single 20 nt match was identified in the Snf7 240 bp ortholog from *Drosophila pseudoobscura* (DpSnf7) and has not been previously evaluated against WCR or SCR. The DvSnf7_21.7 dsRNA construct consists of a single 21 nt match to the DvSnf7 dsRNA embedded in a neutral dsRNA carrier (240 bp total length) and served as the positive control. Previously, single 21 nt match dsRNA constructs, such as DvSnf7_21.7 dsRNA, were shown to induce significant mortality in SCR but were tested at concentrations at least an order of magnitude greater than the full length DvSnf7_240 dsRNA (Bolognesi et al., 2012).

For each bioassay, three replicates of a single treatment targeting 1,000 ng/ml were prepared for the CqSnf7 and DpSnf7 dsRNA test substances. The DvSnf7_21.7 dsRNA treatment was conducted at a single concentration level prepared at 374 ng/ml. Based upon the known response of SCR to DvSnf7_21.7 dsRNA and the known differences between WCR and SCR response to the DvSnf7_240 dsRNA in our laboratory, the concentration of 374 ng/ml assured a positive response in WCR (Bolognesi et al., 2012). Additionally, three water (diet only) replicates were prepared as an assay control for each bioassay. For the assessment of the minimum sequence necessary for activity, differences for insect mortality between treatments were evaluated with a generalized linear model and a binomial distribution under PROC GLIMMIX, and differences in insect mass between treatments were evaluated with a linear model under PROC MIXED (SAS, 2012). Pairwise comparisons were made between each treatment and the assay control for survival and mass using t-tests. All significance tests were determined at the 0.05 level.

Examination of Number of 21 nt Matches Influence on Biological Activity

To examine the influence of the number of 21 nt matches on activity, orthologous Snf7 dsRNAs from a selection of chrysomelid beetles were fed to WCR and CPB in 12-day diet bioassays to characterize the concentration–response relationship and to estimate LC_{50} values (**Table 1**). dsRNAs lacking 21 nt matches were tested at a single limit dose of 5,000 ng/ml. Along with the DvSnf7_240 dsRNA, orthologous dsRNAs from four species closely related to WCR were utilized in these assays; *Acalymma vittatum (AvSnf7), Cerotoma trifurcata (CtSnf7), Galerucella*

TABLE 1 | Relationship between the number of 21 nt matches and activity based on concentration–response bioassays performed with dsRNAs that contained zero to 221 possible 21 nt matches against WCR and CPB in 12-day diet bioassays.

dsRNA ortholog Source Species, Subfamily	WCR LC_{50} and 95% confidence interval (ng/ml)	% Shared identity with DvSnf7_240	# 21 nt matches	Longest contiguous nt sequence(s)
Tested against WCR				
DvSnf7 *Diabrotica virgifera virgifera*; (WCR) **Galerucinae**	7.0 (6.0–8.1)	100	221	240
AvSnf7 *Acalymma vittatum* **Galerucinae**	9.1 (7.3–11.4)	95.0	69	38, 38, 47, 26
CtSnf7 *Cerotoma trifurcata* **Galerucinae**	128 (80–210)	90.8	18	32, 26
GcSnf7 *Galerucella calmariensis* **Galerucinae**	421 (239–632)	90.8	3	23
CqSnf7 *Chrysolina quadrigemina* **Chrysomelinae**	No Activity	82.1	0	19
Tested against CPB				
LdSnf7 *Leptinotarsa decimlineata* (CPB) **Chrysomelinae**	11.2 (9.9–12.6)	100	221	240
MoSnf7 *Microtheca ochroloma* **Chrysomelinae**	105 (90–123)	84.2	12	32
CqSnf7 *Chrysolina quadrigemina* **Chrysomelinae**	1,860 (1,496–2,406)	87.5	12	23, 29
GcSnf7 *Galerucella calmariensis* **Galerucinae**	704 (584–841)	80.8	3	23
AlSnf7 *Aphthona lacertosa* **Galerucinae**	No Activity	77.1	0	14

Significant mortality ($p > 0.05$) was not observed when dsRNA sequences lacking 21 nt matches were fed to WCR and CPB. dsRNAs lacking 21 nt matches were tested at a single limit dose of 5,000 ng/ml. For WCR, survival in the CqSnf7 treatment was 88%, and survival in the water-only control was 94%. For CPB survival in the AlSnf7 treatment was 93%, and survival in the water-only control was 91%.

calmariensis (GcSnf7), and *Chrysolina quadrigemina (CqSnf7)*. These orthologs were previously described in Bachman et al. (2013) and evaluated for sequence alignment against DvSnf7_240 dsRNA and biological activity at a single high concentration. *A. vittatum, C. trifurcata*, and *G. calmariensis* were demonstrated to contain 21 nt matches to DvSnf7 and to be active against WCR, whereas *C. quadrigemina* was shown to lack any 21 nt matches and activity against WCR (Bachman et al., 2013). Concentration–response bioassays and synthesis of the dsRNAs used in these bioassays followed the method described in Bachman et al. (2013). For CPB, orthologous dsRNAs from *Microtheca ochroloma* (MoSnf7) and *Aphthona lacertosa* (AlSnf7) were tested in addition to the LdSnf7, CqSnf7 and GcSnf7 dsRNAs. LC_{50} values were estimated by logistic regression with responses that were corrected for control mortality (Van Ewijk and Hoekstra, 1993).

RESULTS

Examination of Minimum Sequence Necessary for Biological Activity

Mean WCR survival was 80, 83, 82, and 29% for the water only control, CqSnf7, DpSnf7 and DvSnf7_21.7 dsRNA single dietary concentration treatments, respectively. Mean mass for WCR was 0.32, 0.31, 0.32, and 0.22 mg, for the water only control, CqSnf7, DpSnf7 and DvSnf7_21.7 dsRNA positive control treatment, respectively. There was no significant difference in survival and mean mass between either the CqSnf7 and DpSnf7 treatments *versus* the control ($p > 0.05$), whereas there was a highly significant difference between survival and mean mass in the DvSnf7_21.7 dsRNA *versus* the water-only control ($p < 0.001$). Results from these assays demonstrate that continuous dietary exposure to dsRNAs containing only 19 or 20 nt contiguous matches have no effect on survival or growth of WCR. Decreased survival and inhibited growth of WCR larvae fed the DvSnf7_21.7 dsRNA was consistent with previous reports (Bolognesi et al., 2012; Bachman et al., 2013).

Examination of Number of 21 nt Matches Influence on Biological Activity

To characterize the relationship between the number of 21 nt matches in a dsRNA sequence and activity, concentration–response bioassays were performed with dsRNAs that contained possible 21 nt matches against WCR and CPB in 12-day diet bioassays (**Figures 1A, B**). Where dsRNAs lacked 21 nt matches, tests were conducted at a single limit dose, and significant mortality ($p > 0.05$) was not observed when these sequences were fed to WCR and CPB (**Table 1**). Results from WCR, demonstrate a strong positive relationship between an increasing number of 21 nt matches and mortality (**Table 1**; **Figure 1A**). However, for CPB, there was only a general relationship between the number of 21 nt matches and mortality (**Table 1**; **Figure 1B**); the lowest LC_{50} value was observed for the LdSnf7 (11.2 ng/ml), which had a 240 bp (*i.e.*, 100%) contiguous sequence match with the CPB *snf7* gene. However, two different orthologs each with 12 possible 21 nt matches had a large difference in activity (105 ng/ml *vs.* 1860 ng/ml), and a fourth ortholog with 3 possible 21 nt matches had an LC_{50} value that was between the values for the orthologs with 12 possible 21 nt matches (704 ng/ml). Overall, these results are largely consistent with the findings of Miller et al. (2012) with the beetle *T. castaneum*, where greater activity was observed with

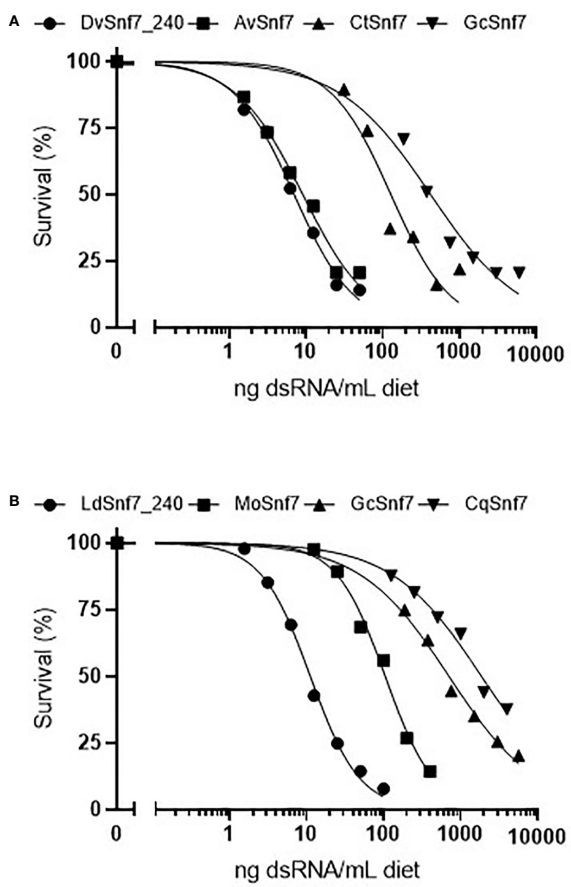

FIGURE 1 | Concentration–response curves with orthologous dsRNAs demonstrate that the activity of dsRNA with **(A)** the western corn rootworm (WCR, *Diabrotica virgifera virgifera*) (WCR) and **(B)** the Colorado potato beetle (CPB, *Leptinotarsa decimlineata*) is related to the degree of shared sequence including the number of potential 21 nt matches. Survival in the water only controls for the DvSnf7, AvSnf7, CtSnf7, LdSnf7, MoSnf7, GcSnf7, and CqSnf7 treatments were 92, 92, 83, 81, 92, 93, 88, and 99%, respectively. Data is reported and analyzed as corrected for control mortality. Concentration–response curves and LC_{50} estimates were performed in Prism GraphPad v8.2.0.

longer dsRNAs with a contiguous sequence match (*e.g.* 520 bp) injected into larvae *versus* shorter dsRNAs with a contiguous sequence match (*e.g.* 69 bp). Similar results demonstrating the relationship between the number of 21 bp matches and activity was reported with the WCR and dietary exposure (Bolognesi et al., 2012).

DISCUSSION

In cases where responsiveness to environmental dsRNA and the presence of 21 nt matches are known for an NTO, understanding the relationship between the degree of sequence match and activity can inform an ERA and testing plan that evaluates the potential for adverse effects in NTOs. Haller et al. (2019) fed dsRNAs with multiple 21 nt matches to NTO ladybeetles, *Adalia bipunctata*, and *Coccinella septempunctata*. Activity against these two species was predicted, based upon previous confirmation of responsiveness in ladybeetles, and confirmed in dietary bioassays. Additionally, Haller et al. (2019) reported on the difference in response of these two species in regard to the number of 21 nt matches present, with *C. septempunctata* (34 matches) showing more responsiveness than *A. bipunctata* (six matches). These results are consistent with our findings here, where the potency of the dsRNA with 21 nt matches was related to the number of 21 nt matches with one exception. The reason why comparatively low activity was observed for one of the two CPB orthologs with 12 possible 21 nt matches is unclear. However, one explanation for the differences in activity between these two orthologs may be related to the exceptionally high rate of polymorphisms in CPB relative to vertebrates and other beetles (Schoville et al., 2018). Therefore, CPB was not an ideal species to characterize the relationship between the number of 21 nt matches and activity. Combined with an exposure assessment (*e.g.* environmental concentration of the dsRNA), this general relationship can be used predictively in an ERA for responsive species to aid in an understanding of potential adverse effects to NTOs be assessed to meet the protection goals (*e.g.* population level effects for a valued NTO).

Further, we have demonstrated that for WCR, dsRNAs with only 19 nt or 20 nt matches are not active and that a minimum of a 21 nt sequence match is necessary for activity of a long (*e.g.* ≥60 bp) dsRNA. This information can also be used to inform an ERA, especially in regard to the selection of NTOs for evaluation. Bachman et al. (2013) suggested that when bioinformatics data for NTOs is available and indicates that the minimum sequence requirements for dsRNA activity (<21 nt matches) are not met, toxicity testing may not be necessary as the likelihood of adverse effects is low. The activity of DvSnf7 dsRNA against WCR was demonstrated to follow phylogeny within Chrysomelida with ≥21 nt matches not found outside the family Chrysomelidae (Bachman et al., 2013). Tan et al. (2016) compared sequence analysis and toxicity testing with the NTO, honey bee (*Apis mellifera:* Hymenoptera: Apidae), and the DvSnf7 dsRNA. No ≥21 nt matches exist between the DvSnf7 dsRNA and the honey bee ortholog leading to a prediction of no toxicity, and laboratory bioassays with both adult and larval honey bee at high concentrations confirmed the predicted lack of activity. Further NTO screening with taxa (outside of Chrysomelidae) representing different ecological receptors (*e.g.* pollinators, detritivores, natural enemies, vertebrates) and a bioinformatics screening of NTOs associated with maize agroecosystems confirmed the lack of activity and lack of ≥21 nt matches beyond Chrysomelidae (Bachman et al., 2016). While these data were used to inform the ERA for the DvSnf7 dsRNA-expressing maize product and supported the conclusion of negligible risk to the environment (Bachman et al., 2016; U.S. EPA, 2017), some species could be eliminated from testing as no mechanism exists for an RNAi effect to occur.

While identifying the presence or absence of 21 nt matches can be used to predict activity in species known to be responsive

to environmental dsRNA, it is not a reliable standalone tool for predicting activity in species that have unknown responsiveness or are recalcitrant to environmental dsRNA. Multiple studies with arthropods ranging from springtails (Pan et al., 2016) to lepidoptera (Pan et al., 2017) have demonstrated no adverse effects of environmental dsRNA even in the presence of a high degree of sequence match. The lack of responsiveness is likely linked to biological barriers such as nucleases in saliva or hemolymph that degrade the dsRNA or inefficient uptake mechanisms from these arthropod's midguts (Baum and Roberts, 2014; Ivashuta, 2015).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

PB and SL conceived the studies and designed the experiments. JF and GM performed the experiments and SL analyzed the results. PB, WM, and SL conceptualized and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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The Induction of an Effective dsRNA-Mediated Resistance Against Tomato Spotted Wilt Virus by Exogenous Application of Double-Stranded RNA Largely Depends on the Selection of the Viral RNA Target Region

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Tomato spotted wilt virus (TSWV) is a devastating plant pathogen, causing huge crop losses worldwide. Unfortunately, due to its wide host range and emergence of resistance breaking strains, its management is challenging. Up to now, resistance to TSWV infection based on RNA interference (RNAi) has been achieved only in transgenic plants expressing parts of the viral genome or artificial microRNAs targeting it. Exogenous application of double-stranded RNAs (dsRNAs) for inducing virus resistance in plants, namely RNAi-based vaccination, represents an attractive and promising alternative, already shown to be effective against different positive-sense RNA viruses and viroids. In the present study, the protection efficacy of exogenous application of dsRNAs targeting the nucleocapsid (*N*) or the movement protein (*NSm*) coding genes of the negative-sense RNA virus TSWV was evaluated in *Nicotiana benthamiana* as model plant and in tomato as economically important crop. Most of the plants treated with *N*-targeting dsRNAs, but not with *NSm*-targeting dsRNAs, remained asymptomatic until 40 (*N. benthamiana*) and 63 (tomato) dpi, while the remaining ones showed a significant delay in systemic symptoms appearance. The different efficacy of *N*- and *NSm*-targeting dsRNAs in protecting plants is discussed in the light of their processing, mobility and biological role. These results indicate that the RNAi-based vaccination is effective also against negative-sense RNA viruses but emphasize that the choice of the target viral sequence in designing RNAi-based vaccines is crucial for its success.

Keywords: RNAi-based vaccination, double-stranded rnas, orthotospovirus, ambisense RNA, nucleocapsid protein, cell-to-cell movement protein

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INTRODUCTION

Tomato spotted wilt virus (TSWV), genus *Orthotospovirus*, family *Tospoviridae*, belongs to the list of the ten most economically important viruses in the world (Scholthof et al., 2011) and is able to cause high yield losses in a variety of crops and ornamentals, in tropical and subtropical regions (Pappu et al., 2009; Mitter et al., 2013; Turina et al., 2016). It has a wide host range and is transmitted by thrips in a persistent manner (Whitfield and Rotenberg, 2015). The TSWV particles are spherical and surrounded by a host-derived membrane with a diameter of 80–120 nm. The TSWV genome is of negative/ambisense polarity and consists of three linear single-stranded RNAs (ssRNA) named large (L), medium (M), and small (S) according to their sizes (Turina et al., 2016). The L segment codes for the RNA-dependent RNA polymerase (RdRp) in negative sense, while M and S segments are ambisense in their genome organization; the M RNA encodes the glycoprotein precursor (GP) of the mature membrane glycoproteins G_N and G_C and the cell-to-cell movement protein (NSm) while the S RNA codes for the nucleocapsid protein (N), and the silencing suppressor protein (NSs).

Up to now, few resistance genes against TSWV have been identified and introgressed in commercial cultivars (Turina et al., 2016); however, the frequent appearance of resistance-breaking isolates, together with the difficulty of selecting and incorporating new resistance genes stress the needs of developing new strategies for protecting plants against TSWV infection.

RNA interference (RNAi) is an RNA-mediated regulatory mechanism, conserved in most eukaryotes, consisting in the sequence-specific degradation of target RNA guided by the complementary small RNAs (sRNAs; Meister and Tuschl, 2004). Beside its crucial activity in regulating growth and development, this mechanism plays a critical role in host defense against subcellular pathogens (Baulcombe, 2004; Padmanabhan et al., 2009; Agius et al., 2012). RNAi is triggered by double-stranded RNAs (dsRNA) or hairpin RNAs (hpRNA) that are processed by specific nucleases called DICER or DICER-LIKE (DCL) into sRNAs able to guide the cleavage of complementary single-stranded RNAs, such as messenger RNAs or viral genomic/antigenomic RNAs (Meister and Tuschl, 2004).

Virus infection is associated with the accumulation of viral small RNAs (vsRNAs), originated by the host plant RNAi machinery from viral dsRNAs or hpRNAs. VsRNAs are able to direct the degradation of complementary viral single-stranded RNAs through the plant RNAi pathways (Agius et al., 2012). As a consequence, viruses are both inducers and targets of RNAi, through a process regarded as a natural antiviral defense mechanism in plants (Guo et al., 2016). Since the discovery of such siRNA-mediated antiviral defense mechanism, a number of transgene- or virus-based silencing technologies have been developed in order to protect plants from pathogens (Dietzgen and Mitter, 2006; Guo et al., 2016). DsRNAs or hpRNAs transcribed from engineered inverted repeats were shown to be potent inducers of a gene silencing response when directed against transgenes or viral pathogens (Smith et al., 2000; Marjanac et al., 2009; Hameed et al., 2017). Different RNAi approaches, all based on plant transformation, were established to induce resistance against TSWV, including the expression of viral genomic sequences (Yazhisai et al., 2015), artificial microRNAs (miRNAs; Mitter et al., 2016), or synthetic *trans*-acting siRNAs (tasiRNAs; Carbonell et al., 2019). However, transgenic approaches are time-consuming, expensive and require efficient plant transformation protocols; moreover, they are prone to significant regulation and acceptance issues, particularly in the case of food crops.

A promising technique is the RNAi-based vaccination, that exploits the RNAi machinery of the plants to protect them from viral infection. Several studies have shown that dsRNAs homologous to viral sequences, when topically applied to plants, can induce RNAi-mediated defense, interfering with infection by plant positive RNA viruses and viroids in a sequence-specific manner [reviewed in Mitter et al. (2017b); Dubrovina and Kiselev (2019))]. Exogenous application of naked dsRNAs (Tenllado and Díaz-Ruíz, 2001; Tenllado et al., 2004; Petrov et al., 2015) or spraying of bacterially expressed dsRNAs (Tenllado et al., 2003; Gan et al., 2010) were demonstrated to protect plants up to 5 days post treatment. Moreover, loading dsRNAs onto clay nanosheets increased dsRNA stability and efficacy up to 20 days post spray (Mitter et al., 2017a). These approaches interfered with the infection of potyviruses, bromoviruses, tobamoviruses, and potexviruses (Tenllado and Díaz-Ruíz, 2001; Tenllado et al., 2004; Petrov et al., 2015; Mitter et al., 2017b).

In the present study, we tested the protective effect of exogenous application of dsRNAs against TSWV, a negative sense RNA virus, belonging to the *Tospoviridae* family, both in the model plant *Nicotiana benthamiana* and in tomato (*Solanum lycopersicum* L.), one of the most important horticultural crops worldwide. For this purpose, we selected two genomic regions, one covering the N gene, which encodes a structural protein, and another covering the NSm gene encoding a non-structural protein. The dsRNAs were synthesized *in vitro* and applied on plant leaves that were further inoculated with TSWV. Plants were monitored for local and systemic symptom development and for the presence of TSWV. Persistence and movement of the dsRNAs in plants were also studied.

MATERIALS AND METHODS

Biological Material

Nicotiana benthamiana and tomato plants were maintained in a growth chamber at 24°C with a light/dark cycle of 16/8 h. Plants with 4–5 fully expanded leaves were used for the bioassays. Plants were inoculated with a TSWV pepper isolate [P105, PLAVIT collection, IPSP-CNR Torino, Italy, World Data Center for Microorganism (WDCM) no. 1057, http://www.wfcc.info/ccinfo/collection/col_by_country/i/39/].

In vitro dsRNA Synthesis

To produce dsRNA molecules, a two-step PCR approach followed by *in vitro* transcription was used (Voloudakis et al., 2015). In the case of *N*- and *NSm*-targeting dsRNAs, total RNA from TSWV-infected *N. benthamiana* plants was extracted

using Trizol (Life Technologies, United States) according to manufacturer's instructions. 1 μg of total RNA extracted from TSWV-infected *N. benthamiana* plants was used as template for cDNA synthesis, using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, United States), according to manufacturer's instructions. Viral fragments were amplified with specific primers designed using Primer3 software[1]. The T7 RNA polymerase promoter/binding site sequence was added at the 5 ì end of both the forward and the reverse primers (**Supplementary Table 1**). PCR was carried out in a final volume of 50 μl, containing 10X reaction buffer, 1 μl cDNA template (diluted 1:5), 200 μM dNTPs, 0.2 μM of each primer, 1.5 mM $MgCl_2$, and 2.0 units of Platinum *Taq* DNA polymerase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, United States). After an initial denaturation at 95°C for 10 min, the first 10 cycles were performed as follows: 95°C for 30 s, 40°C for 45 s, 72°C for 1 min, followed by 35 cycles each of 95°C for 30 s, 55°C for 45 s, and 72°C for 1 min. After a final extension at 72°C for 7 min, PCR fragments were loaded on 1% agarose gel and purified by the DNA Clean and Concentrator kit (Zymo Research, Irvine, United States). To *in vitro* synthesize sense and antisense viral derived ssRNAs, 1 μg of purified DNA template was used in 50 μl transcription reaction containing T7 reaction buffer, 500 μM rNTPs, 5 mM DTT, 50 units of T7 RNA polymerase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, United States), conducted at 37°C for 2 h. After removing DNA template by TURBO RNase-free DNAse (Ambion, Thermo Fisher Scientific, Waltham, MA, United States), dsRNAs were obtained by mixing the specific sense and antisense ssRNAs, then incubating at 95°C for 3 min, and at 37°C for 30 min. The formation of dsRNAs was confirmed by treatment with Mung Bean Nuclease (New England Biolabs, MA, United States).

As negative control, a dsRNA targeting the replication initiator protein (Rep) coding region of the geminivirus *tomato yellow leaf curl Sardinia virus* (TYLCSV) was used. In this case, DNA was extracted from TYLCSV-infected *N. benthamiana* plants using the TLES method described in Maffei et al. (2014). The *Rep*-targeting dsRNA was synthetized as described above, using specific primers reported in **Supplementary Table 1**.

Virus Inoculation and dsRNAs Exogenous Application

In order to ensure the uniformity of the TSWV inoculum in all the experiments, a TSWV inoculum stock was prepared from systemically infected *N. benthamiana* leaves. For this, symptomatic leaf tissue was cut in small slices, split into 500 mg aliquots and stored in liquid nitrogen until use. Inoculation was performed by homogenizing each aliquot in 50 ml of inoculation buffer (5 mM diethyldithiocarbamic acid, 1 mM ethylenediaminetetraacetic acid, and 5 mM sodium sulfite), and applying a 50 μl-dose to the upper side of two different leaves of each plant previously dusted with carborundum. When required, 10 μg of dsRNAs were added to the inoculum, just before inoculation.

[1]http://bioinfo.ut.ee/primer3-0.4.0/

Nicotiana benthamiana plants were used to evaluate the effect of dsRNAs on local lesions. Systemic infection induced by TSWV was evaluated in *N. benthamiana* and tomato plants, at different time points from the inoculation. Plants were inoculated with TSWV together with dsRNAs (*N* and *NSm*). The positive control group was inoculated with TSWV only and water. As negative controls, one group of plants received TSWV and the *Rep*-targeting dsRNAs originated from TYLCSV, while another one received only water. A total of thirteen *N. benthamiana* and seven tomato plants per treatment were analyzed in three and one different experiments, respectively. Symptoms development was monitored until 40 days post inoculation (dpi) in the case of *N. benthamiana* and 63 dpi in the case of tomato. Presence of viral RNA was checked by PCR in newly emerged leaves that did not receive the inoculum.

In planta dsRNA Movement

In order to evaluate the persistence/movement of dsRNAs in the plants that were not inoculated with TSWV, 10 μg of dsRNAs were mixed with 100 μl of sterile water and mechanically inoculated on two carborundum-dusted fully expanded leaves (50 μl per leaf) by gentle rubbing. Two leaf disks from each treated (local) and first expanded leaf from the apex (systemic) were collected at 1, 3, 6, and 9 dpi. Three plants were used for each time point. Just before sampling, leaves were washed with Triton X-100 (0.05%) and water to eliminate residual dsRNAs present on the leaf surface. Total RNA was extracted by Trizol reagent (Life Technologies, United States). cDNA synthesis was performed with random primers using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, United States), according to manufacturer's instructions.

Detection of TSWV and dsRNAs by RT-PCR

Tomato spotted wilt virus infection was evaluated by RT-PCR with a primers pair (TSWV_L_fw/TSWV_L_rv, amplicon size 646 bp; **Supplementary Table 1**) designed on the L RNA, a genomic component unrelated to the N and NSm genes used to design the dsRNAs. In the dsRNAs mobility experiment, the presence of *N*- and *NSm*-targeting dsRNAs was estimated by PCR using primers TSWV_N_fw/TSWV_N_rv and TSWV_NSm_fw/TSWV_NSm_rv, respectively (**Supplementary Table 1**). PCR was performed in a final volume of 25 μl, containing 2.5 μl reaction buffer, 1 μl cDNA template, 200 μM each dNTPs, 0.4 μM each primer, 2 mM MgCl2, and 1 unit Platinum *Taq* DNA polymerase (Invitrogen). After an initial denaturation step for 4 min at 95°C, 30 cycles consisting each of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C were performed, followed by a final extension step for 7 min at 72°C.

Detection of dsRNAs by Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was carried out using iCycler iQTM Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, United States), with the following cycling parameters: 1 cycle at 95°C for 5 min, 45 cycles, each

consisting of 15 s at 95°C and 1 min at 60°C. A melting curve was recorded at the end of each run to assess the specificity of amplification. All reactions were performed with three technical replicates. RT-PCR efficiency was calculated using standard curves constructed with serial dilutions of cDNA extracted from infected plants. Data acquisition and analysis were handled by the BioRad iCycler software (version 3.06070) that calculates Ct values and standard curves. qRT_TSWV_N_fw/qRT_TSWV_N_rv (Mason et al., 2002) and qRT_TSWV_NSm_fw/qRT_TSWV_NSm_rv primers pairs (**Supplementary Table 1**) were used to amplify exogenous dsRNAs, while the primer pair qRT_NbCOX_fw/qRT_NbCOX_rv (**Supplementary Table 1**) was used for the amplification of the *N. benthamiana* gene Niben101Scf02399 coding for the cytochrome c oxidase (NbCOX), used as reference gene (Nerva et al., 2017). The relative dsRNAs amount was estimated for each sample using the ΔCt method, where ΔCt is $|Ct_{dsRNA} - Ct_{cox}|$.

Small RNA Analysis by High Throughput Sequencing (HTS)

Small RNA populations originating from *N*- and *NSm*-targeting dsRNAs were analyzed in samples collected for the dsRNA movement investigation at 1 dpi. Small RNA libraries preparation and sequencing were performed by Novogene (United Kingdom) Company Limited (United Kingdom). After adapter removal with fastp (Chen et al., 2018) and low-quality filtering and artifact removal with fastx-toolkit[2], clean reads were mapped to the TSWV genome segment targeted by the applied exogenous dsRNA (S segment, Acc. Num. DQ376178.1; M segment, Acc. Num. KJ575621.1) using bowtie v1-3-0 (Langmead et al., 2009), with 0 mismatches. Mapping results were visualized using Misis (Seguin et al., 2014). Sequence data have been submitted to the Sequence Read Archive (SRA) with the BioProject ID PRJNA672300.

RESULTS

Selection of Viral Genomic Sequences for dsRNA Production

In order to select the genomic regions of TSWV most suitable as target by dsRNAs, we first considered the vsRNAs profile of TSWV available in the literature (Mitter et al., 2013; Margaria et al., 2015), starting from the hypothesis that the genomic regions characterized by a high number of mapping vsRNAs could be more subjected to RNAi-mediated degradation. According to Mitter et al. (2013), most vsRNAs detected in TSWV-infected *N. benthamiana* plants mapped to the M segment, followed by the S segment, while the L RNA had the least number of mapping vsRNAs. Therefore, we focused our attention on the M and S segments. Among the two open reading frames (ORFs) of the M segment, *GP* and *NSm*, the *GP* ORF has the higher number of mapping vsRNAs (Mitter et al., 2013). However, we decided to select the *NSm* coding region as dsRNA target, since the movement protein has an important effect on viral systemic spread while the glycoproteins are not needed for *in planta* replication and intercellular spread of the virus; indeed, amiRNAs targeting the *GP* fragment did not show any antiviral protective effect (Carbonell et al., 2019). In the case of the S segment containing the *N* and *NSs* ORFs, we focused on the *N* coding region, since it codes for the nucleocapsid protein, a structural protein essential for virions formation. In addition, artificial miRNAs targeting the *NSs* ORF were not effective in protecting plants against TSWV infection (Mitter et al., 2016; Carbonell et al., 2019).

Since previous work showed that the exogenous dsRNAs must be longer than 300 bp to effectively interfere with virus infection (Tenllado and Díaz-Ruíz, 2001), we designed to produce dsRNAs longer than this threshold, i.e., 761 bp for the *N*-targeting dsRNA and 603 bp for the *NSm*-targeting dsRNA.

Finally, to better evaluate the dsRNAs protective effect, we decided to include a dsRNA homologous to a portion of the gene encoding the Rep pf TYLCSV as a negative control. The length of the *Rep*-targeting dsRNA (598 bp) was similar to the *N*- and *NSm*-targeting dsRNAs, but, due to the lack of homology with the TSWV genome, this dsRNA was not expected to show any protective effect against TSWV.

Production of dsRNAs

The method employed to produce dsRNAs, based on two sequential PCR reactions, coupled with *in vitro* transcription (Voloudakis et al., 2015) typically yielded 50–80 μg of dsRNAs, starting from 1 μg of DNA template. The double-stranded nature of the molecules obtained was confirmed by incubating the *in vitro*-transcribed RNAs before and after the annealing step with Mung Bean Nuclease, an enzyme that specifically degrades single-stranded nucleic acids (**Figure 1**).

Different Antiviral Efficacy of Exogenous Application of dsRNAs Targeting the *N* or *NSm* Genomic Regions of TSWV

At 7 dpi, typical chlorotic and necrotic local lesions occurred on leaves of *N. benthamiana* plants inoculated with TSWV alone, with no significant difference (p-value < 0.05) in their average number compared to plants inoculated with TSWV + *NSm*-targeting dsRNAs and with TSWV + *Rep*-targeting dsRNA. At the same time point, plants inoculated with TSWV + *N*-targeting dsRNAs showed drastic reduction in the number of local lesions (**Figure 2**), highlighting a protective effect of the *N*-targeting dsRNAs.

All the *N. benthamiana* plants inoculated with TSWV alone showed typical systemic symptoms of chlorosis, necrosis, stunting, and wilting at 11 dpi. At the same time point, similar symptoms were observed on 92% of plants treated with TSWV + *NSm*-targeting dsRNAs and 75% of plants treated with TSWV + *Rep*-targeting dsRNAs; conversely, only 15% of plants treated with TSWV + *N*-targeting dsRNAs appeared symptomatic. At later observation (25 dpi), all plants treated with TSWV + *NSm*-targeting dsRNAs or with TSWV + *Rep*-targeting dsRNAs showed the systemic TSWV symptoms. In the case of plants treated with TSWV + *N*-targeting dsRNAs, the

[2]http://hannonlab.cshl.edu/fastx_toolkit

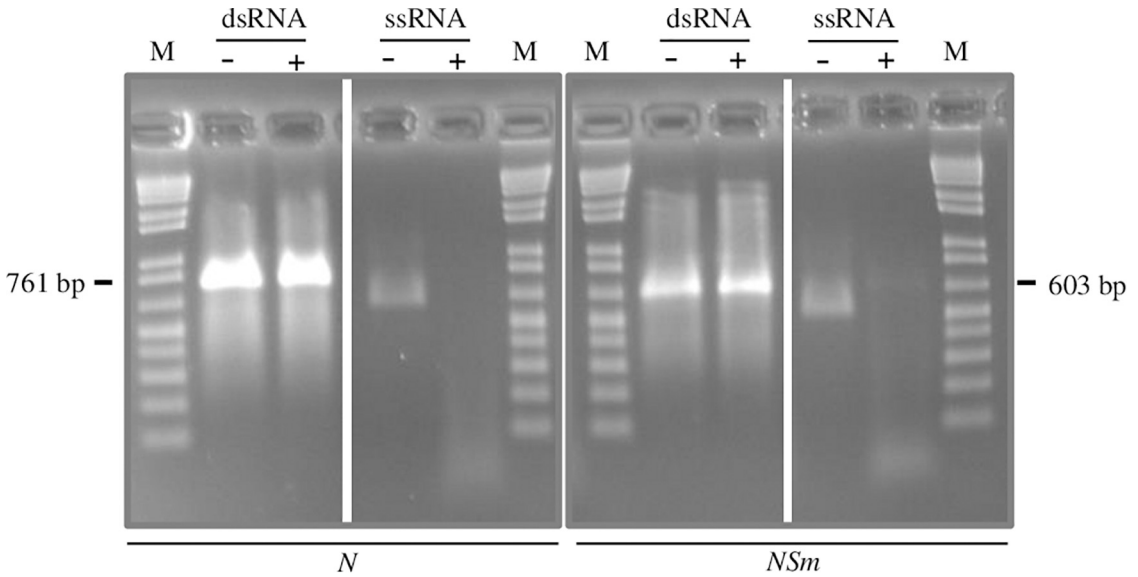

FIGURE 1 | Confirmation of double-strand structure of *N*- and *NSm*-targeting dsRNAs by specific degradation with Mung Bean Nuclease. A single-stranded RNA transcript is used as control. +, Mung Bean Nuclease treatment; -, mock treatment; and M, 100 bp DNA Ladder (New England Biolabs, MA, United States). Nucleotide sequence length of synthetized fragments is shown on the sides.

rate of symptomatic plants reached 46% at 17 dpi and remained unchanged until the end of the experiment (40 dpi; **Figure 3**). The RT-PCR performed on a subset of *N. benthamiana* plants confirmed visual symptoms evaluation (not shown).

In the case of tomato plants, most of the plants (71%) treated with TSWV + *NSm*-targeting dsRNAs and 100% of the plants inoculated only with TSWV were already infected at 21 dpi, but none of the plants inoculated with TSWV and treated with *N*-targeting dsRNAs became infected for the entire duration of the experiment, at 64 dpi (**Figure 4A**).

In both species, we didn't observe any difference in the reproductive stage; all the asymptomatic plants, including those treated with dsRNAs, exhibited a normal flowering, while no flowering was observed in symptomatic plants, due to the heavy symptomatology.

The virus was detected in young leaves of all the symptomatic plants while it was absent in all asymptomatic plants treated with TSWV + *N*-targeting dsRNAs (**Figure 4B**), confirming visual inspection (**Figure 4C**).

Taken together, these results show that exogenous application of dsRNAs homologous to the *N* gene can completely suppress or robustly contrast viral replication and protects plants from TSWV infection, both in *N. benthamiana* and tomato, by inhibiting or strongly delaying symptom development, while dsRNAs targeting the *NSm* gene have almost no protective effect.

Systemic Movement of the Applied dsRNAs

In order to investigate the persistence and the systemic movement of *N* and *NSm*-targeting dsRNAs applied on the leaves, in the absence of virus infection, we analyzed by RT-PCR the presence of both dsRNAs at 1, 3, 6, and 9 dpi in both treated (local persistence) or young untreated leaves (systemic movement) of *N. benthamiana* plants (**Figure 5A**). Both dsRNAs could be detected until 9 dpi in the treated leaves indicating that they persist there for several days after application. Moreover, we detected both *N* and *NSm*-targeting dsRNAs also in untreated leaves at all time points, although with a lower intensity compared to treated tissues.

When the quantitative evaluation of the dsRNAs was performed by qRT-PCR, we observed that most of the applied *N*- or *NSm*-targeting dsRNAs remained in the treated leaves and only a limited amount of them was transported systemically to the young untreated leaves. Interestingly, the dsRNAs systemic movement, even if very limited, could be observed at 1 dpi and remained steadily low until 9 dpi (**Figure 5B**).

These results indicate that both dsRNAs considered are able to move systemically within the plant, but that the majority of them remains in treated leaves. No significant differences in persistence or systemic movement capacity were observed between the *N*- and *NSm*-targeting dsRNAs.

Analysis of the sRNAs Populations Originating From *N*- and *NSm*-Targeting dsRNAs

In order to evaluate if *N*- and *NSm*-targeting dsRNAs were processed by Dicer and were able to originate the silencing effector molecules (siRNAs), we sequenced the sRNAs populations of treated (local), and newly emerged non-treated (systemic) *N. benthamiana* leaves, 1 day post exogenous dsRNAs application. After adapter removal and quality filtering, 18 to 28 million of reads were obtained (**Supplementary Tables 2, 3**). Sequences with length ranging from 20 to 25 nt were selected for

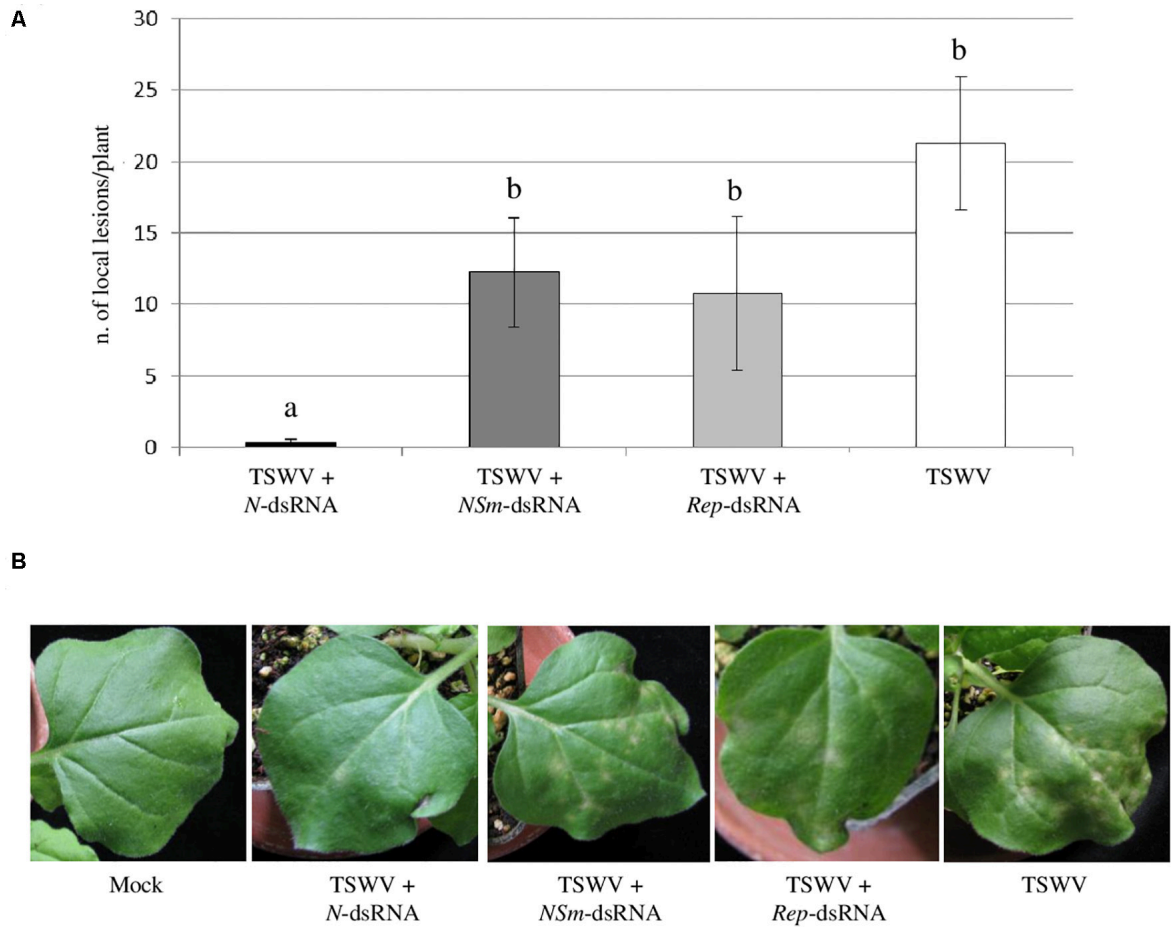

FIGURE 2 | Effect of dsRNAs on production of TSWV-induced local lesions on *N. benthamiana* leaves. **(A)** Histogram reporting the number of local lesions per plant. Results are expressed as mean values of local lesions per plant counted at 7 dpi on 2 inoculated leaves for each plant, for a total of 13 plants per treatment, except for *Rep*-targeting dsRNAs treated plants for which 4 plants were considered. Different letters indicate significant differences at $P < 0.05$ (Kruskal–Wallis test followed by *post hoc* Wilcoxon test). **(B)** Local lesions at 7 dpi on *N. benthamiana* plants treated with *N*-, *NSm*- and *Rep*-targeting dsRNAs and inoculated with TSWV or inoculated with TSWV alone. Different letters indicate statistically significant differences.

further analyses. In the case of leaves treated with dsRNAs (local), the percentage of reads mapping to the *N*- and *NSm*-targeting dsRNAs regions was 0.39 and 0.50, respectively (**Figure 6**); when newly emerged non-treated leaves were considered, only few reads mapped to the corresponding viral region, i.e., 53 reads for the *N*-targeting dsRNA and 89 reads for *NSm*-targeting dsRNA. These results indicate that both dsRNAs are effectively introduced into the plants and processed, thus originating siRNAs that can guide the cleavage of cognate sequences. However, in agreement with the very limited amount of dsRNAs that move systemically into the plant, only few dsRNA-derived sRNAs were found in the newly emerged non-treated leaves.

The size distribution of sRNAs originating from the dsRNAs was basically uniform; in both cases, even if the amount of 21- and 22-nt reads was slightly higher than the number of other reads, no well-defined peaks related to a particular read size was observed (**Figure 6**). This may suggest that the inoculated dsRNA was not processed into sRNAs and the protective effect was possibly dsRNA- and not RNAi-mediated.

DISCUSSION

Previous work on engineering RNAi-mediated transgenic resistance indicated that constructs targeting the *N* and *NSm* genes of TSWV successfully protected plants against viral infection (Mackenzie and Ellis, 1992; Vaira et al., 1995; Prins et al., 1996; Herrero et al., 2000). Subsequent work showed that *N* gene segments as short as 110-nt were sufficient to efficiently induce RNA silencing, and hence virus resistance (Jan et al., 2000). Recently, the use of chimeric transgenes derived from different orthotospoviruses further extended the usefulness of this approach generating a broad-spectrum resistance against this virus group at the genus level (Bucher et al., 2006; Peng et al., 2014). However, even if the use of transgenic plants has been

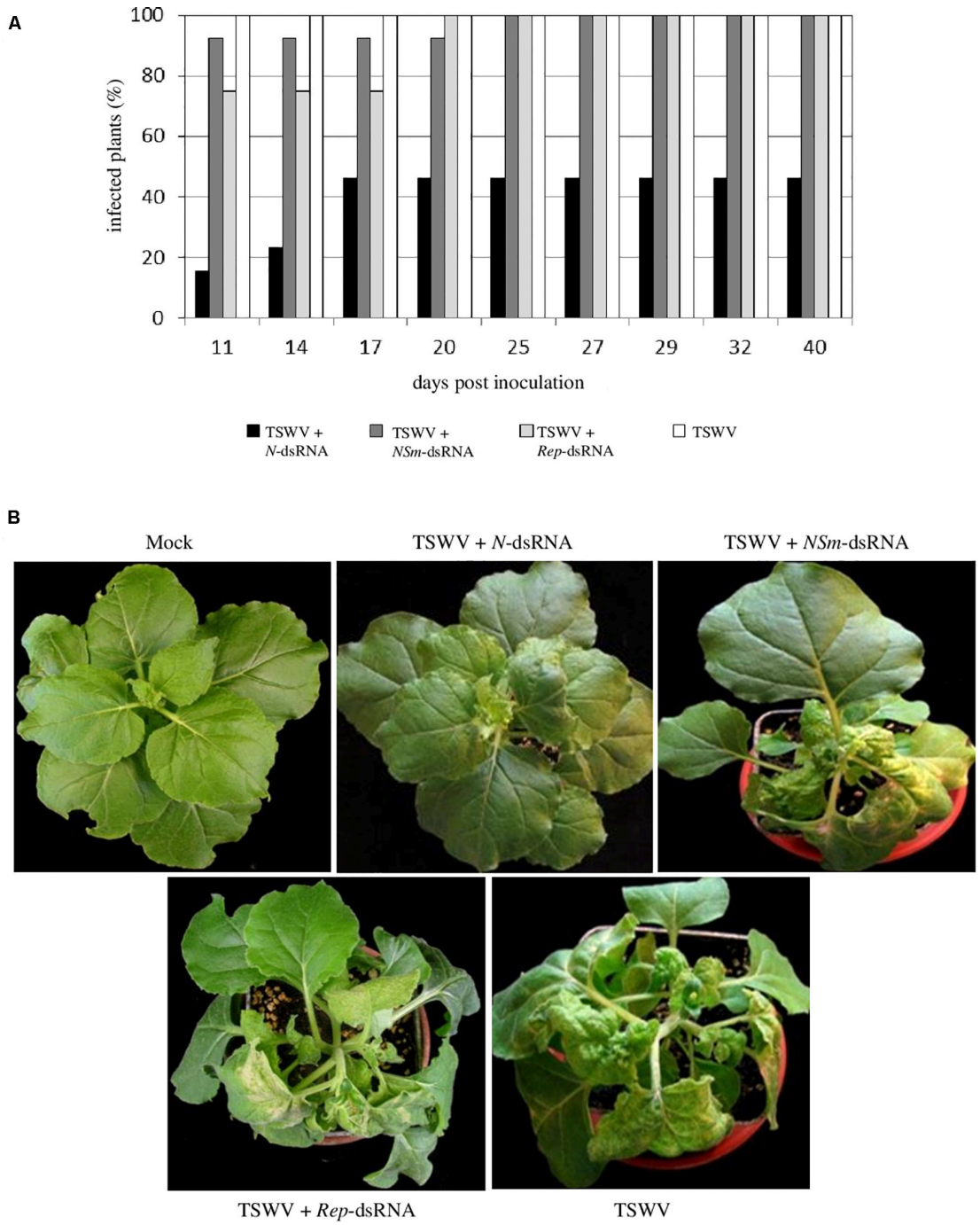

FIGURE 3 | Efficacy of *N*-, *NSm*- and *Rep*-targeting dsRNA treatments against TSWV infection in *N. benthamiana*. **(A)** Percentage of TSWV-infected plants treated or not with dsRNAs. **(B)** Systemic symptoms at 14 dpi on *N. benthamiana* plants treated with *N*-, *NSm*- and *REP*-targeting dsRNAs and inoculated with TSWV or inoculated with TSWV alone.

established as a powerful tool for plant protection, concerns about potential negative effects on human health and environment, together with limited acceptance by consumers dictated the development of alternative non-transgenic strategies exploiting RNAi, such as exogenous application of dsRNAs. In the present study, we tested the efficacy of the RNAi-based vaccination against the economically important virus TSWV, by applying exogenous synthetic dsRNAs on plant leaves.

We established that the RNAi-based vaccination approach is effective also against negative-strand RNA viruses, both in the model plant *N. benthamiana* and in the economically important tomato crop. However, we found that, in our conditions, the

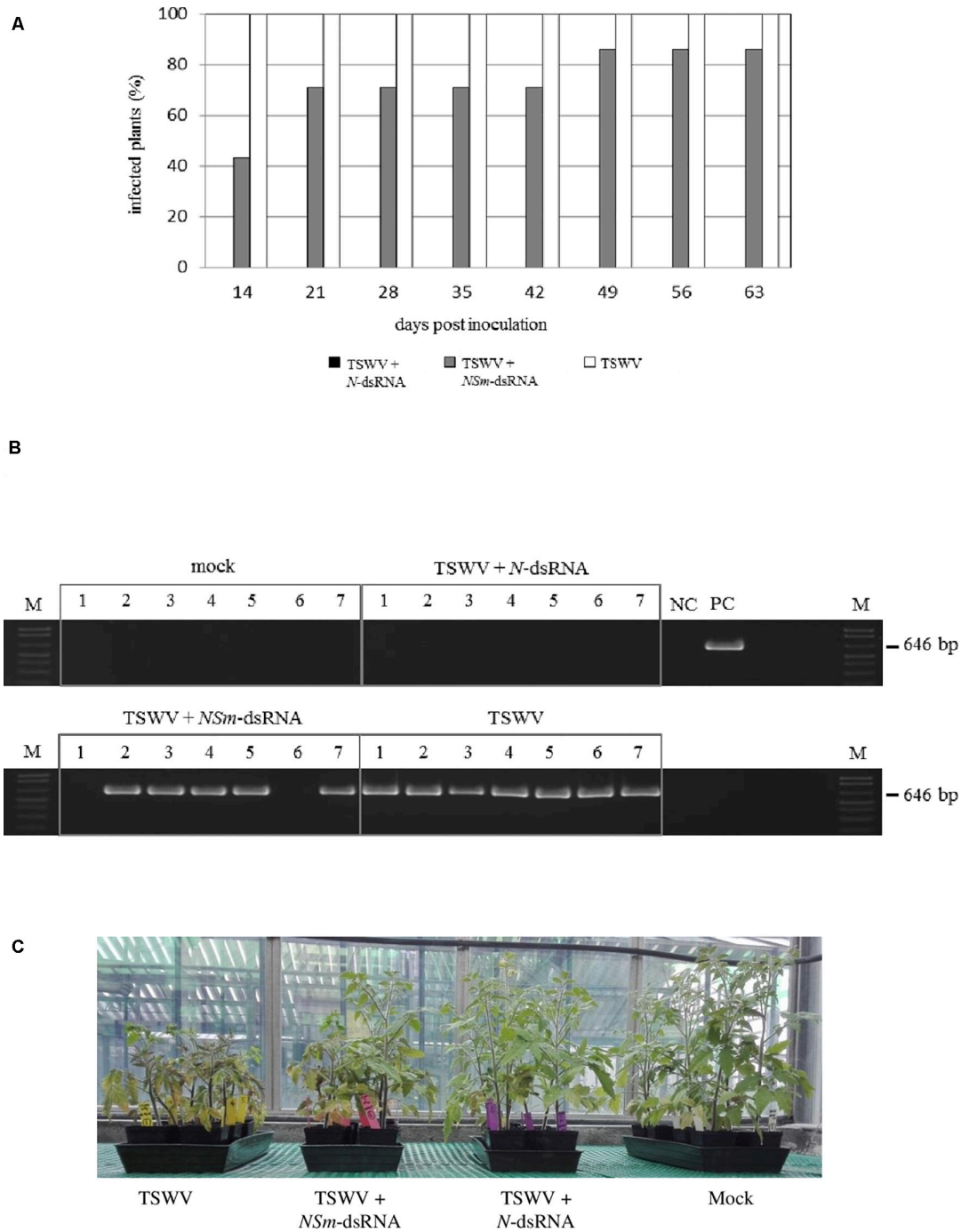

FIGURE 4 | Efficacy of *N*- and *NSm*-targeting dsRNA treatments against TSWV infection in tomato; seven plants (1–7) were used for each thesis. **(A)** Percentage of TSWV infection in plants treated or not with dsRNAs at several time points. **(B)** RT-PCR for the evaluation of TSWV infection in tomato plants at 28 dpi; primers targeting TSWV L genomic segment were used. NC, negative control (Mock-inoculated plant); PC, positive control (TSWV-infected plant); and M, 100 bp DNA Ladder (New England Biolabs, MA, United States). Size of amplified fragment is shown on the side. **(C)** Tomato plants treated with *N*- and *NSm*-targeting dsRNAs and inoculated with TSWV or inoculated with TSWV alone, at 30 dpi.

dsRNAs targeting the *N* gene are able to protect the plant, while those targeting the *NSm* gene are not. It is interesting to note that a slight reduction in the percentage of infected plants among those treated with *Rep*- and *NSm*-targeting dsRNAs was observed in respect to plants inoculated only with the virus. Such reduction is possibly related to the ability of exogenous dsRNAs to activate a sequence-unrelated pattern-induced immunity response (Niehl et al., 2016).

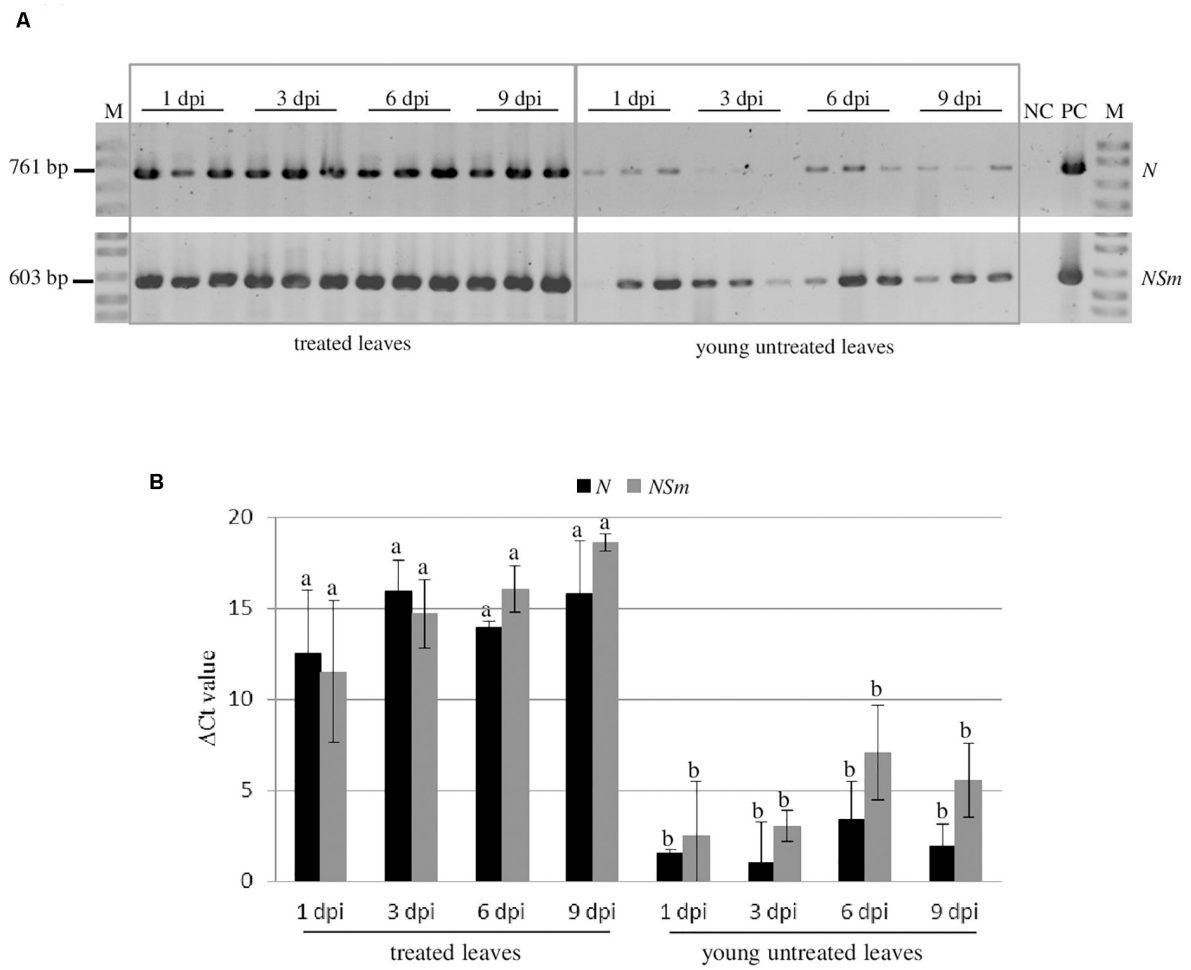

FIGURE 5 | Persistence and systemic movement of *N*- and *NSm*-targeting dsRNAs. Three biological replicates were used. **(A)** Detection of the *N*- and *NSm*-targeting dsRNAs in treated leaves and untreated young leaves *of N. benthamiana* at different time points by end-point RT-PCR. NC, negative control (Mock-inoculated plant); PC, positive control (TSWV-infected plant); and M, 100 bp DNA Ladder (New England Biolabs, MA, United States). Sizes of amplified fragments are shown on the side. **(B)** Quantification of *N*- and *NSm*-targeting dsRNAs by qRT-PCR. The Ct values obtained for the dsRNAs were normalized with the Ct values obtained for the COX transcript used as reference. Vertical lines on each bar represent standard deviations. Different letters indicate statistically significant differences ($p < 0.05$, ANOVA). Different letters indicate statistically significant differences.

Overall, the different behavior of the *N*- and *NSm*-targeting dsRNAs requires some considerations.

First, we can exclude a possible effect of the dsRNA length, since the two dsRNAs are similar in size and include almost the entire coding region. Indeed, previous work on other plant viruses showed that dsRNAs with a size range from 600 to 900 nt can be very effective (Tenllado and Díaz-Ruíz, 2001).

Second, the RNAi-based vaccination has been demonstrated to be dose-dependent (Tenllado and Díaz-Ruíz, 2001; Dubrovina and Kiselev, 2019). Actually, in our experiments, the dose of dsRNAs applied to leaves (10 μg/plant) was in line with those currently used by other authors (Dubrovina and Kiselev, 2019). Furthermore, even when we increased the amount of the *NSm*-targeting dsRNAs up to 30 μg/plant, no protection against the virus was obtained (data not shown), demonstrating the intrinsic inefficacy of targeting this region.

The accessibility of dsRNAs to DCLs, frequently estimated by the abundance of originating vsRNAs, is another important aspect to consider regarding the effectiveness of RNAi-based vaccination. In our case, we can exclude that the viral sequences targeted by dsRNAs induce different response in terms of vsRNA abundance, since the amount of vsRNAs mapping to the *N* and *NSm* coding regions was similar (Mitter et al., 2013; Margaria et al., 2015; Ramesh et al., 2017). Indeed, the analysis of sRNAs populations in dsRNAs-treated plants confirmed that both *N*- and *NSm*-targeting dsRNAs were successfully recognized and processed by the endogenous RNAi machinery.

The ability of dsRNAs to move systemically in the plant can also be relevant for their efficacy. Actually, the systemic transport of exogenously applied dsRNAs is not well understood. Konakalla et al. (2016) observed that dsRNAs can move systemically in tobacco plants already 1 day following leaf application

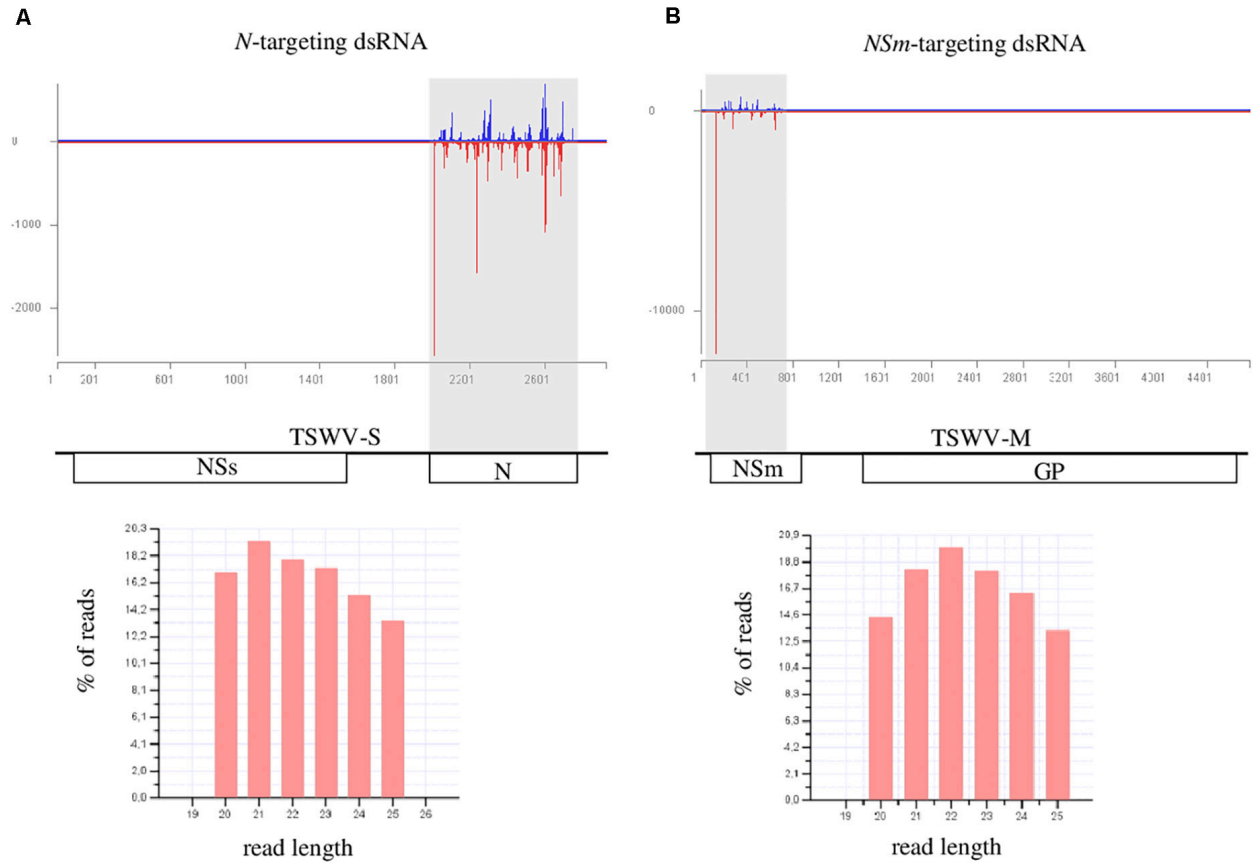

FIGURE 6 | HTS analysis of small RNAs in dsRNAs-treated *N. benthamiana* leaves, 1 day post treatment. **(A)** upper panel: distribution of TSWV-targeted small RNAs along the S segment of TSWV genome (TSWV-S; Genbank acc. N. DQ376178.1); viral sense and antisense small RNAs are reported in blue and red, respectively; *N* (nucleoprotein); and *NSs* (non-structural protein) genes are represented along the viral RNA S segment; lower panel: length distribution of the small RNAs reads mapping to the TSWV S segment. **(B)** upper panel: distribution of TSWV-targeted small RNAs along the M segment of TSWV genome (TSWV-M; Genbank acc. N. KJ575621.1); NSm (non-structural protein); and GP (glycoproteins precursor) genes are represented along the viral RNA M segment; lower panel: length distribution of the small RNAs reads mapping to the TSWV M segment.

(Konakalla et al., 2016), a result also confirmed in tomato plants (Gogoi et al., 2017). Moreover, rice and maize roots soaked in a dsRNAs-containing solution can absorb dsRNAs (Li et al., 2015). On the other hand, other groups reported that exogenous dsRNAs remain mostly in treated leaves, at least in squash and watermelon plants (Kaldis et al., 2018), or in tomato (San Miguel and Scott, 2016). Such contradictory reports could be explained by the existence of an active dsRNA long-distance movement process mediated by proteins binding specific RNA-motifs (Kehr and Kragler, 2018). Our results showed that both *N*- and *NSm*-targeting dsRNAs behave similarly: a small quantity of dsRNAs can move systemically and reside in young untreated leaves already 1 day after their application on the older treated leaves, but the majority of them persist in the treated leaves. According to this, dsRNAs-derived sRNAs were mainly recovered in the leaves treated with exogenous dsRNAs and only few of them were found in newly emerged non-treated leaves. These results may suggest that, as well as dsRNAs, sRNAs also remain mostly in the treated leaves and only a small fraction is subjected to systemic transport. However, we cannot exclude the possibility that the detected sRNAs were produced in the systemic leaves after the processing of the dsRNAs coming from inoculated tissues. Taken together these data allow to rule out the possibility that the lack of protection from TSWV infection of the *NSm*-targeting dsRNA is connected with its movement ability or to the mobility of derived sRNAs.

The limited mobility of dsRNAs and derived sRNAs point out the possible limits in the large-scale use of the dsRNA-vaccination for crop protection against viruses and suggests that more effective application techniques such as the use of high-pressure spraying (Dalakouras et al., 2016) or the conjugation of dsRNAs with protein carriers (Numata et al., 2014), nanostructures (Mitter et al., 2017a), or abrasive substances could be developed.

Finally, the different antiviral efficacy of the *N*- and *NSm*-targeting dsRNAs could reside on the biological role of the *N* and *NSm* gene products. A successful infection by TSWV requires primarily the replication and transcription of the genetic viral elements to produce massive amounts of infectious

ribonucleocapsid proteins (RNPs), the minimal infectious unit containing the three genomic RNAs tightly packed by the N protein and few copies of the viral RdRp. RNPs then associate with NSm and move intra- and inter-cellularly through a continuous endoplasmic reticulum network, supported by NSm-derived tubule structures that allow systemic spread (Zhu et al., 2019). Since the N protein is involved in RNPs aggregation immediately after RNA genome replication, it is likely that a certain amount of *N* coding RNA is required to synthesize enough N protein molecules to form new RNPs. Indeed, *N* gene is constitutively expressed since early time points of infection (Kormelink et al., 1994). Conversely, the *NSm* viral gene is only transiently expressed at the early stage of infection and its product is required for the RNPs to disseminate from the first initially infected cells. Therefore, one can argue that lower amount of NSm protein could be sufficient for this step. In this perspective, the degradation of the *N* coding RNA could have a crucial impact on viral replication due to the central role of its encoded protein; on the other hand, the NSm protein could play a secondary role and, even if targeted by vsRNAs, a small quantity of *NSm*-coding RNA could be sufficient to produce the required amount of movement protein. It is worth noting that, in a natural context, the inducers of antiviral RNAi and R gene-based host defense, namely dsRNAs and viral effector proteins, are deployed in the initial stages of the viral cycle (Zhu et al., 2019). The importance of the link between the biological role of *N* and NSm proteins and the ability of their encoding sequences to confer resistance is also supported by the observation that plants transformed with the *N* sequence were resistant at both plant and cellular level while plants transformed with the *NSm* sequence showed resistance at plant but not at cellular level, since the virus was still able to replicate in protoplasts of *NSm*-transgenic plants (Prins et al., 1996). However, *NSm*-transformed plants were resistant to TSWV infection probably because the *NSm* RNA was constitutively expressed in the whole plant at a level sufficient to contrast the systemic movement of the virus. Finally, it is worth mentioning that most of the successful reports on RNAi-based vaccination involved dsRNAs targeting capsid coding genes, except for one case where up to 66% of tobacco plants were protected from tobacco mosaic virus infection using dsRNAs targeting the movement protein (Sun et al., 2010).

To conclude, we demonstrated that the RNAi-based vaccination is effective also against membrane-encapsidated, multi-component, negative-sense RNA viruses and we added the *Tospoviridae*, a family including one of the most economically important plant viruses, to the list of viral families potentially targeted by this approach. Noteworthy, we showed that the choice of the viral region targeted by dsRNAs was crucial to induce resistance, highlighting that the viral cycle is a fundamental aspect to consider in the RNAi-based vaccination design.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**. Sequence data are available in the Sequence Read Archive (SRA) with the BioProject ID PRJNA672300.

AUTHOR CONTRIBUTIONS

LM, ST, GA, AV, and EN conceived and designed the experiments. ST, MJ, and LM conducted the experiments. DM gave technical support in laboratory and greenhouse activities. SAAB supported ST activities. LM, ST, EN, GA, and AV wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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High-Pressure-Sprayed Double Stranded RNA Does Not Induce RNA Interference of a Reporter Gene

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In plants, RNA interference (RNAi) is an effective defense mechanism against pathogens and pests. RNAi mainly involves the micro RNA and the small interfering RNA (siRNA) pathways. The latter pathway is generally based on the processing of long double stranded RNAs (dsRNA) into siRNAs by DICER-LIKE endonucleases (DCLs). SiRNAs are loaded onto ARGONAUTE proteins to constitute the RNA-induced silencing complex (RISC). Natural dsRNAs derive from transcription of inverted repeats or of specific RNA molecules that are transcribed by RNA-directed RNA polymerase 6 (RDR6). Moreover, replication of infecting viruses/viroids results in the production of dsRNA intermediates that can serve as substrates for DCLs. The high effectiveness of RNAi both locally and systemically implicated that plants could become resistant to pathogens, including viruses, through artificial activation of RNAi by topical exogenous application of dsRNA. The most preferable procedure to exploit RNAi would be to simply spray naked dsRNAs onto mature plants that are specific for the attacking pathogens serving as a substitute for pesticides applications. However, the plant cell wall is a difficult barrier to overcome and only few reports claim that topical application of naked dsRNA triggers RNAi in plants. Using a transgenic *Nicotiana benthamiana* line, we found that high-pressure-sprayed naked dsRNA did not induce silencing of a green fluorescence protein (GFP) reporter gene. Small RNA sequencing (sRNA-seq) of the samples from dsRNA sprayed leaves revealed that the dsRNA was, if at all, not efficiently processed into siRNAs indicating that the dsRNA was insufficiently taken up by plant cells.

Keywords: double stranded RNA, small RNA sequencing, GFP silencing, RNA interference, RNA delivery

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INTRODUCTION

Plant pests cause a significant decline in quantity and quality of crops as well as forestry products. The climate change alters the spreading of insect species, which may induce damage by feeding and/or by transmitting plant pathogens. For example, several studies have reported the recent invasion of Mediterranean plant pests like *Thaumetopoea processionea* in Northern European countries, including England, Denmark, and Sweden (Wagenhoff et al., 2014). Moreover, there are many regulatory restrictions on the use of conventional pesticides because of potential ecological and environmental hazard upon application (Robin and Marchand, 2019). Therefore and in view of the controversial discussions on the employment of genetically modified (gm) plants, novel versatile

and gm-free eco-friendly approaches have become fundamental for pest control, including defense against viruses, in agriculture.

One of the strategies that plants, as sessile organisms, use to cope with pests is RNA interference (RNAi). RNAi comprises two main pathways: the micro RNA (miRNA) and the siRNA pathways (Ruiz-Ferrer and Voinnet, 2009; Borges and Martienssen, 2015). MiRNA production is initiated by transcription of endogenous miRNA genes, which are subsequently processed to typically 21-nt long miRNAs. Mature miRNAs bind to complementary transcripts for degradation or translational inhibition (Brodersen et al., 2008; Lanet et al., 2009; Li et al., 2018). It has been shown that plant-derived miRNAs, e.g., members of the miRNA 166 family, are taken up by aphids and this uptake correlates with resistance of melons to the aphid *Aphis gossypii* (Sattar et al., 2012).

The siRNA pathway is initiated by cleavage of double stranded RNA (dsRNA). DsRNA is subsequently processed into 21-nt, 22-nt, and 24-nt small interfering RNAs (siRNAs) (Fusaro et al., 2006). 21-nt and 22-nt siRNAs lead to post-transcriptional gene silencing mainly *via* degrading complementary transcripts, whereas 24-nt siRNAs mediate epigenetic modifications at complementary DNA for inducing transcriptional gene silencing (TGS) (Wassenegger et al., 1994; Wierzbicki et al., 2012; Dalakouras et al., 2020). The siRNA pathway blocks viral infections as well as transposable element activity. In addition, transgene expression is also frequently suppressed by siRNA-mediated TGS (Baulcombe, 2004).

Virus infections trigger RNAi upon formation of viral dsRNA replication intermediates or viral RNA secondary double stranded structures. Viral dsRNA is recognized by the RNA silencing machinery and is accordingly processed by Dicer-like enzymes (DCLs) into siRNAs. Argonaute (AGO) proteins binding these siRNAs to constitute the RNA-induced silencing complex (RISC) lead to the degradation of viral RNAs. Recruitment of RNA-directed RNA polymerase 6 (RDR6) to specific target RNAs (e.g., aberrant RNAs lacking a 5′ cap and/or a polyA-tail) leads to further dsRNA production (Dalmay et al., 2000; Vaistij et al., 2002; Gazzani et al., 2004). These dsRNAs are subsequently cleaved into secondary siRNAs, a process that is termed "transitivity." Secondary siRNAs yield augmented defense against viruses and serve as footprints of the RNAi machinery (Baulcombe, 2004; Dunoyer and Voinnet, 2005). However, most viruses encode RNA silencing suppressors that impair the RNAi machinery by, for example, sequestering siRNAs or inhibiting AGOs (Silhavy and Burgyán, 2004). Hence, viral infection cannot be prevented by the plant defense in all cases.

In order to prevent virus infections it is essential to deliver dsRNA, exhibiting complementarity to the infecting virus already before the virus enters the plant cell. This strategy was successfully and numerously put into practice by the generation of gm plants expressing virus-specific RNAi-inducing transgene constructs (Wang et al., 2012; Pooggin, 2017). In recent years, alternative approaches that are based on exogenous delivery of dsRNA were employed to protect plants against virus infections. Exogenous RNAs of different origins such as *in vitro* and chemical synthesis or bacterial expression have been used (Lau et al., 2014; Dubrovina and Kiselev, 2019). These RNAs are delivered to plants using various methods, including low-pressure spraying, spreading by brushes, infiltration, biolistic approaches, trunk injections, mechanical inoculation, and high-pressure spraying (Dubrovina et al., 2019; Dalakouras et al., 2020). These methods appeared to improve plant defense against viruses slightly (Carbonell et al., 2008; Gan et al., 2010; Yin et al., 2010; Konakalla et al., 2016; Kaldis et al., 2018).

Different classes of adjuvants, including cationic nanoparticles, surfactants, clay nanosheets, peptide-based agents, and carbon dots have been used to boast plant defense against pests by facilitating the delivery of exogenous dsRNAs through the cell wall and subsequently cell membrane (Jiang et al., 2014; Mitter et al., 2017a; Schwartz et al., 2019; Worrall et al., 2019; Zheng et al., 2019). Indeed, it has been shown that these adjuvants improved plant defense against virus infection by increasing the uptake of dsRNA into plant cells and by protecting the dsRNAs from early degradation (Unnamalai et al., 2004; Mitter et al., 2017b).

Beside improving plant defense, it has been shown that naked dsRNA can be taken up by plant cells reducing the expression of transgenes in *Arabidopsis thaliana (*Mitter et al., 2017b; Dubrovina et al., 2019). However, the lack of molecular fingerprints of RNAi such as phased siRNAs in target sequences upon exogenous dsRNA applications raises questions about the underlying activity mechanism of the exogenous dsRNAs (Uslu and Wassenegger, 2020).

In this study, we investigated the RNA silencing efficacy of exogenously applied dsRNA and the processing of the dsRNA into siRNAs by the plant RNAi machinery using deep sequencing. For this purpose, the green fluorescence protein (GFP) - expressing *Nicotiana benthamiana* line 16C (Nb-16C) as a highly sensitive RNAi reporter system was treated with exogenous dsRNA to search for processed dsRNAs. *N. benthamiana* wild type (Nb-WT) plants were taken as controls to filter out the degradation products of the sprayed dsRNAs and water sprayed Nb-16C plants to eliminate the degradation products of endogenous target sequence. In order to deliver the dsRNAs into the plant cells, we employed the high-pressure spraying procedure (HPSP), which is reported to be the only method inducing transgene silencing via efficient activation of RNAi in *N. benthamiana* (Dalakouras et al., 2016, 2018). In this study, we demonstrate that dsRNA delivery by HPSP did not induce transgene silencing. In concordance with these finding, sRNA-seq revealed that the dsRNAs were not processed into specific siRNAs by RNAi machinery.

RESULTS

DsRNA Synthesis and Monitoring of GFP Expression Upon HPSP

322nt-long dsRNA (dsRNA-5′GFP) and 139nt-long dsRNA (dsRNA-midGFP) matching the GFP sequence (position 1 to 322 and 294 to 432, respectively) expressed in the transgenic *N. benthamiana* line 16C (Nb-16C) were synthesized *in vitro* and annealed subsequently. Single stranded (ss) RNA and possible DNA contaminations were eliminated by DNAse and RNase

treatment (**Supplementary Figure 1**). As a positive control, 22nt-long synthetic siRNA#164 matching the GFP sequence (position 164 to 187) has been used (Dalakouras et al., 2016).

High-pressure spraying creates a radial gradient of pressure. The center of sprayed area has the highest pressure and leads to wounded areas with 2–3 mm radii. The further the distance from the central region, the lower the pressure gets. Therefore, the periphery of the sprayed area recapitulates foliar spraying whereas the center of the area is subjected to high-pressure spraying. Noteworthy that the integrity of the dsRNAs sprayed onto the walls of a 15 ml-falcon tube under six-bar pressure was not affected (**Supplementary Figure 1**).

Leaves and buds of Nb-16C plants have been sprayed with 200 μl of dsRNA-midGFP at four different concentrations (10, 20, 200, and 240 ng/μl). In addition, 200 μl of dsRNA-5′GFP has also been sprayed on Nb-16C plants leaves and buds at three different concentrations (24, 48, and 240 ng/μl). As a positive control 200 μl of 22nt long synthetic siRNA#164 was sprayed at two different concentrations (1.4 and 14 ng/μl) onto the leaves and buds of Nb-16C of the same stage. 200 μl of water spraying is used as a negative control for GFP silencing.

Green Fluorescence Protein expression in sprayed plants was monitored under UV-light for in total 3 weeks. Since the early silencing in the positive controls takes place in a very restricted area, which is less than 5% of the leaf surface and the tissue damage is variable across different leaves, silencing phenotype was evaluated only qualitatively based on silenced spots but not quantitatively (**Figure 1**). Starting from 3 days and more visibly 5 days after spraying 6/11 of the positive controls with 1.4 ng/μl (0.1 μM) siRNA#164 and 12/12 of the positive controls with 14 ng/μl (1 μM) siRNA#164 showed local silencing spots (**Figure 1**). On the other hand, none of the samples sprayed with dsRNA-midGFP (0/15), dsRNA-5′GFP (0/9) or water (0/9) showed silencing up to 3 weeks after spraying (**Figure 1**). In order to understand whether the processed dsRNAs could not induce silencing or the dsRNAs were not processed to siRNAs in the first place, we performed an sRNA-seq experiment.

Small RNA Sequencing

Leaf material from Nb-16C sprayed with water only and with 200 μl of 20 ng/μl dsRNA-midGFP has been collected 5 days post spraying (dps) for small RNA sequencing (sRNA-seq). As control leaf materials, three wildtype (WT) *N. benthamiana* plants sprayed with 200 μl of 20 ng/μl dsRNA-midGFP has been used (WT-ds). Due to the absence of the GFP transgene, WT plants, in contrast to Nb-16C plants, lack the potential to produce RDR6-transcribed secondary dsRNA, which are cleaved into secondary siRNAs by DCLs. Thus, in sprayed WT plants, secondary siRNA cannot accumulate and all the small RNAs matching the GFP sequence must be degradation product of the sprayed dsRNA-midGFP outside the leaf cells. One Nb-16C plant was sprayed with water to see the degradation products endogenously expressed GFP.

Small RNA sequencing reads mapping to the GFP sequence in the dsRNA-midGFP-sprayed Nb-16C (16C-ds) and the dsRNA-midGFP-sprayed WT (WT-ds) samples exhibited an exponential decay curve resulting in higher read counts of shorter read lengths ($R > 0.99$ for all samples) (**Supplementary Figure 2**). Comparison of the reads of 16C-ds samples with same size reads of WT-ds did not show enrichment of any particular size of sRNAs, suggesting that no secondary siRNA was produced in 16C-ds plants (**Figure 2A**). When the reads of 16C-ds and WT-ds samples mapping to the GFP sequence were normalized to 16C-w samples, specific accumulation of 21-nt, 22-nt, or 24-nt RNAs was not detected, suggesting that neither in WT nor in Nb-16C, the sprayed dsRNA-midGFP was processed by DCLs (**Figure 2B**).

Comparison of normalized read count profiles between WT-ds and 16C-ds in midGFP region reads did not show any major differences, ruling out an amplification of siRNAs even in the presence of GFP target sequence in 16C-ds plants (**Figures 3A,B**). More importantly, a very characteristic indication of siRNA production is transitivity, which appears as phased siRNAs mapping to the adjacent sites of the target sequence on both strands. However, when sRNAs mapping to the 5′ and 3′ neighboring regions of midGFP were investigated in 16C-ds samples, there was clearly no accumulation of phased siRNAs outside of midGFP area, ruling out secondary siRNA-mediated transitivity (**Figure 3C** and **Supplementary Figure 3**). The sRNAs mapping to the GFP sequence outside of midGFP area were simply degradations products of endogenous GFP as they were observed in water sprayed 16C (16C-w) samples (**Figure 3C**).

DISCUSSION

In the face of climate change, current public opinion on the commercialization of transgenic plants, and regulatory restrictions on conventional pesticides, exogenous dsRNA-based applications gain further importance for pest control including viruses. However, the presence of an intact cell wall makes the delivery of dsRNAs into the plant cells challenging. In recent studies it has been claimed that naked dsRNAs can be taken up by intact leaf cells by foliar spraying or by simply spreading it by a brush. The dsRNAs delivered to plants subsequently led to transgene silencing and viral resistance. However, due to the fact that no siRNAs could be detected by sRNA-seq upon dsRNA treatment, the precise nature of the mode of action of exogenous dsRNA remains elusive (Mitter et al., 2017a). Therefore, in this study, we addressed the effectiveness of dsRNA delivery into mature plant leaves and analyzed if the dsRNA is processed into siRNAs by combining sRNA-seq and our well-established high-pressure spraying protocol in *N. benthamiana*.

For qualitative silencing analysis by visualizing GFP expressing plants under UV light, we used up to 26-times higher concentration of 139-nt dsRNA and 11 times higher concentration of 322nt dsRNA when compared to the 22-nt siRNA, which was sprayed as a low-concentration positive control (1.4 ng/μl). Considering that one molecule of 139nt dsRNA can be processed into 6 phased molecules of 22-nt siRNAs and 322nt dsRNA into 14 phased sRNA molecules of 22-nt siRNA, the effective dsRNA molarities were roughly 150 times more than the low concentration positive control siRNA.

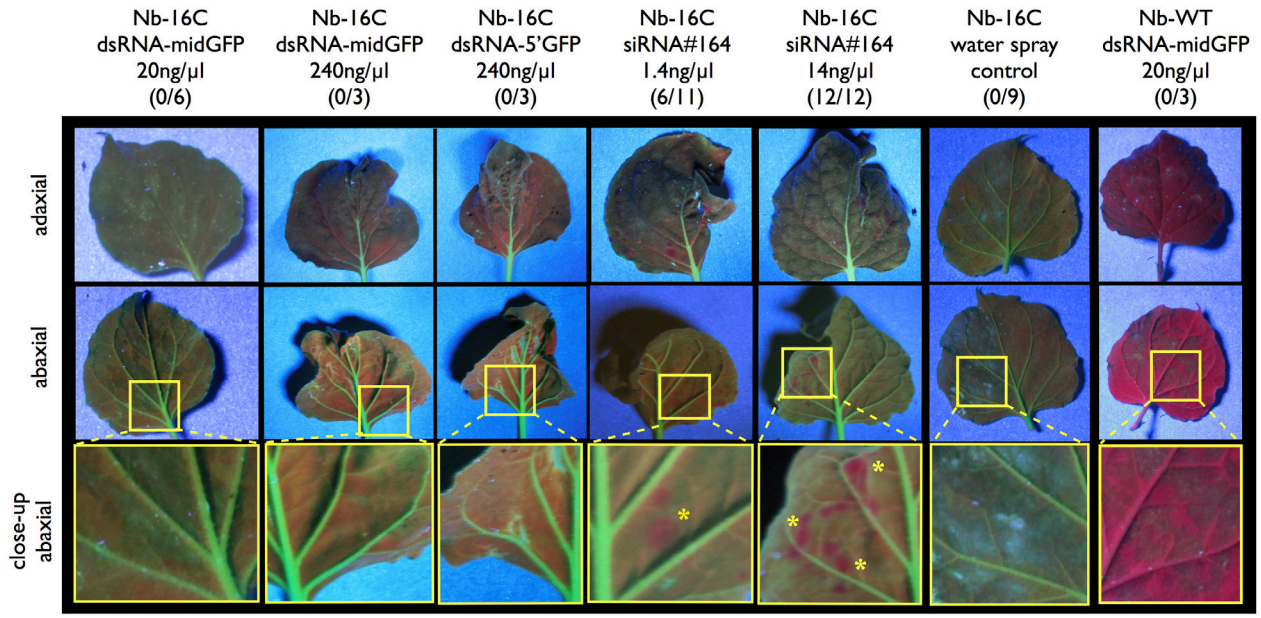

FIGURE 1 | UV-light monitoring of GFP expression after dsRNA and siRNA spraying. Nb-16C plants were sprayed with 20 ng/µl dsRNA-midGFP, 240 ng/µl dsRNA-midGFP, 240 ng/µl dsRNA-GFP5′, 1.4 ng/µl siRNA#164, 14 ng/µl siRNA#164, water only and Nb-WT plants were sprayed with 20 ng/µl dsRNA-midGFP. One to four leaves per plant were sprayed. The three rows show adaxial and abaxial sides of matching leaves, and close up views of abaxial sides, visualized under the UV light, respectively. The area shown in close-up view is shown in yellow rectangle in abaxial view. Nb-WT sample is completely red due to the chloroplasts and Nb-16C samples are green due to the presence of GFP. Only the positive control leaves sprayed with low and high concentration of siRNA#164 showed silencing spots, highlighted with yellow stars in the close-up view. The number of plants showing silencing over the total number of plants treated in given condition is given in parenthesis.

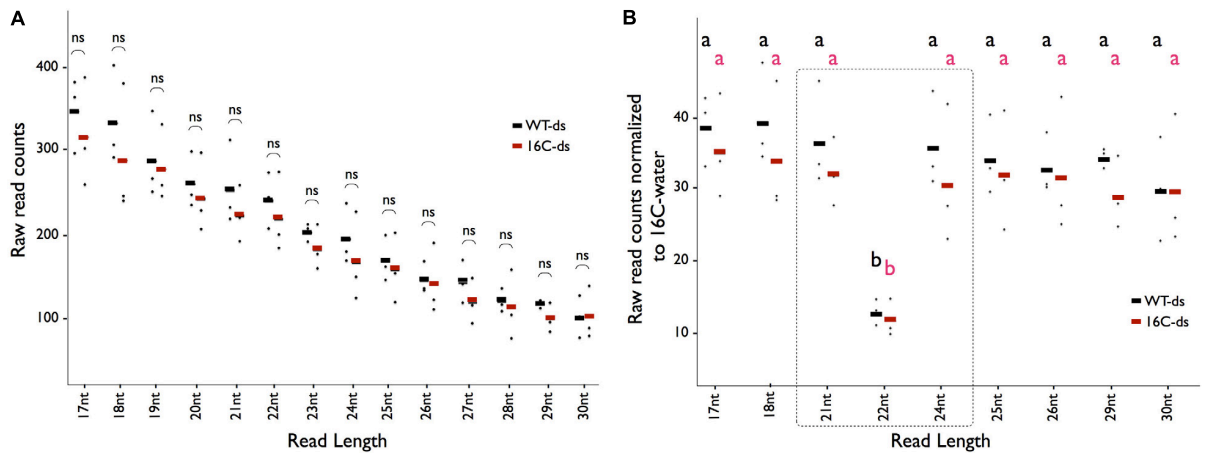

FIGURE 2 | Distribution of sRNAs matching the GFP sequence. **(A)** The average abundance of sRNA-seq reads mapping to the GFP sequence for the given read length (*X*-axis) in WT-ds (black), 16C-ds (red) lines. Each dot represents one biological replicate in the given condition. Student *t*-test shows that there is no significant (ns) enrichment of an sRNA in 16C-ds samples when compared to WT-ds samples. **(B)** The number of reads with specific length mapping to the GFP in 16C-ds (red) and WT-ds (black) samples normalized to 16C-water samples does not show any enrichment of a specific class of sRNA. One-Way ANOVA test shows that there is only depletion of 22nt-long siRNA but no enrichment of sRNAs associated with gene silencing (shown in the dotted square). All data are based on the evaluation of three biological replicates and each dot represents one data point. Significant differences (a and b) were calculated by one-way ANOVA, Bonferroni's post test $P < 0.05$.

Yet, only the spraying of 22-nt siRNAs led to GFP transgene silencing in Nb-16C (**Figure 1**).

For sRNA-seq, 200 µl of 20 ng/µl (0.22 µM) dsRNA-midGFP was used for spraying three 16C and three WT *N. benthamiana*. At the initial step of RNAi, DCLs process dsRNAs into 21-, 22-, or 24-nt long distinct siRNAs. However, sRNA-seq showed the distribution of these particular sRNAs in 16C-ds and WT-ds was almost uniform when compared to the 16C-w with the exception

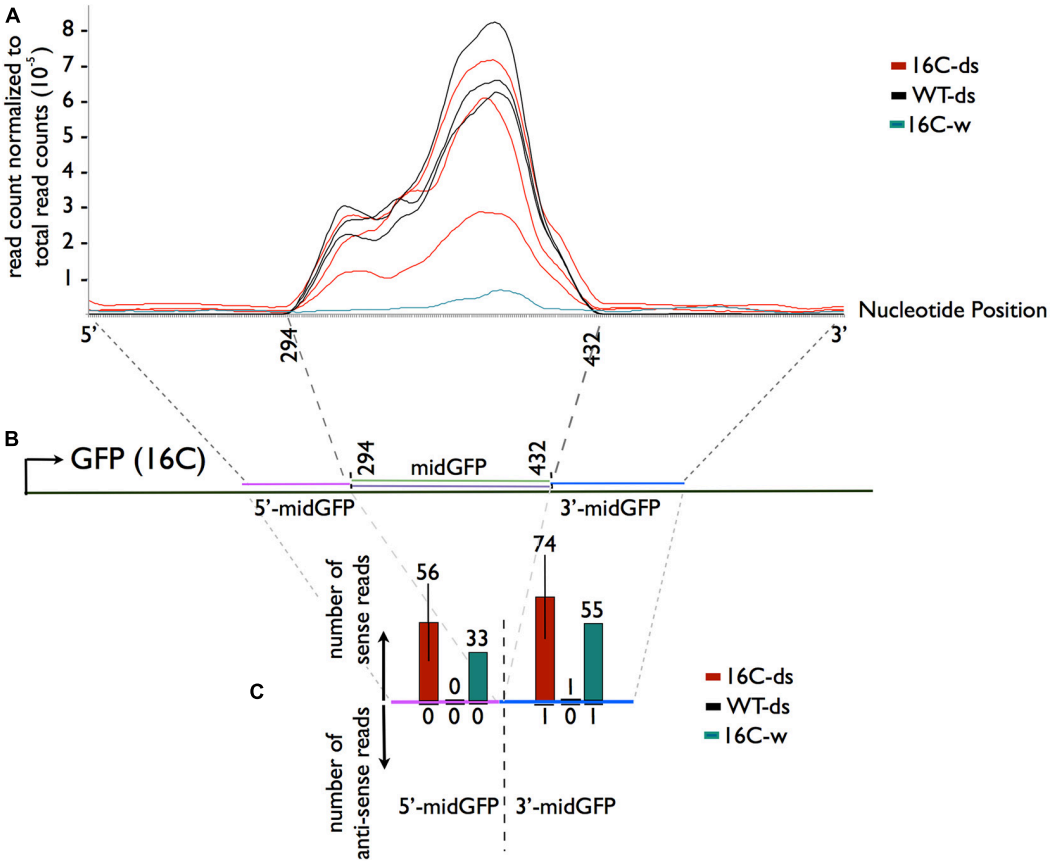

FIGURE 3 | Sequence specific distribution of sRNAs. **(A,B)** SRNA reads mapping to the midGFP region and to the 5′ (magenta) and 3′ (blue) regions flanking the midGFP sequence regardless of the direction were normalized to the total read counts. There is no enrichment of 16C-ds reads over the WT-ds reads in the midGFP region, suggesting that there is no further amplification of the sRNAs in the presence of GFP transcript in 16C-ds. **(C)** The average raw read counts in the neighboring regions were shown to analyze transitivity. There are no anti-sense reads accumulating in 16c-ds samples outside of the sprayed area (downward arrow). The reads matching to the sense strand are degradation products of the GFP transcript, as they can be observed in 16-w sample but not WT-ds samples (upward arrow).

of significantly lower 22-nt long siRNAs (**Figure 2B**). Since the whole leaf was used for sRNA extraction, this suggests that the initial step of RNAi upon dsRNA-midGFP spraying took place neither in the high-pressure (central) nor in the low-pressure sprayed areas (peripheral).

The RNAi machinery has the potential to amplify the plant defense by producing secondary siRNAs through RDR6 activity in the presence of a target complementary sequence, which in this case, is the GFP. Comparing WT-ds and 16C-ds samples allowed us to separate the contribution of the primary siRNAs, which are direct cleavage products of the sprayed dsRNA-midGFP, and secondary siRNAs, which are derive from cleavage products of the RDR6 transcribed secondary dsRNA-midGFP. However, this comparison showed that the presence of GFP target in 16C-ds sample did not increase the production of secondary siRNAs. Moreover, secondary siRNAs display transitivity and thus they map to the regions outside of the trigger dsRNA region. Previously, transitive secondary siRNAs were detected 6 days post agrobacterium mediated infiltration on 16C plants (Dalakouras et al., 2019). Therefore, we focused on early establishment of transitivity at 5 dps in this work. However, we couldn't detect any sRNAs mapping to the complementary strand outside of the dsRNA-midGFP area (**Figure 3C** and **Supplementary Figure 3**). SRNAs mapping to the leading strand of the GFP in 16C-ds samples were detectable. However, these sRNAs derived from degradation of the GFP mRNA, since they were also present in the 16C-w sample.

In addition, the ratio of the longer reads (>24 nt) to shorter reads (<25 nt) was significantly higher outside of the dsRNA-midGFP area when compared to the dsRNA-midGFP area (**Supplementary Figure 4**). This observation suggests that the degradation of the exogenously delivered dsRNA differs significantly from degradation of the endogenous GFP mRNA.

Previous studies showed transgene silencing *via* dsRNA application in *A. thaliana* but we have not observed this phenomenon in *N. benthamiana* (Mitter et al., 2017b; Dubrovina et al., 2019). One possible explanation is that differences in the anatomy of the leaves and structure of the cell wall between *A. thaliana* and *N. benthamiana* led to contradictory results. However, in the same line with our results, the absence of siRNAs after bioclay-associated dsRNAs delivery in *Nicotiana*

tabacum suggests that dsRNA-based transgene silencing, as well as plant protection against pests and viruses may be an indirect effect of the dsRNA.

Despite being a promising approach for plant protection, the mechanisms underlying the effect of exogenous dsRNA application on viral resistance, pest control, and transgene silencing remain controversial and elusive. However, successful applications of new generation adjuvants, e.g., carbon dots are promising approaches for improving dsRNA delivery and efficient pest control in the near future.

MATERIALS AND METHODS

Synthesis and Purification of dsRNA

The 139bp-long GFP-mid fragment was amplified using the GFP139-F (*TAATACGACTCACTATAGGGAGA*gtcgacTATGAAGCGGCACGACTTCT) and the GFP139-R (*TAATACGACTCACTATAGGGAGA*gagctcGATCCTGTTGACGAGGGTGT) primers, both containing a 23 bp long *T7 promoter* and a *Sal*I (5′ end, GFP139-F) and *Sac*I (3′ end, GFP139-R) recognition sequences. The 322bp-long GFP-5′ fragment as amplified using GFP5′-F (*TAATACGACTCACTATAGGGAGA*ATGAAGACTAATCTTTTTCTCTTT) and GFP5′-R (TAATACGACTCACTATAGGGAGACTCAGGCATGGCGCTCTTGA) primers, both containing the 23bp-long *T7 promoter*.

Both PCR products (200 ng) were used as a template to produce dsRNA using the MEGAscript® RNAi Kit[1] according to manufacturer's instructions. DNA template and single stranded RNAs were digested with DNaseI and RNase (provided by the kit) for 1 h. dsRNA was purified using a filter cartridge (provided by the kit) and eluted in 10 mM Tris–HCl buffer containing 1 mM EDTA (pH = 7.0). In six reactions, 358.4 μg of dsRNA-midGFP was produced in total (33.4, 36.7, 36.1, 35.3, 99.1, and 117.8 μg). 227.0 μg of dsRNA-GFP-5′ was synthesized in three reactions combined (80.6, 30.7, and 115.7 μg).

High-Pressure Spraying

For each plant, 200 μl of aqueous dsRNA solutions at given concentrations were sprayed from a 0.5–1 cm distance at the abaxial surface of leaves with an airbrush pistol (CONRAD AFC-250A, 0.25 mm nozzle)[2] and at a pressure of 5–6 bar provided by the METABO Elektra Beckum Classic 250 compressor[3]. 10–12 cm tall *N. benthamiana* wildtype and Nb-16C plants were sprayed with dsRNA and as a control Nb-16C plants were sprayed with water, using the same airbrush type. Each treatment was conducted with a separate airbrush to avoid cross contamination. 11 Nb-16C plants were sprayed with 1.4 ng/μl siRNA#164, 12 plants were sprayed with 14 ng/μl siRNA#164, three plants with 10 ng/μl dsRNA-midGFP, six plants with 20 ng/μl dsRNA-midGFP, three plants with 200 ng/μl dsRNA-midGFP, three plants with 240 ng/μl dsRNA-midGFP, three plants with 24 ng/μl dsRNA-5′GFP, three plants with 48 ng/μl dsRNA-5′GFP, three plants with 240 ng/μl dsRNA-5′GFP, nine plants only with water for monitoring silencing under UV-light. For each plant, 1–4 leaves and one apical meristem bud were sprayed.

RNA Extraction and Small RNA Sequencing

Two leaves per plant from three Nb-16C plants and three Nb-WT plants, sprayed with 20 ng/μl dsRNA-midGFP and two leaves from one Nb-16C sprayed with water were harvested for RNA 5 dps using the mirVana miRNA extraction kit (see text footnote 1) according to manufacturer's instructions. For 16C-ds and WT-ds, three biological replicates were sequenced and evaluated for the experiments and one 16C-w sample was sequenced for normalization purposes (**Figures 2B**, **3C**). 250 ng of RNA per sample was used in library preparation and small RNA libraries were prepared by GenXPro GmbH using the TrueQuant SmallRNA Seq Kit according to the manual of the manufacturers (GenXPro GmbH, Germany). The libraries were sequenced on an Illumina NextSeq500 instrument using 75 cycles of sequencing. sRNA-seq quality control was performed by plotting the read counts of sRNAs longer than 16 bp and shorter than 30 bps. The accumulation of 24-nt long and 21-nt long sRNAs in both Nb-16C and Nb-WT show that the sequencing quality is good and consistent among different samples (**Supplementary Figure 5**).

Bioinformatic Analysis

Small RNA sequencing reads in FASTQ files are used to filter out the 3′ sequencing adapter and quantified FASTA files are obtained. FASTA reads are mapped to the 16C-GFP and midGFP sequences and number of reads per region of interest is measured (Philips et al., 2017). TABLET software was used

[1]www.thermofisher.com

[2]www.conrad.de

[3]www.metabo.com

TABLE 1 | Samples analyzed by sRNA-seq, the total read numbers and specific reads of specific sized mapping to the region of interest of GFP, indicated in **Figure 2**.

Sample name	Total number of reads	Reads matching to the ROI	20nt-long	21nt-long	22nt-long	23nt-long	24nt-long	25nt-long
16C-ds_1	7186316		208	194	186	162	152	156
16C-ds_2	7511064		299	260	276	214	229	204
16C-ds_3	7375715		230	222	202	179	127	122
WT-ds_1	8363721		300	314	275	214	239	201
WT-ds_2	8824820		237	234	246	209	171	164
WT-ds_3	8434532		249	220	209	193	182	148
16C-w	7718149		9	14	37	5	11	10

for qualitative analysis of the data and graphical representation of the mapped sRNA reads (Milne et al., 2013; **Supplementary Figure 3**). Total read counts and mapped read counts are given in **Table 1**.

SRNA-Seq Quantification and Normalization

The comparison of siRNAs mapping to the GFP was done based on raw read counts without any normalization (**Figure 2A**). The enrichment analysis of siRNAs of the given sizes was performed by dividing the raw read count numbers mapping in 16C-ds and WT-ds to 16C-w (**Figure 2B**). The normalized read count at a given position and sample is calculated by the average read count in a sliding window of ten nucleotides divided by the total read count of the given sample (**Figure 3**).

However, there are no established protocols for normalization for quantifying the efficiency of dsRNA processing into siRNAs upon HPSP. Considering that the amount of dsRNA on the leaf surface may alter by the fluctuations in the pressure, the angle, the distance, and the duration of spraying an optimal normalization approach reflecting the efficiency of dsRNA processing into siRNA is lacking. Therefore, additional normalization tests were performed (**Supplementary Figure 6**). For a functional normalization, we took the reads mapping to miR159 as a reference, because miR159 is also processed by RNAi machinery that is also involved in the cleavage of dsRNA (**Supplementary Figure 6A**). In addition, we used all 24-nt long-reads as a global functional normalization reference for each sample (**Supplementary Figure 6B**) as most of 24-nt reads are also products of RNAi machinery.

Statistical Analysis

Pairwise comparisons between same length reads counts of 16C-ds and WT-ds was performed by student *t*-test with significance cut-off of $p < 0.05$ (**Figure 2A**). Statistical comparison among multiple normalized read counts of 16C-ds and WT-ds has been performed with One-Way ANOVA test with Bonferroni post-test $p < 0.05$ (**Figure 2B**). The comparison of degradation products of exogenous and endogenous RNAs has been done with Fisher's Exact Test (**Supplementary Figure 4**).

Ultraviolet (UV) Monitoring

Green Fluorescence Protein fluorescence of Nb-16C plants was monitored using the Black-Ray B-100 UV Lamp[4]. At least three plants per treatment were analyzed. The photos are taken by Canon EOS700D (18–55 mm), aperture priority mode ($A = 10$).

DATA AVAILABILITY STATEMENT

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE160110 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE160110).

[4]www.fishersci.de

AUTHOR CONTRIBUTIONS

VVU, GK, and MW conceived the experiments and wrote the manuscript. VVU and AB conducted the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.534391/full#supplementary-material

Supplementary Figure 1 | DsRNA integrity after high pressure spraying. DsRNA-midGFP shows no indication for degradation and dissociation of the sense and antisense stands after spraying with a six bar pressure [lane (3)]. High-pressure sprayed dsRNA-midGFP was melted at 95°C for 4 min and rapidly cooled-down to show the gel electrophoresis pattern of dissociated sense and antisense strands [lane (4)]. The banding patterns indicated that the high-pressure sprayed dsRNA-midGFP [lane (3)] is composed of dsRNA just like, not sprayed dsRNA-midGFP [lane (2)] rather than ssRNA [lane (4)]. Low-range RNA ladder is loaded to lane (1).

Supplementary Figure 2 | Exogenous dsRNA decay. The exponential decay curve of the average sRNA-seq reads of WT-ds (black line) and 16C-ds (red line) mapping to the GFP sequence. Both graphs are exponential with very high *R* value, in consistent with the lack of sRNAs of specific sizes such as 21, 22, and 24nt. Raw read counts are used as in **Figure 2**.

Supplementary Figure 3 | Strand Specific Distribution of sRNAs. SRNA reads mapping to the midGFP region and to the 5′ (magenta) and 3′ (blue) regions flanking the midGFP sequence are shown. The reads aligning to the sense strand are shown in green and reads mapping to the antisense strand are show in purple. All three sequence samples (biological replicates) from 16C-ds to WT-ds are plotted. Antisense strand reads only match to the midGFP sequence. The graphical representations are screenshots obtained by TABLET software (Milne et al., 2013).

Supplementary Figure 4 | Size distribution of the sense sRNAs mapping to the GFP within and outside of the midGFP area in 16C-ds. sRNAs mapping to sense midGFP sequence are predominantly shorter than 25-nt. These sRNAs possibly originate from the dsRNA-midGFP degradation on the leaf surface. However, when the midGFP area is excluded, the rest of the sense sRNA reads derived from the degradation of the GFP mRNAs are enriched for reads longer than 24-nt (<24 nt). All data are based on the evaluation of three biological replicates normalized to the <24-nt counts. The statistical comparison is performed by Fishers exact Test ($p < 0.05$).

Supplementary Figure 5 | Quality control of the sRNA-seq experiment. The percent of the reads (*Y*-axis) of the given read length (*X*-axis) are plotted. There is a clear enrichment of 24nt-long and 21nt-long sRNAs in both WT-ds and 16C-ds, ensuring the quality of the sRNA-seq for sRNA analysis.

Supplementary Figure 6 | Alternative normalization scheme for sRNA-analysis. **(A)** the reads matching to the midGFP area and the neighboring sites are normalized with respect to the miRNA159 level. miRNAs are also processed by the RNAi machinery, this normalization scheme is used as a functional normalization. **(B)** the reads matching to the midGFP area and the neighboring sites are normalized with respect to the total 24nt-long sRNA abundance. 24nt-long sRNAs are mostly also processed by RNAi machinery, therefore, this normalization scheme is used an alternative global functional normalization.

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