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Host-induced gene silencing – mechanisms and applications

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Summary

Host-induced gene silencing (HIGS) technology has emerged as a powerful alternative to chemical treatments for protecting plants from pathogens or pests. More than 170 HIGS studies have been published so far, and HIGS products have been launched. First, we discuss the strengths and limitations of this technology in a pathosystem-specific context. Next, we highlight the requirement for fundamental knowledge on the molecular mechanisms (i.e. uptake, processing and translocation of transgene-expressed double-stranded RNAs) that determine the efficacy and specificity of HIGS. Additionally, we speculate on the contribution of host and target RNA interference machineries, which may be incompatible depending on the lifestyle of the pathogen or pest. Finally, we predict that closing these gaps in knowledge will lead to the development of novel integrative concepts, precise risk assessment and tailor-made HIGS therapy for plant diseases.

I. Introduction

The drastic loss of biodiversity has increased socio-political demand for pesticide-free agriculture that protects nontarget organisms and the environment. Moreover, many plant pathogens and pests have become less sensitive or even resistant to chemical treatments. These drivers have led to the development of novel, innovative and selective plant-protection measures that rely on RNA interference (RNAi), which mediates small RNA (sRNA)-directed post-transcriptional gene silencing (PTGS). PTGS is triggered by double-stranded RNA (dsRNA) molecules that are processed by Dicer-like proteins (DCLs) into small interfering RNAs (siRNAs) with lengths of 21–24 nucleotides (nt). Subsequent binding of siRNAs to Argonaute proteins (AGOs) leads to the formation of RNA-induced silencing complexes (RISCs) that target complementary RNAs for degradation or translational inhibition (Hammond *et al.*, 2001). Plants have evolved RNAi as an effective primary defence system against pathogens and pests (Zhang *et al.*, 2016), particularly viruses (Kørner *et al.*, 2018). Counteracting this defence, pathogens across kingdoms have coevolved strategies to suppress host RNAi (Hou *et al.*, 2019). The biotechnological exploitation of RNAi is termed host-induced gene silencing (HIGS) (a term coined by Nowara *et al.*, 2010). HIGS can most effectively be induced by dsRNA-producing transgene constructs (typically hairpin RNA) (Waterhouse *et al.*, 1998). Over the last 2 decades, impressive numbers of studies have shown the enormous

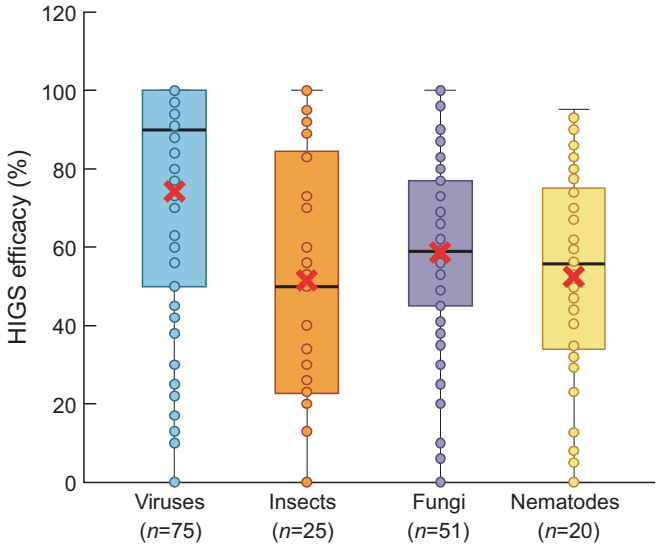

Fig. 1 Application: Summary of all published HIGS application studies (NCBI: December 2020). Host-induced gene silencing (HIGS) efficacies (disease resistance/mortality rate) were extracted from each study with max. 100% and min. 0% (no effects). Total number of studies: viruses, $n = 75$ (mean: 90%); insects, $n = 25$ (mean: 50%); fungi, $n = 51$ (mean: 59%); nematodes, $n = 20$ (mean: 56%). x = mean; circles = data (ranges from 0% (minimum) to 100% (maximum)); horizontal lines (from bottom to top) first quartile, median, third quartile.

potential of PTGS-mediated disease control (Fig. 1). The high number of publications on HIGS-based plant protection within the last year reflects the continued scientific interest in this area and clearly indicates the relevance of RNAi technology applications for plant protection.

II. Applications in plant pathology

HIGS was shown to be effective against a variety of different pathogens and pests: viruses (Fig. 1; $n = 75$), insects (Fig. 1; $n = 25$), fungi (Fig. 1; $n = 51$), and nematodes (Fig. 1; $n = 20$) (for reviews see Rosa *et al.*, 2018; Gaffar & Koch, 2019; Liu *et al.*, 2020). As prokaryotes lack conventional RNAi machinery, HIGS cannot be achieved in bacteria or phytoplasmas. Currently, HIGS-insensitive eukaryotic organisms include only *Saccharomyces cerevisiae* and *Ustilago maydis*, which, through evolutionary conditioning, have lost key RNAi enzymes. However, the presence of RNAi machinery alone does not guarantee success when applying HIGS technology (Fig. 1; '0'-studies: $n = 11$), and the reasons for this can include target selection (expected mortality), target gene silencing (not sufficient), and the RNAi trigger (design aspects). Addressing the question of why some HIGS approaches confer 100% disease resistance or immunity, while others do not, is challenging and will require an in-depth understanding of the molecular basis of HIGS and other RNAi-based technologies. In other words, we do not know whether HIGS can be efficiently applied to all pathosystems even if the above-mentioned technical problems are solved.

All HIGS approaches follow the same procedure, starting with target gene identification. In this regard, the most relevant criterion is that RNAi-mediated knockdown of the selected target gene will ideally lead to 100% lethality, durability (low risk of resistance), and no or minimal side effects on off-target organisms (the ecosystem and humans). Notably, the expansion of sequencing technologies and bioinformatics has opened up new opportunities for target gene selection. In addition, target gene identities promise broad application spectra, but increase the risk for off-target effects (conserved targets). Therefore, designing an RNAi trigger (e.g. dsRNA sequence complementarity and length) that leads to the production of the most effective siRNAs is of central importance. To select the best HIGS inducer, several design platforms as well as off-target prediction tools are available (Lück *et al.*, 2019). However, tool-based design of dsRNA does not necessarily lead to higher silencing efficiencies than manual design (random selection) (Werner *et al.*, 2020). More important in conceiving the best HIGS strategy is the integration of current knowledge on the molecular properties (e.g. pathogen- or pest-specific RNAi mechanism) and strengths and limitations (e.g. routes of dsRNAs and siRNAs) of the chosen pathosystem. For example, HIGS is highly effective in the control of plant viruses (Fig. 1; mean 90%) because they replicate intracellularly and are, therefore, accessible to antiviral plant RNAi (Fig. 2b). In turn, plant viruses evolved silencing suppressors to overcome RNAi-mediated resistance. When biotrophic fungi and oomycetes take up RNA from the host cell, this RNA must cross the plasma membrane of both the host and fungus. How this is accomplished is not yet clear, nor is it clear whether biotrophic fungi take up only siRNAs, or can also take up dsRNAs, as suggested for some necrotrophic fungi such as *Fusarium graminearum* (Fig. 2c). The question is whether their RNAi machinery is compatible to respond to plant siRNA triggers (length, 5′ nt, modifications) in the same way that they respond to their endogenous siRNAs. The same consideration applies to chewing and sap-sucking insects (Fig. 2). To what extent do host and pathogen or pest RNAi machineries overlap regarding functionality and responsiveness or sensitivity, and how does this limit the outcome of HIGS approaches? To answer these questions, we need to understand the molecular mechanisms that underlie HIGS technologies (i.e. the contributions and specificities of host and target RNAi machineries, and the uptake and translocation of RNAi trigger molecules) to define the stability, specificity, and durability of HIGS strategies required for appropriate risk assessment and future field applications.

III. Uptake mechanism: dsRNA vs siRNA

Not every pest or pathogen is capable of efficient uptake of long and/or small dsRNA (Qiao *et al.*, 2021). Among the criteria that determine HIGS efficacy, uptake of the RNAi trigger molecule (long, unprocessed dsRNAs vs short, plant DCL-processed siRNAs) may have the greatest effect on the outcome of each HIGS approach. This idea can be explored by comparing the degree of HIGS-mediated disease control in pests or pathogens with different lifestyles (biotrophic vs necrotrophic fungi) and feeding behaviour (chewing vs sap-sucking insects). HIGS has been shown to be less effective (< 40% efficiency) in controlling diseases caused by sap-sucking insects, particularly aphids, than diseases

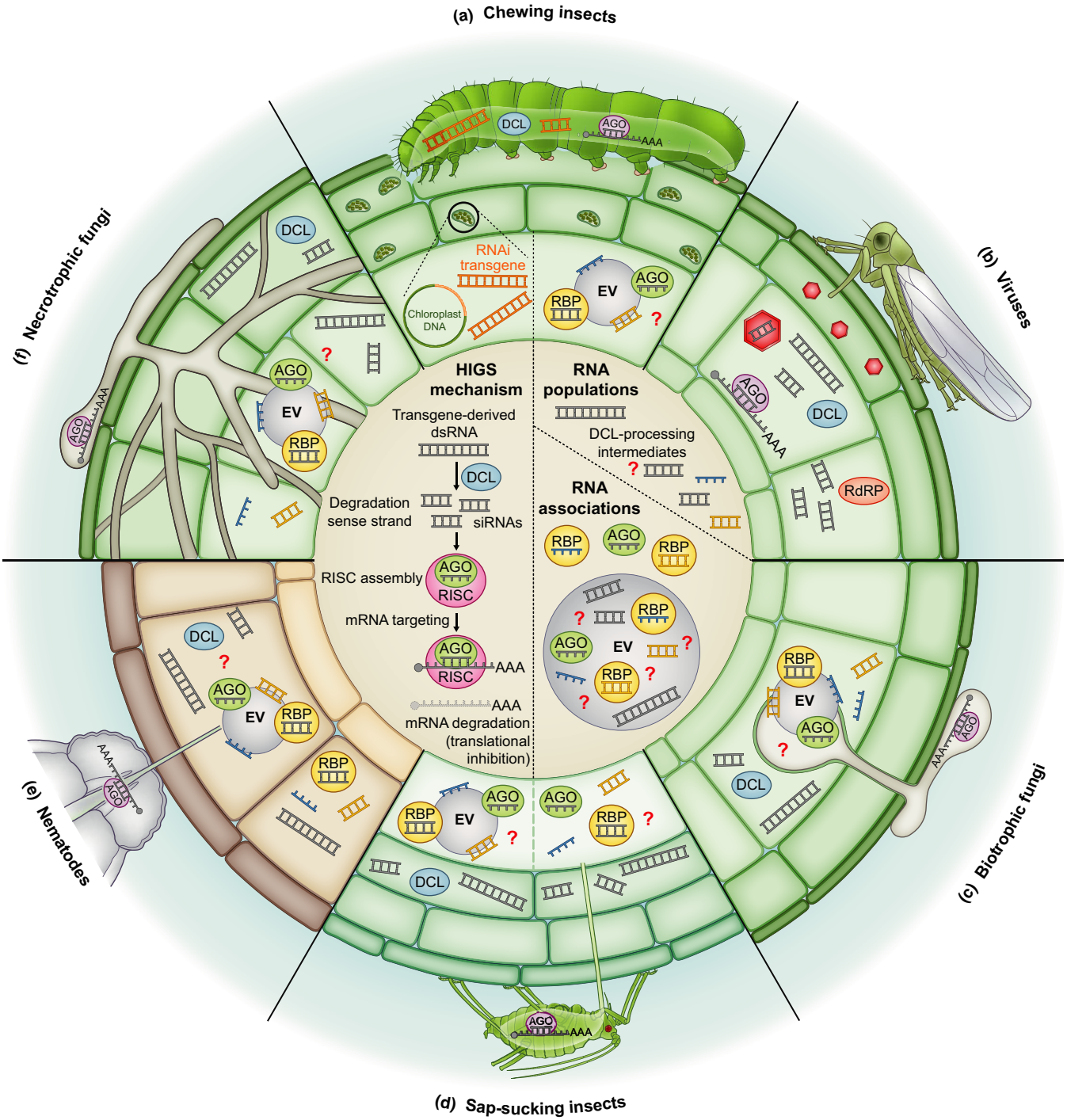

Fig. 2 Mechanism: Overview of diverse host-induced gene silencing (HIGS) mechanisms in different pathogens and pests. (Centre, left) Transgene-derived dsRNA triggers the plant's RNAi machinery to produce siRNAs that direct PTGS of complementary mRNA transcripts in the interacting pathogen or pest. (Centre, right) Diverse classes or populations of intracellular RNAs produced during RNAi and their possible association with RNA-binding proteins (AGOs, DRBs, other RBPs) and/or EVs (red question mark). (Outer circle) (a) Chewing insects have access to chloroplast-expressed dsRNA that were processed by insect DCLs. (b) Viruses (red hexagon), for example transmitted by insect vectors, replicate intracellularly triggering PTGS of viral proteins (e.g. movement proteins, coat proteins etc.). (c) Biotrophic fungi soak up intercellular nutrients through haustoria. How EVs contribute to haustorial siRNA uptake is speculated. (d) Sap-sucking insects take up siRNAs from the phloem of their host plants. Whether they take up naked ds-siRNA and/or ss-siRNA and/or siRNA–protein complexes is unknown. (e) Nematodes take up siRNAs produced by host DCLs that may interact with RBPs as discussed for phloem-feeders (d). (f) Necrotrophic fungi disrupt cellular boundaries and therefore may take up a diverse set of HIGS inducers. Uptake mechanisms as well as the requirement for EV-mediated siRNA transfer between biotrophs and necrotrophs may differ substantially. Contribution of EVs in all HIGS-systems remains questionable as it is unclear whether dsRNA/siRNA concentrations in EVs are sufficient to induce HIGS. AGO, Argonaute protein; DCL, Dicer-like protein; dsRNA, double-stranded RNA; EV, extracellular vesicle, RBP, RNA-binding protein; siRNA, small interfering RNA.

caused by other HIGS-targeted insects. Aphids possess functional RNAi machinery and have been shown to respond to environmental RNAi with diffusion of the silencing signal to the whole insect body (systemic RNAi and cell-autonomous RNAi) (Wang *et al.*, 2015). However, aphids require phloem sap for nutrition; therefore the nature of the HIGS trigger relies mainly on siRNA uptake (Biedenkopf *et al.*, 2020) as HIGS-introduced dsRNA will be processed into siRNAs by the plant RNAi machinery. Therefore, the quantity and quality (target gene accessibility) of phloem-mediated siRNA uptake are important determinants of HIGS efficacy in controlling aphids. Interestingly, higher efficiencies based on dsRNA vs siRNA uptake were also found for other (nonsap-sucking) insects or pests such as *Diabrotica virgifera* (Western corn rootworm (Li *et al.*, 2015) and drosophilid species using liposome-encapsulated dsRNA delivery (Whyard *et al.*, 2009). Of note, other sap-sucking insects such as *Bemisia tabaci* (whitefly) and *Adelphocoris suturalis* (plant bug) were more sensitive (up to 83% mortality) to HIGS (Thakur *et al.*, 2014; Luo *et al.*, 2017). Whether these findings reflect aphids' RNAi specificities in response to HIGS (apart from their feeding behaviour) needs further verification. However, one application strategy to circumvent plant DCL-mediated generation of siRNAs might be the use of phloem-specific promoters that ensure expression and formation of dsRNAs in sieve elements. It should be noted that, in addition to preferentially utilizing dsRNA vs siRNAs, hemipterans inject nucleases into the plant tissue, which may result in extensive degradation of any applied RNA (Lomate & Bonning, 2016).

The question of whether to use dsRNA or siRNA may have important implications for future disease-control strategies based on dsRNA, because uptake of dsRNA and its processing into siRNAs that are highly effective in the target organism may increase target gene silencing. Moreover, in contrast with using only one specific siRNA, processing of long dsRNA into many different inhibitory siRNAs by the targeted pathogen may reduce the chance of RNA resistance developing under field test conditions. Supporting this possibility, a breakthrough was achieved by demonstrating that expression of dsRNA in chloroplasts (DCL-free organelles) targeting the *β-actin* gene of *Leptinotarsa decemlineata* (Colorado potato beetle) caused larval lethality (Zhang *et al.*, 2015). However, such a strategy will not succeed in the control of sap-sucking insects or biotrophs. In addition, the more siRNAs with different sequences that are produced, the higher will be the risk of off-target effects. Finally, resistance against HIGS may be based not only on adaptation of target gene sequences but also on modifications of RNA uptake mechanisms of the target organism (Khajuria *et al.*, 2018).

Like insects, fungal pathogens seem to respond more efficiently to dsRNA than to siRNA. This conclusion was based on the finding that exogenous application of dsRNA triggering PTGS (termed spray-induced gene silencing, SIGS) of *CYP51* genes in the necrotrophic fungus *Fusarium graminearum* (*Fg*) was more efficient compared with HIGS (Koch *et al.*, 2019). Previously, it has been shown that SIGS involves the direct uptake of the long, unprocessed precursor dsRNAs and their subsequent processing by the fungal RNAi machinery (Koch *et al.*, 2016; Gaffar *et al.*, 2019), which may explain the higher silencing efficacy of the fungal target genes that was observed. Supporting this notion, SIGS was found to be *c.* 50% more effective in the control of *Tuta absoluta* (tomato leafminer) and *c.* 30% more effective against *Tetranychus urticae* (two-spotted spider mite) than HIGS (direct comparison in the same study with the same constructs) (Camargo *et al.*, 2016; Suzuki *et al.*, 2017). However, whether SIGS is limited by the length of applied dsRNAs that may interfere with fungal uptake mechanisms needs to be clarified (Höfle *et al.*, 2020). Moreover, the high efficacy of SIGS may rely on the necrotrophic lifestyle of target fungi such as *Fg* and *Botrytis cinerea* (Wang *et al.*, 2016). By feeding on dead plant tissue, necrotrophic fungi may take up topically applied dsRNA or dsRNA that was delivered to the xylem (Dalakouras *et al.*, 2018). Finally, the nature of plant DCL-produced siRNAs can differ from the nature of functional siRNAs of the targeted pathogen. For example, several insects accumulate 20 nt siRNAs and not 21 nt siRNAs, which are predominantly found to be loaded onto AGO1 in plants (Santos *et al.*, 2019). Contrary to this observation, a recent study has demonstrated that HIGS-based and SIGS-based control of the obligate fungal pathogen *Phakopsora pachyrhizi* (Asian soybean rust) reached similar disease restriction (HIGS 58–80% and SIGS 75%) (Hu *et al.*, 2020). Nonetheless, the lifestyle and feeding behaviour of the pathogen or pest influence the routes that dsRNA or siRNA will take to induce RNAi of their molecular targets. However,

IV. Transfer of HIGS-inducing RNAs: extracellular vesicles

While nematodes and some insects possess a transmembrane channel-mediated mechanism for RNA uptake and RNA cell-to-cell movement that is related to the *Caenorhabditis elegans* systemic RNAi deficient (SID) proteins system (Huvenne & Smagghe, 2010), fungi lack SID proteins. Recent data have suggested that, analogous to the role of mammalian exosomes in cell-to-cell communication, fungi instead develop a bidirectional sRNA transport system via extracellular vesicles (EVs) (Rutter & Innes, 2018). However, the mechanisms underlying the transfer and uptake of transgene-derived or HIGS-derived RNAs during plant–fungal interactions are still elusive. It has been shown that EVs purified from *Arabidopsis thaliana* leaf extracts and apoplastic fluids contain transgene-derived sRNAs. EVs from plants expressing CYP3RNA, a 791-nt long dsRNA, which was originally designed to target the three *CYP51* genes of the fungal pathogen *Fg*, contained CYP3RNA-derived siRNAs as shown by RNA sequencing (RNA-seq) analysis (Koch *et al.*, 2020). Notably, the EVs' cargo retained the same CYP3RNA-derived siRNA profile as the respective leaf extracts, suggesting that there was no selective uptake of specific artificial sRNAs into EVs. In addition, mutants of the ESCRT-III complex were impaired in HIGS, and EVs were free of CYP3RNA-derived siRNAs, further indicating that endosomal vesicle trafficking supports the transfer of transgene-derived siRNAs between donor host cells and recipient fungal cells. Although the number of EV-contained siRNAs was low, we have no information on the minimum concentration of siRNAs inside an EV that is required to induce HIGS. At the same time, EVs emerged as novel players in plant–pathogen interaction, serving as

shuttles for proteins and RNAs that interfere with defence responses (Rutter & Innes, 2020). However, multiple questions have arisen that need to be addressed to unravel the molecular mechanisms of EV uptake, loading and release, as well as the role of RNA-binding proteins (AGOs, DRBs, and others) in stabilizing dsRNAs and siRNAs during HIGS (Fig. 2). In addition to EVs' role in HIGS, alternative routes for siRNA delivery also may exist, including: (1) uptake during fungal nutrient acquisition via specific transporters, or (2) passive crossing via trans-cell wall diffusion or through various transmembrane channels or pores.

V. Contributions of RNAi machineries

In addition to identification of the most effective RNAi inducers (dsRNA or siRNA), we lack insight regarding the contributions of host and target RNAi machineries in HIGS. siRNA profiles generated by sRNA-seq are needed to determine the nature of the most effective siRNAs for each target organism. For example, sRNA profiles from HIGS plants targeting *FgCYP51* genes (CYP3RNA; Koch *et al.*, 2013) revealed a hotspot of siRNA production that matched the middle of the CYP3RNA triple construct (Koch *et al.*, 2020). Notably, this hotspot was also found when CYP3RNA was applied by spray (SIGS) as well as *in vitro* treatment of *Fg* axenic cultures (Koch *et al.*, 2016), indicating that processing of CYP3RNA in *A. thaliana* (HIGS), *Hordeum vulgare* (SIGS), and *Fg* (*in vitro*) led to similar siRNA profiles. Subsequent generation of transgenic Arabidopsis plants that expressed this 100-nt hotspot sequence of *FgCYP51A* exhibited a similar resistance to *Fg* infection compared with plants that expressed the 791-nt CYP3RNA (Koch *et al.*, 2020). In line with this finding, another study has revealed several siRNA hotspots distributed along the hairpin stems (Yang *et al.*, 2013). So far, we can only speculate whether hotspot siRNAs exhibit higher activities or indicate RISC stability (i.e. active vs inactive RISC siRNAs). In addition, sequencing accuracy may influence the interpretation of results, as we cannot be certain that all siRNAs are captured by sRNA-seq techniques. However, these considerations clearly illustrate the lack of mechanistic understanding, and a greater understanding is required to further develop HIGS technology. Using 100-nt dsRNA instead of 800-nt dsRNA will reduce possible off-target hits, which is crucial for field applications. The same consideration applies to single-target vs multiple-target constructs and species specificity vs broad-spectrum disease resistance. Even previous publications are contradictory, as some studies found differences between the efficacies of single-target vs double-target or triple-target constructs (Koch *et al.*, 2019) and others did not (Chen *et al.*, 2016).

In addition to understanding the processing of transgene-derived dsRNA by plant DCLs and the significance of siRNA profiles, to improve HIGS technology, we need to identify siRNA-stabilising factors. For example, we lack insight into the role of RNA-binding proteins (RBPs), AGOs, dsRNA-binding proteins and others in HIGS. Based on sRNA-seq data that revealed 5′-identities and lengths of HIGS-derived siRNAs, we can speculate on contributing RBPs, as far as they are known for the specific pathosystem. Given the varied mixture of transgene-derived RNA populations (dsRNA, ds-siRNA, single-stranded (ss) siRNA, ds/ss-DCL intermediates) that may interact with certain RBPs for loading onto AGOs and/or in EVs (Fig. 2), we need further research to prove their relevance for HIGS-mediated pathogen and pest control.

VI. Conclusions

Although many questions regarding the underlying molecular mechanisms of HIGS remain open, commercialisation in the United States started in the mid-1990s with products to control viral plant diseases (papaya, potato, cucurbits) and, recently, RNAi-based control of Western corn rootworm (Head *et al.*, 2017). Given the launch of RNAi products, a risk assessment by the European Food Safety Authority (EFSA) concluded that oral uptake of RNAi products by consumers bears a low risk for interference with gene expression in humans, as too many biological and physical barriers have to be overcome (Schiemann *et al.*, 2019; Kleter, 2020) and exogenously applied dsRNA showed a low potential for persistence in the environment (Bachman *et al.*, 2020). Moreover, technical advances, such as cell-free dsRNA production, have made RNAi technologies competitive regarding the costs (< \$0.50 g^{-1} dsRNA) (http://www.globalengage.co.uk/pgc/docs/PosterMaxwell.pdf). Another important aspect, most off-target predictions are generated *in silico*; therefore, we need additional 'omics approaches for adequate and precise risk assessment. We should be asking 'How we can use HIGS and SIGS technologies to improve and complement plant protection (e.g. combinatory effects to increase sensitivity to low doses of pesticide application)?' instead of discussing RNAi technologies as the sole saviour for the challenges faced related to changing global environments (climate, politics, etc.). We need to find smart and tailor-made solutions to which RNAi technologies may contribute, rather than considering RNAi as the full solution. Consistent with this idea, we need combinatory and integrative strategies and concepts to address socio-political demand for pesticide-free plant production systems to achieve sustainability and to prevent further loss of biodiversity.

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