Host-induced Gene Silencing for Pest/Pathogen Control

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Advanced article

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Host-induced gene silencing (HIGS) is an approach that shows promise for the control of a variety of problematic crop-damaging organisms, ranging from nematodes and insects, to fungi and parasitic plants. In general, HIGS utilises ribonucleic acid interference (RNAi) molecules produced by the plant, which then target key genes in pests/pathogens, ideally leading to improved resistance of the plant and a reduction in damage. As this area of research is still very much in development, the possible off-target and nontarget effects need to be assessed, as do the long-term stability and effectiveness. Practical implementation of HIGS to commercial crop production will rely on extensive field-testing, as well as regulatory and marketplace acceptance of new varieties.

Introduction

Crop loss owing to damage by pests and pathogens is a significant issue. For example, damage to *Triticum aestivum* (wheat) and *Hordeum vulgare* (barley) in the USA alone owing to *Fusarium* species was estimated to be 2.7 billion dollars between 1998 and 2000 (Nganje *et al*., 2004). There are a variety of possible strategies for pest and pathogen control, including seeking naturally resistant cultivars, breeding for improved resistance of susceptible cultivars, the application of commercial pesticides, crop rotation and genetic engineering of resistance. These approaches can be integrated as part of pest management strategies. For further reading see **Integrated Pest Management**.

Genetic engineered resistance can take the form of expressed proteins, such as those from the bacterial species *Bacillus thuringiensis* (commonly known as Bt proteins) to inhibit the growth of the juvenile forms of coleopteran and lepidopteran insects. Farmers commonly grow Bt varieties of many large commodity crops, such as *Zea mays* (corn) and *Gossypium hirsutum* (cotton). As with other approaches to insect control, emerging resistance to Bt crops is becoming problematic (Tabashnik *et al*., 2013), leading to interest in more effective utilisation of this technology, such as the combination of Bt fields with non-Bt refuge plantings and the development of alternate approaches to pest control.

One of these alternatives, a distinctive genetic engineering approach that has demonstrated effectiveness for controlling pests and pathogens, is host-induced gene silencing (HIGS). This approach is unique as the host plant's resistance is improved by RNA (ribonucleic acid)-mediated silencing of genes in the pest/pathogen. Examples of some of the successes of this system in protecting various plant species against pests and pathogens can be found in **Table 1**. In this system, the host plant expresses RNAs that are designed to interfere with the expression of specific pest/pathogen genes. Regulation of genes by RNAs is part of a highly conserved biological process – for further reading see **RNA Interference (RNAi) and MicroRNAs**. HIGS utilises the ribonucleic acid interference (RNAi) pathway, a process by which translation of messenger ribonucleic acids (mRNAs) is reduced by RNA-mediated neutralisation of the mRNAs, or where transcript production is reduced following epigenetic modifications, via RNA-induced transcriptional silencing (RITS). An overview of the molecular interactions between plants and fungi or insects during HIGS is shown in **Figure 1**.

RNAi can lead to inhibition of gene expression by these two distinct mechanisms (reviewed by Wilson and Doudna, 2013). Following transcription, double-stranded ribonucleic acid (dsRNA) or microRNA precursors are cleaved by the ribonuclease DICER or DICER-like homologs into short interfering ribonucleic acid (siRNA) or miRNA fragments no more than 26 nucleotides (nt) in length. These short RNAs may be amplified by an RNA-dependent RNA polymerase, leading to a stronger silencing effect. By post-transcriptional gene silencing, siRNAs or miRNAs complex with enzymes including the nuclease ARGONAUTE (AGO) to form the RNA-induced silencing complex (RISC), which catalyses degradation of transcripts complementary to the bound RNA. In pretranscriptional gene silencing, RNA complexes with AGO and *de novo* DNA (deoxyribonucleic acid) methyltransferases, leading to DNA methylation and epigenetic

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Table 1 Summary of example HIGS studies cited

Transgenic host	Target species	Target gene(s)	Role(s) of target(s) gene(s)	Reference
HIGS targeting nematodes				
Arabidopsis thaliana (*Arabidopsis*)	*Meloidogyne incognita, Meloidogyne javanica, Meloidogyne arenaria, Meloidogyne hapla* (root-knot nematode)	*16D10*	Effector	Huang *et al.* (2006)
Arabidopsis thaliana (*Arabidopsis*)	*Meloidogyne chitwoodi* (root-knot nematode)	*16D10*	Effector	Dinh *et al.* (2014)
Solanum tuberosum (potato)	*Meloidogyne chitwoodi* (root-knot nematode)	*16D10*	Effector	Dinh *et al.* (2014)
Vitis vinifera cv. Chardonnay (chardonnay grape)	*Meloidogyne incognita* (root-knot nematode)	*16D10*	Effector	Yang *et al.* (2013)
Nicotiana tabacum (tobacco)	*Meloidogyne incognita* (root knot nematode)	*Isocitrate lyase*	Metabolism	Lourenco-Tessutti *et al.* (2015)
Nicotiana tabacum (tobacco)	*Meloidogyne incognita* (root-knot nematode)	*Heat shock protein 90*	Chaperone protein	Lourenco-Tessutti *et al.* (2015)
Glycine max (soybean)	*Meloidogyne incognita* (root-knot nematode)	*Tyrosine phosphatase*	Metabolism	Ibrahim *et al.* (2011)
Glycine max (soybean)	*Heterodera glycines* (soybean cyst nematode)	*Major sperm protein*	Sperm motility	Steeves *et al.* (2006)
Glycine max (soybean)	*Heterodera glycines* (soybean cyst nematode)	*L-Lactate dehydrogenase*	Metabolism	Youssef *et al.* (2013)
Glycine max (soybean)	*Heterodera glycines* (soybean cyst nematode)	*J15*	Actin-related protein	Tian *et al.* (2016)
Glycine max (soybean)	*Heterodera glycines* (soybean cyst nematode)	*J20*	Signal transduction	Tian *et al.* (2016)
Glycine max (soybean)	*Heterodera glycines* (soybean cyst nematode)	*J23*	Actin-binding protein	Tian *et al.* (2016)
Solanum melongena (eggplant)	*Meloidogyne incognita* (southern root-knot nematode)	*msp-18*	Pharyngeal gland-specific effector	Shivakumara *et al.* (2017)
Solanum melongena (eggplant)	*Meloidogyne incognita* (southern root-knot nematode)	*msp-20*	Pharyngeal gland-specific effector	Shivakumara *et al.* (2017)
HIGS targeting insects				
Hordeum vulgare (barley)	*Sitobion avenae* (grain aphid)	*Sheath protein*	Salivary channel integrity	Abdellatef *et al.* (2015)
Arabidopsis thaliana (Arabidopsis)	*Helicoverpa armigera* (cotton bollworm)	*CYP6AE14*	Gossypol metabolism	Mao *et al.* (2007)
Zea mays (maize)	*Diabrotica virgifera virgifera* (western corn rootworm)	*H + ATPase*	Proton pump	Baum *et al.* (2007)
Zea mays (maize)	*Diabrotica virgifera virgifera* (western corn rootworm)	*Snf7*	Signal transduction for regulation of lysozyme formation	Bachman *et al.* (2013)

Table 1 (*continued*)

Transgenic host	Target species	Target gene(s)	Role(s) of target(s) gene(s)	Reference
HIGS targeting fungi and oomycetes				
Arabidopsis thaliana (*Arabidopsis*)	*Blumeria graminis* (barley powdery mildew)	*Avirulence a10*	Effector	Nowara *et al.* (2010)
Solanum tuberosum (potato)	*Phytophthora infestans* (potato blight)	*Avirulence protein 3a*	Effector, virulence factor	Bos *et al.* (2010)
Triticum aestivum (wheat)	*Puccinia triticina* (wheat leaf rust)	*MAP kinase* (*PtMAPK1*) *cyclophilin* (*PtCYC1*) *calcineurin B* (*PtCNB*)	Predicted pathogenicity or virulence	Panwar *et al.* (2013)
Solanum tuberosum (potato)	*Phytophthora infestans* (potato blight)	*G protein* β*-subunit* (*GPB1*)	Signal transduction associated with sporangium development	Jahan *et al.* (2015)
Solanum tuberosum (potato)	*Phytophthora infestans* (potato blight)	*Cellulose synthase A2*	Synthesis of cellulose for oomycete cell walls	Jahan *et al.* (2015)
Solanum tuberosum (potato)	*Phytophthora infestans* (potato blight)	*Pectinesterase*	Degradation of pectins in plant cell walls	Jahan *et al.* (2015)
Solanum tuberosum (potato)	*Phytophthora infestans* (potato blight)	*Glyceraldehyde 3-phosphate dehydrogenase*	Glycolysis	Jahan *et al.* (2015)
Arabidopsis thaliana (*Arabidopsis*), *Hordeum vulgare* (barley)	*Fusarium graminearum* (fusarium ear blight)	*Cytochrome P450 51A, 51B and 51C*	Ergosterol biosynthesis	Koch *et al.* (2013)
Hordeum vulgare (barley)	*Blumeria graminis* (barley powdery mildew)	Eight candidate effectors, including two ribonucleases	Unknown	Pliego *et al.* (2013)
Musa musa x paradisiaca (banana)	*Fusarium oxysporum* f. sp. *cubense* (fusarium wilt)	*Velvet*	Regulation of development, mycotoxin production	Ghag *et al.* (2014)
Musa musa x paradisiaca (banana)	*Fusarium oxysporum* f. sp. *cubense* (fusarium wilt)	*Fusarium transcription factor 1*	Regulation of gene expression	Ghag *et al.* (2014)
Arabidopsis thaliana (*Arabidopsis*) *Solanum lycopersicum* (tomato)	*Verticillium dahliae* (verticillium wilt)	*Ave1, SIX gene expression 1* (*Sge1*) *and necrosis- and ethylene-inducing-like protein* (*NLP1*)	Transcription factor, effectors	Song and Thomma (2016)
Lactuca sativa (lettuce)	*Bremia lactucae* (downy mildew)	*Highly abundant message #34* (*HAM34*)	Effector	Govindarajulu *et al.* (2015)
Lactuca sativa (lettuce)	*Bremia lactucae* (downy mildew)	*Cellulose synthase 1*	Synthesis of cellulose for oomycete cell walls	Govindarajulu *et al.* (2015)
Medicago truncatula (barrelclover)	*Glomus species*	*Monosaccharide transporter 2*	Monosaccharide transport, endosymbiosis	Helber *et al.* (2011)
Arabidopsis thaliana (*Arabidopsis*)	*Botrytis cinerea* (grey mould)	*DICER-LIKE 1 and DICER-LIKE 2*	RNAi, filamentous-organism-induced gene silencing (FIGS)	Wang *et al.* (2016)

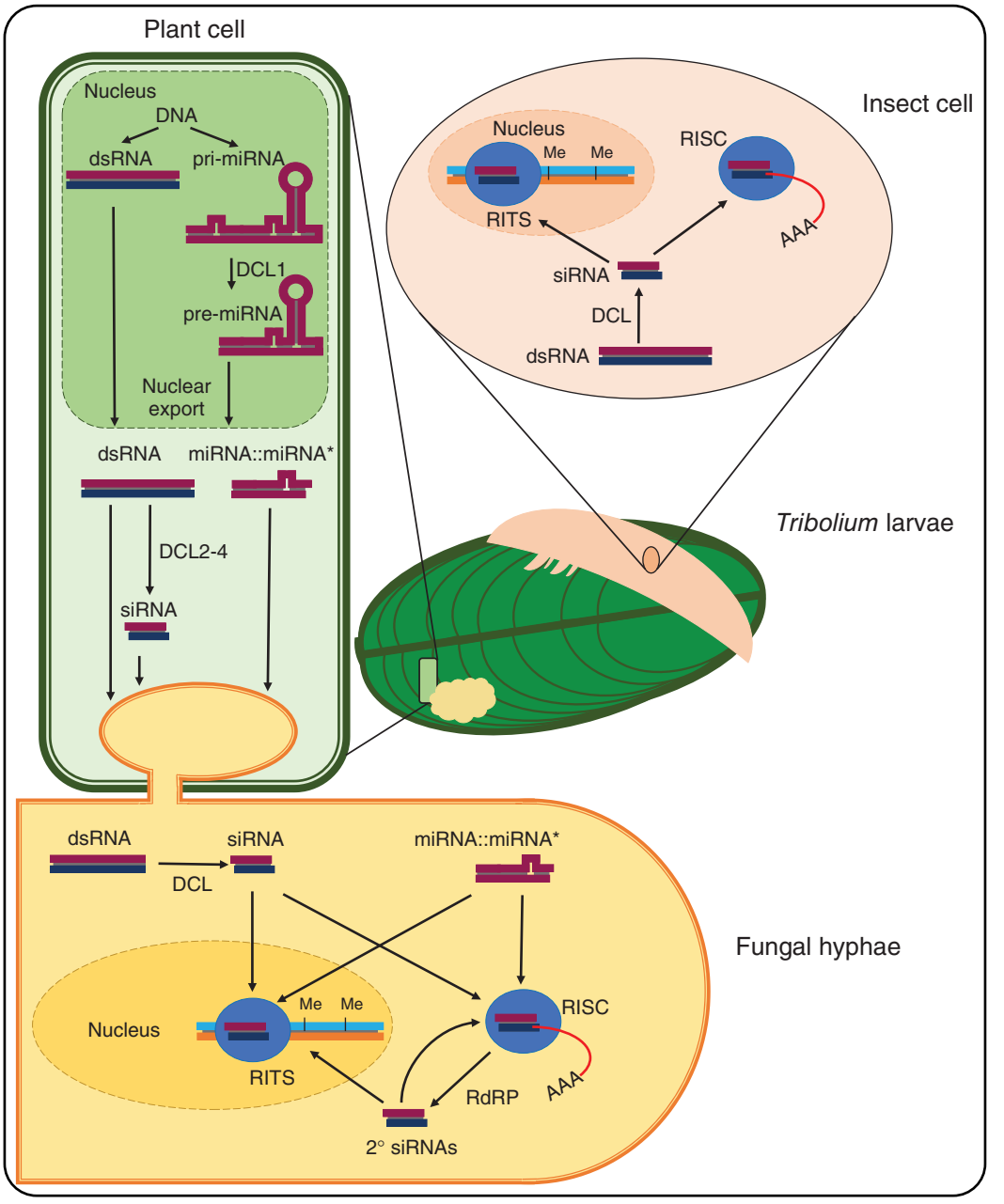

Figure 1 **Overview of HIGS molecular interactions between plant cells and insect pests or fungal pathogens**. Natural dsRNAs/hpRNAs or pre-miRNAs, or synthetic variants (such as amiRNAs), are first transcribed from genomic DNA (deoxyribonucleic acid) in the nucleus of the plant cell. Cropping of miRNA/amiRNA occurs within the nucleus before, or during export, and is catalysed by DICER-LIKE1 (DCL1). The end product of cropping is an miRNA bound to a passenger strand, termed miRNA*. In contrast, cropping of dsRNA to produce siRNAs occurs after nuclear export and can be catalysed by any of the three functionally redundant DICER LIKE genes DCL2-4. Some fungi and oomycytes, such as *P. infestans*, may uptake interfering RNA (ribonucleic acid) in the forms of dsRNA, siRNA and miRNA::miRNA*. These RNAs associate with the RNA-induced silencing complex (RISC) in the cytoplasm, or with the RNA-induced transcriptional silencing (RITS) complex in the nucleus. RNA-dependent RNA polymerase (RdRP) catalyses synthesis of secondary (2°) siRNAs from siRNA complexed with RISC. In contrast, insects including *Tribolium* lack RdRP as well a lack of susceptibility to RNAi from exogenous siRNAs. During HIGS (host-induced gene silencing), the outcome of the molecular interactions shows results in the reduced expression of targeted genes in the pest/pathogen, such as though methylation (Me) of genomic DNA. Representative organisms are not drawn to scale.

silencing of target genes. Both of these mechanisms contribute to knockdown, a decrease in gene expression resulting from interference directed by RNA.

In nature, these mechanisms provide a means for organisms to regulate gene expression. In the context of plant protection, this genetic silencing can be directed towards genes of pathogens or pests, thus impairing the growth of the targeted disease-causing organism and reducing plant damage. HIGS has been tested in numerous plants species and is known to be effective for reducing insect, fungi, nematode, viral and parasitic plant associated damage (Koch *et al*., 2016). This article will focus on HIGS-targeting nematodes, insects and fungi. The relative effectiveness for targeting each organism and reducing crop damage is determined on a case-by-case basis, as there is variability both within and between organisms. While the exact mechanisms of the interaction between plants and pests/pathogens leading to gene suppression are still under investigation, the RNA-based nature of the interference may be a key reason that this approach is effective against organisms from different kingdoms. At the same time, this approach has the potential to be highly specific to the organism of interest, depending on the target gene or genes.

Therefore, a key feature of HIGS is the careful selection of target genes. Ideally, gene targets are specific, essential for pathogen/pest function and lack homologs in nontarget species, thus limiting potential off-target and nontarget effects. In many cases, there is extensive research on the target pest/pathogen and its interaction with the plant species of interest, revealing to researchers a number of putative effectors that may be targets for effective RNAi. Some targets are selected as they are previously identified virulence factors (Song and Thomma, 2016), or known key parasitism genes (Huang *et al*., 2006). A summary of gene targets and their putative roles can be found in **Table 1**.

One advantage to the HIGS system is that potential gene targets can be tested in model organisms, such as *Arabidopsis thaliana* (*Arabidopsis*), a readily transformable substitute for the crop of interest. An early success of the HIGS system with *Arabidopsis* demonstrated that the expression of dsRNA targeting the *Helicoverpa zea* (cotton bollworm) gene *CYP6AE14*, involved in metabolism of gossypol, resulted in reduced damage by the insect. Larvae fed on dsRNA leaves were found to be deficient in gossypol metabolism after switching from the leaves to an artificial diet that included gossypol (Mao *et al*., 2007). Similarly, suppression of a vacuolar H^+-ATPase from *Diabrotica virgifera* (western corn rootworm, WCR) led to reduced damage in specific genetic lines of transformed corn (Baum *et al*., 2007). These groundbreaking demonstrations of HIGS as a means of dsRNA delivery expanded prospects for RNAi-mediated pest/pathogen control.

Potential for HIGS against fungus-like organisms was shown by transgenic *Arabidopsis* expressing dsRNAs targeting the *Blumeria graminis* (powdery mildew, PM) effector *Avra10*. A study of 76 candidate PM genes screened by HIGS in barley resulted in reduced haustorial growth from independent knockdowns of 16 of these genes. To confirm that HIGS of *Avra10* was the cause of reduced haustorial growth, a variant of Avra10 with polymorphisms at the target site was expressed in RNAi:Avra10 barley. This resulted in the rescue of haustorial growth (Nowara *et al*., 2010).

The model species *Caenorhabditis elegans* (*C. elegans*) was the subject of a breakthrough study on the mechanisms of RNAi (Fire *et al*., 1998), leading to the award of the Nobel Prize in Physiology or Medicine in 2006. The genetic resources of *C. elegans* have been leveraged as a basis for identifying potential genes to target in crop-damaging nematodes. For example, alignment of vital *C. elegans* genes against the genome of *Heterodera glycines* (soybean cyst nematode, SCN) was used to identify genes expected to have lethal knockdown phenotypes (Alkharouf *et al*., 2006).

HIGS of Nematodes

Nematodes have the distinction of being both a considerably problematic plant pests and a highly useful model organism for research. Plant parasitic nematodes, including cyst nematodes and root-knot nematodes (RKNs), reportedly cause approximately US$80 billion in crop damage annually (Handoo, 1998).

Early research into the use of HIGS for RKN control targeted the secreted effector 16D10, which interacts with host transcription factors to promote root growth. In *Arabidopsis* hosting RKNs, significant reductions in root-knot gall formation were first observed with RKN-fed dsRNA targeting 16D10 *in vitro* before transfer to roots, followed by *in plantae* demonstrations of the effectiveness by HIGS (Huang *et al*., 2006). This was followed by reports of HIGS of 16D10 by transgenic *Vitis vinifera* (grape) and *Solanum tuberosum* (potato). Reduced proliferation of RKN in these transgenic crops is indicated by reduced egg counts, but resistance is not sufficient to prevent some level of crop damage (Yang *et al*., 2013; Dinh *et al*., 2014).

A number of experiments with transgenic soybean have explored HIGS of genes involved in metabolism, reproduction, signalling and other processes in nematodes. However, several methods of measuring nematode infection exist, complicating the task of comparing the efficacy of HIGS against various targets that are tested in separate experiments. Comparison of multiple methods of measuring infection shows that silencing of metabolic genes, including *Isocitrate lyase*, may result in decreased egg count, but no significant reduction in root knots (Lourenco-Tessutti *et al*., 2015). A 68% reduction in SCN egg count was reported in soybean lines, generating RNAi against *Major Sperm Protein* (Steeves *et al*., 2006). Targeting of a RKN tyrosine phosphatase, an essential signalling protein, reduced the egg count by 92% while *L-Lactate dehydrogenase* suppression reduced the egg count by only 57% (Ibrahim *et al*., 2011). Silencing of a metabolic gene encoding aldolase in SCN reduced the number of mature females on soybean roots by 58% (Youssef *et al*., 2013). These results demonstrate the ability of HIGS to affect SCN and RKN life stages in a crop species of interest. Whether these targets are effective in reducing damage in a commercial setting would be very valuable to determine. One future challenge will be conferring sufficient nematode resistance to plants. A recent study demonstrated similarly limited resistance in *Solanum melongena* (eggplant) expressing dsRNAs targeting two *Meloidogyne incognita* (southern RKN) pharyngeal genes (Shivakumara *et al*., 2017). Insights into the limited successes of HIGS against nematodes may be obtained from further studies

in *C. elegans*. Silencing of genes expressed in some *C. elegans* organs, such as the pharynx, is reportedly inefficient owing to some cell types lacking SID-1, a transmembrane protein needed for dsRNA uptake. Although not efficient in the parental generation, RNAi is reportedly potent in the pharynx of offspring owing to nuclear RNAi (Shiu and Hunter, 2017). Future studies of HIGS in nematodes may aim to investigate intergenerational increases in potency owing to DNA methylation and other forms of nuclear RNAi.

HIGS of Insect Pests

Insects are a major source of crop damage. As there are increasing reports of insecticide resistance and Bt resistance, additional means of controlling insect pests are needed (Tabashnik *et al*., 2013; Bass *et al*., 2014). Control of aphids (numerous insect species of the superfamily Aphidoidea), a pest of particular interest, is a challenge limited by factors including the persistent survival of a minority of aphids and the rapid development of resistance to pesticides. A recent review describes seven characterised mechanisms of resistance to neonicotinoids, pyrethroids, carbamates and other pesticides in *Myzus persicae* (peach aphid) (Bass *et al*., 2014).

Advances in high-throughput sequencing have enabled researchers to identify promising gene targets for implementing HIGS against insect species including aphids. For example, comparative transcriptomics of *Sitobion avenae* (grain aphid), before and after feeding on wheat, revealed thousands of differentially expressed genes. This strategy led to the discovery of five novel genes with knockdown phenotypes of increased mortality, as determined by an *in vitro* dsRNA feeding assay (Zhang *et al*., 2013). Genomic and transcriptomic data have also been used to identify possible off-target sites in nontarget insects, such as *Coleomegilla maculata* (lady beetle) (Allen, 2017).

In wheat, HIGS of nuclear receptor proteins may be a promising avenue for increased control of early aphid infection. A dimer of *ecdysone receptor* (*EcR*) and *ultraspiracle protein* (*USP*) transduces signals associated with all stages of growth, while each monomer is most heavily expressed in early stages of development in the nymph (juvenile aphid). Knockdown of genes encoding either monomer is reported to be fatal to aphids at early stages of development, which are thus promising targets (Yan *et al*., 2016).

The grain aphid consumes sap through a feeding channel while secreting saliva through a separate channel. To prevent sap from entering the salivary channel, a membrane that includes sheath protein (SHP) is required. Aphids feeding on SHP:RNAi barley were reported to have stunted development and a reduction in average lifespan; despite this, engineering resistance against the early stages of insect infection remains a challenge (Abdellatef *et al*., 2015). A contributing factor to this challenge may be the secretion of ribonucleases (RNAses) in the saliva of the pest, as reported in *Acyrthosiphon pisum* (pea aphid) (Christiaens *et al*., 2014).

Advances in the understanding of how plants and insects process dsRNA provide insight into how HIGS to control insect pests can be made more effective. In *Arabidopsis*, simultaneous loss of vital dsRNA-processing proteins *DICER-LIKE 2, 3* and *4* was reported to have no significant effect on HIGS-mediated resistance to *Helicoverpa armigera* (cotton bollworm). This finding suggests that HIGS-mediated resistance to some insects works by a mechanism where dsRNA is secreted from plant tissues and processed into siRNA by Dicer-like proteins expressed in the insect (Mao *et al*., 2007). Many insect pests, including bollworms and aphids, reportedly lack RNA-dependent RNA polymerase and may therefore be unable to produce secondary siRNAs from siRNA in complex with RISC and mRNA. For this reason, delivery of dsRNA from the host to pest in an adequate concentration for silencing is essential for HIGS of insect genes (Gordon and Waterhouse, 2007). For effective silencing in these species, the silencing ribonucleotides passed from plants to insects must be dsRNA rather than siRNA. This was demonstrated by injection of both siRNAs and dsRNAs targeting green fluorescent protein (GFP) into GFP-expressing *Tribolium castaneum* (red flour beetle), in which fluorescence is silenced by injection of dsRNAs, but not by siRNAs (Miller *et al*., 2012). The necessity of dsRNA, rather than siRNA, for HIGS presents a challenge because dsRNAs are subject to rapid turnover, dsRNAs are prone to be processed into siRNAs by plant DICER-like proteins before uptake by the pest. Recent experiments demonstrate that silencing rates may be improved by expressing interfering RNAs in the chloroplast genome rather than the main plant genome (Zhang *et al*., 2015). This finding was attributed to a lack of RNAi components expressed in the chloroplast, and thus an accumulation of dsRNA in greater concentrations. Resistance to *D. virgifera* (WCR) is improved in transgenic maize expressing dsRNAs silencing WCR *Snf7,* a housekeeping gene encoding a signalling protein that regulates lysozyme formation. *RNAi:Snf7* maize engineered Monsanto Company is pending regulatory approval and anticipated to reach the market by 2020 (Bachman *et al*., 2013).

HIGS of Fungi and Oomycetes

Potato late blight caused by the oomycete pathogen *Phytophthora infestans* (*P. infestans*) can rapidly lead to the total loss of a crop (Fry, 2008). The European Potato Failure of the 1840s was caused by this pathogen, which led to widespread starvation and a death toll estimated in excess of one million (Boyle and Grada, 1986). Epidemics returned to North America in the 1990s, contributing to demand for research into control of late blight, as well as the use of *P. infestans* as a model organism for oomycete pathogens (Whisson *et al*., 2005).

In an early report of *in vitro* RNAi against *P. infestans* genes, effective silencing was indicated by reduced fluorescence in transgenic GFP-expressing *P. infestans* incubated with dsRNA targeting *GFP* (Whisson *et al*., 2005). Similarly, a GUS reporter signal was suppressed in transgenic *Fusarium verticillioides* cultured on *Arabidopsis* expressing dsRNAs targeting GUS (Tinoco *et al*., 2010). A similar silencing assay using GFP was used to investigate possible HIGS of *Phytophthora parasitica* genes by transgenic *Arabidopsis*. Unexpectedly, *P. parasitica* appears to be unable to uptake siRNA or dsRNA from a host plant (Wang *et al*., 2011).

In *P. infestans,* G protein β subunit 1 (*GPβ1*) is a critical part of a signalling cascade that triggers sporangium development. *GPB1* is heavily expressed towards the end of the *P. infestans* disease cycle, at which point the pathogen shifts from a biotrophic to a necrotrophic phase. Transgenic potato generating RNAi against *GPB1* is highly resistant to late-stage disease symptoms such as necrosis. More modest levels of resistance are reported for RNAi against several metabolic and catabolic genes (Jahan *et al*., 2015) as well as the effector AVR3a, which is essential for infection (Bos *et al*., 2010). Limited effects of HIGS in reducing infection might be improved by stacking of HIGS against multiple targets, including *Phytophthora suppressors of RNA silencing* (*PSRs*). For example, PSR1 complexes with a transporter of DICER-like 1 (DCL1), leading to mislocation of DCL1 and reduced RNAi ability (Qiao *et al*., 2015). Other examples of successes in the use of HIGS to limit damage by fungi include targeting of conserved virulence genes of *Verticillium* wilt in both *Arabidopsis* and tomato (Song and Thomma, 2016), as well as of *Puccinia triticina* (wheat leaf rust) on wheat (Panwar *et al*., 2013), and of *B. graminis*, the causative agent of PM, on both wheat and barley (Nowara *et al*., 2010).

For control of neurotoxin-producing necrotrophic *Fusarium* fungi, azole pesticides that inhibit activity of ergosterol biosynthesis enzymes are widely used. However, resistant strains have emerged, with enhanced abilities to mitigate the toxic effects of these pesticides by metabolism or sequestration. In transgenic *Arabidopsis* and barley, the expression of dsRNA targeting three genes encoding enzymes in the ergosterol biosynthesis pathway conferred complete or near-complete resistance, with mycelial growth limited to the site of inoculation. While these results demonstrate promise for control of fungal species by RNAi suppression of ergosterol biosynthesis, this pathway is not found in oomycetes (Koch *et al*., 2013).

In addition to disease management, HIGS is commonly used as a tool to gain insight into the molecular basis of host–pathogen interactions involving nontransformable species such as *B. graminis* (PM). HIGS of two secreted RNAses secreted by PM is reported to reduce haustorial growth in barley. One of these was found to inhibit apoptosis of cells penetrated by haustoria (Pliego *et al*., 2013). Whether these or other secreted RNAses robustly cleave host siRNAs remains unclear. Additional experiments may clarify whether targeting of various RNAses can increase the efficacy of HIGS of other target genes.

With an experimental system of transgenic *Arabidopsis* hosting transgenic *Botrytis cinerea* (grey mould), it was found that the mould secretes siRNAs which bind to *Arabidopsis ARGONAUTE 1* and direct RNAi against host immunity genes (Weiberg *et al*., 2013). Knockout of *B. cinerea DICER-LIKE 1*(*BcDCL1*) and *B. cinerea DICER-LIKE 2* (*BcDCL2*) is associated with reduced host disease symptoms, as is knockout of *Arabidopsis ARGONAUTE 1* (Weiberg *et al*., 2013). Simultaneous knockdown of *BcDCL1* and *BcDCL2* was reported to significantly reduce symptoms, demonstrating the role of filamentous-organism-induced gene silencing (FIGS) in overcoming plant resistance and indicating that RNAi against RNAi machinery may be a promising strategy to repress FIGS (Wang *et al*., 2016).

This report of pathogenic hijacking of host RNAi machinery was built upon by a study on HIGS of miRNA1918. This miRNA is highly conserved between tomato and *P. infestans*, possibly owing to a horizontal gene transfer event (Luan *et al*., 2016). In transgenic tomato, ectopic expression of *P. infestans miRNA1918* confers increased susceptibility to infection, perhaps owing in part to reduced expression of a protein with a RING finger domain (Luan *et al*., 2016). Similar RING finger domains are an essential part of the ubiquitination complex that marks plant proteins for degradation, with targets including a number of immunity-related genes described in a recent review (Sharma *et al*., 2016) as well as *ARGONAUTE 1* (Csorba *et al*., 2010).

Fusarium wilt outbreaks may spoil entire plantations of banana; HIGS of Fusarium developmental genes by transgenic *Musa musa x paradisiaca* (banana) shows promise for control of this disease (Ghag *et al*., 2014). *Bremia lactucae* (downy mildew) is a pathogen causing disease of crops including *Lactuca sativa* (lettuce) and *V. vinifera* (grape). A recent study (Govindarajulu *et al*., 2015) demonstrated marked resistance in transgenic lettuce expressing dsRNA targeting a cellulose synthase gene and the effector *HIGHLY ABUNDANT MESSAGE #34* (*HAM34*). HIGS continues to be used as an investigational tool for molecular plant–pathogen interactions and explored as the pest control strategy for diverse crop species threatened by various fungal and oomycete pathogens.

In recent years, bidirectional cross-kingdom RNAi has been demonstrated to be an important factor not only for plant–pathogen interactions, but also for interactions between plants and mutualists (reviewed by Chaloner *et al*., 2016). Although HIGS of a *Glomus* sp. sugar transporter was reported to reduce mycorrhizal development in *Medicago truncatula* (barrelclover) (Helber *et al*., 2011), research in this area has been limited and the prospects of further improvement of yields and pest resistance in symbiotic crop systems by RNAi of negative regulators of mycorrhizal or endophytic growth or adaptation remain to be seen.

Future Directions

There are many potential benefits of using traditional RNAi-based HIGS approaches for controlling pests/pathogens. These include: (1) the demonstrated effectiveness against a variety of pests/pathogens; (2) RNAi does not need to precisely match the target sequence one construct could be effective against sequence variants of the target gene, which could slow the acquisition of resistance; (3) it is self-limiting to those organisms which eat or interact with the plant host in a manner sufficient for the transfer of the RNAi; and (4) reduced ecological impact through reduced pesticide use.

One development which provides more options for the HIGS approach to pest control includes the usage of artificial micro ribonucleic acids (amiRNAs) for gene silencing. In a recent study, HIGS of SCN genes by transgenic soybean expressing amiRNAs led to partial resistance similar to host-derived dsRNAs, but with enhanced silencing specificity (Tian *et al*., 2016). The development of amiRNA strengthens the promise of HIGS as a highly specific form of pest control by reducing the risk of effects on nontarget genes and species. Unlike dsRNA, design of amiRNA depends on substitution of a region on miRNA with a sequence

complementary to the target site, while keeping the secondary structure of pre-miRNA intact. Whereas dsRNAs are cleaved by Dicer at many different positions and can therefore be processed into siRNAs with various sequences, pre-amiRNAs are processed into specific, predictable amiRNAs (Sablok *et al*., 2011). Thus, RNAi methods using amiRNA have been proposed to be free of environmental biosafety issues that may result from nontarget effects (Liu and Chen, 2010). Additionally, while dsRNA-based RNAi methods generally rely on longer stretches of sequence, the short length of amiRNA is associated with reduced potential for off-target and nontarget effects. However, the high specificity of amiRNAs may correspond to the ability of target species to more rapidly acquire sufficient mutations to become resistant. A proposed solution to this concern is the use of multiple amiRNAs targeting the same gene (Sablok *et al*., 2011).

A key limitation to HIGS is the requirement for a robust transformation system for the plant species and cultivars of interest. While it is possible to obtain transgenic plants through a variety of methods, including *Agrobacterium*-mediated transformation, biolistic transformation (particle bombardment or 'gene gun') and viral-mediated transformation, not all plants of interest are amenable to some or any of these approaches. Many species of economic importance, such as wheat, are particularly recalcitrant to transformation (He *et al*., 2015), as are cultivars of interest of specific crops such as corn, although new methods are leading to improvements in this area (Lowe *et al*., 2016). Having a limited number of transformable cultivars can greatly slow down plant improvement via transformation followed by breeding (Altpeter *et al*., 2016). New transformed varieties of crops must undergo regulatory approval before commercial deployment. In the United States, each genetic transformation event is considered independently, rather than on a per-trait basis, which slows the development of new genetically engineered (GE) crops and imposes a financial burden on both development and deployment (Strauss and Sax, 2016). Another issue is that the interfering RNA molecule is typically brought into contact with the target organism by damage to the host plant. While this aspect of the system would help to limit effects on nontarget organisms, some damage to the plant itself may be a necessary cost.

One possible alternate variation of HIGS is the topical application of purified dsRNA molecules, termed spray induced gene silencing (SIGS). In this situation, the plant need not be GE, as the dsRNA is applied to the plant, rather than being produced from transgenes within the plant itself. Whether or not food, fibre or feed harvested from such crops would be regulated as if they were GE would be an interesting consideration for regulatory agencies. However, SIGS may be acceptable to a broader range of growers than conventional GE and may be subject to fewer marketplace restrictions than GE crops. Another advantage is that, in theory, any crop or cultivar could be treated, thus allowing for the protective benefits of HIGS/SIGS without the need for plant transformation. SIGS has already been shown to be effective in protecting barley against fusarium (Koch *et al*., 2016). If this approach is effective at a large scale, then it could be very beneficial to growers. The spray could be applied as needed, both in terms of timing and location. The labile nature of such applied dsRNA is both beneficial and detrimental. The RNA would tend to degrade rapidly in the environment and therefore may not persist on harvested food; however, the same feature means that treatment would also be transient, and reapplication of the spray may be needed. As with other control measures, there could be spot applications to areas of infestation, and this approach could be integrated with other management techniques, including crop rotation. Some current scientific challenges to making SIGS a viable option are improving the cost-effectiveness of producing large amounts of purified dsRNAs, formulation of the purified dsRNAs for deployment and sufficient coverage of the plant material by the spray itself.

In general, HIGS shows great promise as an effective means for controlling pests/pathogens and helping to reduce crop losses. The complex interplay of the host and pest/pathogen means that new methods and approaches for controlling diseases are likely to be needed. Research has demonstrated the feasibility of HIGS approaches in both model and agriculture plant species.

Glossary

Crop Plants such as corn or cotton, which are grown for food, animal feed, fibre or other purposes.

Nontarget Refers to organisms other than the one(s) intended.

Off-target Refers to gene(s) other than the one intended for suppression.

Pathogen Microorganisms, such as bacteria, fungi, nematodes and viruses, which can cause plant diseases.

Pest Plant eating (herbivorous) organisms such as insects which damage plants.

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