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Review article

New wind in the sails: improving the agronomic value of crop plants through RNAi-mediated gene silencing

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Summary

RNA interference (RNAi) has emerged as a powerful genetic tool for scientific research over the past several years. It has been utilized not only in fundamental research for the assessment of gene function, but also in various fields of applied research, such as human and veterinary medicine and agriculture. In plants, RNAi strategies have the potential to allow manipulation of various aspects of food quality and nutritional content. In addition, the demonstration that agricultural pests, such as insects and nematodes, can be killed by exogenously supplied RNAi targeting their essential genes has raised the possibility that plant predation can be controlled by lethal RNAi signals generated *in planta*. Indeed, recent evidence argues that this strategy, called host-induced gene silencing (HIGS), is effective against sucking insects and nematodes; it also has been shown to compromise the growth and development of pathogenic fungi, as well as bacteria and viruses, on their plant hosts. Here, we review recent studies that reveal the enormous potential RNAi strategies hold not only for improving the nutritive value and safety of the food supply, but also for providing an environmentally friendly mechanism for plant protection.

RNA interference: discovery of a novel mechanism for gene regulation

RNA interference (RNAi) is a conserved and integral aspect of gene regulation that utilizes small RNAs (sRNAs) to direct the silencing of gene expression at the transcriptional or post-transcriptional level. The consequence of RNAi is a loss-of-function phenotype that, ideally, is identical to that of a genetic null mutant. RNAi is associated with diverse regulatory processes, including regulation of gene expression at the transcriptional and translational levels, protection against viral infection, control of epigenetic modifications, regulation of genome stability, curbing of transposon movement and regulation of heterochromatin formation (for reviews see Castel and Martienssen, 2013; Ketting, 2011). The term RNAi was coined by Craig Mello and co-workers (Rocheleau *et al.*, 1997) to describe the unknown mechanism through which exogenously supplied sense and antisense RNAs effectively silenced gene expression in *Caenorhabditis elegans*. Subsequent efforts to elucidate the requirements for RNAi structure and delivery revealed that injection of double-stranded RNA (dsRNA) corresponding to a target gene in *C. elegans* suppressed the accumulation of endogenous mRNA transcripts more effectively than injecting either sense or antisense strands individually (Fire *et al.*, 1998). The ability of a low number of injected dsRNA molecules to silence the target gene further led these authors to speculate that there must be an amplification component in the interference process.

Prior to these studies in *C. elegans*, researchers working with plants and fungi had noticed that the introduction of transgenes sometimes induced post-transcriptional gene silencing (PTGS), manifested by reduced accumulation of transcripts for the transgene or, if the transgene contained homology to an endogenous gene, a reduction in transcripts for both genes (Hammond *et al.*, 2001a). This latter phenomenon was termed co-suppression in plants and quelling in fungi. PTGS also could be induced in plants by cytoplasmically replicating viruses (Hammond *et al.*, 2001a). Given the similar phenotypes associated with PTGS and RNAi-mediated gene silencing, Hamilton and Baulcombe (1999) investigated whether endogenously generated dsRNAs also were associated with PTGS. Analysis of tomato or *Nicotiana* species transformed with endogenous or foreign genes, or infected with Potato virus X, revealed a correlation between the activation of gene silencing and the accumulation of short antisense RNA molecules (estimated at 25 nt in length) whose sequences were complementary to the targeted transcript (Hamilton and Baulcombe, 1999). The subsequent demonstration that plants, fungi and animals contain related proteins that are required for PTGS, quelling and RNAi, respectively, provided further evidence that the mechanisms associated with these phenomena are highly related, if not identical (Fagard *et al.*, 2000).

The ability of short antisense RNAs to regulate gene expression has since been described in a wide variety of organisms, including mammalian cells (Elbashir *et al.*, 2001a,b), fungi (Romano and Macino, 1992), Drosophila (Hammond *et al.*, 2001b), nematodes (Fire *et al.*, 1998), algae (Cerutti *et al.*, 2011), moss (Bezanilla *et al.*, 2003) and plants (Napoli *et al.*, 1990). A similar phenomenon also has been described in prokaryotes (Mizuno *et al.*, 1984). Three classes of noncoding (nc)RNAs have been shown to silence different targets: small interfering RNAs (siRNAs) are involved in post-transcriptional regulation of gene transcripts and transposons (Ghildiyal and Zamore, 2009; Moazed, 2009), micro RNAs (miRNAs) modulate the translational potential and the stability of their mRNA targets (Carthew and Sontheimer, 2009; Yeo and Chong, 2011), and PIWI-interacting RNAs (piRNAs) regulate the silencing of transposable elements in the germ-line

(Siomi *et al.*, 2011; Thomson and Lin, 2009). In addition, enhancer RNAs (eRNA) (Orom *et al.*, 2010), promoter-associated RNAs (PAR) and long noncoding RNAs (lncRNA) (Mercer *et al.*, 2009) have been identified, but their specific function in gene regulation is widely unresolved (Kaikkonen *et al.*, 2011).

Studies have shown that RNAi-mediated gene silencing occurs via two pathways: PTGS and transcriptional gene silencing (TGS) (Vaucheret *et al.*, 2001; Vaucheret and Fagard, 2001; Castel and Martienssen, 2013). Both mechanisms start with initial processing or cleavage of a precursor dsRNA into short 21–25 nucleotide siRNA or miRNA duplexes (Hamilton and Baulcombe, 1999) by an RNaseIII-like enzyme called Dicer (Baulcombe, 2004; Ketting, 2011). PTGS proceeds in the cytoplasm acting at the mRNA level. Double-stranded siRNAs are incorporated into an RNA-induced silencing complex (RISC) containing an Argonaute (e.g. AGO-1 of *A. thaliana*) protein that has a sRNA-binding domain and an endonucleolytic activity for cleavage of target RNAs (Vaucheret *et al.*, 2004). The activated RISC subsequently unwinds siRNAs, thereby generating a sense (or passenger) and an antisense (or guide) strand in an ATP-requiring reaction. Whereas the sense strand becomes degraded, the RISC containing the antisense strand then targets a complementary mRNA transcript via base pairing interaction, degrades the mRNA and thereby inhibits protein biosynthesis (for more details, see Brodersen and Voinnet, 2006; Ghildiyal and Zamore, 2009; Liu and Paroo, 2010). In contrast to PTGS, TGS occurs in the nucleus. Epigenetic modifications directed by small RNAs, including RNA-dependent DNA methylation (RdDM), histone modification and chromatin remodelling have been shown to cause transcriptional repression in plants, fungi and animals (Castel and Martienssen, 2013; Mette *et al.*, 2000). The role of TGS in nuclear processes and its mechanistic interaction with PTGS pathways is an emerging field, that is, attracting immense scientific attention. Despite the evolutionary conservation and the mechanistic consistency of RNAi pathways, there are great variations regarding silencing machinery across the different kingdoms (for review see Ketting, 2011).

RNAi—a molecular tool for breeding high-value crops

Over the past decade, there has been exceptional scientific interest in RNAi (for overview, see Lindbo, 2012). RNAi has been widely used as a key strategy in functional genomics (see review by Mohr and Perrimon, 2012). In addition, RNAi technology has been used to enhance or reduce the accumulation of specific metabolites in food and feed crops, thereby altering their nutritional value. For instance, the carotenoid and flavonoid content in tomato (Davuluri *et al.*, 2005); ß-carotene, zeaxanthin, violaxanthin and lutein levels in rapeseed (Yu *et al.*, 2008); lignans in Forsythia cell suspension (Kim *et al.*, 2009); and phenylpropene aroma compounds in cultivated strawberry (Hoffmann *et al.*, 2011) were elevated via RNAi. By contrast, nornicotine levels were reduced in tobacco (Gavilano *et al.*, 2006), and, in Brassica species, the unwanted glucosinolate progoitrin was reduced, while the glucosinolate glucoraphanin was enhanced (Liu *et al.*, 2012).

RNA interference strategies also have a high potential for generating plants with significant medical value, such as plants that are hypoallergenic or that exhibit reduced autoimmunogenic activity. For example, in genetically predisposed individuals, gluten (a complex mixture of prolamin seed storage proteins found in wheat and other grains) triggers a severe autoimmune disorder of the small intestine known as coeliac disease. Currently, the only therapy for patients with coeliac disease is a totally gluten-free diet. However, this diet not only is difficult to follow, but also has adverse effects on the gut. To circumvent these problems, Diter von Wettstein and his group at the Washington State University, Pullman, have sought to develop wheat lines that accumulate reduced levels of immunoreactive prolamins. Using a *DEMETER* (*DME*) silencing hairpin construct, they concomitantly suppressed the expression of the three wheat *DME* homoeologs in the hexaploid wheat genome (Wen *et al.*, 2012). The *DME* genes encode 5-methylcytosine DNA glycosylases, which are responsible for demethylating, and thereby activating, the promoters of a subset of seed proteins, including the immunogenic prolamins, in wheat endosperm. Silencing of *DME* led to reduced *DME* transcript abundance (up to 85%) in different wheat transformants, and up to a 76% reduction in the amount of total immunogenic prolamins, with the reduction/elimination of specific immunogenic prolamin family members varying between the transgenic lines.

Similar strategies that harness the power of plant biotechnology to resolve medical issues may improve the lives of a vast number of patients suffering from food allergies and thus may have a high economic impact. There are several examples in which RNAi technology has been used to reduce allergenic proteins and/or toxic compounds in foods, including tomato, apple, rice and peanuts (for review see Perrimon *et al.*, 2010). The clinical results from a study combating carrot allergy provide further support for the feasibility of creating low-allergenic foods (Peters *et al.*, 2011). Concomitant RNAi-mediated knock-down of the two carrot allergens, Dau c 1.01 and Dau c 1.02, resulted in carrot lines that provoked only minor allergenic reactivity in patients with carrot allergy during skin prick tests. Notably, Dau c 1 proteins belong to the pathogenesis-related 10 (PR 10) family of plant proteins; this family of ubiquitous, small plant proteins contributes to the allergenicity of various fruits, vegetables and tree nuts. However, while silencing PR10 proteins may provide a strategy for generating certain hypoallergenic crops, it remains unclear whether the loss of this defence-associated protein confers increased susceptibility to pest/pathogen infection.

Host plant-induced gene silencing

In addition to metabolic engineering, RNAi technology has been employed to develop plants with improved resistance to abiotic and/or biotic stresses. Plants and other eukaryotes have evolved RNA silencing machineries that not only regulate developmental programs, but also provide protection from invasion by foreign nucleic acids, such as viruses (reviewed by Sidahmed and Wilkie, 2010; Voinnet, 2005). This natural phenomenon can be exploited to control agronomically relevant plant diseases, based on the demonstration that *in vitro* feeding of dsRNA can signal PTGS of target genes in various plant pests and pathogens, such as insects (Price and Gatehouse, 2008; Zhang *et al.*, 2013), nematodes (Lilley *et al.*, 2012) and fungi (Nunes and Dean, 2012). Indeed, expression of such dsRNAs in the corresponding host plant conferred protection from predation or infection. This biotechnological method, termed host-induced gene silencing (HIGS), has emerged as a promising alternative in plant protection because it combines high selectivity for the target organism with minimal side effects, as compared with chemical treatments.

A key step in developing a successful HIGS strategy is the identification of suitable target genes in the infectious agent. Genes with known lethal knockout phenotypes can be highly efficient targets for HIGS (Table 1). Effective targets also may be identified by screening cDNA libraries for highly expressed genes. As an extension of cDNA library screening, next-generation sequencing technologies, such as RNA-seq, could allow the rapid identification of target genes in nonmodel organisms. Once a potentially essential target gene has been identified within a plant pest or pathogen, the next step is to produce the corresponding dsRNA and assess its *in vitro* activity on cultures of the target organism. It also is critical to ensure that the dsRNA and corresponding siRNA species do not exert off-target effects that negatively impact host plant physiology, potential nontarget host colonizers and/or mammals that feed on the modified crop. To avoid this pitfall, bioinformatics programs, such as the open-access SI-FI software (http://labtools.ipk-gatersleben.de), can be used to screen the candidate dsRNA sequence for complementarity to other genes. In the next paragraphs, we will review recent applications of the HIGS strategy in crop plants. We will not discuss HIGS strategies against viruses, as the list of research studies in this field is almost continuous and belongs in a review dedicated to RNAi for viral control.

HIGS against insects

Worldwide, preharvest losses of six major food and cash crops (rice, wheat, maize, potatoes, soybean and cotton) caused by animal pests (arthropods, nematodes, rodents, birds, slugs and snails) amount to an average of 11% (Oerke, 2006). More than that, over 500 species of insects and mites are resistant to one or more insecticides (Elzen and Hardee, 2003). These alarming numbers demonstrate that novel strategies for insect control are urgently required. Over the last 10 years, great strides have been made in elucidating the mechanisms involved in dsRNA uptake and the subsequent development of systemic RNAi in insects (Tomoyasu *et al.*, 2008), as well as the feasibility of using RNAi feeding as a control strategy (for review see Huvenne and Smagghe, 2010). At the time we write this review, RNAi has been tested in more than 30 species from eight insect orders (for review, see Zhang *et al.*, 2013). In *C. elegans*, SID-1 (SYSTEMIC RNA INTERFERENCE DEFECTIVE-1), a multispan transmembrane protein, and SID-2, an intestinal transmembrane protein, have been shown to play critical roles in dsRNA uptake and systemic RNAi (Winston *et al.*, 2002, 2007). The identification of SID-1 and SID-2 orthologs in many, but not all, insects suggests the presence of a dsRNA uptake mechanism in insects that could be exploited for HIGS (reviewed by Gatehouse and Price, 2011; Zhang *et al.*, 2013).

One of the first demonstrations that HIGS successfully controls insect predation was reported by Baum *et al.* (2007). After performing feeding assays to identify the most effective targets for RNAi in the coleopteran western corn rootworm (*Diabrotica virgifera*), they generated maize plants expressing a dsRNA construct targeting the vacuolar H+ATPase (V-ATPase subunit A). Subsequent analyses revealed that the lines accumulating detectable levels of RNAs derived from the dsRNA construct had a higher level of pest resistance. Notably, V-ATPases also are possible targets for antifungal drugs, as their chemotherapeutic inhibition affects fungal viability and virulence (see review by Zhang and Rao, 2012). Concurrently, Mao and co-workers designed a HIGS strategy that indirectly controlled insect predation by enhancing the insect's sensitivity to plant phytotoxins. Following insect attack, plants synthesize a variety of secondary metabolites aimed at reducing predation. In response, some insects have developed the ability to detoxify these compounds, often via the activity of cytochrome P450 monooxygenase superfamily members. Through genetic and biochemical analyses, CYTOCHROME P450 (*CYP6AE14*) expression in the midgut of cotton bollworm (*Helicoverpa armigera*) larvae was found to be essential for tolerance to the cotton phytotoxin gossypol (Mao *et al.*, 2007). Strikingly, larvae reared on transgenic Arabidopsis, tobacco or cotton expressing a *CYP6AE14* dsRNA exhibited reduced expression of this gene and enhanced sensitivity to gossypol (Mao *et al.*, 2007, 2011). Recently, these authors showed that the level of bollworm protection could be further enhanced by co-expressing the *CYP6AE14* dsRNA with a plant cysteine protease (Mao *et al.*, 2013). This effect was attributed to the ability of plant cysteine proteases, such as GhCP1 from cotton and AtCP2 from Arabidopsis, to attenuate the cotton bollworm midgut peritrophic matrix, which presumably improves transmission of dsRNA from the plant to midgut cells.

Host-induced gene silencing targeting of other cytochrome P450 genes from the cotton bollworm has been used to combat cotton bollworm resistance to pyrethroid insecticides, an emergent problem due to pyrethroid overuse in the field. Arabidopsis expressing a dsRNA construct targeting *CYP9A14* reduced larval resistance to deltamethrin, a pyrethroid insecticide widely used for cotton (Tao *et al.*, 2012). In a related study, injection of dsRNAs corresponding to *CYP6B7*, *cytochrome P450 reductase* or *cytochrome* b_5 into the midgut of cotton bollworm larvae restored susceptibility to the pyrethroid insecticide fenvalerate (Tang *et al.*, 2012). While these results further suggest that targeting the cytochrome P450 enzyme systems is an effective strategy for reducing pyrethroid resistance, the efficacy of *in planta* expression of these dsRNAs on cotton bollworm has not been tested.

HIGS against nematodes

In planta expression of dsRNAs that target the genes of parasitizing nematodes was recently summarized by Lilley *et al.* (2012). As these authors note, there are many aspects of this complex host—parasite interaction that are not fully understood. In particular, the inability to transform plant parasitic nematodes or generate mutant lines has hindered efforts to understand gene function, which, by extension, impedes the identification of essential genes that could serve as effective RNAi targets. In an effort to circumvent this problem, Alkharouf *et al.* (2007) compared EST databases from the soybean cyst nematode (SCN) *Heterodera glycines* and *C. elegans*, searching for candidate lethal target genes in *H. glycines* based on their identity with known essential genes in *C. elegans*. Microarray analyses were then used to identify a subset of 32 genes that not only shared high-level identity with essential *C. elegans* genes, but also were highly expressed during *H. glycines* parasitic stages (Klink *et al.*, 2009). Soybean roots expressing RNAi constructs targeting four of these genes exhibited a ~80%–90% reduction in the number of SCN females reaching maturity (Klink *et al.*, 2009). It should be noted that while this strategy was highly successful, other efforts to select SCN target genes based on homology with *C. elegans* genes required for viability or development have met with varying levels of success (Lilley *et al.*, 2012). For example, Youssef *et al.* (2013) recently employed a HIGS strategy to target *HgALD*, which

Table 1 Summary of host plant-induced gene silencing (HIGS) applied to several agricultural pests. Pest species, target genes, target selection and documented effects are summarized

Pathogen	Species	Target gene	Target selection	Host plant	Effect/Comments	References
Insect	*Diabrotica virgifera*	V-ATPase A	cDNA library	Maize	Obvious reductions in root damage	Baum *et al.* (2007)
	Helicoverpa armigera	*CYP6AE14* (cytochrome P450)	cDNA library	*Nicotiana benthamiana* and *Arabidopsis thaliana*	Suppressed *CYP6AE14* expression and reduced growth on gossypol-containing diet	Mao *et al.* (2007)
				Gossypium hirsutum (cotton)	Enhanced resistance to cotton bollworms	Mao *et al.* (2011)
	Myzus persicae	*Rack1* (gut) and *MpC002* (salivary glands)	Homologous genes	*Nicotiana benthamiana* and *Arabidopsis thaliana*	Silenced *M. persicae* produced less progeny	Pitino *et al.* (2011)
	Nilaparvata lugens	*NlHT1* (hexose transporter), *Nlcar* (carboxypeptidase), *Nltry* (trypsin-like serine protease)	cDNA library	*Oryza sativa* L. (rice)	Reduction of targeted gene transcripts in the midgut; lethal phenotypic effects after dsRNA feeding were not observed	Zha *et al.* (2011)
	Helicoverpa armigera	*CYP9A14* (cytochrome P450 monooxygenases)	Known functional gene	*Gossypium hirsutum* (cotton)	Reduced the larval tolerance to the insecticide deltamethrin	Tao *et al.* (2012)
		EcR (ecdysone receptor)	Known functional gene	*Nicotiana tabacum* (tobacco)	Resistance to *H. armigera*; EcR dsRNA also confers resistance to another lepidopteran pest, *Spodoptera exigua*	Zhu *et al.* (2012)
		CYP6AE14 and *GhCP1* (cysteine protease)	Known functional gene	*Gossypium hirsutum* (cotton)	Cotton plants co-expressing dsRNA and cysteine protease exhibit enhanced bollworm resistance	Mao *et al.* (2013)
		HaHR3 (moult-regulating transcription factor)	Known functional gene	*Nicotiana tabacum* (tobacco)	Developmental deformity and larval lethality	Xiong *et al.* (2013)
	Sitobion avenae	*CbE E4* (carboxylesterase)	Homologous genes	*Triticum aestivum* (wheat)	Reduced progeny production and reduced resistance to phoxim insecticides	Xu *et al.* (2014)
Parasitic nematodes	*Meloidogyne incognita*	Splicing factor and integrase	Orthologous genes, conserved functions	*Nicotiana tabacum* (tobacco)	Resistance	Yadav *et al.* (2006)
	Meloidogyne javanica	*MjTis11* (transcription factor)	Known functional gene		Down-regulation of *MjTis11* did not result in a lethal phenotype	Fairbairn *et al.* (2007)
	Meloidogyne species: *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla*	*16D10* (parasitism gene)	cDNA library, homologous genes	*Arabidopsis thaliana*	Resistance effective against the four major RKN species	Huang *et al.* (2006)
	Heterodera glycines	*MSP* (major sperm protein)	cDNA library	*Glycine max* (soybean)	Development of SCN females and number of eggs per cyst were reduced	Steeves *et al.* (2006)
	Heterodera schachtii	*B05*, *4G06*, *8H07* and *10A06* (parasitism genes)	cDNA library	*Arabidopsis thaliana*	Reduction in the number of mature females	Sindhu *et al.* (2009)
	Heterodera glycines	*Cpn-1*, *Y25* and *Prp-17* (reproduction	Homologous genes	*Glycine max* (soybean)	Suppression comparable to conventional resistance	Li *et al.* (2010)

Table 1 Continued

Pathogen	Species	Target gene	Target selection	Host plant	Effect/Comments	References
		or fitness-related genes)				
	Meloidogyne incognita	*Mi-Rpn7* (essential for the integrity of 26S proteasome)	Homologous genes	*Glycine max* (soybean)	Reduced motility and infectivity; no complete resistance	Niu *et al.* (2012)
		Mi8D05 (parasitism gene)	Previously identified	*Arabidopsis thaliana*	Up to 90% reduction in infection by *M. incognita*	Xue *et al.* (2013)
		flp-14 and *flp-18* (FMRFamide-like peptide genes)	Homologous, known functional genes	*Nicotiana tabacum* (tobacco)	50%–80% reduction in infection	Papolu *et al.* (2013)
	Pratylenchus vulnus	*Pv010* (spliceosome subunit)	Orthologous gene	Walnut	Reduced nematode infection	Walawage *et al.* (2013)
	Meloidogyne incognita	*16D10* (parasitism gene)	Known functional gene	*Vitis vinifera* (grape)	Less susceptibility	Yang *et al.* (2013)
	Heterodera glycines	*HgALD* (aldolase)	Previously identified	*Glycine max* (soybean)	Decrease in the number of mature SCN females	Youssef *et al.* (2013)
Parasitic plants	*Striga asiatica*	Fatty acid—aromatic amino acids—and AMP biosynthesis, vacuole morphogenesis	Herbicides target	Maize	No resistance; some differences in *Striga* growth rate	de Framond *et al.* (2007)
	Orobanche aegyptiaca (broomrape)	*M6PR* (mannose 6-phosphate reductase)	Previously identified	Tomato	Significant increase in the percentage of dead *O. aegyptiaca* tubercles on the transgenic tomato plants	Aly *et al.* (2009)
	Triphysaria versicolor	*GUS* (reporter gene)	Proof of concept	Lettuce, Triphysaria, Arabidopsis	GUS silencing; proof of concept	Tomilov *et al.* (2008)
	Cuscuta pentagona	*STM* (SHOOT MERISTEMLESS)	Known functional gene	*Nicotiana tabacum* (tobacco)	Silencing disrupts dodder growth	Alakonya *et al.* (2012)
Bacteria	*Agrobacterium tumefaciens*	*iaaM* and *ipt* (oncogenes)	Known functional gene	*Arabidopsis thaliana* and *Lycopersicon esculentum*	Transformed plants retained susceptible to *Agrobacterium* transformation, but were highly refractory to tumorigenesis	Escobar *et al.* (2001)
			Known functional gene	Walnut	Crown gall control	Walawage *et al.* (2013)
Fungi/ Oomycetes	*Blumeria graminis* f. sp. *tritici*	*MLO*	Known functional gene	*Triticum aestivum* (wheat)	Resistance	Riechen (2007)
	Phytophthora parasitica var. *nicotianae*	*GST* (glutathione S-transferase gene)		*Nicotiana tabacum* (tobacco)	Resistance; GST negative regulator of defence response	Hernández *et al.* (2009)
	Blumeria graminis	*Avra10* (effector gene)		*Hordeum vulgare* (barley) and *Triticum aestivum* (wheat)	Reduced fungal development in the absence of the matching resistance gene *Mla10*	Nowara *et al.* (2010)
	Fusarium verticillioides (= *F. moniliforme*)	*GUS* (reporter gene)	Proof of concept	Tobacco (cv Xanthi)	GUS silencing; proof of concept	Tinoco *et al.* (2010)

Table 1 Continued

Pathogen	Species	Target gene	Target selection	Host plant	Effect/Comments	References
	Puccinia striiformis f. sp. *tritici* or *P. graminis* f. sp. *tritici*	*PSTha12J12* (haustorial Pst transcript)	cDNA library	*Hordeum vulgare* (barley) and *Triticum aestivum* (wheat)	No obvious reductions in rust development or sporulation	Yin *et al.* (2011)
	Phytophthora parasitica	*PnPMA1* (H^+-ATPase) and *GFP* (reporter gene)	Previously identified	*Arabidopsis thaliana*	Not sufficient; No reduction in GFP and PnPMA1 transcripts	Zhang *et al.* (2011)
	P. triticina, P. graminis and P. striiformis	*PtMAPK1* (MAP kinase), *PtCYC1* (cyclophilin) and *PtCNB* (calcineurin B)	Functional orthologs	Wheat	Disease suppression, compromising fungal growth and sporulation	Panwar *et al.* (2013)
	Fusarium graminearum	*CYP51A*, *CYP51B* and *CYP51C*	Fungicides target	*Arabidopsis thaliana* and *Hordeum vulgare* (barley)	Resistance	Koch *et al.* (2013)

encodes fructose-1,6-diphosphate aldolase, a key enzyme of gluconeogenesis. Although this gene was identified as a potential SCN target by Alkharouf *et al.* (2007), the reduction in mature females grown on soybean roots expressing an RNAi construct targeting *HgALD* was 58%.

An alternative HIGS strategy for controlling nematodes involves targeting the genes required for parasitism (Papolu *et al.*, 2013; Yang *et al.*, 2013). In the root-knot nematode (RKN) *Meloidogyne incognita*, the FMRFamide-like peptide genes *flp-14* and *flp-18* encode neuropeptides that have been implicated in nematode migration and invasion of host roots (Papolu *et al.*, 2013). HIGS-mediated targeting of either gene in transgenic tobacco reduced *M. incognita* infection (as measured by a decreased number of females and/or galls) in most, but not all lines. Female fecundity, based on the number of egg masses and eggs per mass, also was reduced by ~50%–80%. Intriguingly, decreased fecundity was observed even in tobacco lines that supported elevated levels of females after infection (Papolu *et al.*, 2013). Parasitism can also be disrupted by HIGS targeting of genes encoding nematode effectors, which are secreted proteins that play critical roles in establishing a successful parasitic relationship with the host. In transgenic Arabidopsis, expression of an RNAi construct targeting the conserved RKN effector gene *16D10*, which encodes a small secretory peptide that helps establish feeding sites, conferred broad-spectrum resistance to the four major RKN species (Huang *et al.*, 2006). Decreased susceptibility to *M. incognita* also was observed in transgenic grape hairy roots expressing hairpin-based silencing constructs with either a 42-bp or a 271-bp stem sequence of the *16D10* gene (Yang *et al.*, 2013). To gain insight into the parameters influencing siRNA production and potency, small RNA libraries generated from six transformed grape hairy root lines were sequenced. Over 100-fold greater levels of *16D10*-specific siRNAs were detected in four lines transformed with the 42-bp stem construct as compared with two lines transformed with the 271-bp stem construct. However, as the number of eggs per root or number of eggs per gram root was generally comparable between lines transformed with either construct, siRNA accumulation does not appear to be directly proportional to *M. incognita* resistance levels. Analysis of the *16D10*-specific siRNA species derived from either construct also revealed that they tend to be generated at specific hot spots that have a higher GC content than the remaining stem sequences, and they are preferentially generated from sequences near the loop terminus, as compared with the stem base. These findings may provide clues for optimizing the efficiency of RNAi constructs, but they also underscore how little is understood about the factors influencing siRNAi potency.

HIGS against parasitic plants

Parasitic plants, such as Dodder and certain genera from the family Orobanchaceae (Orobanche, Striga), cause considerable ecological damage and crop losses worldwide. Parasitic plants are hard to control by chemical means because their life cycle is inevitably coupled with that of their plant host (Sauerborn *et al.*, 2002). The discovery that long-distance movement and exchange of RNA molecules occur between parasitic plants and their hosts (Westwood *et al.*, 2009) raised the possibility that a HIGS strategy targeting specific gene sequences in parasitic plants could be effective. Efforts to employ HIGS to control species from the Orobanchaceae, particularly the genus *Striga*, have yielded varying levels of success (Runo, 2011; Runo *et al.*, 2011). More recently, HIGS was shown to disrupt the growth of dodder (*Cuscuta pentagona*), which belongs to the family Convolvulaceae. To control this parasitic plant, transgenic tobacco expressing an RNAi directed against the dodder *STM* (*SHOOT MERISTEMLESS*) gene was constructed (Alakonya *et al.*, 2012). As dodder develops vascular connections with its host upon parasitism, a phloem-specific promoter, *SUC2* (*SUCROSE-PROTON SYMPORTER2*), was used to facilitate delivery of this RNAi into the parasite. Analysis of dodder grown on these transgenic plants revealed the presence of sRNAs corresponding to the RNAi construct, strongly down-regulated *STM* expression, and a substantial reduction in growth that was associated with defects in the formation of haustorial plant—parasite vascular connections, which are essential for parasitic nutrient and water acquisition. Thus, this technology has

the potential to be an effective method of biological control of parasitic plants.

HIGS against bacteria

Crown gall disease, caused by the soil bacterium *Agrobacterium tumefaciens*, is responsible for substantial economic losses in many perennial nut, fruit and ornamental crops. During the infection process, the bacterial *TRYPTOPHAN 2-MONOOXYGENASE* (*iaaM*) and *ISOPENTENYLTRANSFERASE* (*IPT*) genes are transferred to the plant genome via the bacterial Ti plasmid. Following their integration, expression of these genes results in the overproduction of auxin and cytokinin, respectively, which together mediate uncontrolled plant cell growth and lead to the formation of a gall (also called a plant tumour) (Zhu *et al.*, 2000). In 2001, Escobar and co-workers developed a HIGS strategy to improve crown gall resistance based on the knowledge that *iaaM* and *ipt* do not share significant sequence similarity to any plant genes. They generated *Arabidopsis* and tomato plants expressing two self-complementary RNA constructs designed to initiate RNAi targeting of both genes. Following *A. tumefaciens* infection, transformed *Arabidopsis* and tomato lines exhibiting complete functional resistance to crown gall disease were identified. As this resistance mechanism relies on mRNA sequence homology, rather than the receptor—ligand interaction associated with traditional plant resistance genes, the authors speculated that it might be highly durable, provided *A. tumefaciens* does not possess viral-type suppressors of gene silencing. However, Dunoyer *et al.* (2006) subsequently reported that *A. tumefaciens*-induced gall formation is associated with a functional antisilencing state (i.e. repression of the plants' RNAi silencing response). This conclusion was based on the combined observations that i) gall tissue, unlike *A. tumefaciens*-infiltrated leaves, did not accumulate siRNAs corresponding to the bacterial genes transferred to the plant genome, and ii) RNAi-deficient plants exhibited enhanced susceptibility to *A. tumefaciens* infection. As the silencing suppression in galls appears to be related to phytohormone overproduction, however, HIGS targeting of *iaaM* and *ipt* may yet prove to be an effective strategy for controlling crown gall disease.

HIGS against fungi

Over the last few years, several studies have been published that document efforts to use HIGS to control fungal diseases (for review, see Nunes and Dean, 2012). In 2010, Tinoco *et al.*, provided proof of concept for HIGS in phytopathogenic filamentous fungi by demonstrating that tobacco plants expressing a β-GLUCURONIDASE (GUS) gene-interfering cassette [hairpin (hp)GUS] specifically silenced GUS transcripts in a GUS-expressing strain of necrotrophic *Fusarium verticillioides* during plant colonization. More recently, HIGS of essential fungal ergosterol biosynthetic genes of the *STEROL 14α-DEMETYLASE* (*CYP51*) family was shown to be a highly efficient strategy for controlling the growth and development of another *Fusarium* species, mycotoxin-producing *F. graminearum* (*Fg*) (Koch *et al.*, 2013). *In vitro* feeding of *CYP3RNA*, a 791 nucleotide dsRNA complementary to the three *Fg* genes, *CYP51A*, *CYP51B* and *CYP51C*, resulted in fungal growth inhibition (half maximum growth inhibition [IC_{50}] = 1.2 nM). Moreover, fungal morphology was altered, resembling the morphological alterations that are observed after treatment with the azole fungicide tebuconazole, for which CYP51 enzymes are a target. Consistent with this finding, *CYP3RNA* expression in both Arabidopsis and barley rendered susceptible plants highly resistant to fungal infection. Microscopic analysis revealed that mycelium formation on *CYP3RNA*-expressing leaves was restricted to the inoculation sites, and *Fg*-inoculated barley caryopses were virtually free of fungal hyphae. These results demonstrate that HIGS of the fungal *CYP51* genes is an efficient method for inhibiting fungal mycelium formation and plant infection. Further research is required to assess the potential for RNAi to at least partly supplant application of azole fungicides in plant disease control.

Efforts to control rust fungi have produced mixed results. To develop a HIGS strategy that would reduce susceptibility to the biotrophic leaf rust fungus *Puccinia striiformis* f. sp. *tritici* (*Pst*), Yin *et al.* (2011) selected targets from a subset of genes based on their strong expression in *Pst* haustoria. Among these were putative fungal effector genes, a chitinase gene and an HXT1p hexose transporter. *Barley stripe mosaic virus* (BSMV) was used as the vector to induce RNAi in wheat leaves via virus-induced gene silencing (VIGS). Although silencing of the target genes was detected, it did not correlate with any effect on rust disease development or sporulation (Yin *et al.*, 2011), which suggests that the targeted genes were probably not the best choices for essential genes. Consistent with this conclusion, an *A. tumefaciens*-mediated transient transformation system induced HIGS against the rust fungus *P. triticina* in wheat leaves (Panwar *et al.*, 2013). Following superinfection with *P. triticina*, wheat leaves transiently expressing hairpin RNA-generating constructs targeting the *P. triticina* pathogenicity genes *MITOGEN-ACTIVATED PROTEIN KINASE 1* (*PtMAPK1*), *CYCLOPHILIN* (*PtCYC1*) or *CALCINEURIN B* (*PtCNB*) exhibited a 51%–68% reduction in disease symptoms (based on pustule density) and a 59%–69% reduction in fungal biomass at 10 days postinfection as compared with the empty vector controls. Wheat leaves transiently expressing these constructs also exhibited reduced disease symptoms following superinfection with stem rust (*P. graminis*) or stripe rust (*Pst*). Thus, while this agroinfiltration strategy only reduced *P. triticina* susceptibility in the agroinfiltrated areas, it provides a convenient method for identifying potential targets for HIGS, including genes that might confer broad-spectrum resistance to rust fungi.

A strong HIGS effect also has been demonstrated in cereals infected with the powdery mildew fungus *Blumeria graminis* (Nowara *et al.*, 2010). Analysis of barley or wheat plants expressing dsRNA targeting fungal glucanosyltransferase genes derived from an RNAi construct (barley) or BSMV-induced VIGS (wheat), respectively, revealed reduced *B. graminis* disease symptoms or haustoria formation/development and thus higher resistance to the biotrophic pathogen. In addition, HIGS-mediated targeting of the fungal effector gene *Avra10* reduced the number of functional haustoria inside epidermal cells in the powdery mildew-susceptible barley cultivar Pallas, which lacks the *Mla10* resistance gene, but not in a nearly isogenic line containing *Mla10*. This finding is consistent with the concept that the fungal effector *Avra10* promotes virulence; thus, its silencing enhances resistance in susceptible barley lines. In the *Mla10*-expressing barley line, silencing *Avra10* enabled the fungus to circumvent resistance protein-mediated recognition, but this advantage was counteracted by a reduced ability to develop haustoria.

In comparison with the above results, HIGS-mediated gene silencing was not initiated in the oomycete pathogen *Phytophthora parasitica* during colonization of Arabidopsis expressing

dsRNA sequences targeting either a GFP transgene or the endogenous *P. parasitica PnPMA1* gene (Zhang *et al.*, 2011). The authors argued that oomycetes might lack the machinery required for the uptake of silencing signals as one possible explanation for their failure. However, other examples demonstrate that HIGS works in oomycetes (Table 1).

RNAi uptake into fungal/oomycete cells: Possible pathways

While the uptake of RNAi signals by insects (reviewed by Huvenne and Smagghe, 2010), nematodes (reviewed by Lilley *et al.*, 2012) and even parasitic plants (Alakonya *et al.*, 2012) is well described, nothing is known about the transfer of small RNAs from plant cells into fungal cells. The plant—fungal interface, which is comprised of the fungal haustorial plasma membrane (HPM), the fungal cell wall (HCW), the extrahaustorial matrix (EHMx), the extrahaustorial membrane (EHM) and the plant plasma membrane (PPM) (Figure 1; and Micali *et al.*, 2011; Voegele and Mendgen, 2003), is the primary site for plant—fungal recognition. Uptake of nutrients into the pathogen, delivery of enzymes and toxins into plant cells, secretion of fungal effector proteins and plant antimicrobial molecules (e.g. PR proteins) and biogenesis of cell surface sensors for mutual detection also occur at this interface (Casadevall *et al.*, 2009; Lu *et al.*, 2012; Micali *et al.*, 2011; Wang and Dong, 2011). Because most plant

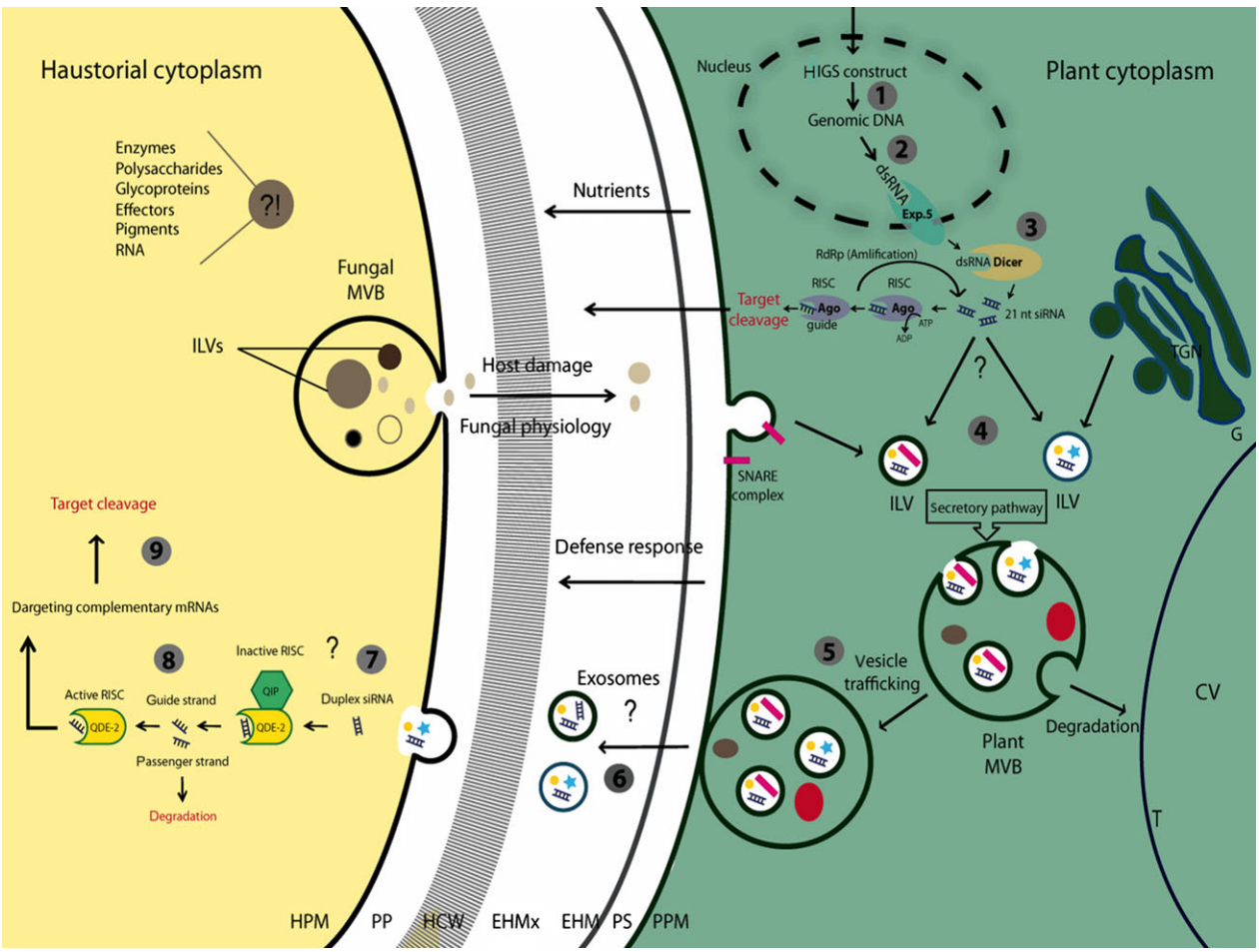

Figure 1 Plant–fungal cellular interface. A potential siRNA translocation pathway is indicated (1–9). A plant-induced gene silencing (HIGS) construct is integrated (1) and transcribed in dsRNA (2). The precursor dsRNA is translocated into the cytoplasm, probably by support of a nuclear pore protein, such as exportin 5 (Exp5), where it is loaded and processed by ribonuclease III (RNaseIII) enzymes, called Dicer (3). The resulting siRNA duplexes are either delivered to the plant's RNAi machinery or are incorporated as duplexes into intraluminal vesicles (ILVs) (4) that originate either from Golgi body (G) via trans-Golgi network (TGN) or from membrane endocytosis, respectively. The ILVs, consisting of several cargos, are internalized by multivesicular bodies (MVBs) that enter the secretory pathway. Cargo of MVBs is often destined for degradation following fusion with lytic vacuoles (shown on the right CV, central vacuole; T, tonoplast). Other cargos (e.g. defence-related proteins, blue star and yellow circle), destined for secretion, follow a retrograde vesicle trafficking pathway (5). MVBs fuses to the PPM followed by subsequent release of ILVs (now called exosomes) (6). Exosomes cross the cellular interface, entering the fungal cell and release their cargo, possibly including plants siRNAs (process unknown) (7). The siRNAs may subsequently enter the fungal RNAi machinery, where they are wrenched by the argonaute protein QDE-2 (quelling deficient-2), while the passenger strand is removed by the exonuclease QIP (8) (Maiti *et al.*, 2007). The guide strand remains in the RNA-induced silencing complex (RISC) which is activated and targets complementary mRNAs, resulting in degradation and gene silencing, respectively (9) (reviewed by Chang *et al.*, 2012). HCW, haustorial cell wall; HPM, haustorial plasma membrane; PP, periplasm; EHMx, extrahaustorial matrix; EHM, extrahaustorial membrane; PPM, plant plasma membrane; PS, paramural space; siRNAs, small interfering RNAs. Unknown and speculative processes are indicated by question marks (?).

pathogens are extracellular, the plant possesses a specialized secretion system which enables delivery of defence molecules to the site of attack. This secretion pathway mediates the transport of low molecular weight compounds required for immune responses, for example cell surface proteins, which reach their cellular destination via exocytosis (Bednarek *et al.*, 2010), and it is involved in synthesizing the EHM. This latter function indicates that the plant is actively exporting many molecules to the plant—fungal interface, possibly including siRNAs. Translocation of sRNAs via exosomes might require membrane-associated receptors for attachment at the fungal cell layers and for further entry into the fungal RNAi machinery. However, alternative routes for siRNA delivery also may exist, including (i) converted bidirectional trafficking of RNA species across the plant—fungal cellular interface via several exocytic/endocytic receptors; (ii) uptake during fungal nutrient acquisition via specific transporters (Divon and Fluhr, 2007; Voegele and Mendgen, 2003); or (iii) passive crossing via trans-cell wall diffusion or through various transmembrane channels or pores. The latter could be a reasonable possibility if the siRNA size is sufficiently small (Casadevall *et al.*, 2009).

Concluding remarks

Biotic stress disproportionately affects farm productivity around the world with immense annual yield losses. *In planta* mediated RNAi has promising potential for pest and disease control. A great number of basic research studies have enabled the rapid increase of knowledge in dsRNA-mediated silencing of target genes. Whereas the first investigations focused on the use of model organisms, it is now becoming possible to apply this knowledge towards modifying specific traits in agriculturally relevant crop plants. In addition to metabolic engineering and HIGS-mediated enhancement of disease resistance, RNAi strategies may be used to improve food safety by controlling the growth of phytopathogenic, mycotoxin-producing fungi. More research is required to optimize practical application strategies and to assess safety aspects; work to resolve these issues is ongoing and, when fully developed, should allow HIGS approaches to at least partly replace traditional chemical protection measures.

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Conflict of interest

No conflict of interest.

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