RNA interference in plant parasitic nematodes: a summary of the current status

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(*Received 17 August 2011; revised 10 October 2011; accepted 26 October 2011; first published online 5 January 2012*)

SUMMARY

RNA interference (RNAi) has emerged as an invaluable gene-silencing tool for functional analysis in a wide variety of organisms, particularly the free-living model nematode *Caenorhabditis elegans*. An increasing number of studies have now described its application to plant parasitic nematodes. Genes expressed in a range of cell types are silenced when nematodes take up double stranded RNA (dsRNA) or short interfering RNAs (siRNAs) that elicit a systemic RNAi response. Despite many successful reports, there is still poor understanding of the range of factors that influence optimal gene silencing. Recent *in vitro* studies have highlighted significant variations in the RNAi phenotype that can occur with different dsRNA concentrations, construct size and duration of soaking. Discrepancies in methodology thwart efforts to reliably compare the efficacy of RNAi between different nematodes or target tissues. Nevertheless, RNAi has become an established experimental tool for plant parasitic nematodes and also offers the prospect of being developed into a novel control strategy when delivered from transgenic plants.

Key words: RNA interference, RNAi, nematode, plant parasitic, gene silencing, efficacy, resistance.

INTRODUCTION

Plant parasitic nematodes occur globally and have a severe economic impact on agriculture through the infestation of agronomically important crops. Current methods of control, such as nematicides or crop rotations are inadequate and can have adverse environmental or economic effects (Lilley *et al*. 2011). The most economically important species are the sedentary endoparasites of the genus *Meloidogyne* (root-knot nematodes) and the cyst nematodes *Heterodera* and *Globodera*. Sedentary endoparasitic nematodes induce the formation of specialised feeding sites in plant roots, which support the development and reproduction of nematodes throughout the life cycle (Davis *et al*. 2004). The syncytia induced by cyst nematodes and giant cells formed by root-knot nematodes have a shared function in nutrient supply, but differ in their structure and the mechanism of their establishment. Syncytia are initiated by second stage juvenile nematodes (J2) from a single procambial, pericycle, endodermal or cortical parenchyma cell and expand into or along the vascular cylinder through the dissolution of adjacent cell walls. This results in the formation of a single hypertrophied multinuclear cell complex, which can contain up to 200 cells and is in close contact with xylem and phloem elements (Sobczak and Golinowski, 2009). In contrast, J2 of *Meloidogyne* spp. select up to 12 parenchymal cells within the stele that are induced to undergo mitosis without concomitant cytokinesis to form large, multinucleate giant cells from which the nematode feeds in turn (Berg *et al*. 2009). The formation of syncytia and giant cells requires substantial re-programming of root cell development, which arises from extensive changes in host gene expression (Gheysen and Fenoll, 2002). This process is triggered by secretions from the nematode pharyngeal gland cells that are introduced into the initial feeding cells via the nematode stylet. The establishment and maintenance of feeding sites are fundamental to the survival of endoparasitic nematodes. This makes them an obvious target of interest for novel control strategies and much research in the field of plant nematology is directed towards understanding the basis for their induction and development. A number of molecular approaches have elucidated some of the processes occurring during feeding site development (Gheysen and Mitchum, 2009) and the nematode effector proteins that may play a role in this (Rosso and Grenier, 2011). However, there are still many aspects of the complex host-parasite interaction that are not fully understood. The challenges of understanding gene function in plant parasitic nematodes that lack specific mutants and transformation systems, combined with the need for effective control strategies, led to the application of RNA interference (RNAi) in these nematodes. Since it was first demonstrated that RNAi could be used to silence genes in J2 cyst nematodes (Urwin *et al*. 2002) double-stranded RNA (dsRNA) has been delivered to a range of plant parasitic nematode species both *in vitro*, as a tool for

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Parasitology (2012), **139**, 630–640.
doi:10.1017/S0031182011002071

functional genomics, and *in planta* as a strategy for transgenic control. Despite the successful silencing of a range of genes there are a number of limitations of RNAi in plant parasitic nematodes that mean the high-throughput screens used to identify gene function in *Caenorhabditis elegans* are likely to be an unrealistic expectation. Variable results have been reported both in terms of gene silencing efficiency and resulting phenotypic effects. Differences in the methods used to deliver RNAi *in vitro* make it difficult to compare results between studies and thus determine those factors most crucial for achieving successful RNAi. The *in planta* delivery of RNAi to feeding nematodes has been reported to affect the successful establishment and/or development of cyst and root-knot nematodes. This offers the prospect of transgenic delivery of dsRNA from the feeding cell to target specific, essential nematode genes as a novel means for plant parasitic nematode control. However, the level of resistance achieved so far using this technique has been variable and may differ between cyst and root-knot nematodes.

Here, we provide an overview of both the *in vitro* and *in planta* RNAi studies carried out to date for plant parasitic nematodes, identify aspects of the technology that would benefit from further, systematic investigation or optimisation and assess the potential for RNAi to be a more widely used tool than at present.

Mechanism of RNAi

Homology-dependent transcriptional gene silencing by RNAi was first described in *C. elegans* (Fire *et al.* 1998) and has since been demonstrated, with varying degrees of efficacy, in a range of eukaryotes (for reviews see e.g. Silva *et al.* 2004; Brodersen and Voinnet, 2006; Perrimon *et al.* 2010). The mechanisms of RNAi in *C. elegans* are described in detail elsewhere in this Special Issue. Briefly, RNAi occurs when double-stranded RNA (dsRNA) is recognised by an organism as foreign, triggering a chain of processes in which both the dsRNA and its mRNA homologue are degraded, leading to sequence-specific silencing. The basic RNAi pathway can be considered a two-step process (Sontheimer, 2005). In the first stage, dsRNA is cleaved by the RNaseIII enzyme, Dicer, into 21–23 bp RNA duplexes called small interfering RNAs (siRNAs). The primary siRNAs are guided to the multi-component RNA-induced silencing complex (RISC) and in the second stage, the strands separate and only the antisense strand is incorporated into the active RISC. Complementary base pairing with the target transcript leads to endonucleolytic cleavage of the mRNA by a member of the Argonaute family of proteins (Grishok, 2005). The cleaved mRNA is subsequently degraded by exonuclease activity leading to a gene silencing effect.

The potency and persistence of RNAi in *C. elegans* can be attributed to an amplification step that is accompanied by transitive RNAi in which the silencing effect spreads mainly upstream along the target RNA strand from the primary trigger sequence. This is achieved by the unprimed synthesis of a distinct class of secondary siRNAs by an RNA-dependent RNA polymerase (RdRP) that may be recruited to the RNA template by guide primary siRNAs (Pak and Fire, 2007; Sijen *et al.* 2007). Secondary siRNAs are more abundant than primary siRNAs. They also induce silencing more efficiently; possibly due to their association with a specific class of Argonaute proteins, in particular CSR-1 that has a prominent Slicer activity (Aoki *et al.* 2007).

RNAi IN PLANT PARASITIC NEMATODES

Although RNAi silencing effects, triggered by either dsRNA or siRNAs, have been observed in plant parasitic nematodes, the molecular details of the pathways have not been elucidated. This situation may improve as genome sequence becomes available for more species and potential pathway components are identified based, at least initially, on homology to well-characterized *C. elegans* genes. Nevertheless, our inability to transform plant parasitic nematodes or to generate mutant lines will limit the detailed molecular characterization likely to be achieved. Thus our current insight into the mechanism of RNAi in plant parasitic nematodes relies mainly on the observed outcomes of the various *in vitro* and *in planta* experiments that have been reported. Interpretation of these results requires careful analysis of the experimental detail as a range of variables is likely to influence gene-silencing success. The majority of published studies report positive outcomes, with negative results remaining largely anecdotal. Whilst this is understandable, it may limit the utility of information that can be drawn from the literature.

There is now published data for the successful *in vitro* RNAi targeting of more than 40 plant parasitic nematode genes representing nine species within five genera. The majority of studies have been carried out with the sedentary endoparasitic cyst or root-knot nematodes but genes of the migratory endoparasites *Radopholus similis* (Haegeman *et al.* 2009) and *Bursaphelenchus xylophilus* (Park *et al.* 2008; Cheng *et al.* 2010; Kang *et al.* 2011; Li *et al.* 2011) have also been successfully targeted. These studies allow a number of factors to be considered when assessing the efficiency of RNAi in plant parasitic nematodes.

In vitro *delivery method*

In *C. elegans* dsRNA can be delivered directly into the worm through microinjection, by feeding with *Escherichia coli* expressing the target gene dsRNA or

https://doi.org/10.1017/S0031182011002071 Published online by Cambridge University Press

by soaking worms in a solution of dsRNA (Kamath and Ahringer, 2003). Microinjection is technically difficult and has not been routinely achieved for plant parasitic nematodes. This, coupled with their obligatory parasitic nature, limits the options for *in vitro* delivery. They do not normally feed outside of the host plant and for sedentary plant parasitic nematodes, J2s and eggs are the only stages of the life-cycle that can survive outside the host root. The majority of *in vitro* studies have therefore been undertaken with these stages, using the soaking method of delivery. Soaking could potentially lead to uptake of dsRNA via a number of routes, including transcuticular, ingestion through the secretory/excretory pore or amphids that are exposed to the external environment. For J2s, oral ingestion via the stylet is considered the most likely route. Although J2 cyst and root-knot nematodes are non-feeding, the addition of a pharyngeal neurostimulant, such as octopamine, resorcinol, serotonin or carbamoylcholine chloride to the soaking solution has been used to induce dsRNA ingestion by J2s in a number of species including *Globodera pallida* (Urwin *et al*. 2002), *G. rostochiensis* (Chen *et al*. 2005), *Heterodera schachtii* (Vanholme *et al*. 2007), *H. glycines* (Urwin *et al*. 2002; Lilley *et al*. 2005*b*; Alkharouf *et al*. 2007; Bakhetia *et al*. 2007, 2008), *Meloidogyne incognita* (Bakhetia *et al*. 2005*a*; Rosso *et al*. 2005; Huang *et al*. 2006; Dubreuil *et al*. 2007; Shingles *et al*. 2007) and *M. javanica* (Adam *et al*. 2008; Gleason *et al*. 2008). In some studies, uptake of the dsRNA along the pharynx and intestinal tract has been monitored through either the co-ingestion of the fluorescent dye fluorescein isothiocyanate (FITC) (e.g. Urwin *et al*. 2002; Huang *et al*. 2006; Sukno *et al*. 2007) or by the use of fluorescently-labelled dsRNAs (e.g. Rosso *et al*. 2005; Adam *et al*. 2008). Although stimulants are frequently included to induce ingestion, adverse effects have occasionally been reported from their use. Adam *et al*. (2008) found that overnight incubation of *M. javanica* J2s in either 0·5% or 1% resorcinol was lethal, whereas 0·5 mg/ml carbamoylcholine chloride stimulated oral uptake over the same time period with no detrimental effects.

Addition of pharyngeal stimulants is not always necessary to achieve an RNAi effect in cyst and root-knot nematode J2s (Kimber *et al*. 2007; Dalzell *et al*. 2009, 2010a). FMRFamide-like peptides (FLPs) are a family of neuropeptides that modulate sensory and motor function in *C. elegans* and plant parasitic nematodes (Kimber and Fleming, 2005). *C. elegans flp* genes are refractory to RNAi, however silencing of five *flp* genes in *G. pallida* caused severe effects on nematode migration (Kimber *et al*. 2007). An effect on motility was observed in worms soaked for 24 h in 0·1 mg/ml dsRNA diluted in DEPC-treated water only. Knockdown of the target *flp* genes was confirmed by RT-PCR. The effect is not limited to dsRNA or to *Globodera* spp. as soaking in siRNAs targeting either *G. pallida flp-12* or *M. incognita flp-18* also resulted in RNAi-mediated silencing (Dalzell *et al*. 2010*a*). It was proposed that dsRNA/siRNA was gaining entry through the amphids, secretory/excretory pore or the cuticle. Urwin *et al*. (2002) reported the occasional presence of the dye FITC in the amphids of cyst nematode J2s following soaking in the absence of octopamine. FITC has also been observed in the amphids of *M. incognita* incubated in collagenase or M9 buffer (Rosso *et al*. 2005). The phenomenon of dye uptake by amphidial and phasmid neurons is well documented in *C. elegans* (Hedgecock *et al*. 1985) and other free-living nematodes (Srinivasan *et al*. 2008). A recent study has shown the potential for larger molecules to be taken up from the amphidial pouches of cyst nematodes and transported along chemosensory neurons. A fluorescently labelled peptide that binds nicotinic acetylcholine receptors was taken up from an aqueous environment by the primary cilia of chemoreceptive sensilla of *G. pallida* before undergoing retrograde transport to their neuronal cell bodies and a limited number of connecting neurons (Wang *et al*. 2011). dsRNA/siRNA may potentially be transported in a similar manner. The success of RNAi targeted to *flp* genes in the absence of pharyngeal stimulants in *G. pallida* was suggested to reflect an increased sensitivity of neuronal tissue to RNAi in plant parasitic nematodes. A *G. rostochiensis* gene encoding a secreted amphid protein of unknown function (*gr-ams-1*) was targeted by dsRNA (Chen *et al*. 2005). Although octopamine was included in the soaking solution, *gr-ams-1* was more susceptible to RNAi than a gland cell expressed endoglucanase. Possibly the two genes were being targeted via different routes, with more efficient dsRNA uptake from the amphids. Previous attempts to target other tissues of J2s with dsRNA in the absence of a pharyngeal stimulant failed to cause an RNAi effect. Soaking *H. glycines* and *G. pallida* in just dsRNA targeted to an intestinal cysteine proteinase did not reduce transcript abundance as determined by virtual Northern analysis (Urwin *et al*. 2002). Similarly, resorcinol was required for suppression of *16D10* transcript in the subventral pharyngeal glands of *M. incognita* (Huang *et al*. 2006).

RNAi effects have been achieved by soaking eggs of *Meloidogyne* spp. in either dsRNA or siRNA (Fanelli *et al*. 2005; Dalzell *et al*. 2010*b*). Eggs of *M. artiellia* contained within the gelatinous matrix were incubated in dsRNA targeted against a chitin synthase gene involved in the synthesis of the chitin layer in eggshells (Fanelli *et al*. 2005). The treatment resulted in a reduction in chitin synthase transcripts and a delay in the hatching of juveniles, along with reduced staining of chitin in the eggshells. This suggested that both the gelatinous matrix and the egg shells with their chitin layer are permeable to dsRNA. Two key factors in the processing of micro (mi)RNAs, the

https://doi.org/10.1017/S0031182011002071 Published online by Cambridge University Press

RNase III enzyme drosha and its cofactor pasha, were silenced in embryos of *M. incognita* by soaking undifferentiated eggs in siRNA solutions (Dalzell *et al.* 2010*b*) again showing that RNA can cross the egg shell. It remains to be seen if eggs of other species are similarly susceptible or if those of cyst nematodes would require pre-stimulation, as the permeability of the egg shell is known to change in preparation for hatching (Jones *et al.* 1998).

Migratory parasites that do not have an obligate sedentary phase can be targeted for RNAi at later stages of the life-cycle. Mixed stages of *R. similis* were incubated with dsRNA targeting a xylanase gene expressed only in adults. Octopamine was used to stimulate ingestion and reduction in transcript was correlated with subsequent reduced infection rates (Haegeman *et al.* 2009). A 24 h incubation was necessary for ingestion of FITC by almost 100% of soaked *B. xylophilus,* but octopamine was not required to achieve this (Cheng *et al.* 2010). This is the only plant parasitic nematode species for which non-soaking methods of dsRNA delivery have been evaluated *in vitro*. Soaking and electroporation of L2/L3 larvae resulted in similar RNAi effects for four essential genes but intestinal microinjection of adult females was found to be the most efficient delivery route, based on a reduced hatching rate in the F1 generation (Park *et al.* 2008).

A number of general factors are commonly recognised as improving the efficiency of RNAi by soaking. Many studies include 3 mM spermidine and 0·05% gelatin in the soaking buffer, as recommended for work with *C. elegans* (Maeda *et al.* 2001). Improved efficacy arising from the addition of spermidine has been confirmed for the cyst nematodes *H. glycines* (Sukno *et al.* 2007) and *G. rostochiensis* (Chen *et al.* 2005). Nematode samples are routinely agitated during soaking with some studies specifically demonstrating that this is essential for efficient uptake and viability of the nematodes (Adam *et al.* 2008; Cheng *et al.* 2010).

Characteristics of the inducing molecule

The majority of *in vitro* RNAi experiments carried out with plant parasitic nematodes have used dsRNA as the inducing molecule with sizes ranging from 42 bp (Huang *et al.* 2006) to >1000 bp (Urwin *et al.* 2002; Bakhetia *et al.* 2007). The discrepancies between studies in terms of the type of gene targeted, incubation time, dsRNA concentration and method for assessment of silencing make it difficult to draw meaningful conclusions as to either the optimum size of dsRNA or its preferred sequence characteristics. The few studies that compare different dsRNAs targeting the same gene may shed some light on the matter. *Gp-flp-6* of *G. pallida* was silenced by both a 316 bp dsRNA and its 227 bp 3′ region, with the shorter molecule consistently proving more potent. The 88 bp 5′ region was insufficient to induce any silencing effect (Kimber *et al.* 2007). Possibly the position within the targeted gene was more important than the length of the dsRNA as both 271 bp and 42 bp dsRNAs covering the full-length transcript or the coding region of the oesophageal gland peptide 16D10, led to >90% transcript reduction in *M. incognita* J2 (Huang *et al.* 2006). A 267 bp dsRNA targeting the 3′ region of the *H. glycines pel-1* pectate lyase transcript induced more potent silencing than a 285 bp dsRNA targeted to the 5′ region of the same gene (Sukno *et al.* 2007) and a similar effect was reported for two dsRNAs homologous to 5′ and 3′ regions of a xylanase gene from *R. similis* (Haegeman *et al.* 2009). In this case, no silencing or significant phenotypic effect was observed with the dsRNA targeting the 5′ catalytic domain.

More recently, synthetic 21 bp siRNAs were reported to induce efficient silencing of *G. pallida* and *M. incognita flp* genes in J2 worms (Dalzell *et al.* 2010*a*). As with dsRNAs, efficacy of individual siRNAs targeting different regions of the same transcript was highly variable. This was the first example of siRNAs being used as silencing triggers in plant parasitic nematodes and was followed by further evidence of their efficacy when drosha and pasha transcripts were subjected to successful knockdown in eggs of *M. incognita* (Dalzell *et al.* 2010*b*). The use of siRNAs for functional analysis has the potential advantage of increasing target specificity and thus minimising off-target effects or allowing selective silencing of gene-family members. However, there is currently insufficient knowledge of the RNAi mechanisms of plant parasitic nematodes to determine if the production of secondary siRNAs by RdRPs might compromise this aspect.

Off-target and non-specific effects

Use of siRNAs as RNAi triggers was explored partly as a way of circumventing problems of non-specific phenotypic effects observed with high soaking concentrations of long dsRNAs (Dalzell *et al.* 2009). After the initial demonstrations of RNAi in plant parasitic nematodes, most studies have included a control incubation with non-nematode dsRNA to assess any effects that may be unrelated to specific gene silencing. A green fluorescent protein (*gfp*) sequence is commonly used, but other controls have been soybean Rubisco subunit (Alkharouf *et al.* 2007), plasmid vector sequence (Kang *et al.* 2011) and tomato chloroplast ribosomal protein (Kimber *et al.* 2007). Despite the use of non-target dsRNA concentrations of up to 4 mg/ml (e.g. Rosso *et al.* 2005) there were, until recently, no reports of any adverse phenotypic or gene-silencing effects related to these non-nematode dsRNAs. Concentrations of control dsRNA >2 mg/ml were found to be

https://doi.org/10.1017/S0031182011002071 Published online by Cambridge University Press

detrimental to *B. xylophilus* after a 24 hr incubation (Cheng *et al*. 2010). Dalzell *et al*. (2009) reported an unexpected aberrant phenotype and reduced motility for *M. incognita* J2s treated with nine different non-nematode derived dsRNAs (including *gfp*) at a concentration of 0·1 mg/ml for 24 hrs. An increased concentration of 1 mg/ml elicited a similar response after 4 hrs. The same phenotypic effect was observed in *G. pallida*, although the concentration of non-nematode dsRNA required to induce a phenotype was 10-fold higher (1 mg/ml dsRNA for 24 h). No pharyngeal stimulants were used in the experiments. Despite the phenotypic effects, there was no general impact on transcript abundance in either nematode, in agreement with previous studies. There was a significant recovery of normal phenotype 24 or 48 hrs after removal from the dsRNA solution. This may partly account for the lack of reported effects in earlier studies, where RNAi phenotypes were frequently assessed only after parasitism of plants, at least 10 days after treatment (e.g. Bakhetia *et al*. 2005*a*; Lilley *et al*. 2005*b*; Bakhetia *et al*. 2007; Dubreuil *et al*. 2007). The study of Dalzell *et al*. (2009) underlined the limited extent of our knowledge regarding the mechanisms of RNAi in plant parasitic nematodes. One hypothesis for the observed effect is that rate-limiting components of the RNAi pathway become saturated, thus inhibiting processing of endogenous small RNAs and affecting normal gene regulation.

Unexpected, but potentially related effects were reported when *H. glycines* was exposed simultaneously to two distinct dsRNAs aimed at silencing two different genes expressed in the dorsal pharyngeal gland cell (Bakhetia *et al*. 2008). Contrary to expectation, no additive or synergistic effects were observed and phenotype penetrance was actually reduced for four genes when targeted in combination rather than individually. This result was consistent with reduced transcript suppression following the combinatorial RNAi treatment and was suggested to reflect competition between siRNAs for RISC binding. An unexpected increase in transcript abundance for the dorsal gland gene *dg13* when targeted in combination with a second gene, *dg14*, was also observed (Bakhetia *et al*. 2008).

Effect of incubation time

The duration of soaking in dsRNA/siRNA can have a considerable effect on the efficacy of RNAi. RNAi effects have been observed following exposure of preparasitic J2 nematodes to dsRNA for time periods ranging from 1 hr to 7 days with 16–24 hrs becoming the most common soaking period used. In general, increasing the incubation time leads to greater ingestion/uptake and subsequent transcript reduction and enhanced phenotypic effects, particularly for cyst nematodes (Chen *et al*. 2005; Kimber *et al*. 2007; Sukno *et al*. 2007). A 4 hr incubation is sufficient to induce RNAi for some cyst nematode genes (Urwin *et al*. 2002; Lilley *et al*. 2005*b*) but soaking for 24 hr was necessary to induce silencing in *G. rostochiensis* (Chen *et al*. 2005). For RNAi of the *flp-12* gene of *G. pallida*, incubation in dsRNA for 12 hr or more was required to elicit an aberrant phenotype. A dramatic increase in the number of worms displaying an RNAi effect occurred between 18 and 24 hr when almost complete inhibition of migration was observed. For other *flp* genes, prolonged incubations of 2 or 7 days were required to achieve this maximum effect (Kimber *et al*. 2007). It is likely that the required incubation times are related to the speed and efficiency of uptake in different nematodes and different experimental systems. For *Meloidogyne* spp. effective silencing has been consistently observed following a 4 hr incubation of J2 in dsRNA (Bakhetia *et al*. 2005*a*; Rosso *et al*. 2005; Huang *et al*. 2006; Shingles *et al*. 2007). In one study with *M. incognita*, just 1 hr incubation was required to cause 90% transcript reduction of a glutathione-S-transferase gene, with a subsequent reduction in the number of egg masses produced by treated nematodes (Dubreuil *et al*. 2007). Bakhetia *et al*. (2005*a*) noted that ingestion of FITC by J2 *M. incognita* after 4 hrs was considerably more extensive than that observed for cyst nematodes over the same time period, with fluorescence frequently visible along the entire intestinal lumen. Possibly the combination of two neurostimulants (resorcinol and serotonin) used by Dubreuil *et al*. (2007) further enhanced the rate of ingestion.

Effect of dsRNA/siRNA concentration

RNAi soaking experiments with plant parasitic nematodes typically use dsRNA at concentrations from 1–5 mg/ml. Only a few studies have investigated concentration-dependent effects in an attempt to optimise *in vitro* RNAi techniques. *G. pallida* J2 were incubated for 24 hr in a 10-fold serial dilution of dsRNA targeting the *flp-12* gene, with concentrations from 0·1 mg/ml to 1 pg/ml. (Kimber *et al*. 2007). The migratory ability of nematodes, assessed as an indication of RNAi efficacy, was profoundly impaired at 0·1 mg/ml, with a significant effect still evident at 0·1 μg/ml. Nematodes incubated in $\leqslant$10 ng/ml dsRNA were not significantly affected. Later experiments targeting the same gene found the transcript suppression induced by 21 bp siRNAs to be even more potent with motility still significantly inhibited at 10 ng/ml siRNA. A similar potency was observed when the *M. incognita flp-18* gene was silenced by siRNA (Dalzell *et al*. 2010*a*). In all cases there appears to be a threshold concentration below which a rapid decrease in efficacy is observed. Conflicting data arose from experiments to silence two pharyngeal gland genes of *H. glycines*. Whilst

https://doi.org/10.1017/S0031182011002071 Published online by Cambridge University Press

5 mg/ml of the most potent dsRNA caused greater silencing of *Hg-pel-1* than 2·5 mg/ml, the effect was reversed using a less potent dsRNA targeted at the same gene. Transcript suppression of *Hg-4E02* was also greater with 2·5 mg/ml dsRNA than with 5 mg/ml (Sukno *et al*. 2007).

In the light of these studies, and considering the non-specific phenotypes reported when using higher concentrations of non-nematode dsRNAs, it would seem prudent for further investigations to be carried out for a range of nematodes, target genes and tissue types. It is possible that lower concentrations of RNAi inducers could be routinely used in functional studies, providing reassurance that non-specific effects were minimised.

Persistence of the RNAi effect

Following removal of the RNAi trigger, the timing and duration of transcript suppression seem highly variable, possibly relating to target gene expression pattern, and initial level and turnover rate of the endogenous transcript. In most studies, transcript depletion tends to be assessed immediately after the soaking period, however this may not be when maximum silencing occurs. A pharyngeal gland-expressed calreticulin (*mi-crt*) gene of *M. incognita* displayed maximum transcript repression 20 hr after removal from a 4 hr exposure to dsRNA whereas transcript of polygalacturonase (*mi-pg-1*) was unaffected at the same time point and displayed optimal silencing after 44 hr. The transcript level of both genes had returned to normal after a 68 hr recovery period (Rosso *et al*. 2005). For a third gene (*Mi-gsts-1*) expressed in the same cells, silencing was detectable immediately after the 1 hr dsRNA incubation and remained stable for 28 hrs, with transcript returning to normal levels after 48 hrs (Dubreuil *et al*. 2007). Similar results have been observed for cyst nematodes. Transcript repression of a β-1,4-endoglucanase was observed immediately following a 16 hr dsRNA treatment of *H. glycines* J2 and after a 5 day recovery period. Transcripts then increased unexpectedly at 10 days before returning to pre-treatment levels by 15 days after dsRNA exposure (Bakhetia *et al*. 2007). Reduced motility of treated J2s was used to monitor persistence of *Gp-flp-12* gene silencing. In this case, there was a small but insignificant recovery of phenotype after 24 hrs and a significant but not complete recovery after 6 days (Kimber *et al*. 2007). A delayed phenotypic effect may arise if the protein target is particularly stable. For instance, whilst immediate suppression of the *Mi-gsts-1* transcript was apparent, GST enzyme activity was still present after 16 hrs and was not abolished until 24 hr post-treatment (Dubreuil *et al*. 2007).

In *C. elegans*, an RNAi phenotype can be inherited; however, only when germline genes are targeted does the RNAi effect extend beyond the F1 generation (Grishok *et al*. 2000). RNAi-induced silencing of a germline expressed green fluorescent protein (*gfp*) transgene was maintained over 80 generations (Vastenhouw *et al*. 2006). It appears that RNAi effects can also be inherited in plant parasitic nematodes without the constraint of germline expression. The *M. javanica* gene *Cg-1* encodes a putative avirulence factor, with nematodes expressing this gene unable to develop on tomato plants carrying the *Mi-1* resistance gene. Following treatment of *M. javanica* J2s with dsRNA, some individuals were able to multiply on resistant plants. Their progeny displayed increasingly successful reproduction over at least five generations whilst selection was maintained on resistant plants. The penetrance of the phenotype was reduced following growth on susceptible plants suggesting that the RNAi effect was lost in some progeny but persisted in the population due to the strong selection pressure (Gleason *et al*. 2008).

DETERMINATION OF AN RNAi PHENOTYPE

The obligate parasitic lifecycle of these nematode species poses a major challenge in determining an RNAi phenotype. The majority of studies treat J2s with dsRNA before allowing them to infect a host plant. Typically, the phenotype is determined by examining the number of nematodes that successfully establish an infection in the host, the subsequent fecundity of females or the proportion of cyst nematodes that develop as male or female. Unfavourable conditions, such as inadequate nutrition, increase the proportion of cyst nematodes developing as male (Lilley *et al*. 2005*a*). An increase in the male:female ratio occurred when RNAi was targeted against genes expressed in the pharyngeal gland cells of *H. glycines* (Bakhetia *et al*. 2007). This indicated that feeding site development was compromised in the RNAi treated nematodes. The limited number of phenotypes that can be identified post infection may result in subtle phenotypes going unnoticed (Lilley *et al*. 2007). Moreover, whilst a reduction in the number of nematodes indicates an important role for a target gene in establishing nematode infection, the exact function of the gene is not elucidated. Specifically targeted phenotypic analysis can be undertaken when the target gene has a putative function based on sequence homology and expression patterns. For instance, *flp* genes encode neuropeptides involved in motor activities, thus an assessment of migratory ability is an appropriate test for effective RNAi of these genes (Kimber *et al*. 2007; Dalzell *et al*. 2010*a*). Silencing of *M. artiellia* chitin synthase was assessed by staining chitin in egg shells and testing the hatching rate of treated eggs (Fanelli *et al*. 2005). A detoxification role was proposed for *B. xylophilus* acetylcholinesterase *ace-3* when

https://doi.org/10.1017/S0031182011002071 Published online by Cambridge University Press

silencing of this gene increased susceptibility to xenobiotic compounds (Kang *et al*. 2011).

Obviously, not all instances of successful gene silencing will necessarily lead to an observable phenotype. The gene may not have an essential function in the stage targeted or there may be functional redundancy between similar genes that have insufficient sequence conservation to be silenced by the RNAi trigger. It has yet to be determined if the phenotype arising from *in vitro* RNAi is always a true indication of *in vivo* gene function, particularly when non-specific effects of dsRNA need to be considered. Soaking *M. incognita* with dsRNA targeting a signal peptidase complex of the nematode caused a reduction in established nematodes at 14 days post infection when aduki beans were challenged with treated juveniles (Charlton *et al*. 2010). When tomato hairy roots were subsequently produced to deliver the same dsRNA *in planta* an additional effect on nematode development was observed. This difference may be related to the dsRNA delivery method, with *in planta* delivery allowing for gene silencing at later stages of development after an *in vitro* effect may have been lost.

DELIVERING RNAi *IN PLANTA* AS A STRATEGY FOR CROP PROTECTION

Whilst the majority of RNAi studies with plant parasitic nematodes have used *in vitro* delivery methods, an increasing number of experiments are utilising *in planta* production of dsRNA to target genes of feeding nematodes. The feeding sites of sedentary plant parasitic nematodes provide an ideal route to deliver dsRNA to nematodes. Plants can be engineered to express dsRNA molecules by cloning the sense and antisense cDNA sequences of the target gene, separated by an intron or spacer, into a binary vector under the direction of a plant promoter. The transcribed RNA forms into a self-complementary hairpin structure with either the spacer region forming a loop or, more commonly, the intron sequence being removed by splicing. The dsRNA can then be processed by the plant DICER enzymes into siRNA (Lilley *et al*. 2007). It is not clear if the nematode ingests these plant-derived siRNAs or the unprocessed dsRNA, which is subsequently processed into siRNAs by nematode DICER enzymes (Gheysen and Vanholme, 2007). As the nematode feeds from the plant throughout its development, this enables continual delivery of dsRNA/siRNA throughout the nematode life cycle. One use of this technique is for functional genomic analysis, particularly of the so-called "parasitism genes" whose products are secreted into the host plant via the stylet and are involved in initiation and maintenance of the feeding site. Not surprisingly, given the current lack of effective control measures, host plant-delivered RNAi is also being explored by a number of groups as a novel resistance strategy for plant parasitic nematodes.

Efficacy of host plant-delivered RNAi

To date, the approach has shown potential against both cyst and root-knot nematodes, despite early concerns that the feeding tubes of sedentary endoparasitic species would prevent the ingestion of dsRNA and possibly siRNAs (Bakhetia *et al*. 2005*b*).

Some proof-of-concept studies have shown extremely promising results; a high level of resistance to root-knot nematode was achieved by targeting a parasitism gene expressed in the subventral gland cells of *M. incognita* (Huang *et al*. 2006). dsRNA complementary to the *16D10* gene was expressed in transgenic *A. thaliana* resulting in a significant reduction [63–90%] in the number of galls and a corresponding reduction in total egg production. The high level of homology between the *16D10* sequences of different *Meloidogyne* species led to broad-range resistance against *M. incognita, M. javanica, M. arenaria* and *M. hapla*. Almost complete resistance to *Meloidogyne* infection was reported in tobacco plants expressing dsRNA corresponding to a nematode-specific collagen gene (patent application by Michaeli *et al*. 2005), splicing factor or integrase (Yadav *et al*. 2006) and of four *M. incognita* genes targeted from transgenic soybean roots, two reduced gall number by >90% (Ibrahim *et al*. 2011). Plants that expressed dsRNA targeting a *M. incognita* tyrosine phosphatase gene supported 92% fewer galls than control plants with a similar reduction reported when a mitochondrial stress-70 protein precursor was targeted. Of the four genes examined, the lowest reduction in gall number was 57% when L-lactate dehydrogenase was targeted. In all cases, female nematodes that did develop within the galls were smaller than the controls (Ibrahim *et al*. 2011). These latter studies all targeted genes presumed to be essential for nematode development based on the lethal RNAi phenotypes of their *C. elegans* orthologues. This may explain the potent phenotypic effects observed.

Partial resistance to *Meloidogyne*, based on a reduction in established nematodes, resulted from targeting either a dual oxidase gene (*Miduox*) with a probable role in cuticle formation or a subunit of signal peptidase (*Mispc3*), a protein complex required for the processing of secreted proteins. Crossing transgenic *Arabidopsis* lines expressing the two separate dsRNAs provided higher levels of resistance to *M. incognita* than either parent plant. The number of nematodes able to establish on the F2 plants was not different from the parental lines, however the proportion of nematodes reaching the adult female stage was lower when both dsRNAs were expressed together indicating further impediment of nematode development (Charlton *et al*. 2010). Not all

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host-delivered RNAi results in a resistance phenotype of *Meloidogyne*. Partial silencing of a putative transcription factor of *M. javanica* (*MjTis11*) did not significantly affect either nematode development or fecundity, perhaps because the degree of silencing was insufficient or the gene product does not have an essential, non-redundant role (Fairbairn *et al*. 2007).

Cyst nematodes can also be targeted by expression of dsRNAs in plant roots. Nematodes infecting transgenic soybean plants expressing a hairpin construct targeting the major sperm protein (MSP) gene of the soybean cyst nematode *H. glycines* displayed an overall 68% reduction in egg production (Steeves *et al*. 2006). However, the experimental set-up precluded clear interpretation of these results (Gheysen and Vanholme, 2007). The *in planta* delivery of dsRNA from *Arabidopsis* to feeding *H. schachtii* reduced transcript abundance of a range of targeted parasitism genes in the pharyngeal gland cells although in some cases this reduction was only small and either non-significant or non-specific (Patel *et al*. 2008, 2010; Sindhu *et al*. 2009). For six of eight genes tested there was a significant reduction in female numbers of between 23–64% but with considerable variation between lines for some constructs (Sindhu *et al*. 2009). Variable, non-significant effects were also observed when a fibrilin gene of *H. glycines* was targeted from chimeric soybean plants whilst in the same study, RNAi of a coatomer subunit of this nematode resulted in a significant reduction in egg production (Li *et al*. 2010a). Soybean composite plants engineered to silence either of two ribosomal proteins, a spliceosomal protein or synaptobrevin, of *H. glycines* by RNAi resulted in 81–93% fewer females developing on the transgenic roots (Klink *et al*. 2009). A similarly high reduction in egg production was achieved by targeting *H. glycines* mRNA for splicing factor *prp-17* or an uncharacterized gene *cpn-1* from soybean roots (Li *et al*. 2010*b*).

The success of RNAi *in planta* can depend on a number of factors, such as the use of a promoter able to drive a sufficiently high level of dsRNA expression and the pattern of expression of the target gene. There is evidence to suggest that a high level of hairpin RNA expression in feeding cells is required to produce gene silencing in feeding nematodes (Fairbairn *et al*. 2007). Although based on the analysis of a relatively small number of studies, it appears that *in planta* RNAi elicits more potent phenotypes when it targets nematode genes involved in essential cellular processes. The only parasitism gene target to confer a similarly high level of control is the *16D10* peptide of *M. incognita*. Possibly the majority of parasitism genes have combinatorial roles in the parasitic interaction or have redundant functions as members of gene families. Whilst off-target silencing in the nematode may not be problematic for RNAi-based resistance strategies, a lack of silencing specificity may influence results when RNAi is used as a tool for functional analysis. In contrast to *in vitro* studies, few of the *in planta* experiments (Sindhu *et al*. 2009; Charlton *et al*. 2010; Patel *et al*. 2010) have included a non-nematode dsRNA control as standard. There is also the additional constraint that expressed dsRNAs should have no homology with sequences in the host plant.

Confirmation of an in planta *RNAi effect*

Although a number of studies have reported a phenotype for nematodes parasitizing plants expressing dsRNAs, it has proved difficult to clearly demonstrate that the phenotype is due to an RNAi effect. Most commonly, qRT-PCR has been used to demonstrate a decrease in transcript level of the target gene, although the desired down-regulation has not always been detected (e.g. Patel *et al*. 2008). Ibrahim *et al*. (2011) reported that control gene transcripts were also reduced in nematodes parasitising dsRNA-expressing plants, this may reflect the fact that the few nematodes that had succeeded in developing to the point of assay were starting to lose viability. Given that only five studies have shown the accumulation of target gene siRNAs in the plant roots (Huang *et al*. 2006; Steeves *et al*. 2006; Fairbairn *et al*. 2007; Sindhu *et al*. 2009; Li *et al*. 2010*b*), the route by which gene silencing occurred cannot be determined. Other studies have used RT-PCR of the intron or spacer region of the hairpin construct to demonstrate the presence of unprocessed transcript (e.g. Patel *et al*. 2008, 2010). Charlton *et al*. (2010) detected low levels of dsRNA in transgenic lines by northern blot analysis but reported a failure to detect corresponding siRNAs, despite observing an RNAi phenotype in the nematodes.

In *C. elegans* and plants, primary siRNAs derived from cleavage of exogenous dsRNA initiate the *de novo* synthesis of secondary siRNAs from the target transcript. These molecules are more abundant than the primary siRNAs and highly effective in gene silencing. With *in planta* RNAi, the target transcript is not present in the plant cell and this amplification step cannot occur. Simultaneous expression of the nematode gene and its corresponding hairpin construct in the same transgenic plant may lead to a higher level of nematode resistance, especially if siRNAs are the main form of RNA ingested by the feeding nematodes. When an *Arabidopsis* line expressing the *Meloidogyne 16D10* transgene was crossed with a line expressing the *16D10* dsRNA, the F1 plants contained higher levels of *16D10* siRNAs (Huang *et al*. 2006).

CONCLUSIONS

Despite the increasing number of RNAi experiments and associated phenotypic effects reported for plant

https://doi.org/10.1017/S0031182011002071 Published online by Cambridge University Press

parasitic nematodes there is still poor understanding of all the factors that influence successful gene silencing. Recent *in vitro* studies have highlighted significant variations in the RNAi phenotype that can occur with different dsRNA concentrations, construct size and duration of soaking. Wide discrepancies in silencing have also been reported for the same experimental set-up carried out on different occasions (Haegeman *et al*. 2009). One may speculate that this situation could be more common than implied by the current body of published work. The community has also recently been alerted to the potential problems of phenotypic effects associated with non-nematode dsRNA/siRNAs. This emphasizes the need for more consistency in the methodology used for *in vitro* RNAi experiments. For example, the concentration of dsRNA used ranges from 0·1–10 mg/ml. Such discrepancies in methodology thwart efforts to reliably compare the efficacy of RNAi between different nematodes or target tissues. Moreover, the effects of construct size on RNAi efficacy highlight the importance of matching the size of control and target dsRNA constructs and maybe selecting regions of genes with similar relative positions if possible.

Nevertheless, RNAi has become an established experimental technique in plant parasitic nematodes and seems more reliable than for many animal parasitic species (Geldhof *et al*. 2007; Viney and Thompson, 2008). The levels of plant resistance that have been reported with *in planta* RNAi signify an exciting prospect for transgenic nematode control. However, there is significant variation in the reported levels of resistance and the high level of specificity of RNAi means crops may only be resistant to one nematode species. This is a disadvantage given that soils are typically infected with multiple species of plant parasitic nematodes. Broader resistance could be achieved by targeting genes that are highly conserved among multiple species, as demonstrated by Huang *et al*. (2006), or stacking RNAi with other strategies of transgenic control. Elucidation of the RNAi pathway in plant parasitic nematodes will increase confidence in the RNAi effect. Whilst this remains some way in the future given the limited techniques available for functional molecular studies, the availability of plant parasitic nematode genome sequences (Abad *et al*. 2008; Opperman *et al*. 2008) is starting to aid our understanding. A recent bioinformatic study to identify orthologues of 77 *C. elegans* RNAi pathway proteins in 13 nematode species, including the root-knot nematodes *M. incognita* and *M. hapla*, concluded that none of the species had any particular RNAi effector deficiencies that were associated with reduced RNAi susceptibility (Dalzell *et al*. 2011). In the meantime, maybe the plant parasitic nematode community should consider following the lead of insect biologists. A public database has been created to bring together all information concerning RNAi experiments carried out with Lepidoptera species from both published and unpublished studies. Importantly, experiments producing negative results are also included and experimental details conforming to MIARE (Minimum Information Criteria for RNAi Experiments) specifications are available (Terenius *et al*. 2011).

FINANCIAL SUPPORT

This work was supported by the Biotechnology and Biological Sciences Research Council of the UK (CJL, grant number BB/F000642/1) and a BBSRC studentship to LJD.

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