9

CATHERINE J. LILLEY*, WAYNE L. CHARLTON*, MANJULA BAKHETIA AND PETER E. URWIN*

THE POTENTIAL OF RNA INTERFERENCE FOR THE MANAGEMENT OF PHYTOPARASITIC NEMATODES

Centre for Plant Sciences, University of Leeds, Leeds, LS2 9JT, UK

Abstract. RNA interference (RNAi) is a natural cellular phenomenon in which double stranded RNA (dsRNA) is recognised as foreign by virtue of its conformation and thus sets in motion a chain of events in which the dsRNA and its mRNA homologue are degraded. This leads to silencing of the targeted gene. First described for the microbivorous nematode *Caenorhabditis elegans*, RNAi has emerged as a powerful tool for investigating gene function in a range of organisms. Practical applications proposed for RNAi include the genetic improvement of crop plants to create novel resistance to plant pathogens. Recent studies have described the successful application of RNAi to plant parasitic nematodes. Key developments in the last year have demonstrated that *in planta* expression of a double-stranded RNA can target a gene of a feeding plant parasitic nematode, inducing a silencing effect. When the targeted gene has an essential function this leads to a level of nematode resistance, paving the way for the potential use of RNAi technology to control plant parasitic nematodes.

1. INTRODUCTION

Plant parasitic nematodes represent one of the major biotic constraints in world agriculture causing global yield losses estimated to be around US$70 billion in 1987 (Sasser & Freckman, 1987). Adjusting for inflation, this figure was revised to US$125 billion in 2003 (Chitwood, 2003). No recent, comprehensive surveys of nematode losses have been carried out and the real figures may be higher than this, as a lack of clear disease symptoms can lead some growers to underestimate yield loss. Yield reductions may also be wrongly attributed to the secondary diseases suffered by crop plants already weakened by nematode attack.

Nematodes of the order Tylenchida are responsible for the majority of crop damage. Agronomically important species include both migratory parasites such as *Radopholus* spp. and *Pratylenchus* spp. that feed sequentially from plant cells in a destructive manner, and the more specialised sedentary endoparasites. These nematodes each form a unique, biotrophic interaction with the host plant, modifying root cells to establish a permanent feeding site that provides a sustained

*These authors contributed equally to this review

A. Ciancio & K. G. Mukerji (eds.), Integrated Management and Biocontrol of Vegetable and Grain Crops Nematodes, 185–203.

source of nutrition. As shown in other chapters of this volume, the most economically important are the many species of the *Meloidogyne* genus (root-knot nematodes) and the *Heterodera* and *Globodera* genera of cyst nematodes. Root-knot nematodes have a very wide host range that potentially covers most flowering plants. Consequently they are responsible for a large part of the global yield loss to nematodes with the most widespread species, *M. incognita*, being possibly the single most damaging crop pathogen worldwide (Trudgill & Blok, 2001). Each individual cyst nematode species has a much narrower host range. The cyst nematode species that attack potato (*Globodera pallida* and *G. rostochiensis*), sugar beet (*Heterodera schachtii*) and soybean (*H. glycines*) are of particular economic importance (Lilley, Atkinson, & Urwin, 2005a).

2. CURRENT CONTROL MEASURES

Effective control of many nematode species is problematic as each of the current measures has aspects that limit its utility. Consequently a number of strategies are usually deployed in an integrated approach. Nematicides are widely used, but chemical control is often limited by factors such as grower preference, economic constraints or legislation to restrict the use of harmful chemicals. Nematicides impose substantial costs and are often not economic for some crops. The compounds applied are amongst the most toxic and environmentally hazardous pesticides in widespread use. Some have already been withdrawn, due to their mammalian toxicity and environmental concerns.

Cultural systems of control are widely practised and the relatively narrow host ranges of cyst nematodes ensure that crop rotation is an effective means of partial control for these parasites. Long rotations are required, however, to decline populations of species such as *G. pallida* that can remain dormant for many years. (Phillips & Trudgill, 1998). Crop rotation is not a practical solution for controlling species with wide host ranges such as *Meloidogyne* spp. and *Rotylenchulus reniformis*.

The most sustainable method of nematode control, requiring no changes to existing cultural practices, is the use of resistant plants that suppress nematode reproduction. Although naturally resistant cultivars have been a commercial success for some crops, the approach is unable to control many nematode problems. No useful source of resistance has been identified for crop plants affected by a range of important nematode species, particularly the migratory ectoparasites (Starr, Bridge, & Cook, 2002). Resistance also tends to be highly specific to certain nematode species or even pathotypes and can be compromised by virulent nematode populations. For example, the natural resistance gene H1 present in potato cultivars such as Maris Piper is useful in controlling populations of *G. rostochiensis* in the United Kingdom (Atkinson, 1995). However, these cultivars do not control *G. pallida* which has now become the prevalent potato cyst nematode in the UK as a direct consequence of selection. The lack of a comparable single dominant natural resistance gene for *G. pallida* has resulted in emphasis on multi-trait quantitative resistance that is often overcome by virulent pathotypes. Only a limited number of crop plants have been identified with resistance to

Meloidogyne species and for many of these the resistance can be overcome by virulent biotypes (Hussey & Janssen, 2002).

2.1. Potential for Biotechnological Control of Plant Parasitic Nematodes

The limitations of conventional control measures provide an important opportunity for plant biotechnology to deliver effective and durable forms of nematode resistance. The approach has a number of advantages. Utilisation of the new crop varieties would not require other changes to agronomic practices and there would be a reduction in nematicide use and its associated toxicological and environmental risks. Resistant crops could be developed to control more than one nematode species, including pathotypes that display virulence to currently used sources of resistance.

Biotechnological approaches have already delivered useful levels of resistance for a range of plant parasitic nematode species. The most advanced strategy to date is the transgenic expression of plant proteinase inhibitors that act to impair function of nematode digestive enzymes. A gene encoding a rice cysteine proteinase inhibitor (cystatin), Oc-I, was engineered to have an enhanced inhibitory activity (Urwin, Atkinson, Waller, & McPherson, 1995). Expression of the engineered variant (Oc-IΔD86) in *Arabidopsis* plants was the first transgenic technology shown to work against both root-knot and cyst nematodes (Urwin, Lilley, McPherson, & Atkinson, 1997a). Using the same approach, resistance has also been demonstrated against *Rotylenchulus reniformis* (Urwin, Levesley, McPherson, & Atkinson, 2000), *Radopholus similis* infecting banana (Atkinson, Grimwood, Johnston, & Green, 2004), and the potato cyst nematode *Globodera*. For the latter species, the technology progressed to the stage of field trials where full resistance to *G. pallida* was achieved by stacking natural partial and transgenic resistance (Urwin, Green, & Atkinson, 2003).

Inhibitors of serine proteinases also have proven potential for the control of plant parasitic nematodes. The trypsin inhibitor sporamin inhibited development of *Heterodera schachtii* when expressed in sugar beet hairy roots (Cai et al., 2003) and a serine proteinase inhibitor expressed in wheat provided protection against the cereal cyst nematode *H. avenae* (Vishnudasan, Tripathi, Rao, & Khurana, 2005). In a different approach, peptides that disrupt chemoreception in cyst nematodes afforded protection from *G. pallida* when engineered to be secreted from potato roots (Liu et al., 2005).

RNA interference (RNAi) technology is now routinely used as an investigative tool for understanding gene function in a range of organisms including plant parasitic nematodes. This review will consider the utility of RNAi technology both to identify potential target genes for novel control strategies and to provide resistance to plant parasitic nematodes.

2.2. The RNAi Mechanism

RNAi is a fundamental mechanism for controlling the flow of genetic information first described, in work with *Caenorhabditis elegans,* by Andrew Fire and Craig Mello (Fire et al., 1998), who in 2006 won the Nobel prize in physiology or

medicine. As the mechanism has been elucidated it has become clear that it shares mechanistic similarities to post transcriptional gene silencing in plants (Jorgensen, Cluster, English, Que, & Napoli, 1996; Waterhouse, Graham, & Wang, 1998). The RNAi process has a role in guarding against viral and transposable elements in the genome (Voinnet, 2001; Waterhouse, Wang, & Lough, 2001). The RNAi mechanism recognises double-stranded RNA (dsRNA) that subsequently causes a chain of events in which both the dsRNA and its mRNA homologue are degraded, leading to sequence-specific, homology-dependent gene silencing. Since the discovery of RNAi the effect has been described in mammals (e.g. Hannon, 2002; Silva, Chang, Hannon, & Rivas, 2004; Zamore, Tuschl, Sharp, & Bartel, 2000), insects (e.g. Kennerdell & Carthew, 2000) and amphibians (Dirks, Bouw, Huizen, Jansen, & Martens, 2003; Li & Rohrer, 2006). Additionally, the phenomenon of quelling in *Neurospora crassa* may also prove to be mechanistically similar to mammalian RNAi (Cogoni & Macino, 2000; Forrest, Cogoni, & Macino, 2004). It is now widely accepted that the process is also an integral part of normal gene regulation processes (Voinnet, 2002). Several reviews are available that concern themselves with the intricacies of RNAi in mammalian, insect and plant systems and describe them in some depth (e.g. Brodersen & Voinnet, 2006; Denli & Hannon, 2003; Hammond, 2005; Qi & Hannon, 2005; Sen & Blau, 2006; Tomari & Zamore, 2005).

3. THE MECHANISM OF RNAi IN NEMATODES

The basic mechanism of RNAi in eukaryotic organisms appears to be conserved, although there are differences in the systemic nature and heritability of the effect. RNAi is triggered by dsRNA molecules that have homology with an endogenous gene. In *C. elegans*, any dsRNA longer than 100bp can induce silencing, with fragments in the range 500–1500 bp commonly used (Kaletta & Hengartner, 2006).

The RNAi pathway is comprised of two basic steps (Sontheimer, 2005). In the initiation phase the Dicer complex recognises the exogenous dsRNA molecules and cleaves the dsRNA in a processive, ATP-dependent manner into short (21–23 bp) interfering RNA duplexes (siRNAs) by way of its multidomain RNaseIII enzyme activity. The resulting siRNAs have 5' phosphate groups and 2 bp overhangs at their 3' ends. *Caenorhabditis elegans* has only one Dicer enzyme that functions in a complex with the dsRNA binding protein Rde-4, the PAZ-PIWI domain family protein Rde-1 and the Dicer-related helicase Drh-1 (Tabara, Yigit, Siomi, & Mello, 2002). Following dsRNA cleavage, it has been postulated that Rde-1 may bind the siRNAs to direct them to the second, effector phase of the RNAi pathway (Fig. 1) (Grishok, 2005).

The multicomponent RNA-induced silencing complex (RISC) degrades specific mRNAs in a targeted manner. During assembly of the active RISC, the siRNA duplex is unwound, the strands separate and only the anti-sense (guide) strand is incorporated into the RISC. Base pairing between the single-stranded siRNA and the complementary mRNA results in the activation of RISC complex that then recognises the target transcript. Endonucleolytic cleavage of the mRNA involves a member of the Argonaute family of proteins (Hammond, 2005) some of which have endonuclease activity in their PIWI domain. The identity of this key

RISC component is unknown in *C. elegans* but around 25 candidate Argonaute homologues exist (Grishok, 2005). The cleaved mRNA is subsequently degraded by exonuclease activity, leading to a gene silencing effect.

Other components of the *C. elegans* RISC have been identified, including an RNA binding protein Vig-1, and Tsn-1, a protein with a Tudor domain and five staphylococcal nuclease domains that may be responsible for the exonuclease activity of RISC (Caudy et al., 2003).

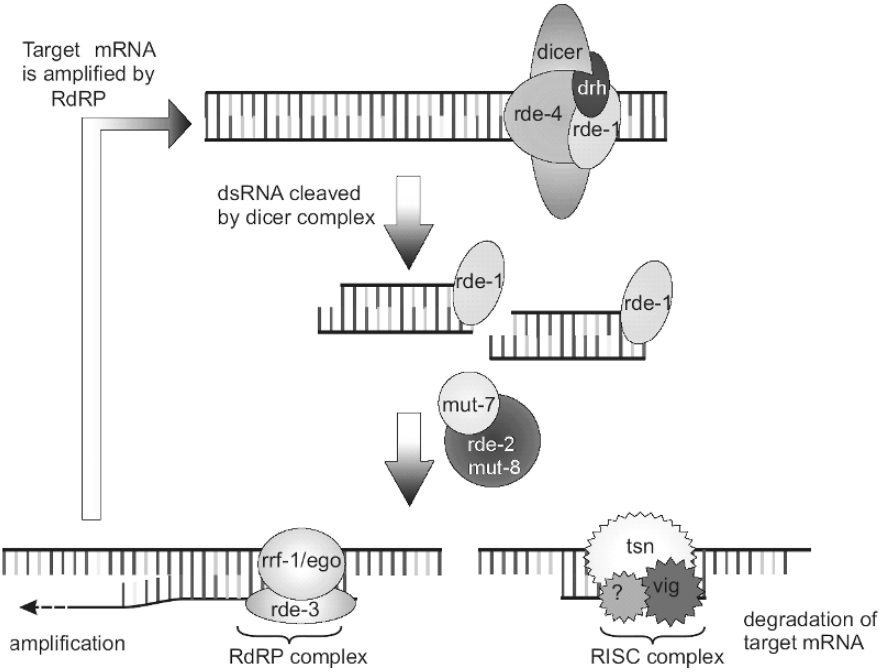

Figure 1. The RNAi gene silencing pathway in C. elegans*. In the initiation phase exogenous dsRNA is recognised by the Dicer complex and processed into siRNAs. The Dicer complex includes the multidomain RNaseIII Dicer enzyme itself, the dsRNA binding protein Rde-4, the PAZ-PIWI domain family protein Rde-1 and the Dicer-related helicase Drh-1. Following dsRNA cleavage, Rde-1 may bind the siRNAs to direct them to the second, effector phase of the RNAi pathway. Assembly of the active RISC complex may involve the helicase/exonuclease domain protein Mut-7 and Rde-2/Mut-8. The siRNA strands separate and the guide strand is incorporated into the active RISC. The activated RISC complex that also contains an unidentified Argonaute family protein, the RNA binding protein Vig-1 and the Tudor-SN protein Tsn-1, recognises and cleaves the target mRNA leading to a gene silencing effect. In a separate amplification step, siRNAs act as primers for the RNA-dependent RNA polymerases Ego-1 or Rrf-1 that may function in a complex with the Rde-3 polymerase. The dsRNA thus produced can enter the Dicer complex and trigger further, transitive, gene silencing.*

Silencing of abundant transcripts by only a few introduced molecules of dsRNA is achieved in *C. elegans* through an amplification step. The initial siRNAs produced by the Dicer complex can act as primers for an RNA-dependent RNA polymerase (RdRP), using the target mRNA as a template. The two RdRPs, EGO-1 and RRF-1, active in the germline and somatic cells respectively, have been implicated in this process. They may function in a complex with the RDE-3 polymerase (Chen et al., 2005a). The dsRNA made in this manner can enter the Dicer complex and trigger further, transitive, gene silencing.

For many species, including the model system Drosophila, dsRNA must be introduced directly into cells by microinjection or electroporation in order to elicit an RNAi response. Following reports of the microinjection technique (Fire et al., 1998) it was later shown that RNAi can be effectively induced in *C. elegans* by simple soaking (Tabara, Grishok, & Mello, 1998) or by feeding the worms with bacteria expressing dsRNA (Timmons & Fire, 1998). This facilitates large-scale functional genomic analysis of RNAi in *C. elegans*. The dsRNA moves systemically from the gut or injected tissue throughout most cells of the worm including the germline. This leads to induced gene-silencing in any cells that express the target mRNA. The systemic movement to the germline results in an RNAi phenotype that can be inherited, with progeny of the treated worms displaying a strong effect. The RNAi effect only extends beyond the F1 generation, however, when germline genes are targeted (Grishok, Tabara, & Mello, 2000).

The isolation of mutants defective in systemic RNAi has led to the identification of some of the genes involved in this process. The *sid-1* gene encodes a protein with 11 membrane spanning domains that localises to cells in contact with the environment, including some neurons (Winston, Molodowitch, & Hunter, 2002). Sid-1 promotes passive uptake of dsRNA with longer molecules transported more efficiently than siRNAs (Feinberg & Hunter, 2003). Three RNAi spreading defective (*rsd*) mutants are all deficient in transmission of the RNAi effect to the germline. A role in vesicle trafficking is predicted for RSD3, suggesting that dsRNA may be transported within endocytotic vesicles (Tijsterman, May, Simmer, Okihara, & Plasterk, 2004). The role of the endocytic pathway in uptake and translocation of dsRNA has recently been confirmed. Several *C. elegans* gene products with roles in intracellular vesicular transport and lipid modification were found to be essential for systemic RNAi (Saleh et al., 2006).

3.1. Plant Parasitic Nematodes RNAi

Reports in the literature provide evidence for the efficacy of RNAi in plant parasitic nematodes but the molecular detail of the RNAi process in plant parasitic nematodes has yet to be elucidated. A range of genes have been targeted for silencing in cyst and root-knot nematode species, and both the phenotypic and molecular effects were documented.

3.1.1. Uptake of dsRNA

The obligatory parasitic nature and small size of infective stages of plant parasitic nematodes makes them refractory to microinjection. Prior to infection of plant roots the non-feeding, pre-parasitic nematodes do not normally ingest. Octopamine was first used to stimulate oral ingestion by pre-parasitic 2nd stage juveniles (J2) of the cyst nematodes *G. pallida* and *H. glycines* leading to uptake of dsRNA from the soaking solution (Urwin, Lilley, & Atkinson, 2002). The same method has been used successfully to induce uptake of dsRNA by J2 of the root-knot nematode *M. incognita* (Bakhetia, Charlton, Atkinson, & McPherson, 2005; Shingles, Lilley, Atkinson, & Urwin, 2007). Resorcinol and serotonin also induce dsRNA uptake by J2 of *M. incognita* and may be more effective than octopamine for this nematode (Rosso, Dubrana, Cimbolini, Jaubert, & Abad, 2005). The addition of spermidine to the soaking buffer and an extended incubation time were reported to increase the efficiency of RNAi for the cyst nematode *G. rostochiensis* (Chen, Rehman, Smant, & Jones, 2005b).

Genes that are expressed in a range of different tissues and cell types have been targeted by RNAi. The ingested dsRNA can silence genes in the intestine (Urwin et al., 2002; Shingles et al., 2007); female reproductive system (Lilley et al., 2005b), subventral and dorsal oesophageal glands (Bakhetia, Urwin, & Atkinson, 2007; Chen et al., 2005b; Huang et al., 2006a; Rosso et al., 2005) and sperm (Urwin et al., 2002; Steeves, Todd, Essig, & Trick, 2006). In *C. elegans* the uptake of dsRNA from the gut has been shown to lead to systemic RNAi. When plant parasitic nematodes ingest dsRNA a systemic response is seen in other tissues. This suggests that plant parasitic nematodes share similar uptake and dispersal pathways with *C. elegans*.

There are other reports in the literature of alternate routes for uptake of dsRNA by plant parasitic nematodes. Soaking intact eggs of *M. artiellia* contained within their gelatinous matrix, in a solution containing dsRNA allowed successful targeting of the chitin synthase gene (Fanelli, Di Vito, Jones, & De Giorgi, 2005). The enzyme plays a role in the synthesis of the chitinous layer in the egg shell. The reduction of its transcript by RNAi led to a reduction in the amount of chitin in the eggshells and a subsequent delay in hatching of juveniles from treated eggs. The results imply that the eggs of this nematode are permeable to dsRNA.

RNAi of genes that are expressed in the neuronal system of *C. elegans* can be difficult to achieve (Kamath, Martinez-Campos, Zipperlen, Fraser, & Ahringer, 2000; Timmons, Court, & Fire, 2001) although RNAi effects in these cells can be enhanced by using a mutant strain defective in the RdRP *rrf-3* (Simmer et al., 2002, 2003). A recent study describes the silencing of five FMRFamide-like (*flp*) neuropeptide genes of *G. pallida*, each with a unique neuronal expression pattern (Kimber et al., 2007). Absence of transcript in treated worms and abnormal behavioural phenotypes were observed when the genes were targeted by RNAi, demonstrating the susceptibility of these neuronal genes to RNAi. The effect occurred for pre-parasitic J2 nematodes soaked only in water containing dsRNA. RNAi of intestinal and pharyngeal gland cell expressed genes has a requirement for stimulated oral uptake to achieve transcript knockout (Urwin et al., 2002; Rosso

et al., 2005). RNAi-mediated silencing of the neuronal *flp* genes must therefore use an alternative route to take up dsRNA. The dsRNA may be gaining access via the secretory/excretory pore, the cuticle, or the amphids. Amphids are paired sense organs of nematodes at the anterior of the animal. The amphidial cavity has sensory neurons that are exposed to the external environment. These neurons demonstrate uptake of fluorescein isothiocyanate (FITC), a feature common to both *C. elegans* (Hedgecock, Culotti, Thomson, & Perkins, 1985) and cyst nematodes (Winter, McPherson, & Atkinson, 2002). Fluorescent dextran conjugates of 12 kDa but not 19.5 kDa are also taken up by the sensory neurons of *C. elegans*, suggesting a size constraint. It is postulated that the retrograde transport along cyst nematode chemosensory dendrites can provide a route for uptake of soluble compounds such as peptides from the external environment (Winter et al., 2002). The exposed nerve processes could also take up dsRNA molecules.

A gene encoding a secreted amphid protein of unknown function (*gr-ams-1*) has been targeted by RNAi of *G. rostochiensis* (Chen et al., 2005b). Although octopamine was included in the soaking solution on this occasion, *gr-ams-1* was more susceptible to an RNAi effect than a gland cell expressed endoglucanase, raising the possibility that neuronal retrograde transport offers more efficient dsRNA uptake that forced ingestion.

3.2. Comparative Observation of Reported Strategies

A range of techniques has been used both to deliver the RNAi effect and to determine the phenotype. The RNAi experiments described in the literature with plant parasitic nematodes have used a range of approaches. While the basic methodology is similar, adjustments are continually being made in order to maximise the strength of the observed phenotype. Comparison between experiments is difficult when the methodology differed, but some observations regarding the persistence and duration of RNAi in these nematodes can be made.

RNAi effects have been observed following exposure of preparasitic nematodes to dsRNA for time periods ranging from 4hr to 7 days. While a 4 hr incubation leads to effective RNAi for some cyst nematode genes (Lilley et al., 2005b; Urwin et al., 2002) increasing the incubation period generally results in increased silencing and stronger phenotypic effects for cyst nematodes (Chen et al., 2005b; Kimber et al., 2007). An incubation of 24 hr was found to be critical for RNAi-induced silencing in *G. rostochiensis* (Chen et al., 2005b). In *G. pallida,* incubation periods in excess of 18 hr were critical when targeting the *flp* genes to produce an aberrant phenotype. The severity of the effect was greatest after 7 days incubation (Kimber et al., 2007). Efficient silencing has been consistently observed in *Meloidogyne* spp. following 4 hr incubation of J2 in dsRNA (Huang et al., 2006a; Rosso et al., 2005; Shingles et al., 2007).

Successful RNAi has been observed in both cyst and root-knot nematodes treated with double stranded RNA molecules ranging in size from 42 bp to 1300 bp. A 309 bp dsRNA targeting a β-1,4-endoglucanase of *G. rostochiensis* induced weaker silencing than 244 bp dsRNA targeting an amphid secreted protein in the

same nematode (Chen et al., 2005b). It is difficult to draw any conclusions from such observations. A number of factors may influence this outcome: differing spatial expression patterns, level and turnover rate of the endogenous transcript in addition to length of the dsRNA. 88 bp, 227 bp and 316 bp of dsRNA have been used to target the same gene, *Gp-flp-6* in *G. pallida* (Kimber et al., 2007). The shortest length was insufficient to induce any silencing effect. Both the 277 bp and 316 bp dsRNAs silenced the target transcript and resulted in reduced motility, but the shorter molecule consistently induced stronger effects. Further studies are required to determine if this is a general phenomenon for RNAi of plant parasitic nematodes, or if the effects are gene specific. Both 42 bp and 271 bp dsRNAs covering the coding region or the full-length transcript of the oesophageal gland peptide 16D10, led to a 93–97% reduction in target transcript in *M. incognita* J2 (Huang et al., 2006a). This suggests that different nematode species and/or different genes may have dissimilar requirements for inducing dsRNA molecules.

The nature of soaking J2 nematodes in dsRNA limits the strength of the RNAi effect, as the RNAi-induced gene silencing is time-limited, once nematodes are removed from exposure to dsRNA. Calreticulin (*mi-crt*) and polygalacturonase (*mi-pg-1*) genes of *M. incognita* targeted by RNAi, in a 4 hr incubation of dsRNA, displayed maximum transcript repression after a further 20 hr and 44 hr respectively. After a 68 hr recovery period the transcript level of both genes had returned to normal (Rosso et al., 2005). Similar results have been observed for cyst nematodes: transcript repression of a β-1,4-endoglucancase was observed immediately following a 16 hr dsRNA treatment of *H. glycines* J2 and after a 5 day recovery period. Transcript abundance increased at 10 days and had returned to pre-treatment levels by 15 days after dsRNA exposure (Bakhetia et al., 2007). The persistence of *Gp-flp-12* gene silencing was monitored by the reduced motility of treated J2s. There was no recovery of phenotype after 24 hrs and a significant but not complete recovery after 6 days (Kimber et al., 2007). If dsRNA-treated juveniles are allowed to invade plants and continue development, the phenotypic consequences of RNAi can be evident after a number of weeks (Bakhetia et al., 2005; Bakhetia et al., 2007; Chen et al., 2005b; Huang et al., 2006a; Lilley et al., 2005b; Urwin et al., 2002). If nematodes are compromised during the early invasion of plants or during induction of the feeding cell, then subsequent development will be affected. Time-limitation is not an issue in *C. elegans* when RNAi is achieved by feeding because the nematodes are being continuously exposed to dsRNA. A similar situation would arise in plant parasitic nematodes if dsRNA was produced in the feeding cell. This would similarly prolong the effective exposure and maximise the RNAi effect.

3.3. Observation of Phenotype

Careful analysis of the phenotypic effect of any RNAi experiment is a key challenge. Due to the obligate parasitic lifecycle of these species, many RNAi phenotypes can only be revealed after the treated pre-parasitic juveniles have been allowed to infect host roots and develop to adulthood. Subtle phenotypic effects may be missed. RNAi can be a powerful approach for functional analysis of

nematode-specific or species-specific genes, with no putative homologues in current databases. Study of such genes, that may be good targets for novel control strategies, could provide insight into unique aspects of the plant-nematode interaction. Experiments to date have commonly analysed impact on the numbers of nematodes able to invade roots and successfully initiate feeding sites, or the proportion of cyst nematodes that develop as either males or females. Effects on female size and fecundity can also be measured as can the size and shape of developing nematodes at a given time point post infection.

In the cyst nematode *Heterodera glycines* a range of genes have been targeted by soaking the infective juvenile animals in dsRNA. These include a cysteine proteinase, C-type lectin (Urwin et al., 2002), β-1,4-endoglucanase, pectate lyase, chorismate mutase, the secreted peptide SYV46 and a gland protein of unknown function (Bakhetia et al., 2007). The phenotypic outcome of all these experiments was a reduced parasitic burden when the treated nematodes were used to infect a host plant.

RNAi using the soaking technique in *Globodera* species has been used to determine the phenotypic effect of targeting a cysteine proteinase, β-1,4-endoglucanase, a secreted amphid protein and FMRFamide-like peptides (Urwin et al., 2002; Chen et al., 2005b; Kimber et al., 2007). Again these lead to a reduction in the parasite burden on the host plant with the exception of the latter that impaired motility. In the root knot nematode *M. incognita*, soaking has been used to target a cysteine proteinase, dual oxidase and a secreted peptide 16D10, the observed phenotypes of which all showed a detrimental effect on pathogenesis (Shingles et al., 2007; Bakhetia et al., 2005; Huang et al., 2006a). Soaking of egg masses has been carried out to target the *M. artiellia* chitin synthase gene resulting in delayed egg hatch (Fanelli et al., 2005).

Gross population analysis of this sort can define the importance of a gene, but not necessarily help elucidate its particular role. If the gene of interest has a putative function based on sequence homology, often corroborated with in situ hybridisation studies, then more directed phenotypic analysis can be carried out. The *flp* genes of *G. pallida* are expressed in the nervous system (Kimber et al., 2002) with a likely role in coordinating motor activities. Consequently, phenotypic effects of RNAi silencing were analysed using migration assays to detect impaired motility (Kimber et al., 2007). RNAi combined with detailed analysis of gene expression by quantitative PCR helped to elucidate stages in the infection process of *H. glycines* when particular oesophageal gland secreted proteins were required (Bakhetia et al., 2007). Molecular and biochemical characterisation of a cathepsin L cysteine proteinase of *M. incognita* targeted by RNAi was correlated with the effect on parasitism (Shingles et al., 2007).

4. *IN PLANTA* DELIVERY OF dsRNA TO TARGET GENES OF PLANT PARASITIC NEMATODES

Delivery of dsRNA from the feeding cell to target specific, essential nematode genes has been proposed as a novel means for plant parasitic nematode control since the first demonstration that RNAi is effective in these nematodes (Urwin

et al., 2002; Atkinson, Urwin & McPherson, 2003; Lilley et al., 2005a). The mode of feeding, particularly of sedentary endoparasitic nematodes, is ideally suited to such an approach. The nematode feeds exclusively from one or a few plant cells and continues to feed throughout development to a mature male or egg-laying female. This ensures constant ingestion of plant cell derived molecules and potentially enables targeted expression of the dsRNA construct in the feeding cells.

RNAi is widely used in plants as a research tool for analysis of gene function. More recently there has been interest in using it to engineer novel traits (Kusaba, 2004; Mansoor, Amin, Hussain, Zafar, & Briddon, 2006) with a number of potential commercial applications already described (e.g. Ogita, Uefuji, Morimoto, & Sano, 2004; Byzova, Verduyn, De Brouwer, & De Block, 2004; Davuluri et al., 2005). RNAi has also been used in plants to confer resistance to viruses (Waterhouse et al., 1998; Pooggin, Shivaprasad, Veluthambi, & Mohn, 2003) and the bacterial pathogen *Agrobacterium tumefaciens* (Escobar, Civerolo, Summerfelt, & Dandekar, 2001).

A variety of vectors are now available for induction of RNAi in plants by production of a dsRNA molecule. The general approach is to clone both sense and anti-sense cDNA sequences of the target gene, separated by a spacer region or intron into a binary vector under the control of a strong plant promoter. The transcribed RNA then forms into a self complementary hairpin structure. Use of an intron sequence as the spacer increases the silencing efficiency (Smith et al., 2000; Wesley et al., 2001).

4.1. The Feeding Strategy of Sedentary Endoparasitic Nematodes

A nematode establishes a feeding site through the modification of root cells to create a specialised feeding cell. This process is well described, particularly for cyst and root-knot nematode species. Following invasion of a host root, the infective juvenile ($J2_i$) migrates either intracellularly (cyst species) or intercellularly (root-knot species) through cortical cells, towards the vascular cylinder where an initial feeding cell is selected. One or more plant cells are then modified to re-differentiate into a specialised feeding site. Nematode proteins from the three pharyngeal gland cells are secreted through the bore of the stylet into the initial feeding cell, and induction of the feeding site is triggered. This results in dramatic changes in gene expression and considerable re-programming of root cell development. Interestingly, although there are morphological similarities and a shared function, the nature of the transformations differs between the syncytia induced by cyst nematodes and the giant cells induced by root-knot nematodes (Davis et al., 2000).

Cyst nematodes initiate a syncytium from a single cell located at the periphery of the formed vasculature (Golinowski, Sobczak, Kurek, & Grymaszewska, 1997). The syncytium then expands by recruitment of up to 200 adjacent cells from the stele through cell wall dissolution. This seems to be a modification of a normal root morphogenesis process (Jones, 1981a; Golinowski et al., 1997). In contrast, the $J2_i$ of *Meloidogyne* spp. selects a small number of parenchymal cells in the differentiating stele and induces them to undergo repeated cycles of acytokinetic mitoses. This results in enlarged and multinucleate giant cells from which the

parasite feeds in turn (Sijmons, Grundler, Von Mende, Burrows, & Wyss, 1991). The cell architecture of these two feeding sites differs considerably but some common features occur. Both have a reduced number of smaller secondary vacuoles and high metabolic activity with increased numbers of organelles and nuclei. There is also a low plasmodesmatal density in cell walls adjacent to unmodified cells and a large number of wall ingrowths into xylem vessels (Jones, 1981b, de Almeida Engler et al., 2004). These feeding sites, if continually stimulated by the nematode, function as sinks that supply the nematode with all its nutritional requirements during the parasitic stages of its life cycle. The sequestration of plant material by the nematode results in serious, deleterious consequences for the host plant.

The nematodes feed using a hollow, protrusible stylet that pierces the giant cell or syncytium to allow pharyngeal gland secretions to pass into the cells and cell contents to be removed. A semi-permeable blind-ended structure known as the feeding tube extends into the cytoplasm of the feeding cell from the stylet orifice. The feeding tube acts as a molecular sieve, permitting the uptake of certain molecules and excluding others. Nematode secretions, as yet unidentified, are probably involved in formation of this tube. This is a unique, self-assembling structure that is reformed each time the stylet is reinserted for a cycle of feeding. The feeding tubes of root-knot and cyst nematodes differ in their structure. The former have thick, electron dense, crystalline walls (Hussey & Mims, 1991) and the latter have a thinner, uneven wall (Sobczak, Golinowski, & Grundler, 1999). The dsRNA or siRNA produced by a plant must pass into the nematode gut if *in planta* RNAi is to be effective. The divergent feeding tube structures lead to differences in size exclusion limits between nematode species. The cyst nematode feeding tube of *H. schachtii* permits uptake of 20 kDa dextrans but not 40 kDa (Böckenhoff & Grundler, 1994) and proteins of 11 kDa but not 23 kDa and 28 kDa (Urwin, Møller, Lilley, McPherson, & Atkinson, 1997b; Urwin, McPherson, & Atkinson, 1998). Uptake of 28 kDa green fluorescent protein (GFP) by *G. rostochiensis* could only be visualised using sensitive detection techniques (Goverse et al., 1998) whereas it was readily ingested by *M. incognita* (Urwin et al., 1997b).

After production of a dsRNA molecule in the plant cell, the RNAi trigger could be available for uptake by the feeding tube in one of three conformations. The first is unprocessed full-length dsRNA. In this form uptake would be possible by those nematodes possessing feeding tubes with pore apertures greater than 26Å in diameter assuming the molecule is drawn lengthwise through the pores. As GFP is a barrel-shaped protein with a diameter of 30Å, any nematode capable of ingesting GFP should also ingest dsRNA. If ingested in this conformation, it is envisaged that the dsRNA would pass into the gut cells, be processed by the nematode Dicer complex and induce silencing via the RNAi pathway.

Alternatively, siRNAs processed by the plant RNAi machinery could be available for ingestion via the feeding tube. This would require the same pore size as full length dsRNA, however lengthwise entry into the pore would be favoured. After cleavage of dsRNA by the plant cell Dicer, problems with uptake may arise if the siRNAs are immediately complexed with the multi-component RISC. It would appear impossible for this large protein-nucleic acid complex to pass through the wall of the feeding tube.

4.2. In Planta RNAi as a Biotechnological Strategy

The year 2006 saw three publications that described the feasibility of silencing nematode genes using dsRNA produced in the host plant (Huang, Allen, Davis, Baum, & Hussey, 2006a; Steeves et al., 2006; Yadav, Veluthambi, & Subramaniam, 2006). Nevertheless, questions still remain concerning the precise mode of action and the form in which the RNAi trigger is taken up by the nematodes. Yadav et al. (2006) demonstrated silencing of *Meloidogyne* genes by RNAi delivered from host tobacco plants. Nematode splicing factor and integrase genes were targeted based on their RNAi phenotype in *C. elegans* and their presumed essential role in *Meloidogyne*. Plants expressing hairpin dsRNA for each of the two sequences displayed >95% resistance to *M. incognita*. The few nematodes that formed galls appeared developmentally compromised and lacked detectable transcript for the targeted genes (Yadav et al., 2006). Unfortunately, no evidence was presented for the presence of either dsRNA or siRNAs in the transgenic plants, so the route by which silencing occurred cannot be deduced.

The second report came from the group of R. S. Hussey. They targeted a 13 amino acid peptide (16D10) that is secreted from the subventral oesophageal gland cells of *M. incognita* (Huang et al., 2006a). The peptide is highly conserved among four *Meloidogyne* species (*M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*) and appears to mediate an early signalling event required for giant cell formation, possibly through interaction with a plant transcription factor (Huang et al., 2006b). *In vitro* delivery of 16D10 dsRNA to J2 *M. incognita* suppressed their subsequent development by up to 81% when inoculated onto *Arabidopsis* roots. Transgenic *Arabidopsis* plants expressing the 16D10 sequence as a hairpin construct were found to contain both intact dsRNA and approx. 21bp siRNAs corresponding to 16D10. Infection of these plants with all four *Meloidogyne* species revealed a 63–90% reduction in the number of galls that developed with a decrease in gall size and corresponding reduction in total egg production. This clearly demonstrates that uptake by the feeding nematode of either full length dsRNA or processed siRNAs can occur and is sufficient to induce an RNAi phenotype. These results also highlight the utility of targeting highly conserved, nematode specific sequences to protect against more than one nematode species.

Most recently, evidence emerged that cyst nematodes can also be targeted by expressing dsRNA molecules in plant roots (Steeves et al., 2006). This is an important finding given the differences in feeding tube structure of the cyst and root-knot genera. Transgenic soybean plants were shown to accumulate siRNAs arising from expression of a hairpin construct targeting the major sperm protein (MSP) gene of the soybean cyst nematode *H. glycines.* Nematodes infecting these plants displayed an overall 68% reduction in egg production. Remarkably, the progeny hatched from the eggs that did develop displayed an overall 75% reduction in egg production when allowed to infect wildtype susceptible soybean plants. These results suggest that RNAi can be transmitted to the F1 progeny in a similar manner to that documented for *C. elegans* (Grishok et al., 2000).

4.3. Future Prospects for RNAi-Based Control of Plant Parasitic Nematodes

RNAi silencing of a gene that plays a key role in the development of the nematode, either directly or indirectly can adversely affect the progression of pathogenesis. Good targets for this technology are likely to be those genes that are nematode specific and have sequence conservation with orthologues from related species to maximise the spectrum of resistance. Putative parasitism genes such as that targeted by Huang et al. (2006a) may be targets of choice but are likely to be selective for particular nematode genera. Cross-species RNAi in nematodes has however, recently been demonstrated with sequences from the animal parasite *Ascaris suum* inducing gene silencing of their *C. elegans* counterparts (Gao et al., 2006).

The limited data that are currently available suggest that levels of plant resistance from RNAi biotechnology are generally comparable to those observed with other transgenic strategies. Transgenically expressed cysteine proteinase inhibitors (cystatins) have typically delivered 70–80% resistance against a number of nematode species (Atkinson et al., 2003). Total protection however, is achievable by pyramiding cystatins with partial natural resistance (Urwin et al., 2003). Additive effects can be achieved by introducing a number of transgenes into a single plant (Urwin et al., 1998). RNAi biotechnology may also benefit from being stacked with other defences. Expressing hairpin constructs targeting more than one gene by RNAi may increase the level of resistance. Co-expressing a number of dsRNA sequences to target multiple genes is highly effective in *Drosophila* (Schmid, Schindelholz, & Zinn, 2002) and up to five genes can be silenced simultaneously in *C. elegans* (Geldhof, Molloy, & Knox, 2006) RNAi based strategies have the advantage that no novel protein is produced. This may ease the progress of this new technology from development to a commercial product.

The possibility of siRNAs inducing "off-target" gene silencing effects presents a concern with RNAi-based technologies. These occur in animal systems (Jackson et al., 2003; Scacheri et al., 2004) but have not yet been reported in plants, even when specifically sought (Kumar, Gustafsson, & Klessig, 2006). Those siRNAs that play a crucial role in homologous sequence gene silencing via the RNAi pathway are, in many respects, analagous to regulatory microRNAs (miRNAs) that are typically 19–24 nucleotides long. In animal cells these miRNAs can trigger translational repression by imperfect annealing to the 3' untranslated regions (UTRs) of genes. Most reported off-target effects are considered to result from siRNAs with partial homology to non-target gene 3' UTRs acting in a similar manner (Birmingham et al., 2006). Plant miRNAs, in contrast to animal miRNAs, have almost perfect complementarity to their target sequences. Plant miRNAs also differ from the animal counter-part by triggering local transcript cleavage rather than translational arrest (Du & Zamore, 2005). This increased sequence specificity of plant miRNA mechanisms should, with bioinformatic input, facilitate selection of nematode genes that minimise the risk of off-target effects.

REFERENCES

Atkinson, H. J. (1995). Plant nematode interactions: molecular and genetic basis. In K. Kohmoto, U. S. Singh, & R. P. Singh (Eds.), *Pathogenesis and host specificity in plant diseases: Eukaryotes* (Vol. II, pp. 355–370). Pergamon Press Oxford, UK.

Atkinson, H. J., Grimwood, S., Johnston, K., & Green, J. (2004). Prototype demonstration of transgenic resistance to the nematode *Radopholus similis* conferred on banana by a cystatin. *Transgenic Research, 13,* 135–142.

Atkinson, H. J., Urwin, P. E., & McPherson, M. J. (2003). Engineering plants for nematode resistance. *Annual Review of Phytopathology, 41,* 615–639.

Bakhetia, M., Charlton, W., Atkinson, H. J., & McPherson, M. J. (2005). RNA interference of dual oxidase in the plant nematode *Meloidogyne incognita. Molecular Plant-Microbe Interactions, 18*, 1099–1106.

Bakhetia, M., Urwin, P. E., & Atkinson, H. J. (2007). qPCR analysis and RNAi define pharyngeal gland cell-expressed genes of *Heterodera glycines* required for initial interactions with the host. *Molecular Plant-Microbe Interactions,* 20, 306–312.

Birmingham, A., Andersen, E. M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., et al. (2006). 3'UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nature Methods, 3*, 199–204.

Böckenhoff, A., & Grundler, F. M. W. (1994). Studies on the nutrient uptake by the beet cyst nematode *Heterodera schachtii* by *in situ* microinjection of fluorescent probes into the feeding structures in *Arabidopsis thaliana*. *Parasitology, 109*, 249–254.

Brodersen, P., & Voinnet, O. (2006). The diversity of RNA silencing pathways in plants. *Trends in Genetics, 22*, 268–280.

Byzova, M., Verduyn, C., De Brouwer, D., & De Block, M. (2004). Transforming petals into sepaloid organs in *Arabidopsis* and oilseed rape: implementation of the hairpin RNA-mediated gene silencing technology in an organ-specific manner. *Planta, 218*, 379–387.

Caudy, A. A., Ketting, R. F., Hammond, S. M., Denli, A. M., Bathoorn, A. M. P., Tops, B. B. J., et al. (2003). A micrococcal nuclease homologue in RNAi effector complexes. *Nature, 425*, 411–414.

Cai, D., Thurau, T., Tian, Y., Lange, T., Yeh, K-W., & Jung, C. (2003). Sporamin-mediated resistance to beet cyst nematodes (*Heterodera schachtii* Schm.) is dependent on trypsin inhibitory activity in sugar beet (*Beta vulgaris* L.) hairy roots. *Plant Molecular Biology, 51*, 839–849.

Chen, C.-C. G., Simard, M. J., Tabara, H., Brownell, D. R., McCollough, J. A. & Mello C. C. (2005a). A member of the polymerase nucleotidyltransferase superfamily is required for RNA interference in *C. elegans*. *Current Biology*, 15, 378–383.

Chen, Q., Rehman, S., Smant, G., & Jones, J. T. (2005b). Functional analysis of pathogenicity proteins of the potato cyst nematode *Globodera rostochiensis* using RNAi. *Molecular Plant-Microbe Interactions, 18*, 621–625.

Chitwood, D. J. (2003). Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture-Agricultural Research Service. *Pest Management Science, 59*, 748–753.

Cogoni, C., & G. Macino, G. (2000). Post-transcriptional gene silencing across kingdoms. *Current Opinion in Genetics & Development, 10*, 638–643.

Davis, E. L., Hussey, R. S., Baum, T. J., Bakker, J., Schots, A., Rosso, M. N., & Abad, P. (2000) Nematode parasitism genes. *Annual Review of Phytopathology 38*, 365–396.

Davuluri, G. R., van Tuinen, A., Fraser, P. D., Manfredonia, A., Newman, R., Burgess, D., et al. (2005). Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nature Biotechnology, 23*, 890–895.

de Almeida Engler, J. D., Van Poucke, K., Karimi, M., De Groodt, R., Gheysen, G., & Engler, G. (2004). Dynamic cytoskeleton rearrangements in giant cells and syncytia of nematode-infected roots. *Plant Journal, 38*, 12–26.

Denli, A.M., & Hannon, G. J. (2003). RNAi: An ever-growing puzzle. *Trends in Biochemical Sciences, 28*, 196–201.

Dirks, R. P., Bouw, G. B., Huizen, R. R., Jansen, E. J., & Martens, J. M. (2003). Functional genomics in *Xenopus laevis*: Towards transgene-driven RNA interference and cell-specific transgene expression. *Current Genomics, 4*, 699–711.

Du, T., & Zamore, P.D. (2005). MicroPrimer: The biogenesis and function of microRNA. *Development, 132*, 4645–4652.

Escobar, M. A., Civerolo, E. L., Summerfelt, K. R., & Dandekar, A. M. (2001). RNAi-mediated oncogene silencing confers resistance to crown gall tumorigenesis. *Proceedings of the National Academy of Sciences of the USA, 98*, 13437–13442.

Fanelli, E., Di Vito, M., Jones, J. T., & De Giorgi, C. (2005). Analysis of chitin synthase function in a plant parasitic nematode, *Meloidogyne artiellia*, using RNAi. *Gene, 349*, 87–95.

Feinberg, E. H., & Hunter, C. P. (2003). Transport of dsRNA into cells by the transmembrane protein SID-1. *Science, 301*, 1545–1547.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature, 391*, 806–811.

Forrest, E. C., Cogoni, C., & Macino, G. (2004). The RNA-dependent RNA polymerase, QDE-1, is a rate-limiting factor in post-transcriptional gene silencing in *Neurospora crassa*. *Nucleic Acids Research, 32*, 2123–2128.

Gao, G., Raikar, S., Davenport, B., Mutapcic, L., Montgomery, R., Kuzmin, E., et al. (2006). Cross-species RNAi: Selected *Ascaris suum* dsRNAs can sterilize *Caenorhabditis elegans*. *Molecular and Biochemical Parasitology, 146*, 124–128.

Geldhof, P., Molloy, C., & Knox, D. P. (2006). Combinatorial RNAi on intestinal cathepsin B-like proteinases in *Caenorhabditis elegans* questions the perception of their role in nematode biology. *Molecular and Biochemical Parasitology, 145*, 128–132.

Golinowski, W., Sobczak, M., Kurek, W., & Grymaszewska, G. (1997). The structure of syncytia. In C. Fenoll, F. M. W. Grundler, & S. A. Ohl (Eds.), *Cellular and molecular aspects of plant-nematode interactions* (pp. 80–97). Dordrecht, Netherlands: Kluwer Academic Publishers.

Goverse, A., Biesheuvel, J., Wijers, G. J., Gommers, F. J., Bakker, J., Schots, A., et al. (1998). *In planta* monitoring of the activity of two constitutive promoters, CaMV 35S and TR2', in developing feeding cells induced by *Globodera rostochiensis* using green fluorescent protein in combination with confocal laser scanning microscopy. *Physiological and Molecular Plant Pathology, 52*, 275–284.

Grishok, A. (2005). RNAi mechanisms in *Caenorhabditis elegans*. *FEBS Letters, 579*, 5932–5939.

Grishok, A., Tabara, H., & Mello, C. C. (2000). Genetic requirements for inheritance of RNAi in *C. elegans*. *Science, 287*, 2494–2497.

Hammond, S. M. (2005). Dicing and slicing – The core machinery of the RNA interference pathway. *FEBS Lettsers, 579*, 5822–5829.

Hannon, G. J. (2002). RNA interference. *Nature, 418*, 244–251.

Hedgecock, E. M., Culotti, J. G., Thomson, J. N., & Perkins, L. A. (1985). Axonal guidance mutants of *Caenorhabditis elegans* identified by filling sensory neurons with fluorescein dyes. *Developmental Biology, 111*, 158–170.

Huang, G., Allen, R., Davis, E. L., Baum, T. J., & Hussey, R. S. (2006a). Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. *Proceedings of the National Academy of Sciences of the USA, 103*, 14302–14306.

Huang, G., Dong, R., Allen, R., Davis, E. L., Baum, T. J., & Hussey, R. S. (2006b). A root-knot nematode secretory peptide functions as a ligand for a plant transcription factor. *Molecular Plant-Microbe Interactions, 19*, 463–470.

Hussey, R. S., & Janssen, G. J. W. (2002). Root-knot nematodes: *Meloidogyne* species. In J. L. Starr, R. Cook, & J. Bridge (Eds.), *Plant resistance to parasitic nematodes* (pp. 43–70). Oxford, UK: CAB International.

Hussey, R. S., & Mims, C. W. (1991). Ultrastructure of feeding tubes formed in giant-cells induced in plants by the root-knot nematode *Meloidogyne incognita*. *Protoplasma, 162*, 99–107.

Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., et al. (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotechnology, 21*, 635–637.

Jones, M. G. K. (1981a). The development and function of plant cells modified by endoparasitic nematodes. In B. M. Zuckerman, W. F. Mai, & R. A. Rohde (Eds.), *Plant parasitic nematodes* (pp. 255–280). New York, USA: Academic Press.

Jones, M. G. K. (1981b). Host cell responses to endoparasitic nematode attack: structure and function of giant cells and syncytia. *Annals of Applied Biology, 97*, 353–372.

Jorgensen, R. A., Cluster, P. D., English, J., Que, Q., & Napoli, C. A. (1996). Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. *Plant Molecular Biology, 31*, 957–973.

Kaletta, T., & Hengartner, M. O. (2006). Finding function in novel targets: *C. elegans* as a model organism. *Nature Reviews Drug Discovery, 5*, 387–398.

Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G., & Ahringer, J. (2000). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biology, 2*(1), research0002.1–0002.10.

Kennerdell, J. R., & Carthew, R. W. (2000). Heritable gene silencing in *Drosophila* using double stranded RNA. *Nature Biotechnology, 18*, 896–898.

Kimber, M. J., Fleming, C. C., Prior, A., Jones, J. T., Halton, D. W., & Maule, A. G. (2002). Localisation of *Globodera pallida* FMRFamide-related peptide encoding genes using *in situ* hybridisation. *International Journal of Parasitology, 32*, 1095–1105.

Kimber, M. J., McKinney, S., McMaster, S., Day, T. A., Fleming, C. C., & Maule, A. G. (2007). *flp* gene disruption in a parasitic nematode reveals motor dysfunction and unusual neuronal sensitivity to RNA interference. *The FASEB Journal,* 21, 1233–1243.

Kumar, D., Gustafsson, C., & Klessig, D. F. (2006). Validation of RNAi silencing specificity using synthetic genes: salicylic acid-binding protein 2 is required for innate immunity in plants. *Plant Journal, 45*, 863–868.

Kusaba, M. (2004). RNA interference in crop plants. *Current Opinion in Biotechnology, 15*, 139–143.

Li, M., & Rohrer, B. (2006) Gene silencing in *Xenopus laevis* by DNA-vector based RNA interference and transgenesis. *Cell Research, 16*, 99–105.

Lilley, C. J., Atkinson, H. J., & Urwin, P. E. (2005a). Molecular aspects of cyst nematodes. *Molecular Plant Pathology, 6*, 577–588.

Lilley, C. J., Goodchild, S. A., Atkinson, H. J., & Urwin, P. E. (2005b). Cloning and characterisation of a *Heterodera glycines* aminopeptidase cDNA. *International Journal of Parasitology, 35*, 1577–1585.

Liu, B., Hibbard, J. K., Urwin, P. E., & Atkinson, H. J. (2005). The production of synthetic chemodisruptive peptides *in planta* disrupts the establishment of cyst nematodes *Plant Biotechnology Journal*, 3, 487–496.

Mansoor, S., Amin, I., Hussain, M., Zafar, Y., & Briddon, R. W. (2006). Engineering novel traits in plants through RNA interference. *Trends in Plant Science, 11*, 559–565.

Ogita, S., Uefuji, H., Morimoto, M., & Sano, H. (2004). Application of RNAi to confirm theobromine as the major intermediate for caffeine biosynthesis in coffee plants with potential for construction of decaffeinated varieties. *Plant Molecular Biology, 54*, 931–941.

Phillips, M. S., & Trudgill, D. L. (1998). Population modelling and integrated control options for potato cyst nematodes. In R. J. Marks & B. B. Brodie (Eds.), *Potato cyst nematodes biology, distribution and control* (pp. 153–163). Oxford, UK: CAB International.

Pooggin, M., Shivaprasad, P. V., Veluthambi, K., & Mohn, T. (2003). RNAi targeting of DNA virus in plants. *Nature Biotechnology, 21*, 131–132.

Qi, Y., & Hannon, G. J. (2005). Uncovering RNAi mechanisms in plants: Biochemistry enters the foray. *FEBS Letters, 579*, 5899–5903.

Rosso, M. N., Dubrana, M. P., Cimbolini, N., Jaubert, S., & Abad, P. (2005). Application of RNA interference to root-knot nematode genes encoding esophageal gland proteins. *Molecular Plant-Microbe Interactions, 18*, 615–620.

Saleh, M-C., van Rij, R. P., Hekele, A., Gillis, A., Foley, E., O'Farrell, P. H. et al. (2006). The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nature Cell Biology, 8*, 793–802.

Sasser, J. N., & Freckman, D. W. (1987). A world perspective on nematology: the role of the society. In J. A. Veech & D. W. Dickerson (Eds.), *Vistas on nematology* (pp. 7–14). Hyatsville, USA: Society of Nematologists.

Scacheri, P. C., Rozenblatt-Rosen, O., Caplen, N. J., Wolfsberg, T. G., Umayam, L., Lee, J. C. et al. (2004). Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proceedings of the National Academy of Sciences of the USA, 101*, 1892–1897.

Schmid, A., Schindelholz, B., & Zinn, K. (2002). Combinatorial RNAi: a method for evaluating the functions of gene families in Drosophila. *Trends in Neuroscience, 25*, 71–74.

Sen, G. L., & Blau, H. M. (2006). A brief history of RNAi: the silence of the genes. *The FASEB Journal, 20*, 1293–1299.

Shingles, J., Lilley, C. J., Atkinson, H. J., & Urwin, P. E. (2007). *Meloidogyne incognita*: molecular and biochemical characterisation of a cathepsin L cysteine proteinase and the effect on parasitism following RNAi. *Experimental Parasitology, 115*, 114–120.

Sijmons, P. C., Grundler, F. M. W., Von Mende, N., Burrows, P. R., & Wyss, U. (1991). *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *Plant Journal, 1*, 245–254.

Silva, J., Chang, K., Hannon, G. J., & Rivas, F. V. (2004). RNA-interference-based functional genomics in mammalian cells: reverse genetics coming of age. *Oncogene, 23*, 8401–8409.

Simmer, F., Tijsterman, M., Parrish, S., Koushika, S. P., Nonet, M. L., Fire, A., et al. (2002). Loss of the putative RNA-directed RNA polymerase RRF-3 makes *C. elegans* hypersensitive to RNAi. *Current Biology, 12*, 1317–1319.

Simmer, F., Moorman, C., van der Linden, A. M., Kuijk, E., van den Berghe, P. V. E., Kamath, R. S., et al. (2003). Genome-wide RNAi of *C. elegans* using the hypersensitive *rrf-3* strain reveals novel gene functions. *PLoS Biology, 1*, e12, 077–084.

Smith, N. A., Singh, S. P., Wang, M.-B., Stoutjesdijk, P. A., Green, A. G., & Waterhouse, P. M. (2000). Total silencing by intron-spliced hairpin RNAs. *Nature, 407*, 319–320.

Sobczak, M., Golinowski, W., & Grundler, F. M. W. (1999). Ultrastructure of feeding plugs and feeding tubes formed by *Heterodera schachtii*. *Nematol*ogy *1*, 363–374.

Sontheimer, E. J. (2005). Assembly and function of RNA silencing complexes. *Nature Reviews Molecular Cell Biology, 6*, 127–138.

Starr, J. L., Bridge, J., & Cook, R. (2002). Resistance to plant-parasitic nematodes: History, current use and future potential. In J. L. Starr, R. Cook, & J. Bridge (Eds.), *Plant resistance to parasitic nematodes* (pp. 1–22). Oxford, UK: CAB International.

Steeves, R. M., Todd, T. C., Essig, J. S., & Trick, H. N. (2006). Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress *Heterodera glycines* reproduction. *Functional Plant Biology, 33*, 991–999.

Tabara, H., Grishok, A., & Mello, C. C. (1998). RNAi in *C. elegans*: soaking in the genome sequence. *Science, 282*, 430–431.

Tabara, H., Yigit, Y., Siomi, H., & Mello, C. C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in *C. elegans*. *Cell, 109*, 861–871.

Tijsterman, M., May, R. C., Simmer, F., Okihara, K. L., & Plasterk, R. H. A. (2004). Genes required for systemic RNA interference in *Caenorhabditis elegans*. *Current Biology, 14*, 111–116.

Timmons, L., & Fire, A. (1998). Specific interference by ingested dsRNA. *Nature, 395*, 854.

Timmons, L., Court, D. L., & Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene, 263*, 103–112.

Tomari, Y., & Zamore, P. D. (2005). Perspective: machines for RNAi. *Genes & Development, 19*, 517–529.

Trudgill, D. L., & Blok, V. C. (2001). Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annual Review of Phytopathology, 39*, 53–77.

Urwin, P. E., Atkinson, H. J., Waller, D. A., & McPherson, M. J. (1995). Engineered oryzacystatin-1 expressed in transgenic hairy roots confers resistance to *Globodera pallida*. *Plant Journal, 8*, 121–131.

Urwin, P. E., Green, J., & Atkinson, H. J. (2003). Expression of a plant cystatin confers partial resistance to *Globodera*, full resistance is achieved by pyramiding a cystatin with natural resistance. *Molecular Breeding, 12*, 263–269.

Urwin, P. E., Levesley, A., McPherson, M. J., & Atkinson, H. J. (2000). Transgenic resistance to the nematode *Rotylenchulus reniformis* conferred by *A. thaliana* plants expressing proteinase inhibitors. *Molecular Breeding*, *6,* 257–264.

Urwin, P. E., Lilley, C. J., & Atkinson, H. J. (2002). Ingestion of double-stranded RNA by pre parasitic juvenile cyst nematodes leads to RNA interference. *Molecular Plant-Microbe Interactions, 15*, 747–752.

Urwin, P. E., Lilley, C. J., McPherson, M. J., & Atkinson, H. J. (1997a). Resistance to both cyst and root-knot nematodes conferred by transgenic *Arabidopsis* expressing a modified plant cystatin. *Plant Journal, 12*, 455–461.

Urwin, P. E., McPherson, M. J., & Atkinson, H. J. (1998). Enhanced transgenic plant resistance to nematodes by dual protease inhibitor constructs. *Planta, 204*, 472–479.

Urwin, P. E., Møller, S. G., Lilley, C. J., McPherson, M. J., & Atkinson, H. J. (1997b). Continual green-fluorescent protein monitoring of cauliflower mosaic virus 35S promoter activity in nematode-induced feeding cells in *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions, 10*, 394–400.

Vishnudasan, D., Tripathi, M. N., Rao, U., & Khurana, P. (2005). Assessment of nematode resistance in wheat transgenic plants expressing potato proteinase inhibitor (*PIN2*) gene. *Transgenic Research, 14*, 665–675

Voinnet, O. (2001). RNA silencing as a plant immune system against viruses. *Trends in Genetics, 17*, 449–459.

Voinnet, O. (2002). RNA silencing: Small RNAs as ubiquitous regulators of gene expression. *Current Opinion in Plant Biology, 5*, 444–451.

Waterhouse, P. M., Graham, M. W., & Wang, M. B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proceedings of the National Academy of Sciences of the USA, 95*, 13959–13964.

Waterhouse, P. M., Wang, M. B., & Lough, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature, 411*, 834–842.

Wesley, S. V., Helliwell, C. A., Smith, N. A., Wang, M.-B., Rouse, D. T., Liu, Q., et al. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant Journal, 27*, 581–590.

Winston, W. M., Molodowitch, C., & Hunter, C. P. (2002). Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science, 295*, 2456–2459.

Winter, M. D., McPherson, M. J., & Atkinson, H. J. (2002). Neuronal uptake of pesticides disrupts chemosensory cells of nematodes. *Parasitology, 125*, 561–565.

Yadav, B. C., Veluthambi, K., & Subramaniam, K. (2006). Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. *Molecular and Biochemical Parasitology, 148*, 219–222.

Zamore, P. D., Tuschl, T., Sharp, P. A., & Bartel, D. P. (2000). RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell, 101*, 25–33.