Opinion TRENDS in Plant Science Vol.10 No.8 August 2005

RNA interference and plant parasitic nematodes

Manjula Bakhetia, Wayne L. Charlton, Peter E. Urwin, Michael J. McPherson and Howard J. Atkinson

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

RNA interference (RNAi) has recently been demonstrated in plant parasitic nematodes. It is a potentially powerful investigative tool for the genome-wide identification of gene function that should help improve our understanding of plant parasitic nematodes. RNAi should help identify gene and, hence, protein targets for nematode control strategies. Prospects for novel resistance depend on the plant generating an effective form of double-stranded RNA in the absence of an endogenous target gene without detriment to itself. These RNA molecules must then become available to the nematode and be capable of ingestion via its feeding tube. If these requirements can be met, crop resistance could be achieved by a plant delivering a dsRNA that targets a nematode gene and induces a lethal or highly damaging RNAi effect on the parasite.

Plant parasitic nematodes

Plant parasitic nematodes generally fall into two main groupings: sedentary and migratory parasites. The sedentary forms include many of the economically important genera such as the root knot nematodes (e.g. *Meloidogyne*), the cyst nematodes (e.g. *Heterodera* and *Globodera*), plus several other genera (e.g. *Rotylenchulus*, *Nacobbus* and *Tylenchulus*). Following infection of the host root, the nematode modifies one or more plant cells, which causes their re-differentiation into a specialized feeding site that supports development of a sedentary, feeding and reproductively competent female. Root knot nematodes form giant cells by inducing plant cell mitosis without concomitant cytokinesis. By contrast, cyst nematodes induce syncytia by the dissolution of adjacent cell walls [1,2]. Both these forms of feeding site occur when root knot nematodes and cyst nematodes establish on one plant species. This suggests that the parasite defines the physiological and morphological changes in plant cell form. Now that some of the genes involved have been characterized, an understanding of the molecular roles of nematode secretions has begun to emerge [3].

Migratory plant parasitic nematodes remain mobile throughout their development and feed sequentially from many plant cells, often without prior cell modification. Some important genera such as *Radopholus* spp. and *Pratylenchus* spp. are endoparasites, whereas others are ectoparasites (e.g. *Longidorus* and *Belonolaimus*). Most plant parasitic nematodes have a hollow stylet through which they inject secretions into the plant cell and remove plant cell contents.

Corresponding author: McPherson, M.J. (m.j.mcpherson@leeds.ac.uk).

Importance of plant nematodes

The cost to world agriculture of nematode parasitism was estimated recently to be US$125 billion annually [4], although the lack of clear disease symptoms might lead some growers to under estimate yield loss. Integrating several control strategies is often necessary to limit economic losses. Chemical control is restricted by economic constraints, by grower preference or by government restrictions to limit the environmental harm that nematicides cause. Crop rotation is deployed as a pest management measure for those species with a restricted host range but it imposes hidden losses when alternative non-host crops provide less economic returns to the grower. Resistant cultivars are of value for the control of some nematodes such as several *Meloidogyne* species on tomato, *Globodera rostochiensis* on potato and *Heterodera glycines* on soybean [5].

The inadequacy of current control measures provides an opportunity for transgenic approaches to make an important contribution to an integrated pest management strategy. For example, transgenic plants expressing proteinase inhibitors can impart effective resistance against several nematode species [5]. An exciting and emerging strategy is the use of RNA interference technology as an investigative tool for target identification that might also provide a further basis for transgenic resistance.

Occurrence of RNA interference

The range of experimental systems that have been used to study homology-dependent gene silencing has led to different terms being used for similar processes [6]. RNA silencing triggered by double-stranded RNA (RNA interference or RNAi) was first demonstrated in 1998 for the free-living nematode *Caenorhabditis elegans* [7]. The effect can also be induced in insects [8], amphibians [9] and mammals [10–12]. A process termed quelling in *Neurospora crassa* might be mechanistically similar to RNAi in mammals [13,14]. Although the underlying mechanism of RNAi was first deciphered in *C. elegans*, a similar phenomenon in plants had previously been described as post-transcriptional gene silencing [15,16]. This might protect plants from mobile genetic elements such as dsRNA viruses or

www.sciencedirect.com 1360-1385/$ - see front matter doi:10.1016/j.tplants.2005.06.007

adverse consequences of transposon activity [1,17–21]. The detailed molecular mechanisms and various component proteins involved in RNAi have been thoroughly reviewed in mammalian and insect systems [22–27].

RNAi provides an experimental tool to help determine gene function in many organisms and might offer new approaches to pathogen control. The systemic RNAi mechanism in plants [28,29] has been manipulated to control viral pathogens [30]. The approach is being investigated for control of the human parasites *Plasmodium falciparum* and *Trypanosoma brucei* [31], plus human pathogenic and non-pathogenic fungi such as *Candida albicans* and *Neurospora crassa* [32].

RNAi mechanism in nematodes

The events involved in the RNAi response in nematodes are illustrated in Box 1. Plants display a similar RNAi mechanism. RNAi functions autonomously in mammalian cells but is transmitted systemically to neighbouring cells, both locally and to different tissues, in nematodes and in plants. In *C. elegans*, the RNAi-deficient (*sid*) locus involved in transmitting the silencing signal between cells has been identified. It encodes a protein of 11 transmembrane domains (SID1) and has a structure suggestive of an import–export channel that probably functions as a receptor [33]. *C. elegans* and presumably other nematodes have a mildly alkaline gut lumen that does not digest nucleic acids rapidly [34]. In *C. elegans*, RNAi can be achieved by feeding on bacteria expressing dsRNA [34–36], by oral uptake of the dsRNA from solution [37] or by microinjection [7,38]. The dsRNA moves systemically from the gut or injected cell, inducing gene silencing in other cells that express a homologous mRNA sequence. The RNAi phenotype can be inherited for at

Box 1. RNAi mechanism in *Caenorhabditis elegans*

A double-stranded RNA (>300 bp) is introduced into a cell following microinjection or feeding. This dsRNA is recognized by DICER (step 1 in Figure I), which cleaves the molecule in a processive ATP-dependent manner into a series of 21–23 bp duplexes that display 2 nt overhangs at their 3′ ends (step 2). These small duplexes, called siRNAs, can be transmitted systemically by interaction with the SID protein (product of *sid-1*) (Step II). The siRNA duplex complexes with the RISC, another nuclease protein (step 3). In a further ATP-dependent step, the duplex is unwound to give single-stranded (ss) siRNA leading to activation of the RISC (step 4). At this stage, release of ss siRNA might result in amplification of the RNAi effect (step I). The activated RISC searches for homologous mRNA transcripts by a base-pairing mechanism and cleaves the mRNA ~12–nt from the 3′-end of the siRNA (step 5) leading to mRNA degradation (step 6). There is also an amplification cycle in *Caenorhabditis elegans* that is carried out by RNA-dependent RNA polymerase, RdRP (product of *ego-1*) (step I). In this process, ss siRNA molecules derived from dissociated dsRNA or released from the activated RISC act as primers on complementary mRNA leading to the production of new dsRNA molecules that become substrates for DICER (step 1).

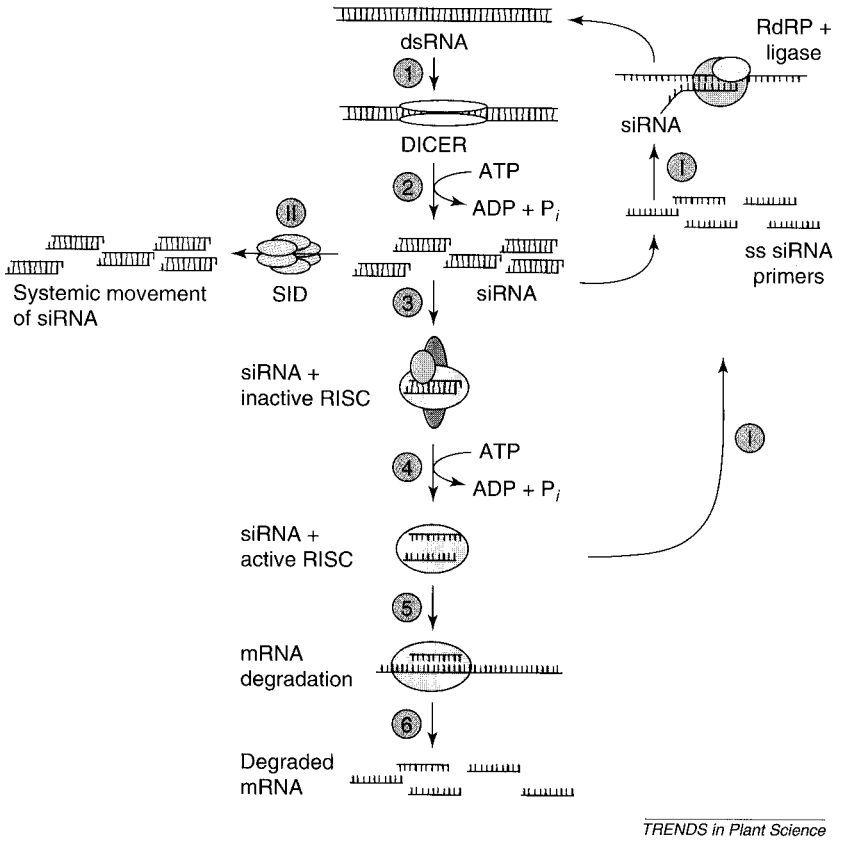

Figure I. Components involved in the RNAi response are: siRNAs, 21–23 bp small interfering RNAs; DICER, an RNase III-type enzyme; RISC, an RNA-induced silencing complex; RdRp, an RNA-dependent RNA polymerase; SID, a transmembrane 'channel' implicated in systemic silencing.

least two generations [39], a feature that might be advantageous for transgenic control if this effect also occurs for plant parasitic nematodes. A useful mutation, *rrf-3*, has been identified in *C. elegans*. This gene encodes the RNA-directed RNA polymerase RRF-3, which leads to a substantial increase in sensitivity to RNAi in a range of tissues [40,41]. If such a phenomenon also occurs in plant nematodes then co-suppression of *rrf-3* together with the target gene might lead to an enhanced RNAi effect.

Infective stages of plant parasitic nematodes are sufficiently small to make their microinjection with dsRNA a major technical challenge. In addition, they do not normally ingest fluid until they have infected a host plant. However, RNAi effects have been achieved using octopamine to stimulate oral ingestion by preparasitic second stage juveniles of *Heterodera glycines*, *Globodera pallida* [42] and *Meloidogyne incognita* [43]. Inclusion of fluoroscein isothiocyanate (FITC) in a dsRNA solution provides a visual marker to check uptake and, when necessary, select individuals for subsequent experiments. RNAi targeting of cysteine proteinase transcripts did not reduce the number of parasites that established on plants but it did alter their sexual fate in favour of males at 14 days post-infection (dpi) [42]. Exposure of *H. glycines* to dsRNA corresponding to a gene of unknown function that encodes a protein with homology to C-type lectins did not affect sexual fate, but 41% fewer nematodes were recovered from the plants [42]. As expected, treatment with dsRNA corresponding to the major sperm protein (MSP) had no effect on nematode development or sexual fate 14 days after treatment. Northern analysis showed reduced transcript abundance for the first two targeted mRNAs in the infective juvenile and for MSP transcripts when males reached sexual maturity and sperm are produced [42]. Other experiments in our laboratory showed efficient FITC uptake by soaking *Meloidogyne incognita*: 90–95% of individuals swallowed the dye. In these experiments, the target was a dual oxidase, a large enzyme comprising a peroxidase domain, EF-hands and NADPH oxidase domain, and potentially involved in extracellular matrix development. Feeding *M. incognita* juveniles on dual oxidase-derived dsRNA led to a reduction in the number and size of established females at 14 and 35 dpi with an overall reduction of ~70% in egg production relative to controls [43]. RNAi has also been induced for a chitin synthase gene that is expressed in the eggshells of *Meloidogyne artiellia* after soaking its developing eggs in a dsRNA [44].

RNAi experiments with juveniles result in phenotypic consequences that are evident up to 5 weeks after treatment. MSP is not expressed until >10 days after treatment. Consequently, the timing of delivery of dsRNA does not seem crucial for efficacy of the RNAi effect. This is pertinent with regard to the prospects of inducing an RNAi effect *in planta*, as discussed below. Now that the RNAi effect has been established for plant nematodes, a high-throughput assay is needed for rapid genome-wide gene function searches, as achieved for *C. elegans* [35]. However, the obligate parasitism and relatively long-life cycles of plant parasitic nematodes are additional limiting factors on the rate of progress of such studies.

Potential for *in planta* RNAi technology to target plant parasitic nematodes

Use of RNAi technology to control plant parasitic nematodes requires *in planta* expression of a dsRNA homologous to a gene that is essential for the parasite and its ingestion by the nematode. Swallowing might occur during invasion but a better approach is to target one or more genes whose expression is essential after feeding starts. The aim is to induce a highly detrimental or lethal RNAi phenotype. Key questions are whether delivery of dsRNA from plant cells to nematodes during feeding can mediate an RNAi response in the nematode and, if so, is this of sufficient magnitude to result in a lethal phenotype?

In mammals, a true knockout mutation does not result and genes in which null mutations display a lethal phenotype can be studied. Such a partial effect would be a disadvantage for qualitative plant resistance to nematodes. This might not be an issue because the process of RNAi differs between mammals and nematodes. Both nematodes [45] and plants [46] have RNA-dependent RNA polymerases that amplify small interfering (si) RNAs to elicit a systemic RNAi response [47]. Consequently, simply feeding the nematodes an appropriate dsRNA can induce an RNAi effect in cells remote from the intestine. Normally a dsRNA is expressed within an environment that also contains the target gene, leading directly to an RNAi phenotype. However, the fate of the dsRNA is unclear if the plant lacks a target gene (Figure 1). A key

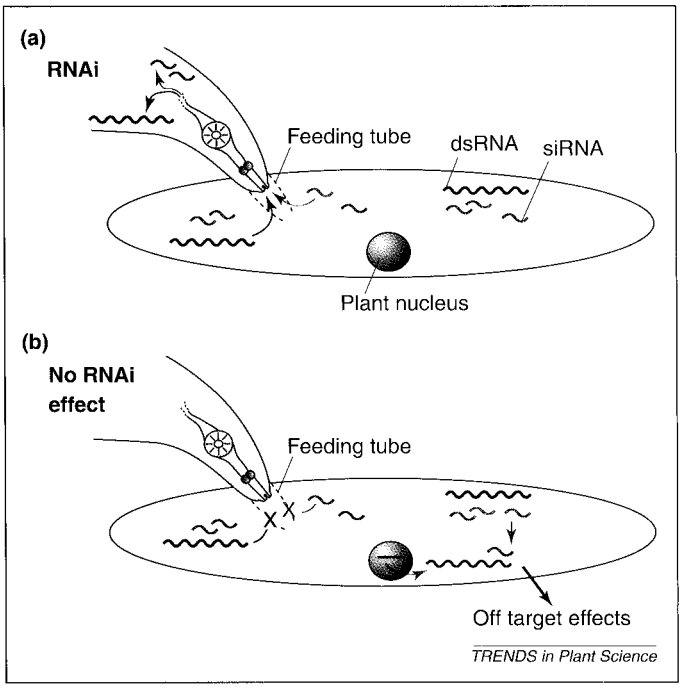

Figure 1. Schematic diagram showing possible fates of dsRNA and siRNA in a nematode–plant feeding cell interaction. **(a)** Productive RNAi in the nematode. dsRNA (blue) is produced in the feeding cell and might be processed by DICER to siRNA (red). dsRNA and/or siRNA might enter the nematode during feeding depending on the size exclusion limit of the feeding tube. Inside the nematode, RNAi is then mediated by RISC and the systemic response as detailed in Box 1. **(b)** Failure of RNAi in the nematode. Neither dsRNA nor siRNA enter the nematode, either because they are not available at sufficient concentration or are excluded from uptake by the pore size of the feeding tube. Consequently no RNAi effect is elicited in the nematode. Sequestration of siRNA by homologous non-target mRNAs transcribed from plant genes in the feeding cell nuclei might lead to off-target effects.

question is whether a true knockout can be achieved for plant parasitic nematodes.

The feeding tube and uptake of dsRNA

Oral uptake of dsRNA or siRNAs by established plant parasitic nematodes requires their passage through the feeding tube. This acts as a molecular sieve and it is not certain that dsRNA is efficiently ingested by all plant nematodes. In *Heterodera schachtii*, the feeding tube permits entry of dextrans of M_r 20 kDa but not M_r 40 kDa [48]. It also allows entry of cystatins of M_r 11 kDa but not a fusion protein of two proteinase inhibitors of M_r 22 kDa [49] or green fluorescent protein (GFP; M_r 28 kDa) [50]. *Globodera pallida* excludes much GFP uptake whereas *M. incognita* readily ingests this protein [50]. *M. incognita* or nematodes that do not form feeding tubes are therefore the favoured species for initial experiments. We estimate that a normal thread-like duplex structure of A-form RNA has a maximum diameter of 26 Å, irrespective of its length. Given that it is a base-paired duplex, a linear form is expected rather than the complex structures that can occur for single-stranded RNAs. Therefore, dsRNA or siRNA might be drawn lengthwise through the pores of the feeding tube. In this orientation its diameter is less than the 30 Å of the barrel-shaped GFP protein. However, the siRNAs are likely to be excluded if they form complexes with the RISC (RNA-induced silencing complex) before ingestion (Figure 1). Even so, some siRNAs might be taken up when they are not associated with the RISC complex.

Specificity of RNAi

RNAi has been considered to be highly gene specific but siRNAs can induce 'off-target' gene silencing effects [51–53] (Figure 1). Silencing is also induced by precursor microRNAs of ~70 nucleotides. They have a hairpin structure of imperfect complementarity within the genome and are processed to provide microRNAs of 21–25 nucleotides [54]. Their mode of action in mammalian cells depends upon the level of complementarity between the target and microRNA. If it is almost perfect, complementarity results in cleavage of the target RNA in a manner similar to that described for siRNA. Imperfect annealing occurs predominantly at the 3′ UTR and this triggers translational repression. In both cases, siRNAs and microRNAs are processed by Dicer-like proteins [55,56] associated with similar RISCs [57] (Box 1). MicroRNAs are normally involved in regulating endogenous gene expression during development. The off-target effects they induce were first recorded for *C. elegans* [58]. The discovery of microRNAs and the need to avoid possible off-target effects requires that the chosen dsRNAs have no unwanted consequences on the host plant. It might be best to avoid targeting a gene family that is highly conserved across the plant and animal kingdoms. Mis-targeting could also arise for genes that have full sequence similarity for just the 21 bp length of the causative agent in RNAi, with little similarity to the targeted gene over the majority of their sequence.

The silencing of genomic sequences situated 5′ of the targeted gene has also been observed in *C. elegans* [59]. The spreading of RNAi targeting from the initiator region into the adjacent 5′ and 3′ regions of the target gene also occurs in transgenic *Nicotiana benthamiana* and *Arabidopsis thaliana* [60].

Efficacy and durability

Development of transgenic nematode-resistant plants based on cysteine proteinase inhibitors (cystatins) provides information that is helpful for developing RNAi-mediated resistance. Transgenically expressed cystatin delivers 70–80% resistance against a range of nematodes on several host plants in containment glasshouses [61–65] and potato in the field [66]. The consequences of almost full inhibition of cysteine proteinases are probably mitigated by the other classes of nematode digestive proteinases [67]. By analogy, a full RNAi effect might depend on the ability of the nematode to compensate for loss of function of a target gene. There are several precedents of RNAi-induced lethality for *C. elegans* [35] that indicate homologous genes might provide high priority targets for parallel studies with plant parasitic nematodes.

High levels of resistance have been achieved by stacking more than one transgene into a construct for additive effects [50]. Co-expression of the dsRNA from several unrelated genes is also highly effective in *Drosophila* [68]. High-level efficacy might therefore be achieved by using a multi-component dsRNA targeting two or more plant parasitic nematode genes. As the RNAi mechanism is homology based, a single construct might reduce the expression of different genes from one family. This has been demonstrated for *Trypanosoma brucei* [69]. The possibility of targeting several genes by either of these methods provides a basis for pyramided, durable resistance against plant parasitic nematodes. In particular, inclusion of an *rrf-3* RNA-directed RNA polymerase homologue as part of a dsRNA chimera to enhance the efficacy of the RNAi effect is an attractive proposition. An alternative approach would be to transform a plant expressing partial natural resistance to achieve full resistance with a dsRNA, as already demonstrated for transgenic expression of a cystatin [62]. A further possibility is to achieve additive effects with a dsRNA and a transgene that expresses a bionematicide.

Biosafety

A key advantage of RNAi-mediated resistance is that dsRNA has no inherent translational ability to produce a functional protein. This means that non-target effects should be minimal and lower than those of even highly specific transgenic proteins such as the δ-endotoxins of *Bacillus thuringiensis* (*Bt*), which are already deployed to control insects in some crops. In addition, dsRNA is not new to the human diet. Virus-infected crop plants are commonly consumed and one consequence of infection with some viruses is that plants produce molecules similar to those used in RNAi technology. Work to underpin registration of RNAi-based plant defence as a biosafe approach will be needed but a favourable outcome is likely. A further level of safety could be provided by delivering dsRNA targeted at nematodes by using promoters that are

preferentially active within feeding cells relative to green tissue or the plant yield [70]. Promoters whose activity is restricted to roots might offer some biosafety advantage, particularly for migratory nematodes such as *Radopholus* that do not induce feeding cells. Protection of non-target species that also feed on the plant can be provided by using dsRNA that has no effect when ingested by other invertebrates such as insects. Any evaluation of non-target effects must also establish that the delivered dsRNA has no off-target effects on any plant tissues.

Concluding remarks

An RNAi effect has been demonstrated by pre-soaking juvenile cyst nematodes [42] and *M. incognita* [43] in dsRNA before infection of the host plant and by inducing the effect in eggs of *M. artiellia* [44]. This work establishes RNAi as a valuable tool for functional analysis of plant nematode genes. It should also help to identify candidate targets for control by chemical, transgenic protein or potentially RNAi-based approaches.

Several issues arise for bioengineering an RNAi-mediated defence against plant parasitic nematodes. They include the need to produce dsRNA *in planta* and the consequences of such expression in the absence of an endogenous, homologous gene sequence. One possibility is that this might induce off-target effects. The state of any RNA ingested by the nematode is important given that siRNA or siRNA bound to plant RISC might not induce an RNAi effect after ingestion. Even if the RISC complex form is active, its uptake or that of full length (~500 bp) dsRNA might not dose nematodes effectively if the feeding tube prevents uptake even partially (Figure 1). If none of these issues prove to be crucial constraints, RNAi might provide an approach to future nematode control that is more biosafe than are chemical or other transgenic approaches to control. Another key feature is that a wide range of genes can be targeted when a systemic RNAi response occurs in the nematode. A combination of the new technology with existing technologies or treatments might provide the most effective and durable basis for future control of these important plant pathogens.

Acknowledgements

We thank BBSRC for research funding.

References

1 Williamson, V.M. and Gleason, C.A. (2003) Plant–nematode interactions. *Curr. Opin. Plant Biol.* 6, 327–333
2 Davis, E.L. *et al.* (2000) Nematode parasitism genes. *Annu. Rev. Phytopathol.* 38, 365–396
3 Vanholm, B. *et al.* (2004) Secretions of plant-parasitic nematodes: a molecular update. *Gene* 332, 13–27
4 Chitwood, D.J. (2003) Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture-Agricultural Research Service. *Pest Manag. Sci.* 59, 748–753
5 Atkinson, H.J. *et al.* (2003) Engineering plants for nematode resistance. *Annu. Rev. Phytopath* 41, 615–639
6 Hammond, S.M. *et al.* (2001) Post-transcriptional gene silencing by double-stranded RNA. *Nat. Rev. Genet.* 2, 110–119
7 Fire, A. *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811
8 Kennerdell, J.R. and Carthew, R.W. (1998) Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. *Cell* 95, 1017–1026
9 Oelgeschlager, M. *et al.* (2000) The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling. *Nature* 405, 757–763
10 Hannon, G.J. (2002) RNA interference. *Nature* 418, 244–251
11 Silva, J. *et al.* (2004) RNA-interference-based functional genomics in mammalian cells: reverse genetics coming of age. *Oncogene* 23, 8401–8409
12 Zamore, P.D. *et al.* (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101, 25–33
13 Cogoni, C. and Macino, G. (2000) Post-transcriptional gene silencing across kingdoms. *Curr. Opin. Genet. Dev.* 10, 638–643
14 Forrest, E.C. *et al.* (2004) The RNA-dependent RNA polymerase, QDE-1, is a rate-limiting factor in post-transcriptional gene silencing in *Neurospora crassa*. *Nucleic Acids Res.* 32, 2123–2128
15 Jorgensen, R.A. *et al.* (1996) Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs antisense constructs and single-copy vs complex T-DNA sequences. *Plant Mol. Biol.* 31, 957–973
16 Waterhouse, P.M. *et al.* (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl. Acad. Sci. U. S. A.* 95, 13959–13964
17 Voinnet, O. (2001) RNA silencing as a plant immune system against viruses. *Trends Genet.* 17, 449–459
18 Waterhouse, P.M. *et al.* (2001) Gene silencing as an adaptive defence against viruses. *Nature* 411, 834–842
19 Tijsterman, M. *et al.* (2002) The genetics of RNA silencing. *Annu. Rev. Genet.* 36, 489–519
20 Vazquez Rovere, C. *et al.* (2002) RNA-mediated virus resistance. *Curr. Opin. Biotechnol.* 13, 167–172
21 Goldbach, R. *et al.* (2003) Resistance mechanisms to plant viruses. An overview. *Virus Res.* 92, 207–212
22 Agrawal, N. *et al.* (2003) RNA interference: biology, mechanism and applications. *Microbiol. Mol. Biol. Rev.* 67, 657–685
23 Elbashir, S.M. *et al.* (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 15, 188–200
24 Bernstein, E. *et al.* (2001) Retroviral role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363–366
25 Hammond, S.M. *et al.* (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 404, 293–296
26 Denli, A.M. and Hannon, G.J. (2003) RNAi: an ever-growing puzzle. *Trends Biochem. Sci.* 28, 196–201
27 Medema, R.H. (2004) Optimizing RNA interference for application in mammalian cells. *Biochem. J.* 380, 593–603
28 Palauqui, J.C. *et al.* (1997) Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J.* 16, 4738–4745
29 Voinnet, O. *et al.* (1998) Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* 95, 177–187
30 Pandolfini, T. *et al.* (2003) Expression of self-complementary hairpin RNA under the control of the *rolC* promoter confers systemic disease resistance to plum pox virus without preventing local infection. *BMC Biotechnol.* 3, 7
31 Ngo, H. *et al.* (1998) Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. U. S. A.* 95, 14687–14692
32 Cottrell, T.R. and Doering, T.L. (2003) Silence of the strands: RNA interference in eukaryotic pathogens. *Trends Microbiol.* 11, 37–43
33 Winston, W.M. *et al.* (2002) Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science* 295, 2456–2459
34 Timmons, L. and Fire, A. (1998) Specific interference by ingested dsRNA. *Nature* 395, 854
35 Kamath, R.S. *et al.* (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol.* 2, research0002.1–2.10
36 Timmons, L. *et al.* (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* 263, 103–112
37 Tabara, H. *et al.* (1998) RNAi in *C. elegans*: soaking in the genome sequence. *Science* 282, 430–431

38 Mello, C.C. and Conte, D., Jr. (2004) Revealing the world of RNA interference. *Nature* 431, 338–342
39 Grishok, A. *et al.* (2000) Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* 287, 2494–2497
40 Simmer, F. *et al.* (2002) Loss of the putative RNA-directed RNA polymerase RRF-3 makes *C. elegans* hypersensitive to RNAi. *Curr. Biol.* 12, 1317–1319
41 Simmer, F. *et al.* (2003) Genome-wide RNAi of *C. elegans* using the hypersensitive rrf-3 strain reveals novel gene functions. *PLoS Biology.* doi: 10.1371/journal.pbio.0000012 (http://biology.plosjournals.org/)
42 Urwin, P.E. *et al.* (2002) Ingestion of double-stranded RNA by preparasitic juvenile cyst nematodes leads to RNA interference. *Mol. Plant–Microbe Interact.* 15, 747–752
43 Bakhetia, M. *et al.* (2005) RNA interference of dual oxidase in the plant nematode *Meloidogyne incognita*. *Mol. Plant–Microbe Interact.* (in press)
44 Fanelli, E. *et al.* (2005) Analysis of chitin synthase function in a plant parasitic nematode, *Meloidogyne artiellia*, using RNAi. *Gene* 349, 87–95
45 Sijen, T. *et al.* (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* 107, 465–476
46 Dalmay, T. *et al.* (2000) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* 101, 543–553
47 Nishikura, K. (2001) A short primer on RNAi: RNA-directed RNA polymerase acts as a key catalyst. *Cell* 107, 415–418
48 Bockenhoff, A. and 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, 249–255
49 Urwin, P.E. *et al.* (1998) Enhanced transgenic plant resistance to nematodes by dual protease inhibitor constructs. *Planta* 204, 472–479
50 Urwin, P.E. *et al.* (1997) Continual green-fluorescent protein monitoring of cauliflower mosaic virus 35S promoter activity in nematode-induced feeding cells in *Arabidopsis thaliana*. *Mol. Plant–Microbe Interact.* 10, 394–400
51 Jackson, A. *et al.* (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21, 635–637
52 Saxena, S. *et al.* (2003) Small RNAs with imperfect match to endogenous mRNA repress translation: implications for off-target activity of small inhibitory RNA in mammalian cells. *J. Biol. Chem.* 278, 44312–44319
53 Scacheri, P.C. *et al.* (2004) Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 101, 1892–1897
54 Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297
55 Ketting, R.F. *et al.* (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev.* 15, 2654–2659
56 Grishok, A. *et al.* (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* 106, 23–34
57 He, L. and Hannon, G.J. (2004) MicroRNAs small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* 5, 522–531
58 Reinhart, B.J. *et al.* (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 403, 901–906
59 Alder, M.N. *et al.* (2003) Gene silencing in *Caenorhabditis elegans* by transitive RNA interference. *RNA* 9, 25–32
60 Vaistij, F.E. *et al.* (2002) Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell* 14, 857–867
61 Urwin, P.E. and Lilley, C.J. (1999) Resistance to plant parasitic nematodes conferred by GM-crops. *Pestic. Outlook* 10, 145–149
62 Urwin, P.E. *et al.* (2003) Expression of a plant cystatin confers partial resistance to Globodera, full resistance is achieved by pyramiding a cystatin with natural resistance. *Mol. Breed.* 12, 263–269
63 Urwin, P.E. *et al.* (1997) Resistance to both cyst and root-knot nematodes conferred by transgenic *Arabidopsis* expressing a modified plant cystatin. *Plant J.* 12, 455–461
64 Urwin, P.E. *et al.* (1997) Characterization of two cDNAs encoding cysteine proteinases from the soybean cyst nematode *Heterodera glycines*. *Parasitology* 114, 605–613
65 Atkinson, H.J. *et al.* (2004) Prototype demonstration of transgenic resistance to the nematode *Radopholus similis* conferred on banana by a cystatin. *Transgenic Res.* 13, 135–142
66 Urwin, P.E. *et al.* (2001) Effective transgenic resistance to *Globodera pallida* in potato field trials. *Mol. Breed.* 8, 95–101
67 Lilley, C.J. *et al.* (1996) Characterization of intestinally active proteinases of cyst-nematodes. *Parasitology* 113, 415–424
68 Schmid, A. *et al.* (2002) Combinatorial RNAi: a method for evaluating the functions of gene families in Drosophila. *Trends Neurosci.* 25, 71–74
69 Zoraghi, R. and Seebeck, T. (2002) The cAMP-specific phosphodiesterase TbPDE2C is an essential enzyme in bloodstream form *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. U. S. A.* 99, 4343–4348
70 Lilley, C.J. *et al.* (2004) Preferential expression of a plant cystatin at nematode feeding sites confers resistance to Meloidogyne incognita and *Globodera pallida*. *Plant Biotech. J.* 2, 3–12