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Review

RNA interference in plant parasitic nematodes

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RNA interference (RNAi, also called RNA-mediated interference) is a mechanism for RNA-guided regulation of gene expression in which double-stranded ribonucleic acid inhibits the expression of genes with complementary nucleotide sequences. Conserved in most eukaryotic organisms, the RNAi pathway is thought to have evolved as a form of innate immunity against viruses and also plays a major role in regulating development and genome maintenance. 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.

Key words: RNA interference, RNAi, gene expression, plant parasitic nematodes.

INTRODUCTION

Plant parasitic nematodes are found as pests of crops throughout the world with many having a severe economic effect. The cost to world agriculture of nematode parasitism was estimated recently to be US$125 billion annually (Chitwood, 2003), 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. 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 *Meloidogyne incognita* on tomato, *Globodera rostochiensis* and *Globodera pallida* on potato, *Heterodera glycines* on soybean and *Heterodera schachtii* on sugar beet (Roberts, 1992; Atkinson, 1995; Castagnone-Sereno, 2002; Atkinson et al., 2003). The most sustainable method of nematode control requiring no changes to existing cultural practices in the use of resistant plants that suppress nematode reproduction (Starr et al., 2002). Chemical control is restricted by economic constraints by grower preference or by government restrictions to limit the environmental harm that nematicides cause. It is not only costly in the developing world but involves application of compounds including carbamates such as Aldicarb, which is one of the most toxic and environmentally hazardous pesticides in widespread use. Toxicological problems and environmental damage caused by nematicides have either resulted in their withdrawal or severely restricted their use (Williamson and Gleason, 2003; Lilley et al., 2007).

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 import effective resistance against several nematode species (Atkinson et al., 2003). An exciting and emerging strategy is the use of RNAi technology as an investigative tool for target identification that might also provide a further basis for transgenic resistance in a range of organisms, including plant parasitic nematodes (Gheysen and Vanholme, 2007).

The limitations of conventional control procedures provide an important opportunity for plant biotechnology to produce effective and durable forms of nematode control. Principle advantages are (a) an approach to pest control that does not require other changes to agronomic practices, (b) a reduction in toxicological and environmental

Abbreviations: RNAi, RNA interference; **dsRNA**, double-stranded RNA; **RISC**, RNA-Induced Silencing Complex; **mRNA**, messenger RNA; **siRNA**, small interfering RNA; **miRNA**, micro RNA; **FITC**, Fluoroscein Isothiocyanate; **MSP**, Major Sperm Protein; **EF**-hands, Helix-turn-helix structural domain; and **NADPH**, Nicotinamide Adenine Dinucleotide Phosphate.

risks associated with chemical control and (c) the provision of effective, appropriate and inexpensive crop protection (Lilley et al., 2007).

BASIS OF RNA INTERFERENCE

The RNAi pathway is initiated by the enzyme dicer, which cleaves double-stranded RNA (dsRNA) to short double-stranded fragments of 20-25 base pairs. One of the two strands of each fragment, known as the guide strand, is then incorporated into the RNA-induced silencing complex (RISC) and base-pairs with complementary sequences. The exogenous dsRNA molecules are recognized by the dicer complex. The mostwell-studied outcome of this recognition event is a form of post-transcriptional gene silencing (Hammond, 2005). This occurs when the guide strand base pairs with a messenger RNA (mRNA) molecule and induces degradation of the mRNA by argonaute, the catalytic component of the RISC (Gregory et al., 2005). The short RNA fragments are known as small interfering RNA (siRNA) which are perfectly complementary to the gene to which they are suppressing as they are derived from long dsRNA of that same gene or microRNA (miRNA) which are derived from the intragenic regions or an intron and are thus only partially complementary (Gregory et al., 2006; Lion et al., 2006; Sijen et al., 2007).

The RNAi pathway has been particularly well-studied in certain model organisms. The first discovery of RNAi is credited to Fire and colleagues working with *Caenorhabditis elegans* (Fire et al., 1998). It had earlier been described as post-transcriptional gene silencing in plants (Jorgensen et al., 1996; Waterhouse et al., 1998). Over the past decade, research has shown an RNAi effect to be inducible in mammals (Zamore et al., 2000; Hannon, 2002; Silva et al., 2004), insects (Kennerdell and Carthew, 1998, 2000) and amphibians (Dirks et al., 2003; Li and Rohrer, 2006). The phenomenon of quelling in the fungus *Neurospora crassa* may also provide to be mechanistically similar to mammalian RNAi (Cogoni and Macino, 2000; Zamore et al., 2000; Hannon, 2002; Forrest et al., 2004; Silva et al., 2004).

In plants, RNAi was initially believed to be solely a defence mechanism against the deleterious effects of transposon movement or infection by dsRNA viruses (Voinnet, 2001; Waterhouse et al., 2001). It is now widely accepted that the process is also an integral part of normal gene regulation processes, a function that is not only limited to plants (Voinnet, 2002).

RNAi MECHANISM IN PLANT PARASITIC NEMATODES

Organisms vary in their ability to take up foreign dsRNA and use it in the RNAi pathway. The effects of RNAi can be both systemic and heritable in flowery plant *Arabidopsis thaliana* and nematode worm *C. elegans,* although not in fruit fly *Drosophila melanogaster* or mammals.

There is accumulating evidence for the efficacy of RNAi in plant parasitic nematodes. A range of genes have been targeted for silencing in cyst and root-knot nematode species and both the phenotypic and the molecular effects listed in Table 1. Nevertheless, the molecular detail of the RNAi process in plant parasitic nematodes has yet to be elucidated.

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 cyst nematodes *H. glycines*, *G. pallida* (Urwin et al., 2002) and root-knot nematode *M. incognita* (Bakhetia et al., 2005). Resorcinol and serotonin also induce dsRNA uptake by second stage juvenile of *M. incognita* and may be more effective than octopimine for this nematode (Rosso et al., 2005). Alterations to the original method, including 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 et al., 2005a).

The genes targeted by RNAi to date are expressed in a range of different tissues and cell types. The ingested dsRNA can silence genes in the intestine (Urwin et al., 2002; Shingles et al., 2007) and also in the female reproductive system (Lilley et al., 2005b), sperm (Urwin et al., 2002; Steeves et al., 2006), and both subventral and dorsal oesophageal glands (Chen et al., 2005a; Rosso et al., 2005; Huang et al., 2006a; Bakhetia et al., 2007). Uptake of dsRNA from the gut is a proven route to systemic RNAi in *C. elegans*. The systemic nature of RNAi in plant parasitic nematodes following ingestion of dsRNA suggests they share similar uptake and dispersal pathways.

Alternative routes to dsRNA uptake may exist for plant parasitic nematodes. RNAi of a chitin synthase gene expressed in the eggs of root-knot nematode *Meloidogyne artiella* was achieved by soaking intact eggs contained within their gelatinous matrix in a solution containing dsRNA (Fanelli et al., 2005). The enzyme plays a role in the synthesis of the chitinous layer in the eggshell. Depletion of its transcript by RNAi led to a reduction in stainable chitin in eggshells and a delay in hatching of juveniles from treated eggs. The results imply that the eggs of this nematode and possibly others are permeable to dsRNA.

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) (Urwin et al., 2002). Exposure of *H. glycines* to dsRNA correspond-

Table 1. Plant parasitic nematode genes targeted by RNAi*.

Nematode species	Gene function	RNAi effect	Site of gene expression
M. incognita	Cysteine proteinase	Delayed development. Decrease in number of established nematodes.	Intestine
	Dual oxidase	Decrease in number of established nematodes. Decrease in fecundity.	Presumed role in extracellular matrix
	Splicing factor	Reduced galling. Reduction in number of females.	Unknown
	Integrase	Reduced galling. Reduction in number of females.	Unknown
	Secreted peptide 16D 10	Reduced galling. Decrease in number of established nematodes.	Subventral pharyngeal glands
H. glycines	Cysteine proteinase	Increased male: female ratio.	Intestine
	C-type lectin	Decrease in number of established nematodes.	Hypodermis
	Major sperm protein	Reduction in mRNA-no phenotypic effect at 14 dpi.	Sperm
	Aminopeptidase	Decrease in number of established nematodes. Increased male: female ratio.	Female reproductive system
	β-1,4-endoglucanase	Decrease in number of established nematodes.	Subventral pharyngeal glands
	Pectate lyase	Increased male: female ratio.	Subventral pharyngeal glands
	Chorismate mutase	Increased male: female ratio.	Subventral and dorsal glands
	Secreted peptide SYV46	Decrease in number of established nematodes.	Dorsal pharyngeal gland
G. pallida	Cysteine proteinase	Increased male: female ratio.	Intestine
	FMR Famide-like peptides	Inhibition of motility.	Nervous system
M. artiellia	Chitin synthase	Delayed egg hatch.	Eggs
G. rostochiensis	β-1,4-endoglucanase	Decrease in number of established nematodes.	Subventral pharyngeal glands
	Secreted amphid protein	Reduced ability to locate and invade roots.	Amphids

*Taken in part from data of Lilley et al. (2007).

ing 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 (Urwin et al., 2002). 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 (Urwin et al., 2002). Other experiments showed efficient FITC uptake by soaking *M. 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 of and size of established females at 14 and 35 dpi with an overall reduction of 70% in egg production relative to controls (Bakhetia et al., 2005). RNAi has also been induced for a chitin synthase gene that is expressed in the eggshells of *M. artiella* after soaking its developing eggs in a dsRNA (Fanelli et al., 2005).

CONCLUDING REMARKS

Imported advances have been made in the biotechnological application of RNAi towards plant parasitic nematode control. 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. Genes that are good targets for this technology are likely to be nematode specific and have sequence conservation with orthologues from related species to maximize the spectrum of resistance.

RNAi effect has been demonstrated by pre-soaking juvenile cyst nematodes and root-knot nematodes in dsRNA before infection of the host plant and by inducing the effect in eggs of root-knot nematode. 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.

A combination of the RNAi technology with existing technologies or treatments might provide the most effective and durable basis for future control of these important plant parasitic nematodes.

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