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Environmental RNA interference in animals

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Animals interact with their environments all the time, and must react to all kinds of factors present in the environments. Environmental RNA interference refers to the phenomenon that exogenous RNA molecules can enter cells of certain animal species and interfere with gene expression of these animals. These exogenous RNAs may be molecules in animal food, or may be present in the living environment from other sources. Molecular pathways for the cell entry of environmental RNAs and further for their functionality in gene interference have started to be revealed in the animal model *Caenorhabditis elegans*. Here we highlight some known examples of environmental RNA interference (RNAi) in animals and introduce the molecular mechanisms underneath.

environmental RNAi, noncoding RNA, *Caenorhabditis elegans*, systemic RNAi

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Publication of the landmark article by Andrew Fire, Craig Mello, and colleagues in 1998 has linked double-stranded RNA (dsRNA) with the RNA interference (RNAi) phenomenon [1]. DsRNAs targeting specific gene sequences were injected into *Caenorhabditis elegans* for this work [1]. Andrew Fire and Craig Mello labs later developed alternative ways for triggering RNAi in *C. elegans* with exogenous dsRNAs [2,3]. What they did was soaking worms in dsRNA solution or feeding them with recombinant *E. coli* engineered to express dsRNA (feeding RNAi) [2,3]. These findings indicated that exogenous RNA, specifically dsRNA, could enter cells of *C. elegans* and initiate downstream RNAi effects. The fact that dsRNAs both injected into and fed to worms could trigger a systemic RNAi effect in most cells also indicated the existence of a spreading mechanism for cell to cell transportation/uptake of RNAi signals, presumably also dsRNAs. Actually this kind of environmental and cell nonautonomous RNAi effects have now been found in many organisms including animals, plants, and protozoans [4–6]. In the past decade, molecular pathways for the cell entry of environmental RNAs and the downstream RNAi effects have been substantially revealed in the animal model *C. elegans*. Efforts have also been made to develop biotechnologies based on the environmental RNAi phenomenon.

1 Uptake of environmental RNA and intercellular RNA movement in *C. elegans*

For environmental RNAi, exogenous RNAs have to be taken up into animal cells. Forward genetic screens with *C. elegans* have identified a number of genes that function in environmental RNAi, and some of them turned out to be involved in the uptaking of dsRNAs from the environments into intestinal cells [7–11] (Figure 1). Among them *sid-1* and *sid-2* are the first genes cloned functioning in environmental RNAi [4]. *sid-1* or *sid-2* single mutants lost the ability to trigger RNAi effect in intestinal cells upon feeding RNAi [4]. Actually *sid-1* mutant did not exhibit any systemic RNAi response to dsRNA injection, soaking, or feeding, while *sid-2* mutant exhibited defect in RNAi by soaking and feeding but remained sensitive to RNAi by injection. *sid-1* encodes a multipass transmembrane protein highly

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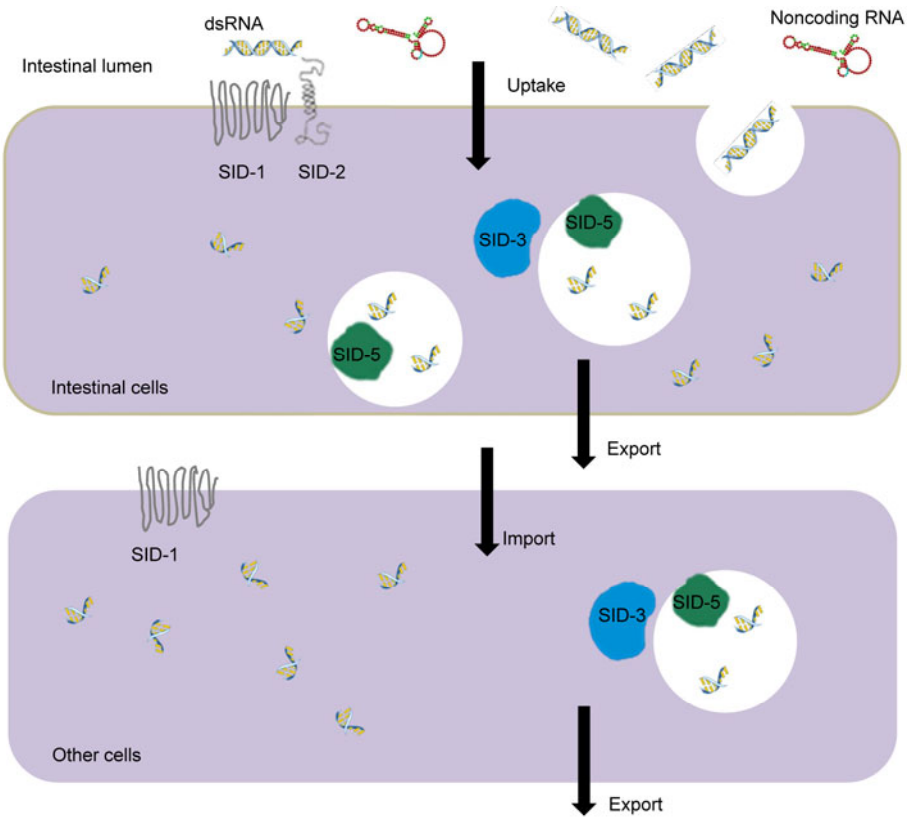

Figure 1 (Color online) Proposed pathway for uptaking, exporting, and importing exogenous environmental RNAs in *C. elegans*.

conserved from nematode to mammals [7,12]. *sid-2* encodes a single-pass transmembrane protein with homologs identified only in two sister species of *C. elegans*, *C. briggsae* and *C. remanei* [8]. *sid-1* is expressed in most non-neuronal cells including intestinal cells. *sid-2* is expressed in intestinal cells, and SID-2::GFP fusion localizes to intestinal cell apical membranes. Heterologous expression of either SID-1 or SID-2 in *Drosophila melanogaster* S2 cells led to cells gaining the ability of importing dsRNA from culture medium [12,13]. According to the structures and functions of SID-1 and SID-2, SID-1 may act as dsRNA channel or a component of channel for uptaking dsRNAs, and SID-2 may act as a dsRNA receptor or a component of receptor to aid the intestinal uptaking by SID-1 of environmental dsRNAs in intestinal lumen. Indeed, it is true that *sid-2* is required only for the uptaking of exogenous dsRNAs but is not necessary for the spreading of RNAi signal from intestinal cells to other cells.

Conversely, *sid-1* is required for both the initial import of dsRNA into worms and the spread of systemic RNAi signal, but not necessary for cells to export systemic RNAi signal [14]. SID-2-dependent dsRNA uptaking requires an acidic extracellular environment in intestinal lumen, and is selective for dsRNAs 50 base pairs or longer. SID-2-dependent transport could be suppressed inhibitory drugs of vesicle transportation. Therefore, both acidic environment and endocytosis seem vital in the uptaking of environmental dsRNA [13].

Reverse genetic screen with *C. elegans* and *Drosophila* S2 cells had identified multiple genes such as *arl-1*, *vps-34*, and *vps-41* in the endocytic pathway to be related to environmental RNAi [11]. Recently, *sid-3* and *sid-5*, two environmental RNAi genes related to endocytosis have been cloned by forward genetics [15,16]. In *sid-3* null mutants RNAi effects triggered by feeding were defective although not abolished [15]. *sid-3* encodes a conserved Ack (the activated Cdc-42-associated kinase) protein with a tyrosine kinase domain, a SH3 domain, and a Cdc42/Rac interactive binding domain. It seems that SID-3, a widely expressed cytoplasmic protein, is involved in the importing of silencing signals into cells but not for their exporting out of cells [15]. ACK in mammalian cells is well associated with endocytosis [17], although a direct link between *sid-3* with endocytosis in *C. elegans* is not established yet. *sid-5* encodes a worm specific small protein of 67 amino acids with a single predicted transmembrane domain [16]. It is clear that SID-5 protein is colocalized with several late endosomal proteins such as RAB-7 and LMP-1. *sid-5* is expressed in essentially all somatic cells, but an intestinal specific expressing of *sid-5* could rescue feeding RNAi defect in *sid-5* mutants. This result indicated that *sid-5* might mainly function to transport silencing signals across intestine by participating in dsRNA export out from intestinal cells and/or dsRNA uptake from the intestinal lumen [16].

2 *C. elegans* RNAi pathway triggered by exogenous RNA

Once inside *C. elegans* cells, exogenous dsRNAs follow a similar pathway to endogenous RNAi. Basically these dsRNAs are cut by DCR-1 with the assistance of RDE-4, RDE-1, DRH-1, and DRH-2 [18–22] (Figure 2). RDE-1 then directs the so-called primary siRNA to its targeting mRNA, resulting in the degradation of target mRNA and triggering the amplification of secondary siRNA by RNA dependent RNA polymerases (RdRP) such as RRF-1 and EGO-1 with the involvement of DRH-3 [23–27]. CSR-1 and some other secondary Argonaute proteins (SAGO) may be responsible for the gene silencing effect of secondary siRNA, and a number of factors such as MUT-7/-14/-15/-16, RDE-2/-3, SAGO-1/-2, and PPW-1 may play crucial roles in the effect of secondary siRNA [28–32] (Figure 2).

Nine out of 61 *C. elegans* ATP-binding cassette (ABC) transporters are also involved in the environmental RNAi effect [33]. Mutants of ABC transporter genes *abt-1*, *pgp-4*, *pgp-11*, *haf-2*, *haf-6*, *mrp-1*, *pmp-1*, *C16C10.12*, and *C05D10.3* showed strong defective in feeding RNAi [33]. Among these nine genes, *haf-6* was characterized further. *haf-6*(*ne335*) displayed dosage sensitive in injection and soaking induced RNAi. Injection or soaking with high concentrations of dsRNA could induce the RNAi effect, while lower concentrations were less effective. *haf-6* mutant also showed defect in intestinal and germline cells but not in other tissues in feeding RNAi. Research had shown that *haf-6* was expressed specifically in intestinal cells and germline cells, and HAF-6 protein might localize to endoplasmic reticulum [33]. All these 9 ABC transporters were later found to be interact genetically for their effects in RNAi with *rde-2* and *mut-7*, two genes known to be involved in the effects of secondary siRNA [34]. Just like *haf-6* mutant, mutants of two genes *rde-10* and *rde-11* were recently identified to be only sensitive to high dosage of dsRNA [35]. RDE-10, RDE-11, RSD-2, RSD-6, and HAF-6 may function together to promote the accumulation of secondary siRNAs originated from both exogenous and endogenous dsRNAs, although detailed molecular mechanism about exactly how these factors perform their job needs further investigation [34,35] (Figure 2). Secondary siRNAs from both exogenous and endogenous RNAs could also enter cell nucleus to inhibit gene transcription with the function of NRDE-2/-3 complex [36,37] (Figure 2). Exogenous environmental RNAi may also interfere with the endogenous RNAi in *C. elegans* by competing shared factors such as secondary Argonautes in the RNAi pathway [38].

3 Natural feeding RNAi in *C. elegans*

Essentially all experiments used to elucidate the molecular mechanisms underlying environmental RNAi were with artificially synthesized or recombinantly expressed dsRNAs under the laboratory condition. Investigators have recently found examples of natural feeding RNAi triggered by noncoding RNAs encoded by bacterial food of *C. elegans*. Ge Shan and colleagues identified two *Escherichia coli* noncoding RNAs, OxyS and DsrA, which could initiate feeding RNAi effect in *C. elegans* [39]. OxyS, a noncoding

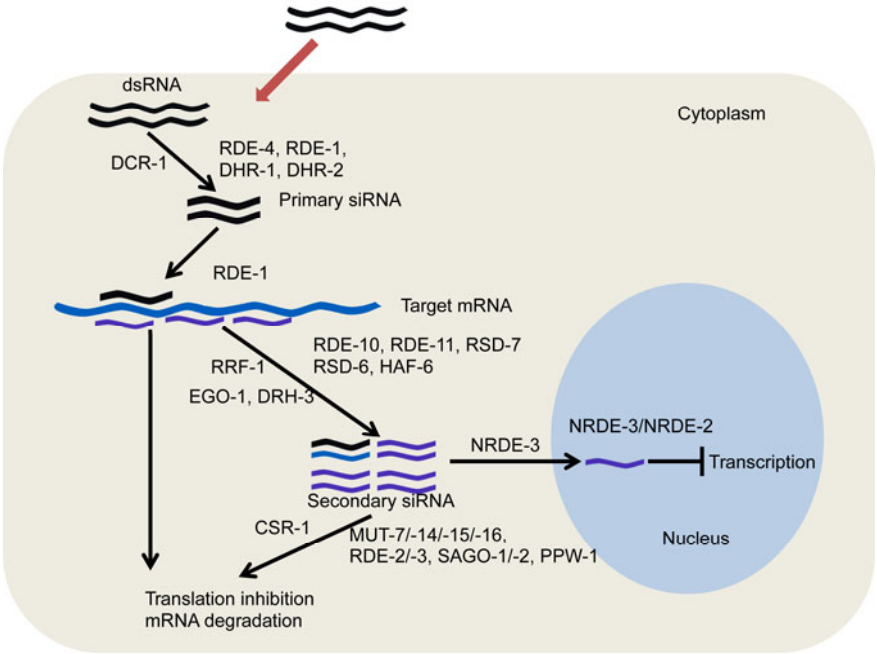

Figure 2 (Color online) Proposed molecular pathway for environmental RNA interference triggered by exogenous RNAs in *C. elegans*.

RNA expressed in *E. coli* under oxidative stress, impaired *C. elegans* olfactory ability by downregulating a chemosensory gene *che-2*. DsrA, a noncoding RNA expressed in *E. coli* under 25°C or below, decreased *C. elegans* longevity by suppressing diacylglycerol lipase gene *F42G9.6*, Other bacteria such as *Bacillus mycoides* might also utilize noncoding RNAs to interfere with gene expression in *C. elegans* [39]. These results demonstrated that *E. coli* noncoding RNAs could regulate gene expression and physiological conditions of *C. elegans* and indicated that noncoding RNAs might have interspecies ecological roles with natural feeding RNAi [39,40].

OxyS and *che-2* have 17 bp complementary sequences, and the inhibition of *che-2* by OxyS requires *alg-1*, *rde-4*, and the ABC transporter gene *haf-2*. DsrA and *F42G9.6* share a stretch of 27 nt sequence. For DsrA, *rde-4* and *haf-6* were required for its effect on *F42G9.6*. It was surprising that multiple other genes previously identified in the environmental RNAi pathway including *sid-1* and *sid-2* might be irrelevant or redundant for the RNAi effect of OxyS and DsrA [39]. It is possible that noncoding RNAs in real nature actually utilize an overlapping yet distinct molecular pathway from artificial dsRNAs in environmental RNAi.

4 Environmental RNAi in other animals

Besides *C. elegans*, environmental RNAi has been observed in a wide range of animal species [41,42]. Environmental RNAi is effective in a number of animal- and plant-parasitic nematodes [42–44]. In the planarian nematode *Dugesia japonica*, soaking of animals in a dsRNA solution could trigger gene knockdown both in cells with direct contact to the environment and in tissues inside the body [45]. Feeding bacteria expressing dsRNA to another planarian nematode *Schmidtea mediterranea* could also trigger an RNAi effect [46]. Efficient environmental RNAi could be achieved in the filarial nematode *Brugia malayi* by application of exogenous siRNA or dsRNA both *in vitro* (cultured separately from the host) and *in vivo* (in an intermediate host, the mosquito *Aedes aegypti*) [47].

Environmental RNAi by feeding or soaking has also been demonstrated in species of insects [48]. In the model organism *D. melanogaster*, soaking embryos in dsRNA solutions could trigger environmental RNAi effects [49]. There was even one report about induction of robust RNAi in *Drosophila* S2 cells with E. coli expressing dsRNA, and the authors believed that phagocytic uptake was underlying this RNAi effect [50]. However, there has been no report of environmental RNAi in *Drosophila* larvae or adults. Oral dsRNA delivery initiated RNAi effect of gene silencing in apple moth (*Epiphyas postvittana*) larvae but not in the oriental leafworm moth (*Spodoptera litura*) [51], although a previous study showed that dsRNA injected could silence of midgut aminopeptidase N in *Spodoptera litura* [52].

Environmental RNAi has also been observed in hydra, which belongs to cnidarians, a sister group to the bilaterians (vertebrates, insects, nematodes). Repeated feedings of dsRNA-expressing bacteria specifically suppressed the expression of target gene in hydra, and eventually phenocopied the phenotype shown in mutants of the target gene [53].

Unlike *C. elegans* and other invertebrates, mammals have complex digestive system and circulatory system, which may make them infeasible for environmental RNAi. Nevertheless a successful example has been reported, in which recombinant nonpathogenic short-hairpin RNA expressing *E. coli* suppressed the expression of an oncogene, *CTNNB1*, in the intestinal epithelium and in human colon cancer xenografts in mice [54]. Oral delivery of siRNA particles encapsulated with β-1,3-*D*-glucan, a component of yeast cell wall, could silence gene expression in mouse macrophages both *in vitro* and *in vivo* [55]. Both these examples are with engineered artificial delivery system of exogenous RNAs.

5 Features and perspectives of animal environmental RNAi

Mechanisms for the entry of environmental dsRNA into intestinal cells and the subsequent intercellular exporting and importing of environmental RNAi signal have only been investigated systematically in *C. elegans* for several years. Exact roles of *sid-1* and *sid-2* in uptaking of environmental dsRNA by intestinal cells are still elusive [7,8,12,14]. Is SID-1 a channel and SID-2 a receptor for dsRNA, or both of them just bind to dsRNA and promote the subsequent endocytosis of dsRNA? Genes such as *sid-3* and *sid-5* related to endocytosis and exocytosis have been linked to environmental RNAi in *C. elegans* and *Drosophila* cells, although a direct role of endocytosis and exocytosis in the uptaking, importing, and exporting of exogenous RNAs has not been assigned [15,16]. Further investigations must be performed to answer these questions.

While the general frame of functional mechanism for RNA interference is relatively conserved in multicellular organisms, a lot of details associated with environmental RNAi are not well conserved. *C. briggsae*, a sister species of *C. elegans*, is responsible for injected dsRNA, while it is incompetent in environmental RNAi by feeding or soaking [8]. Sequence divergence of SID-2 protein between *C. elegans* and *C. briggsae* may be underneath the differential sensitivity to environmental RNAi between these two species. *C. briggsae* SID-2 has relatively low sequence similarity to *C. elegans* SID-2, and this is especially the case for their outmembrane N termini, which share only 23% identity between them. Expressing the *C. elegans sid-2::gfp* fusion in *C. briggsae* resulted its sensitivity to environmental RNAi triggered by dsRNA [8]. On the other hand though, *C. briggsae* is sensitive to natural feeding RNAi initiated by *E.*

coli noncoding RNAs such as OxyS and DsrA, and actually *sid-1* and *sid-2* are irrelevant or redundant for this natural feeding RNAi phenomenon [39]. Both mice and human genomes harbor two *sid-1* homologs, SidT1 and SidT2, and SidT1 has a role in the uptake of dsRNA by human cells, as blocking or knocking down of SidT1 resulted in defect in internalization of cholesterol-conjugated siRNA by human hepatocyte *in vitro* [56].

A comparative analysis of the draft genome of the filarial nematode *Brugia malayi* with the *C. elegans* genome revealed that there were apparently no *sid-1* and *sid-2* homologs in *Brugia malayi* despite that this parasitic nematode is competent for environmental RNAi [57]. A survey has been performed for orthologs of 77 *Caenorhabditis elegans* RNAi pathway proteins in 13 nematode species (transcriptomes of *Ancylostoma caninum* and *Oesophagostomum dentatum*, genomes of *Trichinella spiralis*, *Ascaris suum*, *Brugia malayi*, *Haemonchus contortus*, *Meloidogyne hapla*, *Meloidogyne incognita*, *Pristionchus pacificus*, as well as four *Caenorhabditis* species *C. brenneri*, *C. briggsae*, *C. japonica* and *C. remanei*) [58]. The result revealed that most of the *C. elegans* genes responsible for uptaking and spreading of the exogenous dsRNA are absent from parasitic species, including those nematodes competent for environmental RNAi [58]. It is possible that these genes might be fast evolving, or animals might have evolved alternative pathways for the uptaking of environmental dsRNA.

The functional mechanism of environmental RNAs in animals or even in *C. elegans* is not fully understood neither. For example, defined roles of ABC transporters in environmental RNAi are missing [33–35]. These transporters may import some small molecules crucial for the generation of secondary siRNA in *C. elegans*, or they may be responsible for generating optimal condition (e.g. pH) for the effect of RNAi, and these possibilities have to be tested.

The functional pathway for exogenous environmental RNAi shares many components with that of the endogenous siRNA in *C. elegans*, and actually they may compete for shared factors such as secondary Argonautes [38]. Is there any regulatory mechanism to coordinate the effects of exogenous and endogenous RNAi? Can *C. elegans* actually differentiate exogenous RNAs from endogenous siRNAs? These intriguing questions demand vigorous researches. Functional pathway for environmental RNAi also shows some divergence, as secondary Argonautes are poorly conserved among nematodes with the nuclear AGO NRDE-3 missing in any parasitic nematode [58]. Functional mechanism of environmental RNAi shows divergence even within the *C. elegans* species. *C. elegans* wild isolate CB4856 is resistant to germline RNAi with exogenous dsRNA, and this resistance is linked to a single significant locus on chromosome I that includes the Argonaute gene *ppw-1* [59].

Functional mechanisms underlying environmental RNAi were basically elucidated with synthesized or recombinantly expressed dsRNAs. Natural feeding RNAi triggered by noncoding RNAs OxyS and DsrA utilize an overlapping but distinct molecular pathway from exogenous dsRNAs [39]. Are dsRNAs, or instead noncoding RNAs such as OxyS and DsrA, the major catalog of RNAs in real nature for triggering environmental RNAi? Careful and systematical investigations have to be carried out to answer this important question.

6 Environmental RNAi in a broader sense

Dependent on how we define environmental RNAi, any phenomenon related to the interfering of gene expression by exogenous RNA could be considered as environmental RNAi in a broader sense. It is highly possible that interspecies RNAs may take part in the interactions between parasites (or even infectious bacteria) and their hosts. Although RNAi effect has been observed in parasitic animals with artificially administrated exogenous RNAs, essentially there is still no report about "real" environmental RNAi elicited between parasites and hosts by their own RNAs. To stretch it further, even certain interactions between viruses specifically RNA viruses and their hosts may be considered as environmental RNAi, as genomic RNAs or RNA transcripts of virus often manipulate gene expression of host cells via RNAi pathway. More details about virus, host cell, and RNAi could be found in several review articles [60–62].

There is another more intriguing example of environmental RNAi if you like. Investigators recently reported that exogenous plant miRNAs were present in the sera and tissues of mice and human [63]. mir-168a, which is abundant in rice, was detected in microvesicles of animals after having taken rice as food. Plant mir-168a could silence low-density lipoprotein receptor adapter protein 1 (LDLRAP1) expression in mice [63]. Because plants and mammals are different in their functional mechanisms of microRNAs, and mammals have complex digestive system, it would be more convincing for researchers to reveal the uptaking/importing and the functional mechanisms of these exogenous plant microRNAs. It is already known that certain cells in mammals such as peripheral blood mononuclear cells can generate exosomes or microvesicles packed with RNAs inside [64]. Some miRNAs have been identified in these exosomes and they have been shown to have intercellular gene regulatory effects [65]. This could be viewed as a kind of systemic RNAi in mammals, but whether exogenous small RNAs could utilize these microvesicles for intercellular transportation remains to be examined.

7 Research and biotechnology application of environmental RNAi

Feeding dsRNA-expressing *E. coli* can be conveniently applied in *C. elegans* for knocking down gene expression of individual genes, or at whole-genome scale, to identify

candidate genes involved in specific biological events [66]. Whole-genome RNAi feeding libraries have been developed, and many successful systematic RNAi screenings have been performed [67–71]. In *C. elegans*, feeding RNAi can also be fine tuned to knockdown gene expression for a specific developmental stage [72]. Systematic RNAi screening also enables the application of reverse genetics in *Drosophila* S2 cells, since these cells can take up dsRNA from their culture medium [73,74].

A prominent field for application of environmental RNAi is in the control of pests of animals and plants. Soaking in dsRNA solutions could result in environmental RNAi effect in *Schistosoma mansoni*, a parasitic worm of human [75]. Multiple successful examples have been reported about exogenous dsRNA delivered from transgenic plants resulting in gene silencing in nematodes such as *Meloidogyne* species (root-knot nematodes)[76]. Ingestion of dsRNAs conditioned artificial diet triggered RNAi in the western corn rootworm (WCR, *Diabrotica virgifera virgifera LeConte*). Transgenic expressing dsRNAs against WCR gene in corn plants also showed protection to the crop again WCR feeding damage [77]. When cotton bollworm (*Helicoverpa armigera*) larvae were fed with plant material expressing dsRNA specific to a cytochrome P450 gene of cotton bollworm, decreased expression of this gene and retarded larval growth were observed [78]. Transgenic plants expressing dsRNA targeting to specific animals may be a general strategy to control agriculture pests.

Since the discovery of RNAi pathway, lot efforts have been made to develop RNAi-based therapeutics [66,79–81]. Attempts to apply short-hairpin RNA expressed *E. coli* for the suppressing oncogene in mice, deliver orally siRNA particles to silence gene expression in mouse macrophages, and inject siRNAs in saline solution for the knocking down of disease related genes are essentially application of environmental RNAi for the treatment for human diseases [54,55,79]. Although great optimism is hold for RNAi-based therapy, hurdles have to be overcome to bring it into reality.

8 Conclusion

A substantial amount of knowledge in the field of environmental RNAi has been accumulated so far, while we are still just at the beginning to uncover all the mysteries associated with this phenomenon. One thing we need to point out is that environmental RNAi has also been found in plants and protozoans [6,82–85]. Environmental RNAi has been extensively applied in research with *C. elegans*, but its application in biotechnology and therapeutics requires further development. Research in environmental RNAi will no doubt be an active branch to continuously bring novel insights in the years to come, as the whole field of noncoding RNA research is moving fast forward [86–90].

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