Chapter 4
RNAi for Resistance Against Biotic Stresses in Crop Plants

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Abstract RNA interference (RNAi)-based gene silencing has become one of the most successful strategies in not only identifying gene function but also in improving agronomical traits of crops by silencing genes of different pathogens/pests and also plant genes for improvement of desired trait. The conserved nature of RNAi pathway across different organisms increases its applicability in various basic and applied fields. Here we attempt to summarize the knowledge generated on the fundamental mechanisms of RNAi over the years, with emphasis on insects and plant-parasitic nematodes (PPNs). This chapter also reviews the rich history of RNAi research, gene regulation by small RNAs across different organisms, and application potential of RNAi for generating transgenic plants resistant to major pests. But, there are some limitations too which restrict wider applications of this technology to its full potential. Further refinement of this technology in terms of resolving these shortcomings constitutes one of the thrust areas in present RNAi research. Nevertheless, its application especially in breeding agricultural crops resistant against biotic stresses will certainly offer the possible solutions for some of the breeding objectives which are otherwise unattainable.

Keywords RNA interference · RNAi · Biotic stresses · Insect resistance · Disease resistance

4.1 Introduction

RNA interference (RNAi) is an invaluable technology for unraveling gene function in the area of functional genomics. It has been utilized in basic research ranging from functional studies to gene knockdown in plants and vertebrates and to suppression of cancer and viral diseases in medicine. Moreover, from application point of
view, it is being used extensively for trait modification by selective inhibition of gene expression universally across the organisms. In agriculture, RNAi has been extensively employed particularly for imparting resistance against biotic stresses including insects, bacteria, nematodes, fungal infection, and viruses (Tan and Yin 2004; Yanagihara et al. 2006; Good and Stach 2011; Banerjee et al. 2017; Majumdar et al. 2017; Zhang et al. 2017). This chapter focuses on how RNAi has been extensively used in managing various biotic stresses which constitute serious impediments to crop productivity. Damage due to insects, fungus, parasitic weeds, and plant-parasitic nematodes is a major biotic constraint causing significant yield losses in agriculture year-round.

4.2 History of RNAi

The basic concept involves a double-stranded RNA (dsRNA) molecule which potentially silences the gene with complementary sequences post-transcriptionally. RNAi phenomenon was first discovered in a free-living nematode, *Caenorhabditis elegans* (Fire et al. 1998). They coined the term “RNAi” for describing effective silencing of gene expression by exogenously supplied sense and antisense RNAs in the model nematode, *Caenorhabditis elegans*. This phenomenon, conserved among eukaryotes, was described as post-transcriptional gene silencing (PTGS) (Carthew and Sontheimer 2009; Berezikov 2011). Historically the roots of this exciting development can be traced back to 1990 when chsA gene was overexpressed in transgenic petunia plants and the silencing of endogenous as well as transgene of chalcone synthase in the transgenic plants was observed (Napoli et al. 1990). Loss of endogenous as well as transgene-derived mRNAs was described as co-suppression, a term formulated by Napoli. Soon, importance of this technology was well understood by the scientific community, and since then, phenomenal growth in this technology has taken place. In fungi, this mechanism of PTGS is known as quelling (Agrawal et al. 2003). In nature, viruses mediate PTGS in plants, and the effect is amplified in cytoplasm or in the nucleus.

4.3 Biogenesis and Mechanism of RNAi Pathway

The major small noncoding RNAs (ncRNAs) include microRNAs (miRNAs), small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs) which are all involved in downregulation of gene expression (Aalto and Pasquinelli 2012). Each class of small RNA is unique in its biogenesis and mechanism of action, but there are a few similarities too. Both miRNAs and siRNAs are processed from larger dsRNAs through cleavage by Dicer (a ribonuclease III enzyme). Both are associated with Argonaute proteins (AGO) (Ketting 2011) forming RNA-induced silencing complex (RISC). RISC basically is an Argonaute protein bound to a single strand of
noncoding RNA. Varied ribonucleoprotein complexes arise due to several ncRNAs and Argonautes involved in formation of RISC (Darrington et al. 2017).

The RNAi-mediated gene silencing occurs basically in three stages (Siomi and Siomi 2009). First one involves processing of long dsRNA into small dsRNA by ribonuclease III; in the second stage, unwinding of these small RNAs leads to formation of one guide strand, which is loaded into the RISC, whereas the other strand known as passenger strand gets degraded. Finally, the RISC, directed by the guide strand, locates mRNAs containing sequences complementary to the guide, binds to these sequences, and either degrades the mRNA or blocks its translation (Winter et al. 2009). The mechanism of RNAi is emerging with all its complexity, but with clarity, as more and more players involved in the interference are getting identified and characterized.

The involvement of siRNA molecules as important intermediates of the RNAi process became evident through independent investigations carried out by researchers around the world. The first report of accumulation of siRNAs was confirmed by Hamilton and Baulcombe (1999) while studying tomato lines transformed with 1-aminocyclopropane-1-carboxyl oxidase (ACO) and later in *Drosophila* syncytial blastoderm embryo (Tuschl et al. 1999). Two other independent studies experimentally exhibited the 21–23 nucleotide small RNAs as intermediates for degradation of mRNA (Zamore et al. 2000; Elbashir et al. 2001). But how these small RNA molecules are excised from their precursor was yet to be discovered. As the role of RNase III enzymes had been recognized as dsRNA nucleases already, the RNase III domain-containing proteins were searched as one of the factors in siRNA biogenesis. Recently only, different experimental studies revealed the involvement of RNA-processing enzymes in chopping off the dsRNAs into siRNA molecules. One of the crucial enzymes, Dicer, was identified in *Drosophila*, by browsing its genome for the proteins dedicated for functioning like RNase III endonuclease activity (Bernstein et al. 2001). In another study, Dicer protein in *C. elegans* (a bidentate nuclease) was characterized revealing its functional role in small RNA regulatory pathways (Ketting et al. 2001). It was also deduced to be the ortholog of *Drosophila* DCR-1 protein. Ketting et al. (2001) in this study also showed the requirement of ATP for regulating the rate of siRNA synthesis. In yet another experiment reduction in ATP levels by 5000-fold in *Drosophila* revealed a decrease in the rate of siRNA production (Nykanen et al. 2001). It is now believed that Dicer acts as a complex of proteins with domains for dsRNA binding at its C terminus which are separable from motifs like helicase and PAZ. It was experimentally found to co-localize with an endoplasmic reticulum protein, calreticulin (Caudy et al. 2002). However, the role of ATP in the biogenesis of siRNA is abstruse due to its varied functions among different Dicer proteins in different organisms. An imperative involvement of ATPase in siRNA production was exhibited by *Drosophila* Dicer-2 and *C. elegans* Dcr-1 (Tomari and Zamore 2005) in contrast to human Dicer wherein an ATPase-defective mutant showed regular processing (Carthew and Sontheimer 2009). A comprehensive biochemical, molecular, genetic, and structural study revealed the presence of two main domains, namely, PAZ and RNaseIII, performing a crucial role in excising the siRNAs (Zhang et al. 2004; Macrae et al. 2006).
Once Dicer cuts off the dsRNA, synthesized siRNAs then enter the RISC complex. The double-stranded siRNAs act as a template for the RISC to recognize the complementary mRNA aided by Argonaute proteins. Argonaute proteins are required for the RISC assembly and have been biochemically characterized in *Drosophila*. Amplification of siRNAs has been reported in nematodes, fungus, plants and amoeba (Dykxhoorn et al. 2003). RNA-dependent RNA polymerase (RdRP) is proposed to be involved in augmenting the siRNA molecules on the basis of biochemical studies (Lipardi et al. 2001; Sijen et al. 2001). Sijen demonstrated the fundamental role of *rrf1* gene having sequence homology to RdRP for the production of secondary siRNAs in *C. elegans*. In this study, the concept of transitive RNAi pathway induced by secondary siRNAs came into the picture. Thus, catalytic nature of RNAi was proposed.

4.4 RNAi in Insect Resistance

The direct loss in crop productivity due to damage by insect pest and the input-cost accrued in agrochemical based protection amount to billions of dollars every year worldwide. In spite of alarming environmental hazard directly due to residual toxicity of insecticides in food chain, the consumption of insecticides has been ever incremental. This is primarily due to resistance development in insect-pest population and lack of awareness among the farming community. The worldwide consumption of insecticide increases by almost 30% in every 4 years. Therefore, insect-pest management, preferably through an integrative approach and without indiscriminate use of insecticide, has become a most sought-after area in research planning worldwide. Millions of dollars were granted for researching on sustainable and low-cost alternate avenues of pest control strategies in five most important agricultural crops. Development of resistant cultivars in crops seems to be the most acclaimed alternative for minimizing the application of insecticides. Unfortunately, for most of the major crop- insect damage, either such resistant cultivars are not available or the resistance has been broken down. Further insight into such examples reveals that lack of resistance source maneuverable either through classical breeding or through transgenesis has been the major constraint.

Accessing unrelated gene pool through development of transgenics has emerged as the most potential avenue for overcoming this bottleneck. Success of *Bacillus thuringiensis* (*Bt*) toxin-mediated protection of a large number of crops has been celebrated widely and in fact demonstrated for the first time the potential of biotechnological means in developing genetic resistance. However, applicability of Bt-mediated protection is limited as many of the insect pests are not affected by Bt toxin, and also this technology has faced second-generation challenge of some major insect species developing resistance to *Bt* (Tabashnik 2008; Tabashnik et al. 2008). It has been realized that lack of useful insecticidal transgenes is the major
limitation in transgenic-based engineering of genetic resistance. In contrary, through RNAi, any important gene can be precisely targeted to elicit lethality in the insect species. Use of RNAi has rapidly progressed for gene function analysis in various insect orders, including Diptera (Lum et al. 2003; Dietzl et al. 2007), Lepidoptera (Tian et al. 2009; Terenius et al. 2011), Coleoptera (Baum et al. 2007; Zhu et al. 2011; Bolognesi et al. 2012), and Hymenoptera (Nunes and Simoes 2009; Meer and Choi 2013; Zhao and Chen 2013).

4.5 RNAi Pathway in Insects

Like in plants, RNAi is primarily involved in antiviral defense mechanisms of insects as a part of its innate immunity. However, a number of studies indicate several branches of RNAi involved in endogenous gene regulation in addition to silencing of genetic elements of pathogen invaders and transposons (Van Rij and Berezikov 2009). Gene silencing through RNAi is systemic and transitive as originally described in *C. elegans*. A host-derived RNA-dependent RNA polymerase (RdRp) amplifies the RNAi post-elicitation by dsRNA. In contrast to nematodes, in insects, there is no definite proof of the presence of RdRp. In the absence of RdRp-mediated amplification of dsRNA in insects, the silencing is expected to be more localized. Therefore, elicitation of an effective silencing will require delivery of the dsRNA directly to the target cells and tissues in a continuous manner. The administered dsRNA enters the insect cells via siRNA pathway in which a complex consisting of the RNAase III enzyme (Dicer-2) and TRBP cuts the dsRNA into small 21–23 bpsiRNAs. The RISC bound to AGO recognizes the guide strands of the siRNAs. This complex then binds to complementary sequences of target RNAs which are eventually degraded.

Two types of RNAi pathway are known to occur in insects: cell-autonomous and non-cell-autonomous RNAi. Cell-autonomous RNAi is limited to the cells in which the dsRNA is administered or delivered. In contrary, when the silencing occurs in cells different from the cells delivered with or producing the dsRNA, it is called non-cell-autonomous RNAi. Depending on how the dsRNA is acquired by the cell, non-cell-autonomous RNAi can be grouped in two kinds: environmental RNAi and systemic RNAi. In environmental RNAi, dsRNA is absorbed by a cell from the surrounding environment. Therefore, this is seen in unicellular organisms or any cell lines when administered with dsRNA. Environmental RNAi does not necessarily result into systemic spread of the response. In multicellular organisms, silencing signal is transported from one cell to another by systemic RNAi.

In case of transgenic host-mediated delivery of dsRNA, the dsRNA is delivered into the gut lumen of insects. For eliciting effective RNAi, dsRNA must be taken up by gut cells from the gut lumen which is known as environmental RNAi. If the transcripts of target genes are prevalently expressed in tissues outside the gut cells, the systemic RNAi has to occur for spreading of silencing signal. However, there is no definite study on assessing systemic RNAi in insects.
4.6 RNAi in Plant-Parasitic Nematodes (PPNs)

Plant-parasitic nematodes (PPNs) are grouped on the basis of different type of lifestyles, i.e., sedentary, including root-knot nematode (RKN) and cyst nematodes, and migratory, including root-lesion nematodes. Sedentary endoparasites interact with the host through secretions which are vital cues for plant-nematode interactions. These secretory proteins are thus of major interest as targets for modulating the interaction. RNAi has been extensively used in functional genomics performed on *C. elegans* and opened up the possibility of deciphering the function of uncharacterized genes in other parasitic nematodes. Recent discoveries focused on unraveling the role of different components of RNAi in parasitic nematodes has eventually led to increasing our understanding of RNAi mechanism.

There are overwhelming reports on managing PPNs using RNAi. In nematodes, systemic RNAi can be observed resulting in a gene knockout that spreads throughout the organism. This is because RNA-dependent RNA polymerase (RdRP) is present in nematodes which interact with RISC and leads to production of new dsRNAs which are acted upon by Dicer enzymes and further produces new siRNAs (secondary siRNAs) in a well-coordinated amplification reaction. Therefore, the effect of dsRNA persists over development and also can be exported to neighboring cells thereby leading to silencing effect all over the organism (Daniel and John 2008). *C. elegans* displays systemic RNAi wherein the dsRNA/siRNAs entering from the environment can spread from one cell to another. Studies on identification of effectors of systemic RNAi revealed presence of protein SID-1 in *C. elegans* (Winston et al. 2002; Feinberg and Hunter 2003). Interestingly, *M. incognita* and *M. hapla*, along with other parasitic nematodes, despite exhibiting successful RNAi, were found deficient in SID-1 and other related proteins having a key role in dsRNA uptake and its spread. Several detailed comparative studies have postulated the presence of RNAi components in different PPNs and animal parasitic nematodes that were reported in *C. elegans* (Lendner et al. 2008; Dalzell et al. 2011; Haegeman et al. 2011). All these studies found rare proteins taking part in RNAi pathway. Seventy-seven orthologous effectors in *C. elegans* were searched in 13 nematode species, *Ancylostoma caninum*, *Oesophagostomum dentatum*, *Ascaris suum*, *Brugia malayi*, *C. brenneri*, *C. briggsae*, *C. japonica*, *C. remanei*, *Haemonchus contortus*, *Meloidogyne hapla*, *M. incognita*, *Pristionchus pacificus*, and *Trichinella spiralis*, using reciprocal BLAST followed by domain structure verification (Maule et al. 2011). It was concluded that effector deficiencies cannot, in any way, be associated with reduced susceptibility in parasitic nematodes. Surprisingly, minimum diversity was observed among these parasitic nematodes in most of the orthologous genes belonging to different functional groups (Table 4.1). Thus it was evident that all the species possess varied proteins from across the RNAi spectrum each with alternative proteins which are yet to be fully identified and characterized.
4.7 Mode of dsRNA Delivery

The efficacy of gene silencing substantially depends on the method of dsRNA uptake. In absence of systemic RNAi, gene silencing shall be limited to the cells that take up the dsRNA. Therefore, appropriate delivery system is pivotal (Terenius et al. 2011). Different delivery methods of dsRNA that have been used for successful RNAi in insects and nematodes include microinjection, feeding on either artificial diet (Table 4.2), and/or host-mediated delivery through transgenic plants (Fig. 4.1). Each of these methods has its own advantages and limitations.
Table 4.2 Summary of targeted genes silenced by RNAi approach in plant-parasitic nematodes

| Target gene                  | Nematode       | Host plant | Phenotype                                      | Method of delivery | References               |
|------------------------------|----------------|------------|-----------------------------------------------|--------------------|--------------------------|
| Hgctl                        | *H. glycines*  |            | 41% reduction in number of nematodes          | Soaking            | Urwin et al. (2002)      |
| Hgcp-1                       | *H. glycines*  |            | 40% reduction in number of nematodes          | Soaking            | Urwin et al. (2002)      |
| MiDuox1                      | *M. incognita* |            | 70% reduction in number of nematodes          | Soaking            | Bakhetia et al. (2005)   |
| Gr-eng-1 and Gr-ams-1        | *G. rostochiensis* |          | Around 50% reduction in number of nematodes Reduced ability to locate and invade roots | Soaking            | Chen et al. (2005)       |
| Chitin synthase              | *M. artiellia* |            | Delayed egg hatch                             | Soaking            | Fanelli et al. (2005)    |
| Hg-amp-1                     | *H. glycines*  |            | 61% decrease in number of female reproductive | Soaking            | Lilley et al. (2005)     |
| Integrase and splicing       | *M. incognita* | Tobacco    | >90% reduction in number of established nematodes | HD-RNAi            | Yadav et al. (2006)      |
| Secreted peptide 16D10       | *M. incognita* | *Arabidopsis* | 63–90% reduction in number of galls and gall size | Soaking and HD-RNAi | Huang et al. (2006)      |
| Major sperm protein          | *H. glycines*  | Soybean    | Up to 68% reduction in number of eggs         | HD-RNAi            | Steeves et al. (2006)    |
| Putative transcription factor| *M. javanica*  | Tobacco    | None                                          | HD-RNAi            | Fairbairn et al. (2007)  |
| Ribosomal protein 3a, ribosomal protein 4, spliceosomal SR protein | *H. glycines* | Soybean    | 87% reduction in number of female cysts 81% reduction in number of female cysts 88% reduction in number of female cysts | HD-RNAi            | Klink et al. (2009)      |
| Protein/Coding Region | Organism | Species | Effect | Method | Reference |
|-----------------------|----------|---------|--------|--------|-----------|
| 4G06, ubiquitin-like, 3B05, cellulose-binding protein, 8H07, SKP1-like and 10AO6, zinc finger protein | H. schachtii | Arabidopsis | 23–64% reduction in number of developing females | HD-RNAi | Sindhu et al. (2009) |
| Y25, beta subunit of COPI complex | H. glycines | Soybean | 81% reduction in number of nematode eggs | HD-RNAi | Li et al. (2010a, b) |
| Prp-17, pre-mRNA splicing factor and Cpn-1 | H. glycines | Soybean | 79% reduction in number of nematode eggs | HD-RNAi | Li et al. (2010b) |
| Fib-1 | H. glycines | Soybean | 24% and 37% reduction in cyst and eggs, respectively | HD-RNAi | Li et al. (2010a) |
| Rpn7 | M. incognita | Tomato | Reduction in motility and infectivity of J2s | Soaking and HD-RNAi | Niu et al. (2012) |
| AF531170, parasitism gene | M. incognita | Tomato | 54–59% reduction in number of developing females | Choudhary et al. (2012) |
| 8D05, parasitism gene | M. incognita | Arabidopsis | Reduction in number of galls | HD-RNAi | Xue et al. (2013) |
| flp-14 and flp-18, FMRF amide-like peptide | M. incognita | Tobacco | Reduction in parasitic ability from 67–86% | HD-RNAi | Papolu et al. (2013) |
| Mi-ser-1, serine protease, Mi-cpl-1, cysteine protease and Mi-asp-1 + Mi-ser-1 + Mi-cpl-1 (fusion) | M. incognita | Tobacco | Reduction in number of eggs per gram of root | HD-RNAi | Antonino de Souza Júnior et al. (2013) |
| Pv010 | P. vulnus | Walnut | Reduction in number of nematodes | Feeding (bacterial) and HD-RNAi | Walawage et al. (2013) |
| Mc16D10L | M. chitwoodi | Potato | 65–68% reduction in the number of egg masses | HD-RNAi | Dinh et al. (2014a) |
| Mc16D10L | M. chitwoodi | Arabidopsis | 57 and 67% reduction in number of egg masses and eggs, respectively | HD-RNAi | Dinh et al. (2014b) |
| Mc16D10L | M. chitwoodi | Arabidopsis | Reduction in number of egg masses and eggs, respectively | HD-RNAi | Dinh et al. (2014b) |

(continued)
| Target gene         | Nematode            | Host plant | Phenotype                                                                 | Method of delivery | References                      |
|---------------------|---------------------|------------|---------------------------------------------------------------------------|--------------------|---------------------------------|
| Mi-cpl-1            | *M. incognita*      | Tomato     | 60–80% reduction in infection and multiplication                          | Soaking and HD-RNAi | Dutta et al. (2015)             |
| Pp-pat-10 and Pp-unc-87 | *P. penetrans*      | Soybean    | Up to 40% reduction in number of nematodes                                | Soaking and HD-RNAi | Vieira et al. (2015)            |
| Rs-cb-1             | *R. similis*        | Tobacco    | Reduced reproduction and pathogenicity                                    | Soaking and HD-RNAi | Li et al. (2015)                |
| HSP90, heat shock protein | *M. incognita*      | Tobacco    | Delayed gall formation and up to 46% reduction in the number of eggs      | HD-RNAi            | Lourenço-Tessutti et al. (2015) |
| ICL, isocitrate lyase|                     |            | Up to 77% reduction in egg oviposition                                   |                    |                                 |
| Unc-15              | *Ditylenchus destructor* | Sweet potato | 50% reduction in the infection area                                       | HD-RNAi            | Fan et al. (2015a, b)           |
| MiMSP40             | *M. incognita*      | Arabidopsis | Reduction in the number of galls                                          | HD-RNAi            | Niu et al. (2016)              |
| MeTCTP              | *M. enterolobii*    | Tomato     | Reduction in number of nematodes                                          | Tobacco rattle virus-mediated gene silencing | Zhuo et al. (2017) |
| Integrase and splicing | *M. incognita*      | Arabidopsis | 70% reduction in infection                                                | HD-RNAi            | Kumar et al. (2017)            |
| msp-18 and msp-20    | *M. incognita*      | Eggplant   | 43.64–69.68% and 41.74–67.30% reduction in nematode multiplication, respectively | HD-RNAi            | Shivakumara et al. (2017)      |
4.7.1 Microinjection

Microinjection involves injection of dsRNA or siRNA directly into the body of an organism and has been demonstrated as one of the most successful delivery methods for RNAi to validate gene functions (Ober and Jockusch 2006). In this method, dsRNA is produced by in vitro transcription using T7 or Sp6 promoter sequences. It has been employed successfully for suppressing genes in both insects and nematodes.

4.7.1.1 In Insects

In *D. melanogaster*, microinjection has been successfully used for delivering dsRNAs for two genes, viz., *frizzled* and *frizzled2*, into embryos. The silencing resulted in defects in embryonic patterning that was similar to loss of wingless (wg) function. This was the first study proving the function of *frizzle* through dsRNA microinjection in an insect (Kennerdell and Carthew 1998). Since then, microinjection-based delivery has been used in several insect species. A comprehensive list of Hemipteran insects subjected to microinjection for studying RNAi is presented in Table 4.3. Direct injection of dsRNA into the insect body leads to higher efficiency of gene expression attenuation compared to other methods. Nevertheless, there are several limitations in microinjection delivery method. In vitro synthesis of dsRNA is skill intensive and costly. Additionally, recovery of the insects, especially smaller insects, from aftershock of microinjection, is relatively low. The significant aftershock is due to damage of cuticle leading to adverse immune responses in the insect (Roxstrom-Lindquist et al. 2004). Therefore, microinjection is rarely used in functional analysis of large number of genes from the point of view of insect-pest control. It is evident from Table 4.3 that in the microinjection, mediated delivery has been carried out mostly in the case of hemipteran insects.
4.7.1.2 In Nematodes

After injecting dsRNAs into the worms, progeny is counted and recorded for the mutant phenotypes. Usually after 24 h of injection, good RNAi effect is observed (Fire et al. 1998). In *C. elegans*, dsRNAs of genes like *unc-22, unc-54, fem1*, and *hlh-1* were injected into the adult hermaphrodites, and the interference effect was observed. It was also proposed that in an antisense mechanism, interference of endogenous gene is due to the hybridization between the injected RNA and endogenous mRNA (Fire et al. 1998). It is a classical technique, and different target mRNAs can be used for injection simultaneously. However, microinjection has not been very successful in plant-parasitic nematodes in general and particularly in *M. incognita*. This is because of the small size of the infective stages and their inability to ingest fluid without host plant infection (Banerjee et al. 2017). In this process, although the range of dsRNA concentrations can be used, the success rate relies upon ample uptake or absorption by the worms (Hull and Timmons 2004).

4.7.2 Feeding on Artificial Diet

4.7.2.1 In Insects

dsRNA delivery through artificial diet has been the most popular method for delivering dsRNA into the insect gut especially for relatively smaller insects such as Hemipterans, which are sap-sucking. Several insect species of different taxa were studied for RNAi by the administration of dsRNA through artificial diet as presented in Table 4.3. Araujo et al. (2006) fed the blood-sucking *Rhodnius prolixus* with an artificial diet containing dsRNA of the *nitrophorin2* (*Np2*) gene and found that the saliva of control *R. prolixus* prolonged plasma coagulation by approximately fourfold compared with the saliva of *Np2*-knockdown *R. prolixus*. Feeding *A. pisum* with an artificial diet supplemented with dsRNA of the *A. pisum* aquaporin 1 (*ApAQP1*) gene caused attenuated expression of the target gene, which resulted in an increased osmotic pressure of the hemolymph in this insect (Shakesby et al. 2009).

4.7.2.2 In Nematodes

In a nematode, feeding involves ingestion of bacteria expressing dsRNA of the target gene against which RNAi is employed. Timmons et al. (2001) developed engineered bacteria deficient for RNaseIII producing high levels of dsRNA segments of a specific gene. *C. elegans* feeding on these engineered bacteria showed RNAi effect leading to loss-of-function phenotypes for the target genes. One of the advantages of this method is that it can be conducted for stage-specific RNAi experiments as worms of any stage can be fed with dsRNA (Kamath et al. 2001; Ahringer 2006).
Table 4.3  List of genes targeted for gene silencing in different insect orders

| Organism | Target gene | Stage | Assay method | Conc. of dsRNA/siRNA | Phenotype/mRNA silencing | References |
|----------|-------------|-------|--------------|----------------------|--------------------------|------------|
| Coleoptera | Diabrotica virgifera virgifera | Multiple targets | Neonates | Artificial diet | 1–10 ppb | Larval stunting and mortality | Baum et al. (2007) |
| | | Snf 7 | Neonates | Artificial diet | 4.3 ppb | Growth inhibition and mortality | Bolognesi et al. (2012) |
| | | V-ATPase A and E | Neonates | Artificial diet | ~0.1 ppm | Larval stunting and mortality | Baum et al. (2007) |
| | | α-Tubulin | Neonates | Artificial diet | ~0.1 ppm | Larval stunting and mortality | Baum et al. (2007) |
| | | Leptinotarsa decemlineata | V-ATPase A and E | Neonates | Artificial diet | ~10 ppb | Larval stunting and mortality | Baum et al. (2007) |
| | | Multiple targets | Neonates | Leaf tissue | ND | Reduced body weight and mortality | Zhu et al. (2011) |
| | Phyllostreta striolata | Arginine kinase | Adults | Leaf tissue | 0.8 ppb | Growth retardation, reduced fecundity, and increased mortality | Zhao et al. (2008) |
| Diptera | Tribolium castaneum | V-ATPase E | Neonates | Artificial diet | 2.5 ppm | Reduced growth and mortality | Whyard et al. (2009) |
| Aedes aegypti | V-ATPase A | Adults | Artificial diet | ~1000 ppm | Significant transcript knockdown | Coy et al. (2012) |
| | Multiple targets | First instars | Water | 200, 500 ppm | Reduced growth and mortality | Singh et al. (2013) |
| | ATP-dependent efflux pump | Second instars | Water | ~30 ppm | Increased toxicity | Figueira-Mansur et al. (2013) |
| Anopheles gambiae | Chitin synthase 1, 2 | Third instars | Artificial diet | – | Increased susceptibility to insecticides | Zhang et al. (2010) |

(continued)
| Organism                  | Target gene | Stage            | Assay method        | Conc. of dsRNA/siRNA | Phenotype/mRNA silencing                      | References                  |
|--------------------------|-------------|------------------|---------------------|----------------------|----------------------------------------------|-----------------------------|
| *Anopheles stephensi*    | 3-HKT       | First instars    | Transgenic Chlamydomonas | ND                   | >50% mortality                              | Kumar et al. (2013)         |
| *Bactrocera dorsalis*     | Multiple targets | Adults | Artificial diet | 2000 ppm | Affected egg production and 20% mortality | Li et al. (2011b)           |
| *Glossina morsitans morsitans* | Tsetse EP | Male adults | Blood meal | >400 ppm | No mortality | Walshe et al. (2009) |
|                          | Transferrin | Male adults | Blood meal | >400 ppm | No mortality | Walshe et al. (2009) |
| Hemiptera                |             |                  |                     |                      |                                              |                             |
| *Acyrthosiphon pisum*     | Aquaporin   | 6-day-old nymphs | Artificial diet | 1000–5000 ppm | Elevated osmotic pressure of the hemolymph | Shakesby et al. (2009)      |
|                          | V-ATPase E  | First instars    | Artificial diet | 3.4 ppm | Reduced growth and mortality | Whyard et al. (2009)         |
|                          | V-ATPase E  | Neonates | Artificial diet | ND | dsRNA degradation in saliva hemolymph | Christiaens et al. (2014)   |
|                          | Hunchback (hb) | Neonates | Artificial diet | 750 ppm | Increased mortality | Mao and Zeng (2012) |
|                          | Salivary protein COO2 | Adults | Injection | 50 ng | Lethal | Mutti et al. (2006) |
|                          | Gut digestive enzyme cathepsin-L | Adults | Injection or feeding | 92–460 ng 0.9–2.6 μg μL−1 | Higher mortality and impaired molting | Sapountzis et al. (2014) |
|                          | Structural sheath protein SHP | Adults | Injection | 50 ng | Impaired long-term feeding from sieve tubes and reduced fecundity | Will and Vilcinskas (2015) |
|                          | Angiotensin-converting enzymes ACE1 and ACE2 | Adults | Injection | 138 ng | Higher mortality of aphids feeding on plant | Wang et al. (2015) |
| Target Species                      | Gene/Protein                      | Treatment Type | Treatments | Concentration | Effects/Outcomes                                                                 | Reference          |
|------------------------------------|-----------------------------------|----------------|------------|---------------|---------------------------------------------------------------------------------|--------------------|
| *Aphis gossypii* (cotton aphid)    | Peroxiredoxin 1 gene ApPrx1       | Adults Injection | 285.2 ng  | Decreased survival of aphids under oxidative stress | Zhang and Lu (2015) |
|                                   | Macrophage migration inhibitory   | Adults Injection | 100 ng   | Decreased survival and fecundity of aphids feeding on their host plant | Naessens et al. (2015) |
|                                   | factor ApMIF1                     |                |           |               |                                                                                 |
|                                   | Calreticulin, Cathepsin-L         | Adults Microinjection | 5 nl, 23 nl and 46 nl of siRNA (6 μg/μl) | Insignificant RNAi effects | Possamai et al. (2007) |
|                                   |                                   |                |           |               |                                                                                 |
|                                   | Carboxylesterase gene CarE       | Adults Feeding  | 50–500 ng μL⁻¹ | Reduced resistance to organophosphorus insecticides | Gong et al. (2014) |
| *Aphis gossypii* (cotton aphid)    | Cytochrome P450 monooxygenase gene CYP6A2 | Adults Feeding  | 100 ng μL⁻¹ | Increased sensitivity of the resistant aphids to spirotetramat and alpha-cypermethrin | Peng et al. (2016) |
|                                   | Odorant-binding protein 2 AgOBP2  | Adults Feeding  | 62.5–250 ng μL⁻¹ | Impaired host-seeking and oviposition behavior of aphids | Rebijith et al. (2016) |
| *Bactericera cockerelli*           | Multiple targets                  | Adults Artificial diet | 500–1000 ppm | Mortality | Wuriyanghan et al. (2011) |
| *Bemisia tabaci*                   | V-ATPase subunit A, rpL19         | Adults Artificial diet | 3.11 ppm | Mortality | Upadhyay et al. (2011) |
| *Nilaparvata lugens* (brown plant hopper) | Trehalose PO4 synthase             | Third instars Artificial diet | 500 ppm | Lethality | Chen et al. (2010) |
|                                   | V-ATPase E                        | 2nd instars Artificial diet | 50 ppm | Transcript knockdown and no mortality | Li et al. (2011a) |

(continued)
| Organism                          | Target gene                                                                 | Stage       | Assay method | Conc. of dsRNA/siRNA | Phenotype/mRNA silencing                                                                 | References                                      |
|----------------------------------|------------------------------------------------------------------------------|-------------|--------------|---------------------|----------------------------------------------------------------------------------------|------------------------------------------------|
| Grain aphid *(Sitobion avenae)*   | Catalase gene CAT                                                           | Third instar| Feeding      | 7.5 ng μL⁻¹         | Reduced survival rate and ecdysis index                                                  | Deng and Zhao (2014)                           |
|                                  | Acetylcholinesterase gene SaAce1                                            | Adults      | Injection    | 10 ng               | Increased susceptibilities to pirimicarb and malathion and reduced fecundity            | Xiao et al. (2015)                             |
|                                  | Cytochrome c oxidase subunit VIIc precursor; zinc finger protein; three unknown proteins | Third instars | Feeding      | 3–7.5 ppm         | Higher mortality and developmental stunting                                             | Zhang et al. (2013)                            |
|                                  | Secreted salivary peptide DSR32; salivary protein DSR33; serine protease 1 DSR48 | Adults      | Feeding      | 10 ng μL⁻¹         | Higher mortality                                                                       | Wang et al. (2015)                             |
|                                  | Olfactory coreceptor gene SaveOrco                                          | Adults      | Feeding      | 20 ng μL⁻¹         | Impaired response to behaviorally active odors                                          | Fan et al. (2015a, b)                          |
| Bird cherry-oat aphid *Rhopalosiphum padi* | Acetylcholinesterase gene RpAce1                                            | Adults      | Injection    | 10 ng               | Increased susceptibilities to pirimicarb and malathion and reduced fecundity            | Xiao et al. (2015)                             |
| Greenbug *Schizaphis graminum*    | Salivary protein C002                                                       | Adults      | Feeding      | 20 ng μL⁻¹         | Lethal                                                                                 | Zhang et al. (2015a, b)                        |
| *Peregrinus maidis*               | V-ATPase B and D                                                             | Third instars| Artificial diet | 500 ppm          | Reduced fecundity and mortality                                                          | Yao et al. (2013)                              |
| *Rhodnius prolixus*               | Nitrophorin 2                                                                | Second instars| Artificial diet | 1000 ppm          | Changes in aphid saliva content                                                         | Araujo et al. (2006)                           |
| *Lygus lineolaris*                | Inhibitor of apoptosis                                                      | Neonates    | Artificial diet | 1000 ppm          | Digestion of dsRNA                                                                      | Allen and Walker (2012)                        |
| **Hymenoptera** |  |  |  |  |  |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| *Apis mellifera* | Vitellogenin | Second instars | Natural diet | 500–3000 ppm | Developmental stunting | Nunes and Simoes (2009) |
| *Solenopsis invicta* | PBAN/pyrokinin | Fourth instars | Artificial diet | 1000 ppm | Mortality of pupae | Vander Meer and Choi (2013) |
|  | Guanine nucleotide binding GNBP | Worker ants | Artificial diet | 200 ppm | Mortality | Zhao and Chen (2013) |

| **Isoptera** |  |  |  |  |  |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| *Reticulitermes flavipes* | Cellulase | Workers | Paper discs | 5.1 μ g/cm² | Impact on molting and change in feeding behavior | Zhou et al. (2008) |
|  | Hexamerin | Workers | Paper discs | 2.2 μ g/cm² | Impact on molting and change in feeding behavior | Zhou et al. (2008) |

| **Lepidoptera** |  |  |  |  |  |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| *Chilo infuscatellus* | CiHR3 molting factor | Third instars | Corn kernels | 250 ppm | Yes | Zhang et al. (2012) |
| *Epiphyas postvittana* | Carboxylesterase | Third instars | Droplet | 1000 ppm | Yes | Turner et al. (2006) |
|  | Pheromone bp | Third instars | Droplet | 1000 ppm | Yes | Turner et al. (2006) |
| *Helicoverpa armigera* | AchE receptor | Neonates | Artificial diet | ~0.35 ppm | Reduced fecundity, pupal weight reduction, mortality | Kumar et al. (2009) |
|  | AchE receptor | Neonates | Leaf tissue | ~0.35 ppm | Mortality | Kumar et al. (2009) |
|  | Ecdysone receptor EcR | Third instars | Artificial diet (Ec) | ND | Molting defects and larval lethality | Zhu et al. (2012) |
|  | HaHR3 molting factor | Third instars | Artificial diet (Ec) | ND | Developmental deformity and larval lethality | Xiong et al. (2013) |

(continued)
| Organism                        | Target gene         | Stage                  | Assay method       | Conc. of dsRNA/siRNA | Phenotype/mRNA silencing | References                  |
|--------------------------------|---------------------|------------------------|--------------------|----------------------|--------------------------|-----------------------------|
| CYP6B6                         | Third instars       | Artificial diet (Ec)   | ND                 | Yes                  | Zhang et al. (2013b)      |
| Ultraspiracle protein, EcR     | Third instars       | Artificial diet        | 1000 ppm           | Yes                  | Yang and Han (2014)       |
| *Manduca sexta*                | V-ATPase E          | Neonates               | Artificial diet    | 11 ppm               | Yes                       | Whyard et al. (2009)        |
| *Ostrinia nubilalis*           | Chitinase           | Neonates               | Artificial diet    | 2500 ppm             | Reduced body weight and mortality | Khajuria et al. (2010)      |
| *Plutella xylostella*          | CYP6BG1             | Fourth instars         | Droplet            | 800 ppm              | Yes                       | Bautista et al. (2009)      |
|                                | Rieske protein      | Second instars         | Leaf tissue        | 3 μg/cm²             | Mortality                 | Gong et al. (2011)          |
|                                | AchE receptor       | Second instars         | Leaf tissue        | 3 μg/cm²             | Mortality                 | Gong et al. (2013)          |
| *Sesamia nonagrioides*         | JH esterase JHER    | First to sixth instars | Artificial diet (Ec) | ND                   | Yes                       | Kontogiannatos et al. (2013) |
| *Spodoptera exigua*            | Chitin synthase A   | Neonates               | Artificial diet (Ec) | ND                   | Yes                       | Tian et al. (2009)          |
|                                | β1 integrin subunit | Fourth instars         | Injection and leaf tissue | 100–200 ppm | –                       | Surakaki et al. (2011)      |
| *Spodoptera litura*            | Aminopeptidase N    | Neonates               | Injection          | ND                   | No                        | Rajagopal et al. (2002)     |
| **Spodoptera frugiperda** | Allatostatin C | Fifth instars | Droplet | 600 ppm | Yes | Griebler et al. (2008) |
|---------------------------|----------------|---------------|---------|---------|-----|---------------------|
|                           | Allotropin 2   | Fifth instars | Droplet | 600 ppm | Yes | Griebler et al. (2008) |
|                           | SfT6 serine protease | Fourth instars | Droplet | 600 ppm | Yes | Rodriguez-Cabrera et al. (2010) |
| **Orthoptera**            |                |               |         |         |     |                     |
| *Gryllus bimaculatus*     | Sulfakinins    | Adults        | Droplet | 100–600 ppm | – | Meyering-Vos and Muller (2007) |
| *Locusta migratoria*      | Multiple targets | Fourth instars | Artificial diet | ~240 ppm | No | Luo et al. (2013) |
| *Schistocerca gregaria*   | Tubulin, GAPDH | Adults        | Artificial diet | ND | No | Wynant et al. (2012) |
The feeding method has some major advantages over other methods of delivering dsRNA. These are as follows: (i) it is easy to perform; (ii) feeding dsRNA is less traumatic to the nymphs and juveniles than doing so via injections, the nymphs and juveniles remain healthier, and their mortality is comparatively lower (Shakesby et al. 2009); and (iii) perhaps most significantly, delivering dsRNAs in early stages of insects and nematodes is convenient by this method as compared to microinjection which needs special equipment and often causes high rate of mortality due to art effect. However, there are some challenges, viz., low efficiency of this method and requirement of large quantities of dsRNA, which need to be addressed. Moreover, a detailed study in understanding the mechanism of dsRNA delivery by ingestion for inhibiting gene expression is yet to be carried out.

4.7.2.3 Soaking Method for dsRNA Delivery in Nematodes

This method involves soaking of nematodes in concentrated dsRNA solution and subsequently scoring of worms or their progeny for phenotypes. RNAi by soaking is useful for treating a moderately large number of animals (e.g., 10–100). RNAi through soaking method was first employed in C. elegans as a tool for converting its genome sequence information into functional information (Tabara et al. 1998). Apart from C. elegans, silencing of genes in plant-parasitic nematodes (PPN) through soaking technique has been popularly used but with minor modifications. Other techniques like feeding and microinjection possess some limitations with respect to PPNs. In microinjection, successful recovery of injected juveniles is difficult and PPN juveniles do not take up dsRNA orally easily from the solutions. This was overcome by Urwin et al. (2002) by inducing oral uptake of dsRNA using octopamine, a neuroactive compound by cyst nematodes Heterodera glycines and Globodera pallida. This marked a revolution in imparting RNAi-mediated resistance in cyst and root-knot parasitic nematodes.

Since then many reports on successfully governing the nematode growth utilizing RNAi approach came into the picture. In later studies, compounds like resorcinol and serotonin were used for successful uptake of dsRNA in M. incognita (Rosso et al. 2005; Huang et al. 2006). Apart from neuroactive compounds, fluorescein isothiocyanate (FITC) as a marker for observing dsRNA uptake and as a mean of selecting affected individuals was used in many studies (Urwin et al. 2002; Rosso et al. 2005). Intestinal gene cysteine proteinase was suppressed through the soaking method in G. pallida, H. glycines, and M. incognita (Nakai and Horton 1999; Schmidt et al. 1999). Gene silencing by RNAi soaking has led to various abnormalities in processes like nematode hatching and molting and even resulted in reduced reproduction rates. Many genes, namely, chitin synthase, neuropeptides, msp, c-type lectin, and aminopeptidases, were targeted (Kennerdell and Carthew 1998; Schmidt et al. 1999; Dernburg and Karpen 2002; Ischizuka et al. 2002). But the efficiency and duration of the silencing effect were assessed for M. incognita calreticulin (Mi-crt) and polygalacturonase (Mi-pg-1) (Rosso et al. 2005). Other genes targeted by this approach are cellulases, pectate lyase, chorismate mutase, and glutathione-S
transferase (Anandalakshmi et al. 1998; Cogoni and Macino 2000; Hammond et al. 2001; Matzke et al. 2001; Carmell et al. 2002). However, the silencing acquired by soaking in dsRNA solutions is often transient as duration of soaking and the concentration of dsRNAs affect the RNAi mechanism (Banerjee et al. 2017).

### 4.8 Resistance Via Transgenic Plants Expressing dsRNA

Another alternative method of dsRNA delivery is through host-delivered RNAi (HD-RNAi) where gene is silenced in target organism by the host plant. Since there is no synthesis of any gene product in HD-RNAi, it is likely to address the biosafety concerns more favorably.

#### 4.8.1 In Insects

Genetic transformations of crop plants for expressing dsRNA homologous to important insect gene entail several advantages. It delivers the dsRNA to the target insect pest in a continuous fashion that leads to elicitation of RNAi throughout the life cycle of the insects. Host-mediated delivery of dsRNA was first demonstrated against two important agricultural pests, cotton bollworm, *Helicoverpa armigera*, and Western corn rootworm, *Diabrotica virgifera* (Baum et al. 2007; Mao et al. 2007). Transgenic rice was developed by delivering dsRNA targeting hexose transporter gene *NIHT1*, carboxypeptidase gene *Nicar*, and the trypsin-like serine protease gene *Nltry* of *Nilaparvata lugens*. The study revealed reduced transcript levels of these three targeted genes in the insects that fed on these transgenic rice plants. However, insect lethality was not reported (Zha et al. 2011). Subsequently, several attempts have been made for attenuating key genes of the insects through transgenic host-mediated delivery of dsRNA as presented in Table 4.4. The gene construct for expression of the dsRNA essentially consists of 200–500 nucleotide tandem repeats of the target gene sequence under the control of a constitutive promoter. Such strategy also offers the scope of tissue specific expression of the dsRNA. For example, for targeting the phloem-feeding insect pests, phloem-specific expression of the dsRNA and their transport in phloem sieve elements would be more desirable. However, several attempts in this direction clearly indicated the effective level of protection would depend on targeting suitable target genes in addition to desired level of expression and delivery of intact dsRNA to the infesting insect pests (Price and Gatehouse 2008). Further understanding of the uptake process and elicitation of RNAi by dsRNA in insects will facilitate tailoring the gene expression cassette of dsRNA in order to achieve effective protection.

Mao et al. (2007) used RNAi-mediated approach to reduce insect’s ability to cope up when exposed to xenobiotic compounds, for example, gossypol. Transgenic cotton plants expressing a hairpin dsRNA targeting gossypol-inducible
| Insect                | Target gene                                                                 | Stage            | Plant used        | Phenotype                                      | References          |
|-----------------------|------------------------------------------------------------------------------|------------------|-------------------|------------------------------------------------|---------------------|
| Coleoptera            |                                                                              |                  |                   |                                                |                     |
| Diabrotica virgifera  | V-ATPase A                                                                   | Neonates         | Maize             | Significant reduction in WCR feeding          | Baum et al. (2007)  |
| Virgifera             |                                                                              |                  |                   |                                                |                     |
| Western corn          |                                                                              |                  |                   |                                                |                     |
| rootworm              |                                                                              |                  |                   |                                                |                     |
|                       | Snf 7                                                                        | Neonates         | Maize             | Stunted growth                                 | Bolognesi et al. (2012) |
| Lepidoptera           |                                                                              |                  |                   |                                                |                     |
| Helicoverpa armigera  | CYP6AE14 and GST                                                             | Third instars    | Tobacco and Arabidopsis | Retardation of larval growth                  | Mao et al. (2007)   |
| Cotton bollworm       |                                                                              |                  |                   |                                                |                     |
|                       | CYP6AE14                                                                     | Third instars    | Cotton            | Increased tolerance and stunted larval growth | Mao et al. (2011, 2013) |
|                       | Ecdysone receptor (EcR)                                                      | Second instars   | Tobacco           | Growth reduction and mortality                | Zhu et al. (2012)   |
|                       | HaHR3 molting factor                                                         | Third instars    | Tobacco           | Reduced body weight and mortality             | Xiong et al. (2013) |
| Spodoptera exigua     | Ecdysone receptor (EcR)                                                      | Second instars   | Tobacco           | Increased mortality                           | Zhu et al. (2012)   |
| Hemiptera             |                                                                              |                  |                   |                                                |                     |
| Myzus persicae        | Salivary protein MpC002 and receptor of activated kinase C gene MpRack1      | Nymphs           | Tobacco and Arabidopsis | Reduced fecundity                             | Pitino et al. (2011) |
| Green peach aphid     |                                                                              |                  |                   |                                                |                     |
|                       | Effector gene MpC002, MpPIntO1, and MpPIntO2                                 |                  |                   |                                                | Pitino and Hogenhout (2013) |
|                       | Receptor of activated kinase C gene MpRack1, effector gene MpC002 and MpPIntO2 |                  |                   |                                                | Coleman et al. (2015) |
| Acetylcholinesterase 2 gene MpAChE2; V-ATPase E; tubulin folding cofactor D gene TBCD; 40S ribosomal protein S5-like isoform-1 Rps5; ribosomal protein S14 Rps14; mediator complex subunit 31 Med31; SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1-like gene SMARCD1 | Tobacco | Reduced fecundity | Guo et al. (2014) |
|---|---|---|---|
| Gap gene Hunchback (hb) | Neonates | Tobacco | Reduced fecundity | Mao and Zeng (2014) |
| PEMV coat protein-Hv1a | Arabidopsis | | Bonning and Chougule (2014) |
| Serine proteinase gene MySP | Adults | Arabidopsis | Reduced fecundity and parthenogenetic population | Bhatia et al. (2012) |
| Aquaporin gene MpAQP1; sucrase gene MpSUC1; sugar transporter gene MpSt4 | Adults | Tobacco | Reduced fecundity | Tzin et al. (2015) |
| Macrophage migration inhibitory factor MpMIF1 | Adults | Tobacco | Decreased survival and fecundity of aphids feeding on their host plant | Naessens et al. (2015) |
| *Sitobion avenae* Grain aphid | Carboxylesterase gene CbE E4 | Wheat | Impaired tolerance of phoxim insecticide | Xu et al. (2014) |
| Structural sheath protein (shp) | Barley | Reduced fecundity and inhibited feeding behavior | Abdellatef et al. (2015) |
| *Nilaparvata lugens* Brown Plant Hopper | Multiple targets | Neonates | Transgenic plant | Zha et al. (2011) |
cytochrome P450 gene CYP6AE14 of *H. armigera* showed increased tolerance to the cotton bollworm, *H. armigera* (Mao et al. 2011), but were not lethal to the larvae. Interestingly, when a cysteine proteinase which is supposed to damage larval peritrophic matrix leading to higher accumulation of gossypol in the midgut was co-delivered, the tolerance was further enhanced (Mao et al. 2013). The similar strategy may be applicable for restoring insecticide sensitivity among resistant insect species (Bautista et al. 2009; Tanget al. 2012; Figueira-Mansur et al. 2013).

The host-mediated RNAi for controlling insect pest has been considered to be particularly important for phloem-sucking hemipteran insect pests, viz., aphids. In green peach aphid, plant-mediated RNAi of several target insect-specific genes such as salivary proteins *MpC002*, *MpPIntO1*, and *MpPIntO2* and the gut-specific gene *Rack-1* showed reduced fecundity (Table 4.3). In a similar study, stronger aphicidal activity of a hairpin RNA targeting V-ATPase E or the tubulin folding cofactor D (TBCD) was demonstrated (Guo et al. 2014). RNAi-mediated expression attenuation of a serine protease gene *MySP* in the green peach aphid, *Myzus persicae*, led to a remarkable decrease in their fecundity and parthenogeneticity (Bhatia et al. 2012). These studies on host-mediated delivery of dsRNA and elicitation of RNAi in infesting aphids demonstrated potential of RNAi approach for developing genetic resistance against aphids. Mao and Zeng (2014) reported reduced attack by aphids on transgenic tobacco plants expressing dsRNA against the gap gene *hunchback*, and reproduction rate of aphids was also retarded.

Interestingly, aphid nymphs parthenogenetically born from mothers reared on transgenic plants expressing dsRNA continued to show downregulation of the target gene even when transferred on normal plants. An assessment of RNAi effect over three generations of *M. persicae* revealed 60% reduction in aphid reproduction levels in transgenic Arabidopsis plants expressing ds*MpC002* compared to 40% decline on transgensics expressing dsRack1 and ds*MpPIntO2*. Such transgenerational RNAi was found to last over seven generations in *Sitobion avenae* reared on transgenic barley plants expressing shp-dsRNA (Abdellatef et al. 2015). Such parental transmission of RNAi effect adds to potential of the strategy.

### 4.8.2 In Nematodes

RNAi mechanism partly occurs in the host itself and partly in nematodes feeding on the transgenic plant expressing dsRNA for the target gene. The plant RNAi machinery produces siRNAs which are ingested by nematodes feeding upon these plants through stylet (Li et al. 2011). By far HD-RNAi is the most successful methodology for developing resistance against nematodes in important crops. This technique exploits the capability of PPNs of ingesting macromolecules from the host plants. Specifically, the method involves producing dsRNA construct and developing transformed plants by *Agrobacterium*-mediated
transformation. For generating dsRNA, a part of the target gene is cloned in sense and antisense orientation separated by an intron or spacer region and expressed under a constitutive or tissue-specific promoter. Majority of researchers have adopted this time-consuming methodology and have successfully developed transgenics resistant against nematodes. Another new approach with rapid screening system has been developed involving hairy root method for transformation of crops like soybean, tomato, and sugar beet.

Genes involved in various vital processes are mostly targeted by this approach being categorized into effector genes (most targeted), house-keeping genes, developmental genes, and genes associated with mRNA metabolism. Two genes encoding integrase and splicing factor were suppressed in *M. incognita* using host-delivered RNAi. It was the first report eliciting RNAi in *M. incognita* by developing transgenic tobacco lines (Yadav et al. 2006). The lethality of these genes as RNAi targets was further reconfirmed by Kumar et al. (2017) in *Arabidopsis* by utilizing this approach against *M. incognita*. Effective silencing of 16D10 effector genes leads to 63–90% reduction in the infectivity of *M. incognita* in *Arabidopsis* (Huang et al. 2006). Since 16D10 is highly conserved in *Meloidogyne* species, resistance against three other major species was also developed (Li et al. 2011). *M. chitwoodi* also showed a reduction in the number of nematodes and eggs on silencing 16D10L gene via HD-RNAi approach in transgenic Arabidopsis and potato plants (Dinh et al. 2014a, b).

Cyst nematodes also exhibited gene suppression by this technique successfully. The suppression of four parasitism genes, ubiquitin-like (4G06), cellulose-binding protein (3B05), SKP1-like (8H07), and zinc finger protein (10A06), in *Heterodera schachtii* resulted in the reduction of females in RNAi transgenic *Arabidopsis* lines (Sindhu et al. 2009). Silencing of esophageal proteins in *H. glycines* leads to the reduction in reproduction (Bakhetia et al. 2007). In another study, successful suppression of major sperm protein of *H. glycines* resulted in 68% decrease in eggs per gram root tissue when infected on transgenic soybean plants (Steeves et al. 2006). Transgenic tobacco lines expressing dsRNAs of two neuropeptides, flp-14 and flp-18, showed 50–80% decline in the infection of *M. incognita* (Papolu et al. 2013). Other genes silenced using this methodology are Mj-Tisll, Rpn7, tyrosine phosphatase, mitochondria stress 70 protein precursor and neuropeptides against Meloidogyne spp(s) (Hamann et al. 1993; Lindbo et al. 1993; Depicker and Montagu 1997; Pasquinelli 2002; Lim et al. 2003; Valdes et al. 2003). Host-mediated RNAi strategy is more successful in root-knot (RKN) nematodes as compared to cyst nematodes (CN) owing to factors like more RNAi sensitivity and larger size exclusion limit of RKNs than in CNs (Li et al. 2011). Host-delivered RNAi appears to be the most successful technique in controlling nematode infection.

Identification of appropriate target genes based on preliminary diet-based bioassay and ensuring adequate *in planta* expression of the dsRNA in the transgenic host are pivotal requirements for effective host-mediated RNAi. However, further understanding of the mechanisms on dsRNA uptake by insect and nematodes will facilitate the tailoring of dsRNA expression in HD-RNAi.
4.9 dsRNA Uptake Mechanisms

The dsRNA uptake mechanism in insects is known to be achieved by either of the two pathways, viz., a protein-mediated pathway and via endocytic pathway. The major component of protein-mediated pathway is a multi-pass transmembrane protein known as systemic RNA interference deficient-1 (Sid-1) which exports the small interfering RNAs across neighboring cells (Bansal and Michel 2013). The second pathway is receptor-mediated pathway. In case of C. elegans, the endocytic pathway involves a Sid-2 gene localized in intestinal cells. It encodes a membrane protein and is thought to import dsRNA from the intestinal lumen which are then exported to other cells with the help of sid-1 channels (Winston et al. 2007; McEwan et al. 2012). Hence, Sid-1 and Sid-2 proteins must work in conjunction to achieve environmental RNAi. Sid-1 genes have been reported to be evolutionarily conserved among insects orders, but Sid-2 gene is absent in insects. Tribolium is considered as the model insect for studying systemic RNAi with presence of Sid-1 like proteins. However, the Sid-1 gene of Tribolium was found orthologous to Tag-130 gene of C. elegans and not Ce-Sid-1 gene interestingly, where Tag-130 has not been reported to be associated with systemic RNAi in nematodes (Tomoyasu et al. 2008). The presence of Sid-1-like channel proteins varies among different orders of insects. The involvement of Sid-1-like channel proteins in dsRNA uptake has been reported in brown plant hopper [BPH, Nilaparvata lugens (Xu et al. 2013)], the Colorado potato beetle [CPB, Leptinotarsa decemlineata (Cappelle et al. 2016)], and the red flour beetle Tribolium castaneum (Tomoyasu et al. 2008). In 2016, genes involved in RNAi pathway in insects were identified and classified. The study reveals absence of Sid-1/Tag-130 orthologs in Diptera order (Dowling et al. 2016). It was suggested that in Drosophila melanogaster, dsRNA uptake is mediated via endocytic pathway along with pattern recognition receptors (PRRs) based on a study by Ulvila et al. (2006). This study reports more than 90% reduction in the uptake of double-stranded RNA on silencing of these two receptors by RNAi technology. Most of the studies examining dsRNA uptake so far focused on either the endocytic pathway or Sid-1-like dependent system. However, a clear understanding of the roles of these pathways on dsRNA uptake across the insect species is still lacking. Nevertheless, insects belonging to another order have been reported to have both the Sid-1-like channel proteins and receptor-mediated endocytosis pathways playing a role in dsRNA uptake (Cappelle et al. 2016).

However, the dsRNA uptake mechanism in worms is quite different. The components involved in dsRNA uptake have been well studied in C. elegans, and presence of Sid-1 and Sid-2 genes along with other components like rsd-2, rsd-3, and rsd-6 has been well documented in the C. elegans genome. But surprisingly in a study, it was found these proteins were not evolutionary conserved (Dalzell et al. 2011). The dataset recognizes sid-1 orthologs in two parasitic nematodes, viz., in Haemonchus contortus and Oesophagostomum dentatum only. The Sid-2 protein was not found to be present in other nematode species. Intriguingly, the plant-parasitic nematodes
such as *Meloidogyne* and *Globodera* spp. despite the absence of Sid-1 and Sid-2 genes exhibit systemic RNAi when subjected to silencing technology indicating a presence of similar receptor-mediated endocytic process for dsRNA uptake as reported in insects (Dalzell et al. 2011). Though lot of information has been generated over past few years, a clear understanding on dsRNA uptake mechanism(s) in worms is still elusive

4.10 RNAi Resistance in Other Agricultural Pests

Other than insects and nematodes, there are agricultural pests belonging to phylum Arthropoda that affect the crop productivity worldwide, and RNAi-based strategy to control these pests has shown some success. These pests are fire ants, mites, locusts (order Orthoptera), and many more. Systemic RNAi has already been demonstrated in these pests via microinjection. On feeding the worker ants, *Solenopsis invicta*, with 1000 ppm dsRNA targeting PBAN/pyrokinin gene, increased mortality rate of the fourth instar larvae. Direct toxic effect was also observed even when the dsRNA concentration was reduced to 200 ppm (Zhao and Chen 2013). In spider mite, gene silencing and increased mortality rate was observed when 160 ppm of dsRNA, targeting several genes, was employed via permeated leaf disc assay (Kwon et al. 2013). In another mite, *Varroa destructor*, an ectoparasite of the honey bee, *Apis mellifera*, both the delivery methods of dsRNA, i.e., by immersing mites in a dsRNA solution or by host-mediated RNAi, wherein dsRNA was fed to the honey bees and eventually delivered to mites, were found to attenuate the target gene expression through environmental RNAi (Campbell et al. 2010; Garbian et al. 2012).

Interestingly, locust species displayed systemic RNAi response but were refractory to environmental RNAi. Even a considerable concentration of 15 pg of dsRNA per mg body mass (~10 ng/insect) was enough to silence a gene in the desert locust, *Schistocerca gregaria* (Wynant et al. 2012). In case of *Tribolium castaneum*, the systemic response continued to increase over time in a dose-dependent manner and furthermore led to mortality 7 days postinjection. A similar dose-dependent response was also exhibited by the migratory locust, *Locusta migratoria*, leading to target gene suppression and lethality, but was unresponsive to environmental RNAi (Luo et al. 2013).

4.11 RNAi for Fungus Resistance

Fungi are classified as a separate eukaryotic kingdom from plants and animals. The vital RNAi components (RNA-dependent RNA polymerase (RdRP), Dicer, and Argonaute) have been found in different fungi indicating the presence of functional RNAi pathway (Dang et al. 2011). The RNAi phenomenon is termed as
“quelling” in fungi which was first demonstrated in ascomycete *Neurospora crassa* (Romano and Macino 1992). Silencing of fungal genes by RNAi has shown to be desirable for many fungal species like Ascomycota, Basidiomycota, Zygomycota, and Phytophthora species (Nunes and Dean 2012). Several studies have been published reporting the successful use of host-induced gene silencing (HIGS) to control fungal diseases (Table 4.5) (Koch and Kogel 2014). Suppression of GUS transcripts in a GUS-expressing strain of *Fusarium verticillioides* (phytopathogenic filamentous fungi) while colonizing transgenic tobacco plants expressing GUS gene-interfering cassette was reported (Tinoco et al. 2010).

In vitro feeding of dsRNA complementary to three genes involved in ergosterol biosynthetic pathway, viz., CYP51A, CYP51B, and CYP51C, showed reduced growth of *Fusarium graminearum* (Koch et al. 2013). In wheat, mycotoxin-specific genes were silenced in *F. graminearum* and resulted in inhibition of virulence (McDonald et al. 2005). Fungal pathogenicity genes have shown to be an appropriate target for controlling fungal infection. A complete loss of pathogenicity was reported on targeting two of the host-selective ACT-toxin

| Species | Target gene | Host plant | Effect/comments | References |
|---------|-------------|------------|----------------|------------|
| *Blumeria graminis* f. sp. tritici | MLO | Wheat | Resistance | Riechen (2007) |
| *Phytophthora parasitica* var. nicotianae | GST (glutathione S-transferase gene) | Tobacco | Resistance; GST negative regulator of defense response | Hernandez et al. (2009) |
| *Blumeria graminis* | Avra10 (effector gene) | Barley and wheat | Reduced fungal development in the absence of the matching resistance gene Mla10 | Nowara et al. (2010) |
| *Fusarium verticillioides* (F. moniliforme) | GUS (reporter gene) | Tobacco | GUS silencing; proof of concept | Tinoco et al. (2010) |
| *Puccinia striiformis* f. sp. tritici or *P. graminis* f. sp. tritici | PSTha12J12 (haustorial Pst transcript) | Barley and wheat | No obvious reductions in rust development or sporulation | Yin et al. (2011) |
| *Phytophthora parasitica* | PnPMA1 (H + -ATPase) and GFP (reporter gene) | Arabidopsis | Not sufficient; no reduction in GFP and PnPMA1 transcripts | Zhang et al. (2011) |
| *P. triticina, P. graminis, and P. striiformis* | PtMAPK1 (MAP kinase), PtCYC1 (cyclophilin), and PtCNB (calcineurin B) | Wheat | Disease suppression, compromising fungal growth and sporulation | Panwar et al. (2013) |
| *Fusarium graminearum* | CYP51A, CYP51B, and CYP51C | Arabidopsis and barley | Resistance | Koch et al. (2013) |
genes in the fungus *Alternaria alternata* (Miyamoto et al. 2008; Ajiro et al. 2010). Similar reports on silencing of pathogenicity gene or avirulent gene proved successful in inhibiting the fungal growth and development. In *Magnaporthe oryzae*, silencing of 37 genes involved in calcium signaling process adversely affected hyphal growth, sporulation, and pathogenicity (Nunes and Dean 2012). HIGS-mediated silencing of effector gene *Avra10* showed a reduction in the number of haustoria in powdery mildew-susceptible barley cultivar (Koch and Kogel 2014).

To date, there are several successful reports of gene silencing in fungi with varied silencing efficiency. For instance, in *Moniliophthora perniciosa*, the silencing efficiency varied depending upon the targeted gene with reduction rates ranging from 18% to 97% in case of hydrophobin transcripts and 23% to 87% in peroxiredoxin transcripts (Santos et al. 2009), while when RNA hairpin precursor used to transform the Ascomycota *Ophiostoma novo-ulmi*, the expression of 6%, 22%, and 31% relative to the wild type was reported (Carneiro et al. 2010) in three transformants. Although usage of RNAi for managing fungus growth is nowadays a favored approach by researchers, RNAi silencing also leads to some off-target effects as observed by Lacroix and Spanu (2009) on silencing various genes in *C. fulvum*. These off-targets can be avoided by using specific silencing trigger sequence in RNAi vector, by tissue-specific and inducible silencing (Senthil-Kumar and Mysore 2011).

### 4.12 Barriers Limiting RNAi

The potential of RNAi technology for controlling various pests has been well documented over the past decade. However, there are many limitations which need to be taken care of for successful deployment of RNAi technology. There are several factors which need to be carefully looked into while designing RNAi experiments, including the off-target effects, dsRNA design, length and concentration of dsRNA, and many more. Therefore, to ensure a successful and effective RNAi-based silencing, these factors need to be balanced optimally. In case of insects, persistency of RNAi is a major problem due to which an optimum amount of dsRNA needs to be determined for an effective silencing. Interestingly, it is not true for every order of insect which is to be managed. For instance, about 60% (or lower) of gene knockdown was reported in certain recalcitrant insect species, while in coleopterans, 90% knockdown of gene was successfully achieved ensuing a long-lasting hereditary (Baum et al. 2007; Huvenne and Smagghe 2010; Zhu et al. 2011; Bolognesi et al. 2012; Rangasamy and Siegfried 2012; Li et al. 2013). Not only in insects but in nematodes also barriers like off-target effects have been reported while performing RNAi technology based management approaches. Designing an effective siRNA sequence is a major limitation in RNAi technology-based silencing. The following are some major barriers.
4.12.1 Off-Target Effects

Off-target effects result from the knockdown of unintended genes other than the target gene. Therefore, one of the most important aspects is avoidance of nonspecific target effects. It is the sequence used that determines possible off-target effects in the target organism and also in other species. Other than sequence, off-target effects can arise due to wide range of siRNAs being produced from a single dsRNA which increases the chance of nontarget effects. There are many reports of off-target effects, for instance, in triatomid bug *R. prolixus*, two homologous nitroprin genes were silenced other than the targeted gene (Araujo et al. 2006). Thus, selecting a sequence for synthesizing dsRNA is a crucial and limiting step in RNAi technology.

4.12.2 The Design of dsRNA

Selection of target gene is the first step in decision-making process for successful induction of RNAi in an organism. The gene selected should have a crucial role in the concerned organism, and genes involved in parasitism or development are likely candidate genes fulfilling all such requirements. Moreover, it should be highly specific and not conserved across different genera (Danchin et al. 2013) especially in pollinators. Next stage is to choose a suitable target site from the selected target gene. It is necessary to ensure the designing of a species-specific dsRNA. For identifying potential target sites for eliciting effective RNAi, bioinformatic tools are available online. Specificity of the dsRNAs could be conferred by either targeting conserved domain or variable region depending on the candidate gene with the aim to minimize possibility of affecting any unintended genes or organisms. This is particularly important to ensure that dsRNAs targeting agricultural pests should not possess any overlapping similarity to the genes of beneficial pollinators. By targeting the UTR regions, even closely related homologous genes can be selectively silenced through RNAi as demonstrated in *D. melanogaster*, *T. castaneum*, *A. pisum*, and tobacco hornworms, *Manduca sexta*, with respect to vATPase gene (Whyard et al. 2009). The concept of dsRNAs being used as tailor-made pesticides is emerging wherein highly specific dsRNAs are employed against havoc-creating pests and are also eco-friendly to the environment.

4.12.3 Length and Concentration of dsRNA

In general, longer RNA molecules tend to have longer half-life and therefore may be considered desirable while designing dsRNAs. However, size of the dsRNA molecule could be a limiting factor toward efficient uptake by the organisms. In nematodes, 28–140 kDa dsRNA could be efficiently ingested by *Meloidogyne* species (Urwin et al. 1997; Li et al. 2007; Zhang et al. 2012), though the limit is not known for other pests. In red flour beetle, the length and concentration of dsRNA had
4.12.4 **Screening of Target Genes**

For realizing RNAi-mediated gene silencing as an applicable strategy of pest control in agriculture, it remains imperative to achieve significant mortality or growth arrest of the pest population. Therefore, any attenuation of the target gene must be indispensable for the pest organism. This in turn underlines the importance of identifying appropriate target gene for the target pest. Though most of the studies have used limited set of target genes reported earlier, more emphasis should be given on identification of novel candidate genes (Pitino et al. 2011; Zhu et al. 2011). The upcoming genomics and bioinformatics tools, like genome search (Bai et al. 2009), cDNA library (Mao et al. 2007; Baum et al. 2007), RNA-seq and digital gene expression tag profile (DGE-tag) (Wang et al. 2011), and RIT-seq (Alsford et al. 2011), have been used for identification of new target genes.

4.12.5 **Persistence of the Silencing Effect**

The persistence of silencing signal determines the effectiveness of RNAi. Studies on low persistence of silencing effect have been reported in *A. pisum* wherein silencing effect on aquaporin persisted for 5 days of delivery before subsiding (Shakesby et al. 2009) indicating transient nature of RNAi effect. Thus, continuous supply of dsRNA seems to be essential for effective RNAi. It lends support for the transgenic host-mediated expression of the dsRNA for persistent and effective silencing. Persistent RNAi will also be useful in manifesting desired effect on the target organism even in case of inefficient and partial downregulation of the target gene.

4.12.6 **Life Stage of the Target Organism**

Selecting a life stage for larger silencing effects is species dependent that is to be targeted. In most cases, younger stage is preferred despite the efficient handling of older stages. In plant-parasitic nematodes, selecting the pre-parasitic juvenile stage
for delivering dsRNAs shows better silencing effect. Similar observation was reported in insects, for example, in case of \textit{R. prolitus}, no silencing effect was observed after treating its fourth instars compared to 42% silencing when using second instars (Araujo et al. 2006).

### 4.12.7 Methods of Delivery and Uptake Mechanisms

Various methods of dsRNA delivery have been used across the organisms. Such methods include microinjection, feeding with bacteria expressing dsRNA, feeding through diet supplementation, and host-mediated ingestion. The efficiency of RNAi varies significantly among different organisms and when using different delivery methods. In insects, either microinjection or diet supplementation has been the method of choice, though the aftershock effect of microinjection remains a concern in many species. Microinjection-mediated direct delivery bypasses the exposure of the dsRNA molecule to the nucleases present in the digestive tract. However, for realizing true efficacy of the dsRNA, it is desired to deliver through oral delivery that mimics the host-mediated delivery through ingestion.

### 4.12.8 Nucleases and Viruses

Limited success in RNAi in some of the insects has been attributed to rapid degradation of dsRNA by saliva of the insects. The saliva of \textit{Lygus lineolaris} was found to contain RNases which interact with plant material prior to ingestion (Allen and Walker 2012). Presence of nucleases in the saliva and viruses in the hemolymph of insects also limits the silencing efficiency by degrading dsRNAs (Thompson et al. 2012; Christensen et al. 2013).

### 4.13 Improving RNAi

#### 4.13.1 Large Throughput Screening for Selection of Target Genes

An ample number of studies in insect orders of Coleoptera, Diptera, Lepidoptera, Hemiptera, and others comprising of several insect pests have shown that RNAi targeting insect genes can affect growth and development of insects, often leading to insect death (Tables 4.3 and 4.4). The kind of genes for which a relatively high RNAi efficiency could be achieved included genes encoding detoxification enzymes, metabolism and cytoskeleton structure, cell synthesis, nutrition, etc. Alternative pathways of many of these genes in insects as well as relative importance of a
particular pathway in an insect species are not known with certainty. Therefore, use of RNAi as a strategy for pest control will require an essential step of target selection. If an indispensable gene has to be identified for an insect species, it will involve large throughput screening rather than going for homologous genes, effective for other insect species.

Chitin covers the exoskeleton of insect body, and the insect midgut lined by peritrophic membrane (PM) constitutes the major channel for absorption of nutrients as well as orally administered dsRNA. Therefore genes expressed and functioning in the insect midgut have been screened by many researchers (Wang and Granados 2001). For example, a chitinase gene (OnCht) and a chitin synthase gene (OnCHS2) were identified from gut-specific EST of European corn borer (Ostrinia nubilalis) (Khajuria et al. 2010). Chitin content of the PM is regulated by OnCht as demonstrated in feeding experiment with dsRNA- and RNAi-based suppression which led to reduced growth and development of European corn borer larvae (Khajuria et al. 2010). In a similar study, Mao et al. (2007) identified several gossypol inducible genes, including a putative P450 monooxygenase, CYP6AE14, from a midgut-specific cDNA library from fifth-instar larvae exposed to gossypol. Similarly, for screening targets for RNAi in coleopteran insects, a large number of cDNAs from the cDNA libraries of Western corn rootworm (Diabrotica virgifera virgifera) were in vitro transcribed and used in feeding-based bioassays (Baum et al. 2007).

A rapid method of cDNA screening was demonstrated by Wang et al. (2011) by combining Illumina’s RNA-seq and digital gene expression tag profile (DGE-tag) in Asian corn borer (ACB) (Ostrinia furnacalis). In addition to being a rapid and cost-effective method, this method allows monitoring expression of the genes throughout the insect body and thus broadening the base of target selection. Using Illumina parallel sequencing technology, abundance of >90,000 transcripts from trypanosome libraries was scored before and after induction of RNAi. The results led to constitution of non-redundant set of protein-coding sequences (CDS) comprising ~7500 genes (Alsford et al. 2011). Thus these methods can derive core set of essential gene loci if genome sequence of the organism is known. RNAi-mediated attenuation of these core loci is most likely to significantly retard survival and fitness of the insect pests.

In recent years, several modifications and methods for effective delivery and uptake of dsRNA have been proposed. Such methods include chemical modifications of siRNA duplex delivery through nanoparticles and liposomes, sprayable RNAi-based products, root absorption and trunk injection, and bacteria- or virus-based delivery. A few of them with much potentiality have been described below.

### 4.13.2 Nanoparticles

Synthetic, nontoxic nanoparticles could be generated from natural as well as synthetic polymers. Nanoparticles offer ease of surface modifications and biodegradability in addition to more penetration ability, thus an effective vehicle for
delivery of dsRNA (Vauthier et al. 2003; Herrero-Vanrell et al. 2005). In mosquito dsRNA encapsulated in polymer, chitosan was used to achieve RNAi (Zhang et al. 2010). The encapsulation process used the electrostatic forces between the negative charges of the RNA backbone and positive charges of the amino groups of chitosan. Zhang et al. (2015a, b) demonstrated effective knockdown of AgCHS1 and AgCHS2 in A. gambiae and A. aegypti (sema1a) during larval development by using chitosan nanoparticles. He et al. (2013) fed lepidopteran pest, Asian corn borer (Ostrinia furnacalis), with diet containing the mixture of fluorescent nanoparticle (FNP) and CHT10-dsRNA, naked CHT10-dsRNA, FNP and GFP-dsRNA, and GFP-dsRNA. RNAi-mediated gene silencing occurred only in the larvae fed on the diet containing the mixture of FNP and CHT10-dsRNA leading to retarded growth and eventually death of the larvae.

4.13.3 Liposomes

Liposome vesicles composed of nontoxic natural lipids are already being used in drug delivery. Liposomes can cross the cell membrane effectively and deliver the exogenous molecules. Whyard et al. (2009) used cationic liposomes for encapsulating and delivering dsRNA targeting 3′-UTR of the g-tubulin gene in four different species of Drosophila (D. melanogaster, D. sechellia, D. yakuba, and D. pseudoobscura) and demonstrated mortality of the insects only in case of encapsulated dsRNA. In Drosophila, presence of sid1 homologues has never been confirmed, and the uptake of dsRNA is likely to be by receptor-mediated endocytosis (Ulvila et al. 2006). Higher efficiency of RNAi in case of liposome-mediated delivery in certain cases could be attributed to the fact that it bypasses the gut nucleases which reduces the efficacy of orally delivered dsRNA.

4.13.4 Chemical Modifications

Chemical modifications are known to increase the stability of RNA molecules. In case of siRNA also such modifications have been proposed to improve half-life and pharmacokinetic properties of the siRNA duplexes, target-binding affinity, and delivery (Kurreck 2003; Manoharan 2003; Dorsett and Tuschl 2004). Interestingly a couple of examples have demonstrated that such modifications may increase the specificity of dsRNA. For example, methylation at 2′-position of the ribosyl ring of the second base of the siRNA could decrease off-target effects (Jackson et al. 2003), siRNA duplex with 3′-overhangs at each end was more effective in gene silencing compared to blunt-ended duplex (Elbashir et al. 2001), and addition of 3′-TT overhangs (the “Tuschl design”) on both strands of duplex siRNA has been preferred in many cases. A few other designs, for instance, siRNAs without 3′-overhangs and single 3′-overhang structures in the guide strand, have been active in gene silencing (Czauderna et al. 2003; Lorenz et al. 2004).
4.14 Future Perspective and Conclusion

Despite few limitations, the applicability of RNAi in improving crop resistance especially against biotic stresses is expected to be the most reliable and significant approach in the future as evident from a plethora of studies. Certain products based on RNAi-mediated resistance such as Monsanto’s SmartStax Pro, for control of Western corn earworm, and DuPont Pioneer’s Plenish high oleic acid soybean (Majumdar et al. 2017) are likely to be commercialized soon. However, efficacy of these plants remains to be proven in actual field situations. Diverse classes of biotic factors, affecting crop production worldwide, have shown varied levels of susceptibility toward RNAi, which warrants need for modified and improved versions of dsRNA delivery methods. The better understanding of host-pest interaction and the genetic basis of parasitism are likely to generate more potential target genes for effective HD-RNAi. CRISPR/Cas system has come up as a powerful technique in creating knockout mutants to unravel complex mechanism of parasitism and thus paves the way for identification of the key pest genes. Transplastomic expression of dsRNA in the plants would be a further improvement for achieving higher expression. Applying dsRNA through methods with low environmental risks, for instance, irrigation water, root drench, or trunk injection, would obviate the need for genetic transformation. These methods result in localized application along with rapid breakdown of dsRNA and therefore likely to be more acceptable from a biosafety point of view (Joga et al. 2016). Successful demonstration of using layered double hydroxide clay nanosheets for topical application of dsRNA against viruses (Mitter et al. 2017) opens up possibilities of applying dsRNA like any other protective agrochemicals.

To conclude, RNAi has emerged as one of the most potential control mechanisms for pests like insects, nematodes, fungus, etc. Although still a lot remains to be explored and understood about the molecular process of RNAi in plants and their pests, the present available knowledge and the studies reviewed in this chapter have proved RNAi technology as an important tool in identifying gene functions and targeting vital genes for controlling pest development. RNAi-mediated loss-of-function phenotypes not only determine functions of unknown genes but also lead to identification of new specific targets for managing pest or improving agricultural traits. But understanding RNAi mechanism is of utmost importance as RNAi machinery varies from genus to genus. There are several shortcomings that need to be addressed, for instance, persistence of silencing effects, off-target effects of silencing, etc. Not only this, the biosafety, risk assessment, and government regulations related to commercialization of RNAi-based transgenics still have to be developed. The revelation of RNAi technology has revolutionized the area of research in biotechnology. Not only in pest management, the wide range of RNAi application includes modification of agronomic traits, eliminating mycotoxin contamination, improving nutritional value of crops, etc. It is also proving its worth in RNAi-based therapeutics research for human welfare. In toto, this technology is a potential boon in the arsenal of the scientific community to address the challenges associated with climatic changes, burgeoning population, and sustainability of human race.
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