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RNA INTERFERENCE: A NEW SUSTAINABLE APPROACH FOR INSECT PEST MANAGEMENT

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Abstract–RNA interference (RNAi) refers to a group of post-transcriptional or transcriptional gene silencing mechanisms conserved from fungi to mammals. It is a phenomenon primarily for the regulation of gene expression, self or nonself depending upon the surrounding factors or conditions. It is done in nature with the help of non-coding RNA molecules to control cellular metabolism and helps in maintaining genomic integrity by preventing the invasion of viruses and mobile genetic elements. It is a simple and rapid method of silencing gene expression in a range of organisms as a consequence of degradation of RNA into short RNAs that activate ribonucleases to target homologous mRNA. The process of RNAi can be mediated by either small interfering RNA (siRNA) or micro-RNA (miRNA). The RNAi pathway is triggered by the presence of double-stranded RNA, which is cleaved by the ribonuclease-III domain-containing enzyme, Dicer to generate 20-25 nucleotide long siRNA duplexes, siRNA is then loaded onto the RNA- induced silencing complex (RISC), in which an Argonaute (Ago)-family protein, guided by the siRNA, mediates the cleavage of homologous RNAs. Synthetic double-stranded RNA (dsRNA) introduced into cells can selectively and robustly induces suppression of specic genes of interest. Because of its exquisite specicity and efficiency, RNAi is being considered as valuable research tool, not only for functional genomics, but it also has considerable potential for the control of insect pests.

INTRODUCTION

In agricultural systems, insect pests can cause crop damage mainly through loss in yield and quality; it leads to loss in profit of farmers. It has been estimated that the global crop loss due to insect pest was 10.8% during the post green revolution period; it cost billions of dollars (Dhaliwal *et al*., 2015). For the control of insect pests' farmers mostly rely on chemical pesticides. Chemical pesticides are still the major approach for control of insect pests, but they are associated with significant hazards to the environment and human health. The alternative commercial biotechnological system relies mostly on the expression of *Bacillus thuringiensis*, insecticidal proteins (Cry toxins). Its effectiveness however is threatened by the development of resistance in some species such as *Ostrinianubilalis* (Hubner) (Lepidoptera, Pyralidae) and *Heliothisvirescens* (Fabricius) (Lepidoptera: Noctuidae) (Baum *et al*., 2007). In this regard, there is an urgent need to develop and adopt economically and ecologically sound alternative strategy forinsect pest management. Gene silencing has been suggested as one of the new alternatives to reduce damage from insect pests. Gene silencing is a general term that describes epigenetic processes of gene regulation. Various methods of gene silencing methods are present *viz*., Transcriptional, Post-transcriptional, Meiotic, Antisense oligonucleotides, Ribozymes and RNA interference mediate gene silencing. Among these,recently, RNA interference mediate gene silencing contributes successful insect pest management. RNA interference, commonly known as RNAi, regulates gene silencing mostly either at the transcriptional or post-transcriptional

level. It is a sort of ''Reverse Genetics.'' RNAi is the mechanism in molecular biology where the presence of certain fragments of double-stranded RNA (dsRNA) interferes with the expression of a particular gene which shares a homologous sequence with the dsRNA(Bosher and Labouesse, 2000: Kim and Rossi, 2007). In 1998 Andrew Fire and Craig Mello rst described RNAi phenomenon in round worm, *Caenorhabditiselegans* (Maupas) by injecting dsRNA, they also coined the term RNAi (Fire *et al*., 1998) RNAi has become a valuable research tool, both in vitro studies and living organisms because synthetic dsRNA introduced into cells can selectively and robustly induce suppression of specic genes of interest. RNAi may be used for large-scale screens that systematically shut down each gene in the cell, which can help identify the components necessary for a particular cellular process or an event such as cell division. Besides, it has various applications in medicine, biotechnology, and functional genomics. Overview on use of RNAi in insects was presented Table 1.

The Concept of RNA Interference

'RNA interference' refers collectively to diverse RNA- based processes that all result in sequence-specific inhibition of gene expression, either at the transcription, mRNA stability or translational levels (Thakur, 2003). It has most likely been evolved as a mechanism for cells to eliminate foreign genes.The unifying features of this phenomena includes the production of small RNAs 21-26 nucleotides (nts) that act as specific determinants for down-regulating gene expression and the requirement of one or more members of the Argonaute family of proteins. RNAi operates by triggering the action of dsRNA intermediates, which are processed into RNA duplexes of 21-24 nts by a ribonuclease III-like enzyme called Dicer. Once produced, these small RNA molecules or short interfering RNAs (siRNAs) are incorporated in a multi-subunit complex called RNA induced silencing complex or RISC. RISC is formed by a siRNA and an endonuclease among other components. The siRNAs within RISC act as a guide to target the degradation of complementary messenger RNAs (mRNAs). The host genome codifies for small RNAs called miRNAs that are responsible for endogenous gene silencing.

Components of Gene Silencing

Both genetic and biochemical approaches have been undertaken to understand the basis of silencing. Some of the components identied serve as initiators, while others serve as effectors, ampliers, and transmitters of the gene silencing process. In the years to come, many other components as well as their interrelations will be revealed. Here, some common components of RNA interference mediated gene silencing

Small Interfering RNA

Small Interfering RNA (siRNA) are short double-stranded RNA molecules that induce sequence specic post-transcriptional gene silencing (PTGS). The siRNAs act in the nucleus and the cytoplasm and are involved in RNAi as well as chromatin dependent gene silencing (CDGS) pathways, CDGS siRNAs come from long dsRNA precursors derived from a variety of single-stranded RNA (ssRNA) precursors, such as sense and antisense RNAs. siRNAs also come from hairpin RNAs derived from transcription of inverted repeat regions. siRNAs may also arise enzymatically from non-coding RNA precursors. Their role was rst discovered in 1999 by David Baulcombe's group in plants. siRNAs have a well dened structure: two hybridized strands of 18-23 RNA bases (usually 21 bp), each with phosphorylated 5′ ends and hydroxylated 3′ ends with two overhanging DNA nucleotides at the 3′terminus. The easiest way to achieve RNAi is the use of synthetic siRNA molecules. Once siRNA is transfected to a cell, it is incorporated into a nuclease complex called RNA-Induced Silencing Complex (RISC). RISC targets and cleaves mRNA that is complementary to this siRNA, thus interrupting translation of targeted genes.

Micro RNA

Micro RNAs are noncoding genes present in cells of various eukaryotic organisms. These are a form of single-stranded RNA, typically 19-25 nucleotides long, and are thought to regulate the expression of other genes. These RNA genes which are transcribed from DNA but do not form a protein. They were discovered in 1993 by Lee and Feinbaum during a study of the gene lin-14 in *C. elegans* development. Gene silencing may occur either viamRNA degradation or by preventing mRNA from being translated. Since its discovery, miRNA research has revealed its multiple rolesin negative regulation (transcript degradationand sequestering, translational suppression) and possible involvement in positive regulation (transcriptional and translational activation). By affecting gene

Table 1. Overview on use of RNAi in insects:

Organism	Target gene	Location	Stage	Method of application	Amount of dsRNA	dsRNA Length	mRNA silencing	References
Coleoptera								
Diabroticavirgi feravirgifera	Vacuolar ATPase subunit A	Gut	Neonates	Articial diet	<5.4 ng/cm^2	246	Drastic reduction	(Baum*et al*., 2007)
Leptinotarsade cemlineata	Vacuolar ATPase subunit A and E	Gut	Neonates	Articial diet	<52 ng/cm^2	300		
Phyllotretastriolata	Arginine kinage	Gut	Adults	Leaves	0.05-3.2ng/cm^2	332	Significant reduction	(Zhao *et al*., 2008)
Diptera								
Glossinamorsitansm orsitans	Midgut protein TsetseEP	Midgut	Male adults	Blood meal	10 µg	315	30-35%	(Walshe *et al*., 2009)
Hemiptera								
Acyrthosiphonpisum	Water specic aquaporin	Gut	Aphids	Articial diet	1 µg	-	50 %	(Shakesby *et al*., 2009)
Rhodniusprolixus	Nitroporin2	Saliva glands	2nd instars	Artiûcial diet	13 µg	502	20±10%	(Araujo *et al*., 2006)
Hymenoptera								
Apismellifera	Toll-related receptor 18W	Whole organism	Larvae	Soaking	1.2 µg	852	60 %	(Aronstein *et al*.,2006)
Isoptera								
Reticulitermesavipes	Cellulose enzymes	Saliva glands	Workers	Articial diet	13 µg	500	60%	(Zhou *et al*., 2008)
Lepidoptera								
Epiphyaspostvittana	Larval gut carboxylasterase	Gut	3rd instars	Droplet	1 µg	500	50-60%	(Turner *et al*., 2006)
Helicoverpaarmigera	Cytochrome P450 (CYP6AE14)	Midgut	3rd instars	transgenic plant	-	Diced	Decreased	(Mao *et al*., 2007)
Plutellaxylostella	Cytochrome P450 (CYP6BG1)	Midgut + carcass	4th instars	Droplet	0.250mg	345	98%	(Bautista *et al*., 2007)
Spodopterafrugiperda	Allatostatin C	Brain	4th instars	Droplet	3 µg	331	80±10%	(Griebler *et al*., 2007)

regulation, miRNAs are likely tobe involved in most biological processes. miRNAs occasionally also cause histone modication and DNA methylation of promoter sites,which affects the expression of target genes.The process of RNAi can be moderated byeither siRNA or miRNA but there are subtledifferences between the two. Both siRNA and miRNA can play a role in epigenetic through a process called RNA-induced transcriptional silencing (RITS).

RNase III and Dicer

RNase III is divided into three classes on the basis of domain structure *viz*., bacterial RNase III, Drosha family nucleases, and Dicer enzyme. The RNase III enzyme acts as a dimmer, thus digests dsRNA with the help of two compound catalytic centers, whereas each monomer of the Dicer enzyme possesses two catalytic domains, with one of them deviating from the consensus catalytic sequences (Agarwal *et al*., 2003). Dicer is the key enzyme, which initiating the RNA-silencing process. Dicer was identied in Drosophila extract which showed its involvement in the initiation of RNAi. It was named as Dicer (DCR) due to its ability to digest dsRNA into uniformly sized small RNAs (siRNA) by Hannon and a graduate student in his laboratory, Emily Bernstein in 2000 (Bernstein *et al*., 2001). It is a dsRNA specific Ribonuclease III-like endonuclease which cleaves the target dsRNAs into fragments of 21-24 nts, leaving 3'-hydroxyl and 5'-phosphate ends, and 2 nt 3' overhangs at the termini of the duplex. Dicer interacts with the RISC complexand after cleavage, the dsRNA fragments disassociate from dicer and become associated with the RISC.

RNA-Induced Silencing Complex and Guide RNAs

The RISC is a multiprotein complex that incorporates one strand of a siRNA or miRNA. After recognizing the complementary mRNA, it activates RNase to cleave the RNA. The sequence-speciûc nuclease activity observed in the cellular extracts responsible for ablating target mRNAs was termed the RNA-induced silencing complex (RISC) (Agarwal *et al*., 2003; Watson *et al*., 2005; Hannon *et al* 2002). In order to guide suppression of homologous targets, the small (s) RNA duplex must become single-stranded which needs a less stable strand with the 5´phosphate end of the helix that is incorporated as a guide RNA into an effector complex containing an Argonaute (AGO) protein. Two types of effector complexes viz. cytoplasmic and nuclear complexes have been described for induction of RNAi. Cytoplasmic complex, known as the RNA-Induced Silencing Complex (RISC), can mediate both mRNA cleavage and translational inhibition. The nuclear complex known as RNA-Induced Transcriptional Silencing (RITS) complex was first described in fission yeast for repressing DNA expression. Both these complexes contain ARGONAUTE (AGO) proteins, which are specific to each effector complex e.g. RITS have AGO4 and RISC involves AGO1, which are homolog of the translation. The RNA endonuclease, Dicer plays a role in aiding RISC action by providing the initial RNA material to activate the complex as well as the rst RNA substrate molecule.

Argonaute proteins

Argonaute proteins are the direct binding partners of siRNAs and form the core of RISC. These are about 100 kDa, highly basic proteins comprising PAZ and PIWI domains. The PAZ domain, which occurs also in the Dicer enzymes mediate protein-protein interactions, and facilitate binding with the Dicer complex (Tomoyasu *et al*., 2008; Peters and Meister, 2007). Due to their basic characteristics these proteins bind RNAs (e.g. siRNAs), and guide them to functional complexes. It is now well known that AGO1, having slicer activity, is an essential component of RISCs and cleaves the target mRNAs which are homologous to the miRNA or siRNA sequences in the complex and AGO4 is essential for DNA and histone methylation in *Arabidopsis*. One of the protein components of this complex belonged to Argonaute family of proteins and called as Argonaute2 (AGO2). The Argonaute family members have been linked both to the gene-silencing phenomenon and to the control of development in diverse species. The specific Argonaute proteins can transport specific classes of small regulatory RNAs to distinct cellular compartments to regulate gene expression.

Mechanism of RNA Interference

Our knowledge on RNAi has expanded dramatically in short time since its discovery. In the last few years, important insights have been gained in elucidating the mechanism of RNAi, although it seems to be very complicated. RNAi appears to be a highly potent and specic process, which starts when a dsRNA is introduced either naturally or articially in a cell. An endoribonuclease enzyme cleaves the

long dsRNA into small pieces of miRNA or siRNA depending upon the origin of long dsRNA, i.e., endogenous or exogenous, respectively. A dsRNA may be generated by either RNA- dependent RNA polymerase or bidirectional transcription of transposable elements. Based on the in vitro and in vivo experimental results, a two-step mechanistic model for RNAi has been established. The rst step referred to as the RNAi initiating step, involves the binding of RNA nucleases to a large dsRNA and its cleavage into discrete 18 to 23 nucleotide RNA fragments (siRNA). In the second step, these siRNAs join a multinuclease complex, RISC, which degrades the homologous single-stranded mRNAs (Watson *et al*., 2005; Hannon *et al*., 2002). Mechanisms of RNAi mediated gene silencing through siRNA and miRNA is diagrammatically presented in Figure 1 and 2.

Initiation

This stage is characterized by generation ofsiRNA mediated by type III endonuclease Dicer. It appears that the RNAi machinery,once it nds RNA duplexes, cuts it up intosmall molecules with a length of about 21nt. These also have 2-nucleotide, 3'overhangs, and 5' phosphates. Of course, different organisms have different numbers of Dicer genesthat process different sorts of dsRNAs. In general, Dicer works by recognizing theends of dsRNA with its PAZ domain (Dicer has PAZ domain at one end and dual RNase III domains on the opposing end) and then cuttingthe dsRNA with its RNase III domain. Thedistance between the PAZ and RNase III domaindetermines the length of siRNA produced by Dicer which varies by species. Long dsRNA and miRNA precursors are processed to siRNA/miRNA duplexes by the RNase-III-like enzyme Dicer. These short dsRNAs are subsequently unwound and assembled into effector complexes, RISCs, which can direct RNA cleavage, mediate translational repression or induce chromatin modication.

Effector

The second step of RNAi mechanism is referredas effector step; the incorporation of guidestrand. This is characterized by the formation ofsilencing complex. The siRNA and miRNAduplexes that contain ribonucleoprotein particles (RNPs) are now made into RISC. Generally, effector complexes containing siRNAs are known as a RISC, while those containing miRNAs are known as miRNPs. It is thought that RISC undergoes an ATP-dependent step that activates the unwinding of the double-stranded siRNAs. RISC is composed of PPD

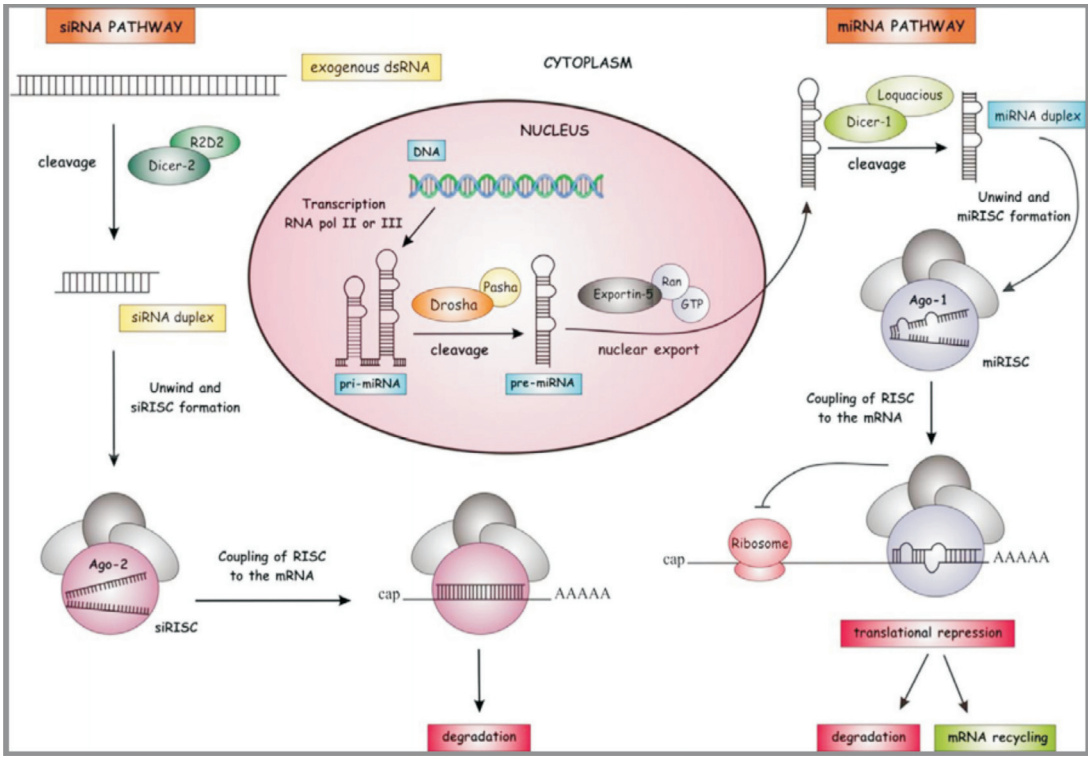

Fig. 1. Gene silencing mechanisms of through siRNA and miRNA

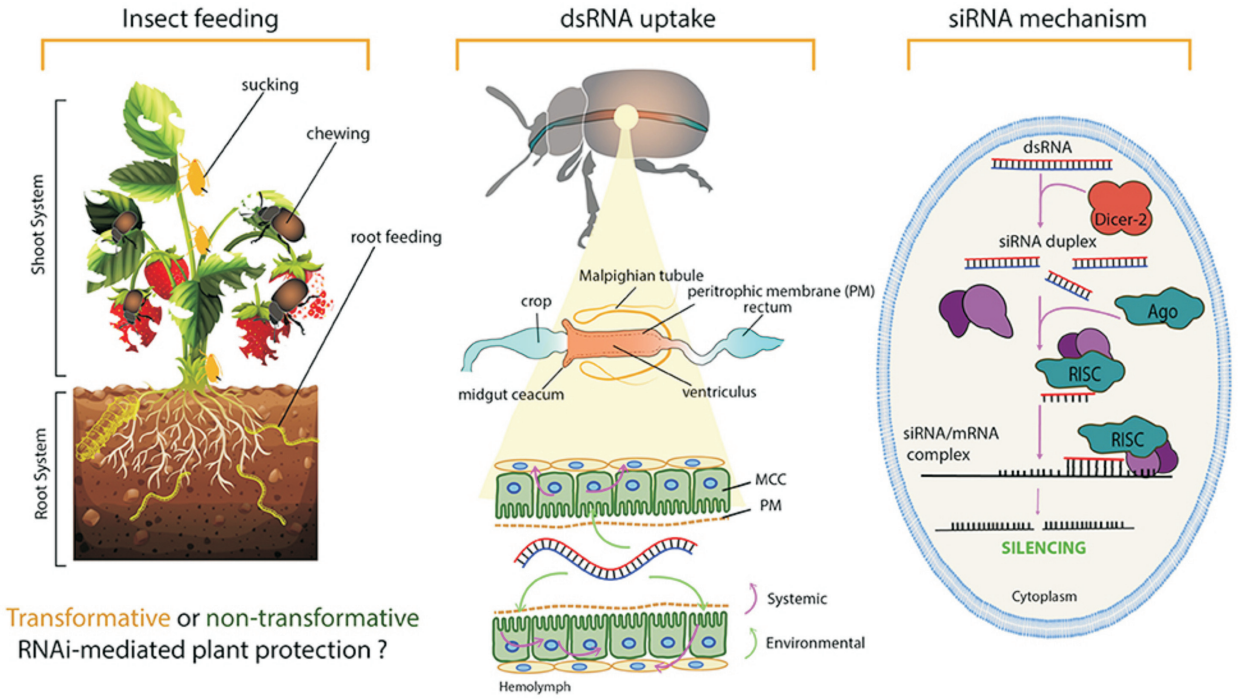

Fig. 2. Overview of RNA Interference
(Photos credited to authors of previously published research articles, names of these authors mentioned in this review citations and acknowledged).

proteins (PAZ PIWI Domain proteins), which are highly conserved super-family. The Argonaute protein and ones trand of the siRNA form the RISC. Once the RISC complex has been formed from 50-phosphorylated siRNAs and endogenous Argonauteprotein, the siRNAs in the RISC complex guide the degradation that is sequence-specic, of the complementary or near complementary mRNAs. RISC binds to the mRNA which is targeted by the single RNA strand within the complex and cleaves the mRNA or represses their translationby homology-dependent m-RNA degradation. The mRNA is cleaved in the middle of its complementary region. This cleaved mRNA cannot be translated. These newly synthesized siRNAs destroy the target with the help of RNA-dependent RNA polymerase (RdRP). Afterwards, RISC dissociates and is ready to cleave other mRNAs. By that even a few numbers of the RISC canlead to high-level gene silencing.

Delivery Methods of dsRNA Into Insects

Delivery of dsRNA is a major challenge in RNAi-based plant protection method. After identifying the target gene, choosing a convenient strategy to deliver the dsRNA into the insect body is very important. Methods of dsRNA delivery in insects can greatly vary and strongly influence the efficiency of gene silencing, thus their potential as insect pest control agent. It is important to note that since gene silencing is only limited to cells that are infected, the main challenge is the selection of the delivery system. Methods of delivery must be first defined, being effectively easier and better understood for cell-autonomous RNAi (Siomi and Siomi, 2009). The main delivery methods include injection, soaking, feeding, transgenic technique and viral infection. The articial RNAs could be exogenously applied onto the plant surface using additional techniques to increase the RNA absorption/uptake *viz*., cationic nanoparticles, clay nanosheets, surfactants and peptide-based RNA delivery systems.

Microinjection

Microinjection in this method direct injection of dsRNA into the body of insects. It is one of the most effective delivery methods for systemic RNAi. Short dsRNA has the most success with this mechanism (Winston and Molodowitch, 2002). The major advantage of injecting dsRNA into the insect body is the high efficiency of inhibiting gene expression. However, there are some limitations with micro-injection. First, the cost for vitro synthesis and

storage of dsRNA is relatively high, and the steps are complicated. In addition, injection pressure and the wound generated inevitably affect the insects. In practice, this delivery method would have very limited application as pest control in the field level.

Soaking

In this method, direct application of the dsRNA solution on the insect body. Soaking dsRNA solution can inhibit gene expression, its effectiveness is comparable to the injection method in that it requires a higher concentration of dsRNA (Eaton *et al*., 2002). The soaking method is suitable only for certain insect cells and tissues as well as for specific insects of developmental stages that readily absorb dsRNA from the solution, and therefore, it is rarely used.

Feeding of artificial diet

Compared to other methods, dsRNA feeding is the most attractive primarily because it is convenient and easy to manipulate. Since it is natural method of introducing dsRNA into insect body, it causes less damage to the insect than microinjection (Chen *et al*., 2010). It is especially popular in very small insects that are more difficult to manipulate using microinjection.

Developing transgenic insects

The advantage of using transgenic insects that carry the dsRNA is that as it is inheritable, the expression can be stable and continuous. The technique has been proposed to help either reduce population through introduction of sterile insects or for population replacement (Scolari *et al*., 2011). In this case, dsRNA must be first injected in the host insect. The transgenic method has been first used in *D. melanogaster* with the GAL4/UAS transgenic system that leads to the expression of hairpin RNA

Virus-mediated uptake

Virus-mediated RNAi methods involve the infection of the host with viruses carrying dsRNA formed during viral replication and targeting the gene of interest in the host (Couto *et al*., 2010). Virus-mediated RNAi studies are still rare. However, this method takes advantage of the infection and ability of the virus to spread rapidly in a host population. Viruses that are commonly employed for this purpose include lentiviruses, adenoviruses, and adeno associated viruses (AAVs).

Application of dsRNA through root absorption or injection into plant

Delivery of dsRNA to phytophagous insects could be achieved by supplying dsRNA through root absorption or injection into plant vessels, where these insects can naturally acquire dsRNA through sucking or chewing. A proof of concept for in planta dsRNA delivery, without a transformation event was rst described (Hunter *et al*., 2012). However, it is important to bear in mind that this strategy will demand mass production of dsRNA, which may be costly using molecular biology kits.

Factors Affect the Silencing Effect and RNA Interference Efficiency in Insect Management

In recent years, the research on the potential of using RNA interference (RNAi) to suppress crop pests has made an outstanding growth. But some factors can affect the efficiency of the dsRNA uptake and systemic silencing spread in different insects. Here, some important points that must be considered in developing an RNAi approach against insect pests.

The RNAi molecule

The success of an RNAi experiment hinges on the production of a specic RNAi molecule (in the form of dsRNA, siRNA, or a hairpin RNA) for a target gene of interest. For example, in Tribolium, the use of RNAi against vermillion (white) or Lac-2 provides rapid phenotypic evidence of RNAi success manifest in white compound eyes or white pupae, respectively (Arakane *et al*., 2005).

Length of the dsRNA fragment

The length and optimal concentration of exogenous dsRNA are very important for eective RNAi. The lengths of dsRNA required to attain an effective RNAi vary relying on insect species (Bolognesi *et al*., 2012). Most of the studies reported that dsRNA ranged from 140 to 500 nucleotides in length are required for successful RNAi(Huvenne and Smagghe, 2010).

Species-specic nucleotide sequence

A further issue to be considered in the design of RNAi molecules is the exquisite specicity of RNAi. In the context of eld applications of RNAi, this property facilitates design of insect-lethal sequences that are highly species-specic. For example, feeding four species of Drosophila with species-specic

vATPasedsRNA resulted in reduced vATPase mRNA and signicant mortality in conspecic, but not heterospecic ies (Whyard *et al*., 2009). The design of the dsRNA determines the one particular target gene to be silenced, but off- target effects can occur if siRNAs have some sequence similarity with unintended genes. This implies that an RNAi- plant can control two or even more pests this can also affect none target insects, becoming a bio-safety issue.

Persistence of the silencing effect

Persistence of the dsRNA in the insect body also affects the silencing effect. The transient effect of dsRNA against the pheromone binding protein in the light brown apple moth may be correlated with the turnover rate of the target protein (Turner *et al*., 2006).

Life stage of the target organism

In order to incorporate the dsRNA into insect body, selection of ideal life stage of insect is most important for the successful pest management. The older life stages are more efficient for handling, but the younger stages often show larger silencing effects. It was reported that the stronger silencing effect was observed in 5th instar larvae compared to adult moths in the fall armyworm (Griebler *et al*., 2008).

CONCLUSION

Widespread increase in the application of RNAi technology in insect research has facilitated the identification of insect gene function. Research has shown that the dsRNA is particularlyconservative; there are various functions and development factors among insect species. Such variations are yet to be fully understood but certainly can serve as a basis for determining their capacity to control insect genes. The main challenge for moving towards larger scale projects remains the development of effective delivery mechanisms. Feeding is very popular in insect RNAi research and may have the most promising future in pest control, especially with the creation of transgenic plants producing dsRNA. Indeed, existing studies have not providedenough evidence that systemic RNAi, with silencing RNA molecules spreading throughout the entire body can be achieved in all insects. Which insects have characteristics promoting systemic RNAi. Such questions need to be answered before moving further in developing largescale pest control systems. Undoubtedly, there is broad potential for the application of RNAi technology in pest control, mainly if combined into IPM strategies.

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