REVIEW ARTICLE

RNA interference: An update on its application in insect control

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Abstract: RNA interference (RNAi) is being used as one of the important technologies for better designing of pest control strategies. It is found to be effective against different insect orders such as Coleoptera, Diptera, Lepidoptera and Hemiptera. The efficiency of RNAi depends on the design of dsRNA and the mode of delivery selected. The delivery of RNAi through transgenic plants is now a reality with some products currently in the market. Topically applied dsRNA/siRNA (Spray induced gene silencing) has attracted attention due to its feasibility & low cost compared to transgenic plants. Sustainable agriculture relies on practices and technologies that combine effectiveness with a minimal environmental footprint. RNAi-based products can revolutionize pest and pathogen management safely and effectively if conception and development are conducted in a precautionary way.

Key words: Gene silencing, Insects, delivery systems, dsRNA

An overview of RNAi in insects

RNAi is a post-transcriptional gene silencing mechanism initiated by introduction of double stranded RNA (dsRNA) leading to generation of loss of function phenotypes by degrading the target gene messenger RNAs (mRNAs). It utilises small RNA (sRNA) as trigger molecules for the manipulation of gene expression. sRNAs are derived from double stranded RNA (dsRNA) and come in a variety of forms each differing in structure, function and biogenesis. E.g, Micro RNA (miRNA, ~22nt long) which is transcribed from plant genomes to regulate expression of endogenous genes (Bosco et al, 2008) and small interfering RNA (siRNA, 20-25nt long) which is derived from exogenous dsRNA sequences or the products of miRNA directed silencing (Broughton *et al.*, 2014). The RNAi pathway involves cleavage of the introduced dsRNA by dicer within cells. The resulting short RNAs or siRNAs get unwound to guide strand and passenger strand, of which the latter degenerates. Guide strand gets incorporated to RNA-induced silencing complex (RISC). The RISC-RNA complex degrades the corresponding mRNA and reduce protein expression (Fig 1).

Sensitivity of insects to RNAi: The efficiency of RNAi varies with insects orders. Coleopterans show high sensitivity to dsRNA, Dipterans show moderate sensitivity while Lepidopterans and Hemipterans show weak sensitivity to dsRNA. This is due to the presence of dsRNA degrading enzymes in their digestive system. RNAi efficiency differs within insects of the same order and also based on the mode of delivery of dsRNA.

Uptake, release and export of dsRNA could affect RNAi efficiency and resistance in insects. Insects can uptake RNA as both dsRNA and siRNA, directly or indirectly. Direct uptake refers to uptake through topical contact or feeding on plant tissues while indirect uptake involves first entering RNA in to plant vascular system and then uptake by insects. The dsRNA reaches insect cells through SID-like transporters or endocytosis or with the help of specific receptors which are found more for long dsRNA when compared to siRNA. After entering the cell, dsRNA should escape from endosomes (internal sorting organelles) to get accessibility to target mRNA which is found to be limiting in some insects. Plants, fungi and nematodes have endogenous RNA dependent RNA polymerase (RdRp) which target

on single-stranded RNA molecules and synthesize a second strand, generating dsRNA consequently. This produces a systemic spread of RNAi signaling in them (Zotti *et al.,* 2017). Since RdRp is absent in insects further amplification of dsRNA is not possible. The dsRNA reaches from cell to cell (Systemic RNAi) through special structures like exosomes, nanotubes or other carrier molecules in insects (Karlikow *et al*., 2016).

CHOICE OF dsRNA TARGETS IN INSECTS

The dsRNA targets selection can be of three types.

1. **Resistance factors:** Targeting genes associated with resistance to existing pesticides or other control measures. E.g, dsRNA targeting sodium channel increased sensitivity to pyrethroids in *Aedes aegypti* (Bona *et al*., 2016), Asian citrus psyllid topically applied with dsRNA against Cyo genes increased sensitivity to imidacloprid (Kilny *et al*., 2014).

2. Developmental and /or arthropod genes: Many insect-specific genes worth considering will reduce the risk of off-target effects in vertebrates. E.g, EcR KD in grain aphids (Yan *et al*., 2016).

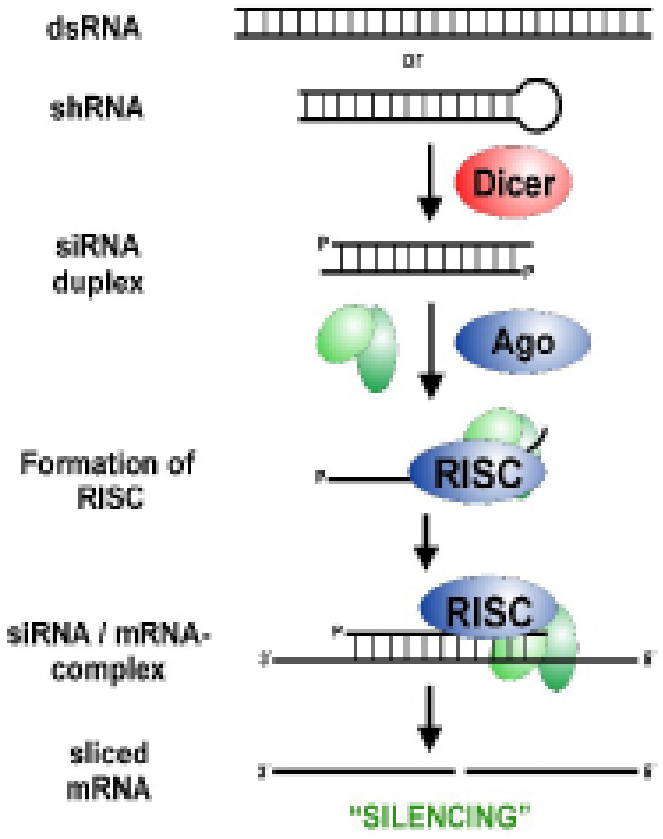

Fig 1: Molecular mechanism of siRNA silencing. Source: http://www.uni-konstanz.de/FuF/chemie/jhartig/

3. Intracellular trafficking pathways: These are tightly regulated processes used by a variety of molecules to cross the membranes of living cells. E.g, ESCART protein including *snf7* (Head *et al*., 2017), *vATPases* (Baum *et al*., 2007), COP pathway proteins (Taning *et al.,* 2018), Endocytosis proteins (Pinheiro *et al*., 2018).

Besides these, dsRNA is also being studied for inducing sterility in insects (Whyard *et al*., 2015).

DIFFERENT DELIVERY SYSTEMS OF dsRNA

After choosing the appropriate target genes, the most suitable delivery system has to be selected for the efficient application of RNAi in pest control. In laboratories, methods like artificial feeding and microinjection are utilized. For application of RNAi in fields, methods like Host-induced gene silencing (HIGS), Spray-induced gene silencing (SIGS), Virus-induced gene silencing (VIGS), Stem injection and Root absorption are used. Except for the first method, all others are non-transformative delivery systems.

1. Host-induced gene silencing (HIGS) (Transgenic inplanta delivery): Entails the creation of transgenic crops that express the dsRNA specific for the pest.

SmartStax Pro is the first commercial RNAi product targeting on insect pests. It is a transgenic corn crop developed by Monsanto (Bayer crop science) against Western corn rootworm (WCR). It employs a pyramid strategy utilizing two different *Bt* proteins (Cry34Ab1/ Cry35Ab1 and Cry3Bb1) as well as dsRNA targeting *snf7* gene expressed in the plant (Head *et al*., 2017). *Bt* protein inserts itself into the gut epithelium and cause gut paralysis and thereby death. Down regulation of *snf7* gene, which plays an essential role in protein trafficking, will also bring mortality of the insect (Bolognesi *et al.*, 2012). This combined strategy lead to the swift death of the pest and less resistance development against the Plant Incorporated Protectant (PIP) (Head *et al.*, 2017). Bt genes also ensure protection from lepidopteran pests like fall armyworm resulting in a healthy crop both above and below ground. This product was approved by the United States Environmental Protection Agency in 2017. In 2016, it was released in Canada and a year later, in 2017, released in the USA.

The advantage of this method is that it provides constant dsRNA exposure thereby achieving long term control with out spray. There are drawbacks associated, like regulation of transgenic crops, high cost of producing transgenic crops, reduced public acceptance, time-consuming production procedure, resistance development due to continuous exposure to dsRNA and absence of established transformation protocols.

2. Spray-induced gene silencing (SIGS) (Topical delivery): This involves the application of dsRNA topically as spray. This is an emerging area under focus due to the restrictions in transgenic approach. It is found to be more effective for insects that are more sensitive to dietary uptake of dsRNA. Given the low persistence of dsRNA in the environment, SIGS most likely need special formulations to increase stability and RNAi efficiency. Different formulations are experimented which includes nanoparticles, synthetic polymer, liposomes, proteinaceous delivery system and chemically modified siRNA.

Nanoparticles: Polyplex based delivery system consisting of either natural or synthetic polymer subunits. The most used nanoparticles are chitosan-based. Zhang *et al*. (2010) demonstrated that *Aedes gambiae* larvae soaked in chitosan-coated dsRNA solution improved RNAi knockdown. The incorporation of dsRNA into such a nanoparticle complex increased the stability and uptake of dsRNA *in vivo* (Zhang *et al*., 2010). This method of oral dsRNA delivery appeared beneficial for the control of African malaria mosquito, *Aedes gambiae* and yellow fever mosquito *A. aegypti* (Zhang *et al*., 2010; Mysore *et al*., 2013).

Synthetic polymer: Guanylated polymers based formulation of dsRNA experimented through oral delivery to *Spodoptera exigua* increased RNAi efficiency (Christiaens *et al*., 2018). Since lepidopterans have a high alkaline gut lumen, rapid nucleolytic degradation of dsRNA takes place in the digestive system resulting in low sensitivity for RNAi. Polymers with high guanidine content provided strong protection against nucleolytic degradation at pH 11. Polymers also enhanced cellular uptake and targeting essential gene *Chitin synthase B*, mortality of the pest also increased.

Liposomes: These are lipid-based transfection agents. Positively charged lipid envelops negatively charged dsRNA forming compact lipid bilayer. RNAi in *Drosophila* sp. using Lipofectamine 2000 and cellfectin improved efficiency (Whyard *et al*., 2009).

Proteinaceous delivery system: Cell-penetrating peptides (CPP) are used here. PTD-DRBD fusion protein (Peptide transduction domain-dsRNA binding domain) is integrated with dsRNA to form RNP (Ribonucleoprotein) improved mortality of cotton boll weevil than naked dsRNA (Gillet *et al*., 2017).

Chemical modifications on siRNA: Modification of one or both strands of dsRNA to improve stability and specificity (Jackson *et al*., 2003). It reduces off-target effects thereby safety concerns and cost-effectiveness are considered.

The advantages of SIGS are that it ensures increased cellular uptake and protection from (nucleolytic) degradation. Since dsRNA is not provided constantly, resistant development is also less. Unlike HIGS, it is easy to regulate. There are drawbacks for this method such as potential implication for biosafety and stability in the environment.

3. Virus-induced gene silencing (VIGS) (using microorganisms): Studies revealed that transgene was not required to trigger silencing pathway in plants. Non-transgenic plants infected by viruses induce dsRNA mediated post-transcriptional gene silencing which degrade pest genome. For VIGS, the viral genomes are modified by removing genes which induce virus symptoms and cloning a fragment (usually 300–500-bp) of the target gene with efficient siRNA generation and no off-target genes into the modified viral genome (Xu *et al*., 2006). The recombinant virus is then introduced into plant cells through *Agrobacterium tumefaciens*- mediated transient expression or *in vitro* transcribed RNA inoculation or direct DNA inoculation. After the recombinant virus is introduced into plant cells, the transgene is amplified along with the viral RNA by either an endogenous or a viral RNA-dependent RNA polymerase (RdRp) enzyme generating dsRNA molecules (Dalmay *et al*., 2000), these dsRNA intermediates in gene silencing. Virus-specific for insects & plants can be engineered to produce dsRNA inside the insects themselves or in plants respectively. Here infection with virus induces dsRNA synthesis and gene silencing. Virus-specific

for insects is more acceptable when compared to the latter.

Flock House Virus (FHS) was engineered to express *Drosophila melanogaster* specific dsRNA (Taning *et al.,* 2018). The advantage of using plant infecting virus is that it can move inside plant systematically through phloem, so recombinant virus can target phloem-feeding insects. Since VIGS is transiently transformed it does not cause alteration of the plant genome. Recombinant TMV expressing RNAi effectors infected *Nicotiana benthamina* plants caused death of citrus mealybug feeding on them (Khan *et al*., 2013). This method is beneficial for woody plants since it takes time to produce GM crops. VIGS can be transmitted to plant progeny & actively co-opts the plant to express dsRNA. Virus simulate natural path of cell entry which is an additional benefit of this method. Cross infection of beneficial insects also challenges biosafety. Since viruses are not accepted widely due to these drawbacks, Virus-like particles (VIP) produced by certain microbes is an alternative.

VIGS-like technology is also employed with other microorganisms like bacteria, yeast, entomopathogenic fungi, microalgae etc. The use of microorganisms ensures sustained release of dsRNA. It also prevents rapid degradation of dsRNA in the digestive system. Since common bacteria like *Escherichia coli* are engineered and used, this bacteria may cause infection on beneficial insects too which is found to be one of the drawbacks. Thus, the use of symbiotic bacteria specific for particular insects is more preferred. Besides using bacteria as the delivery system, they are used to produce a large amount of dsRNA which can be sprayed on crops at low costs. The major drawback of VIGS is that it is considered as a GM product and goes through all related regulatory processes.

Comparison of delivery as naked dsRNA, through *E. coli* and symbiotic bacteria *Rhodococcus rhodnii* in kissing bug *Rhodnius prolixus* was successfully done by Whitten *et al.* (2017). They reported that all these methods invoke a systemic response. Though naked dsRNA and *E. coli* produced dsRNA resulted in only transient systemic RNAi (since *E. coli* get eliminated from the cell after dsRNA ingestion), symbiotic bacteria *Rhodococcus rhodnii* colonised the gut and thereby established sustained systemic RNAi. The engineered *R. rhodnii* were also transmitted horizontally through ingestion of faeces which was found to be an additional benefit.

4. Stem injection and root absorption: Both are non-transgenic *in planta* delivery systems. Stem injection was found to be efficient for perennial trees. Hunter *et al.* (2012) reported that citrus trees treated with dsRNA through stem injection and root drench have shown effective control against citrus psyllid and leafhoppers for up to 57 days. Rice plant roots soaked in a solution containing dsRNA targeting *carboxylesterase* and *CYP18A1* genes from brown planthopper significantly knocked down these genes (Li *et al*., 2015). Delivery of dsRNA molecules through irrigation water is an alternative for crops that use irrigation in normal growing systems (Li *et al*., 2015). Though it allows a constant supply of dsRNA, the short persistence of dsRNA is a drawback associated with this method. Therefore, the use of advanced formulations is needed to protect dsRNA from degradation.

OTHER APPLICATIONS OF RNAi BASED PRODUCTS

Besides using against crop pests, RNAi based products are also developed for mosquito control, the human disease vector. A study on the efficiency of genetically engineered yeast expressing interfering RNA corresponding to mosquito neural genes as lure-and-kill mosquito and oviposition attractants were conducted and reported to be successful (Hapairai *et al*., 2017). RNAi based products against pests of beneficial insects were also developed. Deformed Wing Virus infection on both larvae and adults were reduced by feeding European honeybees with dsRNA (Desai *et al*., 2012). RNAi was also efficient against internal microsporidian parasite, *Nosema* (Paldi *et al*., 2010) and obligatory ectoparasite *Varroa destructor* in *Apis mellifera* (Garbian *et al*., 2012). Since dsRNA are target specific, they are found to be harmless against honeybees, so safety is assured.

RNAi BASED PRODUCTS: ACCOMPLISHMENTS

Currently approved RNAi based GM crops are based on ncRNA (non-coding RNA) to control insects (8%) and diseases (27%) or to improve scientific plant traits (65%). With all the drawbacks that GM products raise, more research is focusing on non-transformative delivery of dsRNA for gene silencing.

Monsanto is developing the use of RNAi through a technology called "Biodirect" in which dsRNA formulation is applied exogenously to protect plants against insect and pathogen attacks (https://monsanto.com/innovations/agricultural-biologicals). Syngenta is also developing lines of biocontrol products based on RNAi to protect potato plants from the attack of the colorado potato beetle (https://www.youtube.com/embed/BiVZbAy4NHw?ecver=1).

Since the production of inexpensive RNA is a primary need, a biotechnology start-up working on sprayable RNAi based insecticides *RNAissance Ag* introduced a technology in which industrial fermenting bacterial species are used to produce RNA in safe and cost-effective manner. Their sprayable RNAi based biopesticide against diamondback moth is under early field trial. Considering the hostile environmental condition to which dsRNA molecules are exposed in the field, the biotech company RNAgri (former APSE) developed a system where APSE RNA containers (ARC) are produced by *E. coli* bacteria, allowing the mass production of encapsulated ready to spray dsRNA. Research is being carried out on improving the potential of different RNAi based products and reducing their limitations by scientists from various countries.

During the last decade several researchers have carried work on RNAi based products, still there are some areas left behind. At field level, the time of application and rate of application of dsRNA has to be investigated. Since it is difficult to establish dsRNA uptake under field condition, new methods has to be discovered. The selection of most appropriate target gene, species specificity, cost of dsRNA, delivery of dsRNA formulations and regulation are major challenges while considering about RNAi based biopesticides. In India, since a more scientifically informed approach is followed for regulation of GM crops, topically applied RNAi based products will be a viable alternative. Recent study on topical application of dsRNA against papaya ring spot virus - Tirupati and Delhi isolate was successful providing 94% and 81% resistance respectively (Vadlamudi *et al*., 2020). More studies in SIGS are in need against other economic pests also.

CONCLUSION

RNAi based products have immense potential in managing crop pests, pests which are a threat to beneficial insects, pathogens and insect vectors of human and plant diseases safely and effectively. Although GM products are not widely accepted, non-transformative approaches in RNAi can be exploited as pest control measures. Realization of the complete benefits of the technique can be achieved only through appropriate dsRNA design and delivery mechanisms. The major limitation associated with the products is their less stability and impact on non-target organisms. The use of suitable formulations will reduce the degradation of dsRNA in both environments and inside the host. Bioinformatics based design of dsRNA sequences to minimize homology with endogenous transcripts in both the host plant and NTOs is an important approach to mitigate the risk of biosafety (species-specific products). Synthesis of broad-spectrum biopesticides targeting a wide range of pests can also be explored in this way.

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