

## RNA Interference: A new Strategy in the Evolutionary Arms Race Between Human Control Strategies and Insect Pests\*

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The relationship between humans and the insect pests of cultivated plants may be considered to be an indirect coevolutionary process, i.e., an arms race. Over time, humans have developed several strategies to minimize the negative impacts of insects on agricultural production. However, insects have made adaptive responses via the evolution of resistance to insecticides, and more recently against *Bacillus thuringiensis*. Thus, we need to continuously invest resources in the development of new strategies for crop protection. Recent advances in genomics have demonstrated the possibility of a new weapon or strategy in this war, i.e., gene silencing, which involves blocking the expression of specific genes via mRNA inactivation. In the last decade, several studies have demonstrated the effectiveness of this strategy in the control of different species of insects. However, several technical difficulties need to be overcome to transform this potential into reality, such as the selection of target genes, the concentration of dsRNA, the nucleotide sequence of the dsRNA, the length of dsRNA, persistence in the insect body, and the life stage of the target species where gene silencing is most efficient. This study analyzes several aspects related to the use of gene silencing in pest control and it includes an overview of the inactivation process, as well as the problems that need to be resolved to transform gene silencing into an effective pest control method.

Key words: Coevolution, crop protection, dsRNA, gene expression, gene silencing, pest control, target gene selection.

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The relationship between humans and the pests of cultivated plants may be viewed as a process that is analogous to an arms race. The term “arms race” is used to describe a coevolutionary relationship where two species generate reciprocal selective pressure. Thus, adaptive changes that occur in one species generate selective pressures on the other. For example, we may highlight the relationships between predators and prey, and/or between plants and herbivores. Species undergo adaptive changes over time, so the consumption rates of prey by predators and/or herbivores remain relatively constant in the same way as the success rate of prey escape (KAREIVA 1999; RAUSHER 2001; ANDERSON *et al.* 2010; STUKENBROCK & BATAILLON 2012; STOUT 2013).

The terms “indirect coevolution” or “arms race” indicate that relationships occur via cultivated plants, where the economic damage caused by insects generate pressures on humans, thereby leading us to develop new strategies to protect these plants and to minimize damage. We use this expression only to describe this specific aspect of our relationship with insect pests, i.e., the interactions between insects, cultivated crops, and human control strategies. The purpose of this analogy is to highlight the continuity of this process (RAUSHER 2001). Humans invest a great deal of intellectual energy into seeking and developing control techniques to reduce agricultural losses. Examples of these weapons include chemical insecticides and,

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more recently, the use of transgenic plants in agriculture. The adaptive responses of insects are relatively rapid and they occur by the evolution of resistant strains in most of the target species subjected to our weapons (WHALON *et al.* 2008; BIELZA 2008). The ability to learn allows us to refine our strategies by applying resistance evolution management techniques, such as that found in plants with genes (Bt) from *Bacillus thuringiensis*, which reduce the speed of response of insects. However, several studies have shown the presence of resistant strains in Bt plants (BRAVO & SOBERÓN 2008; GASSMANN *et al.* 2011; HEAD & GREENPLATE 2012; TABASHNIK *et al.* 2013).

The analysis of our relationship with insect pests clearly shows that, like other coevolutionary relationships, a continuous association demands permanent investment, so the rate of consumption remains relatively constant, or at least within acceptable levels.

Recent advances in the area of genomics indicate the possibility of using a new weapon or strategy in this war, i.e., gene silencing with RNA. This strategy employs a mechanism that eukaryotic cells use naturally to destroy their own non-functional RNA molecules, as well as to defend against viruses and transposons. Gene silencing involves blocking the expression of specific genes by destroying the corresponding mRNA molecules, so that the process does not affect the rate of gene transcription.

This study aims to describe various aspects related to the use of this new strategy for the control of agricultural pests. These aspects include an overview of this inactivation process and the problems that must be resolved to realize the potential of gene silencing in pest control.

## Development

### The process

Originally, RNAi was triggered accidentally in petunia plants in 1990 (VAN DER KROL *et al.*, 1990; NAPOLI *et al.* 1990), although gene silencing as a system was first described by FIRE *et al.* (1998) in *Caenorhabditis elegans*, which demonstrated that this process was triggered by double-stranded RNA molecules (dsRNA). Subsequently, the mechanism has been studied intensively and many reports have described the process, including evaluations of its practical application in different areas (for details of the mechanism see: SHARMA *et al.* 2013; NISHIMURA *et al.* 2013; GAO *et al.* 2014; CHABOT *et al.* 2014; HEATH *et al.* 2014; WILSON & DOUDNA 2013; DU TOIT 2014, and references therein). To achieve silencing, the dsRNA molecule must be complementary to the

target gene and it may originate either in the nucleus or the cytoplasm. Indeed, silencing can be triggered by various processes mediated by RNA molecules that contain 20-30 nucleotides. The process is initiated by the recognition and cleavage of dsRNA into small interfering RNA (siRNA) molecules of 21-26 nucleotides by an enzyme called Dicer (DCR, or Dicer-like). siRNAs comprise two strands: a guide strand and a passenger strand. The silencing complex, which cleaves and inactivates mRNA, is activated by the binding of DICER+siRNA to the RNA-induced silencing complex (RISC) where the argonaute protein (Ago) is the catalytic component. Thus, the guide strand of the siRNA directs the cleavage process and facilitates the binding of mRNA to the RISC complex, whereas the passenger strand is destroyed (Fig. 1). The action of the complex inactivates more than 90% of the mRNA molecules. The efficiency of silencing is greater when the inoculated dsRNA leads to the formation of an RNA hairpin (hpRNA) spaced with an intron (BAUMBERGER & BAULCOMBE 2005; QI *et al.* 2005; BRODERSEN & VOINNET 2009; GHILDIYAL & ZAMORE 2009; RIEDMANN & SCHWENTNER 2010; PERRIMON *et al.* 2010; KATOCH & THAKUR 2013; BURAND & HUNTER 2013).

The RNAi experiments have involved the following types of synthetic RNAi molecules: small RNA (small hairpin RNAs, shRNAs), micro-RNA (small hairpin microRNAs, shmiRNAs), and long molecules of dsRNA. Further details have been reported by ECHEVERRI and PERRIMON (2006), LEE and KUMAR (2009), SIOMI and SIOMI (2009), and BRODERSEN and VOINNET (2009).

### Incorporation of dsRNA into cells

Two mechanisms of dsRNA incorporation into cells have been identified. The first is mediated by two transmembrane proteins, i.e., SID-1 and SID-2 (defective systemic RNAi). The former is essential and is responsible for the systemic spread of RNAi, whereas the latter is specific to the gut and, together with SID1, facilitates the spread of RNAi from the environment (FEINBERG & HUNTER 2003; WINSTON *et al.* 2007; MCEWAN *et al.* 2012). The second mechanism involves receptor-mediated endocytosis-specific RNAi (JOSE & HUNTER 2007; SALEH *et al.* 2006; ULVILA *et al.* 2006; HUVENNE & SMAGGHE 2010; GU & KNIPPLE 2013).

### The potential

The possibility of applying gene silencing to insect pest control was demonstrated by the oral administration of dsRNA (ARAUJO *et al.* 2006; TURNER *et al.* 2006), which significantly reduced

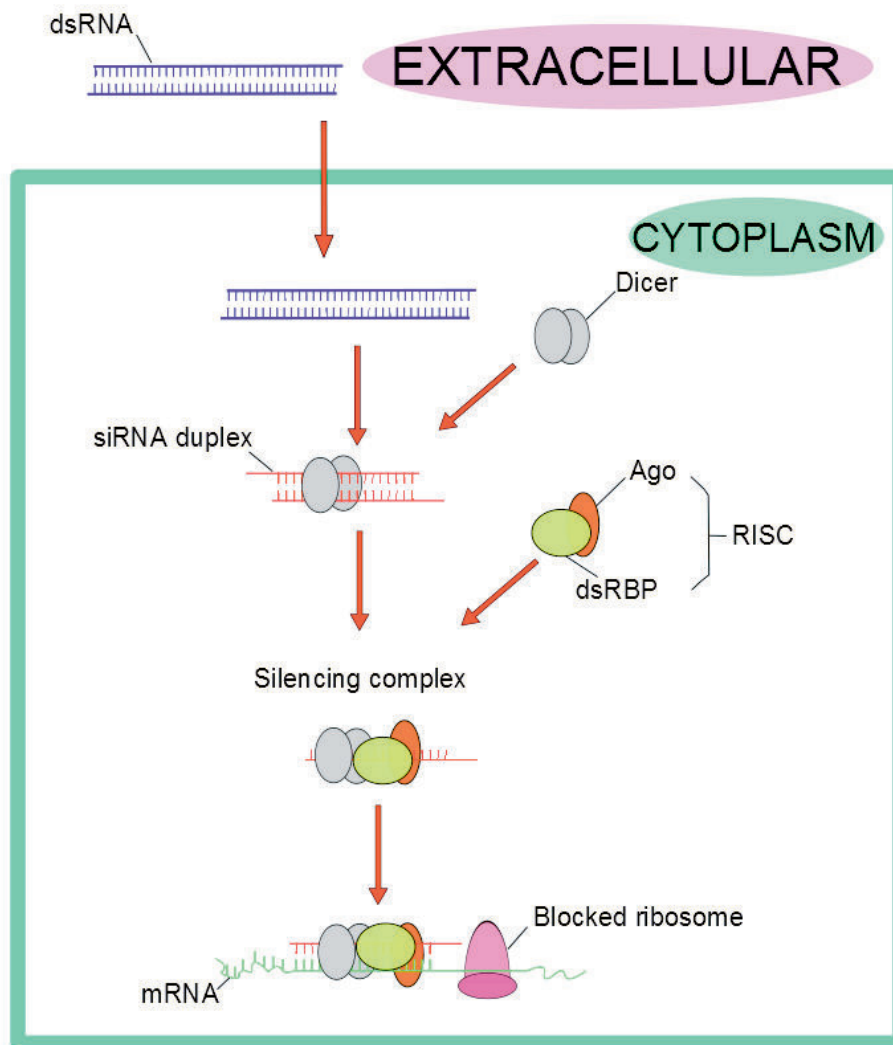


Fig. 1. The general aspects of siRNA pathways of RNA interference. Adapted from WILSON & DOUDNA 2013). Ago: Argonaute protein, dsRNA: Double-stranded RNA, dsRBP: Double-stranded RNA binding protein, mRNA: Messenger Ribonucleic Acid, RISC: RNA-induced silencing complex.

the expression levels of specific genes in the hemipteran *Rhodnius prolixus* (one of the main vectors of *Trypanosoma cruzi*, which is the causative agent of Chagas disease) and the lepidopteran *Epiphyas postvittana* (the light brown apple moth). Experiments using *E. postvittana* demonstrated the presence of ingested dsRNA in larval cells or other tissues and in the adults produced, i.e., oral administration induced the formation of systemic RNAi. Gene silencing using dsRNA has also been demonstrated in several insect orders, including Diptera (LI *et al.* 2011; COY *et al.* 2012; KUMAR *et al.* 2013; SINGH *et al.* 2013), Coleoptera (ZHAO *et al.* 2008; ZHU *et al.* 2011; RANGASAMY & SIEGFRIED 2012; CAO *et al.* 2012; RAMASESHADRI *et al.* 2013; WANG *et al.* 2013), Hymenoptera (WANG *et al.* 2010; HUNT *et al.* 2011; CHOI *et al.* 2012), Orthoptera (DONG & FRIEDRICH 2005; ZHANG *et al.* 2011), Lepidoptera (TERENIUS *et al.* 2007; GRIEBLER *et al.*

2008; TIAN *et al.* 2009; MAO *et al.* 2011; GONG *et al.* 2011, 2013; KUMAR *et al.* 2012; ASOKAN *et al.* 2012; TANG *et al.* 2012; WANG *et al.* 2013), Homoptera (CHEN *et al.* 2010), Hemiptera (ROSA *et al.* 2012), and Isoptera (ZHOU *et al.* 2008).

The difficulties that need to be overcome to transform gene silencing into an effective pest control practice include the transfer of dsRNA into the target cells, selection of target genes, a broader understanding of the mechanisms involved with the silencing process, and knowledge of how these aspects are associated with differences in the effects on different species.

#### Delivery of dsRNA

The delivery of exogenous dsRNA into the cells of several eukaryotic species triggers a mechanism that causes the rapid inactivation of mRNA mole-

cules containing nucleotide sequences complementary to the introduced sequence. The transport of dsRNA (delivery) into target cells is the limiting factor in the development and application of silencing techniques as an effective insect control strategy (ZHANG *et al.* 2013; YU *et al.* 2013).

Several methods have been tested in experiments with insects such as micro-injection, immersion, ingestion (artificial feeding systems and expression by transgenic plants). Artificial feeding systems involve the ingestion of artificial diets, which include dsRNA expressed in bacteria or synthesized *in vitro* and mixed with food. In particular, the transfer of dsRNA via feeding is the most attractive option and it has the greatest potential for field applications (ARAUJO *et al.* 2006; BAUM *et al.* 2007; CHEN *et al.* 2010; TIAN *et al.* 2009).

The presence of chitin in the exoskeleton of insects hinders absorption via the outer surface, but the peritrophic membrane maintains direct contact with the external environment. The cells responsible for the absorption of food in the lumen can absorb dsRNA and this is the most promising method of dsRNA transfer from the external environment into insect cells (VOINNET 2005; WHANGBO & HUNTER 2008; HUVENNE & SMAGGHE 2010).

The transfer of dsRNA to target species via ingestion is a method with many advantages, such as low cost and ease of preparation, while it is less invasive and easier to use in smaller species. However, the efficiency of the process depends on the continuous supply of adequate concentrations of dsRNA (ARAUJO *et al.* 2006; TIAN *et al.* 2009; WALSH *et al.* 2009; YU *et al.* 2013). The limitations of the application of this method include difficulties in defining the required amount of dsRNA to obtain an adequate response and the effects of the midgut environment on their action (RAJAGOPAL *et al.* 2002; ARAUJO *et al.* 2006; TURNER *et al.* 2006; SURAKASI *et al.* 2011; LI *et al.* 2013).

During ingestion, insects can be fed a mixed diet that contains either synthetic dsRNA (WHYARD *et al.* 2009) or food supplemented with *Escherichia coli* cells that express dsRNA (TIMMONS & FIRE 1998; TIMMONS *et al.* 2001; TURNER *et al.* 2006; BAUM *et al.* 2007; TIAN *et al.* 2009; SURAKASI *et al.* 2011). For sucking insects, dsRNA could be provided in solution (CLEMENS *et al.* 2000; WHYARD *et al.* 2009). Another option for agricultural applications is the construction of transgenic plants that express dsRNA targeted at specific pests (MAO *et al.* 2011; PITINO *et al.* 2011; ZHA *et al.* 2011; KUNG *et al.* 2012).

In addition to high costs, the ingestion of synthetic dsRNA by insects still presents several difficulties, particularly providing the appropriate concentrations to obtain positive results. The use

of *E. coli* strain HT115 is an inexpensive means of expressing and producing large amounts of dsRNA. Furthermore, the ingestion of bacteria does not damage the animals, thereby ensuring that death is caused by gene silencing rather than by the treatment process (TIMMONS *et al.* 2001; TIAN *et al.* 2009; LI *et al.* 2011). In addition, vectors that express dsRNA for different genes simultaneously could increase the efficiency of target species control (MCINTYRE *et al.* 2011; WANG *et al.* 2013; ATTASART *et al.* 2013).

The use of transformed plants in agriculture (plant-mediated RNAi) is the main method for continuous transfer because it allows dsRNA to combat the particular species that feed on plant organs such as roots. Several studies have demonstrated that the dsRNA produced by transgenic plants is absorbed by the midgut and it reduces the expression of the target genes. Some studies have shown that this approach can achieve a higher degree of specificity than that obtained with Bt plants (BAUM *et al.* 2007; MAO *et al.* 2007, 2011; PITINO *et al.* 2011; KUMAR *et al.* 2012; KUNG *et al.* 2012).

Two other studies involving the use of ingested dsRNA should also be mentioned. First, chloroplasts of the microalgae *Chlamydomonas* were transformed to express dsRNA complementary to the 3HKT gene, which was then used for mosquito control (KUMAR *et al.* 2013). This approach facilitates the control of aquatic insects, especially disease vectors. Second, the use of nanoparticles in the preparation of dsRNA inocula has been reported to improve the stability and efficiency of RNAi (ZHANG *et al.* 2010).

#### Variation in results

Several studies have reported differences in the intensity of the response among the insect species analyzed, thereby demonstrating that the suppression process is affected by the intrinsic properties of each species, as well as the genes used and the target tissue. In some species, the response is excellent and it persists for several generations of germ cells (BELLÉS 2010; LIU & KAUFMAN 2004; LYNCH & DESPLAN 2006; RONCO *et al.* 2008; MITO *et al.* 2011), whereas the results are not satisfactory in some species of Diptera and Lepidoptera (TERENIUS *et al.* 2011). The silencing process is fairly constant, but the mechanisms and the proteins involved differ among species (HUVENNE & SMAGGHE 2010). The dose required for a significant response using the micro-injection method also varies among species (HIRAI *et al.* 2004; TERENIUS *et al.* 2007) and similar results were observed in experiments where dsRNA was administered via ingestion (YANG *et al.* 2009; BAUTISTA *et al.* 2009; KHAJURIA *et al.* 2010). Additional studies are required to establish a better relation-



ship between the concentration and intensity of silencing dsRNA, as well as the effects of the process on the viability of the target species (IGA & SMAGGHE 2010; TERENIUS *et al.* 2011). Some studies suggest a possible relationship between the amount of dsRNA used and the level of silencing obtained (KUMAR *et al.* 2009).

Variation in the midgut chemical conditions will determine the species in which the transfer of dsRNA via food may work successfully. In some species, the results are negative. The midgut environment contains several digestive enzymes, including nucleases that digest nucleic acids. Thus, the efficiency of the process demands that the dsRNA must pass through the intestine intact before it is absorbed by cells. Therefore, the ability to digest dsRNA is a problem that must be solved to ensure the effective utilization of silencing to control pests on a large scale. Furthermore, evaluations should also test the effects of variation in intestinal pH among species (HAKIM *et al.* 2010; KATOCH & THAKUR 2013). Nanoparticles could increase the half-life of dsRNA by protecting the molecules (ZHANG *et al.* 2010; HE *et al.* 2013). The use of nanoparticles for bioproduct protection has been demonstrated successfully in food production (DURÁN & MARCATO 2012) and in biological insecticides (PÉREZ-DE-LUQUE & RUBIALES 2009; GHORMADE *et al.* 2011; KHATER 2012). Therefore, research in this area could yield positive results and facilitate the development of efficient methods for silencing via the ingestion of dsRNA.

#### Selection of target genes

The selectivity of RNAi is attributable to the identity of segments with specific nucleotides of the target gene. WHYARD *et al.* (2009) showed that the application of dsRNA for the enzyme V-ATPase produced positive results only in the species from which the target sequence was obtained. This specificity has been highlighted in several studies (DOENCH & SHARP 2004; MASLIAH *et al.* 2013; GU & KNIPPLE 2013).

The choice and selection of the target gene used in the silencing process is also a key step that ensures specificity, so careful selection of the target gene is necessary. The size of the dsRNA can also affect the efficiency of the results (YU *et al.* 2013; GU & KNIPPLE 2013). Experiments using 130 different genes have been performed in Lepidoptera and only 38% of these obtained high levels of silencing (TERENIUS *et al.* 2011). Similarly, the results of experiments with 290 different genes in beetles indicated varying levels of efficiency (BAUM *et al.* 2007).

The many factors that may affect the efficiency of RNAi in the control of insect pests include the concentration of dsRNA, the strength of the response, and the length of the sequence. In general, larger RNA molecules with higher similarity to the target mRNA are more efficient. The process is also affected by the type of cell into which the dsRNA is inserted and the enzymes involved in their recognition. Another important point is the possible instability of dsRNA and its degradation after internalization (SIOUD 2007; KIM *et al.* 2005; SIOLAS *et al.* 2005; AKHTAR & BENTER 2007; JERE *et al.* 2009).

#### Final comments

Regardless of the possibilities of using gene silencing for insect pest control, its application in the field will depend on a better understanding of the mechanisms involved with the overall process. This involves selecting genes that are essential for the survival of the target species and that are highly susceptible to the silencing process. Furthermore, it will be necessary to obtain a better understanding of the mechanisms involved with the processing and activation process in different species, or groups of organisms (ZHU 2013; YU *et al.* 2013; LI *et al.* 2013; GU & KNIPPLE 2013; BURAND & HUNTER 2013).

To facilitate the study and evaluation of gene silencing as an efficient tool for controlling agricultural pests, standardized experimental protocols need to be established for groups of target species (particularly via ingestion), allowing for the comparison of results. According to HUVENNE & SMAGGE (2010), the main areas that can affect the efficiency of silencing, which should be examined more closely, are the concentration of dsRNA, the nucleotide sequence of the dsRNA, the dsRNA length, the persistence of silencing, and the life stage of the target species in which silencing is more efficient.

The histories of strategies that have been developed to reduce losses in agricultural production almost always include considerable early success, followed by the development of resistance in some species or strains. The evolution of resistance to chemical insecticides has been reviewed previously (GEORGHIOU & LAGUNES-TEJEDA 1991). This phenomenon has also occurred with herbicides (NEVE 2007; POWLES & YU 2010) and antibiotics (DAVIES & DAVIES 2010), and it is happening at present with insecticides based on *Bacillus thuringiensis*.

Therefore, we must accept that part of what we produce will always be consumed by our competitors (pests), irrespective of the quality of our crop defense strategies. Thus, we must analyze all of the

steps involved with the production process to reduce agricultural losses, i.e., from planting until the arrival of food at the consumer's table, and identify methods that minimize losses during each step. After many years of this relationship (the arms race), it is time to accept that it is impossible to produce food on a large scale without a portion being consumed by other species.

To develop more efficient strategies based on gene silencing, we must remember that its success as a defense strategy is limited by the responsiveness of the target species, i.e., the development of new defense strategies (resistance). This response cannot be predicted but the coevolutionary process means that it will surely happen.

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