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# CONCEPTS OF RNA INTERFERENCE AND ITS USES AS A PEST MANAGEMENT IN PLANTS AND BIOTECHNOLOGY: REVIEW

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## Abstract

RNAi is an RNA-dependent gene silencing process that is controlled by the RNA induced silencing complex (RISC). The process is initiated by short double-stranded RNA molecules in the cell's cytoplasm, where they interact with the catalytic RISC component argonaute. The RNA is cleaved into short fragments by the enzyme dicer. Dicer binds and cleaves double-stranded RNAs to produce double stranded fragments of 20-25 positive base pairs, along with a few unpaired overhang bases on each end. There is an intrinsic mechanism probably present in all multicellular eukaryotes, which converts double stranded (ds) RNA into small (21 nt) RNAs (by cleavage with endo nucleases called Dicers) and uses them to direct sequence-specific degradation of cognate single-stranded RNAs. These small RNAs are termed short interfering (si) RNAs and the process is called RNA interference (RNAi). Curiously, most animals possess only one Dicer gene, whereas in plants Arabidopsis has four and poplar and rice seem to have five and six, respectively. RNAi as its application, it is importantly used for pest and disease management in plants without any negeative impact on the environment and with no intensive labor on human being.

Keyword: RNA Interference, RNA induced silencing complex (RISC), RNAi Mechanism, Pest management, Biotechnology

## Introduction

**Gene silencing (interference):** is a general term describing epigenetic processes of gene regulation. The term gene silencing is generally used to describe the "switching off" of a gene by a mechanism other than genetic modification of the specific organism. That is, a gene which would be expressed (turnedon) under normal circumstances is switched off by machinery in the cell. RNA interference (RNAi) is a promising gene regulatory approach in functional genomics that has significant impact on crop improvement which permits down-regulation in gene expression with greater precise manner without affecting the expression of other genes. RNAi mechanism is expedited by small molecules of interfering RNA to suppress a gene of interest effectively (Adnan et al, 2014).

Genes are regulated at either the transcriptional or post-transcriptional level.Transcriptional gene silencing is the result of histone modifications, creating an environment of heterochromatin around a gene that makes it inaccessible to transcriptional machinery (RNA polymerase, transcription factors, etc). Post-transcriptional gene silencing is the result of mRNA of a particular gene being destroyed or blocked. The destruction of the mRNA prevents translation to form an active gene product (in most cases, a protein). A common mechanism of post-transcriptional gene silencing is RNAi (Ahlquist, 2002). Both transcriptional and post-transcriptional gene silencing are used to regulate endogenous genes. Mechanisms of gene silencing also protect the organism's genome from transposons and viruses. Gene silencing thus may be part of an ancient immune system protecting from such infectious DNA elements (Gregory et al, 2005). Genes may be silenced by DNA methylation during meiosis as in the filamentous fungus Neurospora crassa.

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## 2. Scientific Cencepts of RNA Interference

**RNA interference** (**RNAi**) is a system within living cells that helps to control which genes are active and how active they are. Two types of small RNA molecules microRNA (miRNA) and small interfering RNA (siRNA) are central to RNA interference. RNAs are the direct products of genes, and these small RNAs can bind to specific other RNAs and either increase or decrease their activity for example by preventing a messenger RNA from producing a protein. It has an important role in defending cells against parasitic genes , viruses and transposons but also in directing development as well as gene expression in general. It is also a promising gene regulatory approach in functional genomics that has significant impact on crop improvement which permits down-regulation in gene expression with greater precise manner without affecting the expression of other genes. RNAi mechanism is expedited by small molecules of interfering RNA to suppress a gene of interest effectively (Younis et al, 2014).

RNA interference is also a process in which translation of some of a cell's messenger RNA (mRNA) sequences is prevented, because of the presence of (and consequent destruction of) matching double-stranded RNA sequences. RNA interference is believed to protect the cell against viruses and other threats. Interference refers to the interruption of the cell's translation of its own mRNA. RNA interference is also called posttranscriptional gene silencing, since its effect on gene expression occurs after the creation of the mRNA during transcription (Lodish, et al, 2004). It also imparts its effective and efficient role to knock down the expression of any particular gene through short interfering RNA molecules in any target cell and moreover to assess the changes that occur in signaling pathways (Younis et al, 2014).

### 2.1. History and Discovery of RNA Interference

The discovery of RNAi was preceded 98 y observations of transcriptional inhibition by antisense RNA expressed in transgenic plants and more directly by reports of unexpected outcomes in experiments performed by plant scientists in the U.S. and The Netherlands in the early 1990s. In an attempt to alter flower colors in petunias researchers introduced additional copies of a gene encoding chalcone synthase, a key enzyme for flower pigmentation into petunia plants of normally pink or violet flower color. The overexpressed gene was expected to result in darker flowers, but instead produced less pigmented, fully or partially white flowers, indicating that the activity of chalcone synthase had been substantially decreased; in fact, both the endogenous genes and the transgenes were downregulated in the white flowers. Soon after, a related event termed quelling was noted in the fungus Neurospora crassa, although it was not immediately recognized as related.

Further investigation of the phenomenon in plants indicated that the downregulation was due to post-transcriptional inhibition of gene expression via an increased rate of mRNA degradation (Parker et al, 2006). This phenomenon was called cosuppression of gene expression, but the molecular mechanism remained unknown. Not long after, plant virologists working on improving plant resistance to viral diseases observed a similar unexpected phenomenon. While it was known that plants expressing virus-specific proteins showed enhanced tolerance or resistance to viral infection, it was not expected that plants carrying only short, non-coding regions of viral RNA sequences would show similar levels of protection.

Researchers believed that viral RNA produced by transgenes could also inhibit viral replication. The reverse experiment, in which short sequences of plant genes were introduced into viruses, showed that the targeted gene was suppressed in an infected plant. This phenomenon was labeled "virus-induced gene silencing" (VIGS), and the set of such phenomena were collectively called post transcriptional gene silencing (Macrae et al, 2006).

After these initial observations in plants, many laboratories around the world searched for the occurrence of this phenomenon in other organisms. Nature paper reported a potent gene silencing effect after injecting double stranded RNA into Caenorhabditis elegans (Fire et al, 1998). In investigating the regulation of muscle protein production, they observed that neither mRNA nor antisense RNA injections had an effect on protein production, but double-stranded RNA successfully silenced the targeted gene. As a result of this work, they coined the term RNAi. Fire and Mello's discovery was particularly notable because it represented the first identification of the causative agent for the phenomenon (Daneholt and Bertil, 2006). RNA interference has been found in plants, fungi, insects and a variety of animals, including the roundworm (C. elegans) fruit fly (Drosophila melanogaster), zebrafish, and mouse.

#### 2.1.2. Mechanisms of RNA Interferencing Dicing Up DsRNA

Under most circumstances, RNA in a cell is present as a single stranded molecule only. For instance, mRNA is created in the cell nucleus and transported to the ribosomes in the cytoplasm as a single strand. Double-stranded RNA (dsRNA) in which two complementary strands pair up, is normally present only in circumstances that pose a threat to the cell. This can occur when a dsRNA virus infects the cell, or from infection by some other viruses whose genomes are temporarily copied into dsRNA. It also occurs when certain types of transposable genetic elements (transposons) copy themselves in preparation for reinserting elsewhere in the cell's genome. Though the RNA copies are single-stranded, most transposons have sequences at their ends that, when transcribed into RNA, can fold back on themselves to form dsRNA.When a cell detects dsRNA, it uses a nuclease enzyme to cut it into small fragments, twenty-one to twenty-three nucleotides long (the Drosophila enzyme is whimsically but accurately named "dicer") ( Lee et al, 2004). This inactivates the RNA, so that it cannot be used to carry out the viral replication cycle or be reinserted into the genome (in the case of a transposon), thus protecting the cell from its harmful effects.

Degradation of the dsRNA is not the end of the process, however the presence of these fragments also prevents the expression of mRNA containing the same sequences. That is, if the

host cell has used its own gene to create a single-stranded mRNA, and that mRNA is present in the cytoplasm along with dsRNA fragments with matching nucleotide sequences, the mRNA will be degraded, and the protein it codes for will not be made. This is the "interference" that gives the phenomenon its name. Indeed, it was this process that led to the discovery of RNA interference Scientists found that introducing double stranded RNA reduced rather than increased production of the encoded protein.

Note that not all mRNA activity in the cell is suppressed: Only those mRNAs of similar sequence are targeted. This provides a clue to the mechanism of suppression. Experiments have shown that dicer targets mRNA by using the dsRNA fragments themselves as guides. While the details are not yet clear, it is believed that one side of the dsRNA is matched with the complementary mRNA sequence, making a new dsRNA, which is itself then degraded (Hammond et al, 2000). This mechanism also allows the process to be self-sustaining, as each new round creates new fragments that can target any new mRNA.

The recognition process also lends further credence to the belief that RNA interference is a protective mechanism. By targeting only mRNA sequences previously identified as double-stranded (and therefore dangerous), a cell can avoid creating proteins that may be derived from viruses, albeit at the risk of turning off one or more of its own genes at the same time.

### 2.2. New Developments in DsRNA

Recent research has also shown that a class of similar dsRNA fragments, called small temporal RNAs, play important roles in development in the roundworm, fruit fly, and other animals. Although little is so far known about them, these fragments are made by dicer from the cell's own RNA as a normal part of the developmental process and appear to help control gene expression. This is an exception to the statement that the presence of dsRNA signals a threat to the cell; how these are distinguished from threatening dsRNA is not yet known (Danholt and Bertil, 2006).

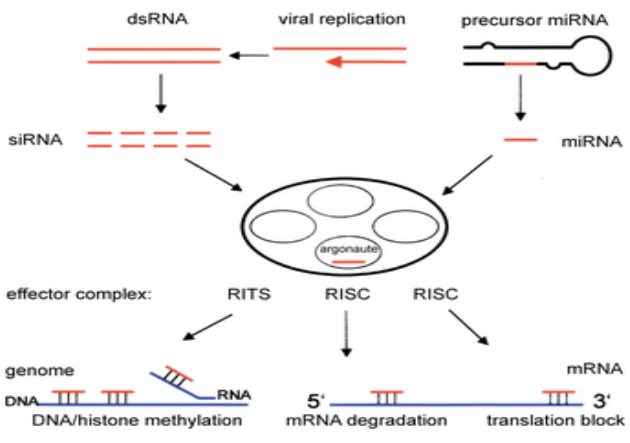


**Figure 1. Mechanism of enzyme dicer works in a cell**

The enzyme dicer trims double stranded RNA, to form small interfering RNA or microRNA. These processed RNAs are incorporated into the RNA-induced silencing complex (RISC), which targets messenger RNA to prevent translation (Lee et al, 2004).

The RNAi pathway is found in many eukaryotes including animals and is initiated by the enzyme Dicer, which cleaves long double-stranded RNA (dsRNA) molecules into short fragments of ~20 nucleotides. One of the two strands of each fragment, known as the guide strand, is then incorporated into the RNA-induced silencing complex (RISC). The most well-studied outcome is post-transcriptional gene silencing, which occurs when the guide strand base pairs with a complementary sequence of a messenger RNA molecule and induces cleavage by Argonaute, the catalytic component of the RISC complex (Hammond et al, 2000). This process is known to spread systemically throughout the organism despite initially limited molar concentrations of siRNA.

The selective and robust effect of RNAi on gene expression makes it a valuable research tool, both in cell culture and in living organisms because synthetic dsRNA introduced into cells can induce suppression of specific genes of interest. RNAi may also 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. Exploitation of the pathway is also a promising tool in biotechnology and medicine. Historically, RNA interference was known by other names, including post transcriptional gene silencing, and quelling. Only after these apparently unrelated processes were fully understood did it become clear that they all described the RNAi phenomenon.

RNAi is an RNA-dependent gene silencing process that is controlled by the RNA-induced silencing complex (RISC) and is initiated by short double-stranded RNA molecules in a cell's cytoplasm, where they interact with the catalytic RISC component argonaute (Daneholt and Bertil, 2006). When the dsRNA is exogenous (coming from infection by a virus with an RNA genome or laboratory manipulations), the RNA is imported directly into the cytoplasm and cleaved to short fragments by the enzyme dicer. The initiating dsRNA can also be endogenous (originating in the cell), as in pre-microRNAs expressed from RNA-coding genes in the genome. The primary transcripts from such genes are first processed to form the characteristic stem-loop structure of pre-miRNA in the nucleus, then exported to the cytoplasm to be cleaved by dicer. Thus, the two dsRNA pathways, exogenous and endogenous, converge at the RISC complex ( Bagasra and Prilliman, 2004).

#### 2.2.1. Gene knockdown

The RNA interference pathway is often exploited in experimental biology to study the function of genes in cell culture and in vivo in model organisms. Double-stranded RNA is synthesized with a sequence complementary to a gene of interest and introduced into a cell or organism, where it is recognized as

exogenous genetic material and activates the RNAi pathway. Using this mechanism, researchers can cause a drastic decrease in the expression of a targeted gene. Studying the effects of this decrease can show the physiological role of the gene product. Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred as a "knockdown", to distinguish it from "knockout" procedures in which expression of a gene is entirely eliminated.

Extensive efforts in computational biology have been directed toward the design of successful dsRNA reagents that maximize gene knockdown but minimize "off-target" effects. Off-target effects arise when an introduced RNA has a base sequence that can pair with and thus reduce the expression of multiple genes at a time. Such problems occur more frequently when the dsRNA contains repetitive sequences. It has been estimated from studying the genomes of H. sapiens, C. elegans, and S. pombe that about 10% of possible siRNAs will have substantial off-target effects (Qiu et al, 2005). A multitude of software tools have been developed implementing algorithms for the design of general, mammal-specific, and virus-specific siRNAs that are automatically checked for possible cross-reactivity.

Depending on the organism and experimental system, the exogenous RNA may be a long strand designed to be cleaved by dicer, or short RNAs designed to serve as siRNA substrates. In most mammalian cells, shorter RNAs are used because long double-stranded RNA molecules induce the mammalian interferon response, a form of innate immunity that reacts nonspecifically to foreign genetic material. Mouse oocytes and cells from early mouse embryos lack this reaction to exogenous dsRNA and are therefore a common model system for studying gene-knockdown effects in mammals. Specialized laboratory techniques have also been developed to improve the utility of RNAi in mammalian systems by avoiding the direct introduction of siRNA, for example, by stable transfection with a plasmid encoding the appropriate sequence from which siRNAs can be transcribed, or by more elaborate lentiviral vector systems allowing the inducible activation or deactivation of transcription, known as conditional RNAi (Lodish, et al, 2004).

### 2.2.2. Double Stranded RNA (DsRNA) Cleavage

Exogenous dsRNA initiates RNAi by activating the ribonuclease protein Dicer, (Bernstein et al, 2001). which binds and cleaves double-stranded RNAs (dsRNA)s to produce double-stranded fragments of 21-25 base pairs with a few unpaired overhang bases on each end (Zamore et al, 2000). Bioinformatics studies on the genomes of multiple organisms suggest this length maximizes target-gene specificity and minimizes non-specific effects (Qiu etal, 2005). These short double-stranded fragments are called small interfering RNAs (siRNAs). These siRNAs are then separated into single strands and integrated into an active RISC complex. After integration into the RISC, siRNAs base-pair to their target mRNA and induce cleavage of the mRNA, thereby preventing it from being used as a translation template ( Ahlquist, 2002).

Exogenous dsRNA is detected and bound by an effector protein, known as RDE-4 in C. elegans and R2D2 in Drosophila, that stimulates dicer activity. This protein only binds long dsRNAs, but the mechanism producing this length specificity is unknown (Parker et al, 2006). These RNA-binding proteins then facilitate transfer of cleaved siRNAs to the RISC complex. This initiation pathway may be amplified by the cell through the synthesis of a population of 'secondary' siRNAs using the dicer-produced initiating or 'primary' siRNAs as templates (Baulcombe, 2007). These siRNAs are structurally distinct from dicer-produced siRNAs and appear to be produced by an RNA-dependent RNA polymerase (RdRP) (Pak et al, 2007).

### 2.3.1. Micro RNA

Micro RNAs (miRNAs) are genomically encoded non-coding RNAs that help regulate gene expression, particularly during development. The phenomenon of RNA interference, broadly defined, includes the endogenously induced gene silencing effects of miRNAs as well as silencing triggered by foreign dsRNA (Macrae et al, 2006). Mature miRNAs are structurally similar to siRNAs produced from exogenous dsRNA, but before reaching maturity, miRNAs must first undergo extensive post-transcriptional modification (Fire et al, 1998).

An miRNA is expressed from a much longer RNA-coding gene as a primary transcript known as a pri-miRNA which is processed, in the cell nucleus,to a 70-nucleotide stem-loop structure called a pre-miRNA by the microprocessor complex. This complex consists of an RNase III enzyme called Drosha and a dsRNA-binding protein Pasha. The dsRNA portion of this pre-miRNA is bound and cleaved by Dicer to produce the mature miRNA molecule that can be integrated into the RISC complex; thus, miRNA and siRNA share the same cellular machinery downstream of their initial processing. The siRNAs derived from long dsRNA precursors differ from miRNAs in that miRNAs, especially those in animals, typically have incomplete base pairing to a target and inhibit the translation of many different mRNAs with similar sequences. In contrast, siRNAs typically base-pair perfectly and induce mRNA cleavage only in a single, specific target. In Drosophila and C. elegans, miRNA and siRNA are processed by distinct argonaute proteins and dicer enzymes.

### 2.3.2. Down Regulation of Genes

Endogenously expressed miRNAs, including both intronic and intergenic miRNAs, are most important in translational repression and in the regulation of development, especially on the timing of morphogenesis and the maintenance of undifferentiated or incompletely differentiated cell types such as stem cells. The role of endogenously expressed miRNA in downregulating gene expression was first described in C. elegans in 1993. In plants this function was discovered when the "JAW microRNA" of Arabidopsis was shown to be involved in the regulation of several genes that control plant shape. In plants, the majority of genes regulated by miRNAs are transcription factors; thus miRNA activity is particularly wide-ranging and regulates entire gene networks during development by modulating the expression of key

regulatory genes, including transcription factors as well as F-box proteins. In many organisms, including humans, miRNAs have also been linked to the formation of tumors and dysregulation of the cell cycle. Here, miRNAs can function as both oncogenes and tumor suppressors (Gregory et al, 2005).

### 2.3.3. RNA Induced Silencing Complex (RISC) Activation and Catalysis

The active components of an RNA-induced silencing complex (RISC) are endonucleases called argonaute proteins, which cleave the target mRNA strand complementary to their bound siRNA (Daneholt and Bertil 2006).

As the fragments produced by dicer are double-stranded, they could each in theory produce a functional siRNA. However, only one of the two strands, which is known as the guide strand, binds the argonaute protein and directs gene silencing. The other anti-guide strand or passenger strand is degraded during RISC activation. Although it was first believed that an ATP-dependent helicase separated these two strands, the process is actually ATP-independent and performed directly by the protein components of RISC.

The strand selected as the guide tends to be the one whose 5' end is least paired to its complement, but strand selection is unaffected by the direction in which dicer cleaves the dsRNA before RISC incorporation. nstead, the R2D2 protein may serve as the differentiating factor by binding the more-stable 5' end of the passenger strand. The structural basis for binding of RNA to the argonaute protein was examined by X-ray crystallography of the binding domain of an RNA-bound argonaute protein (Liu et al, 2003). Here, the phosphorylated 5' end of the RNA strand enters a conserved basic surface pocket and makes contacts through a divalent cation (an atom with two positive charges) such as magnesium and by aromatic stacking (a process that allows more than one atom to share an electron by passing it back and forth) between the 5' nucleotide in the siRNA and a conserved tyrosine residue. This site is thought to form a nucleation site for the binding of the siRNA to its mRNA target (Yuan et al, 2005).

It is not understood how the activated RISC complex locates complementary mRNAs within the cell. Although the cleavage process has been proposed to be linked to translation, translation of the mRNA target is not essential for RNAi-mediated degradation. Indeed, RNAi may be more effective against mRNA targets that are not translated. Argonaute proteins, the catalytic components of RISC, are localized to specific regions in the cytoplasm called P-bodies (also cytoplasmic bodies or GW bodies), which are regions with high rates of mRNA decay; miRNA activity is also clustered in P-bodies. Disruption of P-bodies decreases the efficiency of RNA interference, suggesting that they are the site of a critical step in the RNAi process (Wang, 2007).

## 3. Research Uses of RNA Interference

Because of its ability to turn off individual gene expression, RNA interference provides a remarkably precise tool for studying the effects of individual genes. There are several ways to deliver dsRNA to cells. It can be injected into a single cell or placed in a viral chromosome that infects the cells being studied. Roundworms will absorb dsRNA if they are soaked in a solution containing it, or if they eat bacteria that contain it.

RNA interference has several advantages over the alternative way of "knocking out" a gene, called gene targeting. Unlike gene targeting, administration of dsRNA does not require long and laborious breeding of the target organism carrying the knockout. Even more importantly, the dsRNA knockout is temporary and can be induced at any stage of the life cycle, rather than exerting its effect throughout life, as with gene targeting. This allows short-term studies of gene effects, a feature particularly valuable for studying development, for instance.

RNA interference has been used for applications in biotechnology, particularly in the engineering of food plants that produce lower levels of natural plant toxins. Such techniques take advantage of the stable and heritable RNAi phenotype in plant stocks. For example, cotton seeds are rich in dietary protein but naturally contain the toxic terpenoid product gossypol, making them unsuitable for human consumption (Baulcombe , 2007).

RNAi has been used to produce cotton stocks whose seeds contain reduced levels of delta-cadinene synthase, a key enzyme in gossypol production, without affecting the enzyme's production in other parts of the plant, where gossypol is important in preventing damage from plant pests. Similar efforts have been directed toward the reduction of the cyanogenic natural product linamarin in cassava plants (Baulcombe , 2007). Although no plant products that use RNAi-based genetic engineering have yet passed the experimental stage, development efforts have successfully reduced the levels of allergens in tomato plants and decreased the precursors of likely carcinogens in tobacco plants. Other plant traits that have been engineered in the laboratory include the production of non-narcotic natural products by the opium poppy, resistance to common plant viruses, and fortification of plants such as tomatoes with dietary antioxidants. Previous commercial products, including the Flavr Savr tomato and two cultivars of ringspot-resistant papaya, were originally developed using antisense technology but likely exploited the RNAi pathway.

### 3.1. Biological Functions of RNA Sielencing Immunity

RNA interference is a vital part of the immune response to viruses and other foreign genetic material, especially in plants where it may also prevent self-propagation by transposons (Bagasra and Prilliman, 2004). Plants such as Arabidopsis thaliana express multiple dicer homologs that are specialized to react differently when the plant is exposed to different types of viruses (Matranga, 2000). Even before the RNAi pathway was

fully understood, it was known that induced gene silencing in plants could spread throughout the plant in a systemic effect, and could be transferred from stock to scion plants via grafting. This phenomenon has since been recognized as a feature of the plant adaptive immune system, and allows the entire plant to respond to a virus after an initial localized encounter.

In response, many plant viruses have evolved elaborate mechanisms that suppress the RNAi response in plant cells. These include viral proteins that bind short double-stranded RNA fragments with single-stranded overhang ends, such as those produced by the action of dicer. Some plant genomes also express endogenous siRNAs in response to infection by specific types of bacteria. These effects may be part of a generalized response to pathogens that downregulates any metabolic processes in the host that aid the infection process (Ahlquis, 2002).

Although animals generally express fewer variants of the dicer enzyme than plants, RNAi in some animals has also been shown to produce an antiviral response. In both juvenile and adult Drosophila, RNA interference is important in antiviral innate immunity and is active against pathogens such as Drosophila X virus. A similar role in immunity may operate in C. elegans, as argonaute proteins are upregulated in response to viruses and worms that overexpress components of the RNAi pathway are resistant to viral infection (Zhao and Srivastava, 2007).

The role of RNA interference in mammalian innate immunity is poorly understood, and relatively little data is available. However, the existence of viruses that encode genes able to suppress the RNAi response in mammalian cells may be evidence in favour of an RNAi-dependent mammalian immune response. However, this hypothesis of RNAi-mediated immunity in mammals has been challenged as poorly substantiated. Alternative functions for RNAi in mammalian viruses also exist, such as miRNAs expressed by the herpes virus that may act as heterochromatin organization triggers to mediate viral latency.

### 3.1.2. RNA Interference for Plant Insects/Pests Resistance

Plant breeders and biotechnologists are using different approaches to develop insect/pest resistant varieties. Although classical breeders have been developed various insect/pest resistant cultivars, however, this approach is tedious and time consuming as complexity increases with some added traits. The practice of using pesticides to control pests has become a common approach around the world, but having dramatic health and environmental effects its use seems to be very limited in coming years.

Among transgenic approaches to control specific insect/pest, Bt-based toxins proved effective and replaced chemical insecticides in many crops. Most of the commercially used biotechnological approaches to control insect/pests on crops are subjected to expression of Bt insecticidal proteins which help in the permeabilization of gut epithelial cells membrane in susceptible insects (Younis et al, 2014).

However, this approach is limited for some specific crops to manage some specific pests, and there is also a threat that some insects can develop resistance against Bt. After the successful induction of transgene encoded RNAi in plants, biotechnologists speculated about crop protection from insects through genetic engineering to exhibit dsRNAs target insect genes and recently, application of dsRNA for knocking specific genes has been well-documented (Ingram, 1996).

### 3.1.3. RNAi Effect on Sharpshooter (GWSS) A Vector of Piearce Disease on Grape

The glassy-winged sharpshooter (GWSS) is among the most robust and thus most threatening vectors of Xylella fastidiosa (Xf), the bacterium that causes Pierce's disease (PD) (Davis, Purcell et al, 1982), a devastating disease occurring in wine grapes from California to Texas to Florida (Sutton et a,l 2007). New strategies that will lead to environmentally sound approaches to control GWSS and other insect vectors are needed. RNA interference (RNAi) has been suggested as a strategy to develop "insect-proof plants" (Gordon and Waterhouse 2007) and even referred to as a "genetic insecticide" (Scharf, 2008). RNAi is a eukaryotic gene regulation/defense mechanism in which small RNA segments, small interfering RNAs (siRNAs) (21-25 nt), generated by processing of dsRNA molecules often of viral origin, specifically down-regulate complementary RNA sequences (Meister and Tuschl 2004).

Intra-thoracic injection of dsRNAs has been shown to be the most effective way to induce RNAi in whole insects of many species including Anopheles gambiae (Blandin, Moita et al. 2002; Blair, Sanchez-Vargas et al. 2006), Blattella germanica (Ciudad, Piulachs et al. 2006), Drosophila melanogaster (Dzitoyeva, Dimitrijevic et al. 2001), Spodoptera litura (Rajagopal, Sivakumar et al. 2002), Culex pipiens (Sim and Denlinger 2009), Lutzomyia longipalpis (Mauricio R.V. Sant'Anna), Cecropia pupae (Bettencourt, Terenius et al. 2002), Acyrthosiphon pisum (Mutti, Louis et al.), Rhodnius prolixus (Araujo, Soares et al. 2009), Aedes aegypti (Cooper, Chamberlain et al. 2009), Bemisia tabaci (Murad Ghanim), Dermacentor variabilis (Mitchell Iii, Ross et al. 2007) and Tribolium castaneum (Arakane, Dixit et al.). Oral induction has also been demonstrated in several of these same species.

Our effort demonstrates for the first time that RNAi activity can be induced in a leafhopper species, but also is inducible in GWSS cell lines. In the long term, RNAi can be used as an effective fundamental tool to better understand the dynamics of plant: pathogen: vector interactions as well as GWSS physiology and of course we hope as a strategy to complement overall efforts for PD control (Bryce and Cristina, 2009).

According to Bryce and Cristina 2009, sets of dsRNA molecules were delivered to GWSS cells via lipid-based transfection and to GWSS nymphs via intra thoracic injection or by feeding on cuttings immersed in a solution containing dsRNAs. Real time RT-PCR, semi quantitative RT-PCR, Northern blot of small and large RNA fractions showed that RNAi was achieved in cells and insects injected with dsRNA, where target mRNAs were

partially degraded and specific siRNA, hallmarks of RNAi were detected.

Because there are several potential sharpshooter vectors of Xf, the sequences isolated from GWSS were also amplified from the blue green sharpshooter (BGSS; Graphocephala atropunctata) and from the green sharpshooter (Draeculacephala minerva) and cloned. (Bryce and Cristina , 2009) demonstrated that a high degree of sequence conservation among these distinct sharpshooters and the resulting sequences could be used to develop a general RNAi strategy to control multiple Xf vectors. Western Blot analysis also showed a reduction of actin protein in GWSS nymphs injected with actin dsRNA. GWSS nymph injected with actin dsRNA shows decreased actin protein level.

Fifteen third and fourth instar GWSS nymphs were injected with 1µg actin dsRNA in 1µl volume with 1 µg GFP dsRNA or with 1 µl injection buffer and left on basil plants for five days. Then, proteins were extracted from three living and one dead insect and subjeted to Western blot analysis, using actin antibodies specific for Drosophila melanogaster. Results showed a decrease in actin protein in the nymph injected with actin dsRNA and alive five days post injection In addition, some of the injected nymphs did not complete ecdysis, demonstrating a striking phenotypic effect in whole insects vs. those injected with control gfp dsRNAs. GWSS nymphs injected with actin dsRNA died during molting. Fifteen third and fourth instar GWSS nymphs were injected with 1ug actin dsRNA in 1ul, or with 1 ul injection buffer and left on basil plants for five days. During this period, two of the actin dsRNA injected insects couldn't complete molting and died visible phenotypic effect was also obtained in GWSS cells transfected with actin dsRNA, where aberrations of the actin filaments occurred starting 72 hours post transfection .

## 3.3. Biotechnology Aspects of RNA Interference

### 3.3.1. RNAi As a Tool For Gene Function Analysis in Plants

Although RNAi is not a knockout but a knockdown technology, its high efficiency and ease of application make it applicable to genome-wide analysis of gene function. In plants, RNAi is often achieved by a transgene that produces hairpin RNA (hpRNA) with a dsRNA region

Conventionally, antisense-mediated gene silencing has been widely used in the analysis of gene function in plants. Although antisense-mediated gene silencing is an RNAi-related phenomenon hpRNA-induced RNAi has been shown to be much more efficient (Wang, 2007). In an hpRNA-producing vector, the target gene is cloned as an inverted repeat spaced with an unrelated sequence and is driven by a strong promoter, such as the 35S CaMV promoter for dicots or the maize ubiquitin 1 promoter for monocots. When an intron is used as the spacer, which is essential for stability of the inverted repeat in Escherichia coli, the efficiency becomes very high: almost 100% of transgenic plants show gene silencing. However, the mechanism by which the intron increases silencing efficiency remains unclear . RNAi can be used against a vast range of targets; 30 and 50 untranslated regions (UTRs) as short as 100 nt could be efficient targets of RNAi. For genome-wide analysis of gene function, a vector for high-throughput cloning of target genes as inverted repeats, which is based on an LR Clonase reaction, has been constructed .Another high-throughput RNAi vector is based on 'spreading of RNA targeting' (also called transitive RNAi) from an inverted repeat of a heterologous 30 UTR . For analysis of genes essential to plant viability, a chemically regulated RNAi system has also been developed.

Direct introduction of dsRNA or a plasmid producing hpRNA transiently by particle bombardment has been shown to induce RNAi in plants. This approach is useful for the analysis of gene function in plants in cases where transgenic approaches that require stable transformation are more difficult. Virus-induced gene silencing (VIGS) is another approach often used to analyze gene function in plants. RNA viruses generate dsRNA during their life cycle by the action of virus-encoded RdRP. If the virus genome contains a host plant gene, inoculation of the virus can trigger RNAi against the plant gene. Because this approach does not involve a transformation process, it might be suitable for the functional analysis of essential genes. Amplicon is a technology related to VIGS.

It uses a set of transgenes comprising virus genes that are necessary for virus replication and a target gene. Like VIGS, amplicon triggers RNAi but it can also overcome the problems of host specificity of viruses. Is thought to be useful in regulating the degree of suppression. However, the use of a weak Promoter appears to result in a reduction in the frequency of suppression, rather than the induction of weak suppression. An alternative approach is the use of sequences with various homologies to the target gene. In LGC-1, homology-dependent suppression by RNAi was observed. Such homology dependency could Result from the effectiveness of each sienna to cleave Target mRNA. The degree of suppression of a gene could be 'designed' by using homologous genes isolated from closely or distantly related species that exhibit various Homologies to the target gene. Such an approach could be applied to the improvement of various agronomic traits Such as plant heistrand of the siRNA then hybridizes to mRNA as a guide and the RISC cleaves the mRNA near the center of the siRNA.

The siRNA duplex consists of a 19 nt double stranded region with 2 nt 30 overhangs. In Drosophila, mismatches between siRNA and the target mRNA greatly reduce the efficiency of mRNA cleavage, particularly when these are located near the center of the siRNA .It should be noted that in plants a small number of mismatches can be tolerated.

### 3.3.2. The Molecular Mechanism of RNAi And RNAi Related Phenomena in Plants

PTGS involves the generation of dsRNA by RdRP. Micro RNA (miRNA) is an endogenous siRNA-like RNA known to be involved in the developmental regulation of gene expression in animals and plants . Its precursor (pre-miRNA) is a small hpRNA with 'bulges' in its stem region (Okamura et al, 2004). All dsRNA, hpRNA and pre-miRNA are processed by Dicer into 21 nt

RNA duplexes and the unwound ssRNA is then incorporated into RISC. In plants, dsRNA and pre-miRNA can be processed by distinct DICER-LIKE proteins .

In animals, miRNA, which is partially complementary to mRNA, inhibits translation. In plants, like siRNA, miRNA cleaves mRNA despite a small number of mismatches with the target mRNA. It should be noted, however, that some miRNAs also inhibit translation in plants as well as in animals .strand of the siRNA then hybridizes to mRNA as a guide, and the RISC cleaves the mRNA near the center of the siRNA The siRNA duplex consists of a 19 nt double stranded region with 2 nt 30 overhangs (Gregory et al, 2005). In Drosophila, mismatches between siRNA and the target mRNA greatly reduce the efficiency of mRNA cleavage, particularly when these are located near the center of the siRNA . It should be noted that in plants a small number of mismatches can be tolerated.

**Summary**

**RNA interference** (**RNAi**) is a post transcriptional genetic mechanism of various eukaryotes (as plants, fungi, nematodes, and mammals) which suppresses gene expression and in which double-stranded RNA cleaved into small fragments initiates the degradation of a complementary mRNA. It is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules. Historically, it was known by other names including co-suppression, post transcriptional gene silencing (PTGS), and quelling. It has recently become a highly effective and major tool of functional genomics for silencing the gene expression of crop improvement for pest management and other yielding improvements. The RNAi is a sophisticated technology having revolutionary capabilities could be further exploited for functional analysis of target genes and regulation of gene expression for crop protection and improvement.

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