Chapter 7

The Extraordinary Nature of RNA Interference in Understanding Gene Downregulation Mechanism in Plants

Jorge Ricaño-Rodríguez, Jacel Adame-García, Silvia Portilla-Vázquez, José M. Ramos-Prado and Enrique Hipólito-Romero

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61689

Abstract

Gene silencing (also known as ribonucleic acid [RNA] interference [RNAi] or interfering RNA) was first recognized in plants and is considered one of the most significant discoveries in molecular biology in the last several years. These short-chain ribonucleic acid molecules regulate eukaryotic gene expression. The phenomenon involves a process that promotes RNA transcripts degradation through complementarity between RNA molecules and RNAi transcripts, resulting in the reduction of their translation levels. There are two principal classes of regulatory RNA molecules: small interfering RNAs (siRNA) and microRNAs (miRNA). Both are generated from the cleavage of double-stranded self-complementary RNA hairpins by a DICER enzyme that belongs to the RNase III family. Small RNAs (of about 21–24 nucleotides in size) guide specific effector Argonaute protein to a target nucleotide sequence by complementary base pairing. Thereby, the effector protein complex downregulates the expression of RNA or DNA targets. In plants, *cis*-regulatory RNAi sequences are involved in defense mechanisms against antagonistic organisms and transposition events, while *trans*-regulatory sequences participate in growth-related gene expression. siRNA also performs neutral antiviral defense mechanisms and adaptive stress responses. This document is an attempt to scrutinize the RNAi nature in understanding gene downregulation mechanism in plants and some technical applications.

Keywords: Plant gene silencing, RNAi biosafety, RNA-directed DNA methylation, RNA interference, small interfering RNA

1. Introduction

The discovery of ribonucleic acid (RNA) interference is undoubtedly one of the most important scientific events of the last decades. The beginning of this fascinating story takes place for the

first time in the early 1990s, when a few scientists attempted to increase the color in petunia flowers (*Petunia* sp.), through the addition of target gene copies involved in pigment biosynthesis pathways that were joined to very strong promoters and inserted into the petunia genome. Although respective results showed a decrease in floral color, those expected should be just the opposite. This meant that some transgenic plant lines used in the experiments exhibited suppression or co-suppression (gene silencing) that may be coordinated of both the transgene and the homologous endogenous plant gene. Therefore, it was concluded that plant tissues exhibiting gene suppression (co-suppression) had showed strong evidence of reduced steady-state levels of transgene and homologous messenger RNA (mRNA) [1–2].

Plant RNA silencing is divided into transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) based on its action target. Although the molecular mechanism behind this phenomenon was unrecognized, shortly before, the results of co-suppression assays related to the production of tobacco etch virus (TEV)-resistant plants using transgenic lines that express the TEV coat protein were published [3–5].

Gene silencing was also referred to gene quelling in plants and fungi and later RNAi in animals. It is considered a conserved regulatory mechanism of gene expression and has been mostly characterized in eukaryotic cells. As far as we know, RNA silencing leads to a specific nucleotide sequencing process in plants that induces mRNA degradation or translation inhibition at the posttranscriptional level. On the other hand, in plants, it sometimes can cause epigenetic modifications at the transcriptional level, which depend on a process called RNA-directed DNA methylation (RdDM) [6–7]. In addition, siRNA-mediated RNA silencing also serves as natural antiviral defense mechanism (*e.g.,* virus-induced gene silencing [VIGS]) [8].

Since miRNA-mediated gene silencing pathway has emerged as a key regulatory mechanism for controlling gene expression, recent discoveries have shown that this pathway is composed of a series of different important components. Among others, it starts with a double-stranded RNA (dsRNA) trigger, followed by an intermediary processor called DICER (Argonaute protein) or a DICER-like protein (DCL). This peptide is a member of the endoribonucleases RNase III family that specifically cleaves dsRNA. The processor product, which consists of small RNAs (siRNAs or miRNAs) of about 21–24 nucleotides (nt) in size, activates an effector complex called RISC (RNA-induced silencing complex), where the Argonaute protein (AGO) (*i.e.,* essential catalytic component) works as a key player to initiate gene expression regulation. Posteriorly, RNA-dependent RNA polymerase (RDR) amplifies the dsRNA target (siRNAs-guided AGO) and cleaves the target RNA. These molecular interactions stabilize the dsRNA substrate to produce secondary siRNAs and maximize the silencing process. The entire complex is considered a gene silencing suppressor (GSS) [7].

Due to its effectiveness and relative ease of use, gene silencing technique has become a potential tool in both basic and applied research. Given the fact that phytopathogenic microorganisms are a major cause of plant diseases, RNA silencing-based resistance proves to be an effective biotechnological alternative to engineer resistant crops, among other applications. In either case, it is necessary to generate dsRNA trigger molecules before using RNAi to silence target genes that help to metabolic engineering of transgenic plants and generation of pest-resistant crops by inserting into plants a transgene that will produce homologous miRNA sequences. Finally, the recent discovery of dsRNA in unicellular eukaryotes implies that miRNAs have a

http://dx.doi.org/10.5772/61689

deep evolutionary history. The last indicates dsRNAs have evolved independently within eukaryotes through exaptation of their shared and inherited RNAi machinery [9].

2. RNAi machinery: Brief overview of its biogenesis

It is noteworthy that some authors believe that RNAi was first discovered in plants as "co-suppression" [1–2], but not in worms as PTGS [10]. For creating transgenic plants, several attempts have been made to engineer more desirable characteristics [11]. This is how the "co-suppression" concept was coined to explain the ability of exogenous elements to modify gene expression. Currently, the general comprehension that we have about RNAi emerges from an evolutionarily conserved gene regulatory mechanism in higher organisms.

It is known that some other molecules related to siRNA (*i.e.*, trans-acting siRNA and repeat-associated small interfering RNA commonly abbreviated "TAS," "ta-siRNA" or "tasiRNA" and "rasiRNA") repress gene expression through PTGS in plants. All of these molecules are a class of small RNAs involved in the RNAi pathway [11]. Many times, RNAi is considered a quelling process because it is the result of overexpression or suppression of specific transgenes.

According to some authors [12–13], dsRNA was characterized in detail after injecting anti-sense-stranded RNA into an organism that was an effective way to inhibit gene function. This was the first attempt to use an antisense RNA approach to inactivate a *Caenorhabditis elegans* gene [14]. Due to the above results and thanks to further investigations, it was concluded that the active molecules that triggered this phenomenon could be considerable amounts of dsRNA that interfered in vitro RNA transcripts. dsRNA injection into the nematode acted systematically to cause posttranscriptional depletion of homologous RNA. This methodology offers a way of specific and potent inactivation of gene function. It is also known that RNAi acts systemically when injected into the animal´s tissue, inhibiting gene function.

Through a variety of experiments, it has been suggested that RNAi destabilizes cleaved RNA after its processing. The nature of RNAi inspired Timmons and Fire [15] to perform a simple but efficient experiment that produced an astonishing result. Several nematodes were fed with bacteria that had been engineered to express dsRNA corresponding to *C. elegans unc*-22 gene. The organisms showed a similar phenotype (dependent on their food source) to that of *unc*-22 mutants. The ability to expose a vast number of samples with dsRNA established the basis for the development of a versatile tool to select RNAi-defective *C. elegans* mutants as well as target genes [16]. Small RNA molecules have been described according to their origin and function (*i.e.*, siRNAs, rasiRNAs, and miRNAs). RNA polymerization may produce dsRNA in nature (*e.g.*, viruses).

Although it is very common to observe transcript overlapping from repetitive sequences such as transposons and transgene arrays, dsRNA is rapidly processed into short RNA duplexes of about 21–28 nucleotides in length. A clear example of the natural function of these molecules is mRNAs or viral genomic/antigenomic RNAs that are recognized and split to several particles (translationally repressed). In addition, short RNAs are implicated in guiding chromatin modification [7]. RNA silencing mechanisms have been also recognized as antiviral defense against exogenous RNA viruses and random integration of transposable element transcripts.

The general role of gene silencing only became clear when it was realized that specific genes in plants and animals encode short forms of fold-back dsRNA5 (precursor molecules of miRNAs) [17]. There are three different metabolic pathways that induce RNAi and share a common molecular mechanism. These are currently known as miRNA, siRNA, and Piwi-associated RNA (RNAi that prevents transposons mobility through the genome), although the last one has been only found in animals [18]. Gene silencing is part of an miRNA or siRNA complex that works as splicing pattern to identify nucleotide sequences ready for degradation via RISC machinery.

The RISC complex is the result of several enzyme couplings involved in RNAi mechanism, that mediate target mRNA silencing through degradation or translational inhibition. miRNA production starts from a pre-miRNA (primary miRNA) transcript whose length sequence is about of 1000 nucleotides and create complementary loops, either single or double, as well as complementary sequences (5′–3′) [19]. Since this mechanism involves both endogenous and exogenous microsequences, their precursors produce dsRNA molecules of appropriate size in order to be linked to an effector protein. This phenomenon is mediated by an endoribonuclease enzyme (class III; DICER) with different structural domains, although the most important are those called PAZ (Piwi, Argonaute, and Zwelli) and helicase *(i.e.*, specific amino acid sequence responsible for unpacking genes). After an intensive search for the enzymatic mechanisms of gene silencing, DICER enzymes were first recognized as responsible for processing dsRNA to siRNA in *Drosophila* [20]. These enzymes contain a helicase and a couple of dimerized RNase and PAZ domains, although variability among organisms can be observed.

Helicase domains are RNAi precursors, which are perfectly aligned with dsRNA. Moreover, helicase metabolizes ATP (adenosine triphosphate) to translocate enzymes in order to generate a large number of sequences [21]. In plant genera such as *Arabidopsis*, DICER DCL1 (DICER-like1) proteins converge sequentially with pre-miRNAs for synthesizing loops and posteriorly with dsRNA of about 21 nucleotides in length. Through partial sequence alterations of RNA helicase domains caused by point mutations, it has been observed a reduction phenomenon of the amount of mature miRNA sequences. It is now known that plant DLC1 proteins are essential for a proper embryonic development [22].

In DICER proteins, PAZ domains have been extensively studied. Structurally, they have similarities to oligonucleotide–oligosaccharide structures, and theoretically, PAZ domains recognize the 3′ end of RNA substrates. On the other hand, recent studies have shown that they link not only the 3′- but also their 5′-phosphorylated substrates, where cleavage positions are recognized at a distance of 22 nucleotides [23–24]. In the conventional RNAi model, DICER enzymes interact in the cytoplasm to degrade their substrates prior to the RISC complex linkage.

DICER enzymes are important siRNA and miRNA intermediary pathways and generate dsRNA molecules as imperative substrates for Argonaute proteins. DICER are also considered common effectors of ribonucleoproteinic complexes linked to a single RNA sequence of 20–30 nucleotides complemented to target genes and conduct, at the same time, mRNA degradation [25]. Argonaute proteins contain four domains: terminally-N, PAZ, middle (MID), and Piwi terminally-C. The latter is typical of such complexes [26].

http://dx.doi.org/10.5772/61689

Many organisms express multiple members of this superfamily of proteins. For example, *Homo sapiens*, *Drosophila melanogaster*, and *Arabidopsis thaliana* express up to 8, 5, and 10 peptides, respectively. Individual members of each family are highly specialized in carrying out gene silencing process [23]. One of the most prominent roles of this class is its relationship with pre-ribosomal RNA synthesis (pre-rRNA) [27]. During the miRNA formation, HASTY proteins (exporter miRNA proteins) translocate their precursor into the cytoplasm. Subsequently, double-stranded precursor is dissociated and miRNA guide sequence is incorporated into a containing AUG protein complex, usually to form a specific RISC complex (miRISC) [28]. AGO1 PAZ domain complex links to miRNA and helps to incorporate miRISC. miRISC–miRNA complex prevents target gene expression, by either mRNA cleavage or translation inhibition [29]. In miRNA processing, introns among pre-miRNA sequences are removed through RNA splicing (posttranscriptional RNA maturation).

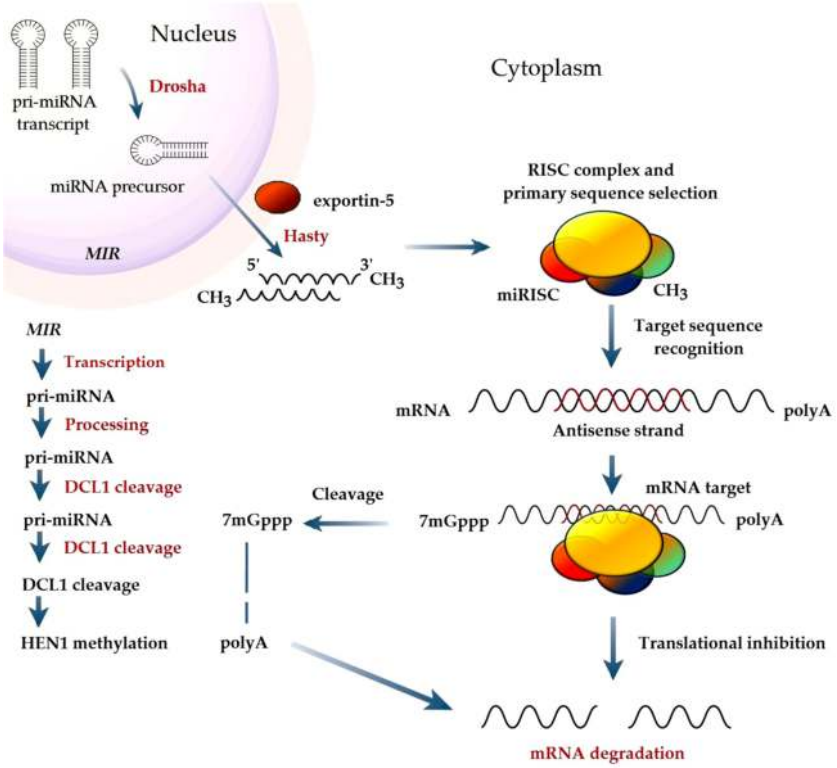

Figure 1. Pathway of siRNA-guided posttranscriptional regulation of gene expression. RNA polymerase II is mediating miRNA genes (*miR)* transcription that generates primary miRNA. DICER (DCL1) processing takes place in the nucleus through cap-binding complexes (CBC), DAWDLE (DDL), dsRNA-binding RNA protein hypnotastic leaves 1 (HYL1), and Hua enhancer 1 (Hen1) protein interactions. The HASTY (HST1; nucleocytoplasmic transporter activity) ortholog transports methylated miRNA to the cytoplasm and miRNA is coupled to RISC complex. miRNA guides miR–RISC complex in order to silence target mRNA by either excision or translational inhibition [99].

It has been recently discovered that there are ribonucleotide structures at the intermediate stage of the metabolic complex that allow the synthesis of specific molecules known as noncoding RNAs (ncRNAs), which are also considered regulatory RNA molecules (of 200 nucleotides) that are not translated into proteins [30]. They are intermediaries of target mRNA degradation that is finally identified by RISC complex, whose function is defined by different protein interactions [25]. Endoribonuclease RNase III DICER enzyme is the majorly involved key in RNAi and miRNA pathways. It plays an important role in assembling the RISC complex in addition to its catalytic function over microsequences [31].

RNase III DICER family enzymes are important intermediaries for siRNA and miRNA pathways. These peptides generate dsRNAs that will be linked to an Argonaute protein. Bacterial RNase III class I enzymes form DICER's active site, and it comprises a terminally-C RNase III domain [18]. In addition, prokaryotic enzymes are capable to dimerize and achieve a cleavage of both strands of dsRNA. DICER enzymes use RNase III pseudodimer domains of a single polypeptide with a single double-stranded RNA-binding domain (dsDRBD) to accomplish a similar dsRNA cleavage [32]. PAZ domain of these paired active sites has a terminal-N domain, and it recognizes the dsRNA end that is characteristic of RNAi intermediaries.

DICER proteins complexity can be attributed to multiple domain levels, ranging from several combinations of catalytic RNase III as well as the number of differently expressed proteins in single organism. In a generic RNAi model, DICER enzymes function in the cytoplasm, where they cleave their substrates before loading into RISC complex [23]. In recent years, DICER enzymes have been receiving much attention because they are capable of playing an important role in transcriptional gene silencing. Limited evidence suggests that DICER may also be found and functional in mammal cells. Among all DICER non-catalytic domains, PAZ has been one of the most intensively studied domains because of its presence in AGO proteins recognizing 3′-nucleotides of siRNAs [33].

3. Role of miRNAs in plant immunity

Eukaryotic cells are capable of modulating the stability of their miRNAs in response to environmental and endogenous stimuli and/or to regulate mRNA transcription levels (regulating mRNA transcript level). Such alterations in reducing mRNA levels are mediated by RNAi *cis* regulator and by RNA-binding proteins [34–35].

miRNA sequences are often related to the regulation of various biological processes such as stress mitigation [36]. *Arabidopsis* has two miR393a and miR393b genes that are processed almost identically when they mature and subsequently become miR393 sequences. This miRNA has been considered a nonfunctional sequence [37]. However, later studies showed the involvement of these molecules in plant immunity because of their interaction with AGO proteins during bacterial infections [38]. The sequence has a target gene called *MEMB12*, which encodes a structural protein of Golgi apparatus involved in vesicular secretion processes.

http://dx.doi.org/10.5772/61689

Plants respond to either biotic or abiotic environmental stresses by differential gene expression and miRNA sequences regulation. In several plant species, increased expression of miR160, miR167, and miR393 have been observed during drought conditions. It is known that miR393 blocks the expression of a gene encoding auxin receptors, while miR167 and miR160 interfere with the expression of some genes related to stress responses [39]. In addition, plant *miR*-sequences play important regulatory roles in many other processes (refer to Table 1 for some detailed examples).

Description	Annotation	Mature sequence	Reference
Arabidopsis thaliana miR156a stem-loop	Regulatory roles through complementary to mRNA	ath-miR156a-5′ (21-40 nt) ath-miR156a-3′ (83-104 nt)	[79]
Arabidopsis thaliana miR167a stem-loop	Target of mRNAs coding for auxin response factors, DNA binding proteins related to control transcription in response to the phytohormone auxin	ath-miR167a-5′ (19-39 nt) ath-miR167a-3′ (101-121 nt)	[80]
Arabidopsis thaliana miR168a stem-loop	Target of mRNAs coding for Argonaute (AGO1) proteins	ath-miR168a-5′ (18-38 nt) ath-miR168a-3′ (103-123 nt)	[79]
Arabidopsis thaliana miR169a stem-loop	Target of mRNA coding for CCAAT binding factor (CBF)-HAP2-like proteins	ath-miR169a-5′ (18-38 nt) ath-miR169a-3′ (190-209 nt)	[81]
Arabidopsis thaliana miR170a stem-loop	Target of mRNAs coding for GRAS domain (family of transcription factors whose members have been implicated in radial patterning in roots, signaling by gibberellin and light signaling	ath-miR170a-5′ (18-38 nt) ath-miR170a-3′ (190-209 nt)	[82]
Arabidopsis thaliana miR172a stem-loop	Target of mRNAs coding for APETALA2-like transcription factors	ath-miR172a (78-98 nt)	[81]
Nicotiana tabacum miR6020b stem-loop	Regulatory roles through complementary to mRNA	nta-miR6020b (21-41 nt)	[83]
Oryza sativa miR156a stem-loop	Regulatory roles through complementary to mRNA	osa-miR172a (7-26 nt)	[80]
Physcomitrella patens miR1049 stem-loop	Regulatory roles through complementary to mRNA	ppt-miR1049 (89-109 nt)	[84]
Populus trichocarpa miR156d stem-loop	Family of plant non-coding RNA	ptc-miR156d (11-30 nt)	[85]
Ricinus communis miR156a stem-loop	Target of mRNAs coding for Argonaute (AGO1) proteins	rco-miR156a (6-26 nt)	[86]

Description	Annotation	Mature sequence	Reference
Saccharum officinarum miR408c stem-loop	Regulatory roles through complementary to mRNA	sof-miR408c (247-267nt)	[87]
Selaginella moellendorffii miR156 stem-loop	Regulatory roles through complementary to mRNA	smo-miR156c (11-31 nt)	[84]
Solanum tuberosum miR6022-stem-loop	Regulatory roles through complementary to mRNA	stu-miR6022 (197-217 nt)	[83]
Zea mays miR156b stem-loop	Regulatory roles through complementary to mRNA	zma-miR156b-5′ (21-40 nt) zma-miR156b-3′ (86-106 nt)	[88]

Table 1. Examples of representative microsequences and their role in plant physiology

Plants require at least 14 essential minerals coming from the soil for proper development; therefore, RNAi is involved in both regulation and homeostasis of nutrients [40]. It is worth mentioning that constructions of genomic libraries have proved to be very valuable for studies of miRNAs associated with these metabolic processes [41]. Thereby, biotechnological applications of miRNAs might require microarray studies helping to discover important miRNA-associated metabolic responses to water, heat, salt, biotic stress, and UV radiation, as well as stress-mediated hormonal regulation and nutrient homeostasis, and resulting in future creations of "biotech" lines resistant to adverse environmental conditions.

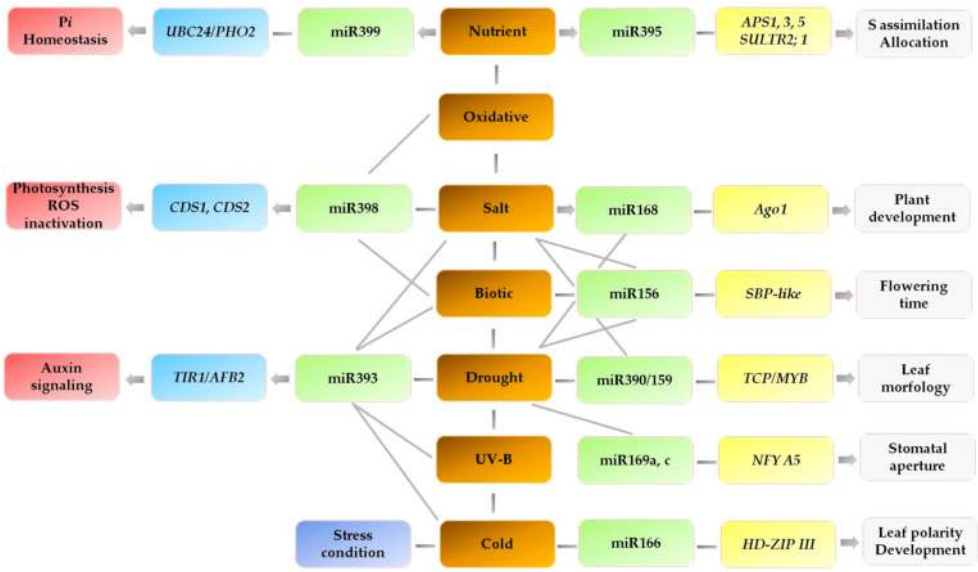

Figure 2. Summary of representative plant miRNAs involved in response to stresses. Modified from Kruszka et al., 2012 [40].

http://dx.doi.org/10.5772/61689

4. RNAi in crop protection against pest insects

As mentioned above, one of the first researches showing that RNAi could degrade specific mRNA sequences, resulting in blocking of the expression of certain insect genes, was conducted in *C. elegans*, a rhabditoid nematode [14]. The responsible researchers behind the project shared the Nobel Prize for Medicine in 2006 for what they called "a fundamental mechanism for controlling the flow of genetic information." To date, functional genomics using RNAi technology has been studied in several insect species, including orders such as Diptera, Coleoptera, Lepidoptera, Isoptera, Orthoptera, Hymenoptera, and Hemiptera, among others [42].

The functional approach of this tool has been successful in characterizing genes related to different physiological processes, including development, reproduction, behavior, and immune systems [43–44]. A viable biological control strategy based on RNAi application should target a gene that is vital for a proper physiological process as well as require an efficient delivery method for RNAi triggers. Recent research in insects has shown the in vitro microinjection effect of synthetic double-stranded sequences in embryos [45]. However, although this delivery method provides a tool for understanding gene function, dsRNA microinjection may not be feasible for pest control due to its high cost. RNAi potential as biotechnological tool for controlling insect populations was first demonstrated after oral introduction of dsRNA into insect body [46]. The study was conducted using *Rhodnius prolixus* larvae, which were fed with a dsRNA developed from the genes sequence coding protein called nitrophorin 2 (anticoagulant encoding transcripts), after which a significant decrease of anticoagulant activity levels on insect´s salivary glands was observed.

In the same year, a research that involved *Epiphyas postvittana*, a lepidopteran that is capable of attacking up to 123 different species of dicotyledonous, was conducted [47]. Oral introduction of dsRNA target encoding intestinal proteins as well as intermediary pheromone-like protein synthesis in adult antennas decreased mRNA transcript levels in both tissues. In addition, assays related to *Aedes aegypti* showed that RNAi may be induced in insects through topical application [48]. In this study, dsRNA diluted in acetone caused *AaeIAP1* gene transcription that encodes an inhibitor protein of programmed cell death (apoptosis) in adult females, remained blocked. Thus, a significant increase in insect's mortality was observed.

Posteriorly, topical application of such molecules in borer moth larvae *Ostrinia furnacalis* showed similar effect. It was observed when RNAi inducer was introduced into larvae by direct spray of an aqueous solution containing double-stranded ribonucleotides, after which insects showed stunted growth as well as early death. Moreover, a significant reduction in egg hatchability compared to controls was observed. Besides, fluorescently labeled dsRNA molecules persisted in larval stages once they reached the intestine and hemocytes [49].

As mentioned above, artificial in vitro RNAi is expensive. Alternatively, a construction of a target gene-specific dsRNA vectors, its insertion into insect genomes and subsequent in vivo expression could be economically beneficial approach. Several recent investigations have allowed obtaining silencing vectors in bacteria host plants and plant viruses, which have been successfully implemented to study the expression of specific insect genes [50–53].

In addition, one way to generate genetically modified nematode-resistant plants is to produce copies (repeated and inverted) of target gene sequences in the plant tissue so that worms eating dsRNA-bearing plant material suffer from rapidly induced and triggered RNAi of important insect gene (s) under target. Although the results of RNAi potential to control insect pests as well as beneficial insects from parasites and diseases are encouraging, more research is necessary to understand the barriers and an efficient application. In the last several years, technical problems were uncovered, although a lot of concerns still remain. Future scientific efforts will help to solve current obstacles, which should allow this technology to be applied for integrated pest management (IPM) strategies as a novel way of action [54–57].

5. Gene silencing and viral immunity

Although there is little scientific background related to RNAi potential against various types of viruses capable of infecting animal cells (*e.g.,* dengue virus and *Drosophila*) [58–59], some studies suggest RNAi involvement in plant pathogenicity. Silencing viral suppressors affect the accumulation and function of siRNAs, including *trans*RNAi-mediated posttranscriptional gene silencing process that was recently discovered (tasiRNA; trans-acting siRNA). As a result, abnormal development of host organisms is often triggered [60–61]. As mentioned above, it can be considered that the effectiveness of RNAi technology was first demonstrated in 1998 [14]. In past decades, RNAi application was a successful tool for controlling various "difficult-to-eradicate" viral strains causing different pathologies in the wide range of economically important crops [62].

Plant gene silencing induced by viral agents (*i.e.,* VIGS; virus-induced gene silencing in plants) is one of the most common techniques that involves RNAi as immune mediator [63]. This technology allows implementing a system that releases dsRNA sequences in order to identify target viral genes, which generate multiple resistance mechanisms. In stable transgenic plants, this manipulation may require sequential processing or cross-linking among dsRNA sequences for considerable periods of time [64].

In addition, using RNAi has resulted in increasing immune resistance against viruses in different plant species, for example, (1) bean golden mosaic geminivirus (BGMV) [65], (2) rice dwarf virus (RDV) [66], (3) white leaf disease of rice (RHBV) [67], (4) rice tungro baciliform virus (RTBV) [68], (5) African cassava mosaic virus (ACMV) [69], (6) tobacco rattle virus (TRV) [70], and (7) citrus tristeza virus (CTV) [71], among others.

Functional approach of VIGS tool proves to be successful in characterizations of various physiological processes, including gene expression, development, reproduction, behavior, and immune system [43]. Presence of gene expression inhibitors in development of such diseases has to be consistent with the fact that inhibitors usually determine pathogenicity [72–73]. However, RNAi interaction in host metabolic pathways may not be the leading cause of infection symptoms because most of viral suppressors show no affection to plant metabolism [74].

http://dx.doi.org/10.5772/61689

In the conventional RNAi-mediated pathogenicity models, short ribonucleotide sequences are derived from infectious viruses, and host subviral RNA-induced gene silencing is carried out through random sequence complementarities. For example, transcribed gene expression related to self-complementary RNA hairpins (self-complementary hairpin RNA) encoding potato spindle tuber viroid sequences (PSTVd) is also capable of inducing viral symptoms in tomato (*Solanum lycopersicon*) [75]. Furthermore, RNAi-mediated pathogenicity models have shown that a darkening effect of tobacco plant, associated to the tobacco mosaic virus (TMV), is caused by a satellite RNA (*i.e.,* pathogenic RNA molecule). It is strongly inhibited by a silencing suppressor called P1/HC-Pro. Such wilt symptoms are due to a silencing effect on the chlorophyll biosynthetic-encoding *(*CHLI*)* gene [76–77].

RNAi-mediated gene silencing could be considered a general mechanism for pathogenicity of subviral RNA because such infective molecules may conduct gene silencing in various ways. siRNAs have high sequence identity degree with host´s promoter regions, and it may induce cytosine methylation by RNA-directed DNA methylation (RdDm), leading to transcriptional inactivation [78–82] as well as gene downregulation [83–87].

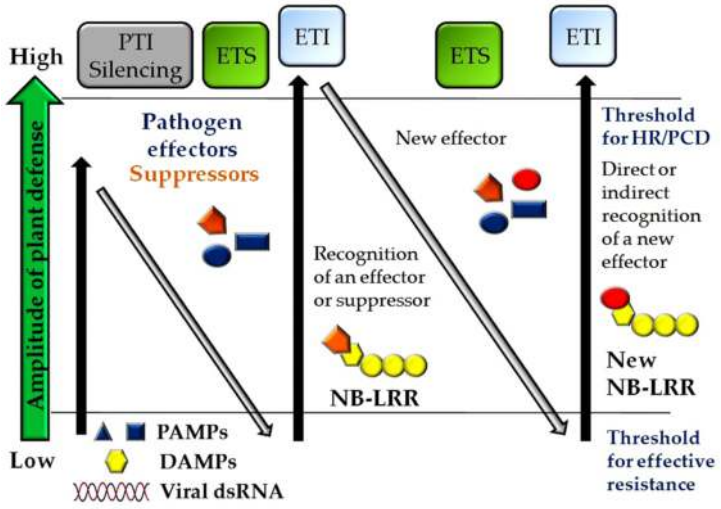

Figure 3. Zigzag model for evolution of innate immunity and silencing-based plant defense against viral and non-viral pathogens. Modified from Jones and Dangl, 2006 [88]. Susceptibility is proportional to PTI + silencing + ETS + ETI. Plants detect pathogen-associated molecular patterns (PAMPs) as well as host danger-associated molecular patterns (DAMPs) via pattern recognition receptors (PRRs) to induce pattern-triggered immunity (PTI). Plants additionally detect viral dsRNA to trigger RNA silencing. Effector-triggered susceptibility (ETS) results from the recognition of NB–LRR protein activating effector-triggered immunity (ETI; amplified version of PTI that passes a threshold for induction of hypersensitive response (HR) and programmed death cell (PDC). Pathogens that have lost the specifically recognized effector/suppressor are selected to help isolates to suppress ETI. NB–LRR plan alleles have evolved and selected to recognize newly acquired effectors resulting in ETI.

The zigzag model proposed by Jones and Dangl [88] shows the initial perception of pathogen-associated molecular patterns (PAMPs) as triggered immunity (TI)-based defense response

(*i.e.*, PAMP-TI) that regulates pathogenic growth and subsequent host infection. However, successful pathogens promote effector/virulence factors through PTI suppression. As a specific counteract action for pathogenic effectors, plants have evolved effector-triggered immunity (ETI), which is considered multiple rounds of effector-triggered susceptibility (ETS) followed by ETI.

On the basis of the above background, Zvereva and Pooggin [89] considered to extend this model to plant–virus interactions. On the other hand, because RNA silencing is an evolutionary conserved mechanism that defends organisms against transgenes and viruses, zigzag model may be related to specific *miR*-gene expression linked at the same time to plant innate immunity.

6. Human health approaches in gene silencing: biosafety and final considerations

The convention of biological diversity is intended to protect species from potential risks of genetic modified organisms (GMO), which are the result of applying modern biotechnological tools. On January 2000, Cartagena Protocol on Biosafety was signed by most of the developed countries. According to the Article 1 of this document, primary aim is to ensure a proper protection level in the field of safe transfer and handling of living modified organisms that may show adverse effects on conservation and sustainable use of biological diversity, considering also risks to human health, and specifically focusing on migration of species.

It is known that plant small RNAs help regulate several physiological processes such as growth and stress responses by attaching target mRNAs to modify their translation. Most people in the earth live on plant-based diets, and their food contains small RNAs from 19–24 nucleotides in size, among other bioactive molecules. Due to this fact, it is common that scientific community may ask the following: are plant small RNAs capable of regulating gene expression into the consumer´s genome? [90–91]. Before giving our opinion, some cases of small RNAs/miRNAs application for customized human gene therapy as well as RNAi relationship to food security and environmental biosafety will be discussed.

Over 800 human miRNAs have been discovered to date, and exploiting new platforms for controlling their expression are of urgent need. For example, nanotechnology and biomaterial synthesis have developed solid knowledge of sensing treatments using miRNAs against cancer. It is important to understand that human systemic administration using optimized delivery systems of interfering molecules is critical for proper functioning of *miRs*. Thereby, liposome-based nano-vehicles are capable of efficient transporting of miRNAs and antisense RNA helping to accumulate them easier in the liver, spleen, and kidney [92–94].

If plant-implemented glyco-engineering techniques based on RNAi silencing could reduce target glycosyltransferases transcripts, virus-like particles (VLPs) production in transgenic plants may be a reliable path to develop CHIKV (chikungunya) vaccines, for example [95]. Transgenic rice seeds as bioreactor for molecular pharming systems show great promise for

http://dx.doi.org/10.5772/61689

producing and processing recombinant proteins. Some of the advantages over conventional plant host or animal bioreactors are the following: (1) high capacity to obtain considerable expression levels, (2) production cost is lower than that of conventional fermentation, and (3) high capacity of seed reproduction [96–97].

About two years passed since it was demonstrated the ability of dietary miRNAs to regulate an animal gene in the liver [98]; however, while a few opinions suggested this was a possible way of cross-kingdom gene regulation, majority of data suggest gastrointestinal uptake of dietary plant miRNAs is not possible due to fast acid digestion [99]. On the other hand, measured tissue and blood dietary miRNA levels reported are so few that their dietary impact is insignificant.

Since plants can be modified by engineering RNAi pathways to alternatively generate small RNA molecules, RNAi could generate new crop lines for providing protection against pest insects (including nematodes), without cross-linking new protein varieties into food. Due to this fact, credible ecological risk assessments (ERAs) that are primordial tasks for stakeholders should be constructed. ERAs will allow the characterization of exposure pathways and potential hazards for RNAi crops (*e.g.*, off- and nontarget effects, genetic mutations, and polymorphism) [100]. Risks are also associated with genome direct changes in plants for human consumption, commonly related to newly expressed proteins that eventually show toxicity and allergenicity. However, when aversely a target gene decreased its expression, safety implications in particular cases such as when a silenced enzyme substrate accumulates to toxic levels may be observed [101]. Currently, optimal threshold doses for most food allergens remain unknown, thereby oral challenges test capable of evaluating the effects of RNAi consumption should be carried out in the future [102].

Another major concern about using RNAi-transformed plants for improving crops selection is the use of antibiotic resistance markers because antibiotic resistance genes could raise environmental risks as these genes may trigger horizontal transfer. In that sense, gene horizontal transfer will lead to generating antibiotic resistant microorganisms [103]. On the other hand, transgenic lines such as siRNA-mediated virus-resistant plants may provide a solution to reduce the indiscriminate use of toxic pesticides [97]. It is worth mentioning that during an international scientific workshop (June 2014) organized by the European Food Safety Authority (EFSA), some of the selected key outcomes suggested that bioinformatic analyses will play an imperative role in the identification of possible human and environmental risk assessments of RNAi-based plants [104].

According to Yang and colleagues [90], summary of evidence regarding dietary miRNAs uptake and functionality in mammalian consumers may be divided into two parts: (1) **evidences against:** inconsistent exogenous levels in serum typically low, various feeding studies failed to show absorption of dietary microRNAs, target suppression is shown only in the initial study, in silico analyses suggest that crossed contamination may be the main cause of plant microRNA reads in animal tissue; (2) **evidences for:** oral uptake of miRNAs is well characterized in nematodes and insects (indirect evidence), detection of RNAi from different kingdoms (including mammalian organisms), detection of *miR*-sequences in mice fed with

cabbages, microRNAs absorbed by humans and mice fed milk, tumor suppression observed when miRNAs were orally delivered into mice.

7. Conclusions

The general understanding about RNAi nature is an evolutionary conserved gene regulatory mechanism on superior organisms with several interspecific variations, which allows the survival of species through the reduction of the number of homologous RNA silencing proteins.

RNAi molecular bases that are implemented for fighting several diseases caused by biological agents or extreme abiotic conditions are vital for sustainable agriculture. It has been found that the existence of several virulence factors caused by phytopathogens related to blocking recognition patterns and signaling in immune responses. However, despite knowing the outcome of these physiological processes, it was not entirely clear which could be the molecular mechanisms that trigger such phenomena. Just a few years ago, the principal pathway was discovered and now we know that gene silencing is caused by RNAi, whereby it may regulate gene expression in eukaryote organisms.

It is true that plant metabolic pathways regulate their gene expression through a silencing phenomenon that emerges from siRNA, miRNA, and tasiRNA; however, all these interfering molecules share common elements in their biogenesis and structural characteristics, as well as in action mechanisms involved in common cellular components. Although miRNAs discovery has delved into the role that RNAi plays in plant gene regulation, more questions arise about its nature; for example, how exactly trans-acting elements repress gene expression and how RNA interference is completely involved in the model for evolution of innate immunity and silencing-based plant defense against viral and nonviral pathogens proposed by Jones and Dangl? [88]. Likewise, it would be highly interesting to understand why some similar nature microsequences block the expression of genes encoding auxin receptors while others interfere stress responses (*e.g.,* miR393, miR167, and miR160, respectively) [39].

Small RNAi-directed gene regulation mechanism was independently discovered in plants, fungi, worms, and mammalian cells, and scientific attention has been focused mainly on the regulation of development, biotic and abiotic stress responses, as well as genome stability through controlling plant gene expression. In addition, the siRNA-mediated RNA silencing also functions as a neutral antiviral defense mechanism.

Some authors consider the future possibility of having a better approach on the exact location of target genes from agricultural interest organisms (*i.e.,* crops and insects) by means of artificial microRNAs generation (amiRNA) [105]. Such projections could improve research in crop plants and metabolic engineering through developing better predictable and artificial manipulable microsequences. miRNAs are also being exploited recently as new platforms for developing solid knowledge in different science fields such as medicine, nanotechnology, and integrated pest management. Thus, synthesis of RNAi in plant-based biofactories could be effective in several disciplines involved in forthcoming experiments.

http://dx.doi.org/10.5772/61689

Recent advances have shown the potential of RNAi for its future role in transgenic plants against pest insects in the environment [100]. Perhaps the most relevant application will be in modifying crop–pest interactions so that transgenic lines are capable of producing secondary metabolites against nematodes and some other pathogens. In fact, some researchers have proposed to extend this approach for controlling mammalian diseases.

The recent discovery of some of the most important RNAi molecular mechanisms is useful to discuss future applications in agricultural biotechnology, and attending the resulting food security concerns emerged from the *in situ* application of such tool must be imperative. As a result of a couple of studies on human effects of the consumption of plant foods with high levels of interfering microsequences, considerable uncertainties become noticeable, for example, the effect of these microarrays on the metabolism of those who directly consume engineered plant foods [90].

So far, limited reports related to food security as well as environmental risks involving RNAi are available, since RNAi biotechnological approaches are very difficult to scrutinize and, consequently, proofs of concept are difficult to obtain. In the future, potential and limitations of engineered plants, including alternative strategies for generating low allergic supplies like low weight proteins, should be studied by using bioinformatic tools followed by the respective studies (*i.e.*, physiological characterization of transgenic plants, toxicity and allergenicity of expressed proteins, as well as metabolites production and nutritional characteristics) [102].

Author details

Jorge Ricaño-Rodríguez[1*], Jacel Adame-García[2], Silvia Portilla-Vázquez[3], José M. Ramos-Prado[1] and Enrique Hipólito-Romero[1]

*Address all correspondence to: jorgericano@gmail.com

1 Center for Eco-Literacy and Dialogue of Knowledge. University of Veracruz. Campus USBI. Col Emiliano Zapata. Xalapa, Veracruz, México

2 Cell Biology Laboratory. Technological Institute of Úrsulo Galván. Úrsulo Galván, Veracruz, México

3 Food Research and Development Unit (UNIDA). Laboratory of Genetics. Technological Institute of Veracruz, Veracruz, México

References

[1] Napoli C, Lemieux C, Jorgensen R. Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible cosuppression of homologous genes in trans. Plant Cell. 1990;2:279-289. DOI: 10.1105/tpc.2.4.279

[2] van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR. Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell. 1990;2:291-299. DOI: 10.1105/tpc.2.4.291

[3] Lindbo JA, Dougherty WG. Pathogen derived resistance to a potyvirus: Immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence. Molecular Plant-Microbe Interactions. 1992;2:144-153. DOI: 10.1094/MPMI-5-144

[4] Lindbo JA, Dougherty WG. Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. Virology. 1992;189:725-733. DOI: 10.1016/0042-6822(92)90595-G

[5] Lindbo JA, Siolva-Rosales L, Proebsting WM, Dougherty WG. Induction of a highly specific antiviral state in transgenic plants: Implications for regulation of gene expression and virus resistance. Plant Cell. 1993;5:1749-1759. DOI: 10.1105/tpc.5.12.1749

[6] Prins M, Laimer M, Noris E, Schubert J, Wassenegger M, Tepfer M. Strategies for antiviral resistance in transgenic plants. Molecular Plant Pathology. 2008;9:73-83. DOI: 10.1111/j.1364-3703.2007.00447.x

[7] Peragine A, Yoshikawa M, Wu G, Albrecht HL, Poethig RS. SGS3 and SGS2/ SDE1/ RDR6 are required for juvenile development and the production of trans-acting siRNAs in *Arabidopsis*. Genes & Development. 2004;18:2368-2379. DOI: 10.1101/gad.1231804

[8] Ding SW. RNA-based antiviral immunity. Nature Reviews Immunology. 2010;10:632-644. DOI: 10.1038/nri2824

[9] Tarver JE, Donoghue PCJ, Peterson KJ. Do miRNAs have a deep evolutionary history? Bioessays. 2012;34:857-866. DOI: 10.1002/bies.201200055

[10] Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. Nature. 2004;431:343-349. DOI: 10.1038/nature02873

[11] Jorgensen RA, Cluster PD, English J, Que Q, Napoli CA. Chalcone synthase cosuppression phenotypes in petunia flowers: Comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. Plant Molecular Biology. 1996;31:957-973. DOI: 10.1007/BF00040715

[12] Hunter GP. Gene silencing: Shrinking the black box of RNAi. Current Biology. 2000;10:R137-R149. DOI: 10.1016/S0960-9822(00)00325-0

[13] Duan CG, Wang CH, Guo HS. Application of RNA silencing to plant disease resistance. Silence. 2012;3:5. DOI: 10.1186/1758-907X-3-5

[14] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 1998;391:806-811. DOI: 10.1038/35888

[15] Timmons L, Fire A. Specific interference by ingested dsRNA. Nature. 1998;395:854. DOI: 10.1038/27579

[16] Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, Fire A, Mello CC. The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. Cell. 1999;99:123-132. DOI: 10.1016/S0092-8674(00)81644-X

[17] Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell. 2004;116:281-297. DOI: 10.1016/S0092-8674(04)00045-5

[18] Shabalina SA, Koonin EV. Origins and evolution of eukaryotic RNA interference. Trends in Ecology and Evolution. 2008;23:578-587. DOI: 10.1016/j.tree.2008.06.005

[19] Saini HK, Griffiths-Jones S, Enright AJ. Genomic analysis of human microRNA transcripts. Proceedings of the National Academy of Sciences of the United States of America. 2007;104:19-24. DOI: 10.1073/pnas.0703890104

[20] Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001;409:363-366. DOI: 10.1038/35053110

[21] Cenik ES, Fukunaga R, Lu G, Dutcher R, Wang Y. Phosphate and R2D2 restrict the substrate specificity of Dicer-2, an ATP-driven ribonuclease. Molecular Cell. 2011;42:172-184. DOI: 10.1016/j.molcel.2011.03.002

[22] Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, Carrington JC. P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. Developmental Cell. 2003;4:205-217. DOI: 10.1016/S1534-5807(03)00025-X

[23] Doyle M, Jaskiewicz L, Filipowicz W. Dicer proteins and their role in gene silencing pathways. In: Guo F, Tamanoi F, editors. The Enzymes: Eukaryotic RNAses and their partners in RNA degradation and biogenesis.1st ed. Oxford: Academic Press; 2012. p. 1-28. DOI: 10.1016/B798-0-012-404741-9.00001-5

[24] Park JE, Heo I, Tian Y, Simanshu DK, Chang H, Jee D, Patel DJ, Kim VN. Dicer recognizes the 5′ end of RNA for efficient and accurate processing. Nature. 2011;475:201-205. DOI: 10.1038/nature10198

[25] Wilson RC, Doudna JA. Molecular Mechanism of RNA Interference. Annual Review of Biophysics. 2013;42:217-239. DOI: 10.1146/annurev-biophys-083012-130404

[26] Tolia NH, Joshua-Tor L. Slicer and the argonautes. Nature Chemical Biology. 2007;3:36-46. DOI: 10.1038/nchembio848

[27] Nicholson AW. Function, mechanism and regulation of bacterial ribonucleases. FEMS Microbiology Reviews. 1999;23:371-390. DOI: 10.1111/j.1574-6976.1999.tb00405.x

[28] Voinnet O. Origin, biogenesis, and activity of plant microRNAs. Cell. 2009;136:669-687. DOI: 10.1016/j.cell.2004.06.017

[29] Meng Y, Shao C, Wang H, Chen M. The regulatory activities of plant microRNAs: A more dynamic perspective. Plant Physiology. 2011;157:1583-1595. DOI: 10.1016/j.cell.2004.06.017

[30] Perkel JM. Visiting noncodarnia. Biotechniques. 2013;6:301-304.DOI: 10.1016/j.cell.2004.06.017

[31] Pattanayak D, Solanke AU, Kumar PA. Plant RNA interference pathways: Diversity in function similarity in action. Plant Molecular Biology Reporter. 2013;31:493-506. DOI: 10.1016/j.sbi.2006.12.002

[32] MacRae IJ, Doudna JA. Ribonuclease revisited: Structural insights into ribonuclease III family enzymes. Current Opinion in Structural Biology. 2007;17:138-45. DOI: 10.1016/j.sbi.2006.12.002

[33] Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W. Single processing center models for human DICER and Bacterial RNase III. Cell. 2004;118:57-68.DOI: 10.1016/j.cell.2004.06.017

[34] Staiger D, Korneli C, Lummer M, Navarro L. Emerging role for RNA-based regulation in plant immunity. New Phytologists. 2013;197:394-404. DOI: 10.1111/nph.12022

[35] Streitner C, Simpson CG, Shaw P, Danisman S, Brown JWS, Staiger D. Small changes in ambient temperature affect alternative splicing in *Arabidopsis thaliana*. Plant Signaling and Behavior. 2013;8:e24638.1-e24638.7. DOI: 10.4161/psb.24638

[36] Sunkar R, Li YF, Jagadeeswaran G. Functions of microRNAs in plant stress responses. Trends Plant Science. 2012;17:196-203. DOI: 10.1016/j.tplants.2012.01.010

[37] Jones-Rhoades MW, Bartel DP, Bartel B. MicroRNAs and their regulatory roles in plants. Annual Review of Plant Biology. 2006;57:19-53. DOI: 10.1146/annurev.arplant.57.032905.105218

[38] Zhang X, Zhao H, Gao S, Wang WC, Katiyar-Agarwal S, Huang HD, Raikhel N, Jin H. *Arabidopsis* Argonaute 2 regulates innate immunity via miRNA393- mediated silencing of a Golgi localized SNARE gene, MEMB12. Molecular Cell. 2011;42:356-366. DOI: 10.1016/j.molcel.2011.04.010

[39] Sunkar R, Zhu JK. Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. Plant Cell. 2004;16:2001- 2019. DOI: 10.1105/tpc.104.022830

[40] Kruszka K, Pieczynski M, Windels D, Bielewicz D, Jarmolowski A, Szweykowska-Kulinska Z, Vazquez F. Role of microRNAs and other sRNAs of plants in their

changing environments. Journal of Plant Physiology. 2012;169:1664-1672.DOI: 10.1016/j.jplph.2012.03.009

[41] Wang L, Zheng J, Luo Y, Xu T, Zhang Q, Zhang L, Xu M, Wan J, Wang MB, Zhang C, Fan F. Construction of a genome wide RNAi mutant library in rice. Plant Biotechnology Journal. 2013;11:997-1005. DOI: 10.1111/pbi.12093

[42] Burand JP, Hunter WB. RNAi: Future in insect management. Journal of Invertebrate Pathology. 2013;112:568-574. DOI: 10.1016/j.jip.2012.07.012

[43] Belles X. Beyond Drosophila: RNAi *in vivo* and functional genomics in insects. Annual Review of Entomology. 2010;55:111-128. DOI: 10.1146/annurev-ento-112408-085301

[44] Roether S, Meister G. Small RNAs derived from longer non-coding RNAs. Biochimie. 2011;93:1905-1915. DOI: 10.1016/j.biochi.2011.07.032

[45] Gu L, Knipple DC. Recent advances in RNA interference research in insects: Implications for future insect pest management strategies. Crop Protection. 2013;45:36-40. DOI: 10.1016/j.cropro.2012.10.004

[46] Araujo RN, Santos A, Pinto FS, Gontijo NF, Lehane MJ, Pereira MH. RNA interference of the salivary gland nitrophorin 2 in the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) by dsRNA ingestion or injection. Insect Biochemistry and Molecular Biology. 2006;36:683-693. DOI: 10.1016/j.ibmb.2006.05.012

[47] Turner CT, Davy MW, MacDiarmid RM, Plummer KM, Birch NP. RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. Insect Molecular Biology. 2006;15:383-391. DOI: 10.1111/j.1365-2583.2006.00656.x

[48] Pridgeon JW, Zhao L, Becnel JJ, Strickman DA, Clark GG, Linthicum KJ. Topically applied AaeIAP1 double-stranded RNA kills female adults of *Aedes aegypti*. Journal of Medical Entomology. 2008;45:414-420. DOI: 10.1603/0022-2585(2008)45[414:TAADRK]2.0.CO;2

[49] Wang Y, Zhang H, Li H, Miao X. Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. PLoSOne. 2011;6:e18644. DOI: 10.1371/journal.pone.0018644

[50] Li X, Zhuo K, Luo M, Sun L, Liao J. Molecular cloning and characterization of a calreticulin cDNA from the pinewood nematode *Bursaphelenchusxylophilus*. Experimental Parasitology. 2011;128:121-126. DOI: 10.1016/j.exppara.2011.02.017

[51] Zhu F, Xu J, Palli R, Ferguson J, Palli SR. Ingested RNA interference for managing the populations of the Colorado potato beetle, *Leptinotarsa decemlineata*. Pest Management Science. 2011;67:175-182. DOI: 10.1002/ps.2048

[52] Zha W, Peng X, Chen R, Du B, Zhu L, He G. Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the Hemipteran insect *Nilaparvatalugens*. PLoSOne. 2011;6:e20504.DOI: 10.1371/journal.pone.0020504

[53] Kumar P, Pandit SS. Baldwin IT. Tobacco rattle virus vector: A rapid and transient means of silencing *Manduca sexta* genes by plant mediated RNA interference. PLoSONE. 2012;7:e31347.DOI: 10.1371/journal.pone.0031347

[54] Fairbairn DJ, Cavallaro AS, Bernard M, Mahalinga-Iyer J, Graham MW, Botella JR. Host-delivered RNAi: An effective strategy to silence genes in plant parasitic nematodes. Planta. 2007;226:1525-1533.DOI: 10.1007/s00425-007-0588-x

[55] Yadav BC, Veluthami K, Subramaniam K. Host-generated double-stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. Molecular and Biochemical Parasitology. 2006;148:219-222. DOI: 10.1016/j.molbiopara.2006.03.013

[56] Huang G, Allen R, Davis EL, Baum TJ, Hussay RS. Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. Proceedings of the National Academy of Sciences of the United States of America. 2006;103:14302-14306. DOI: 10.1073/pnas.0604698103

[57] Steeves RM, de Bruin R, Kenter S, van der Hoorn R, van Blokland R. Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress *Heterodera glycines* reproduction. Functional Plant Biology. 2006;33:991-999.DOI: 10.1071/FP06130

[58] Kakumani PK, Ponia SS, Rajgokul KS, Chinnappan M, Banerjea AC, Medigeshi GR, Malhotra P, Mukherjee SK, Bhatnagar RK. Role of RNAi in dengue viral replication and identification of NS4B as a RNAi suppressor. Journal of Virology. 2013;87:8870-8883. DOI: 10.1128/JVI.02774-12.

[59] Karlikow M, Goic B, Saleh MC. RNAi and antiviral defense in *Drosophila*: Setting up a systemic immune response. Developmental and Comparative Immunology. 2014;42:85-92. DOI: 10.1016/j.dci.2013.05.004

[60] Shivaprasad PV, Rajeswaran R, Blevins T, Schoelz J, Meins F, Hohn T, Pooggin MM. The CaMV transactivator/viroplasmin interferes with RDR6-dependent *trans*-acting and secondary siRNA pathways in *Arabidopsis.* Nucleic Acids Research. 2008;36:5896-5909. DOI: 10.1093/nar/gkn590

[61] Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, Carrington JC. P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. Developmental Cell. 2003;4:205-217. DOI: 10.1016/S1534-5807(03)00025-X

[62] Cakir C, Tor M. Factors influencing barley stripe mosaic virus-mediated gene silencing in wheat. Physiological and Molecular Plant Pathology. 2010;74:246-253. DOI: 10.1016/j.pmpp.2010.04.001

[63] Senthil-Kumar M, Mysore KS. Virus-induced gene silencing can persist for more than two years and also be transmitted to progeny seedlings in *Nicotiana benthamiana* and tomato. Plant Biotechnology Journal. 2011;9:797-806. DOI: 10.1111/j.1467-7652.2011.00589.x.

[64] Fernández LC, Donaire L, Livia D, Cisar L. RNA silencing and the interplay between plants and viruses. Recent Advances in Plant Virology. 2011;6:121-136. ISBN: 978-1-904455-75-2

[65] Bernstein E, Caudy AA. Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001;409: 363-366. DOI: 10.1038/35053110;

[66] Takumi S, Motoyasu Y, Taiyun W, Hirohiko H, Toshihiro O. Silencing by RNAi of the gene for Pns12, a viroplasm matrix protein of Rice dwarf virus, results in strong resistance of transgenic rice plants to the virus. Plant Biotechnology Journal. 2008;7:24-32. DOI: 10.1111/j.1467-7652.2008.00366.x

[67] Hemmes H,Kaaij LD, Prins M, Goldbach R, Schnettler E. Binding of small interfering RNA molecules is crucial for RNA interference suppressor activity of rice hoja blanca virus NS3 in plants. Journal of General Virology. 2009;90:1762-1766. DOI: 10.1099/vir.0.010488-0

[68] Tyagi H, Rajasubramaniam S, Rajam MV, Dasgupta I. RNA-interference in rice against Rice tungro bacilliform virus results in its decreased accumulation in inoculated rice plants. Transgenic Research. 2008;17:897-904. DOI:10.1007/s11248-008-9174-7

[69] Vanderschuren H, Alder A, Zhang P, Gruissem W. Dose-dependent RNAi-mediated geminivirus resistance in the tropical root crop cassava. Plant Molecular Biology. 2009;70:265-272. DOI: 10.1007/s11103-009-9472-3

[70] Velásquez AC, Chakravarthy S, Martin GB. Virus-induced gene silencing (VIGS) in *Nicotiana benthamiana* and tomato. Journal of Visualized Experiments. 2009;28:1292-1301. DOI: 10.3791/1292

[71] Soler S, Fagoaga C, Chiibi S, López C, Moreno P, Navarro L, Flores R, Peña L. RNAi mediated protection against citrus tristeza virus in transgenic citrus plants. In: Erdmann VA, Barciszewski J, editors. Non coding RNAs in plants. 1st ed. Berlin: Heidelberg; 2011. p. 447-460. DOI: 10.1007/978-3-642-19454-2_27

[72] Li HW, Lucy AP, Guo HS, Li WX, Ji LH, Wong SM, Ding SW. Strong host resistance targeted against a viral suppressor of the plant gene silencing defense mechanism.

The European Molecular Biology Organization Journal. 1999;18:2683-2691. DOI: 10.1093/emboj/18.10.2683

[73] Qiu W, Park JW, Scholthof HB. Tombusvirus P19-mediated suppression of virus-induced gene silencing is controlled by genetic and dosage features that influence pathogenicity. Molecular Plant-Microbe Interactions. 2002;15:269-280. DOI: 10.1094/MPMI.2002.15.3.269

[74] Chen J, Li WX, Xie D, Peng JR, Ding SW. Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of microRNA in host gene expression. Plant Cell. 2004;16:1302-1313. DOI: 10.1105/tpc.018986

[75] Wang MB, Bian XY, Wu LM, Liu LX, Smith NA, Isenegger D. On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. Proceedings of the National Academy of Sciences of the United States of America. 2004;101:3275-3280. DOI: 10.1073/pnas.0400104101

[76] Smith NA, Eamens AL, Wang MB. Viral small interfering RNAs target host genes to mediate disease symptoms in plants. PloS Pathogens. 2011;7:e1002022. DOI: 10.1371/journal.ppat.1002022

[77] Shimura H, Pantaleo V, Ishihara T, Myojo N, Inaba J, Sueda K, Burgyán J, Masuta C. A viral satellite RNA induces yellow symptoms on tobacco by targeting a gene involved in chlorophyll biosynthesis using the RNA silencing machinery. PLoS Pathogens. 2011;7:1002021. DOI: 10.1371/journal.ppat.1002021

[78] Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. Prediction of plant microRNA targets. Cell. 2002;110:513-520. DOI: 10.1016/S0092-8674(02)00863-2

[79] Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP. MicroRNAs in plants. Genes & Development. 2002;16:1616-1626. DOI:10.1101/gad.1004402

[80] Xie Z, Allen E, Fahlgren N, Calamar A, Givan SA, Carrington JC. Expression of *Arabidopsis* miRNA genes. Plant Physiology. 2005;138:2145-2154. DOI: 10.1104

[81] Rajagopalan R, Vaucheret H, Trejo J, Bartel DP. A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. Genes Development. 2006;20:3407-3425. DOI: 10.1101/gad.1476406

[82] Li F, Pignatta D, Bendix C, Brunkard JO, Cohn MM, Tung J, Sun H, Kumar P, Baker B. MicroRNA regulation of plant innate immune receptors. Proceedings of the National Academy of Sciences of the United States of America. 2012;109:1790-1795. DOI: 10.1073/pnas.1118282109

[83] Axtell MJ, Snyder JA, Bartel DP. Common functions for diverse small RNAs of land plants. Plant Cell. 2007;19:1750-1769. DOI: 10.1105/tpc.107.051706

[84] Lu S, Sun YH, Shi R, Clark C, Li L, Chiang VL. Novel and Mechanical Stress-responsive MicroRNAs in *Populus trichocarpa* that are absent from *Arabidopsis*. Plant Cell. 2005;17:2186:2203. DOI: 10.1105/tpc.105.033456

[85] Zeng C, Wang W, Zheng Y, Chen X, Bo W, Song S, Zhang W, Peng M. Conservation and divergence of microRNAs and their functions in Euphorbiaceous plants. Nucleic Acids Research. 2010;38:981-995. DOI: 10.1093/nar/gkp1035

[86] Dezulian T, Palatnik JF, Huson DH, Weigel D. Conservation and divergence of microRNA families in plants. Genome Biology. 2005;6:13-18.DOI: 10.1186/gb-2005-6-11-p13

[87] Zhang L, Chia JM, Kumari S, Stein JC, Liu Z, Narechania A, Maher CA, Guill K, McMullen MD, Ware D. A Genome-wide characterization of microRNA genes in maize. PLoS Genetics. 2009;5:e1000716. DOI: 10.1371/journal.pgen.1000716

[88] Jones JD, Dangl JL. The plant immune system. Nature. 2006;444:323–329. DOI: 10.1038/nature05286

[89] Zvereva AS, Pooggin MM. Silencing and innate Immunity in plant defense against viral and non-viral pathogens. Viruses. 2012;4:2578-2597. DOI: 10.3390/v4112578

[90] Yang J, Hirschi KD, Farmer LM. Dietary RNAs: New Stories Regarding Oral Delivery. Nutrients. 2015;7:3184-3199. DOI: 10.3390/nu7053184

[91] Bartel DP. MicroRNAs: Target Recognition and Regulatory Functions.Cell.2009;136: 215-233. DOI: 10.1016/j.cell.2009.01.002

[92] Conde J, Edelman ER, Artzi N. Target-responsive DNA/RNA nanomaterials for microRNA sensing and inhibition: The jack-of-all-trades in cancer nanotheranostics? Advanced Drug Delivery Reviews. 2015;81:169-183. DOI: 10.1016/j.addr.2014.09.003

[93] Strasser R, Stadlmann J, Svoboda B, Altmann F, Glossl J, Mach L. Molecular basis of N-acetylglucosaminyltransferase I deficiency in Arabidopsis thaliana plants lacking complex N-glycans. Biochemistry Journal. 2005;387:385-91. DOI: 10.1042/BJ20041686

[94] Schähs M, Strasser R, Stadlmann J, Kunert R, Rademacher T, Steinkellner H. Production of a monoclonal antibody in plants with a humanized N-glycosylation pattern. Plant Biotechnology Journal. 2007;5:657-63. DOI: 10.1111/j.1467-7652.2007.00273.x

[95] Salazar-Gonzalez JA, Angulo C, Rosales-Mendoza S. Chikungunya virus vaccines: Current strategies and prospects for developing plant-made vaccines. Vaccine. 2015;33: 3650-3658. DOI: 10.1016/j.vaccine.2015.05.104

[96] Ou J, GuoZ, Shi J, Wang X, Liu J, Shi B, Guo F, Zhang C, Yang D. Transgenic rice endosperm as a bioreactor for molecular pharming. Plant Cell Reports. 2014;33:585-594. DOI: 10.1007/s00299-013-1559-2

[97] Lemgo GN, Sabbadini S, Pandolfini T, Mezzetti B. Biosafety considerations of RNAi-mediated virus resistance in fruit-tree cultivars and in rootstock. Transgenic Research. 2013;22:1073-1088. DOI: 10.1007/s11248-013-9728-1

[98] Zhang L, Hou D, Chen X, Li D, Zhu L, Zhang Y, Li J, Bian Z, Liang X, Cai X. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: Evidence of cross-kingdom regulation by microRNA. Cell Research.2012;22:107-126. DOI: 10.1038/cr.2011.158

[99] Ricaño-Rodríguez J, Zavala-González E, Ramírez-Lepe M. Silenciamiento génico en plantas: mecanismos moleculares del ARN de interferencia y aplicaciones biotecnológicas. Revista Fitotecnia Mexicana. 2014;37:339-350.

[100] Auer C, Frederick R. Crop improvement using small RNAs: applications and predictive ecological risk assessments. Trends in Biotechnology. 2009;27:64-651. DOI: 10.1016/j.tibtech.2009.08.005

[101] Casacuberta JM, Devos Y, Jardin Pd, Ramon M, Vaucheret H, Nogué F. Biotechnological uses of RNAi in plants: risk assessment considerations. Trends in Biotechnology. 2015;33:145-147. DOI: 10.1016/j.tibtech.2014.12.003

[102] Scheurer S, Sonnewald S. Genetic engineering of plant food with reduced allergenicity. Frontiers in Bioscience. 2009;1:59-71.DOI: 10.2741/3231.

[103] Saurabh S, Vidyarthi AS, Prasad D. RNA interference: Concept to reality in crop improvement. Planta. 2014;239:543-564. DOI: 10.1007/s00425-013-2019-5

[104] Ramon M, Devos Y, Lanzoni A, Liu Y, Gomes A, Gennaro A, Waigmann E. RNAi-based GM plants: Food for thought for risk assessors. Plant Biotechnology Journal. 2014;12:1271-1273. DOI: 10.1111/pbi.12305

[105] Li JF, Chung HS, Niu Y, Bush J, McCormack M, Sheen J. Comprehensive protein-based artificial microRNA screens for effective gene silencing in plants. Plant Cell. 2013;25: 1507-1522. DOI: 10.1105/tcp.113.112235