International Research Journal of Biotechnology Vol. 14(2) pp. 1-11, April, 2023
Available online http://www.interesjournals.org/IRJOB

Research Article

A Research on RNAi Technology and its Application in Crop Improvement

Derejaw Tesfa* and Tileye Feyissa

Ethiopian Institution of Agricultural Research (EIAR), Debereziet Agricultural Research Center (DZARC), Ethiopia and Institute of Biotechnology in Addis Ababa University (AAU), Ethiopia

*Corresponding Author's E-mail: Derejawt07@gmail.com

Received: 23-Mar-2022, Manuscript No. irjob-23-92552; **Editor assigned:** 25-Mar-2022, PreQC No. irjob-23-92552(PQ); **Reviewed:** 08-Apr-2022, QC No. irjob-23-92552; **Revised:** 13-Apr-2022, Manuscript No. irjob-23-92552(R); **Published:** 20-Apr-2023, DOI: 10.14303/2141-5153.2023.39

Abstract

RNAs together are now found to be a major player in gene expression in all prokaryotic and eukaryotic organisms. Small RNAs regulate genes by targeting transcripts in the cytoplasm and repressing their translation in all organisms. After discovery of RNAi technology in 1990, modifying the genes by the interference of small RNAs is one of the ways through which plants react to the environmental stresses. Endogenously generated and exogenously supplied dsRNAs activate RNAi in a sequence-specific manner. This strategy is a promising path for providing low risk and environmentally safe plant protection. Hence, investigating the role of small RNAs in regulating gene expression assists the researchers to explore the potentiality of small RNAs in abiotic and biotic stress management. Altogether, the history, conserved enzymes like Dicer, RISC (RNA-induced Silencing Complex) and its synthesis, delivery, gene knockdown mechanism, its assay after delivery and ending application of RNAi are reviewed.

INTRODUCTION

RNAs are now found to be a major player in gene expression. In all prokaryotic and eukaryotic organisms, RNAs may be under classes of molecules coding (mRNA) and non-coding (tRNA, rRNA, small RNAs). These all have their own function in organisms. mRNA has polypeptide synthesis function, tRNA decodes the codons of the mRNA and transfers amino acids for polypeptide synthesis and rRNA binds to mRNA region and helps to structurally support and even use to catalase to form the assemblage of amino acids and to form peptide bonds **(Clancy S 2008).**

Small RNAs are several classes of small non-coding RNAs that can regulate gene by targeting transcripts in the cytoplasm and repressing their translation. Some of them are: miRNAs, siRNAs, tasiRNAs, rasiRNAs, vsiRNAs and piRNAs. Most of these molecules form complexes with proteins to form ribonucleoproteins/RNP/ and distributed in the nucleus, in the cytoplasm, or in both. They are synthesized by RNA polymerase II and III and composed of 20 to 300 nucleotide length, and 100,000 to 1,000,000 copies per cell **(Clancy S 2008).**

The gene regulatory ones are a group of 21–25 nucleotides long RNA molecules referred to as "Small RNAs" These small RNAs leads RNA silencing pathway. RNA interference has become a new insight for understanding gene regulation. RNAi is an evolutionarily conserved **(Wilson RC et al., 2013),** rely on distinct populations of sRNAs to regulate mRNA translation/degradation or heterochromatin formation in a sequence-specific manner **(Carthew RW et al., 2009) (Borges F et al., 2015).**

RNA silencing in gene regulation limits the expression of target genes by suppressing transcription (Transcriptional gene silencing [TGS]), inducing sequence specific RNA degradation process (post-transcriptional gene silencing [PTGS]/ RNA interference) or inhibiting the translation of their mRNA into protein **(Sinha SK 2010)** and its process has an important role in defiance against invasive nucleic acids, such as transposable elements and viruses. It has also an important mechanism for regulating endogenous gene expression **(Obbard DJ et al., 2009) (Li C et al., 2019).** This review focuses on the technique and applications of RNAi for crop improvement.

The history of RNAi technology

Before the discovery of RNAi, scientists applied various methods such as insertion of T-DNA elements, and transposons, treatment with mutagens or irradiation and antisense RNA suppression to generate loss-of-function mutations. These approaches allowed scientists to study the functions of a gene or gene family of interest in an organism. Apart from being time-consuming, the above methods did not always work satisfactorily **(Williams M et al., 2004).** For instance, transposons and T-DNA elements were found to occasionally insert randomly in the genome resulting in highly variable gene expression. Furthermore, in many instances the particular phenotype or a trait could not be correlated with the function of a gene of interest. Hence scientists search other technologies and then discovered RNAi technology **(Williams M et al., 2004).**

PTGS/RNAi was first observed in plants but later were observed in almost all eukaryotic organisms including protozoa, invertebrates, vertebrates, fungi and algae **(Sinha SK 2010).** In plants, RNAi was first discovered in 1990 when Jorgensen's laboratory tried to get petunia flowers with darker purple by introducing numerous copies of a gene that code for deep purple flower i.e. Chalcone Synthase (Chs A), but amazingly some of the final plants yielded white or patchy flowers. Somehow the transgene silenced the expression of both homologous endogenous and introduced loci and the phenomenon was then termed as co-suppression. Eventually this finding led to the discovery of RNAi.

Antisense RNA suppression was an early form of RNA silencing employed mainly by plant scientists. This process involved the introduction of the antisense strand of RNA into the cell that corresponded to the target mRNA, the transcript intended to silence **(Brantl S 2002).** After entry into the cell, the introduced antisense RNA and the native target mRNA would bind via complementary base pairing preventing the translation of mRNA. This is due to the inability of ribosomes to bind to dsRNA **(Brantl S 2002) (Agrawal N et al., 2003).**

This process, however, did not always result in a loss of function of a targeted gene. Because of endogenously generated and exogenously supplied dsRNAs, RNAi is activated in a sequence-specific manner. This strategy is a promising technology for providing low risk and environmentally safe plant protection **(Liu S et al., 2020).** dsRNA has complementary sequence that integrates against endogenous mRNA in vivo, and possible catalytic or amplification components play a highlighting role during the interference process **(Chen X et al., 2019).**

This initiated many more scientists have been continued to do using exogenous dsDNA, and they have been looking at the complex process of RNAi in more detail. They studied several forms of RNA as well as two highly conserved enzymes. Because RNAi or dsRNA alone does not degrade the mRNA. It requires the assistance of the conserved enzymes (dicer and RNA-induced Silencing Complex RISC).

Conserved enzymes

Scientists studied two major conserved enzymes which use for RNAi technology. These enzymes are:

- Dicer in animals and Dicer-like elements in plants;
- RISC (RNA-induced Silencing Complex)

Dicer in animals and dicer-like elements in plants

The discovery of siRNA in directing RNAi intensified the search for factors that generate these small RNA species (Figure 1). Based on the physical properties of the siRNA duplex, RNase III family were likely candidates responsible for generating siRNAs.

Typically Dicer has the characteristics of an N-terminal (Piwi-Argonaute-Zwille (PAZ) which is responsible for small RNA binding ,C-terminal PIWI (RNase III motif)which deliberates catalytic activities of double stranded RNA binding domain and also have other supplementary of Dicer (S1 and S2) **(Zhang R et al., 2018).** In contrast to animals, higher plants generate array of small RNAs (sRNA) with specialized functions.

In plant cellular system, dsRNAs are mainly processed into three categories: short interfering RNAs (siRNAs), micro RNAs (miRNAs), and piwi interacting RNAs (piRNAs). These are generated with help of Dicers. For example, the model plant Arabidopsis (Arabidopsis thaliana) consists four Dicer like (DCL) paralogs. DCL2, DCL3 and DCL4 that generate the 22-, 24- and 21-nt siRNAs, respectively after transfer or

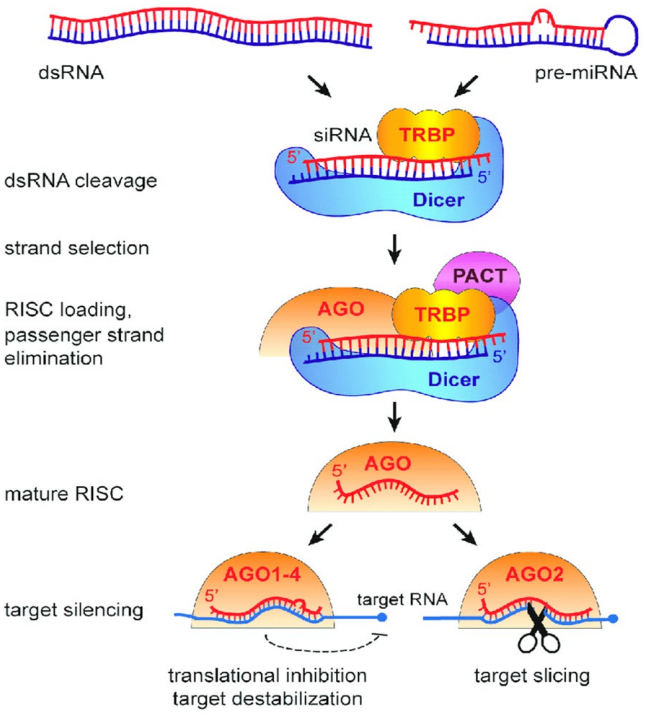

Figure 1. Mechanism of how Dicer cuts the pre mRNA and assembling of RISC.

transcription in the plant cell. These occurs when dsRNAs are processed by DICER-LIKE (DCL) endonucleases into 21-24-nt short interfering RNAs (siRNAs) **(Dalakouras A et al., 2018),** whereas DCL1 recognizes genome-encoded imperfect hairpin RNAs resulting in the biogenesis of 21/22-nt miRNAs **(Borges F et al., 2015).**

These DCL proteins also have some redundant function in A. thaliana. For instance, DCL1 can produce 21-nt tasiRNA in the dcl2, dcl3, dcl4 triple mutant **(Bouche N et al. 2006)** and DCL2 can produce 22-nt tasiRNA when the DCL4 activity is compromised. SiRNA thus produced by Dicer activity is then combined into a various multicomponent ribonuclease called RNA Induced Silencing Complex (RISC) **(Sinha SK 2010).**

RISC (RNA-induced silencing complex)

It is common name for a family of varied molecular complexes (multiprotein). This is specifically a ribonucleoprotein, which is complementary with one strand of a single-stranded RNA (ssRNA) fragment, such as microRNA (miRNA), or double-stranded small interfering RNA (siRNA). The single strand acts as a template for RISC to recognize complementary messenger RNA (mRNA) transcript (Figure 2).

RISC was discovered in 1998 by Andrew Fire, Craig Mello Hannon and their colleagues and biochemical identification of RISC also by Gregory Hannon and his colleagues within the same time. After that, it endeavoured to identify the RNAi mechanisms involved in gene silencing by dsRNAs in Drosophila cells. Once found, one of the proteins in RISC called Argonaute, (family of RNase H) triggers and chops the mRNA. This process is called RNA interference (RNAi) and it is established in many eukaryotes; it is important process in gene silencing and defense viral infections.

Argonaut crystal structure, which has 2–8 bases of the guide strand form a Watson-Crick-paired, A-form double helix with a complementary region of the target RNA. The rest of the duplex is disordered, signifying either that the complete duplex was not formed in the crystals or that the second half of the duplex remains mobile when bound to Argonaut of heterochromatin or by DNA eradication **(Pratt AJ et al., 2010).**

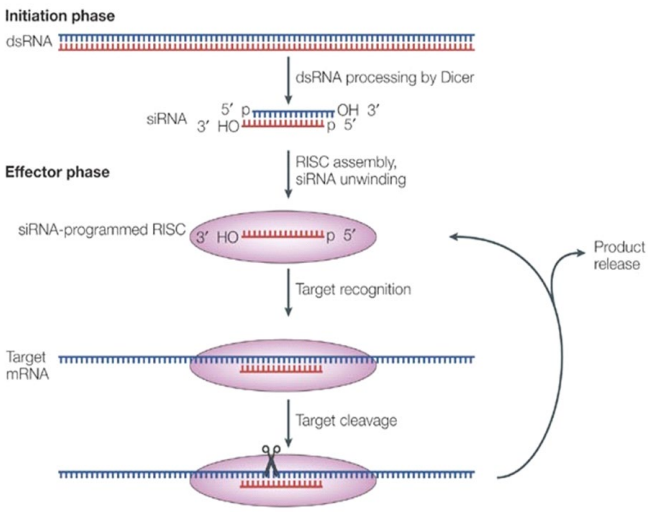

Figure 2. Mechanism of how RISC cut the target sequence.

RNAs (20–30 nucleotides in length) assemble into RISC and guide the complex to complementary RNA targets through base-pairing interactions **(Pratt AJ et al., 2010).** Once programmed by sRNA, RISC can be silence targeted genes with mechanism of:

a) Decrease or stop the level of protein synthesis through repression of translation,

b) Decrease or stop the transcript level through mRNA degradation, or

c) Decrease the level of the genome itself through the formation of heterochromatin (Figure 2).

SIRNA: synthesis, delivery, and gene knockdown

In brief, the application of siRNA for gene silencing involves a careful consideration of the following variables: **(Agrawal N et al., 2003)**

- Selecting the siRNA sequence in the target gene; and synthesis of siRNAs or construction of plasmids bearing DNA sequence encoding for siRNAs;
- Optimizing transfection of the siRNAs or the plasmids expressing siRNAs in the target cells; and
- Monitoring the efficacy of gene silencing

Selection and Generation of siRNA

The parameters used to design or optimize siRNA inducing gene silencing are (**Sakurai K et al., 2010) (Naito Y et al., 2012)**: - length, justifies secondary structure, sugar backbone, and sequence specificity of the siRNA duplex. Even though there is no consensus on choosing the siRNA sequence, the sequence should be selected in the region 50 to 100 bp downstream of the start codon, the 5_ or 3_ translated regions and regions near the start codon should be avoided, and the GC content of the siRNAs should be kept between 30 and 70%.

The computer programs developed by Lin (Jack Lin's siRNA sequence finder; www.lc.sunysb.edu/stu /shiklin/rnai.html) and by Ambion (www.ambion.com) is helpful as guidelines to select potential siRNA sequences. This has been used as a general rule but not be guarantee is that the sequence of one strand should be AA (N19) TT, where N is any nucleotide, i.e., these siRNAs should have a 2-nucleotide 3_ overhang of uridine residues. The siRNAs should be 21 nucleotides long and should have 5_- phosphate and 3_-hydroxyl group for efficiency.

Delivery of dsRNAs

After selection and chemically synthesized RNAi sequence, scientists use different methods to deliver RNAi into the

host. The efficacy of gene silencing substantially depends on the method of dsRNA up take **(Jain PK et al., 2018).** So far, conventional RNAi application has been induced largely based on the use of recombinant viruses (virus-induced gene silencing), Agrobacterium tumefaciens-mediated transiently expressed transgenes, and stably transformed transgenic plants that enable the production of dsRNA molecules against selected targets (host-induced gene silencing) **(Das PR et al., 2020).**

Recently, synthetic, nontoxic nanoparticles might be prepared from natural as well as synthetic polymers. Nanoparticles should be biodegradable and more penetrator. Thus it will be an effective vehicle for dsRNA delivery **(Jain PK et al., 2018).** DNA nano structures such as 3D tetrahedron, 1D hairpin tile. 1D Nano string were used to facilitate the delivery and biological action of 21-nt GFP sRNAs in infiltrated Nicotiana benthamiana leaves **(Zhang R et al., 2018).**

The 3D reveals both mRNA degradation and translational arrest of the GFP, whereas sRNAs attached to 1D nanostructure mainly for translational arrest although the reasons underlying this observation were not elucidated. Among these, the most reliable and commonly used approaches for delivery of dsRNA to plant cells are agro infiltration, micro-bombardment and virus-induced gene silencing **(Abdurakhmonov IY et al., 2016).**

Simplistic and apo plastic delivery: This method (Figure 3) is used at high-pressure to relocate RNAi (siRNA, dsRNA or miRNA) into plant cells and this helps to be targeted with a minimum of RNAi **(Dalakouras A et al., 2018).** Despite it is not a disadvantage, RNA to be in practical over petiole absorption and trunk injection is reserved in the xylem and apoplast and does not generate RNAi. When dsRNAs are applied to plants targeting some insects or fungi, it may convey as

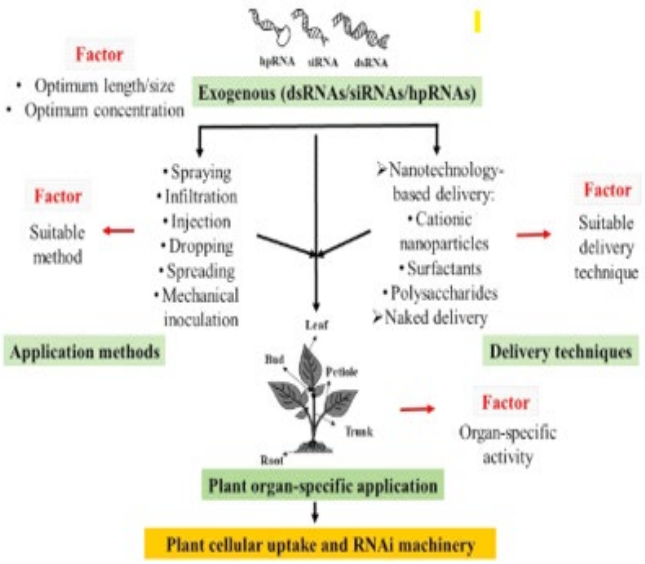

Figure 3. Schematic diagram possible factor that influence the exogenous double strand RNA (dsRNA), short interference RNA (siRNA) and induced RNA interference (RNAi) in plant.

non-processed (by plant DCLs) dsRNAs **(Dalakouras A et al., 2018).** It will be treated by the pathogen/pests DICER proteins. This also leads to greater biological activity against insect or fungal genes. It was achieved when appearance of dsRNA transgenes in chloroplasts was done. Because in chloroplast there is no DCLs and thus the chloroplast-expressed dsRNAs persist intact for pathogen/pest uptake.

Another dsRNA application through petiole uptake and/ or trunk injection could serve as a GMO-substitute to such transplasmic plants. Because they both uphold dsRNA in once present in the plant cell, the applied dsRNAs may be processed not only by DCL4/DCL2 into 21-/22-nt siRNAs but also by DCL3 into 24-nt siRNAs, at DCL localization **(Pumplin N et al., 2016).**

As shown in figure 3, another delivery method of RNAi technology like, non-transformative strategies through a Spray-Induced Gene Silencing (SIGS) process is applicable to protect plants from different insects and pathogens. It is a potential alternative to conventional pesticides **(Koch A et al., 2014).** But this technology has been not adaptable because of high cost of production, long time for development and lack of protocol to use it efficiently **(Smagghe G 2019).** For instance, the commercial availability of "Honey Sweet," a cultivar resistant to the Plum Pox Virus (PPV), took 20 years to reach the market **(Smagghe G 2019).**

DsRNA-treated plants are considered to be GMO-free, and epigenetic since it triggers promoters of sequence and histone modifications that will eventually result in transcriptional gene silencing **(Dalakouras A et al., 2015)** (Figure 3).Schematic diagram possible factor that influence the exogenous double strand RNA (dsRNA), short interference RNA (siRNA) and induced RNA interference (RNAi) in plant.

Mechanisms small RNAs mediate gene silencing: The possible cellular mechanism of dsRNA induced RNAi in plants involves the following steps (Figure 4) **(Meister G et al., 2004) (Dalakouras A et al., 2018) (Liu S et al., 2020):**

(i) First dsRNAs insert to cell and then DICER-LIKE (DCL) endonucleases rapidly cleavages them into 20 to 25-nucleotide siRNAs with 2-nt 3´ overhangs at both ends;

(ii) Next siRNAs is incorporated into an ARGONAUTE (AGO) protein to form an RNA-induced silencing complex (RISC).

• Finally, the siRNA molecules guide the RISC to scan the cytoplasm for recognition and cleavage/ degradation of the complementary transcripts, thus resulting in Post-Transcriptional Gene Silencing (PTGS) Figure 4.

Transitivity and systemic silencing:

Transitivity: A phenomenon that the emerging dsRNA greater than 39 bp in length is slashed by RNase III-related enzymes and produces new siRNAs to amplify mRNA deprivation **(Sinha SK 2010).** In occasion of plants, transitive

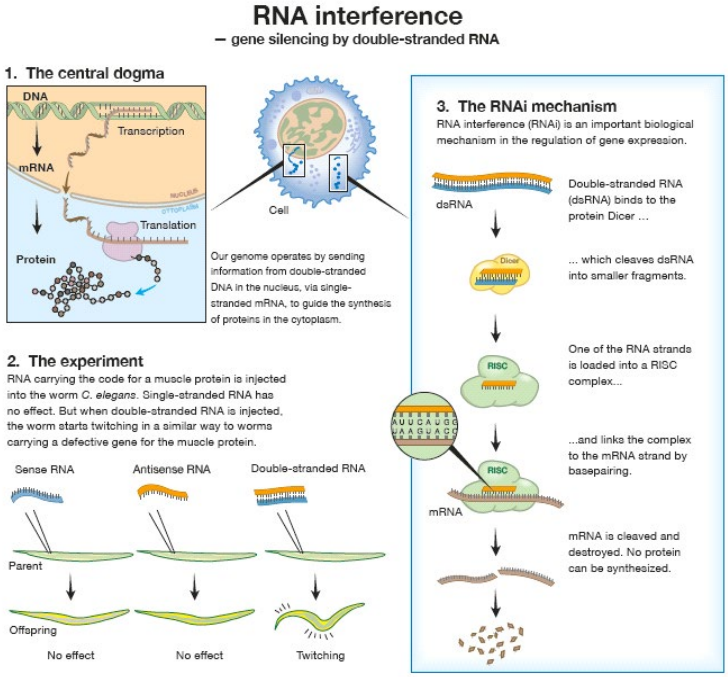

Figure 4. Mechanism of sRNA mediate gene silencing.

RNA silencing is dependent on RNA-dependent RNA polymerase activity and is bidirectional i.e. it can travel both in 5′ → 3′ and 3′→5′ direction **(Sinha SK 2010)**

5′ distribution of the signal is supposed to be intermediated by the primary siRNA produced by the action of the Dicer in response to the prompt.

Systemic: is the process of transformation of RNAi in the body, (cell to cell or tissue to tissue) systemically which directs with RNAi signal. There are two types of RNAi: cell-autonomous RNAi and non-cell- autonomous RNAi **(Joga MR et al., 2016).**

Cell-autonomous RNAi refers to RNAi that happens inside the cell while non-cell-autonomous RNAi requires introduced into the cell and/or transport of the silencing signal from one cell to another or from one tissue to another. In non-cell autonomous RNAi introduced in to cell by helping the phenomenon of environment like soaking or feeding **(Baum JA et al., 2014).** Although RNAi pathways share mostly the same elements among insect species, its systemic nature, if present, may act by different molecular mechanisms across different insect taxa.

Assay of gene knock down after delivery of RNAi

Because of siRNA oligonucleotides target mRNA for degradation; gene knockdown can be used to measure effects on gene expression using reverse negative control siRNA-treated cells **(Han Haiyong H 2019)**. Eg: According to Listanto PCR with specific primers to detect the presence of hptII or the RNAi construct (Figure 5). The authors used thirty-nine of the 168 plantlets for PCR analysis using specific primers for hptII gene and 14 plantlets were PCR positive.

Western blotting and immunofluorescence flow cytometry and phenotypic and/or functional assays:

It used to assess protein levels to ensure efficient knockdown of gene expression and to determine the optimal time point for assessing cellular effects of siRNA knockdown. Even though mRNA levels do not always correlate with protein levels, the protein abundance decreased if siRNA is functional **(Haiyong H 2019).**

Homogeneous cell-based assay: These assay measures phenotypes averaged over a population of cells, such as cell viability. In oppose to that single cell measurements, which are faster to perform and analyses strategies are more widely established. However, they mask the potential phenotypic heterogeneity between cells, e.g., differences in responses due to cell cycle states. Typical homogeneous assays are measurements of biochemical activities or reporter gene assays **(Heigwer F et al., 2018).**

Biochemical assays: In a biochemical assay, belongings of a cell population measures biochemical reactions emitting quantifiable signals after RNAi silencing. A frequently used assay is the measurement of cellular ATP level that targeted for silence. After the lysis of cells, autonomous ATP acts as a substrate for an exogenously provided luciferase enzyme, thus emitting light that can be measured using a multiwall plate reader. The quantity of intracellular ATP is the rate-limiting component and the amount of emitted light is proportional to the number of viable cells in a well **(Heigwer F et al., 2018)**

Reporter gene assays: this analysis often requires the measurement of a second metabolic activities and

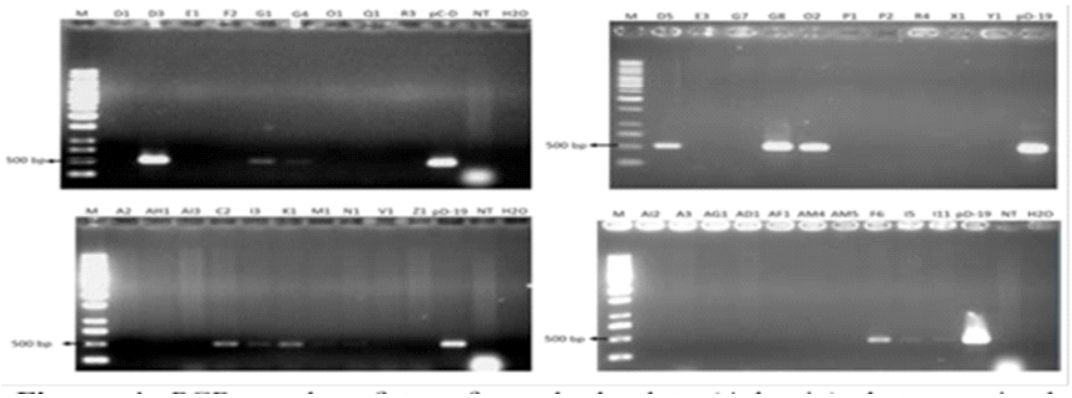

Figure 5. PCR results of transformed plantlets (atlantic that contained RNAi using primers for hpIIgene. m=1kb DNA ladder, pD=plasmid pCombia 1300containing RNAi construct NT=Atlantic non-transforman.

physiological observation that expresses and reporter measuring of cell viability and abundance. But scientists care about false positives resulting from changes in cell viability.

For example, constitutively expressed Renilla luciferase can be used to regulate firefly luciferase signaling reporter using a dual luciferase assay. Caveats of reporter gene screens include difference in the relative sensitivity of the reporter genes and their susceptibility to general changes in cell states and stress **(Heigwer F et al., 2018).**

Importance of RNAi in crop improvement

Applications of RNAi technology as gene silencing producing improved crop varieties in terms of disease-, insect resistance, enhancing nutritional qualities advance agronomic traits, etc.

RNAi improves agronomic traits

Using RNAi technology, improvement of nutritional values and grain yield in different crops is promising. For instance, nutrition level of peanut oil is conventionally qualified by the proportion of oleic and linoleic acids. Oleate desaturase (FAD2) gene is the negative manager to high oleic acid production. Specific sequence of RNAi transfer to cell that has FAD2 gene using Agrobacterium-mediated transformation. And then FAD2 gene was inhibited. Thus a significant increase of oleic acid content was observed in transgenic peanut line **(Abdurakhmonov IY et al., 2016).** Similarly in wheat, starch-branching enzyme encoding genes SBEIIa and SBEIIb, which is accountable to the production of amylose content, was inhibited. On the other hand, other researchers improved yield by RNAi mediated transgenic line for qSW5 to increase seed weight and also use RNAi destruction of the target GA 20-oxidase (OsGA20ox2) gene from original higher rice. This leads increased number of seeds per panicle and larger panicle **(Chen X et al., 2019) (Ansari A et al., 2017).**

RNAi in drought tolerance: According to Gupta et al. (2014) drought has an important cause of constraint of yield and quality of agriculture production. Some applications of RNAi are:

- Using AtHPR1 promoter driving an RNAi construct can effectively down regulate farnesyltransferase in canola. This modification led to an adequate yield production of canola under drought stress.

- In rice receptor of C-kinase 1 is the negative regulator of stomatal closure related to water loss. Liang's team has successfully created a transgenic rice line whose RACK1 gene was suppressed by RNAi. Their transgenic rice line showed a great tolerance against drought stress.

- Another group used virus-induced gene silencing method to suppress the expression of proteinase APRO2 gene and transcription factor JMJC gene in peanuts. The GM peanut was improved to have great production yield and better-quality under water deficit stress. On the other hand, in Arabidopsis, miRNAs such as miR396, miR168, miR167, miR165, miR319, miR159, miR394, miR156, miR393, miR171, miR158, and miR169 were identified as drought responsive. A consistent upregulation of miRNA miR393, miR319 and miR397 has been shown in Arabidopsis in response to drought stress miRNA (Gupta et al., 2014).

Genome wide analysis of miRNAs was carried out in drought stressed rice plants belonging to different developmental stages using a microarray platform fig 6. It was observed that 17 miRNAs (miR156, miR159, miR168, miR170, miR171, miR172, miR319, miR396, miR397, miR408, miR529, miR896, miR1030, miR1035, miR1050, miR1088, and miR1126) were significantly downregulated in response to drought stress while another 14 miRNAs miR159, miR169, miR171, miR319, miR395, miR474, miR845, miR851, miR854, miR896, miR901, miR903, miR1026, and miR1125 were upregulated in response to drought stress **(Gupta K et al., 2014)** Figure 6.

RNAi in insect pests resistance

According to the review of **(Younis et al., 2014),** the practice of using pesticides to control pests has become a common approach in the world, but due to dramatic health and

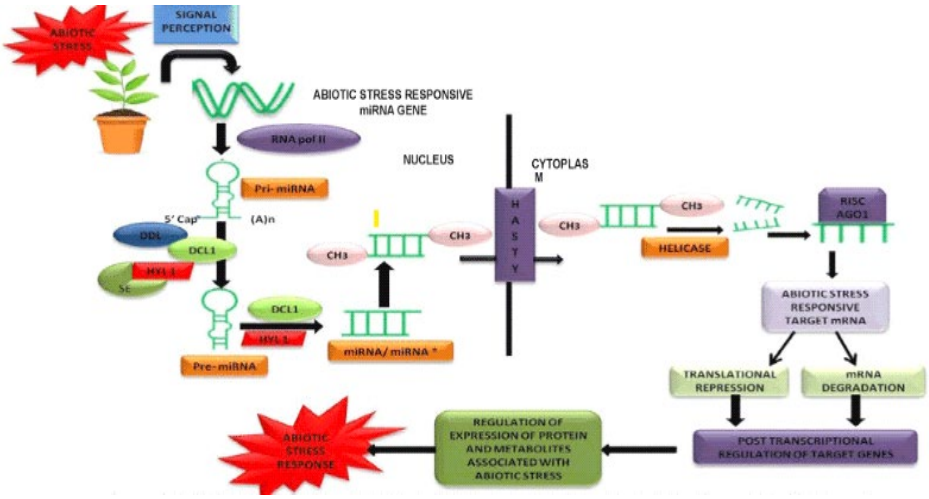

Figure 6. Path way showing post transcriptional regulation mediated by abiotic stress responsive miRNA genes.

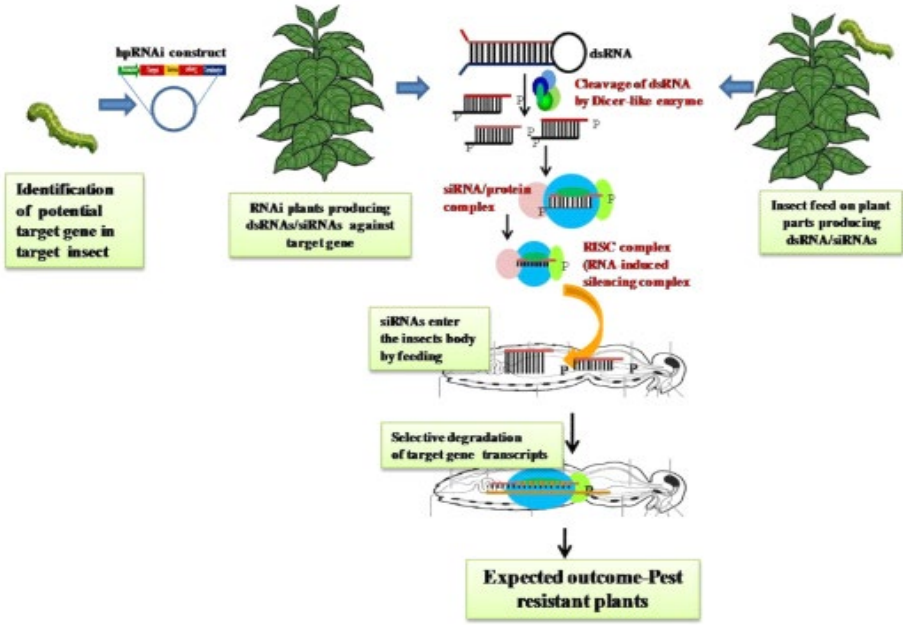

Figure 7. Mechanism of pest gene interference using RNAi technology.

environmental effects, its uses seems to be very limited. Hence scientists search a technology that is environmentally friendly and safe. Regarding insect and pest resistance (Figure 7), researchers identified gene expressed in the insect midgut. It was correlated with larva growth when food contains gossypol. After feeding on plant material revealing dsRNA specific to gene 'CYP6AE14', the result of the transcript diminished in midgut and larva growth also lagging. The gene silencing of 'glutathione-S-transferase' (GST1) can encourage RNAi process when herbivorous insects feed on plant material expressing dsRNA **(Younis A et al., 2014).** When dsRNA was injected in whitefly body cavity, RNAi was induced that knocked-down the genes expression to 70% in midgut as well as salivary glands of whitefly.

Wong's team designed an oral delivered sequence specific dsRNA experiment which can induce RNAi activity and selectively kill target species. Oral administration of dsRNAs targeted 30UTR of the gamma-tubulin gene can selectively kill Drosophila Figure 7.

RNAi in parasites control

Parasites like nematodes and weeds cause substantial loss to important crops like cereals, pulses, and vegetables in most part of the world. Regarding to nematodes, RNAi showed its potential to overcome these losses by silencing either plant genes which are involved in infection process or the essential genes within the nematode (targeting a root-knot nematode (Meloi dogyne javanica)) **(Guo Q et al., 2016)**. On the other hand, weed is controlled by transferring RNAi through host crop to weeds.

For example, Targeted RNAi was transformed in to GUS gene into lettuce. Then their result showed lower GUS activity and transcript quantification confirmed a significant reduction in

GUS mRNA level. This was analyzed by using hemi-parasitic plant Triphysaria versicolor which expressed GUS reporter gene to parasitize the root of either transgenic or non-transgenic lettuce. This result suggested that sequence specific silencing signal can be successfully transmitted through host plant to parasitic plant and induced further destruction of target genes within parasites **(Chen X et al., 2019).**

Applying RNAi molecules in plants against fungi

Transgenic plants expressing dsRNAs against fungal genes is a very promising antifungal approach **(Koch A et al., 2014)** Koch and co-workers demonstrated that spraying of barley (Hordeum vulgare) with in vitro-transcribed 791-bp dsRNA simultaneously targeting three Fusarium graminearum ergosterol biosynthesis genes (CYP51A, CYP51B, and CYP51C) strongly inhibited fungal growth **(Koch A et al., 2014).**

As compared to spraying of siRNAs and dsRNA, in vitro-produced dsRNA had high efficiency to inhibit fungal growth. But siRNA inhibits in lesser extent. dsRNA seemed to be more mobile than siRNAs in barley. But the reasons underlying this phenomenon are unclear. It is also undefined how the dsRNA that first reached the apoplast was transported to the symplast **(Koch A et al., 2014).** In another study, foliar application in Brassica napus of in vitro-transcribed dsRNA targeting various fungal genes deliberated plant protection against Sclerotinia sclerotium and Botrytis cinerea **(McLoughlin AG et al., 2018).**

More recently, spraying of in vitro-transcribed dsRNA targeting the myosine5of Fusarium asiaticum in wounded wheat (Triticum aestivum) coleoptiles resulted in reduced fungal virulence.

RNA transport phenomena between plants and fungi. was discovered that the aggressive fungal pathogen B. cinerea is able not only to deliver siRNAs into host plant cells to suppress host immunity genes, but also uptake exogenously applied dsRNAs and siRNAs that inhibit its growth, providing a typical case of bidirectional trans-kingdom RNAi. More specifically, when in vitro-transcribed dsRNA or siRNAs targeting the Bc-DCL1 and Bc-DCL2 genes were applied on the surface of fruits (tomato [Solanum lycopersicum 'Roma']; strawberry [Fragaria3ananassa]; and fox grape [Vitis labrusca 'Concord']), vegetables (iceberg lettuce [Lactuca sativa]; and onion [Allium cepa]), and flowers.

Applying RNA molecules in plants against viruses

Single-stranded circular RNA viroid's involves important intermolecular folding and similar dsRNA molecules and as such are treated by plant DCLs into siRNAs. Initial reports advised that viroid's are resistant to siRNA-mediated degradation due to their wide-ranging secondary structure **(Dalakouras A et al., 2019)**; but the studies indicate that transgenic plants expressing viroid dsRNAs were viroid-resistant **(Dalakouras A et al., 2019)**. Hence, despite their secondary structure, and similar to viruses, viroid's can most likely be targeted for sRNA-mediated degradation **(Dalakouras A et al., 2015) (Flores R et al., 2017).**

The dsRNA is processed into virus-derived siRNAs resulting in degradation of any homologous RNA, including the single-stranded virus RNA genomes.

The cellular pre-existence of dsRNAs/siRNAs designed to target the virus already before it manages to replicate and generate RNA silencing suppressors is a well-established antiviral crop protection strategy. The GMO method where transgenic plants prompt dsRNAs against viral proteins has been well documented with very satisfactory results **(Mitter N et al., 2017).** Furthermore, RNAi-mediated virus protection is now available in several commercially approved crops **(Rosa C et al., 2018).**

For example: Pepper mild mottle virus, tobacco virus, and alfalfa mosaic virus were mechanically co-inoculated in Nicotiana. benthamiana leaves with in vitro-transcribed 977-bp, 1,483-bp, and 1,124-bp dsRNAs targeting the pepper mild mottle virus replicase, tobacco etch virus helper component, and alfalfa mosaic virus RNA, respectively. Applications of RNAi technology as gene silencing producing improved crop varieties in terms of different agronomic traits (Summarized in Table 1). The technique takes advantage of the heritable and stable RNAi phenotypes in plants.

Limitation of RNAi technology

Although siRNA technology is common and seems promising for agricultural applications, it is still fraught with challenges. These are:

Stability: Because of siRNA is an exogenous molecule, the transport of specific sequence to the host or tissue or cells successfully and silencing still have a problem. Although siRNA is double-stranded and more stable than single-stranded RNA, it can still be quickly degraded by nucleases in plasma and tissues. Therefore, it requires chemical modification to increase its half-life. Chemical modification to optimize a different therapeutics problems, such as increasing silencing potency, improving nuclease resistance, enhancing half-life, inhibiting immune stimulation, reducing off-target effects, etc. However, when optimizing one of these problems, it can also add to other problems. Therefore, the type of chemical that needs to be modified should be considered when the method is optimized **(Chen X, et al., 2019).**

Off-target effect is that siRNA inhibits: during applying RNAi technology, there may be an expression of undesired genes besides expression of desired genes. This may lead to unpredictable genetic changes. Several studies show that off-target gene regulation is a result of degradation of mRNA with partial identity to the "seed region" of the siRNA sequence, the position 2–8 nucleotides from 5, end of the guide strand. Bioinformatics studies have shown that the "seed region" is usually found in 30-UTR, which is suggested

Table 1. Examples of novel plant traits engineered through RNAi.

Type of RNAi molecules	Application methods	Delivery techniques	dsRNA length/ size	dsRNA concentration dose	Plant organ-specific application	Goal/target of RNAi	Detection of siRNAs	Reference
dsRNA	Co-inoculation with target virus	Naked delivery	Viroid-specific dsRNAs	1250 and 5000 molar excess	Young leaves of: Lycopersicon esculentum, Gynura aurantiaca, and Dendranthema grandiflora	Virus resistance		Younis A et al., 2014
dsRNAs	Spraying	Layered double hydroxide (LDH) clay nanosheets based delivery, Naked delivery	977 bp for PMMoVIR54 and 330 bp for CMV2b viral gene, 504 bp for GUS gene ¬	100 µg	Seeds of A. thaliana, Leaves of Vigna unguiculate and Nicotiana tabacum	GUS transgene in A. seedling, Reduced local lesions numbers caused by CMV and PMMoV virus in V. unguiculate and N. tabacum	vsiRNAs from N. tabacum tissue samples with and without CMV inoculation using small- RNA sequencing	Mitter N et al., 2017
dsRNAs Root soaking	dsRNAs Root soaking	Cationic fluorescent nanoparticle G2- based delivery, Naked delivery	450 bp for STM gene, 550 bp for WER gene	1 µg	Roots of Arabidopsis	Down-regulated target STM and WER endogenous gene		Bouche N et al., 2006
dsRNAs	Co-inoculation with target virus	Naked delivery	315, 596, and 977 bp for PMMoV, 1483 bp for TEV, 1124 bp for AMV	5 µl (2.5 µM)	Leaf of N. tabacum	Virus resistance		Bouche N et al., 2006
dsRNAs	Co-inoculation with virus using spraying and mechanical rubbin	Bacterial expression-based delivery, Naked delivery		5 µg	Leaf of N. benthamiana	Virus resistance		McLoughlin AG et al., 2018
dsRNA	Foliar application	Naked-delivery	250–500 bp targeting B. cinereal genes	10–20 ml	Leaves of Brassica napus and Arabidopsis	Fungal resistance		McLoughlin et al., 2018
dsRNA	Spraying	Naked-delivery		30–40 ng/ml	Leaves surface of: C. sativus, Glycine max, Hordeum vulgare, and Triticum aestivum	Fungal resistance		Guo Q et al., 2019
RNAi					Rice, OsSSI2	Leaf blight resistance		Younis A et al. 2014
dsRNA	HIGS				Fusarium graminearum Cytochrome P450 lanosterol C-14a-demethylase (CYP51)	Inhibiting fungal mycelium formation		Koch A et al. 2014
dsRNA	Feeding				Sitobion avenae (insect) Secreted salivary peptide DSR32, salivary protein DSR33, serine protease 1 DSR48	Mortality		Guo Q et al. -2015
			FAD2		Enhanced nutrient content	Increased oleic acid content		

as a miRNA-like mechanism **(Chen X et al., 2019).**

Evaluate the spectrum of off-target sequences, these analyses are currently of limited value for the risk assessment for three reasons:

(i) Scarcity of suitable genome sequence data;

(ii) Rules governing efficient mRNA and/or sRNA recognition by the RNAi machinery are incompletely understood; and

(iii) The capacity of plant sRNA to trigger silencing in non-plant organisms is not always clear and has been estimated for only a few species. Progress in basic research on RNAi mechanisms, production of suitable genome data for relevant species, and design of efficient algorithms to make more reliable predictions.

CONCLUSION

RNAi technology can be considered as safe for environment, organisms and ever green technology. It eliminates even certain risks associated with development of transgenic plants carrying first generation constructs (binary vectors and sense and antisense genes). Conventional transgenic technologies generally need the expression of whole genes, which are comparable. The small size of the RNAi transgene required for silencing, permits multiple genes to be targeted in a single construct. This is because of RNAi is being evolutionary conserved and specificity it applied in distinct population to silence. So using this technology by supporting bioinformatics tools and in vitro dsRNA synthesis will have more advantage in agriculture to improve different important traits in the future.

REFERENCES

1. Clancy S (2008). The central dogma of molecular biology suggests that the primary role of RNA is to convert the information stored in DNA into proteins. In reality, there is much more to the RNA story. Nature Education. 1: 102.
2. Wilson RC, Doudna JA (2013). Molecular mechanisms of RNA interference. Annu Rev Biophys. 42: 217–239.
3. Carthew RW, Sontheimer EJ (2009). Origins and mechanisms of miRNAs and siRNAs. Cell. 136: 642–655.
4. Borges F, Martienssen RA (2015). The expanding world of small RNAs in plants. Nature Rev Mol Cell Biol. 16: 727–741.
5. Sinha SK (2010). RNAi induced gene silencing in crop improvement. Physiol Mol Biol Plants. 16: 321–332.
6. Obbard DJ, Gordon KHJ, Buck AH, Jiggins FM (2009). The evolution of RNAi as a defence against viruses and transposable elements. Philos Trans R Soc Lond Ser B Biol Sci. 364: 99–115.
7. Li C, Zamore PD (2019). RNA interference and small RNA analysis. Cold Spring Harbor Protoc. 4: 247–262.
8. Williams M, Clark G, Sathasivan K, Islam AS (2004). RNA Interference and Its Application in Crop Improvement. Plant Tissue Culture and Biotechnology. 1-18.
9. Brantl S (2002). Antisense-RNA regulation and RNA interference. Biochimica et Biophysica Acta. 1575: 15-25.
10. Agrawal N, Dasaradhi PVN, Mohmmed A, Malhotra P, Bhatnagar RK, et al (2003). RNA Interference: Biology, Mechanism, and Applications. Microbiol Mol Biol Rev. 67: 657–685.
11. Liu S, Jaouannet M, Dempsey DMA, Imani J, Coustau C, et al (2020). RNA-based technologies for insect control in plant production. Biotechnol Adv. 39: 107463.
12. Chen X, Jiang L, Zheng J, Chen F, Wang T, et al (2019). A missense mutation in Large Grain Size 1 increases grain size and enhances cold tolerance in rice. J Exp Bot. 70: 3851-3866.
13. Zhang R, Jing Y, Zhang H, Niu Y, Liu C, et al. (2018). Comprehensive Evolutionary Analysis of the Major RNA-Induced Silencing Complex Members. Scientific reports. 1–7.
14. Dalakouras A, Jarausch W, Buchholz G, Bassler A, Braun M, et al (2018). Delivery of Hairpin RNAs and Small RNAs into Woody and Herbaceous Plants by Trunk Injection and Petiole Absorption. Plant Sci. 1–11.
15. Bouche N, Lauressergues D, Gasciolli V, Vaucheret H (2006). An antagonistic function for Arabidopsis DCL2 in development and a new function for DCL4 in generating viral siRNAs. EMBO J 25: 3347–3356.
16. Pratt AJ, Macrae IJ (2010). The RNA-induced Silencing Complex: A Versatile Gene-silencing Machine. J Biol Chem. 284: 17897–17901.
17. Sakurai K, Chomchan P, Rossi JJ (2010). Silencing of gene expression in cultured cells using small interfering RNAs. Curr Protoc Cell Biol. 47: 1–28.
18. Naito Y, Ui-Tei K (2012). SiRNA design software for a target gene-specific RNA interference. Front Genet. 3: 1–7.
19. Jain PK, Bhattacharya R, Kohli D, Aminedi R, Agrawal PK (2018). RNAi for Resistance Against Biotic Stresses in Crop Plants. Biotechnologies of Crop Improvement. 2: 67-112J.
20. Das PR, Sherif SM (2020). Application of Exogenous dsRNAs-induced RNAi in Agriculture: Challenges and Triumphs. Front Plant Sci 11: 946.
21. Abdurakhmonov IY, Ayubov MS, Ubaydullaeva KA, Buriev ZT, Shermatov SE, et al (2016). RNA Interference for Functional Genomics and Improvement of Cotton (Gossypium sp.).. Plant Sci. 7: 1–17.
22. Pumplin N, Sarazin A, Jullien PE, Bologna NG, Oberlin S, et al (2016). DNA Methylation Influences the Expression of DICER-LIKE4 Isoforms, Which Encode Proteins of Alternative Localization and Function. Plant Cell. 28: 2786-2804.
23. Koch A, Kogel K (2014). New wind in the sails: improving the agronomic value of crop plants through RNAi-mediated gene silencing. Plant Biotechnol J. 12: 821-831.
24. Smagghe G (2019). Management of Pest Insects and Plant Diseases by Non- Transformative RNAi. Plant Sci. 10: 319
25. Dalakouras A, Dadami E, Wassenegger M (2015). Engineering viroid resistance. Viruses. 7: 634-646.
26. Meister G, Tuschl T (2004). Mechanisms of gene silencing by double-stranded RNA. Nature. 431: 343–349.

27. Joga MR, Zotti MJ, Smagghe G, Christiaens O (2016). RNAi Efficiency Systemic Properties, and Novel Delivery Methods for Pest Insect Control: What We Know So Far. Front Physiol. 1–14.

28. Baum JA, Roberts JK (2014). Progress towards RNAi-mediated insect pest management. Advances in insect physiology. 47: 249–295.

29. Haiyong H (2019). RNA Interference to Knock Down Gene Expression. HHS Public Access. 1–9.

30. Heigwer F, Port F, Boutros M (2018). RNA Interference (RNAi). Screening in Drosophila. Genetics. 208: 853-874.

31. Ansari A, Wang C, Wang J, Wang F, Liu P, et al (2017). Engineered Dwarf Male-Sterile Rice: A Promising Genetic Tool for Facilitating Recurrent Selection in Rice. Front Plant Sci. 8: 2132.

32. Gupta K, Sengupta A, Saha J, Gupta B (2014). Gene Technology the Attributes of RNA Interference in Relation to Plant Abiotic Stress Tolerance 3: 1–4.

33. Younis A, Siddique MI, Kim C, Lim K (2014). RNA Interference (RNAi). Induced Gene Silencing: A Promising Approach of Hi-Tech Plant Breeding. Int J Biol Sci. 10: 1150–1158.

34. Guo Q, Liu Q, Smith NA, Liang G, Wang MB (2016). RNA Silencing in Plants: Mechanisms, Technologies and Applications in Horticultural Crops, Current Genomics. Curr Genomics. 17: 476-489.

35. McLoughlin AG, Wytinck N, Walker PL, Girard IJ, Rashid KY, et al (2018). Identification and application of exogenous dsRNA confersplant protection against Sclerotinia sclerotiorum and Botrytis cinerea. Sci Rep. 8: 7320.

36. Flores R, Navarro B, Kovalskaya N, Hammond RW, Di Serio F (2017). Engineering resistanceagainst viroids. Curr Opin Virol. 26: 1-7.

37. Mitter N, Worrall EA, Robinson KE, Li P, Jain RG, et al (2017). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. Nat Plants. 3: 16207.

38. Rosa C, Kuo YW, Wuriyanghan H, Falk BW (2018). RNA Interference Mechanisms and Applications in Plant Pathology. Annu Rev Phytopathol. 56: 581-610.