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REVIEW PAPER

RNA Interference in Crop Improvement

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ABSTRACT

RNA interference is a natural process, which silences specific genes before translation by degrading mRNA. In late 1980s, it was observed that the introduction of certain transgenes into plants could result in homology dependent silencing of an endogenous locus and the mechanism was referred as co-suppression. However, the complete study of this phenomenon and underlying mechanism were clear only after elaborate studies conducted in the model organism *Caenorhabditis elegans* and the process was named as RNA interference (RNAi). RNAi operates by triggering the action of dsRNA intermediates, which are processed into RNA duplexes of 21-24 nucleotides by an enzyme of ribonuclease III family, called Dicer. These short interfering RNAs (siRNAs) are incorporated into RNA induced silencing complex (RISC). The siRNA acts as a guide to target the degradation of complementary mRNAs. The host genome coding for small RNAs, called miRNAs are responsible for endogenous gene silencing. The dsRNAs triggering gene silencing can originate from several sources such as endogenous or transgenic antisense sequences, inverted repeat sequences or viral RNA during replication. Currently, RNA silencing is an area of intense investigations leading to exciting new discoveries in crop improvement programmes. RNAi technology holds the key for future biotechnological applications in the fields of agriculture and medicine.

Key words *siRNAs, Dicer, RISC and miRNAs*

RNA interference is a natural process, which silences specific genes before being translated. It is a natural process that is highly conserved in many organisms and silences genes by degrading messenger RNA (mRNA) before it is translated. In plants, the RNA silencing story unfolded serendipitously during a search for transgenic petunia flowers that were expected to be more purple. In 1990, R. Jorgensen's laboratory wanted to upregulate the activity of a gene for *chalcone synthase* (*chsA*), an enzyme involved in the production of anthocyanin pigments. Surprisingly, some of the transgenic petunia plants harboring the *chsA* coding region under the control of a 35S promoter lost both endogene and transgene *chalcone synthase* activity, and thus many of the flowers were variegated or developed white sectors (Napoli *et al*., 1990). The loss of cytosolic *chsA* mRNA was not associated with reduced transcription, as demonstrated by run-on transcription tests in isolated nuclei. Jorgensen coined the term cosuppression to describe the loss of mRNAs of both the endogens and the transgene.

However, the complete study on this phenomenon and underlying mechanisms were answered after its discovery and characterization in the nematode *Caenorhabditis elegans* by Andy Fire, Craig Mello, and their colleagues in 1998. They named it RNA interference (RNAi), carrying out silencing of gene by degradation of mRNA. It was also shown that the dsRNA injected into the body cavity could produce an interfering effect in distant tissues and in F1 progeny, indicating that cells may have an RNA-transport characteristic of RNAi: (1) the effect of gene silencing is induced by double-stranded RNA, (2) the effect of RNAi is systemic, and (3) RNAi is heritable. These were the landmark findings that encouraged many group of scientists worldwide for RNAi studies in many systems for different functional aspects of different genes.

In nature, the plants and insects have evolved to use RNAi as protection against viruses. Several years ago, it had been reported that antisense RNA targeted to specific endogenous genes in *Caenorhabditis elegans*, when either expressed from a transgene or injected directly into the worm's gonad, could phenocopy a null or hypomorphic mutation in the targeted gene (Fire *et al*., 1998). The experimental results were thoroughly supportive for the RNA-mediated class of mechanisms. The ability of viral RNAs to interfere with a homologous gene in the plant genome was the strong evidence for the existence of RNA-mediated silencing mechanisms. It was confirmed in the subsequent experimentation in which RNAs were introduced in the absence of a DNA template by using RNA viruses as vectors (Pooggin *et al*., 2003).

After the work of several years (even before it was named RNAi) with *C. elegans* for the study of gene functions, it was found that injection of antisense RNA causes dramatic downregulation of gene expression in *C. elegans* germline. The high efficiency of RNAi in worms put forth the existence of novel mechanism(s) underlying this phenomenon. Only a few molecules of double-stranded RNA (dsRNA) per cell were capable of specific inhibition, which was not expected from a simple antisense mechanism as there is simply not enough material to bind to all the endogenous mRNA targeted for destruction. These results generated two fundamental questions: how can dsRNA mediate genes specific interference and what is the

physiological purpose of this process? Later, many queries to this phenomenon were solved after the finding that the genetic interference from injected "sense" and "antisense" RNA is actually mediated by dsRNA that is present at a low level in all in vitro RNA synthesis because of the nonspecific activity of RNA polymerases. Highly purified preparations of antisense (and sense) RNAs were had negligible effects, whereas dsRNA exhibited potent and specific interference against any of a variety of targeted genes.

In the recent years, there had been tremendous growth in the applications of this technology for understanding the mechanism of gene function in animal as well as plant systems. The efficiency of this technique has increased significantly with the availability of genome sequences of number of species. Through regulation of the expression of protein coding genes, this technique is being successfully used as an important tool for functional genomics and manipulation of the expression of desired genes.

Genomic machineries for RNAi

Independently of one another, investigations on diverse organisms, labeled variously as PTGS in plants, RNAi in animals, quelling in fungi, and virus-induced gene silencing, have converged on a universal paradigm of gene regulation. The critical common components of the paradigm are that (i) the inducer is the dsRNA, (ii) the target RNA is degraded in a homology dependent fashion and (iii) the degradative machinery requires a set of proteins which are similar in structure and function across most organisms. In most of these processes, certain invariant features are observed, including the formation of small interfering RNA (siRNA) and the organism- specific systemic transmission of silencing from its site of initiation.

Small RNAs

Small interfering RNA (siRNA)

The siRNA mediate RNAi by downregulating target mRNAs through endonucleolytic cleavage. These small RNAs originate from long dsRNA molecules, which are generally produced from RNA virus replication, convergent transcription of cellular genes or mobile genetic elements, self-annealing transcripts, or experimental transfection process (Jinek *et al*., 2009). Next comes the role of Dicer, which is an endonuclease to cleave the dsRNA at 21–25-nucleotide intervals. Upon cleavage in to small fragments one strand of the siRNA duplex, which is known as the guide strand, is loaded onto an Argonaute protein at the core of an RNA-induced silencing complex known as RISC, which is a ternary complex that consists of an Argonaute protein, Dicer and a dsRNA-binding protein. The duplex is unwound while assembling to RISC, which is ATP dependent process (Fig. 1). During loading of RNA, the other non-guide stand known as the passenger strand is cleaved by an Argonaute protein and ejected out of the complex. The Argonaute protein then uses the sequences of guide siRNA to associate with target RNAs that contain perfectly complementary sequence and then catalyses the slicing of these targets (thereby known as sequence specific gene silencing). Later, the cleaved target RNA is released and the RISC is recycled for another round of slicing. The sense strand is also known to recycle back and result in amplification of silencing effect.

There is another phenomenon of gene silencing mediated by transcriptional gene silencing (TGS). Here, the siRNAs homologous to promoter sequences act as a guide for theRNA-induced transcriptional gene silencing complex,

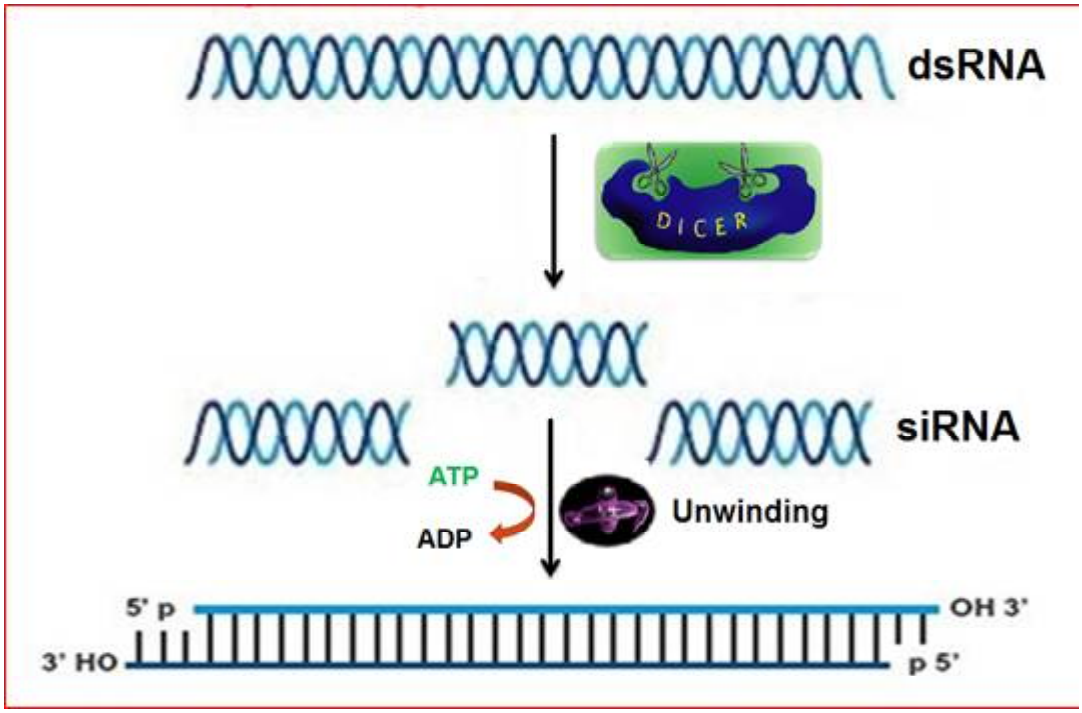


Fig.1. Small interfering RNA pathway

resulting in the methylation of the promoter sequences. Methylation of promoter sequences blocks gene transcription and results in suppression of transcription, thereby named as transcriptional gene silencing.

Micro RNA (miRNA)

MicroRNA is a small non-coding RNA molecule found in plants and animals, which functions in transcriptional and post-transcriptional regulation of gene expression. Encoded by eukaryotic nuclear DNA, miRNAs function via base-pairing with complementary sequences within mRNA molecules, usually resulting in gene silencing via translational repression or target degradation. Micro RNA is synthesis in three steps process.

Nuclear processing

A single pri-miRNA may contain from one to six miRNA precursors. These hairpin loop structures are composed of about 70 nucleotides each. Each hairpin is flanked by sequences necessary for efficient processing. The double-stranded RNA structure of the hairpins in a pri-miRNA is recognized by a nuclear protein known as DiGeorge Syndrome Critical Region 8 (DGCR8 or "Pasha" in invertebrates), named for its association with DiGeorge Syndrome. DGCR8 associates with the enzyme Drosha, a protein that cuts RNA, to form the "Microprocessor" complex (Gregory *et al*., 2006). In this complex, DGCR8 orients the catalytic RNase III domain of Drosha to liberate hairpins from pri-miRNAs by cleaving RNA about eleven nucleotides from the hairpin base (two helical RNA turns into the stem). The product resulting has a two-nucleotide overhang at its 3' end; it has 3' hydroxyl and 5' phosphate groups. It is often termed as a pre-miRNA (precursor-miRNA).

Nuclear export

Pre-miRNA hairpins are exported from the nucleus in a process involving the nucleocytoplasmic shuttle Exportin-5. This protein, a member of the karyopherin family, recognizes a two-nucleotide overhang left by the RNase III enzyme Drosha at the 3' end of the pre-miRNA hairpin. Exportin-5-mediated transport to the cytoplasm is energy-dependent, using GTP bound to the Ran protein (Lund *et al*., 2006).

Cytoplasmic processing

In the cytoplasm, the pre-miRNA hairpin is cleaved by the RNase III enzyme Dicer. This endoribonuclease interacts with the 3' end of the hairpin and cuts away the loop joining the 3' and 5' arms, yielding an imperfect miRNA:miRNA* duplex about 22 nucleotides in length. Overall hairpin length and loop size influence the efficiency of Dicer processing, and the imperfect nature of the miRNA:miRNA* pairing also affects cleavage (Murchison *et al*., 2004). Although either strand of the duplex may potentially act as a functional miRNA, only one strand is usually incorporated into the RNA-induced silencing complex (RISC) where the miRNA and its mRNA target interact (Fig. 2).

The mechanism of miRNA-guided translational regulation is not as well understood as that of target-RNA cleavage. In animals, the binding sites for miRNAs in target mRNAs have inadequate complementarity to allow the target to be cleaved. The first evidence for translational repression by miRNAs was observed in transgenic *C. elegans*, where it was observed that the miRNAs targeted to a specific gene reduced protein synthesis without affecting mRNA levels. In this case, the target mRNA contained in its 32

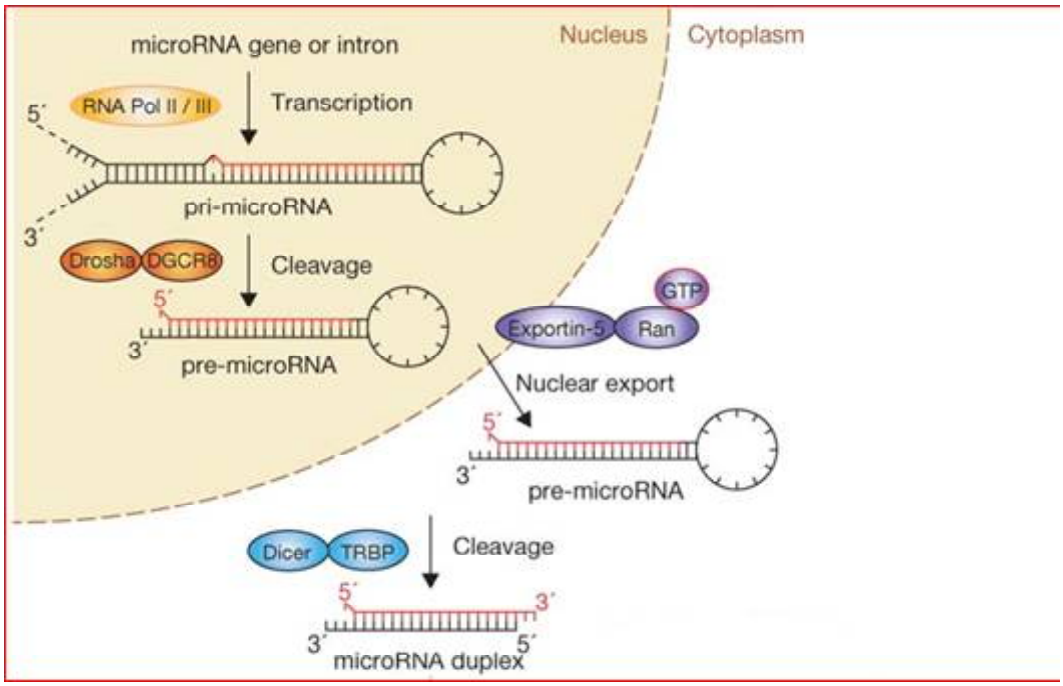


Fig. 2. Micro RNA pathway

UTR several binding sites for the miRNA, and both target and miRNA were found to be associated with polyribosomes. Therefore, it was concluded that the miRNAs block translation elongation or termination rather than translational initiation.

Proteins complex

RNA induced silencing complex (RISC)

The RNA-induced silencing complex, or RISC, is a multiprotein complex that incorporates one strand of a small interfering RNA (siRNA) or microRNA (miRNA). RISC uses the siRNA or miRNA as a template for recognizing complementary mRNA. dsRNA can induce the degradation of homologous mRNA transcripts when it is processed to 21–23 base pairs siRNAs by an endonuclease known as Dicer. The resulting siRNAs function as sequence specific guides to direct an RISC. Ultimately, a RISC-bound endonuclease (called "slicer" an Argonaute protein) to lead to the cleavage of the target (mRNA).

The siRNA and miRNA-duplex-containing ribonucleoprotein particles (RNPs) are rearranged into the RNA induced silencing complex (RISC). The duplex is unwound while assembling to RISC. The functional RNPs contain only single-stranded siRNAs or miRNAs. The assembly of RISC and miRNPs is ATP dependent, which probably reflects the requirement for energy driven unwinding of the siRNA or miRNA duplex and/or other conformational or compositional changes of the preassembled RNA-duplex-containing RNP (Zamore *et al*., 2000). The studies have shown the sequence composition of the siRNA duplex has an impact on the ratio of "sense" (same sequence as the target gene) and "antisense" (complementary to the target gene) siRNAs entering the RISC complex.

Microprocessor

The second endonuclease of RNaseIII family is known as Drosha, which is present in the nucleus for initial processing of pri-miRNA. Drosha being a nuclear protein, have the domain structure consisting of a proline-rich region and an arginine- and serine-rich region at the N terminus, followed by two RNaseIII domains and a dsRBD (Lee *et al*., 2003). It catalyses the initial processing of pri-miRNAs (primary-miRNA with hairpin structure) in the nucleus, thereby yielding pre-miRNAs, which are hairpin structures with phosphorylated 52 ends and 32 dinucleotide overhangs. The purified Drosha cleaves dsRNA (hairpin) nonspecifically; however, specific cleavage of pri-miRNAs requires association with a protein known as DGCR8, which is also known as Pasha in invertebrates. In human, the core region of DGCR8 is composed of a tandem pair of dsRBDs. This process occurs in the complex called the microprocessor. DGCR8 binds to the base of the pri-miRNA hairpin, positioning Drosha to cleave the pri-miRNA stem at a distance of 11 base pairs from the junction between the duplex stem and the flanking ssRNA regions. By its activity, DGCR8 is likely a trans-acting specificity determinant, analogous to the PAZ domain of Dicer. After its export to the cytoplasm, the hairpin (known as a precursor miRNA, pre-miRNA) undergoes another endonucleolytic cleavage, which is catalyzed by Dicer, generating a miRNA-miRNA* duplex (where miRNA is the antisense or guide, strand and miRNA* is the sense or passenger strand) of 21–25 nucleotides.

Enzymes

Dicer

The endonuclease activity of Dicer cuts off the dsRNA molecule. Its processing substrates therefore include long dsRNAs and pre-miRNAs (hairpin structures), which are refined in to short dsRNA fragments known as siRNAs and miRNAs respectively having defined length of typically 21–25 nucleotides (Fire *et al*., 1998). In addition to two copies of the conserved RNaseIII domain and a dsRBD in the carboxyl terminus, this enzyme also have an amino-terminal DEXD/H-box domain, followed by a small domain of unknown function (the DUF283 domain) and a PAZ domain. This PAZ domain is also present in Argonaute proteins. In Dicer, the PAZ domain binds specifically to the 32 end of single-stranded RNA (ssRNA). The crystal structure of Dicer from the unicellular eukaryote Giardia intestinalis revealed that the ability of Dicer enzymes to produce dsRNA fragments of specific length originates from a unique spatial arrangement of the PAZ domain and the RNaseIII domains (Macrae *et al*., 2006).

In the prokaryotes, Dicer is a comparatively simple endonuclease, consisting only of the PAZ domain and two tandem RNaseIII domains viz. RNaseIIIa and RNaseIIIb. It has the structural resembles with wood cutting axe, with the two RNaseIII catalytic domains forming the blade and the PAZ domain making up the base of the handle . The RNaseIIIa domain and the PAZ domain are connected by a long helix running the length of the handle. In Dicer, four conserved acidic amino-acid residues in the active site of each RNaseIII domain coordinate two metal cations, suggesting that Dicer uses a two-metal-ion mechanism to catalyze RNA cleavage (Ma *et al*., 2006). The PAZ domain of Dicer has the same fold and 32 -overhang-binding residues as the PAZ domain of Argonaute proteins

(Lingel *et al*., 2003). The distance between the 32 -overhang-binding pocket of the PAZ domain and the active site of the RNaseIIIa domain is 65 Å, which corresponds to the length of a 25- nucleotide RNA duplex. The domain architecture of Dicer thus suggests that it functions as a molecular ruler, generating products of defined length by anchoring the 32 dinucleotide of the substrate RNA duplex, which is generated by an initial nonspecific cleavage in the PAZ domain and cleaving at a fixed distance from that end (Lingel *et al*., 2003).

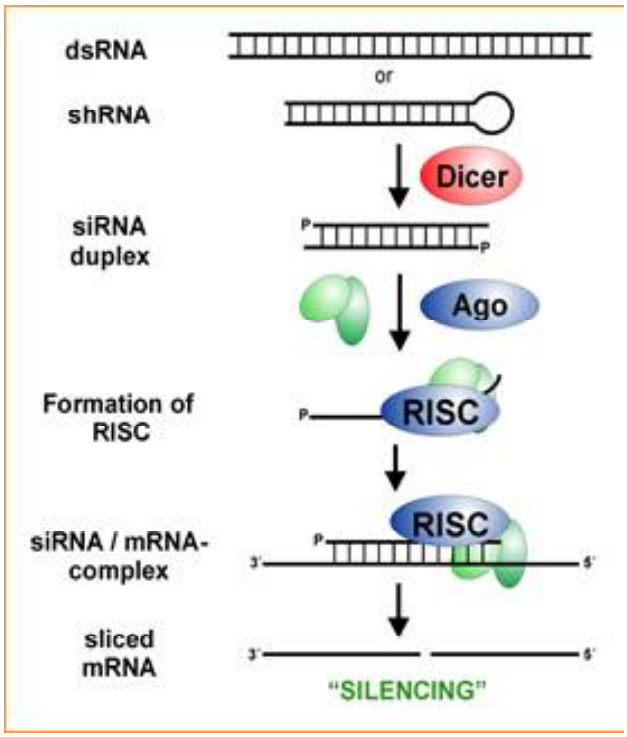
RNA-dependent RNA polymerase

The effects of both RNAi and PTGS are potent and

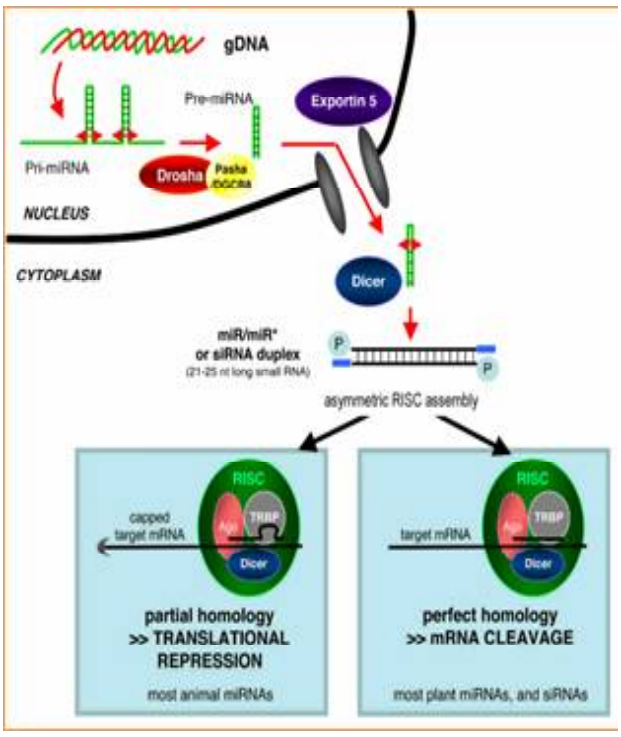
systemic in nature. This has led to a proposed mechanism in which RNA-dependent RNA polymerases (RdRPs) play a role in both triggering and amplifying the silencing effect. Transgenic and virus-infected plants show an accumulation of aberrant transgenic and viral RNAs. The RdRP enzymes might recognize these aberrant RNAs as templates and synthesize antisense RNAs to form dsRNAs that are finally the targets for sequence-specific RNA degradation (Cogoni and Macino, 1999).

Mechanism of RNAi

RNA interference refers collectively to diverse RNA-based processes that all result in sequence-specific inhibition of gene expression at the transcription, mRNA stability or translational levels. It has most likely evolved as a mechanism for cells to eliminate foreign genes. The unifying features of this phenomenon are the production of small RNAs (21-26 nucleotides that act as specific determinants for down-regulating gene expression (Hannon, 2002) and the requirement for one or more members of the Argonaute family of proteins. RNAi operates by triggering the action of dsRNA intermediates, which are processed into RNA duplexes of 21-24 nucleotides by a ribonuclease III-like enzyme called Dicer. Once produced, these small RNA molecules or short interfering RNAs (siRNAs) are incorporated in a multi-subunit complex called RNA induced silencing complex (RISC). RISC is formed by a siRNA and an endonuclease among other components. The siRNAs within RISC acts as a guide to target the degradation of complementary messenger RNAs (mRNAs) (Tang *et al*., 2003). The host genome codifies for small RNAs called miRNAs that are responsible for endogenous gene silencing. Degradation of target mRNA happens in two different pathways on the basis of complementary of guide strand. Perfectly complementary resulted in complete degradation of target mRNA and on the other hand partially complementary resulted in suppression of genes (Fig. 3). The dsRNAs triggering gene silencing can originate from several sources such as expression of endogenous or transgenic antisense sequences, expression of inverted repeated sequences or RNA synthesis during viral replication. When dsRNA molecules produced during viral replication trigger gene silencing, the process is called virus-induced gene silencing (VIGS) (Lu *et al*., 2003). One interesting feature of RNA silencing in plants is that once it is triggered in a certain cell, a mobile signal is produced and spread through the whole plant causing the entire plant to be silenced (Dunoyer *et al*., 2007). After triggering RNA silencing, the mobile signaling molecules can be relay-amplified by synthesis of dsRNAs on the primary cleavage of product templates or by their cleavage into secondary siRNAs. This amplification leads to the transitory nature of silencing reaction that may spread along the mRNA, though initiated by a locally targeted single siRNA and spreads in both the 5′and 3′ directions. This bi-directional transition further have been witnessed by a process where both the 5′ and 3′ cleavage products of the initial target RNA act as aberrant mRNAs to trigger dsRNA synthesis, and induce secondary silencing reactions. This silencing process is also enhanced by the enzymatic activity of the RISC complex, mediating multiple turnover reactions (Hutvagner and Zamore, 2002). Furthermore, production of the



Degradation of mRNA



Suppression of gene

Fig. 3. Mechanism of RNAi

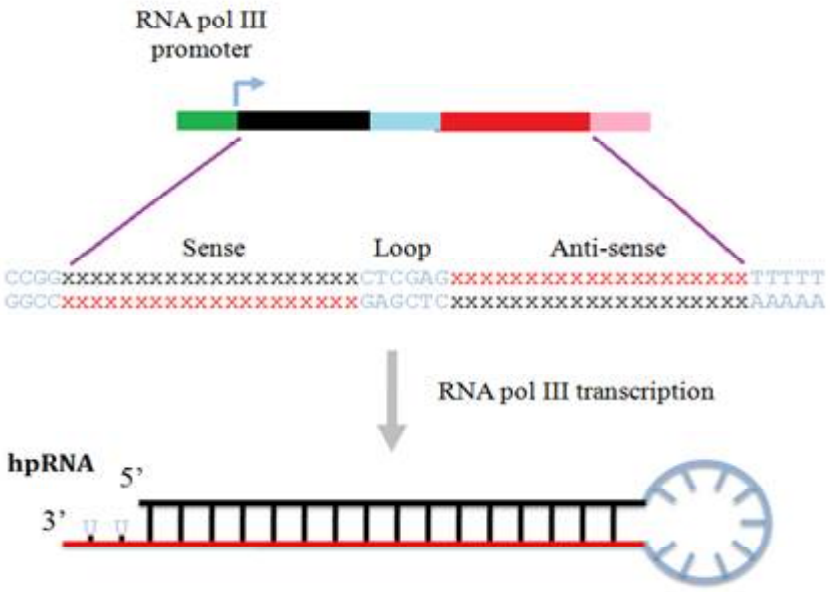


Fig. 4. Gene construct for RNAi

secondary siRNAs leads to enrichment of silencing via its spread from the first activated cell to neighboring cells, and systemically through the system. The cell-to-cell spread can be mediated as passive spread of the small RNAs via plasmodesmata or by the silencing signal complex (Kobayashi and Zambryski, 2007). The systemic spread in phloem is mediated by the 24 nt siRNAs, unloading of the systemic signal appears to be mediated via plasmodesmata, since it does not spread into meristematic cells. The discovery of RNA binding protein (PSRP1) in the phloem and its ability to bind 25 nt ssRNA species add further to the argument that siRNAs (24-26 nt) are the key components for systemic silencing signal. The extent of cell-to-cell movement is dependent on the levels of siRNAs produced at the site of silencing initiation, but is independent of the presence of siRNA target transcripts in either source or recipient cells.

Three step process

1. Processing of dsRNA into small RNA
2. Amplification of small RNA
3. Degradation of mRNA

Two different pathways

1. Degradation of mRNA
2. Suppression of gene

Gene construct for RNAi

RNAi inactivates gene expression in a sequence-specific manner. Double-stranded RNA (dsRNA) is an effective trigger of RNAi. In mammalian cells, nematodes and flies, RNAi can be induced by direct introduction of dsRNAs. In contrast, RNAi in plants is usually established by transformation with a construct that produce hairpin RNAs.

Chuang and Meyerowitz (2000) first demonstrated that efficient RNA silencing in *Arabidopsis thaliana* can be conferred by an RNAi construct. They showed that introduction of an RNAi vector resulted in the silencing of a homeobox gene equivalent to that of the corresponding null mutant. The plant RNAi vector consists of an inverted repeat harboring target sequences under the control of a strong promoter. The inverted repeat sequences are separated by a spacer fragment. The inverted repeat region against the target gene forms a dsRNA structure which serves as the substrate of Dicer. After transcription, inverted repeat sequences form a dsRNA structure (called as stem) and a spacer forms the loop of a hairpin RNA respectively (Fig. 4). The spacer sequence allows us to construct an RNAi vector more easily, since replication of inverted repeat sequences without a spacer is unstable in *Escherichia coli* (Hirai and Kodama, 2008).

Applications in plants

The developments in the RNAi technology has made us to understand the genes functions and their regulation associated with different metabolic pathways controlling the important traits and therefore has prompted for the engineering of these plants for the desired results. In plants, RNAi is induced by introduction of expression vectors, which transcribe a selfcomplementary dsRNA. The selection of transformation method is also one of the factors for the efficacy of this technique in the plant. The *Agrobacterium*-mediated transformation is generally used transformation technique. Besides this, the direct introduction of RNAi vectors via particle bombardment and electroporation has also been employed in plant cells. The effects of RNA silencing could be modulated depending on the architecture of RNAi construct. The RNAi constructs usually have a spacer sequence between an inverted repeat,

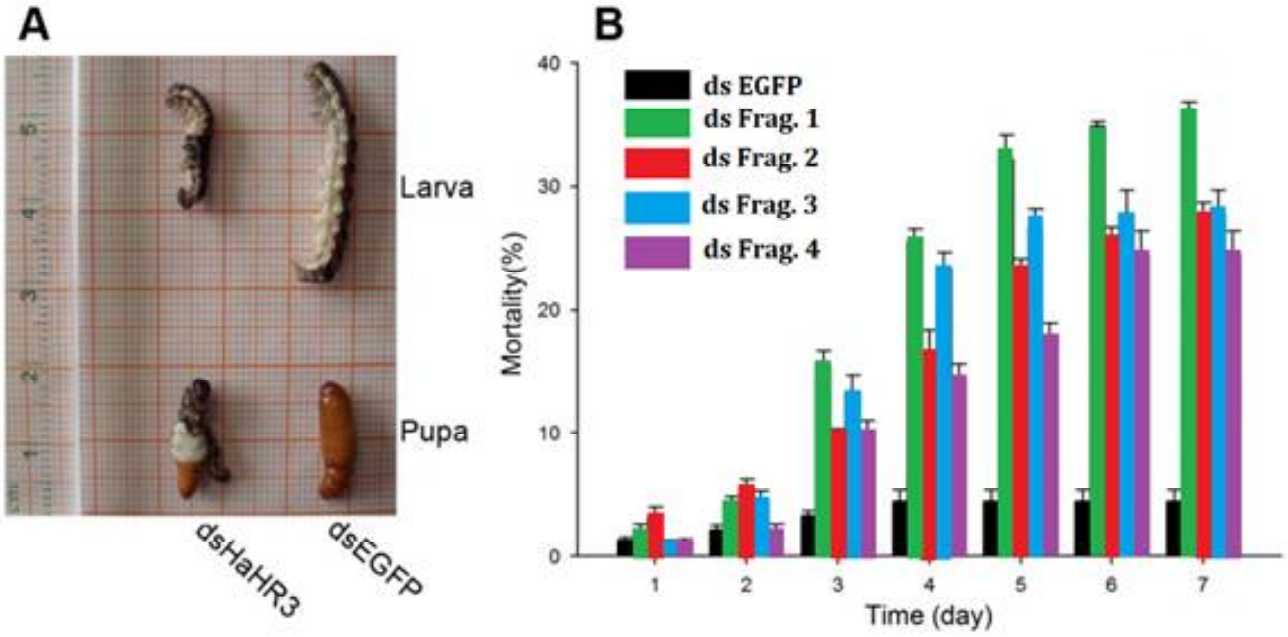


Fig. 5. Phenotype and mortality of *Helicoverpa armigera* feeding with different bacterial-expressed dsRNAs (A: *H. armigera* moulting and pupation failure, B: Ingestion of artificial diet containing different dsRNA fragment)

and the resulting transcript from the RNAi construct takes the shape of stem–loop structure. These transcripts are therefore called hpRNAs. This RNAi constructs with inverted repeat and interrupted with a spacer is inserted downstream of a constitutive promoter. Intron-containing hpRNA-based vectors have been proven to be highly efficient for plant RNAi-based gene silencing (Smith *et al*., 2000). They have been shown to increases gene silencing efficiency by 90–100 % (Wesley *et al*., 2001). In a hpRNAvector, the target gene is cloned as an inverted repeat spaced with an intron and is driven by either a strong whole plant promoter, such as the 35S CaMV (dicots) or the maize ubiquitin1 (monocots) or an organ specific silencing promoter. A spacer fragment between the arms of the inverted repeat is useful for increasing the stability of the vector in Escherichia coli, and using a splicable intron as a spacer, which increased the frequency of strong silencing phenotypes (Smith *et al*., 2000). The degree of silencing with these constructs was much greater than that obtained using either co-suppression or antisense constructs.

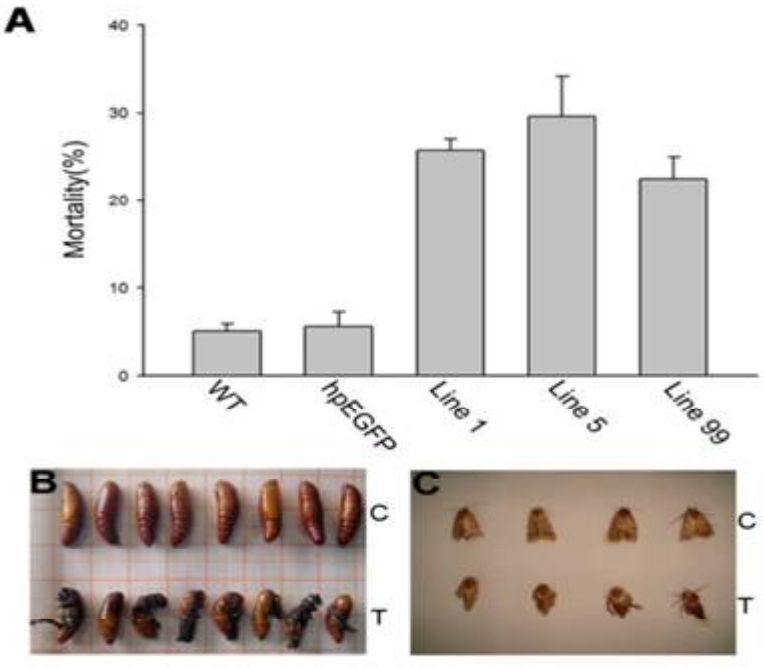


Fig. 6. Mortality and developmental deformity in *Helicoverpa armigera*. (A) Mortality after feeding with different lines of tobacco leaves, (B) Inhibition of pupation after feeding with hpRNA-*HaHR3* tobacco leaves, (C) Deformity in adult emergence.

Table 1. RNA-mediated gene silencing other for insects

Inset	Target region	RNAi effect	Reference
Ostrinia furnalalis	*DS10 & DS28*	Mortality increased up to 73-100%	Wang *et al*., 2011
Phyllotreta striolate	*PsOrl*	Block host-plant-seeking behaviour	Zhao *et al*., 2011
Nilaparvata lugens	*TPS*	Decline expression of target gene	Chen *et al*., 2010

Fig.7. *Agrobacterium* binary vector pDE00.0201 for the expression of self-complementary *iaaM* and *ipt* oncogenes. This vector contains an *nptII*-selectable marker gene driven by the mannopine synthase 2_ promoter (mas5_), a *uid*A scorable marker gene driven by the ubi3 promoter (ubi3), and self-complementary *iaaM* and *ipt* genes driven by 35S cauliflower mosaic virus promoters. Arrows indicate the direction of transcription. LB and RB indicate the left and right T-DNA border sequences.

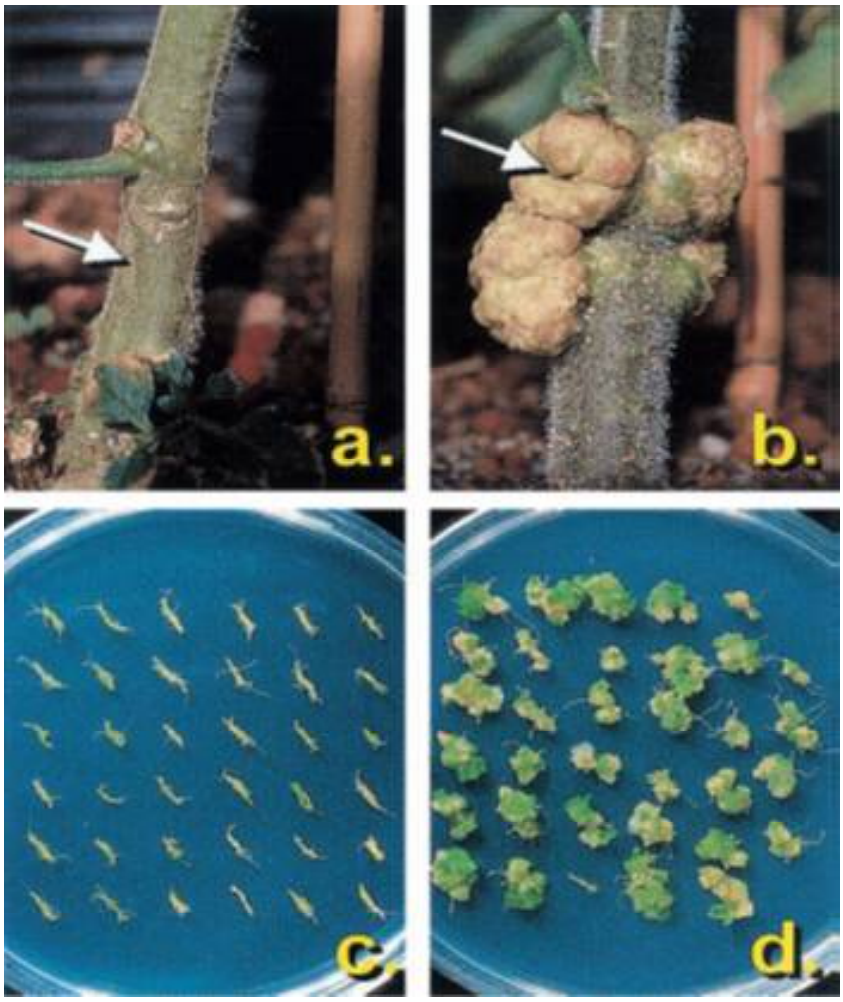


Fig.8. Transgenic (*a*) and wild-type (*b*) tomatoes were infected with *A. tumefaciens* 20W-5A by piercing the stem with a syringe and extruding a small amount of bacterial suspension into the wound. Tumorigenesis was scored 5 weeks after inoculation. Large tumors are evident at the inoculation site (marked with an arrow) on the wild-type plant but not the transgenic plant, indicating suppression of tumorigenesis. In the lower panels, *in vitro* grown roots were harvested from transgenic (*c*) and wild-type (*d*) *Arabidopsis* lines and inoculated with *A. tumefaciens* A208. Tumorigenesis was scored 5 weeks after inoculation. Large teratoma-type tumors are present on wild-type root bundles but not on transgenic root bundles, again indicating suppression of tumorigenesis.

Table 2. Effects of targeted region of RNAi in various plantvirus systems.

Host system	Virus	Target genes	Reference
Soybean	Bean pod mottle virus	*Pds, Actin*	Zhang and Ghabrial, 2006
Cassava	African cassava mosaic virus	*pds, su,cyp79d2*	Fofna *et al*., 2004
Arabidopsis	Cabbage leaf curl virus	*gfp, CH42, pds*	Turange *et al*., 2002

Therefore, it is become the most popular method for genes silencing in plants.

Induction of pest and disease resistance

Insect resistance

RNA interference (RNAi) caused by exogenous double-stranded RNA (dsRNA) has developed into a powerful technique in functional genomics, and to date it is widely used to down-regulate crucial physiology-related genes to control pest insects. A molt-regulating transcription factor gene, *HaHR3*, of cotton bollworm (*Helicoverpa armigera*) was selected as the target gene. Four different fragments covering the coding sequence (CDS) of *HaHR3* were cloned into vector L4440 to express dsRNAs in *Escherichia coli*. After feeding *H. armigera* the artificial diet containing dsRNAs for 7 days, the bioassay results showed that the four different dsRNA-*HaHR3* fragments increased *H. armigera* mortality. The mortality increased rapidly on day 3-4 after feeding dsRNA-*HaHR3*; on day 3, the mortality of feeding with dsRNA-*HaHR3*-fragment 1 (dsFrag.1) and dsRNA- *HaHR3*-fragment 3 (dsFrag.3) showed significant differences with dsRNA-*EGFP*. On day 4, the mortality caused by all four dsRNA-*HaHR3*-fragments showed significant differences compared to dsRNA-*EGFP* ($P<0.05$). The mortality plateaued on day 6-7. dsFrag.1, which caused the highest mortality, showed a significant difference ($P<0.01$) with ds*EGFP* starting on day 5. On day 7 after feeding with dsFrag.1, the mortality went up to 36.22% (Fig. 5). The most effective silencing fragment was then cloned into a plant over-expression vector to express a hairpin RNA (hpRNA) in transgenic tobacco (*Nicotiana tabacum*). When *H. armigera* larvae were fed the *E. coli* or transgenic plants, the *HaHR3* mRNA and protein levels dramatically decreased, resulting developmental deformity and larval lethality (Fig. 6). The results demonstrate that both recombinant bacteria and transgenic plants could induce *HaHR3* silence to disrupt *H. armigera* development, transgenic plant-mediated RNAi is emerging as a powerful approach for controlling insect pests (Xiong *et al*., 2013).

Bacterial disease resistance

Crown gall disease, caused by the soil bacterium *Agrobacterium tumefaciens*, results in significant economic losses in perennial crops worldwide. *A. tumefaciens* is one of the few organisms with a well characterized horizontal gene transfer system, possessing a suite of oncogenes that, when integrated into the plant genome, orchestrate *de novo* auxin and cytokinin biosynthesis to generate tumors. Specifically, the *iaaM* and *ipt* oncogenes which show 90% DNA sequence identity across studied *A. tumefaciens* strains are required for tumor formation. By expressing two self-complementary RNA constructions designed to initiate RNA interference of *iaaM* and *ipt* (Fig. 7). They generated transgenic *Arabidopsis thaliana* and *Lycopersicon esculentum* plants that are highly resistant to crown gall disease development.

In *in vitro* root inoculation bioassays with two biovar I strains of *A. tumefaciens*, transgenic *Arabidopsis* lines averaged 0.0–1.5% tumorigenesis, whereas wild-type controls averaged 97.5% tumorigenesis. Similarly, several transformed tomato lines that were challenged by stem

Fig. 9. Resistance to ACMV infection of cassava (dsAC1-2: Transgenic cassava plant)

Table 3. RNA-mediated improvement of important traits in various crop plants

Host plant	Targeted genes	Nutritional value	Reference
Cotton	*d-cadinese synthase*	Reduction of toxic gossypol in seeds	Sunilkumar *et al*., 2006
Wheat	Starch branching enzyme (SBEIIa & SBEIIb)	High amylase	Regina *et al*., 2006
Coffee	*Theobromine synthase*	Decaffeinated coffee plant	Ogita *et al*., 2004
White pea	Gens responsible for BOAA	Non-toxic	Williams *et al*., 2004

Fig.10. Commercially available blue rose 'Applause' variety.

inoculation with three biovar I strains, one biovar II strain, and one biovar III strain of *A. tumefaciens* displayed between 0.0% and 24.2% tumorigenesis, whereas controls averaged 100% tumorigenesis (Fig. 8). This mechanism of resistance, which is based on mRNA sequence homology rather than the highly specific receptor-ligand binding interactions characteristic of traditional plant resistance genes, should be highly durable. If successful and durable under field conditions, RNAi-mediated oncogene silencing may find broad applicability in the improvement of tree crop and ornamental rootstocks (Escobar *et al*., 2001).

Viral disease resistance

African cassava mosaic virus (ACMV) is a causal organism of cassava mosaic disease (CMD). The transgenic cassava plants resulting from RNA silencing technology by targeting the viral mRNAs of Rep (AC1), TrAP(AC2) and REn (AC3) showed increased ACMV resistance. The full expression of the antisense RNAs was ensured by inserting the full coding sequences of Rep, REn and TrAP separately in antisense orientation, into the 32 untranslated region (32 UTR) of a *hygromycin phosphotransferase* gene *(HPT)* under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The transformed plants were symptomless after ACMV infection of plants at lower infection pressure (100 ng viral DNA/ plant). Transgenic ACMV-resistant plants had significantly reduced viral DNA accumulation in their infected leaves. Short sense and antisense RNAs specific to AC1 were identified in transgenic lines expressing AC1 antisense RNA, suggesting the role of short RNAs mediate interference by posttranscriptional gene silencing (Fig. 9). These results successfully demonstrated resistance to ACMV infection of cassava by expressing antisense RNAs against viral mRNAs encoding essential nonstructural proteins, providing a new tool to combat CMD (Vanderschuren *et al*., 2007).

Enhancing nutritional qualities

Increasing carotenoid and flavonoid content in tomato

The carotenoids and flavonoids are well known for their various health benefits. One of the dietary sources of carotenoids and flavonoids are tomatoes. Thereby, genetic manipulations in tomato were considered as the possible way for increasing their content, ensuring higher levels of these constituents in diet. Generally the overexpression of genes encoding biosynthetic enzymes or transcription factors were known for improving the carotenoid or flavonoid content individually, but not in combination. RNAi-mediated suppression of an endogenous photomorphogenesis regulatory genIe *DET1*using fruit-specific promoters significantly increased carotenoid and flavonoid in the fruit without affecting the level of other components. This was the first report of simultaneous elevation of flux through two independent health-related biosynthetic pathways with no negative collateral effects on fruit yield or quality (Davuluri *et al*., 2005).

Attractive flower colour

Blue rose

Roses are famous for their beautiful colours including

red, pink, orange, yellow and even white. These colours have been developed through traditional breeding but never has a blue rose successfully been bred. One gene involved in flower colour, is the *dihydroflavonol reductase (DFR)* gene. The *DFR* gene makes the enzyme *dihydroflavonol reductase (DFR)* which turns on the manufacturing process in the plant that produces pigment that in turn colours flowers. In roses the *DFR* gene is very good at producing red pigment and hence the range of commonly seen rose colours. However, the rose *DFR* gene is particularly bad at producing blue pigment; hence the difficulty in breeding a blue rose.

The first critical step in producing a blue rose was to stop the rose *DFR* gene making red pigment. CSIRO first developed gene silencing, or hairpin RNAi, in 1997. It was a significant breakthrough allowing scientists to turn down or switch off completely the activity of genes. Florigene and Suntory used CSIRO's gene silencing technology to turn off the activity of the rose *DFR* gene so that it didn't produce red pigment. With the red pigment production turned off using CSIRO's gene silencing technology and the final task was to find a *DFR* gene good at producing blue and placing it in the rose. Florigene and Suntory replaced the rose *DFR* gene with a *DFR* gene from an iris, which is excellent at producing blue pigment. The iris *DFR* gene was inserted into the rose and subsequently a rose with a blue flower was produced.

The Japanese company that successfully created the blue rose, Suntory Ltd. along with Florigene Ltd. of Australia, hold the license for producing it. The first ones were released commercially in Japan in November 2009, with a sales price of $22 to $32 U.S. each. Suntory blue rose Applause represents successful joint development by Suntory Holdings Limited and Florigene, an Australia-based Suntory Group subsidiary (Fig. 10). Never before have roses with nearly 100% blue pigment in the petals been grown anywhere in the world (Katsumoto *et al*., 2007).

Ongoing projects in India on RNAi

Table 4. Ongoing projects in India on RNAi in different institutes

Institutes	Ongoing project (http://dbtindia.nic.in/index.asp)
IARI, New Delhi	Plant-nematode interaction
GKVK, Bangalore, Karnataka	Functional characterization of water stress genes
RGCB Thiruvanantapurm Kerala	RNAi approaches for validation of defence related genes for resistance from wild pepper-*Piper colubrinum*
MAU, Parbhani, Maharashtra	Development of RNAi-mediated resistance in sunflower against sunflower necrosis virus (SNV)
CICR, Nagpur, Maharashtra	Identification of species specific dsRNA or siRNAs in cotton insect pests to explore their use in pest management through RNAi based technologies
TNAU, Coimbatore, Tamilnadu	Identification of molecular targets for management of crop pests using RNAi approach
JNU, New Delhi	Molecular mechanism of PTGS mediated host symptom recovery associated with tomato leaf curl virus infection.

Limitations of RNAi

Despite the impressive list of potential advantages, RNAi is not without a similarly long list of potential disadvantages that need to be borne in mind (and preferably eliminated or worked-around).

Off-target effects

It has become clear from the large-scale screens in animals that significant effect on the expression of genes that were not the predicted targets of RNAi can occur. The major difficulty is the limited sequence specificity of siRNAs as few as seven nucleotides of sequence complementarity between a siRNA and mRNA can lead to the inhibition of expression (Escobar *et al*., 2001). The possibility of productive siRNA–mRNA interactions despite scattered mistmatches in the paired region makes the prediction of potential off-target effects very difficult. It has been often repeated that such problems are much less frequent in plants, but this might be simply because no systematic studies have yet been completed. The isolated reports to date conclude that RNAi in plants exhibits much greater sequence specificity.

A completely different type of off-target effect can occur owing to the inhibition of natural miRNA or siRNA regulation through saturation of the pathways with exogenous or transgene siRNAs. This has again been observed in animal systems, but there are no published reports so far of this effect in plants. In fact, even when specifically searched for, no off-target effects of any type were observed, even when very strong promoters were used to drive hairpin RNA production (Ma *et al*., 2006).

Transitive silencing

A second specificity problem can occur via transitive silencing, whereby RNAi against a gene-specific sequence spreads into neighbouring sequences conserved between the target mRNA and mRNAs from related genes, which become silenced in turn (Bleys *et al*., 2006). Such transitive silencing results from the action of RNA-dependent RNA polymerases that maintain and amplify the RNAi signal in some pathways. However, transitive silencing appears to occur rarely even when suitable conserved sequences exist in the targeted RNAs.

Inefficacy and instability

A mutation at the DNA level (base change, deletion or insertion) is almost always irreversible (except in the special case of some natural transposon insertions) and the effect on the function of the affected gene generally predictable (i.e. premature stop codons or insertions into the middle of a gene usually lead to null phenotypes). By contrast, RNAi inhibition can have widely varying effects depending on the target gene, the region of the transcript that is targeted and even between sibling plants carrying identical RNAi constructs (Wang *et al*., 2005). The reasons for this variability are multiple and need to be considered when interpreting RNAi phenotypes. Firstly, short siRNAs might target a part of the mRNA that is masked by secondary structure or bound proteins, thus reducing efficacy through inaccessibility of the target site to siRNAs. Secondly, inefficacy could result from an inability to reduce translation to the point where a phenotype ensues; for example, some genes can have their expression reduced drastically without generating the phenotype.

Lastly, instability can result from silencing of the transgene long hairpin transgenes appear to be particularly sensitive to transcriptional silencing leading to a loss of RNAi phenotypes over several generations.

CONCLUSION

Plant diseases are critical problems in agriculture. Disease occurrence may be completely excluded if preventive strategies are established. Plant variants that possess increased natural resistance could substitute for susceptible cultivars. Although this "classical breeding" is a powerful method to produce the resistant plants, it is usually costly and time consuming work. In addition, features such as crop quality and quantity may be compromised by breeding for resistance. In contrast, genetic manipulation is a relatively rapid method to introduce the disease resistance. This is especially advantageous for diseases that suddenly emerge. Transgenic plants expressing the RNA-silencing pathway have been shown to efficiently resist pathogen infection. This pathway perhaps represents the most specialised molecular strategy that plants use to combat pathogens. Thus, RNA-silencing-based approaches might be an effective way of reducing crop loss caused by pathogens. The past decade has witnessed a rapid advance in our understanding of RNAi. It is a very efficient knockdown technology in plants as it is thought to be useful for genetic improvement; RNA silencing is an area of intense investigation that is leading to exciting new discoveries in the fields of control of gene expression, development and host defence. An offshoot of this research is the use of RNAi as a tool to engineer plants by specifically silencing target genes or their promoters.

However, recent advances have brought high expectations for the future role of RNA-mediated resistance in crops. Once the novel resistance performances are in line with these expectations, this technology will create a new era in plant disease management, and its application will be extended to the commercial product in agriculture crops, at the same time further feasibility studies are needed for its wider application in future.

DISCUSSION

RNA silencing has emerged as an area of intensive investigations leading to new discoveries in the control of gene expression. This seminar report provides an update on the mechanisms of RNAi-mediated gene silencing and implication of this valuable technology for the development of transgenic crop plants, with a focus on nutritional enrichment and plant protection from bacteria, nematodes, fungi, and insects pests, which are the potential issue in production and productivity of agriculture crops. Since its advent, many advances have been revealed in understanding of RNAi-mediated gene suppression resulting in variety of applications of this technology in the diverse group of organisms.

This technique is being successfully used in the modulation of biosynthetic pathways in a specific tissue to obtain a desired phenotype, which was a rather time-consuming process with the traditional breeding programs. RNAi is a homology-dependent process; thereby, selection of a unique region of the target sequence can ensure that a specific gene family member is silenced. The transgenic strategies involving RNAi seems to be more acceptable as compared to currently used genetically modified crops as they are based solely on the use of plant-derived sequences. Transgenic plants producing Bt toxins have proven to be successful in controlling insect pests of many crop plants; however, there have been sporadic reports of development of resistance against Bt toxin. For the insect control, the RNAi technology could therefore be considered in tandem with Bt technology.

In the area of insect pest control, there are brighter horizons from this technology. There are number of studies on the different categories of insects, which damage the crop plant globally. Although variations in RNA oligonucleotides may be an important factor to explain differences in the amount of dsRNA in mammals and insects, thorough experimental outputs are still required in insects, in cases where high amounts of dsRNA are used to achieve silencing. Viruses have been the obvious targets for RNAi technologies, as most viruses have single-stranded RNA genomes. Currently, transgenic lines of several crop plants have been field tested or commercially released and continue to show very strong resistance to several plant viruses.

Ensuring food security in the present scenario of ever increasing population is the biggest challenge to the global agriculture. The RNAi technology has been continuously applied in the recent past for generation of new crop quality traits and plant from viruses, insects, nematodes, and other pathogens without introduction of new proteins. We have now much more understanding of the endogenous gene-

silencing mechanism, providing knowledge and efficiency that can be used to develop precisely targeted gene-silencing approaches for various requirements. RNAi-mediated gene silencing has advantages over antisense and cosuppression due to their higher silencing efficiency and shortened time period for screening for the targeted plants. The use of tissue-specific and inducible promoters has improved our ability to silence gene expression in only the target tissues and consequently addressed the issue of "off-target" effects. The utilization of RNAi technology with organ or tissue-specific expression is more appealing for the generation of traits, which were not feasible by other conventional approaches. The manipulation of a plant regulatory gene can simultaneously influence the production of several phytonutrients generated from independent biosynthetic pathways and provide a novel way of increasing food value. Now, with the advancement of this technology, there is the possibility of elimination of harmful compounds from the edible plant parts. In the different parts of the world, the traditional crops like fieldbeans, faba beans, and grass pea are still consumed in the routine cuisine by the poor people, which ultimately results in different disorders like lathyrism. Therefore, with the utilization of these novel techniques of RNA interference, we can, to some extent, ensure the removal of naturally occurring toxic/antinutrients compounds from the edible portion of the plant. This will also help in maintaining the cultural food of the people, which are often good source of nutritional components. To emphasize the implication of this technique for the so called less utilized or underutilized crops will somewhat ensure the nutritional security for the people especially in the developing countries where the traditional crop is only cheap source of food. Generally, the occurrence of one or combination of antinutritional components has often overshadowed the nutritional superiority and neutraceutical value of some of the traditional crops. Once the antinutrients level of these crops is dealt with, the nutritional excellence will attract other consumers, too. Therefore, the improvement in the nutritional status of the less known but promising food crops with the RNAi technology is sure to have global humanitarian and economic implications globally. The use of RNAi technology to increase the nutritional value of edible plant parts is continuously increasing, with many successful results being published each year. Moreover, the transgenic plants as outcome of this technique would be cost effective by producing RNAi inducers throughout a plant's entire life. However, continuous research efforts are required for modification of crop plants through RNAi to meet the ever increasing needs of growing human population. This technique is expected for many more deliverables for the nutritional security in coming future.

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