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RNA Interference: An Eco-Friendly Tool for Plant Disease Management

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ABSTRACT

Efficient control of plant pathogens affecting economically important crop species represents one of the major challenges for sustainable agriculture production. Though plant breeding has been the classical means of manipulating the plant genome to develop resistant cultivar for controlling plants diseases, the advent of genetic engineering provides an entirely new approach. Currently, the area planted with crops genetically modified for resistant to disease is less compared with that of crops for tolerance to herbicide, or resistant to insects. Numerous strategies are being pursued to render plants resistant to fungi, bacteria, viruses and nematodes. Recently, RNA interference (RNAi) technology has emerged to be a promising therapeutic weapon to mitigate the inherent risks like use of specific transgenes, marker genes or gene control sequences associated with development of traditional transgenics as disease-resistant transgenic plants can be produced within a regulatory framework. The advantage of RNAi as a novel gene therapy against fungal, viral and bacterial infection in plants lies in the fact that it regulates gene expression via mRNA degradation, translation repression and chromatin remodeling through small non-coding RNAs. Mechanistically, the silencing processes are guided by processing products of the dsRNA trigger, which are known as small interfering RNAs (siRNAs) and microRNAs (miRNAs). The application of tissue-specific or inducible gene silencing, with the use of appropriate promoters to silence several genes simultaneously should enhance researchers' ability to protect crops against destructive pathogens. This review updates the current state on the use of RNAi, molecular principles underlying the biology of this phenomenon, development of RNAi technologies in relation to plants and discusses strategies and applications of this technology in plant disease management to save the green world from pathogenic intruders in eco-friendly manner.

Keywords: disease management, plant pathogens, RNA interference, siRNA, transgenics

Abbreviations: **ABA**, absciscic acid; **ACMV**, *African cassava mosaic virus*; **AGO**, Argonaute; ***AtRdRP***, Arabidopsis RNA-dependent RNA polymerase; **bp**, base pair; **CaLCuV**, *Cabbage leaf curl virus*; ***Chl***, chalcone synthase; **CMV**, *Cucumber mosaic virus*; **CP**, coat protein; **DCL**, dicer-like protein; **dpi**, days post infection; **dsRNA**, double-stranded RNA ; **ds-siRNAs**, double-stranded small interfering RNAs; **eIF2C**, elongation factor 2C; ***GFP***, green fluorescent protein; ***HC-Pro***, helper-component proteinase; **HIV**, *Human immunodeficiency virus*; **hpRNAs**, hairpin RNA; **kDa**, kilo Dalton; **LISW**, laser-induced stress wave; **miRISC**, micro RNA-induced silencing complex; **miRNAs**, microRNAs ; **mRNA**, messenger RNA; **MSP**, major sperm protein; **MSV**, *Maize streak virus*; **MYMIV**, *Mungbean yellow mosaic India virus*; **nt**, nucleotide; **PME**, pectin methylesterase; ***Pol*IV**, RNA *Polymerase* IV; **pri-tasiRNAs**, primary transacting siRNAs; **PTGS**, posttranscriptional gene silencing; ***PVX***, *Potato virus X*; **RDR**, RNA-dependent RNA polymerase; **RdRP**, RNA-dependent RNA polymerase; **RISC**, RNA-induced silencing complex; **RITS**, RNA-induced transcriptional silencing complex; **RLC**, RISC loading complex; **RMD**, RNA-mediated defense; **RNAi**, RNA interference; **rRNA**, ribosomal RNA; **SARS-Cov**, severe acute respiratory syndrome-associated coronavirus; **SDE**, silencing defective; **SGS**, suppressor of gene silencing; **siRISC**, small interfering RNA-induced silencing complex; **siRNAs**, Small interfering RNAs; **ssRNA**, single-stranded RNA; **ta-siRNAs**, transacting siRNAs; **T-DNA**, transfer DNA; **TGMV**, *Tomato golden mosaic virus*; **TGS**, Transcriptional gene silencing; **TMV**, *Tobacco mosaic virus*; **tRNA**, transfer ribonucleic acid; **TRV**, *Tobacco rattle virus*; **VIGS**, virus-induced gene silencing

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Review

INTRODUCTION

Importance of plant disease management in crop production and in food security has never been greater than at present as humankind faces the challenge of feeding the current world population of 6.2 billion with a projected increase of 10 billion by 2050 (US Bureau Census, International Data base 2000). Plant pests and pathogens reduce 30% of the world food (Rhilla *et al.* 2001). To offset these crop losses from pathogens, various attempts have been made in the field of disease management since the inception of the green revolution. However, during the last two decades much attention has been paid to integrated disease management practices which make disease control inexpensive and safe. Plant breeding has been the classical means of manipulating the plant genome to develop resistant cultivars for controlling plants diseases. However, the advent of genetic engineering provides entirely new approaches like use of DNA markers for identification, mapping, cloning and transformation of economically important gene (s). Although, currently the area planted with crops genetically modified for resistant to diseases specially viruses is small (<0.1 mha) compared with that of crops for tolerance to herbicide or resistant to insects (102 mha) (James 2007). Different transformation strategies are being pursued during these days to render plants resistant to various plant pathogens viz. fungi, bacteria, viruses and nematodes. The risks associated with the use of transgenic crop plants for agricultural crop production are related to the use of a specific transgene, marker gene or gene control sequences, whereas other concerns address the entire approach of engineering heterologous genes into plants (de Boer 2003). The study of genetic host resistance fulfils this requirement but is being a continuous endeavor as the boom and bust cycle goes on in the process of co-evolution, though therapeutic tools based on current molecular biology hold the key after the exploitation of traditional breeding and biotechnological methods like use of molecular marker for identification, mapping, cloning of pest and disease resistant genes and their utilization by introgression, pyramiding and development of transgenics. The inherent risks associated with traditional transgenics can be mitigated by new and innovative strategies and transgenic plants can be produced within a regulatory framework. In this context, different RNA molecules are known to carry out multiple functions in the molecular cell biology. The messenger RNA (mRNA) molecules carry the translatable information from DNA to the translational machinery and ribosomal RNA (rRNAs) and transfer RNA (tRNAs) form essential components of this machinery. RNA also has essential role as component of some RNA-processing or DNA repair enzymes (Storici 2008).

During the last decade, our knowledge repertoire of RNA-mediated functions has largely increased, with the discovery of small non-coding RNAs which play a central part in a process called 'RNA silencing'. RNA silencing, induced either by double-stranded RNAs (dsRNAs) or targeted to homologous RNA and DNA sequences, is a complex surveillance and regulatory process. It mediates the post-transcriptional repression of the target gene expression and represses the proliferation and expression of different invading nucleic acids, such as viruses, viroids, transposons or transgenes. Its functional components and mechanisms have been intensively studied in different organisms, such as *Caenorhabtitis elegans*, *Drosophila melanogaster*, and vertebrates, including humans, *Neurospora* fungi and *Saccharomyces pombe* yeast and in plants, using *in vitro* assays and sequence comparisons (Pasquinelli *et al*. 2000; Rhoades *et al*. 2002; Tang *et al*. 2003; Bartel and Bartel 2003; Lewis *et al*. 2003; Moss and Tang 2003; Jones-Rhoades and Bartel 2004). In animals this phenomenon has been named RNA interference (RNAi) (Fire *et al*. 1998; Hammond *et al*. 2000), in fungi it is called quelling, and in plants, co-suppression or post-transcriptional gene silencing (PTGS) (Cogoni and Macino 1999; Catalanotto *et al*. 2000; Fagard *et al*. 2000; Fulci and Macino 2007). Specifically, when induced by replicating viruses, RNA silencing is called RNA-mediated defense (RMD), and when virus-vectors are used as tools to target silencing to an inserted sequence, the process is called virus-induced gene silencing (VIGS) (Baulcombe 1999; Ratcliff *et al*. 1999; Lu *et al*. 2003; Baulcombe 2004).

RNA silencing or PTGS exploits an endogenous gene regulatory mechanism of eukaryotic cell in which dsRNAs interfere with homologous mRNA either by triggering its degradation or inhibiting its transcription or translation (deBakker *et al*. 2002; Almeida and Allshire 2005), whereby susceptible genes can be silenced. This RNA-mediated gene control technology has provided new platforms for developing new eco-friendly molecular tools for crop improvement by suppressing the genes responsible for various stresses susceptibilty and improving novel traits in plants including disease resistance and will become a promising future therapeutic agent to combat different plant invaders. It has emerged as a method of choice for gene targeting in fungi (Nakayashiki 2005), viruses (Baulcombe 2004), bacteria (Escobar *et al*. 2001) and plants (Brodersen and Voinnet 2006) as it allows the study of the function of hundreds of thousands of genes to be tested (Godge *et al*. 2008). Since a gene can be silenced either throughout an organism or in specific tissues (Islam *et al.* 2005), it offer the versatility to partially silence or completely turn off genes, in cultured cells or whole organisms and can selectively silence genes at particular stages of the organism's life cycle (Milhavet *et al.* 2003). Due to all these elegant and unique features of RNAi, our review specifically focuses on the concept of RNAi, its pathways and induction in plants, evolution to a host-pathogen system and we present an overview, when, where and how this technology can be exploited for more effective plant disease management.

RNAi: THE CONCEPT

'RNA interference' refers collectively to diverse RNA-based processes that all result in sequence-specific inhibition of gene expression, either at the transcription, mRNA stability or translational levels. It has most likely been evolved as a mechanism for cells to eliminate foreign genes.

The unifying features of this phenomena includes the production of small RNAs (21-26 nucleotides (nts) that act as specific determinants for down-regulating gene expression (Waterhouse *et al*. 2001; Hannon 2002; Pickford and Cogoni 2003) and the requirement of one or more members of the Argonaute family of proteins (Hammond *et al*. 2001). RNAi operates by triggering the action of dsRNA intermediates, which are processed into RNA duplexes of 21-24 nts by a ribonuclease III-like enzyme called Dicer (Fire *et al*. 1998; Bernstein *et al*. 2001; Wesley *et al*. 2001). Once produced, these small RNA molecules or short interfering RNAs (siRNAs) are incorporated in a multi-subunit complex called RNA induced silencing complex or RISC (Hammond *et al*. 2000; Tang *et al*. 2003). RISC is formed by a siRNA and an endonuclease among other components. The siRNAs within RISC act as a guide to target the degradation of complementary messenger RNAs (mRNAs) (Hammond *et al*. 2000; Tang *et al*. 2003). The host genome codifies for small RNAs called miRNAs that are responsible for endogenous gene silencing. 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 (Voinnet 2005). 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. The silencing process is also enhanced by the enzymatic activity of the RISC complex by mediating multiple turnover reactions (Hutvagner and Zamore 2002; Tang *et al.* 2003). Furthermore, production of the secondary siRNAs leads to enrichment of silencing via its spread from the first activated cell to neighboring cells, and systemically through the system (Himber *et al.* 2003). 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 nts) are the key components for systemic silencing signal (Xie and Guo 2006). 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 (Li and Ding 2006).

KEY PLAYERS OF RNAi

Different RNA molecules provide specificity to guide various activities of RNAi machinery including RNA cleavage (El-bashir *et al*. 2001; Llave *et al*. 2002; Hily and Liu 2007), translational repression (Doench *et al*. 2003) and methylation of chromatin (Volpe *et al*. 2002; Chan *et al*. 2004) are outlined in **Fig. 1**. The biogenesis of various RNA molecules and their roles in RNAi machinery along with other key players are briefly described in the following sections.

Small interfering RNA (siRNA)

This species of RNA is produced as populations from long dsRNAs that result from read through or bi-directional transcription of DNA repeats or transposon loci, and from the action of host-encoded RNA-dependent RNA polymerases that synthesize complementary strands from cellular RNAs (Dalmay *et al*. 2000b; Mourrain *et al*. 2000). siRNAs can be generated from transgenic pan-handled transcripts that are used to provide experimental RNAi (Waterhouse and Helliwell 2003). Endogenous siRNAs either direct the endonucleolytic cleavage of homologous transcripts (trans-acting siRNAs) (Peragine *et al*. 2004; Vazquez *et al*. 2004b) or promote DNA methylation and heterochromatin formation at the genetic loci from which they originate (*cis*-acting siRNAs) (Xie *et al*. 2004), often results in transcriptional gene silencing (TGS). *Cis*-acting siRNAs are produced in the nucleus by DCL3 (dicer-like protein 3), whereas *trans*-acting siRNAs require DCL1 for their biogenesis (Xie *et al*. 2004).

MicroRNA (miRNA)

It constitutes another class of endogenous small RNA. These molecules are excised by DCL1 from nuclear and non-coding precursor transcripts, of approximately 70-200 nts in length, which acquire a partial stem-loop structure (Dunoyer *et al.* 2005). Mature miRNAs are cytoplasmic and direct the cleavage or translational repression of mRNAs that carry discrete complementary target sites (Bartel and Bartel 2003; Bartel 2004). These miRNA sequences reside between protein coding genes or within introns (Bartel and Bartel 2003).

Intially, miRNA targets identified in plants were a series of evolutionarily conserved transcription factors that control important developmental fates (Rhoades *et al*. 2002), but later advancement in the work indicated that miRNAs regulate many other biological processes (Jones-Rhoades and Bartel 2004; Sunkar and Zhu 2004). Moreover, it has been reported that gene inversion or duplication events can generate species-specific miRNAs that probably contribute to the ability of plants to adapt to their environment (Allen *et al*. 2004; Voinnet 2004).

Transacting siRNA (tasiRNA)

tasiRNA is a class of plant endogenous small RNA (sRNA) which evolves from non-coding single-stranded transcripts called the pri-tasiRNAs. These are converted into dsRNA by *RDR6–SGS3*, giving rise to siRNAs as discrete species in a specific 21-nt phase (Peragine *et al*. 2004; Vazquez *et al*. 2004; Brodersen and Voinnet 2006). Much like plant miRNAs, mature tasiRNAs guide cleavage and degradation of homologous cellular transcripts. The tasiRNA generating loci (TAS1–3) have been identified in *Arabidopsis* (Allen *et al*. 2005) and probably also exist in other plant species and organisms that contain RNA-dependent RNA polymerase (RDRs), such as *C. elegans* or *Neurospora crassa*. tasiRNA production involves an interesting mix of miRNA action and the siRNA biogenesis machinery (Brodersen and Voinnet 2006). Pri-tasiRNAs contain a binding site for a miRNA that guides cleavage at a defined point. The initial miRNA-guided cut has two important consequences. First, it triggers RDR6-mediated transitivity on the pri-tasiRNA cleavage products, allowing dsRNA production either 50 or 30 of the cleavage site (Allen *et al*. 2005) and secondly, it provides a well defined dsRNA terminus, which is crucial for the accuracy of a phased dicing reaction performed by DCL4, which produces mature tasiRNAs.

RNA-dependent RNA polymerase (RDR)

Since silencing is triggered by dsRNA, conversion of ssRNA sequences to ds form is prerequisite for silencing of endogenous transcripts. In *Arabidopsis*, RDR function is mediated by proteins encoded by the SILENCING DEFECTIVE1 (SDE1)/ SUPPRESSOR OF GENE SILENCING2 (SGS2) RDR genes, referred as RDR6 (Xie *et al*. 2004) and the SDE3 RNA helicase gene (Mourrain *et al*. 2000; Dalmay *et al*. 2000b, 2001; Tang *et al*. 2003). However, these gene functions are not needed for virus-induced PTGS (Dalmay *et al*. 2000b; Voinnet 2001), or for PTGS of transgenes that are transcribed into sense-antisense RNAs forming dsRNA structures (Waterhouse *et al*. 2001; Beclin *et al*. 2002). But host-encoded and pathogenesis-inducible RDR, identified as NtRdRP1 in tobacco (Xie *et al*. 2001), and as *AtRdRP1* in *Arabidopsis* (Yu *et al*. 2003), are known to enhance the silencing-based defense reaction against different RNA viruses. This suggests that the dsRNA accumulation may be the rate-limiting step in the silencing-based defense reaction.

One of the silencing pathways leading to DNA methyla-

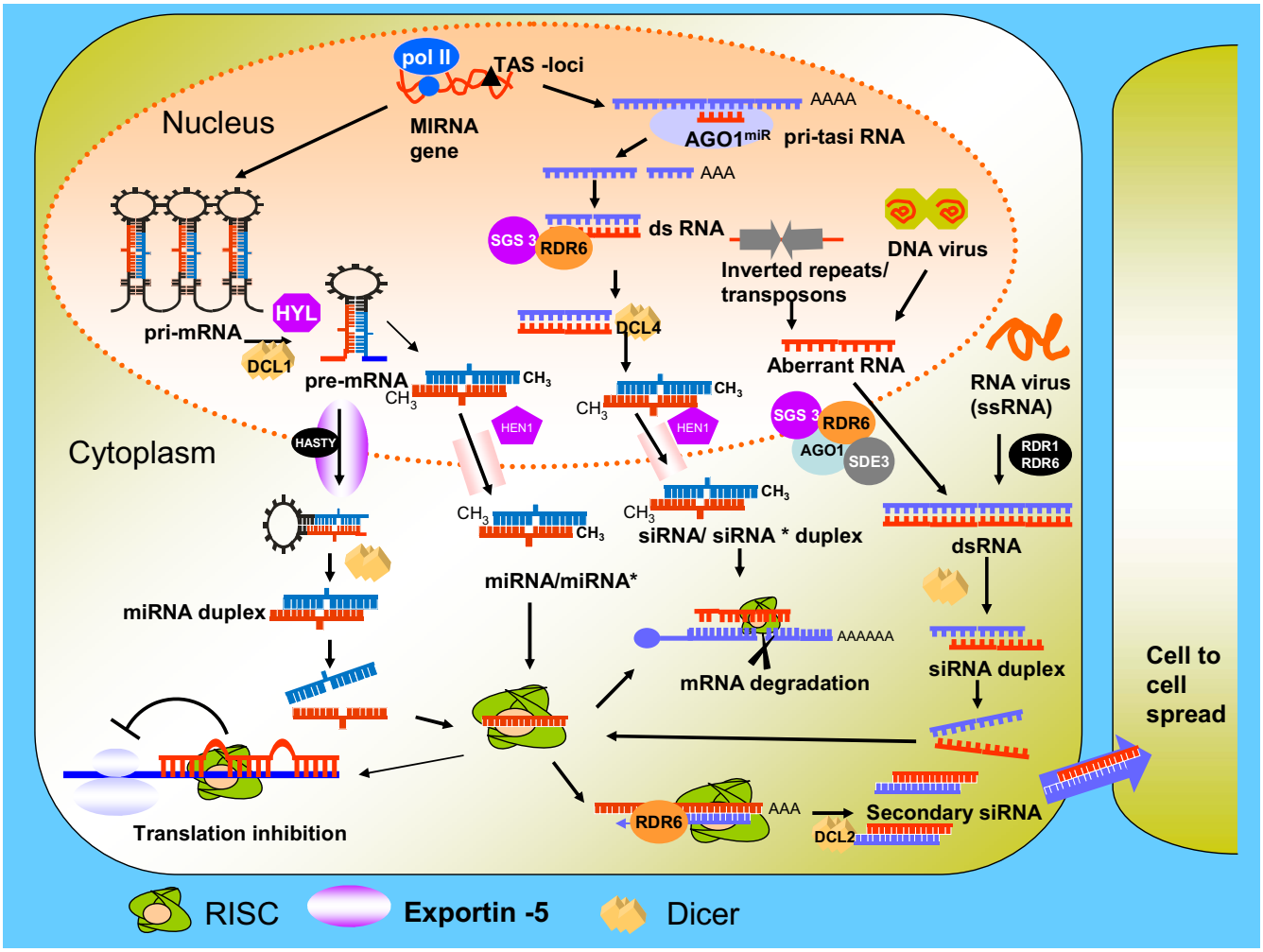

Fig. 1 Different RNAi pathways in plants. 1) miRNA and tasi-RNA biogenesis regulating expression of MIRNA gene and TAS loci. With the help of DCL1 and HYL1, pri-miRNA is processed to short lived miRNA: miRNA* duplex in plants and are methylated by HEN1 within the nucleus. Thereafter, the miRNA is exported to the cytoplasm by HASTY and after maturation, methylated miRNA is incorporated into a RISC. In this complex, the miRNA is capable of targeting complementary RNAs for cleavage by AGO1, and also for translational repression. Similarly in second phase, *trans*-acting small interfering RNAs (ta-siRNA) duplexes can be set by miRNA-directed cleavage of the *TAS* transcript. One strand from each siRNA duplex is stably incorporated into RISC, and the other is degraded. siRNAs in RISCs guide cleavage of complementary RNAs. 2) Heterochromatic siRNAs, endogenous siRNAs/siRNAs from transgenes or viral RNA or DNA are generated through similar or partially overlapping pathways. Long double-stranded RNA, generated through the action of RNA-dependent RNA polymerases (RDRs), and iteratively processed by Dicer-like (DCL) proteins to yield multiple siRNA duplexes. One strand from each siRNA duplex is stably incorporated into a RISC, and the other is degraded. siRNAs in RISCs guide cleavage of complementary RNAs. Pol IV is involved in heterochromatic siRNA production in plants, either transcribing the genomic DNA to produce the single-stranded RNA or transcribing the double-stranded RNA to amplify the single-stranded RNA.

tion is mediated by RNA *Polymerase* IV (Pol IV), *RDR2* and DCL3. In this pathway Pol IV and *RDR2* would synthesize dsRNA which is then cleaved by DCL3 to generate siRNA. In one other pathway, Pol IV acts together with AGO4 and one of the RDR to produce specific siRNAs. These two silencing pathways are independent in the leaves but interdependent in the flowers (Herr *et al*. 2005). Synthesis of small RNAs requires activity of specific RDRs e.g. tasiRNAs production depends on RDR6/SGS3 proteins (Allen *et al*. 2005).

Dicer

Dicer is the key enzyme initiating the RNA-silencing process. It is a dsRNA specific Ribonuclease III-like endonuclease which cleaves the target dsRNAs into fragments of 21-24 nts, leaving 3′-hydroxyl and 5′-phosphate ends, and 2 nt 3′ overhangs at the termini of the duplex (Bernstein *et al*. 2001; Lau *et al*. 2001). Dicer interacts with the RISC complex (**Fig. 2**), and after cleavage, the dsRNA fragments disassociate from dicer and become associated with the RISC. Only one strand of the miRNA fragments (the strand complementary to the target sequence), or short lived ds-siRNAs are incorporated into the RISC, where these RNAs function as sequence specific tags and target the silencing function to the homologous RNAs (Dunoyer *et al*. 2004). It is likely that assembly of these effector complexes follow a pathway that begins with binding of siRNA duplexes by the heterodimer of Dicer-2 and R2D2, a dsRNA binding protein with tandem dsRNA binding motifs, in the RISC loading complex (RLC) (Tomari *et al*. 2004) (**Fig. 2**). The Dicer-2 is required in RISC assembly downstream for siRNA production. Thereafter, RLC delivers the siRNA duplex into AGO2, which subsequently cleaves the passenger strand siRNA, triggering its dissociation from the complex and activation of RISC that contains only the siRNA guide strand (Rand *et al*. 2005).

RNA-induced silencing complex (RISC)

In order to guide suppression of homologous targets, the small (s) RNA duplex must become single-stranded which needs a less stable strand with the 5′ phosphate end of the helix that is incorporated as a guide RNA into an effector complex containing an Argonaute (AGO) protein (Schwarz *et al*. 2003; Herr 2005) (**Fig. 2**). Two types of effector complexes viz. cytoplasmic and nuclear complexes have been described for induction of RNAi. Cytoplasmic complex,

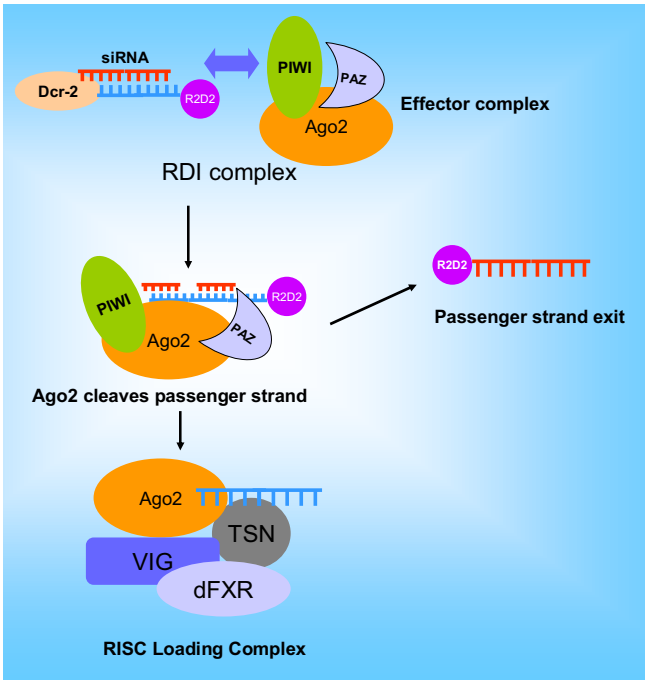

Fig. 2 Formation of RISC assembly complexes. The asymmetric siRNA molecule bound by Dcr2 and R2D2 which sense the stability at both ends of siRNA duplex. This initiation complex is known as RDI complex (Dcr2–R2D2 initiation complex).Dcr2 is eventually exchanged with Ago2, which by virtue of its PIWI domain cleaves the passenger strand which results in the formation of an active RISC-loading complex.

known as the RNA-induced Silencing Complex (RISC) (Hammond *et al.* 2000), can mediate both mRNA cleavage and translational inhibition (Sigova *et al*. 2004). RISC is a multi-subunit, large assembly of 250-500 kDa, associated with the single stranded fragments of the si- or miRNA (Nykänen *et al*. 2001; Omarov *et al*. 2007). However, the nuclear complex known as RNA-induced Transcriptional Silencing (RITS) complex was first described in fission yeast for repressing DNA expression (Verdel *et al*. 2004). Both these complexes contain ARGONAUTE (AGO) proteins, which are specific to each effector complex e.g. RITS have AGO4 and RISC involves AGO1, which are homolog of the translation initiation factor eIF2C (Hammond *et al*. 2000; Hall 2005; Song and Joshua-Tor 2006).

ARGONAUTE proteins

Argonaute proteins are the direct binding partners of siRNAs and form the core of RISC (Meister 2008). These are about 100 kDa, highly basic proteins comprising PAZ and PIWI domains (Paddison 2008). The PAZ domain, which occurs also in the Dicer enzymes mediate protein-protein interactions, and facilitate binding with the Dicer complex. PAZ and PIWI domains are responsible for 3′ 2 nt overhang recognition and endonucleolytic activities, respectively (Hammond *et al*. 2000; Hall 2005; Song and Joshua-Tor 2006) (**Fig. 2**). Due to their basic characteristics these proteins bind RNAs (e.g. siRNAs), and guide them to functional complexes (Tang *et al*. 2003; Bartel and Bartel 2003; Dugas and Bartel 2004; Lecellier and Voinnet 2004; Matzke *et al*. 2004; Vaucheret *et al*. 2004). It is now well known that AGO1, having slicer activity, is an essential component of RISCs and cleaves the target mRNAs which are homologous to the miRNA or siRNA sequences in the complex (Vaucheret *et al*. 2004; Baumberger and Baulcombe 2005; Qi *et al*. 2005; Ronemus *et al*. 2006) and AGO4 is essential for DNA and histone methylation in *Arabidopsis* (Fagard *et al*. 2000; Morel *et al*. 2002; Zilberman *et al*. 2003; Irvine *et al*. 2006). In the absence of small interfering RNAs (siRNAs), NRDE-3 resides in the cytoplasm. NRDE-3 binds siRNAs generated by RNA-dependent RNA polymerases acting on messenger RNA templates in the cytoplasm and redistributes to the nucleus. Nuclear redistribution of NRDE-3 requires a functional nuclear localization signal for nuclear RNAi, and results in NRDE-3 association with nuclear-localized nascent transcripts (Guang *et al*. 2008). The specific Argonaute proteins can transport specific classes of small regulatory RNAs to distinct cellular compartments to regulate gene expression.

Other players of RNAi

Hua enhancer 1 (HEN1), a dsRNA methylase (Park *et al*. 2002; Boutet *et al*. 2003; Xie *et al*. 2004) is required for miRNA accumulation in *Arabidopsis* and for the methylation of miRNA duplexes (Yu *et al*. 2005). HEN1 can also methylate the 3′-OH of siRNAs with less efficiency when they are in duplexes with a 2 nt overhang, and preferably of 23 nt. HEN1 is also involved in the production of siRNAs (Akbergenov *et al*. 2006; Yang *et al*. 2006). HYPONESTIC LEAF 1 (HYL1), a dsRNA binding protein, affects miRNA, but not siRNA accumulation (Anantharaman *et al*. 2002; Han *et al*. 2004a; Vazquez *et al*. 2004a; Wu *et al*. 2007). HYL1 function is also required for hormonal (auxin, ABA, and cytokinin) responses (Lu and Federoff 2000). It is noticed that some cellular proteins are also affecting the silencing processes. For instance enhanced pectin methylesterase (PME) can enhance the degradation of the viral RNAs and the RNA silencing mechanism (Dorokhov *et al*. 2006). Still some other proteins, e.g. dsRNA binding proteins (Han *et al*. 2004; Vazquez *et al*. 2004b; Kurihara *et al*. 2006), SDE3 (Dalmay *et al*. 2001), and SGS3 (Mourrain *et al*. 2000) may operate with different DCL proteins in different RNA silencing pathways (Herr *et al*. 2006).

RNAi PATHWAYS IN PLANTS

In plants, three RNAi pathways are known to occur that seem to be involved in a variety of regulatory and immune functions (Meins *et al*. 2005). The first pathway regulates gene expression by microRNAs (miRNAs) (Jones-Rhoades *et al*. 2006) and *trans*-acting smRNAs (ta-smRNAs) (Vaucheret 2006). Both species predominantly direct cleavage of near-perfect complementary target mRNAs (Vaucheret *et al*. 2004), but suppression of translation has also been observed (Chen 2004). The working of miRNA based RNAi pathway (**Fig. 1**) emerged after the expression of a specific gene "MIRNA" which is predominantly found within genomic segments previously known as intergenic regions (IGRs) (Jones-Rhoades *et al*. 2006). The expression of this gene begins with Pol II transcription to yield a primary miRNA transcript (pri-miRNA) that is capable of forming the characteristic imperfect "foldback" hairpin structure (Xie *et al*. 2005). The pri-miRNA transcript is cleaved by DCL1 in the nucleus with the help of the dsRBP, HYPONASTIC LEAVES1 (HYL1), to produce the shorter precursor miRNA (pre-miRNA) dsRNA molecule. The first DCL1-catalyzed cleavage step in the miRNA biogenesis pathway is made just below the miRNA duplex region of the dsRNA stem loop (Lu and Fedoroff 2000). The miRNA duplex is then released from the pre-miRNA stem loop structure by the second cleavage step of the miRNA pathway, which is again directed by the combined action of DCL1 and HYL1 (Vazquez *et al*. 2004). The two-nucleotide 3′ overhangs of the liberated miRNA duplex are methylated by the sRNA-specific methyltransferase HEN1. The duplexes of siRNAs are also methylated by HEN1 and assumed to protect all sRNA species from polyuridylation and degradation (Yu *et al*. 2005). The miRNA duplex is then transported to the cytoplasm, with several classes of miRNA relying on the action of the *Drosophila* Exportin-5 ortholog HASTY (HST) for nuclear exportation (Park *et al*. 2005). In the cytoplasm, the mature single-stranded miRNA is loaded onto AGO1, the catalytic center of RISC to guide the slicer activity of AGO1 to repress the expression of complementary mRNA transcripts (Jones-Rhoades *et al*. 2006) which

are predominantly mediated by transcript cleavage (Xie and Qi 2008).

In addition, ta-siRNAs are also known to play significant role in RNAi pathway which arises from defined genetic loci (TAS loci) through miRNA-dependent biogenesis pathway (Vaucheret 2006) (**Fig. 1**). The expression of ta-siRNAs is initiated by Pol II transcription to yield TAS transcripts that contain miRNA target site(s). The miRNA-directed cleavage of TAS transcript is thought to generate critical features that are recognized by RDR6 (Allen *et al*. 2005). The cleaved TAS transcript is converted into dsRNA by RDR6, a process that requires the SUPRESSOR OF GENE SILENING 3 (SGS3) (Peragine *et al*. 2004). Successive cleavage of the resulting dsRNA by DCL4 produces a phased array of 21-nt siRNAs (Xie *et al*. 2005). Some of these siRNAs (the ta-siRNAs) are incorporated into RISCs to direct the cleavage of mRNA targets (Peragine *et al.* 2004).

The second, cytoplasmic pathway protects cells against the invasion of foreign nucleic acids, such as viruses and transgenes, through the action of siRNAs that direct the cleavage of homologous RNAs (Lecellier and Voinnet 2004). Viruses engineered to contain an endogenous sequence can trigger silencing of the endogene, which is termed 'virus-induced gene silencing' (VIGS) (Ruiz *et al*. 1998; Baulcombe 1999; Godge *et al*. 2008) (**Fig. 1**). This system involves a branched pathway that converges on the production of dsRNA. Depending on the nature of the transgene and the virus, dsRNA is either formed directly by intra- or intermolecular base pairing (hpRNA constructs, IR transgenes) or indirectly through antisense transgenes or replication intermediates (RNA viruses), or through the action of RDR6 and other proteins (sense transgenes and DNA viruses) (Smith *et al.* 2000). The subsequent processing of the dsRNA into two classes of siRNAs depends on different DCL activities (Tang *et al.* 2003). DCL3 produces the long siRNAs (24-26 nt) that is involved in methylation and systemic silencing while DCL4 produces the short siRNAs (21 nt) (Dunoyer *et al.* 2005) that can either guide RISC-mediated RNA degradation or spread to adjacent cells where they trigger RDR6-dependent systemic silencing (Himber *et al.* 2003; Schwach *et al.* 2005).

The third, nuclear pathway ensures genome integrity and defense against endogenously repeated DNAs, transposons and retroelements. It is related to transcriptional gene silencing (TGS), because it involves DNA methylation and/or chromatin modification by endogenous heterochromatic siRNAs (Zilberman *et al*. 2003; Chan *et al*. 2004). The key player of this RNAi pathway is heterochromatic siRNAs which are typically 24-nt small RNAs that are associated with genomic repetitive sequences such as transposons, retroelements, rDNAs, and centromeric repeats (Hamilton *et al.* 2002; Kasschau *et al*. 2007). Heterochromatic siRNA biogenesis states that Pol IVa produces single stranded RNA transcripts from certain genomic loci including transposable elements and other repetitive regions (Lu *et al*. 2005). The Pol IVa transcripts move to the nucleolus where they are converted into dsRNAs by RDR2, and subsequently processed by DCL3 to yield heterochromatic siRNAs in the nucleolar RNA processing center that colocalizes with Cajal bodies (Li *et al*. 2006). The heterochromatic siRNAs direct DNA and histone methylation in an AGO4-dependent pathway (Zilberman *et al*. 2004). The heterochromatic siRNA-directed DNA methylation also requires the Pol IVb (Herr *et al*. 2005), the SNF2-like ATPase DRD1 (Kanno *et al*. 2005), and the *de novo* cytosine methyltransferase DRM2 (Chan *et al*. 2004). Since AGO4 has been shown to physically interact with the largest subunit of Pol IVb, and DCL3, AGO4, and Pol IVb colocalize in the nucleolar RNA processing center, Pol IVb may be part the AGO4-containing RISC in the chromatin RNA silencing pathway (Li *et al*. 2006). It has been proposed that the AGO4/ NRPD1b/ siRNA complex directs target recognition for DRD1- and DRM2-dependent DNA methylation at specific loci (Pontes *et al*. 2006). AGO6, another member of the *Arabidopsis* AGO family, has been shown to have partially redundant functions with AGO4 in heterochromatic siRNA-directed silencing (Zheng *et al*. 2007).

WORKING MODEL OF RNAi IN PLANTS

RNA interference was discovered in plants as a mechanism whereby invading nucleic acids, such as transgenes and viruses are silenced through the action of small RNA molecules (Brodersen and Voinnet 2006). The most important biochemical features of RNAi pathways in plants include the formation of double stranded (ds)RNA; processing of dsRNA to small (s) 20–26-nt dsRNAs with staggered ends; and inhibitory action of a selected sRNA strand within effector complexes acting on partially or fully complementary RNA or DNA. A simplified model for RNAi in plants is presented (**Fig. 3**), which includes four distinct phases, briefly described next.

Production of small RNAs

Two types of RNA molecules have the potential to serve as a trigger of RNA silencing. dsRNAs are precursors of siRNAs, whereas ssRNAs with step-loop structures serve as precursors of miRNAs (pre-miRNAs). *Arabidopsis* and rice genomes encode at least for four different dicer-like proteins (DCL1-DCL4) (Schauer *et al*. 2002). DCL1 produces miRNAs (Bartel 2004; Chen *et al.* 2005), DCL3 produces 24 nt long siRNAs involved in DNA methylation and in heterochromatin formation (Xie *et al.* 2004) and DCL2 cleaves natural antisense transcripts into siRNAs (Deleris *et al*. 2006). DCL4 generates siRNAs of 21 nt which mediate viral RNA silencing (Dunoyer *et al*. 2005; Gasciolli *et al*. 2005; Herr 2005; Qi *et al.* 2005; Xie *et al*. 2005). If DCL4 is not functional, then DCL2 and DCL3 produce 22 and 24 nt-long siRNAs, respectively, from viral sequences, but only siRNAs produced either by DCL4 or by DCL2 can mediate antiviral silencing. These dicers restrict virus accumulation into inoculated leaves and DCL4 produce the silencing signal which inhibit the virus spread because viral siRNAs are produced by DCL4 and DCL2 partially antagonizes the production of miRNAs by DCL1 in leaf and fully substitutes for DCL4 to produce viral siRNAs when DCL4 is inhibited by viruses (Bouche *et al*. 2006; Dunoyer *et al*. 2007).

Assembly of effector complexes

To trigger, silencing double-stranded siRNAs must be channeled through an ordered RISC assembly pathway that results in the selection of one strand and the destruction of the other (Pham and Sontheimer 2005). RISC, which directs methylation of chromatin, siRISC and miRISC, which guide cleavage and translational arrest, respectively, of target mRNAs play a significant role in assembly of effector complexes (**Fig. 2**). All of these complexes contain one guide strand of the duplex small RNAs as the specific determinants and a member of the Argonaute protein (AGO) family (Li and Ding 2006). The PIWI domain of AGOs has structural similarities to RNaseH, and its ability to cleave the target RNA base-paired with the guide strand siRNA has been demonstrated for *Arabidopsis* AGO1 (Baumberger and Baulcombe 2005).

Amplification and transitive silencing

Fungi, nematodes and plants encode eukaryotic RNA-dependent RNA polymerases (RDR) that generate new sources of dsRNA for dicing, leading to further silencing amplification. This amplification leads to the transitory nature of silencing reaction that may spread along the mRNA, though initiated by a locally targeted single siRNA (Klahre *et al.* 2002) and spreads in both the 5′ and 3′ directions (Tang *et al.* 2003). This bi-directional transition further have been witnessed by a process where both the 5′ and 3′ cleavage

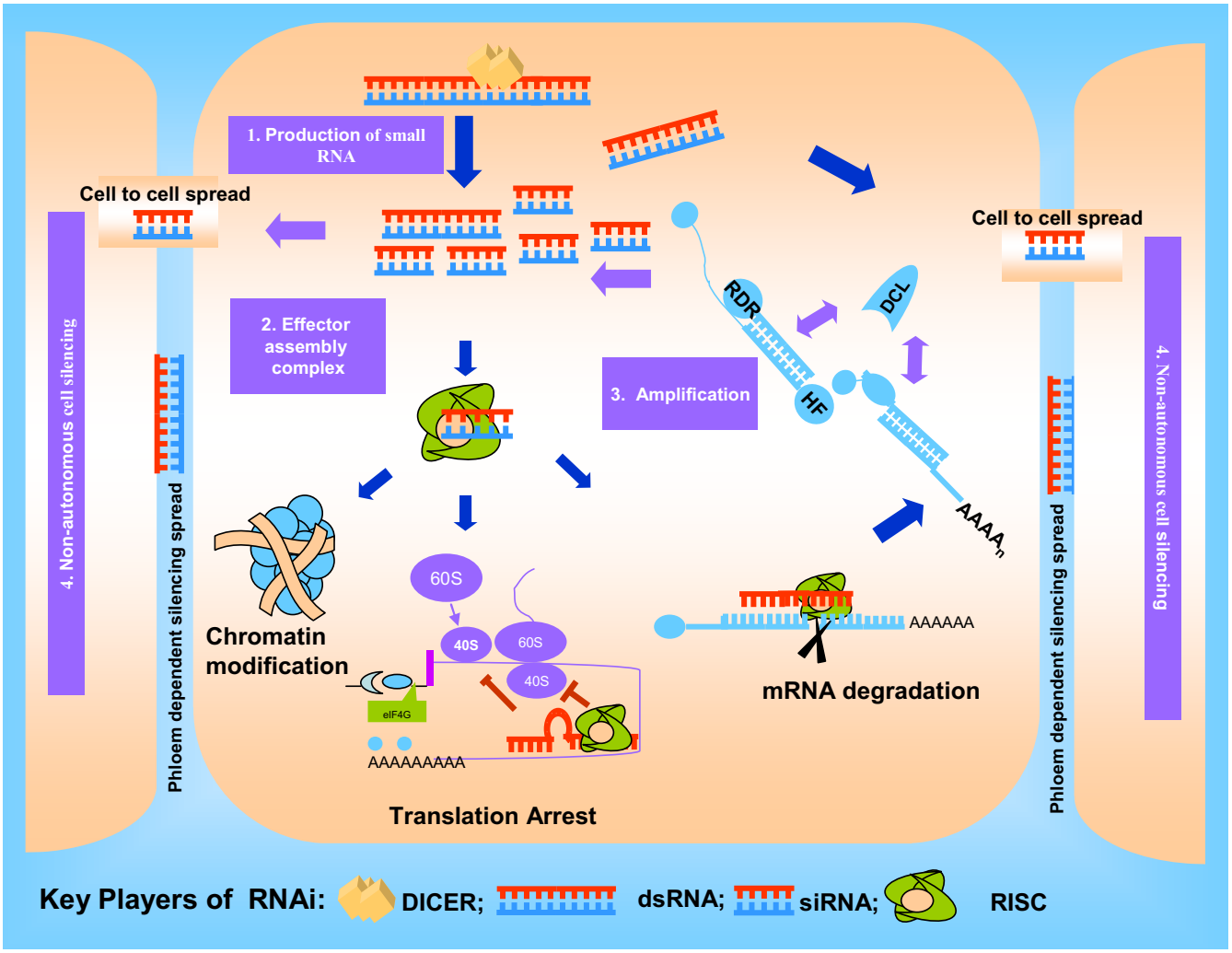

Fig. 3 Simplified working model of RNAi in plants. Most important steps include: 1) Dicing leading to the production of small duplex RNAs (21-30 nt); 2) Assembly of effector complexes guiding chromatin remodeling, translational arrest and mRNA cleavages or degradation; 3) Amplification to generate new sources of dsRNA for dicing, leading to further silencing; 4) Non-cell autonomous cell silencing beyond the sites of silencing initiation via putative silencing signals.

products of the initial target RNA act as aberrant mRNAs to trigger dsRNA synthesis (Herr *et al.* 2006), and induce secondary silencing reactions (**Fig. 4**). In both plants and *Caenorhabditis elegans*, RDR amplification results in the spread of silencing along the target gene beyond the region initially targeted for silencing of transitive RNAi (Sijen *et al*. 2001; Vaistij *et al*. 2002). RDR genes essential for RNA silencing in the germ line (*ego-1*) and somatic tissues (*rrf-1*) in *C. elegans* have been identified (Smardon *et al*. 2000; Sijen *et al*. 2001).

Non-cell autonomous silencing

The effects of RNA silencing can spread beyond the sites of silencing initiation via a putative specific silencing signal (Voinnet 2005). Two distinct steps have been observed in the spread of RNA silencing in plants (**Fig. 4**). Accumulated evidences suggest a role for 21-nt siRNAs in the short-distance spread and 24-nt siRNAs in the phloem-dependent long-distance transport (Palauqui *et al*. 1997; Hamilton *et al*. 2002; Himber *et al*. 2003). Although RDR amplification is not required for the cell-to-cell spread, extensive short-distance spread beyond 10 to 15 cells in plants requires the *RDR6*/*DCL4* pathway and its product, the 21-nt siRNAs (Himber *et al*. 2003; Dunoyer *et al*. 2005; Schwach *et al*. 2005). The cell-to-cell spread is mediated as passive spread of the small RNAs via plasmodesmata or by the silencing signal complex which is between 27 and 54 kDa (Kobayashi and Zambryski 2007). The systemic spread in phloem is mediated by the 24 nt siRNAs (Himber *et al.* 2003), unloading of the systemic signal is mediated via plasmodesmata, since it does not spread into meristematic cells (Voinnet *et al.* 2005). In contrast, a predicted role for the 24-nt siRNAs (or their longer precursor dsRNA) produced by the *RDR2*/*DCL3*/*AGO4* pathway in the long distance silencing spread remains to be rigorously examined. However, both classes of siRNAs are found in the phloem, indicating their potential to mediate silencing spread in plants (Yoo *et al*. 2004). Similarly, it is also not clear if DNA methylation associated with the maintenance or persistent silencing of transgenes play a specific role in non-cell autonomous silencing.

METHODS TO INDUCE RNAi IN PLANTS

In RNAi research field, one of the biggest challenges is the delivery of the active molecules that will trigger the RNAi pathway in plants. In this system, a number of methods for delivery of dsRNA or siRNA into different cells and tissue include transformation with dsRNA-forming vectors for selected gene(s) by an *Agrobacterium* mediated transformations (Chuang and Meyerowtiz 2000; Waterhouse *et al*. 2001); delivery cognate dsRNA of *uidA* GUS (β-glucuronidase) and TaGLP2a:GFP (green fluorescent protein) reporter genes into single epidermal cells of maize, barley and wheat by particle bombardment (Schweizer *et al*. 2000), introducing a *Tobacco rattle virus* (TRV)-based vector in tomato plants by infiltration (Liu *et al*. 2002a); delivery of dsRNA into tobacco suspension cells by cationic oligopeptide polyarginine-siRNA complex; infecting plants with

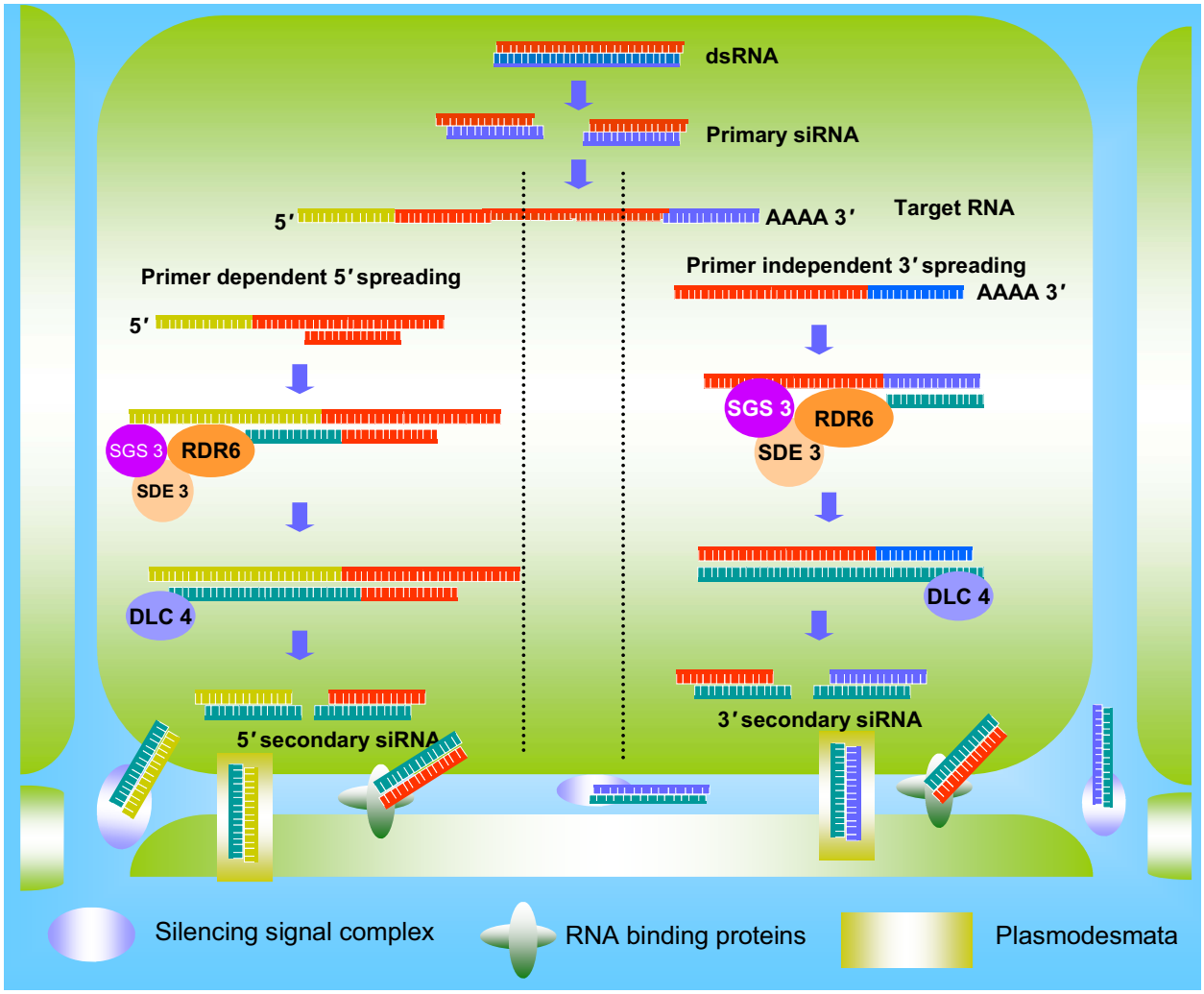

Fig. 4 Model depicting bi-directional mobilization of silencing signal in a plant cell. In transitive RNA silencing the mobile signaling molecules relay-amplified by synthesis of dsRNAs into secondary siRNAs by a process where both the 5′ and 3′ cleavage products of the initial target RNA act as aberrant mRNAs to trigger dsRNA synthesis. siRNAs is produced as shown in scheme (1) showing primer dependent 5′ spreading. Further dsRNA synthesis according to the scheme depicted in (2) primer independent 3′ spreading. DCL4 appears as putatively involved in secondary siRNA biogenesis that induce secondary silencing reactions. The cell-to-cell spread can be mediated as passive spread of the small RNAs via plasmodesmata or by the silencing signal complex.

viral vectors that produce dsRNA (Dalmay *et al*. 2000a) and delivery of siRNA into cultured plant cells of rice, cotton and slash pine for gene silencing by nanosense pulsed laser-induced stress wave (LISW) (Tang *et al*. 2006) are being used. Among these the most reliable and commonly used approaches for delivery of dsRNA to plants cells are agro-infiltration, micro-bombardment and VIGS. These are discussed in the following sections.

Agroinfiltration

Agroinfiltration is a powerful method to study processes connected with RNAi. The injection of *Agrobacterium* carrying similar DNA constructs into the intracellular spaces of leaves for triggering RNA silencing is known as agroinoculation or agroinfiltration (Hilly and Liu 2007). In most cases agroinfiltration is used to initiate systemic silencing or to monitor the effect of suppressor genes. In plants, cytoplasmic RNAi can be induced efficiently by agroinfiltration, similar to a strategy for transient expression of T-DNA vectors after delivery by *Agrobacterium tumefaciens*. The transiently expressed DNA encodes either an ss- or dsRNA, which is typically a hairpin (hp) RNA. The infiltration of hairpin constructs are especially effective, because their dsRNA can be processed directly to siRNAs, while the constructs expressing ssRNA can also be useful to induce silencing (Johansen and Carrington 2001; Voinnet 2001; Mlotshwa *et al*. 2002; Tenllado *et al*. 2003) and for dissecting the mechanism of gene silencing, especially concerned with its suppressors, systemic silencing signal and also for simple protein purification (Johansen and Carrington 2001; Voinnet 2001; Mlotshwa *et al*. 2002; Tenllado *et al*. 2003). Besides, they provide a rapid, versatile and convenient way for achieving a very high level of gene expression in a distinct and defined zone.

Micro-bombardment

In this method, a linear or circular template is transferred into the nucleus by micro-bombardment. Synthetic siRNAs are delivered into plants by biolistic pressure to cause silencing of GFP expression. Bombarding cells with particles coated with dsRNA, siRNA or DNA that encode hairpin constructs as well as sense or antisense RNA, activate the RNAi pathway. The silencing effect of RNAi is occasionally detected as early as a day after bombardment, and it continues up to 3 to 4 days post bombardment. Systemic spread of the silencing occurred 2 weeks later to manifest in the vascular tissues of the non-bombarded leaves of *Nicotiana benthamiana* that were closest to the bombarded ones. After one month or so, the loss of GFP expression was seen in non-vascular tissues as well. RNA blot hybridization with systemic leaves indicated that the biolistically delivered siRNAs induced due to *de novo* formation of siRNAs, which accumulated to cause systemic silencing (Klahre *et al*. 2002).

Table 1 RNAi effects on targeted region in some fungal plant pathogens.

Pathogen	Targeted region	Result	References
Magnaporthae oryzae	*eGFP*	Sequence specific degradation of mRNA	Kadotani *et al*. 2003
Cladosporium falvum	*cgl 1* and *cgl 2*	Blocking disease infection spread	Segers *et al*. 1999
Venturia inaequalis	*Multiple inverted repeats*	?	Fitzgerald *et al*. 2004
Fusarium graminearum	?	?	Nakayashiki 2005
Blumeria graminis	*Mlo*	Immunity	Schweizer *et al*. 2000

Virus-induced gene Silencing (VIGS)

Modified viruses as RNA silencing triggers are used as a mean for inducing RNA in plants. Different RNA and DNA viruses have been modified to serve as vectors for gene expression (Timmermans *et al*. 1994; Pogue *et al*. 2002). Some viruses, such as *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX) and TRV, can be used for both protein expression and gene silencing (Kumagai *et al*. 1995; Angell and Baulcombe 1999; MacFarlane and Popovich 2000; Mallory *et al*. 2002). All RNA virus-derived expression vectors will not be useful as silencing vectors because many have potent anti-silencing proteins such as TEV (*Tobacco etch virus*), that directly interfere with host silencing machinery (Kumagai *et al*. 1995; Palmer and Rybicki 2001). Similarly, DNA viruses have not been used extensively as expression vectors due to their size constraints for movement (Kjemtrup *et al*. 1998). However, a non-mobile *Maize streak Virus* (MSV)-derived vector has been successfully used for long-term production of protein in maize cell cultures (Kumagai *et al*. 1995). Using viral vectors to silence endogenous plant genes requires cloning homologous gene fragments into the virus without compromising viral replication and movement. This was first demonstrated in RNA viruses by inserting sequences into TMV (Dallwitz and Zurcher 1996), and then for DNA viruses by replacing the coat protein gene with a homologous sequence (Kjemtrup *et al*. 1998). These reports used visible markers for gene silencing phytoene desaturase (*PDS*) and chalcone synthase (*CHS*), providing a measure of the tissue specificity of silencing as these have been involved in carotenoid metabolic pathway. The *PDS* gene acts on the antenna complex of the thylakoid membranes, and protects the chlorophyll from photooxidation. By silencing this gene, a drastic decrease in leaf carotene content resulted into the appearance of photobleaching symptom (Liu *et al.* 2002a; Turnage *et al.* 2002). Similarly, over expression of *CHS* gene, causing an albino phenotype, instead of producing the anticipated deep orange color (Cogoni *et al*. 1994). As a result, their action as a phenotypic marker helps in easy understanding of the mechanism of gene silencing. **Table 1** shows some general characteristics for currently available virus-derived gene silencing vectors. Most viruses are plus-strand RNA viruses or satellites, whereas *Tomato golden mosaic virus* (TGMV) and *Cabbage leaf curl virus* (CaLCuV) are DNA viruses. Though RNA viruses replicate in the cytoplasm while DNA viruses replicate in plant nuclei using the host DNA replication machinery. Both types of viruses induce diffusible, homology-dependent systemic silencing of endogenous genes. However, the extent of silencing spread and the severity of viral symptoms can vary significantly in different host plants and host/virus combinations. With the variety of viruses and the diversity of infection patterns, transmission vectors, and plant defenses it is not surprising that viruses differ with respect to silencing (Teycheney and Tepfer 2001). Because the continuing development of virus-based silencing vectors can extend VIGS to economically important plants, it is useful to consider some of the characteristics of successful VIGS vectors.

RNAi AS A THERAPEUTIC TOOL FOR NEW GENERATION PLANT PATHOLOGISTS

Therapeutic applications of RNAi are potentially enormous and the idea was being tested for the last several years against human diseases. siRNA have been shown to inhibit infection by human immunodeficiency virus (HIV), poliovirus and hepatitis C virus in cultured cell lines (McManus and Sharp 2002) and respiratory syncytial virus, an RNA virus that causes severe respiratory diseases in neonates and infants (Bitko and Barik 2001) and was found to reduce the expression of the BCR-ABL oncoprotein in leukemia and lymphoma cell lines, leading to apoptosis in these cells (Wilda *et al*. 2002). In future the potential to combat carcinomas, myeloma, cancer caused by over expression of an oncoprotein or generation of an oncoprotein by chromosomal translocation and point mutations and severe acute respiratory syndrome-associated coronavirus (SARS-Cov), which is responsible for SARS infection are some of the possibilities.

The adaptation of RNAi tool in mammals and thereafter to plants has inspired the hope among plant pathologists throughout the globe that RNAi triggered by siRNAs might form the basis for a new ecofriendly tool for designing molecules or drugs capable of silencing the virus, bacteria, nematode which are destroying green agricultural world by causing serious diseases. Taking innovation from RNAi concept, this has been utilized by various researchers dealing with the management of biotic stress in important agricultural crops. The first case of gene silencing via dsRNA-triggered RNAi technology was noticed when inverted repeats were used to over express dsRNA that triggered highly efficient silencing of flower genes (Chuang and Meyerowtiz 2000). This vector-based RNAi technology was further improved by Waterhouse and colleagues by introducing an intron as the linker (Smith *et al*. 2000). These RNAi vectors were specifically designed to generate long dsRNA species that had the same sequence as the target genes. Similarly, vectors designed to express hairpin RNAs have also been successfully applied to silence the corresponding genes, while constitutive expression of dsRNA or hairpin RNA often leads to unexpected adverse effects on plants growth and development.

In addition, chemically inducible RNAi silencing vectors have been developed to enable temporal and spatial control of gene silencing (Chen *et al*. 2003; Guo *et al*. 2003). The application of such chemically inducible silencing systems to study plant functional genomics is significant, but large-scale use of chemicals for plant improvement is impractical and harmful to the environment. Tissue specific or organ specific control of gene silencing might be a better choice for the development of plant RNAi technologies.

Another approach to silence genes in plants is VIGS (Ratcliff *et al*. 2001; Dinesh-Kumar *et al*. 2003). In this approach, target genes can be transiently inactivated by infecting the plants with a recombinant virus expresses fragments of the endogenous plant gene transcripts, without introducing any genetic change in plants beyond the cells in which it is initiated. They are believed to have a role in conferring viral resistance in plants.

EVOLUTION OF RNAi IN CONTEXT TO HOST-PATHOGEN SYSTEM

The evolutionary story of RNAi began in the early 1990s with the attempts of Napoli and colleagues who tried to deepen the purple colour by introducing a *Chalcone synthase* gene in *Petunia* under a strong promoter. Contrary to expectation, the pigmentation in the flowers of transformed

plants was not enhanced. Instead, the flowers were de-pigmented and endogenous gene mRNA transcript levels were greatly reduced (Napoli *et al*. 1990). Because both the transgene and the endogenous gene were suppressed, the observed phenomenon was termed "co-suppression". Though the mechanistic aspect of this phenomenon remained unknown at that time, post transcriptional gene silencing (PTGS) was not the most accepted proposal (Napoli *et al*. 1990; Jorgensen *et al*. 1996; Cogoni and Macino 2000). This phenomenon of suppression of an endogenous gene by transformation with homologous sequences was also observed in the fungus *Neurospora crassa* where it was termed quelling (Romano and Macino 1992). However, the significance of these observations went unnoticed for several years until the mystery was solved in 1998, when it was demonstrated that dsRNA is even more effective in silencing gene expression than ss antisense RNA, a phenomenon that was termed as RNAi (Fire *et al*. 1998). Although such gene silencing can occur at the transcriptional level, it was recognized that a major mechanism of gene suppression occurs post transcriptionally and that a major mechanism of this PTGS is RNAi, the selective degradation of mRNAs targeted by siRNA (van Blokland *et al*. 1994). This mechanism was later on developed as a VIGS system based on sequence homology studies between a virus and either a transgene or an endogenous gene that would cause PTGS (Lindbo *et al*. 1993; Kumagai *et al*. 1995). In this system, a virus vector carrying a copy of the gene to be silenced is introduced into the cell, the cellular machinery recognizes the viral threat and releases a protective defense to destroy not only viral genes but also any extra-gene being carried by the viral vector, affecting any native or transgenic homologous transcripts (Ruiz *et al*. 1998; Waterhouse *et al*. 2001). Such PTGS via RNAi can occur rapidly with proteins for many genes, being decreased within hours, and completely absent within 24 h. Based upon these and other findings initially made in studies of plants, it seems very likely that RNAi evolved as a mechanism to defend plant cells against fungal, bacterial, viral and nematode infection.

RNAi IN PLANT DISEASE MANAGEMENT

Despite substantial advances in plant disease management strategies, our global food supply is still threatened by a multitude of pathogens and pests. This changed scenario warrants us to respond more efficiently and effectively to this problem. The situation demands judicious blending of conventional, unconventional and frontier technologies. In this sense, RNAi technology has emerged as one of the most potential and promising strategies for enhancing the building of resistance in plants to combat various fungal, bacterial, viral and nematode diseases causing huge losses in important agricultural crops. The nature of this biological phenomenon has been evaluated in a number of host-pathogen systems and effectively used to silence the action of pathogen. Many of the examples listed below illustrate the possibilities for commercial exploitation of this inherent biological mechanism to generate disease-resistant plants in the future by taking advantage of this approach.

Management of plant pathogenic fungi

RNA-mediated gene silencing (RNA silencing) is used as a reverse tool for gene targeting in fungi. Homology-based gene silencing induced by transgenes (co-suppression), antisense, or dsRNA has been demonstrated in many plant pathogenic fungi, including *Cladosporium fulvum* (Hamada and Spanu 1998), *Magnaporthae oryzae* (Kadotani *et al*. 2003), *Venturia inaequalis* (Fitzgerald *et al*. 2004), *Neurospora crassa* (Goldoni *et al*. 2004), *Aspergillus nidulans* (Hammond and Keller 2005), and *Fusarium graminearum* (Nakayashiki 2005) (**Table 1**), whether it is suitable for large-scale mutagenesis in fungal pathogens remains to be tested. Hypermorphic mechanism of RNA interference implies that this technique can also be applicable to all those plant pathogenic fungi, which are polyploid and polykaryotic in nature. And also offers a solution to the problem where frequent lack of multiple marker genes in fungi is experienced. Simultaneous silencing of several unrelated genes by introducing a single chimeric construct has been demonstrated in case of *Venturia inaequalis* (Fitzgerald *et al*. 2004).

HCf-1, a gene that codes for a hydrophobin of the tomato pathogen *C. fulvum* (Spanu 1997), was co-suppressed by ectopic integration of homologous transgenes. Transformation of *C. fulvum* with DNA containing a truncated copy of the hydrophobin gene *HCf-1* caused co-suppression of hydrophobin synthesis in 30% of the transformants. The co-suppressed isolates had a hydrophilic phenotype, lower levels of *HCf-1* mRNA than wild type and contain multiple copies of the plasmid integrated as tandem repeats at ectopic sites in the genome. The transcription rate of *HCf-1* in the co-suppressed isolates was higher than in the untransformed strains, suggested that silencing acted at the post-transcriptional level. This was due to ectopic integration of the transgene next to promoters which initiate transcription to form antisense RNA and that this in turn determines down-regulation of *HCf-1*. But gene silencing was not associated with DNA cytosine methylation (Hamada and Spanu 1998). Similarly, the silencing of *cgl1* and *cgl2* genes using the *cgl2* hairpin construct in *C. fulvum* has also been reported (Segers *et al*. 1999), though the effect was possibly restricted to highly homolougous genes (exons of *cgl1* and *cgl2* are 87% identical). However, the less homologus *cgl3* (53% overall identity to *cgl 2*) was not affected as the target specificity always depends upon the actual sequence alignment and more over, short regions of high density that led to unwanted off-targets effects. Such a strategy could be exploited for protecting the consumable products of vegetables and fruits crops from the postharvest diseases caused by different plant pathogens in future.

Fitzgerald and colleagues (2004), using hairpin vector technology have been able to trigger simultaneous high frequency silencing of a green fluorescent protein (*GFP*) transgene and an endogenous trihydroxynaphthalene reductase gene (*THN*) in *V. inaequalis*. *GFP* transgene, acting as easily detectable visible marker while the trihydroxynaphthalene reductase gene (*THN*) playing role in melanin biosynthesis. High frequency gene silencing was achieved using hairpin constructs for the *GFP* or the *THN* genes transferred by *Agrobacterium* (71 and 61%, respectively). *THN*-silenced transformants exhibited a distinctive light brown phenotype and maintained the ability to infect apple. Silencing of both genes with this construct occurred at a frequency of 51% of all the transformants. All 125 colonies silenced for the GFP gene were also silenced for *THN* (Fitzgerald *et al*. 2004). Similarly, multiple gene silencing has been achieved in *Cryptococcus neoformans* using chimeric hairpin constructs (Liu *et al.* 2002) and in plants using partial sense constructs (Abbott *et al.* 2002).

The first effort towards the systematic silencing of *Magnaporthe grisea*, a causal organism of rice blast was carried out by Kadotani *et al.* (2003) by using the enhanced green florescent protein gene as a model. To assess the ability of RNA species to induce silencing in fungus, plasmid construct expressing sense, antisense and hairpin RNA were introduced into an e*GFP*-expressing transformants. The fluorescence of *eGFP* in the transformants was silenced much more efficiently by hairpin RNA of *eGFP* than by other RNA species. In the silenced transformants, the accumulation of *eGFP* mRNA was drastically reduced. But not methylation of coding or promoter region was involved. The small interfering RNA molecules of 19-23 nucleotides were observed in both sense and antisense strands of *eGFP* gene (Kadotani *et al* 2003). Later on Nakayashiki and collegues (2005) developed a protocol for silencing the *mpg1* and polyketide synthase-like genes. *mpg1* gene is a hydrophobin gene which is essential for pathogenicity as it act as a cellular relay for adhesion and trigger for the development of appressorium (Talbot *et al.* 1996). Their work on this host-

pathogen system revealed that they were successfully able to silence the above mentioned genes at varying degrees by pSilent-1-based vectors in 70–90% of the resulting transformants. Ten to fifteen percent of the silenced transformants exhibited almost ''null phenotype''. This vector was also efficiently applicable to silence a *GFP* reporter in another ascomycete fungus *Colletotrichum lagenarium* (Nakayashiki 2005).

The aforementioned examples demonstrate the genetic mechanism of RNAi in fungi enables it to be a useful method for inhibiting expression of a target fungal virulent gene. However, only two reports were retrieved from the literature where the potential of RNAi as plant defense has been reflected against phytopathogenic fungi. Schweizer and coworkers (2000) have shown that dsRNA interfere with gene function at single cell level in cereals. They delivered dsRNA into single epidermal cells of maize, barley or wheat by particle bombardment and reported transient expression of *C1*- and *b-Peru* genes. In the presence of dsRNA corresponding to dihydroflavanol-4-reducatse gene, *C1*- and *b-Peru* dependent cell autonomous accumulation of red anthocyanin pigment in maize and barley was reduced. dsRNA was demonstrated to be negatively interfere with *Mlo*, which encodes a negative regulator of race non-specific resistance to the powdery mildew fungus in barley. In the presence of *Mlo* dsRNA, transformed cells became more resistant, thereby phenocopying plants that carry a heritable loss-of-function *mlo* resistant allele. Secondly, Roberts and colleagues (2006) patented the information regarding *in planta* RNAi control of fungi where in they reported the introduction of partial or fully, stabilized dsRNA, including its modified forms such as siRNA sequences, to the target phytopathogenic fungi, where the dsRNA inhibits expression of at least one or more target genes of the fungi that exerted deleterious effect upon the pathogen by suppressing the expression of a host plant gene that is necessary for establishment or maintenance of a fungal infection, or development of plant disease symptoms, fungal reproduction and for uptake of nutrients by a fungal cell and eventually results in the death of the organism. In this study, the pathogen was a rust fungus, the causal agent of soybean rust (*Phakopsora pachyrizi*) and they reported that this mechanism will find particular benefit for protecting plants from fungal attack.

Management of plant pathogenic bacteria

One of the striking examples of bacterial disease management where RNAi showed a remarkable type of gene regulation was documented by Escobar *et al*. (2001). They developed a crown gall disease management strategy that targets the process of tumourogensis (gall formation) by initiating RNAi of the *iaaM* and *ipt* oncogenes. Expression of these genes is a prerequisite for wild type tumor formation. Transgenic *Arabidopsis thaliana* and *Lycopersicon esculentum* transformed with RNAi constructs, targeting *iaaM* and *ipt* gene(s) showed resistance to crown gall disease. Transgenic plants generated through this technology contained a modified version of these two bacterial gene(s) required to cause the disease and was the first report to manage a major bacterial disease through RNAi. The extra genes recognize and effectively shut down the expression of the corresponding bacterial gene during infection, thus preventing the spread of infection. The incoming bacteria could not make the hormones needed to cause tumors and plants deficient in silencing were hyper-susceptible to *A. tumefaciens* (Dunoyer *et al*. 2007). Successful infection relied on a potent anti-silencing state established in tumors whereby siRNA synthesis is specifically inhibited. The procedure can be exploited to develop broad-spectrum resistance in ornamental and horticultural plants which are susceptible to crown gall tumorigenesis. This approach can be advocated for the effective management of those pathogens which multiply very rapid and results in tumor formation such as *Albugo candida*, *Synchytrium endobioticum*, *Erwinia amylovora* etc. The natsiRNA (nat-siRNAATGB2) was strongly induced in *Arabidopsis* upon infection by *Pseudomonas syringae* pv. *tomato* and down-regulates a *PPRL* gene that encodes a negative regulator of the *RPS2* disease resistance pathway. As a result, the induction of nat-siRNAATGB2 increases the *RPS2*-mediated race-specific resistance against *P. syringae* pv. *tomato* in *Arabidopsis* (Katiyar-Agarwal *et al.* 2006). Recently, the accumulation of a new class of sRNA, 30 to 40 nucleotides in length, termed long-siRNAs (lsiRNAs), was found associated with *P. syringae* infection. One of these siRNAs, AtlsiRNA-1, contributes to plant bacterial resistance by silencing AtRAP, a negative regulator of plant defense (Katiyar-Agarwal *et al.* 2007). A *Pseudomonas* bacterial flagellin derived peptide is found to induce the accumulation of miR393 in *Arabidopsis*. miR393 negatively regulates mRNAs of F-box auxin receptors, resulting in increased resistance to the bacterium (*P. syringae*), and the overexpression of miR393 was shown to reduce the plant's bacterial titer by 5-fold (Navarro *et al*. 2006).

Management of plant pathogenic viruses

Antiviral RNAi technology has been used for viral disease management in human cell lines (Bitko and Barik 2001; Gitlin *et al*. 2002; Jacque *et al*. 2002; Novina *et al*. 2002). Such silencing mechanisms (RNAi) can also be exploited to protect and manage viral infections in plants (Waterhouse *et al*. 2001; Ullu *et al*. 2002). The effectiveness of the technology in generating virus resistant plants was first reported to PVY in potato, harbouring vectors for simultaneous expression of both sense and antisense transcripts of the helper-component *proteinase* (*HC-Pro*) gene (Waterhouse *et al*. 1998). The *P1/HC-Pro* suppressors from the potyvirus inhabited silencing at a step down stream of dsRNA processing, possibly by preventing the unwinding of duplex siRNAs, or the incorporation into RISC or both (Chapman *et al*. 2004). The utilization of RANi technology has resulted in inducing immunity reaction against several other viruses in different plant-virus systems (**Table 2**).

In phyto-pathogenic DNA viruses like geminiviruses non-coding intergenic region of *Mungbean yellow mosaic India virus* (MYMIV) was expressed as hairpin construct under the control of the 35S promoter and used as biolistically to inoculate MYMIV-infected black gram plants and showed a complete recovery from infection, which lasted until senescence (Pooggin *et al*. 2003). RNAi mediated silencing of geminiviruses using transient protoplast assay where protoplasts were co-transferred with a siRNA designed to replicase (Rep)-coding sequence of *African cassava mosaic virus* (ACMV) and the genomic DNA of ACMV resulted in 99% reduction in Rep transcripts and 66% reduction in viral DNA (Vanitharani *et al*. 2003). It was observed that siRNA was able to silence a closely related strain of ACMV but not a more distantly related virus.

About more than 40 viral suppressors have been identified in plant viruses (Ruiz and Voinnet 2007). Results from some of the well-studied virus suppressors indicated that suppressors interfere with systemic signaling for silencing (Mlotshwas *et al*. 2002). During last few years, the *p69* encoded by *Turnip yellow mosaic virus* has been identified as silencing suppressors that prevented host RDR-dependent secondary dsRNA synthesis (Chen *et al*. 2004). *P14* protein encoded by aureus viruses suppressed both virus and transgene-induced silencing by sequestering both long dsRNA and siRNA without size specificity (Merai *et al*. 2005). Multiple suppressors have been reported in *Citrus tristeza virus* where *p20* and coat protein (*CP*) play important role in suppression of silencing signal and *p23* inhibited intracellular silencing (Lu *et al*. 2003). Multiple viral components, viral RNAs and putative RNA replicase proteins were reported for a silencing or suppression of *Red clover necrotic mosaic virus* (Takeda *et al*. 2005). In this case, the RNA silencing machinery deprived of DICER-like enzymes by the viral replication complexes appears to be the cause of the suppression. *Pns10* encoded by *Rice dwarf virus* sup-

Table 2 Effects of targeted region of RNAi in various plant-virus systems.

Host system	Virus	Targeted region	References
N. benthamiana, *M. esculenta*	*African cassava mosaic virus*	*pds*, *su*, *cyp79d2*	Fofana *et al*. 2004
Barley, wheat	*Barley stripe mosaic virus*	*pds*	Holzberg *et al*. 2002; Scofield *et al*. 2005
Soybean	*Bean pod mottle virus*	*pds*	Zhang and Ghabrial 2006
Barley, rice, maize	*Brome mosaic virus*	*pds*, *actin 1*, *rubisco activase*	Ding *et al*. 2006
Arabidopsis	*Cabbage leaf curl virus*	*gfp*, *CH42*, *pds*	Turnage *et al*. 2002
P. sativum	*Pea early browning virus*	*pspds*, *uni*, *kor*	Constantini *et al*. 2004
N. benthamiana	*Poplar mosaic virus*	*gfp*	Naylor *et al*. 2005
N. benthamiana, *S. tuberosum*	*Potato virus X*	*pds*, *gfp*	Ruiz *et al*. 1998; Faivre-Rampant *et al*. 2004
Nicotiana tabacum	*Satellite tobacco mosaic virus*	*Several genes*	Gossele *et al*. 2002
N. benthamiana, *N. tabacum*	*Tobacco mosaic virus*	*pds*, *psy*	Kumagai *et al*. 1995
N. benthamiana, *Arabidopsis*, tomato, *Solanum* species, chilli pepper, opium poppy, *Aquilegia*	*Tobacco rattle virus*	*Rar1*, *EDS1*, *NPR1/NIM1*, *pds*, *rbcS*, *gfp*	Liu *et al*. 2002b; Ratcliff *et al*. 2001; Brigneti *et al*. 2004; Chung *et al*. 2004; Hileman *et al*. 2005; Gould and Kramer 2007
N. benthamiana	*Tomato bushy shunt virus*	*gfp*	Hou and Qiu 2003
N. benthamiana	*Tomato golden mosaic virus*	*su*, *luc*	Peele *et al*. 2001
N. benthamiana, *Lycopersicon esculentum*, *N. glutinosa*, *N. tabacum*	*Tomato yellow leaf curl China virus-associated b DNA satellite*	*pcna*, *pds*, *su*, *gfp*	Tao and Zhou 2004

(Modified after Godge *et al*. 2008)

Table 3 RNAi effect on targeted region of plant parasitic nematodes.

Nematode	Targeted region	RNAi affect
M. incognita	Cysteine proteinase	Delayed development, Decrease in established nematodes population
	Dual oxidase	Decrease in established nematodes population and fecundity.
	Splicing factor, Integrase	Reduction in gall formation and Female nematode population
	Secreted peptide 16D 10	Reduction in gall formation and established nematode population
H. glycines	Cysteine proteinase	Increased male: female ratio.
	C-type lectin	Reduction in established nematodes population
	Major sperm protein	Reduction in mRNA level
	Aminopeptidase	Decrease in established nematodes population and increase in male: female ratio.
	β-1,4-endoglucanase	Decrease in established nematodes population
	Pectate lyase, chorismate mutase	Increase in male: female ratio.
	Secreted peptide SYV46	Decrease in established nematode population
G. pallida	Cysteine proteinase	Increase in male: female ratio.
	FMR Famide-like peptides	Motility inhibited
G. rostochiensis	Chitin synthase	Delay in egg hatch
	β-1,4-endoglucanase	Decrease in established nematodes population
	Secreted amphid protein	Reduction in invasion ability to locate and invade plant roots
Heterodera schachtii	*Suc* transporter genes	Reduction of female nematode development

(Modified after Karakas 2008)

pressed local and systemic S-PTGS but not IR-PTGS suggesting that *Pns10* also targets an upstream step of dsRNA formation in the silencing pathway (Cao *et al*. 2005).

Niu and colleagues (2006) used a 273-bp (base pair) sequence of the *Arabidopsis* miR159 a pre-miRNA transcript expressing amiRNAs against the viral suppressor genes *P69* and *HC-Pro* to provide resistance against *Turnip yellow mosaic virus* and *Turnip mosaic virus* infection, respectively. In addition, a dimeric construct harboring two unique amiRNAs against both viral suppressors conferred resistance against these two viruses in inoculated *Arabidopsis* plants.

Similarly, Qu *et al*. (2007) used a different amiRNA vector to target the 2 b viral suppressor of the *Cucumber mosaic virus* (CMV), a suppressor that interacted with and blocked the slicer activity of *AGO1* had also shown to confer resistance to CMV infection in transgenic tobacco. A strong correlation between virus resistance and the expression level of the 2 b-specific amiRNA was shown for individual plant lines.

It is evident from above-mentioned reports that the RNA components, such as single strand template RNA, dsRNA and/or siRNA of the silencing pathways are the preferred targets of most viral suppressors. However, plant viruses are known to have evolved a counter-silencing mechanism by encoding proteins that can overcome such resistance (Li and Ding 2006; Díaz-Pendón and Ding 2008). These suppressors of gene silencing are often involved in viral pathogenicity, mediate synergism among plant viruses and result in the induction of more severe disease. Simultaneous silencing of such diverse plant viruses can be achieved by designing hairpin structures that can target a distinct virus in a single construct (Díaz-Pendón and Ding 2008).

Contrarily, the RNAi system may cause an increase in the severity of viral pathogenesis and/or encode proteins, which can inactivate essential genes in the RNAi machinery (Elbashir *et al*. 2001) that helps them in their replication in the host genome (Hannon 2002).

Management of plant parasitic nematodes

Several major plant parasitic nematodes such as the root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* spp.) along with other minor nematodes cause significant damage to important crops like legumes, vegetables and cereals in most parts of the world and continue to threaten these agricultural crops. So a natural, eco-friendly defense strategy that delivers a cost-effective control of plant parasitic nematodes is needed which is difficult to achieve through conventional approaches. However, the birth of RNAi technology from classical *C. elegans* studies has shown the ways and means to explore the possibilities of this mechanism for protecting plants from nematode damage. In this context, two approaches have been advocated, one of them relies on targeting plant genes that are involved with the infection process, and the second approach targets essential genes within the nematode. RNAi can be induced in *C. elegans* by feeding it dsRNA, so it was reasoned that expressing hpRNAs containing sequences of vital nematode genes in

the host plant might deliver dsRNA to a feeding nematode to incapacitate or kill it.

After the demonstration of gene silencing using siRNA duplexes in the nematode (Fire *et al*. 1998), the use of RNAi has rapidly emerged as the technique of choice for plant nematologists to put their efforts, especially for nematode management in agriculture. RNAi-mediated suppression of a gene plays an indispensable role in hampering the nematode development and may adversely affect the progression of pathogenesis in direct or indirect ways. There are accumulating evidences for the efficacy of RNAi in plant parasitic nematode management and a wide range of genes have been targeted for silencing in cyst and root-knot nematode species (**Table 3**).

RNAi in the context of phyto-parasitic nematodes was used as early as the beginning of this century, when stimulation of oral ingestion by second-stage juveniles of cyst nematodes *H. glycines*, *G. pallida* (Urwin *et al*. 2002) and root-knot nematode *M. incognita* (Bakhetia *et al*. 2005) was achieved by using octopamine. Later on, resorcinol- and serotonin-inducing dsRNA uptake by second stage juvenile of *M. incognita* was found to be more effective than octopimine (Rosso *et al*. 2005). The genes targeted by RNAi to date are expressed in a range of different tissues and cell types. The ingested dsRNA can silence genes in the intestine (Urwin *et al*. 2002; Shingles *et al*. 2007), female reproductive system (Lilley *et al*. 2005), sperm (Urwin *et al*. 2002; Steeves *et al*. 2006), and both subventral and dorsal oesophageal glands (Chen *et al*. 2005; Rosso *et al*. 2005; Huang *et al*. 2006; Bakhetia *et al*. 2007). Uptake of dsRNA from the gut is a proven route to systemic RNAi in *C. elegans*. The systemic nature of RNAi in plant parasitic nematodes following ingestion of dsRNA suggests that they share similar uptake and dispersal pathways.

However, RNAi of a *chitin synthase* gene expressed in the eggs of *Meloidogyne artiella* was achieved by soaking intact eggs contained within their gelatinous matrix in a solution containing dsRNA (Fanelli *et al*. 2005). The enzyme plays a role in the synthesis of the chitinous layer in the eggshell. Depletion of its transcript by RNAi led to a reduction in stainable chitin in eggshells and a delay in hatching of juveniles from treated eggs. Similarly, RNAi targeting for *cysteine proteinase* transcripts did not reduce parasitic population of established nematodes on plants but result into the alteration of their sexual fate in favour of males at 14 days after invasion (Urwin *et al*. 2002).

On the other hand *H. glycines* exposed to dsRNA corresponding to a protein with homology to C-type lectins did not affect sexual fate, but 41% fewer nematodes were recovered from the plants. But treatment with dsRNA corresponding to the major sperm protein (MSP) had no effect on nematode development or sexual fate 14 days after treatment. In addition to this, reduction in transcript abundance for targeted mRNAs in the infective juvenile and for MSP transcripts when males reached sexual maturity and sperm are produced was observed (Urwin *et al*. 2002). In further extension of such types of experiments showed efficient FITC uptake by soaking *M. incognita*, 90-95% of individuals swallowed the dye when the target was a dual oxidase (an enzyme comprised with a peroxidase domain EF-hands and NADPH oxidase domain and potentially involved in extracellular matrix development). The effect of RNAi was observed when root knot nematode (RKN) juveniles were fed on dual oxidase-derived dsRNA, the reduction in the number and size of established females at 14 and 35 days post infection with an overall reduction of 70% in egg production was observed (Bakhetia *et al*. 2005).

Heterodera schachtii induces syncytial feeding structures in the roots of host plants, and this requires the up-regulation of *Suc* transporter genes to facilitate increased nutrient flow to the developing structure. Targeting these genes and down-regulating them with RNA silencing resulted in a significant reduction of female nematode development (Hoffman *et al*. 2008). Indeed, tobacco plants transformed with hpRNA constructs against two such root-knot nematode genes have shown such an effect: the target mRNAs in the plant parasitic nematodes were dramatically reduced, and the plants showed effective resistance against the parasite (Fairbairn *et al*. 2007).

FUTURE OUTLOOK OF RNAi AS AN ECO-FRIENDLY AND BIOSAFE TOOL FOR PLANT PATHOLOGISTS

The field of RNAi is moving at an impressive pace and generating exciting results associated with RNAi, transgene silencing and transposon mobilization. The RNA silencing has practical use because of the ability to reduce gene expression in a manner that is highly sequence specific as well as technologically facile and economical, having potential in agriculture specifically for the management of mascotous plant diseases. However, the major obstacles hindering its immediate applications include selection of targeting sequences and in the delivery of siRNA. The key issues are 1) how to select silencing targets for a particular disease and 2) how to efficiently deliver siRNAs into specific cell types *in vivo*?

Besides, RNAi technology can be considered an eco-friendly, biosafe and ever green technology as it eliminates even certain risks associated with development of transgenic plants carrying first generation constructs (binary vectors and sense and antisense genes). As witnessed from earlier strategies for obtaining viral resistant plants, the expression of protein product from the transgene of interest risked hetero-encapsidation through protein-protein interactions between target and non-target viral gene product, resulted in the development of a non-aphid transmissible strain of *Zucchini yellow mosaic virus* to aphid-transmissible strain from a transgene expressing a plum pox capsid protein (Lecoq *et al*. 1993). Since RNAi triggers the formation of dsRNA molecules that target and facilitate the degradation of the gene of interest as well as the transgene itself to avoid problems arising from the synthesis of gene sequences as well as non-coding regions of gene, thus limiting undesirable recombination events. Keeping in view the potentialities of RNAi technology and lesson from this classical example demonstrated that why and how this technology has emerged to combat plant pathogens in the near future as it has already added new dimensions in the chapter of plant disease management. However, a better and comprehensive understanding of RNAi should allow future plant pathologists to work effectively and efficiently in order to manage various mascotous intruders of crop plants.

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