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, abscisic 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; elF2C, 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, Mangbean yellow mosaic India virus; nt, nucleotide; PME, pectin methylesterase; PolIV, 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; RAI, 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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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 homeostasis is a challenging task but it is becoming a necessary 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 (Störici 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 Caenorhabditis 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;Rhodes 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; Catalano 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; Ratcliffe 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 (de Bakker et al. 2002; Almeida and Allshire 2005), whereas 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 susceptibility and improving novel traits in plants or in specific tissues (Islam et al. 2005), it offers the versatility of RNAi as an eco-friendly and biosafe tool for plant pathologists.

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 include 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 DNA duplexes of 21-24 nts by the RNase 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 (Voornet 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 (PSPRP1) 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 mRNA sequences reside between protein coding genes or within introns (Bartel and Bartel 2003). Initially, 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 sRNA sequences to ds form is prerequisite for silencing of endogenous transcripts in Arabidopsis. This 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 pairs forming dsRNA structures (Waterhouse et al. 2001; Beclin et al. 2002). But host-encoded and pathogenesis-inducible RDR, identified as NrRdRP1 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-

...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 of the pathways, Pol IV acts together with AGO4 and one of the RDRs 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 dissociate 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 dsRNA strands 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,
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 (Fagarad 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 NRDE-3, 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 probably is a 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 this silencing processes. For instance enhanced pectin methyl esterase (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; Kurthara 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 miRNAs (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 dsRBPs, 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 Federoff 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 siRNA-specific methyltransferase HEN1. The duplexes of siRNAs are also methylated by HEN1 and assumed to protect all RNA 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 Via de novo cleavage of TAS transcripts at through 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 SUPPRESSOR OF GENE SILENCING 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 Voitnet 2004). Viruses engineered to contain an endogenous sequence can trigger silencing of the endogene, which is termed “virus-induced gene silencing” (VIGS) (Rines 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 transgenese) or indirectly through antisense transgenase and replication intermediates (RNA viruses), or through the action of RDR6 and other proteins (sense transgenase 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 heterochromatin siRNAs (Zilberman et al. 2003; Chan et al. 2004). The key player in this RNAi pathway is heterochromatin siRNAs, which are typically 24-nt RNAs that are associated with genomic repetitive sequences such as transposons, retroelements, rDNAs, and centromere repeats (Hamilton et al. 2002; Kasschau et al. 2007). Heterochromatin 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 heterochromatin 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 DRM (Chan et al. 2004). Since AG0 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). AG06, another member of the Arabidopsis AGO family, has been shown to have partially redundant functions with AG04 in heterochromatin 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 Voitnet 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; Gascioli 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 in 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 determinant 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
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 (Palaqugi 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 transformation (Chuang and Meyerowitz 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...
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 agroinfiltration, 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).
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 phytoreuss 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 photooxidation symptom (Liu et al. 2002a; Turnage et al. 2002). Similarly, over expression of CHS gene, causing an abino phenotype, instead of producing the anticipated deep orange color (Cognini et al. 1994). As a result, their action as a phenoletic 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 Tobacco 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 Tepler 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), polio-virus 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 (Birko and Baric 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 Meyerowitz 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 plant 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 (Ratliff 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 expressing 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 develop 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

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

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Targeted region</th>
<th>Result</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnaporthe oryzae</td>
<td>eGFP</td>
<td>Sequence specific degradation of mRNA</td>
<td>Kadotani et al. 2003</td>
</tr>
<tr>
<td>Cladosporium falvum</td>
<td>cgl 1 and cgl 2</td>
<td>Blocking disease infection spread</td>
<td>Segers et al. 1999</td>
</tr>
<tr>
<td>Venturia inaequalis</td>
<td>Multiple inverted repeats</td>
<td>?</td>
<td>Fitzgerald et al. 2004</td>
</tr>
<tr>
<td>Fusarium graminearum</td>
<td>?</td>
<td>?</td>
<td>Nakayashiki 2005</td>
</tr>
<tr>
<td>Blumeria graminis</td>
<td>Mlo</td>
<td>Immunity</td>
<td>Schweizer et al. 2000</td>
</tr>
</tbody>
</table>
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; 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 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 biologic 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), Magnaporthe 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 polypolykaryotic 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 effect of cgl1 and cgl2 hairpin construct in C. fulvum has also been reported (Segers et al. 1999), though the effect was possibly restricted to highly homologous genes (exons of cgl1 and cgl2 are 87% identical). However, the less homologous cgl3 (53% overall identity to cgl2) 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 an 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 transformants. The cgl2 hairpin construct 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. (2000) by using the enhanced green fluorescent 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 eGFP-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 noneptylation 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 colleagues (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 et al., 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 interferes 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 Cl- and b-Peru genes. In the presence of dsRNA corresponding to dihydroflavanol-4-reductase gene, Cl- 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 fungus, 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 reported by Escobedo et al. (2001). They developed a crown gall disease management strategy that targets the process of tumourogenesis (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 siRNA (30 to 40 nucleotides in length), termed long siRNAs, 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).

Antiviral RNAi technology has been used for viral disease management in human cell lines (Bitko and Barik 2001; Novina et al. 2002; Novina et al. 2004). 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 inhabitated 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 RNAi 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 biologically 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 replicate (Rep)-coding sequence of African cassava mosaic virus (ACMV) and the dsDNA 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 these suppressors interfere with the RNA silencing machinery deprived of DICER-like enzymes and transgene-induced silencing by sequestering both long and short RNA components, viral RNAs and putative RNA replicase proteins (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. 2006). Multiple suppressors have been reported for silencing of 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 suppress...
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 amRNA 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 amRNA 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; Diaz-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.

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

<table>
<thead>
<tr>
<th>Host system</th>
<th>Virus</th>
<th>Targeted region</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. benthamiana, M. esculenta</td>
<td>African cassava mosaic virus</td>
<td>pds, su, cyp7d2</td>
<td>Fofana et al. 2004</td>
</tr>
<tr>
<td>Barley, wheat</td>
<td>Barley stripe mosaic virus</td>
<td>pds</td>
<td>Holzberg et al. 2002; Scofield et al. 2005</td>
</tr>
<tr>
<td>Soybean</td>
<td>Bean pod mottle virus</td>
<td>pds</td>
<td>Zhang and Ghabrial 2006</td>
</tr>
<tr>
<td>Barley, rice, maize</td>
<td>Brome mosaic virus</td>
<td>pds, actin 1, rubisco activase</td>
<td>Ding et al. 2006</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>Cabbage leaf curl virus</td>
<td>gfp, CH42, pds</td>
<td>Turnage et al. 2002</td>
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<tr>
<td>P. sativum</td>
<td>Pea early flowering virus</td>
<td>psps, unii, kor</td>
<td>Constantini et al. 2004</td>
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<td>N. benthamiana</td>
<td>Poplar mosaic virus</td>
<td>gfp</td>
<td>Naylor et al. 2005</td>
</tr>
<tr>
<td>N. benthamiana, S. tuberosum</td>
<td>Potato virus X</td>
<td>pds, gfp</td>
<td>Ruiz et al. 1998; Faivre-Rampant et al. 2004</td>
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<tr>
<td>Nicotiana tabacum</td>
<td>Satellite tobacco mosaic virus</td>
<td>Several genes</td>
<td>Gossele et al. 2002</td>
</tr>
<tr>
<td>N. benthamiana, N. tabacum</td>
<td>Tobacco mosaic virus</td>
<td>pds, psy</td>
<td>Kumagai et al. 1995</td>
</tr>
<tr>
<td>N. benthamiana, Arabidopsis, tomato, Solanum species, chilli pepper, opium poppy, Aquilegia</td>
<td>Tobacco mosaic virus</td>
<td>pds, psy</td>
<td>Kumagai et al. 1995</td>
</tr>
<tr>
<td>N. benthamiana</td>
<td>Tomato golden mosaic virus</td>
<td>su, luc</td>
<td>Hou and Qiu 2003</td>
</tr>
<tr>
<td>N. benthamiana, Lycopersicon esculentum, N. glutinosa, N. tabacum</td>
<td>Tomato yellow leaf curl China</td>
<td>pcn1, pds, su, gfp</td>
<td>Tao and Zhou 2004</td>
</tr>
</tbody>
</table>

(Modified after Godge et al. 2008)

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

<table>
<thead>
<tr>
<th>Nematode</th>
<th>Targeted region</th>
<th>RNAi affect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. incognita</td>
<td>Cysteine protease</td>
<td>Delayed development, Decrease in established nematodes population</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dual oxidase</td>
<td>Decrease in established nematodes population and fecundity.</td>
<td></td>
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<tr>
<td></td>
<td>Splicing factor, Integrase</td>
<td>Reduction in gall formation and Female nematode population</td>
<td></td>
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<tr>
<td>H. glycines</td>
<td>Secreted peptide 16D 10</td>
<td>Reduction in gall formation and established nematode population</td>
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<td></td>
<td>C-type lectin</td>
<td>Reduction in established nematodes population</td>
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<td></td>
<td>Major sperm protein</td>
<td>Reduction in mRna level</td>
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<td></td>
<td>Aminopeptidase</td>
<td>Decrease in established nematodes population and increase in male: female ratio.</td>
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<td></td>
<td>β-1,4-endoglucanase</td>
<td>Decrease in established nematodes population</td>
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<tr>
<td>G. pallida</td>
<td>Pectate lyase, chomalone mutase</td>
<td>Increase in male: female ratio.</td>
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<td></td>
<td>Secreted peptide SYV46</td>
<td>Decrease in established nematodes population</td>
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<td></td>
<td>Cysteine protease</td>
<td>Increase in male: female ratio.</td>
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<td>FMR Famide-like peptides</td>
<td>Motility inhibited</td>
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<td>G. rostochiensis</td>
<td>Chitin synthase</td>
<td>Delay in egg hatch</td>
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<td></td>
<td>β-1,4-endoglucanase</td>
<td>Decrease in established nematodes population</td>
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<td></td>
<td>Secreted amphid protein</td>
<td>Reduction in invasion ability to locate and invade plant roots</td>
<td></td>
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<tr>
<td>Heterodera schachtii</td>
<td>Suc transporter genes</td>
<td>Reduction of female nematode development</td>
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( Modified after Karakas 2008)

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 hptRNAs 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 conversely 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 octopamine (Rosso et al. 2005). The genes targeted by RNAi to date are expressed in a range of different tissues and cell types. Once targeted dsRNA can silenced 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 staminate 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 of PthF1 dsRNA, 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 72-78% in egg production was observed (Bakhetia et al. 2005).

Heteroder a 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 macuous 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 the use of potentially harmful chemicals and 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 macuous intruders of crop plants.

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