An Overview of Antiviral RNA Silencing in Plant: Biogenesis, Host–Virus Interaction and Potential Applications

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Abstract

Small RNA molecules play a crucial regulatory role in maintaining genome stability as well as developmental regulations through a set of complex and partially overlapping pathways in a wide range of eukaryotic organisms. Active in both cytoplasm and nucleus, RNA interference regulates eukaryotic gene expression through transcriptional repression by epigenetic modification and interaction with transcription machinery. Small interfering RNAs (siRNAs/miRNAs) of 21–24 nucleotides constitute the innate defence arm against a variety of pathogens, especially viruses. Plant viruses with either DNA or RNA genomes are subjected to small RNA-directed RNA degradation. Additionally, DNA viruses are subjected to another line of defence through 'RNA-directed DNA methylations' (RdDM). On the other hand, viral-encoded proteins, called silencing suppressors (VSRs), are known to counter the defence machinery, and therefore the virus can evade the host surveillance system. Some plant viruses additionally adopt certain strategies like acquiring silencing resistant structures (some RNA virus) to evade the RNA silencing machinery and thereby shaping the viral as well as the host genome. Recently, it has been reported that particular viral proteins and viral siRNAs contribute directly to pathogenicity by interacting with certain host proteins or RNAs. Transcriptional regulation of host gene by small RNA of viral origin plays important role in pathogenesis and symptom development. Small regulatory RNAs of cellular rather than pathogen origin have also been found to play a broad role in improving the basal defence in the case of plant–virus interaction. This chapter provides key insights into the complex intricate machinery of diverse RNA silencing mechanisms, describes various evolutionary diverse strategies of viral

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silencing suppressors at various steps, offers a broader view of host recovery following virus infection and finally suggests the possible applications of RNA silencing to generate virus resistant plants.

Keywords
siRNAs • miRNAs • RdDM • VSRs • Pathogenesis • Host recovery

Introduction

RNA silencing is an evolutionary conserved gene regulatory mechanism active in a majority of eukaryotic system (e.g. plants, animals, yeast and insects). The versatile mechanism involves inhibition both at translational level (through the degradation of the target mRNA and/or by inhibiting its translation in a sequence-specific manner, i.e. posttranscriptional gene silencing, PTGS) and during transcription of transposons and repetitive DNA elements (transcriptional gene silencing, TGS). The concept of RNA silencing was born in early 1990 when transgenic plants expressing an extra copy of chalcone synthase (CHS) unexpectedly resulted in the suppression of both transgene and endogenous CHS mRNA giving birth to a phenomenon called co-suppression in scientific landscape (Napoli et al. 1990; van der Krol et al. 1990). Subsequently, in 1996, in the fungus *Neurospora crassa*, a similar phenomenon was noticed (Cogoni et al. 1996). The direct study of RNA interference was performed in *C. elegans,* where delivery of exogenous dsRNA resulted in sequence-specific degradation of cognate cellular mRNAs (Fire et al. 1998). Similar effects were observed in the majority of other eukaryotes including mouse, *Drosophila* and human (Elbashir et al. 2001; Billy et al. 2001). From the inception of the concept, the intricacy and importance of this process channelized persistent efforts in detailed exploration of the mechanism.

Three classes of small RNA regulate gene expression in cytoplasm. These are microRNAs (miRNAs), small RNAs which have imperfect complementarity with target and are generated from long RNA with hairpin structure causing translational repression of target mRNA; small interfering RNAs (siRNAs), with perfect complementarity to targets and cause transcript degradation; and PIWI-interacting RNAs (piRNAs) targeting transcripts in animal germ lines. Plants produce miRNA and siRNA but no piRNA. The term RNAi is conventionally used for siRNA-mediated silencing but convergence of different small RNA pathway prompted us to use RNAi as an umbrella term in this chapter to describe small RNA-dependent silencing. In plants, the RNA silencing machinery includes at least three complex yet partially overlapping pathways: (1) siRNA-mediated cytoplasmic RNA silencing (PTGS), (2) silencing mediated by miRNAs and (3) DNA methylation-dependent silencing at the transcriptional level (Baulcombe 2004).

Viruses, one of the most important causative agents of infectious diseases in both plants and animals, encode few multitasking proteins to support their life cycle. For successful replication of the genome, viruses use self-encoded replicase along with various replication factors (Mori et al. 1992) or reverse transcriptase (Laco and Beachy 1994) in case specific manner. Interestingly, viruses are also known to efficiently use host-encoded RNA-dependent RNA polymerase (Dalmay et al. 2000; Mourrain et al. 2000). Most plant viruses have a narrow host range and capable of manipulating developmental pathways of the hosts leading to striking and elaborate array of symptoms formation (Hull 2002). Plant viral symptoms were also observed and commercially exploited long before the concept of virus came into existence. For example, the flame-like streaks of tulip flower caused by infection with tulip-breaking virus (Dekker et al. 1993) achieved high prices. The autumnal yellow

appearance of eupatorium plants caused by *Eupatorium yellow vein virus* and its cognate betasatellite was praised by a Chinese princess (Saunders et al. 2003).

Recent studies have demonstrated 'RNA silencing' as a major contributor of plant defence response against viruses (Wang and Metzlaff 2005; Pallas and Garcia 2011; Waterhouse et al. 2001). Plant viruses induce PTGS in a homology-dependent manner (Baulcombe 2004; Meister and Tuschl 2004; Eamens et al. 2008). During the initial stage of RNA silencing, long double-stranded RNAs (dsRNAs) (produced by the transcripts of inverted repeat sequence as in transposons or by the transcription from convergent promoters or by host RDRs (either in primer-dependent or primer-independent mechanism) (Dalmay et al. 2000; Mourrain et al. 2000; Sijen et al. 2001)) are cleaved into small interfering RNAs (siRNAs) of 21–24 nucleotides (Bernstein et al. 2001; Hamilton et al. 2002) which play diverse and redundant functions (Xie et al. 2004; Gasciolli et al. 2005; Blevins et al. 2006). Duplex siRNAs then undergo unwinding in ATP-dependent manner (Nykanen et al. 2001) and one of the two strands called guide strand gets incorporated into complex machinery of proteins called RNA-induced silencing complex (RISC) to carry out sequence-specific degradation of complementary target mRNA (Khvorova et al. 2003). A similar strategy has been adopted by both plants and animals to generate endogenous microRNAs (miRNAs). siRNA-directed transcriptional and heterochromatic gene silencing (Lippman and Martienssen 2004) constitute the third branch where the siRNAs (24–26 nt) of slightly larger size can be generated from transcripts of inverted repeats or tandem-repeated sequence and by ectopically expressed RNAs corresponding to the promoter region (Jones et al. 1999; Mette et al. 2000) and function in association with AGO4 and RDR2 (Hamilton et al. 2002; Qi et al. 2005; Xie et al. 2005; Zilberman et al. 2003). Methylation of cytosine residue in DNA (RNA-dependent DNA methylation, RdDM) or at the 9th position of histone H3 (H3K9) also results in suppression of gene expression (Blander and Guarente 2004). This RdDM pathway has been reported to maintain the genome integrity at both centromeric and telomeric repeat regions and suppress the transcription of transposons and other invasive DNAs (Matzke et al. 2009; Haag and Pikaard 2011). On the other hand, viruses in turn develop several strategies to interfere with host defence machinery to establish successful pathogenesis. One of the most fascinating strategies is the evolution of viral suppressors of RNA silencing which interfere with various steps of RNA silencing pathway of host (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington 1998; Llave et al. 2000). Additionally, viruses also adopt different strategies to bypass RNA silencing machinery. Bromoviruses protect their RNA genome from host ribonucleases by accumulating inside the membrane vesicle (Schwartz et al. 2002). Members of the family *Avsunviroidae* undergo chloroplastic replication and thereby protect the viroid genome from RNA silencing (Tabler and Tsagris 2004). Again, the extensive intramolecular fold structures of viroids make them inaccessible to RISC complex (Wang et al. 2004). Defective interfering RNAs, which are devoid of target sequences also help to escape RNA silencing as observed in case of tombusviruses (Dalmay et al. 1995). Transfected siRNAs specific for either influenza A or HIV virus failed to target viral genome because of the occurrence of quasi species by spontaneous mutations in the target region (Ge et al. 2003; Boden et al. 2003; Das et al. 2004). Viruses (e.g. respiratory syncytial virus) also interact with certain cytoplasmic proteins (Biltko and Barik 2001) for encapsidation, high rate of replication and spread that may finally aid the viruses to escape RNA silencing machinery.

Components of RNA Silencing Machinery

RNA silencing is an ancient defence mechanism. While coming down the ladder of evolution, the process has incorporated several unique and functionally diverse proteins as important

contributors in the complexity and specificity of the pathway. Proteins like Dicer-like enzymes (DCLs), ARGONAUTEs (AGOs), HYPONASTIC LEAVES (HYL1) and other dsRNA-binding proteins, HUA ENHANCER1 (HEN1) and RNA-DEPENDENT RNA POLYMERASEs are providing specificity to the RNA silencing machinery of plant.

Dicer-Like Proteins (DCLs): In comparison to other eukaryotes (mammals, worms, flies and fungi) *A. thaliana* encodes different DCLs. DCLs/DICERs are multidomain RNAse III-like ribonucleases which include evolutionary conserved N-terminal RNA helicase domain, central Piwi/Argonaute/Zwille (PAZ) domain and C-terminal dual catalytic and dsRNA-binding domains (Bernstein et al. 2001; Schauer et al. 2002). DCL1, the first RNAse III-like ribonucleases discovered, processes endogenous dsRNAs to miRNAs (21–22 nt) which in turn control diverse set of mRNAs of various transcription factors (Park et al. 2002; Xie et al. 2005). DCL2 produces less abundant 22-nt siRNAs population, and DCL3 produces 24-nt hc-siRNAs to carry out RdRM and to modify cis- and trans-elements of the gene, DNA repeats and transposons loci (Xie et al. 2004; Bouche et al. 2006). DCL4 is involved in the biogenesis of endogenous tasiRNAs (Reinhart et al. 2002; Gasciolli et al. 2005). Recently, DICER-LIKE 4 (DCL4) has been shown to terminate transcription of *Arabidopsis* endogenous FCA gene (a nuclear RNA-binding protein which controls the flowering time in Arabidopsis) by promoting cleavage of the aberrant RNA produced from the locus (Liu et al. 2012). In rice, DCL4 and DCL3 homolog DCL3b are likely to be involved in the generation of phased siRNAs of 21 and 24 nucleotides, respectively (Song et al. 2012). It has been proposed that formation of differently sized siRNAs is probably mediated by a PAZ domain of the DCLs, which configures the single-cleavage centre with respect to long N-terminal RNase III domain (Schauer et al. 2002).

Argonautes (AGOs): The uniqueness of RISC complex is provided by the AGO proteins. They can bind to both siRNA and miRNA. Ten different AGO proteins are encoded by *Arabidopsis* genome (Baulcombe 2004) of which AGO1, AGO2, AGO5 and AGO7 reportedly contribute to the antiviral defence in plant (Wang et al. 2011). Typically RISC-containing miRNAs and 21-nt siRNAs (produced by DCL1 and DCL4, respectively) associate with either AGO1, AGO2, AGO7 or AGO10 to cause post-transcriptional gene silencing (PTGS) of target mRNA by translational repression (Brodersen et al. 2008) or slicing (Baumberger and Baulcombe 2005). In contrast, 24-nt siRNAs, produced by DCL3, associate with AGO4, AGO6 or AGO9 and initiate transcriptional gene silencing (TGS) (Brosnan and Voinnet 2011).

AGO contains four functionally distinct domains to interact extensively with small RNA molecules. PAZ domain recognizes the 3′ end (Lingel et al. 2003, 2004; Ma et al. 2004); PIWI domain adopts an RNase-H fold and confers targeted endonucleolytic activity to certain AGOs to cleave the target mRNA in the region complementary to the guide RNA (Song et al. 2003; Yuan et al. 2005; Kawamura et al. 2008); MID domains of AGOs interact with the 5′ end of small RNAs and can direct the sorting of different classes of small RNAs into the appropriate AGO family members (Frank et al. 2012, 2010; Ma et al. 2005; Parker et al. 2005). Recently, the N-terminal domain of AGO has been proposed to be involved in unwinding of duplex siRNAs/miRNAs (Kwak and Tomari 2012). These AGOs vary in terms of catalytic triad present either in them or in residues that are involved in 5′ phosphate binding (Liu et al. 2004; Rivas et al. 2005). AGOs have been found to coimmunoprecipitate with viral small RNAs, but AGO1, largely considered as the principal slicer, has been found to bind to miRNAs and certain class of siRNAs of endogenous origin but not with the viral siRNAs (Hunter et al. 2003; Fagard et al. 2000; Baumberger et al. 2007), while AGO4 is required for RdDM mediated by 24-nt siRNAs (Zilberman et al. 2004). AGO1 and AGO5 preferentially bind to small RNAs containing 5′ terminal U or C residues, respectively whereas AGO2 and AGO4 have a strong strand bias for

small RNAs with 5′ terminal A. 5′ terminal nucleotide of small RNAs determines strand selection into AGO complexes. Nevertheless, this 5′ end-dependent incorporation is not exclusive (Mi et al. 2008; Montgomery et al. 2008; Takeda et al. 2008; Havecker et al. 2010). DDH residues of AGO1 have been shown to possess cleavage activity (Elbashir et al. 2001; Mallory et al. 2004).

RNA-Dependent RNA Polymerases (RDRs): Host-encoded RNA-dependent RNA polymerase (RDR) uses viral primary siRNA molecules as primers to convert (aberrant) RNA target sequences into new long dsRNAs which in turn are processed into secondary siRNAs. These RDR-dependent amplified pools of viral siRNAs are originated from the entire target RNA sequence causing transitive silencing Sijen et al. (2001)). In *Arabidopsis* 6 RDRs have been reported. Three of them, i.e. RDR1, RDR2 and RDR6, belong to RDRα group containing a catalytic DLDGD motif. Arabidopsis RDR1, RDR*2* and RDR*6*, and orthologs of these genes, are involved in the amplification, and plants from which these genes have been knocked out are highly susceptible to various plant viruses. RDR3, RDR4 and RDR5 contain DFDGD motif and are characterized as members of RDRγ group (Wassenegger and Krczal 2006). RDR2 and RDR6 are found to be the most important members that contribute significantly in endogenous small RNA pathway by converting the ssRNA templates to dsRNA in a primer-independent manner (Curaba and Chen 2008). The role of RDR 3, 4 and 5 are not well explored yet. They are found as tandemly repeated clusters on chromosome II. RDR1 has been found to be induced in response to either viral infection or salicylic acid (Yu et al. 2003). Interplay of different RDRs is important in regulating antiviral response of host. RDR1 from *N. tabacum* suppresses RDR6-mediated antiviral silencing and enhances viral infection in *N. benthamiana* where it is reported to be truncated due to insertion of inframe mutation (Ying et al. 2010). RDR2 also antagonizes the production of RDR6-dependent siRNAs in sense PTGS (Jauvion et al. 2012).

Nonfamily dsRNA-Binding Proteins: HYPONASTIC LEAVES1 (HYL1) and dsRNA-binding proteins (DRB 2–4) bind to DCLs and assist cleavage of double stranded RNAs. HYL1 interacts with and DCL1 and colocalizes in the nuclear bodies along with C2H2 Zn finger protein, Serrate and this complex is required for miRNA processing (Han et al. 2004; Vazquez et al. 2004; Fang and Spector 2007; Song et al. 2007). DRB4 interacts specifically with DCL4 (Hiraguri et al. 2005). R2D2 in *Drosophila* (Liu et al. 2003) and RDE4 in *C. elegans* (Tabara et al. 2002) are two important DRBs helping Dicers to deliver duplex small RNAs to downstream effector complexes.

HUA ENHANCER 1 (HEN1): HEN1, a small methylase, is unique to plants which methylates the 2′ OH of the terminal nucleotide at 3′ end of the small RNAs (Yang et al. 2006; Yu et al. 2005). The small RNA duplexes with 3′ 2-nt overhangs are preferred substrate for HEN1 and get methylated immediately after DCL-mediated cleavage to provide stability and protection to the small RNAs against oligouridylation (Yang et al. 2006).

Origin of Viral siRNAs

Genome of plant viruses interestingly can serve as both the target and trigger of RNA silencing. Earlier it was speculated by the scientific fraternity that double-stranded RNA intermediate generated during replication of positive-strand RNA virus could trigger the production of vsiRNA (Ahlquist 2002) which, if true, would generate equal amount of siRNA from both positive and negative RNA strand. But Molnar and associates found that the genomic strand of the viruses gave rise to greater amount of vsiRNA. Consequently, it was proposed that highly structured, single-stranded viral RNAs could be processed into vsiRNAs to trigger RNA silencing (Molnar et al. 2005). Moreover, certain regions on the viral genome were identified as ‘hot’ which had greater potential of producing vsiRNA over ‘non-hot’ regions. It was further suggested that single-stranded viral RNA with stable

secondary structure is more likely the probable source of vsiRNA than dsRNA replication intermediates (Szittya et al. 2010; Donaire et al. 2009). In the case of plant DNA viruses which replicate through dsDNA intermediate also produce vsiRNAs from foldback structures of RNA transcription units (Moissiard and Vionnet 2006; Vanitharani et al. 2005).

Role of vsiRNAs in Attenuating Expression of Host Transcript

Detailed studies with vsiRNA have indicated that vsiRNAs can posttranscriptionally regulate the host transcripts expression. Bioinformatics study with Potato spindle tuber viroid (PSTVd-RG1) revealed presence of stretches of 19–20-nt sequences from various plant species that share sequence identity with the viroid. Interestingly, most of these sequences corresponded to virulence-regulating region of the pathogen. Analysing the plant sequence divulged presence of number of transcription factors and chromodomain helicase DNA-binding protein that shared sequence homology with the viral sequences (Wang et al. 2004). This result suggested putative role of vsiRNA in regulating expression of host regulatory genes. Study with *Cauliflower mosaic virus* revealed that the CaMV infection greatly reduced the expression of one mRNA from *Arabidopsis* sharing 18–25nt microhomology with 35S RNA leader sequence (Moissiard and Vionnet 2006). The functionality and efficiency of vsiRNA in regulating host genes depends on many cellular factors including activity of the silencing suppressors of viral origin and abundance of vsiRNAs. Recently it has been reported that siRNAs derived from viral satellite RNA can directly regulate the expression of a host gene and hence attenuate the disease symptoms. A 24-nt region of CMV-Y satellite RNA (Y-Sat), called the 'yellow domain', was shown to be responsible for yellow symptoms induced in Y-Sat-infected tobacco plants (Kuwata et al. 1991). Smith et al. (2011) observed that a 22-nt complementary region of this yellow domain was present in the sequence of subunit I of magnesium chelatase, an enzyme involved in chlorophyll biosynthesis. Extensive study revealed that Y-Sat-induced symptoms are elicited by the vsiRNAs-mediated silencing of *CHLI* (2011). This result was further confirmed by Shimura et al. (2011), who showed that *N. benthamiana* plants expressing inverted repeat sequence of Y-Sat also develop yellow symptom mimicking the virus-infected phenotype. Downregulation of CHLI expression in both transgenic and Y-Sat-infected plants further proved the role of Y-Sat-derived vsiRNA in affecting host expression. Finally, it was demonstrated that Y-Sat-derived vsiRNAs could specifically target the 22-nt sequence in *CHLI* mRNA and therefore downregulate *CHLI* mRNA, thus inducing the yellowing symptom by impairing the chlorophyll biosynthesis pathway.

Cell-to-Cell and Long-Distance Movement of Virus-Derived siRNAs in Plants

In plants, RNAi acts non-cell autonomously and spreads in transacting manner. Grafting experiments with transgene-induced rootstocks with non-silenced target shoots or scion showed that a sequence-specific silencing signal is transmitted from rootstocks into shoots (Palauqui et al. 1997). Mobile RNA silencing was found to have two distinct arms in plants: cell-to-cell (through plasmodesmata) (Himber et al. 2003; Dunoyer et al. 2010) and long-distance movement through phloem (Palauqui et al. 1997; Voinnet and Baulcombe 1997; Yoo et al. 2004; Kalantidis et al. 2008). The siRNAs (21 to 24 nt) generated from the processing of the long dsRNAs act as mobile silencing signal for both the cases. Few of the components of this signal transduction pathway including number of small RNAs, proteins and protein channels have been identified.

Local Movement of Silencing Signal From Cell to Cell: Local movement of silencing signal occurs through specialized intercellular channels called plasmodesmata (Lucas and Lee 2004; Oparka 2004; Kim and Zambryski 2005; Maule 2008). In the absence of signal amplification triggered by cellular RDRs, the spread of

silencing signal is limited to 10–15 cells beyond the site of signal initiation. Plasmodesmata can allow the transfer of up to 27 kDa protein (Kobayashi and Zambryski 2007). However, plasmodesmata, upon binding to various virus-encoded proteins, can undergo significant change in their size exclusion limit (Imlau et al. 1999) and thereby allowing larger molecules like viral ribonucleoproteins and transcription factors (e.g. KNOTTED 1) to pass through it (Lucas et al. 1995; Carrington et al. 1996). Spread of 'local' or 'limited' cell-to-cell silencing depends on the 21-nt siRNAs generated by DCL4 in AGO1-dependent cleavage of target endogenous genes (Himber et al. 2003; Parizotto et al. 2004). When SULPHUR gene fragment was expressed using phloem companion cell-specific promoter, mutation in RDR6 failed to interfere with the yellowing of the companion and its adjacent 10–15 cells (Himber et al. 2003) indicating little role of RDR6 in the local silencing process. Mutation of DCL4 leads to loss of non-cell-autonomous silencing indicating that 21-nt but not 24-nt siRNAs are sufficient for non-cell-autonomous RNAi (Hamilton et al. 2002; Dunoyer et al. 2005, 2010).

Extensive Long-Distance Movement of Silencing Signals: Spread of silencing signal beyond 10–15 cells is termed as extensive silencing. The larger siRNAs (24–26 nt) rather than 21 nt are essential for spread of long-distance silencing signals (Himber et al. 2003) which is dependent on signal amplification by RDR6, SGS3 and a putative RNA helicase (SDE3) (Mourrain et al. 2000; Vaistij et al. 2002; Himber et al. 2003) either in primer-dependent (5′–3′ transitivity) or primer-independent way (3′–5′ transitivity). This amplification is predominantly carried out by the secondary siRNAs generated from cleaved dsRNAs that function as repetitive wave of local cell-to-cell signalling of 10–15 cells at a time (Himber et al. 2003).Two important proteins NRPD1a (a component of RNA Pol IV) (Herr et al. 2005; Kanno et al. 2005; Onodera et al. 2005; Pontier et al. 2005) and RDR2 (an RNA-dependent RNA polymerase 2) (Xie et al. 2004; Herr et al. 2005; Pikaard 2006) function as essential components of non-cell-autonomous RNA silencing (Dunoyer et al. 2007; Smith et al. 2007). Phloem acts as a specific highway for transport of long-distance systemic silencing signals through specialized sieve tube cells from source to sink (Palauqui et al. 1997). Additionally, long-distance spread of silencing signal requires high amount of target transcripts (Garcia-Perez et al. 2004; Schwach et al. 2005). Spreading of miR166 expression in phloem tissues during leaf development indicates its involvement in long-distance signal movement to act at distance (Juarez et al. 2004). Abundance of PHO2 and miR399 in the phloem with regard to inorganic phosphate (Pi) alteration and coexpression suggests their involvement in systemic silencing (Lin et al. 2008). miR172 was found to be present in sRNA libraries prepared from phloem exudates and likely to play important role in long-distance signalling (Zeevaart 2008). miR319 gets transported from leaves to roots where it targets a subset of the TCP family of transcription factors that regulates *LOX2* expression (Yoo et al. 2004; Schommer et al. 2008; Buhtz et al. 2010). Phloem small-RNA-binding protein 1 (PSRP1) was subsequently shown to bind and facilitate movement of single-stranded sRNA molecules between cells (Ham et al. 2009). CmPP16 protein from *Cucurbita maxima* was shown to possess properties similar to those of viral movement proteins (Aoki et al. 2005).

Antiviral Defence Pathways in Plants: RNA Silencing

In 1992, Lindbo and Dougherty observed that transgenic plant expressing non-translatable coat protein of tobacco etch virus (TEV) was resistant to cognate virus. Taking clue from this observation, it was rather sagaciously proposed that the resistant phenotype was the consequence of a mechanism active in cytoplasm which target and destroy the mRNA in sequence-specific manner (Lindbo et al. 1993). The hypothesis was the first step in building the concept of RNA silencing as antiviral defence. Later on it was also observed that an infectious viral cDNA clone engineered to carry a part of a host gene,

when mobilized inside the plant, caused silencing of both the specific host gene and the viral sequence. Antiviral RNA silencing in plant has turned out to be an integrated network of at least 3 different mechanisms, namely, cytoplasmic RNA silencing, endogenous mRNA silencing by microRNAs and DNA methylation-dependent silencing at transcriptional level (Baulcombe 2004). These mechanisms not only provide antiviral resistance but also are crucial for cellular functions such as regulation of gene expression, maintenance of genome integrity and stress response. The basic processes of RNA silencing have been well documented in several reviews (Meister and Tuschl 2004; Eamens et al. 2008; Ruiz-Ferrer and Vionnet 2009; Ding 2010; Llave 2010).

Cytoplasmic RNA silencing (Fig. 1a) starts through a process known as virus-induced gene silencing (VIGS). dsRNAs or hpRNAs are targeted by DCLs to produce small RNAs of varying length (21–24 nt). The resulting small RNAs are unwound with the help of an ATP-dependent RNA helicase and subsequently incorporated into RISC-containing AGO1 to perform degradation of viral mRNA and translational repression or methylation of the homologous target genes. Transcriptional gene silencing (Fig. 1c) initiates in the nucleus following infection with viruses or subviral elements which are gradually subjected to inactivation through DNA methylation (TGS). RNA-directed DNA methylation (RdDM) plays a very important role in terms of silencing transposons as well as repetitive DNA elements to maintain genome integrity as well as stability (Matzke et al. 2009; Haag and Pikaard 2011). In RdDM, dsRNAs are synthesized by a DNA-dependent RNA polymerase called RNA polymerase IV (Pol IV) and RDR2 specific to plant and then processed by DCL3 to produce 24-nt siRNAs. These 24-nt siRNAs form an AGO4-containing RISC and interact with the nascent transcript prepared by RNA Pol V (another plant-specific RNA polymerase). This interaction facilitates recruitment of various methylation factors like DRM2, and ultimately de novo cytosine methylation of the target DNA takes place. Therefore, in general RdDM has been known to contribute to plant defence by transcriptional repression of genes from DNA viruses.

The miRNA pathway (Fig. 1b) starts when the miRNA genes are transcribed by RNA polymerase II, and the resulting transcripts contain complementary regions that form short imperfect hairpins. These imperfect hairpins are processed by DCL1 in the nucleus into 21-nt miRNAs with the aid of several other proteins like zinc-finger protein SERRATE and the dsRNA-binding proteins DRB1 and HYL1. The miRNAs play a decisive role in plant development by either repressing or optimizing the expression of various transcription factors associated with developmental processes. miRNAs in plants function through homology-dependent RNA degradation as well as through translational repression (Brodersen et al. 2008) unlike animal miRNAs which usually bind to 3′ UTR. In cytoplasmic siRNA-dependent RNA silencing pathway, the exogenous or endogenous long dsRNAs or short hpRNAs are degraded by either DCL4 or DCL2 to generate 21- and 22-nt siRNAs, respectively. These siRNAs then recruited onto AGO1-containing RISC and RISC-containing guide siRNA cleave target mRNAs. RDR6, one among the six reported RDRs of Arabidopsis, then synthesizes long double-stranded RNA using ssRNAs as template to give rise to trans-acting siRNAs of 21 nt which also have been found to play a role in various stress responses as well as plant developmental processes. Another type of siRNAs called natural antisense siRNAs have been reported in many eukaryotes including plants which are produced from cis-natural antisense transcripts (cis-NATs) in response to various biotic and abiotic stresses (Zhang et al. 2012).

Counter-Defence Response of Virus: Plant Viral Suppressors (VSRS)

Plant viral synergism is defined by a situation, where a plant infected with two or more unrelated viruses shows symptoms much severe than that caused by either of the virus alone. Majority

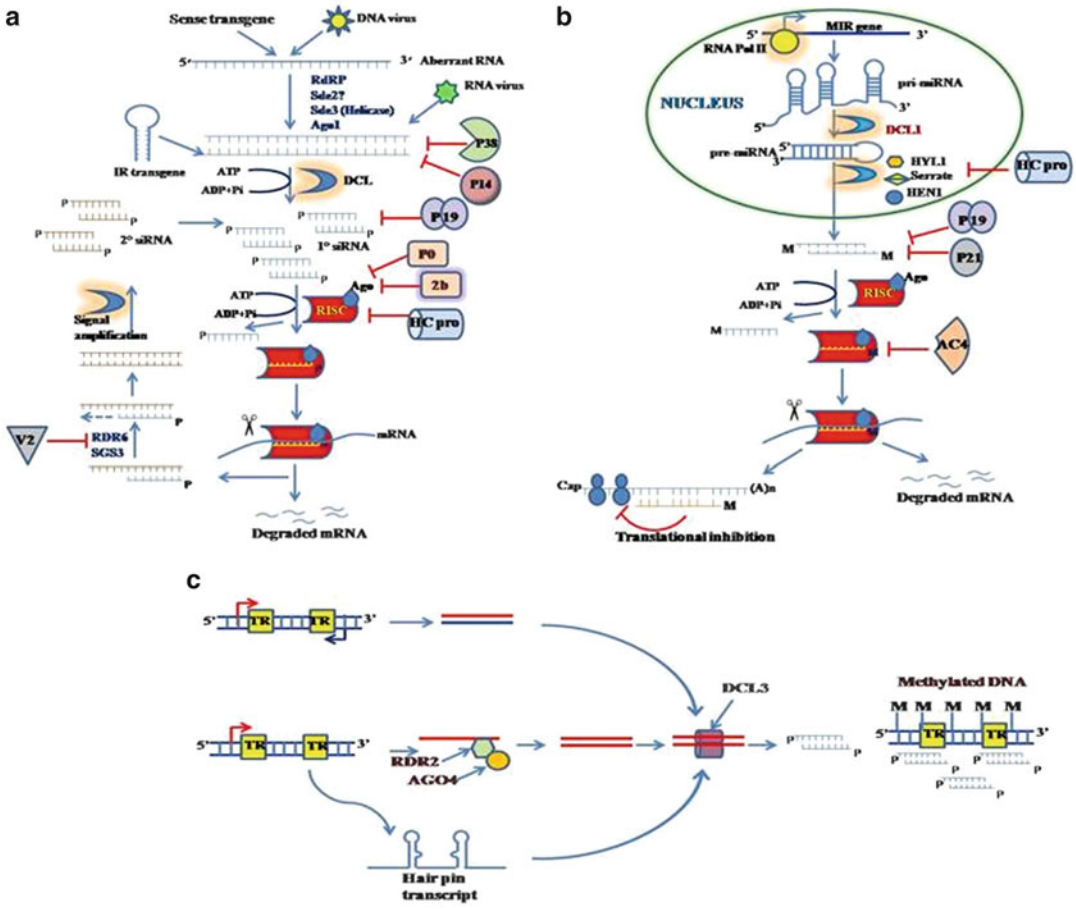

Fig. 1 Anti-viral RNA silencing pathways in plants and their suppression by plant viral encoded suppressors. Three major RNA silencing pathways include (**a**) cytoplasmic RNA silencing, (**b**) endogenous mRNA silencing by mRNAs and (**c**) transcriptional gene silencing through RdDM. Plant VSRs are represented by various shapes, while various steps of inhibition is represented by *block arrows*

of the synergistic interaction between two viruses usually involves potyvirus as one of the co-infecting virus. With the discovery of importance of helper component proteinase (Hc-Pro) of potyvirus in synergism, the concept of viral suppressor working as a counter-defence tool was born. Hc-Pro was designated as viral suppressor of gene silencing (Vance et al. 1995). Viral suppressors (VSRs) have emerged as one of the most potent tricks available with the viruses to invade hosts' defence for establishing successful pathogenesis. VSRs are found in almost all the plant viruses though few reports of suppressors from insects and mammalian viruses are also available at present. Plant VSRs have been found to evolve as diverse group of proteins and share very less sequence homology across the genera. VSRs are presumably evolved to counter the host silencing machinery-mediated defence response and therefore to suppress the host surveillance system. Different suppressors are reported to inhibit different steps of RNA silencing machinery by interacting with different effector components by any of the following ways: (i) through interaction with dsRNA to inhibit their processing by Dicers, (ii) binding to siRNAs and sequestering them to make them unavailable for the RISC, (iii) interacting either directly or indirectly with AGO to degrade or inactivate them and thus preventing functional

RISC assembly and (iv) inhibition of systemic silencing by interacting with either host RDR or DCL4 or DBR4. In addition, some other mechanisms have also been proposed. Some VSRs activate specific group of miRNAs and thereby inhibit some of the important effector molecule of RNA silencing machinery. In other cases, VSRs outcompetes HEN1 for binding to siRNA duplex having 2-nt overhang at 3′ end. There are VSRs, specially found in DNA viruses, which have been shown to inactivate TGS either by transactivating a set of host genes, which in turn act as suppressor or inactivate some of the host methyl transferases. Role of the individual group of suppressors and their mode of action is given in Table 1.

In addition to their primary role in suppression of antiviral RNA silencing, VSRs can act as potent mediator of plant viral diseases by affecting the intrinsic function of essential host factors through direct or indirect interactions. For example, P2 protein, encoded in *Rice dwarf virus* (RDV), interacts directly with rice ent-kaurene oxidases (Zhu et al. 2005) and interferes with gibberellin biosynthesis. Reduced hormone accumulation results in stunting of the infected rice plants. CMV-2b protein has been shown to interact with Arabidopsis catalase (CAT3) and interfere with its scavenging activity (Inaba et al. 2011). At least 10 host proteins were reported to contribute to pathogenicity of tombusviruses (Ishibashi et al. 2010).

The Plant Fights Back: Phenomenon of Host Recovery

The idea of 'host recovery' phenomenon came as a converging mechanism of both natural resistance and host PTGS where the plants infected by virus showed initial symptom on inoculated leaves, but the newly emerging leaves were completely asymptomatic. The systemic and newly emerged leaves provided complete sequence-specific resistance against the virus. The incident of virus-induced symptom recovery was observed for the first time in 1928 when the initial leaves of tobacco plants infected with tobacco ring spot virus showed necrosis and disease symptom. The upper systemic leaves were asymptomatic and consequently showed resistance to secondary infection by the homologous virus (Wingard 1928). Further study suggests that methylation-dependent gene silencing is also associated with host recovery. Such type of recovery has been well documented in geminivirus-infecting host plants. Wild-type *Arabidopsis* and *N. benthamiana* plants inoculated with Beet curly top virus (BCTV) $L2^-$ mutant showed recovery (L2 interferes with methylation by interacting with host methyl transferase) owing to the recovery of host from the mutant virus infection (Hormuzdi and Bisaro 1995; Wang et al. 2003). Geminivirus-induced symptom recovery has also been reported in watermelon, cassava and pepper following infection with cucurbit leaf crumple virus, African cassava mosaic Cameroon virus [ACMV-CM] and pepper golden mosaic virus, respectively (Hagen et al. 2008; Chellappan et al. 2004; Rodriguez-Negrete et al. 2009). Transient expression of dsRNA corresponding to viral IR showed enhanced symptom recovery in Zucchini plants (Hagen et al. 2008). Recovery of plants from virus infection was linked with RNA silencing machinery, and particularly in geminiviruses recovery was correlated with reduced viral titre followed by increased viral siRNA accumulation (Chellappan et al. 2004). Natural recovery of host can also be observed following infection with nepovirus (Ratcliff et al. 1997) and caulimovirus (Covey et al. 1997). In contrary to previous studies as observed in DNA viruses, it was reported that the recovery of *N. benthamiana* carrying functional RDR1 orthologue of *Medicago truncatula* was associated with RNA silencing but not with reduced viral titre from a necrotic response induced by a nepovirus, Tomato ring spot virus (ToRSV). The disappearance of symptoms was not accompanied by reduction of viral mRNA (Jovel et al. 2007). Mutation in AV2 also leads to recovery (Basu et al. unpublished data) because of its inability to bind to SGS3, and therefore, allowing the

Table 1 Different plant viral suppressors and their mode of action

Mechanism of suppression	Virus genus	Name of the virus	VSRs	Other functions	Reference
Binding to dsRNA	Aureusvirus	Pothos latent aureusvirus	P14	Symptom determinant	Merai et al. (2005)
	Carmovirus	Turnip crinkle virus	P38	Coat protein	Thomas et al. (2003)
					Qu et al. (2003)
	Cucumovirus	Tomato aspermy virus	2b	Nuclear localization/	Brigneti et al. (1998)
		Cucumber mosaic virus	2b	Host-specific movement	
	Tenuivirus	Rice hoja blanca virus	NS3	Unknown	Bucher et al. (2003); Yang et al. (2011b)
	Nodavirus	Flock house virus	B2	Plaque formation	Li et al. (2002)
	Tospovirus	Groundnut bud necrosis virus (GBNV)	NSs	Interference in plant defence and development	Goswami et al. (2012)
Sequestering/binding siRNAs duplex	Tombusvirus	Cymbidium ring spot virus	P19	Movement	Silhavy et al. (2002)
		Tomato bushy stunt virus	P19		
	Closterovirus	Beet yellow virus	P21	Replication enhancer	Reed et al., (2003); Ye and Patel (2005)
	Potyvirus	Tobacco etch virus	HC-pro	Movement, polyprotein processing, aphid transmission, pathogenicity determinant	Anandalaxmi et al. (1998), Brigneti et al. (1998); Kasschau and Carrington (1998)
	Pecluvirus	Peanut clump virus	P15	Movement	Dunoyer et al. (2002)
	Hordei virus	Barley stripe mosaic virus	γB	Replication enhancer, movement, seed transmission and pathogenicity determinant	Yelina et al. (2002)
Binding to single-stranded miRNA/siRNA	Geminivirus	African cassava mosaic virus	AC4	Movement , virulence	Chellappan et al. (2005)
Interfering methylation of siRNA/miRNA	Tombusvirus	Carnation Italian ring spot virus	P19	Movement	Lozsa et al. (2008); Yu et al. (2005)
	Tombus	Tobacco mosaic virus	P122, P126 and P130	Replication protein	Kubota et al. (2003)
					Csorba et al. (2007)
					Vogler et al. (2007)
Degrading 21-, 22- and 24-nt siRNAs to 14 nts	Closterovirus	Sweet potato chloratic stunt virus	RNase3	dsRNA-specific endonuclease and helps in viral synergism	Cuellar et al. (2009)

(continued)

Table 1 (continued)

Mechanism of suppression	Virus genus	Name of the virus	VSRs	Other functions	Reference
Interaction with DRB4	Caulimovirus	Cauliflower mosaic virus	P6	Viral translational transactivator , motile inclusion formation and microtubule stabilization, inhibition of signalling responses to salicylic acid and regulation of innate immunity	Hass et al. (2008) Harries et al. (2009) Love et al. (2012)
Targeting AGO1 protein	Cucumovirus	Fny-CMV	2b	Nuclear localization and movement	Mayers et al. (2000) Zhang et al. (2006)
Degradation of AGO1 through SCF complex	Polerovirus	Beet western yellows virus and potato leaf roll virus	P0	Pathogenicity determinant	Pazhouhandeh et al. (2006), Bortolamiol et al. (2007); Baumberger et al. (2007)
Interfering RDR6-SGS3-mediated signal amplification	Begomovirus	Tomato yellow-leaf curl virus	V2	Pre-coat protein	Glick et al. (2008)
Inhibiting RDR6-dependent 2^0 siRNA production	Potexvirus	Potato virus X	P25	Movement, Nucleotide binding and RNA helicase	Vionnet et al. (2000) Kalinina et al. (2002)
		Turnip yellow mosaic virus	P69	Movement, pathogenicity determinant	Chen et al. (2004)
Blocking intercellular spread of silencing	Closterovirus	Citrus tristeza virus	P20, P23 and CP	Replication enhancer, nucleic acid binding and encapsidation, respectively	Lu et al. (2004); Chiba et al. (2006)
Suppressing local and systemic S-PTGS	Phytoreovirus	Rice dwarf virus (RDV)	Pns10	Actin binding, viroplasm assembly	Cao et al. (2005); Wei et al. (2006); Jia et al. (2012)
Interfering long-distance and systemic silencing	Hordeivirus	*Poa semilatent virus*	γb	Movement and virulence	Yelina et al. (2002)
Inactivation of adenosine kinase and sucrose non-fermenting 1 (SNF1)	Begomovirus	Tomato golden mosaic virus	AC2	Transcriptional activator	Bisaro (2006)
		Beet curly top virus			Wang et al. (2003) Hao et al. (2003)
Interaction and attenuation of S-adenosyl methionine (SAMDC1) and its degradation	Curtovirus	Beet severe curly top virus (BSCTV)	C2	Transcriptional activator	Zhang et al. (2011b)
Inhibiting S-adenosyl-L-homocysteine hydrolase (SAHH)	Begomovirus	Tomato yellow-leaf curl china virus [TYLCCV]	βC1	Movement, virulence	Yang et al. (2011a)

amplification of silencing signal in presence of RDR6–SGS3 interaction and the secondary siRNA so produced degrade the viral mRNA and ultimately led to recovery in the newly emerging systemic leaves.

The Role of Plant miRNA in Plant–Virus Interaction

Recently it has been reported that plant miRNAs are responsive to developmental cues and environmental stresses. Tomato plants after infection with Cucumovirus and Tobamovirus showed significant differential expression in 85 % of its total miRNA pool (Fang and Spector 2007). All the differentially expressed miRNA were classified into 25 families. Among all these families, miR159 and miR171 contained most number of miRNAs. Most of these miRNAs were targeted to control expression of transcription factors, plant flower and leaf and height development and reproductive growth. High-throughput sequencing revealed a set of conserved miRNAs. Earlier it was also shown that infection with *Tobamoviridae*, *Potyviridae*, and *Potexviridae* families caused altered accumulation of certain miRNA in *Nicotiana tabucum* in which miRNAs 156, 164, 165 and 167 accumulated to higher levels compared to noninfected tissues (Bazzini et al. 2007). Silencing suppressors of various plant viruses have been reported to change target mRNA level through directly altering the accumulation of endogenous miRNA levels inducing changes also in target mRNA accumulations (Kasschau et al. 2003; Dunoyer et al. 2004; Zhang et al. 2006). Other workers have established the correlation between enhanced expression of miR168 and *AGO1* mRNA in virus-infected plants (Zhang et al. 2006; Csorba et al. 2007; Havelda et al. 2008). These reports also make room to develop a novel strategy where manipulating the host miRNA level holds promise to combat with the viral stress.

Application of RNA Silencing Towards Plant Virus Resistance

Plant pathogens especially viruses are responsible for severe loss in crop production every year throughout the world. Earlier these pathogens were controlled using conventional measures including crop rotation, use of insecticides and breeding with resistant varieties. During 1986, Beachy and his associates demonstrated for the first time the use of pathogen-derived sequence (using TMV coat protein) to engineer resistance in the host (Powell et al. 1986). Since then various strategies based on either protein-mediated or RNA-mediated resistance have been developed. The actual mechanism of protein-mediated resistance is still not clear, and several pathways may be involved based on the type of gene used for engineering resistance. On the other hand, the mechanism of RNA silencing is well understood. During the last two decades, substantial effort has been channelized based on siRNA-mediated RNA silencing to engineer resistance in plants. These approaches differed in varied precursor sequence like pathogen-derived sequences in sense or antisense orientation, shRNA constructs, intron hairpin constructs and artificial miRNA sequences that were used to generate siRNA in plants. The use of intron hairpin RNAi constructs has been shown to be highly effective and caused nearly 100 % silencing of the target gene as compared to sense, antisense or hpRNAi constructs (Smith et al. 2000). It is also possible to target multiple viruses using single-RNAi constructs containing sequences from multiple viruses to generate broad-spectrum resistance (Jan et al. 2000; Bucher et al. 2006). One important hindrance to employ RNAi for engineering resistance is the selection of target and the minimum length of the target sequence for effective silencing. Hutvagner et al. (2000) showed that siRNAs generated by silencing of GUS gene mainly correspond to two-third region of 3′end of mRNA. Now a number of computational algorithms are freely available online for the rational design of siRNA and selection of target

sequence to generate effective silencing of the target gene using several parameters.

Increased knowledge of microRNA (miRNA) biogenesis machinery and their role in regulation of transcript expression has helped to develop synthetic or artificial miRNAs (amiRNAs) to direct efficient silencing of any target transcript. amiRNA-mediated approach is one of the recently developed strategies with wide range of applications especially for conferring viral resistance in crop plants. Several studies have established potential of amiRNAs to target and degrade mRNAs of both viral and plant origin and thereby specifically degrading the target mRNA (Schwab et al. 2006, Niu et al. 2006, Qu et al. 2007, Zhang et al. 2012). Recently, amiRNAs targeting different ORFs of Tomato leaf curl virus AC1 along with AC2/AC4 (Yadava and Mukherjee 2010), the middle region of the AV1 (coat protein) transcript (amiR-AV1-3) and the overlapping region of the AV1 and AV2 (pre-coat protein) transcripts (amiR-AV1-1) (Vu et al. 2013) were designed and expressed in transgenic tomato plants to confer resistance and tolerance to ToLCV, respectively. Seemingly, amiRNA approach has several advantages over conventional siRNA-mediated strategy. In the hairpin RNAi approach, multiple siRNAs are formed from single precursor, and off-target genes are often silenced, while in amiRNA approach only one mature miRNA is produced targeting the specific gene. In amiRNAs mismatches can be introduced to avoid signal amplification and transitivity. siRNA-based gene silencing has been shown to be temperature dependent, while miRNA biogenesis has been shown to occur in various conditions and under extreme temperatures and therefore has wider scope of applications.

Conclusion

RNA silencing is an evolutionary conserved mechanism, which operates in several eukaryotic organisms across kingdoms and involves highly sequence-specific degradation of complementary RNA and transcriptional gene silencing. sRNAs of 21–24 nts in length are the key players of RNA silencing. The major components (players) of RNA silencing machinery in plants include AGO1, RDRs, DCLs, HEN1 and HYL1. These components are required for processing of dsRNA into siRNA and maintenance of RNA silencing. The mobile silencing signal moves from initiating cell to neighbouring cells through plasmodesmata and to long distance through phloem. Viruses are one of the most devastating pathogens of plants causing substantial crop loss every year. Viruses are both inducers and targets of RNA silencing. dsRNAs generated during replication of RNA viruses or transcription of overlapping sequences in DNA viruses induce RNA silencing which leads to sequence-specific degradation of target RNA into 21–24-nt siRNAs. Viruses in turn evolved suppressors of RNA silencing as powerful weapon to counter the host defence machinery. The suppressors encoded by different plant viruses act at different steps of RNA silencing thus inhibiting RNA silencing pathways in plants. Occasionally, infected plants show recovery from virus infection leading to remission of symptoms. Recovered plants remain immune to subsequent infection by a homologous virus through RNA silencing mechanism. Based upon the knowledge of RNA silencing mechanism, it is possible to engineer virus resistance in plants based on RNA silencing using viral-derived sequences as target.

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