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Viral Suppressors of RNA Silencing in Plants

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ABSTRACT

In eukaryotes, small RNAs play a crucial regulatory role in many processes including development, maintenance of genome stability and antiviral responses. These different but overlapping RNA-guided pathways are collectively termed 'RNA silencing'. In plants, RNA silencing serves as a major line of antiviral defense that is induced by, and targeted against viruses. As a counter-defensive strategy, viruses have evolved to encode suppressor proteins that inhibit various stages of the silencing process. These suppressors are diverse in sequence and structure and appear to be encoded by virtually any type of plant viruses. This review focuses on the novel methods of suppressor screening and revealing the characteristics of RNA silencing suppressors. We have also discussed the mechanism of suppression activity, which principally operate by modifying the accumulation, activity, and/or transmission of siRNAs through either direct interaction with the RNA species or components of the RNA silencing machinery. Finally, the biotechnological applications of silencing suppression have been considered.

Keywords: plant viruses, siRNAs

CONTENTS

INTRODUCTION

RNA silencing/interference (RNAi) is a well established phenomenon of regulating gene expression through nucleotide sequence specific interaction mediated by small RNA molecules. The first significant finding that paved the way for the identification of this gene regulatory mechanism was the production of variegated flowers during the engineering of *Petunia* plants for over expression of the chalcone synthase (*chsA*) gene. The *chsA* transgene was supposed to enhance the anthocyanin production and hence flower colour (Napoli *et al.* 1990; van der Krol *et al.* 1990). It was later confirmed that the introduction of the *chsA* transgene led to silencing of the endogenous gene expression, resulting in the development of white patches in the petals (Jorgensen 1995; Meister and Tuschl 2004). A search for the mechanism revealed that the silencing was not due to the reduced nuclear transcription of the transgene but degradation of the transcript in the cytosol through a partial mRNA duplex formation. This silencing phenomenon first identi-

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fied in plants was termed "co-suppression" (Napoli *et al.* 1990; van der Krol *et al.* 1990) and subsequently reported in many organisms- from fungi (Cogoni and Macino 1999) to animals (Jones and Schedl 1995; Pal-Bhadra *et al.* 1997, 1999). This also triggered the discovery of the components and pathways of the silencing machinery. Till date, an array of small 19-30 nt RNA molecules have been identified including the microRNAs (miRNAs), small interfering RNAs (siRNAs) and the Piwi interacting RNA (piRNAs). The former two small RNAs act through two evolutionarily conserved regulatory pathways, viz. transcriptional gene silencing (TGS) and post transcriptional gene silencing (PTGS) while the pi-RNAs cause only TGS. The TGS operates in the nucleus and limits the gene expression by inducing mostly the histone or DNA methylation. On the other hand PTGS, which is also known as RNA interference (RNAi) in animals and gene quelling in fungi, is mainly a cytoplasmic event that acts at the transcript level by way of repression of translation or cleavage of the target mRNA which form W-C pairing with the small RNAs (Agrawal *et al.* 2003; Sanan-Mishra *et al*. 2007).

The key molecules of RNA silencing machinery comprises – Dicer-like enzymes (DCLs), Argonaute proteins (AGO) and RNA-dependent RNA polymerases (RdRPs) Agrawal *et al.* 2003; Sanan-Mishra *et al*. 2007). The key initiator molecule for RNA silencing is the double stranded RNA (dsRNA) which can either be delivered exogenously or produced *in vivo* by RdRPs (**Fig. 1**). The dsRNAs can be produced as a result of transcription through the inverted repeats, convergent transcriptions or intrinsic secondary structure of the transcripts. The dsRNAs are then diced into small duplex RNAs of 20-26 nts with the characteristic 2nt 3' overhangs by the enzyme DICER, which has evolved from a type III RNA endonuclease and is a prime component of the DICER complex (Fire *et al.* 1998; Hamilton and Baulcombe 1999; Elbashir *et al.* 2001; Wesley *et al.* 2001). Each DICER complexes with its cognate RNA binding protein and the absence of latter makes the DICER inactive *in vivo*. Subsequently the diced small RNAs are loaded into an effector complex known as RNA-Induced Silencing Complex (RISC), to guide specific silencing of cognate transcripts by cleavage of the target RNA or repression of translation (Hammond *et al.* 2000; Elbashir *et al.* 2001; Hammond *et al.* 2001; Nykanen *et al.* 2001). In several cases, the primary siRNAs prime the synthesis of secondary long dsRNA by RdRP-mediated activities for generation of secondary siRNA or transitive siRNAs (**Fig. 1**). Thus the siRNAs are amplified and the consequent silencing spreads beyond the location from where silencing is initiated. Within the target transcripts, the silencing generally spreads in the 5'-3' direction and not 3'-5' direction (Alder *et al.* 2003; Haque *et al.* 2007). However, the bidirectional silencing has also been reported in plants (Agrawal *et al.* 2003).

GENE SILENCING IN PLANT DEFENSE

RNA silencing is important for the regulation of development and the control of transposition events in both plants and animals (Baulcombe 2004; Vastenhouw and Plasterk 2004; Matzke and Birchler 2005). In fission yeast, RNA silencing establishes and maintains the heterochromatin structure of the centromere and mating type locus (Grewal and Rice 2004). It is one of the major principles behind 'pathogen-derived resistance (PDR)' towards the plant viruses and thus it acts as a major adaptive immune system (Baulcombe 2004; Voinnet 2005a).

During virus infection long dsRNAs, generated as replicative intermediates or due to secondary structure or convergent transcription of viral RNAs, serve as a potent trigger of RNA silencing. The long dsRNA is converted into functionally different short (21–22 nt) and long (24–26 nt) siRNA duplexes (Hamilton *et al.* 2002) by different DCLs (Tang *et al.* 2003). The elevated siRNA levels of viral transcripts are correlated with the reduction in viral titer (Ratcliff *et al.* 1997; Szittya *et al.* 2002), as the viral siRNA guide a multi-subunit endonuclease, referred to as RISC to sequence specific cleavage of the viral RNA (Hammond *et al.* 2000; Tang *et al.* 2003). Thus, the host RNA silencing mechanism serves as a major line of antiviral defense to protect the plants from viral infection (Voinnet 2001). Moreover, the plant mutants defective in one or several of the RNA silencing pathways are found to show enhanced susceptibility to virus infection (Dalmay *et al.* 2000; Mourrain *et al.* 2000; Boutet *et al.* 2003; Wilkins *et al.* 2005).

In plants the RNA silencing machinery not only elicits antiviral activity at a localized site but the silencing signal spreads systemically, from cell to cell and triggers RNA silencing in the distant tissues of the infected plants. It has been proposed that short siRNAs play a role in the cell-to-cell movement for short distance movement of the silencing signals (Himber *et al.* 2003) while the long siRNAs are involved in DNA methylation and systemic spread of silencing (Hamilton *et al.* 2002; Himber *et al.* 2003). If these signals spread and the silencing condition is established ahead of a viral infection, viral RNAs are degraded before viral replication at the viral infection front (Voinnet *et al.* 2000). This phenomenon has also been described as host 'recovery' phenotype, in which the newly emerging leaves lack viral symptoms and remain substantially free of virus (Baulcombe 2004).

Additionally, the RNA silencing processes in response to one mild virus may cross-protect the infected plant against virulent infection by another related virus carrying sequences homologous to the pre-infecting one (Ratcliff *et al.* 1997), a phenomenon scripted by the plant virologists as early as the 1920s. All such phenomena are now collectively known as virus induced gene silencing (VIGS). The effector siRNA molecule serves as molecular memory which counters the virus on re-infection. In the "post-transient-invasion" phase, the molecular memory of the viral RNA is provided by RDR-replicated fragments of viral RNA or sub-minimal replication of the viral genome (Martín-Hernandez and Baulcombe 2008). Hence, the RNA silencing activated against the infecting virus not only helps the host from the initially virulent infection so that the new growth is both symptom and virus free but also bestows resistance to a secondary challenge by the same or homologous viruses. In recent years, antiviral characteristics of RNA silencing are being exploited to silence or knock off the host genes for functional analysis. The viral open reading frames (ORFs) are used to transmit inverted repeats of target gene sequences with intervening introns to ensure production of siRNAs against the desired transcripts when the host plants mount resistance against the challenge viruses.

DISCOVERY OF PLANT RNA SILENCING SUPPRESSORS

The viruses have also evolved to overcome or suppress the strong host defense response of RNA silencing and cause severe pathogenic symptoms and diseases. The viruses have been found to counter the host RNA silencing defense mechanism by encoding protein(s) known as RNA silencing suppressor (RSS). The viral suppressor is a virus encoded protein or RNA element that blocks silencing of viral nucleic acid sequences guided by siRNAs or miRNAs. The discovery of RSS itself played an important role in establishing RNA silencing as a natural antiviral response.

The initial indication that viruses encode silencing suppressors came from seminal experiments aimed at understanding the phenomenon of synergism during co-infection. Following synergism the weak viral symptoms are aggravated by co-infection of a second, unrelated virus (Vance *et al.* 1995). The study of specific viruses like the Potex virus (*Potato virus X* or PVX) and potyvirus has led to the identification of the potyviral Helper component proteinase (HC-Pro) as the synergism determinant (Pruss *et al.* 1997). This determinant later turned out to be a strong suppressor of RNA silencing.

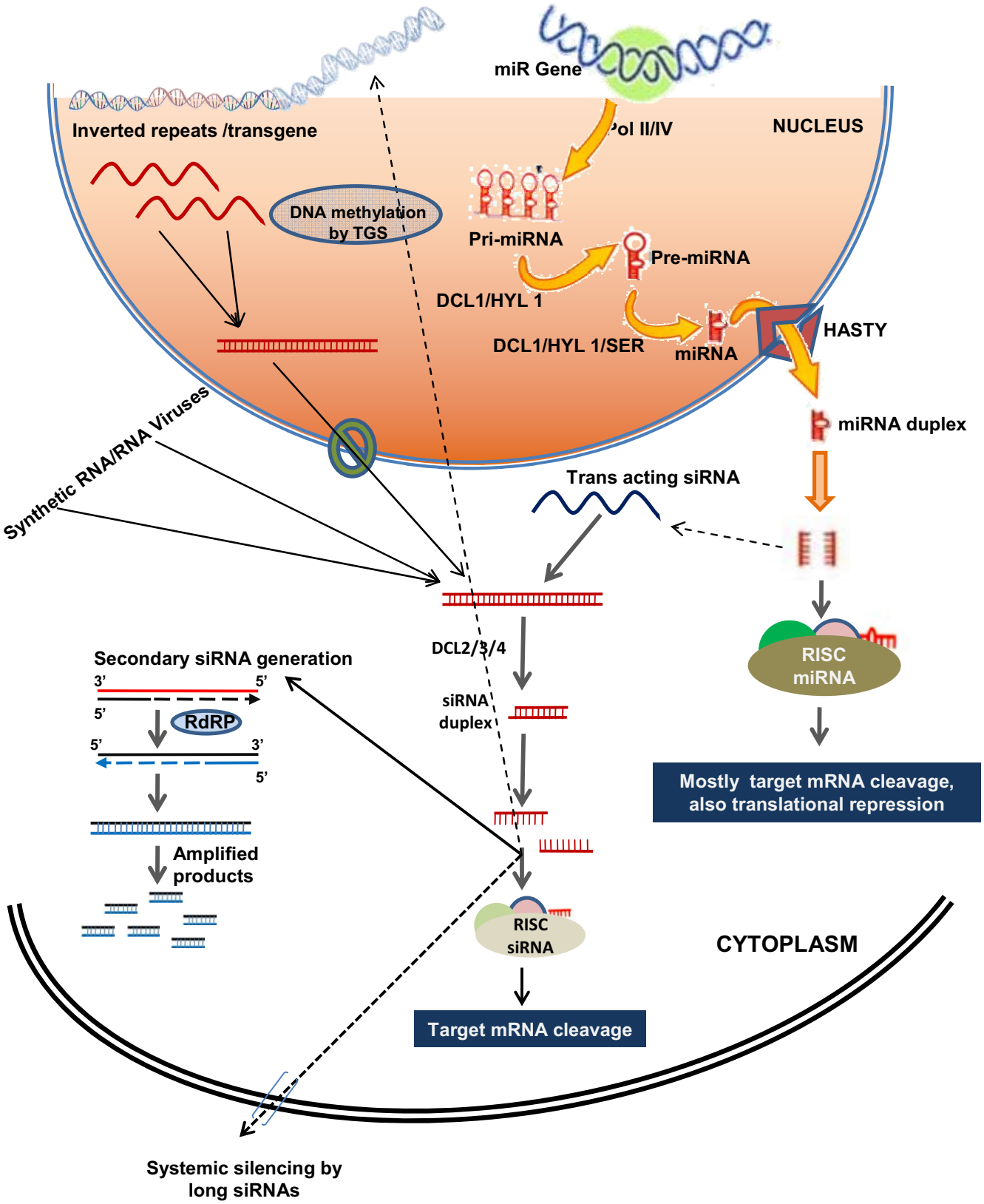

Fig. 1 Mechanism of RNA silencing. The initiator molecule for RNA silencing is the small double stranded RNA (dsRNA). The key effector molecules include miRNAs and siRNAs. The miRNAs are transcribed from endogenous loci by RNA Polemearse (Pol) II or RNA Pol IV as primary miRNAs (pri-miRNA) transcripts having highly folded structures. These are processed to precursor miRNAs (pre-miRNA) having characteristic stem-loop structures with the help of DCL1 and HYL enzymes. The pre-miRNAs are diced into small duplex RNAs of 24 nts with the characteristic 2nt 3' overhangs by the enzyme DCL1, HYL1 and SER1 and other uncharacterised proteins. The miRNA duplex is subsequently transported to cytosol with the help of HASTY proteins. The long double stranded RNAs could also be derived from exogenously delivered RNAs or infecting viruses. These may also be produced *in vivo* as a result of transcription through the inverted repeats, convergent transcriptions or intrinsic secondary structure of the transcripts. They are processed into small 20-24 nt duplexes from long double stranded RNAs by DCL2, DCL3 or DCL4. Subsequently the small RNAs are loaded into an effector complex known as RNA-Induced Silencing Complex (RISC), to guide specific silencing of cognate transcripts by cleavage or translational repression of the target RNA. They may also get transported back to the nucleus to initiate transcription gene silencing (TGS) through DNA methylation. In several cases, the miRNAs or primary siRNAs prime the synthesis of secondary long dsRNA by RdRP mediated activities for generation of trans-acting siRNA or secondary siRNA, respectively resulting in the amplification of silencing signal. The secondary siRNAs are mostly transitive siRNAs that are transported out of the cell thereby spreading silencing beyond the location of initiation.

Synergistic viral diseases of higher plants are caused by the interaction of two independent viruses in the same host and are characterized by a dramatic increase in symptoms and in accumulation of one of the co-infecting viruses. One of the classical examples is PVX/potyviral synergism. PVX, by itself, causes mild symptoms but multiplies vigorously during co-infection and it has been shown that increased pathogenicity and accumulation of PVX are mediated by the expression of potyviral RSS, HC-Pro (Pruss *et al.* 1997). It was proposed that HC-Pro suppresses a host defense response akin to RNA silencing that normally restricts accumulation of these viruses (Ruiz *et al.* 1998). This hypothesis was subsequently verified independently by three research groups. In the course of those experiments, the 2b protein of *Cucumber mosaic virus* (CMV) was also identified as a RSS (Anandalakshmi *et al.* 1998; Brigneti *et al.* 1998; Kasschau and Carrington 1998). Similar synergism has been evidenced in other viruses as well, including the geminiviruses where the differential role of AC2 and AC4 governs the synergism between two different cassava geminivirus strains (Vanitharani *et al.* 2004).

Hence, RNA induced silencing was probably a general constraint to virus accumulation in plants and suppression of silencing was the likely shared property of many plant viruses. These findings were also coupled to the earlier investigations that characterized HC-Pro and CMV2b as pathogenicity determinants, i.e. factors that were not strictly required for viral replication but needed for efficient accumulation at the cellular and/or whole plant level. A reinvestigation of pathogenicity factors from diverse viruses interestingly identified many of them as potent silencing suppressors (Voinnet *et al.* 1999). Since then several additional proteins have now been shown to act as RSSs (**Table 1**). The ubiquitous nature of silencing suppression indicates that it is probably an absolute requirement for plant viruses if they are to accumulate and spread in plants.

Even though the majority of RSSs have been identified from plant viruses, there are a few reported from insect and animal viruses as well (Li *et al.* 2004; Wang *et al.* 2006). It is now well established that the RNA silencing machinery is present in animal systems and it likely functions as an important defense against transposons and viruses. It is therefore not unreasonable to expect that animal viruses would have been exposed to similar selection pressure as plant viruses during the course of evolution and would be expected to encode proteins with RSS activity. It is somewhat surprising, therefore, that the definitive reports of animal viral proteins with such suppressor activity are meager compared with reports on plant viruses. Interestingly, the first insect virus suppressor of RNA silencing described is the B2 protein encoded by *Flock house virus* (FHV) (Li *et al.* 2002). Using plant, insect or mammalian cell-based assays, a number of mammalian viruses encoded dsRNA binding proteins, majority of which serves as interferon or protein kinase R antagonists have been demonstrated to have RNAi suppressor activity. Some of the best studied examples so far are NS1 of *Influenza A virus* (Bucher *et al.* 2004; Li *et al.* 2004), VP35 of *Ebola virus*, E3L of *Vaccinia virus*, TAT of HIV-1, NSs of *La Crosse virus*, TAS of *Primate foamy virus-1* and C of *Hepatitis virus C* (Soldan *et al.* 2005; Schutz and Sarnow 2006; Haasnoot *et al.* 2007). Furthermore, it has been shown that the interferon antagonists like VP35, NS1 and E3L are the RNAi suppressors in human cells and these are capable of restoring the production of a HIV-1 strain, defective in the *Tat* gene (Haasnoot *et al.* 2007). These few examples demonstrate that RNA silencing and other innate antiviral responses are interrelated and a systematic search for RSS activity among the genes of most animal viruses could be very fruitful.

Table 1 List of RNA silencing suppressors identified from plant, animal and insect viruses.

Virus group with representative species	Name of suppressor
Plant viruses	
Tombusvirus *Tomato Bushy stunt virus*	P19
Potyvirus *Potato virus-Y*	HC-Pro
Luteovirus *Beet western yellow virus*	P0
Cucumovirus *Cucumber mosaic virus*	2b
Sobemovirus *Rice yellow mottle virus*	P1
Closterovirus *Beet yellow virus*	P21
Begomovirus *African cassava mosaic virus*	AC2, AC4, AV2
Pecluvirus *Peanut clump virus*	P15
Carmivirus *Turnip crinkle virus*	P38
Potexvirus *Potato virus-X*	P25
Tospovirus *Tomato spotted wilt virus*	NSs
Tenuivirus *Rice hoja blanca virus*	NS3
Hordeivirus *Barley stripe mosaic virus*	λb
Tobamovirus *Tomao mosaic virus*	P130
Closterovirus *Citrus tristeza virus*	P20, P23, CP
Aureusvirus *Pothos latent virus*	P14
Satellite virus associated with begomovirus DNAβ	βC1
Viroid *Hop stunt viroid*	Circular and linear mature RNA forms
Animal viruses	
Orthomyxovirus *Influenza A virus*	NS1
Rhabdovirus Ebola virus	VP35
Vaccinia virus	E3L
Retrovirus HIV-1	Tat
Orthobunyavirus La Crosse virus	NSs
Retrovirus Primate foamy virus-1	Tas
Flavivirus *Hepatitis virus C*	C
Reovirus	σ3
Adenovirus	VAI and VAII
Insect viruses	
Nodavirus *Flock house virus*	B2
Bicistrovirus *Drosophila* C virus	1A
Cricket paralysis virus	N-terminal fragment of 140 amino acids of CrPV nonstructural polyprotein.

CHARACTERISTICS OF RSS

A striking feature that emerges is the huge diversity in sequence and structure of these proteins and the fact that they are found in virtually any type of viruses are suggestive of multifarious activities of such proteins in the RNAi response pathways. Such diversity can be partly explained as evolutionary convergence, since silencing suppression evolved as additional features on unrelated proteins that already had diverse functions (**Table 1**). It seems that evolutionary selection of a particular class of viral proteins to function as silencing suppressor bears little relationship to any other primary function the viral protein might have in the virus life cycle. However, despite their diversity, the RSSs share some important characteristics.

"Synergism"

Co-infection of plants with certain pairs of unrelated viruses results in much more dramatic disease symptoms than those caused by infection with either virus alone and this phenomenon is termed synergism (Damirdagh and Ross 1967). RSSs have been identified as one of the major principle behind this synergism. For example the documented enhanced accumulation of non-potyvirus during co-infection with potyvirus has been realized to be due to RSS encoded by later, viz., HC-Pro (Pruss *et al.* 1997). The RSS potently suppress the siRNA-mediated (Carrington *et al.* 2001; Li and Ding 2001; Baulcombe 2002; Roth *et al.* 2004; Silhavy and Burgyan 2004; Moissiard and Voinnet 2004) and miRNA-mediated (Kasschau *et al.* 2003; Chapman *et al.* 2004; Chen *et al.* 2004; Dunoyer *et al.* 2004) pathways; resulting in breakdown of the host anti-viral defense response. The synergism is even more potent upon infection with

viruses expressing distinct RSS with different mode of suppression activity. For example, potyvirus synergism with two unrelated viruses PVX and CMV, involves co-action of distinct suppressors viz. HC-Pro with p25 or 2b; targeting intracellular and intercellular silencing, respectively (Mlotshwa *et al.* 2002). Similarly, synergistic severe mosaic disease caused by simultaneous infection with isolates of the Cameroon strain of *African cassava mosaic virus* (ACMV-(CM)) and *East African cassava mosaic Cameroon virus* (EACM/CV) in cassava and tobacco is characterized by a dramatic increase in symptom severity and a several-fold increase in viral-DNA accumulation due to synergestic activity of RSS, AC4 and AC2 encoded by the strains, respectively (Vanitharani *et al.* 2004). In fact, the expression of RSS in *trans* has been shown to enhance the severity of the otherwise mild virus to several folds. This phenomenon has been used as one of the screening assays for viral ORFs for RSS activity (Pruss *et al.* 1997; Brigneti *et al.* 1998; Voinnet *et al.* 1999). The severity of the synergism is mainly due to weakening of the host defense by targeting the silencing pathway at multiple points by RSS (Pruss *et al.* 1997; Mlotshwa *et al.* 2002).

Cross kingdom activity

An interesting feature of viral RSS is their ability to suppress RNA silencing in both animal and plant cells, regardless of the preferred host of the virus (Voinnet *et al.* 1999). The known RSS of insect and animal virus origin, including B2 of FHV, NS1 of *Influenza virus*, E3L of *Vaccinia virus*; and TAS of PFV-1 can efficiently suppress RNA silencing in plant systems (Li *et al.* 2002, 2004). Among the few suppressors of plant virus origin, such as p19 of TBSV, CP of TCV, p15 of *Peanut clump virus*, etc., that have been tested for silencing suppression activities in animal cell cultures, only p25 of PVX failed to retain suppressor function (Dunoyer *et al.* 2004; Lakatos *et al.* 2004). In fact, the RSSs of animal origin were able to functionally complement the activity of RSS from insect or plant viruses (Dunoyer *et al.* 2004; Schnettler *et al.* 2008). Similarly, the *Rice hoja blanca virus* encoded RSS NS3 was able to *trans* complement the activity of animal virus RSS TAT protein of HIV (Schnettler *et al.* 2009) in mammalian cells. Given that RNA silencing is a defense mechanism conserved across animals and plants; it seems logical to suggest that suppressors likely target some conserved aspects of the silencing pathways.

However, the ambiguity is escalated by the fact that the characterized protein components of the animal and plant silencing machinery share fairly low sequence identity, except for some conserved amino acid residues within several functionally important regions such dsRNA binding domains and PAZ domains (Carmell *et al.* 2002; Finnegan *et al.* 2003). Although it cannot be completely ruled out that these conserved domains are targeted by the RSS, what seems more likely is that the RSS targeting the RNA components (dsRNA and/or siRNA) of the pathway may have been preferentially selected. Evidence supporting this conclusion comes from the known affinity of p19 of TBSV for siRNA (Silhavy *et al.* 2002), the strong RNA binding activity of TCV CP (Skuzeski and Morris 1995), the established dsRNA binding affinity of NS1 of *Influenza virus* and E3L of *Vaccinia virus*. Extending on this theme, a recent report regarding the p14 silencing suppressor encoded by *Pothos latent virus* (PoLV), showed that it bound to both long and short dsRNA, including the siRNA duplex (Merai *et al.* 2005). Interestingly, although the genome of PoLV is similar to other tombusviruses, its suppressor (p14) is a smaller protein than p19 with higher affinity to long dsRNAs. Most recently, the B2 suppressor of FHV has been shown to bind both long and short dsRNA as well, lending additional support for dsRNA and siRNA as main targets of silencing suppressors (Lu *et al.* 2005). On the other hand, many RSSs, say AC2 protein of begomoviruses, fail to bind any form of RNA and thus they need to target host RNAi factors. Interestingly, though the FHV-B2 protein has strong affinity to dsRNA, it also targets the DICER protein of the insect cells to down regulate siRNA biogenesis (Singh *et al.* 2009).

The suppressor activities differ even among homologous proteins encoded by viruses of the same genus like triple gene block protein 1 (TGBp1) encoded by these potexviruses contributes to the variation in the level of RNA silencing suppression by potexviruses due to its drastically different levels of silencing suppressor activity (Senshu *et al*. 2009).

Pathogenesis by disregulation of miRNA accumulation

Systemic infection by plant viruses frequently results in disease symptoms that resemble developmental defects, including loss of leaf polarity, loss of proper control of cell division, and loss of reproductive functions (Hull 2001). These and other phenotypes are often been found to be due to the expression of the virus-encoded pathogenicity factors, many of which are suppressors of RNA silencing (Voinnet *et al.* 1999). Plants expressing P1/HC-Pro, p21, p19, AC4, AC2 and *Citrus tristeza virus*-CP exhibited moderate to severe defects in leaf and rosette development (**Fig. 2**, Chapmann *et al.* 2004; Chellappan *et al*. 2004; Singh *et al.* 2009). In plants and animals, miRNAs play an important role in the regulation of genes important for growth and development (Carrington and Ambros 2003; Bartel 2004; Wienhold and Plasterk 2005). Interestingly, the multiple developmental defects induced by the expression of RSS resembles with the phenotypes associated with the *dcl1* mutants (Kasschau *et al.* 2003). Therefore, suggesting that the RSS evolved by the viruses to counter the host generated antiviral-siRNA pathway interferes with the miRNA pathway as well (Pasquinelli and Ruvkun 2002; Chapman *et al.* 2004). This is probably due to the superficial similarity that exists between the various aspects like structure, biogenesis and functional pathways of siRNA and miRNA, and thus the RSSs have been found to seriously modulate the cellular

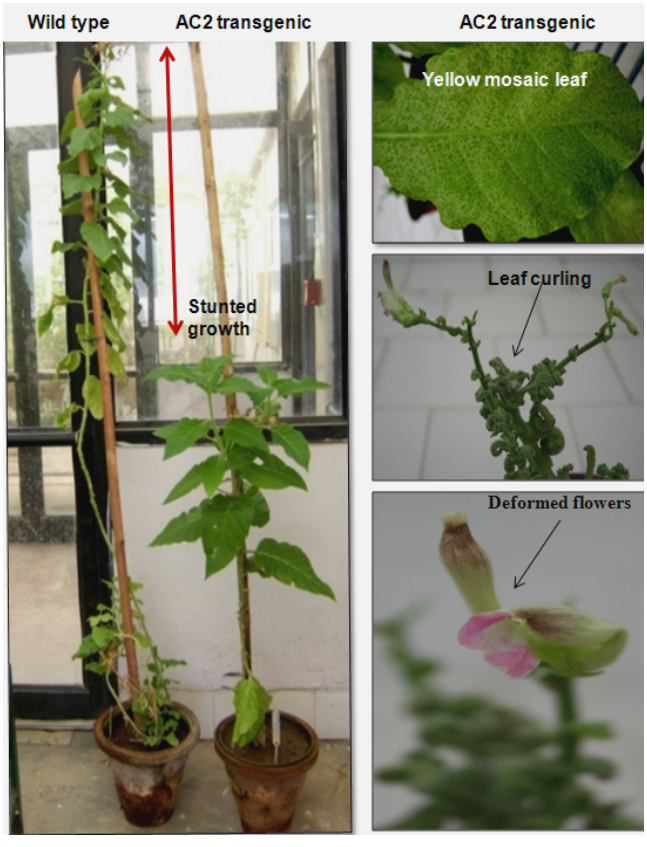

Fig. 2 Developmental anomalies in tobacco plant expressing MYMIV-AC2 protein.

miRNAs (Chapman *et al.* 2004; Chellappan *et al*. 2004; Dunoyer *et al.* 2004; Ren 2005). The suppressors have been found to down-regulate the miRNA activity, as the target mRNA level tested were highly elevated in presence of the RSSs (Chapmann *et al.* 2004). However, there are also few reports where the RSS up regulates the miRNA activity as the pathogenicity mechanism: for example, the case of p69 encoded by *Turnip crinkle mosaic virus* (Chen *et al.* 2004). It is note worthy that the exact mechanism of interference by a few of the RSSs on miRNA pathway has been deciphered, as p19, HC-Pro and p21 lead to developmental anomalies by interfering with the HEN-1 mediated methylation of miRNA (Yu *et al.* 2005). In this way the altered accumulation and/or functioning of miRNAs by RSSs interfere with the development and growth of plant and induce pathogenesis (Chapman *et al.* 2004; Dunoyer *et al.* 2004; Ren 2005). However, a better understanding of suppressor function will be needed to fully appreciate the role of suppressors in blocking the miRNA pathways.

SCREENING FOR RSS IDENTIFICATION

Identification of the RSS has now become an integral part of virus characterization. The major bottleneck in this is probably the unavailability of large array of screening systems. In plants however a number of assays have been developed, based on the suppression of RNA silencing of a reporter transgene. The transgene may be silenced constitutively (Elmayan and Vaucheret 1996) or locally by infiltration with an *Agrobacterium tumefaciens* strain carrying a Ti plasmid harboring the same gene, a process referred to as agro-infiltration (Voinnet and Baulcombe 1997; Voinnet *et al*. 1998). The suppression of silencing is assayed by introducing, the candidate protein into the silenced plants by a replicating virus vector, genetic crosses or agro-infiltration. We describe here some of the standard procedures, including the ones developed in our laboratory, that could be used for identification of the viral-encoded suppressors.

Assay for reversal of established silencing

As the name suggests, it is based on screening for the reversal of silencing of a reporter-gene by a viral protein using transgenic plants. The principle can be developed into different variants for screening viral ORFs for RSS activity, such as:

(a) Crossing a silenced transgenic plant with a second transgenic plant expressing a candidate viral RSS protein (Anandalakshmi *et al.* 1998; Kasschau and Carrington 1998) and screening for the reversal of silencing in the F1 generation (**Fig. 3A** top panel).

(b) Transient ectopic expression of the candidate viral protein in transgenic line silenced for the reporter gene. The ectopic expression of viral protein can be achieved by either of the following methods.

(c) A recombinant viral vector harboring the concerned gene could be inoculated on to the silenced transgenic plants. Initial studies involved PVX as expression vector because, on its own, it appeared to be inactive in the silencing reversal assay (Brigneti *et al*. 1998). However, subsequent experiments involving a different approach revealed that PVX itself encodes a silencing suppressor, the P25 protein (Voinnet *et al.* 2000). Thus, an additive or synergistic effect of P25 on the originally tested suppressors could not be excluded, indicating that the choice of the viral vector can influence the outcome of this approach (**Fig. 3A** middle panel).

(d) The viral ORFs can also be delivered by *Agrobacterium* infiltration (Karjee *et al*. 2008; **Fig. 3A**), where the ORFs would be ectopically expressed and can revert the expression of the silenced gene by suppressing the RNA silencing. It is a simple and reliable method for screening various viral ORF for RSS activity. However, the procedure requires a stable transgenic line silenced for the reporter gene and an incubation period of 7-8 days post infiltration (dpi) for optimal expression of the viral ORF and suppression of silencing to take place (**Fig. 3A** bottom panel).

The major limitation posed by the method is need for the development of stable transgenic line and it can only screen RSS that interfere with the pre-established silencing but might fail to identify those that potentially can suppress only the initiation of silencing.

Assay for reversal of initiation of silencing

In this transient assay, the candidate suppressor protein is co-delivered with a transgene construct that triggers RNA silencing of a reporter transgene, such as one encoding a Green Fluorescent Protein (GFP; **Fig. 3B**). Delivery is via leaf infiltration of recombinant *Agrobacterium* cultures and upon T-DNA transfer, the silencing inducer and the putative suppressor are co-expressed in most cells of the infiltrated zone. In the absence of a silencing suppressor, the transgenic GFP mRNA is rapidly degraded whereas its accumulation is usually stabilized in the presence of a suppressor (Llave *et al.* 2000; Voinnet *et al.* 2000; Hamilton *et al.* 2002; Takeda *et al.* 2002; Bucher *et al.* 2003; Dunoyer *et al.* 2004; Pfeffer *et al.* 2004). The method does not require development of transgenics, which make the assay very simple and allows large numbers of protein variants to be tested for suppression activity, leading to rapid identification of key amino acids or domains involved (Kasschau and Carrington 2001). Moreover, it is comparatively rapid as the suppression activity can be monitored within 4dpi. It helps in the identification of the RSSs that interferes with the initiation of RNA silencing.

Viral amplicon enhancement assay

A recent study describes an elegant alternative to the methods mentioned above, which relies on functional complementation of a candidate suppressor protein by a known silencing suppressor (Yelina *et al.* 2002; Karjee *et al*. 2008). The principle behind the assay is that, upon introduction of viral amplicon into the host plant, the later will recognize it as viral infection and would manifest defense response, viz. RNA silencing (Singh *et al*. 2007; Pandey *et al.* 2009). As a result, the viral amplicon mutant lacking endogenous RSS would show reduced accumulation of the viral amplicon, which can be recovered if a viral ORF with RSS activity could be provided in *trans* (**Fig. 3C**). This principle has been extrapolated to develop screening assay for RSS, where the RNA silencing influenced phenomenon can be restored by *trans* complementation with homologous or heterologous viral ORF with RSS activity (Karjee *et al.* 2008). The method has been successfully used to identify suppressors of both plant and animal viral origin (Schnettler *et al.* 2009). The assay has an advantage that it screens for the RSS that can suppress virus induced gene silencing, which is more relevant for the virus biology.

In vitro assays for suppression of small RNA biogenesis and/or function

Soon after the discovery of RNA silencing, *in vitro* systems were developed from cell extracts (originate from *Drosophila* cells/embryos, wheat germ extract, *Arabidopsis* inflorescence, human cell line, HEK293). Even though initially the *in vitro* systems were developed to understand the biochemistry of the RNA silencing pathway, these *in vitro* systems have been used for identification of RSSs. RNA silencing can be broadly categorized into two major effector processes, viz. dicing and slicing. The dicing generates siRNAs from the long dsRNA substrate while the slicing leads to cleavage of the target RNA by RISC activity. Here we are briefly describing two *in vitro* assay systems for the identification of viral proteins as RSS.

(a) **Dicing assay:** The assay primarily comprises of reconstituted DICER (cellular extract enriched with or

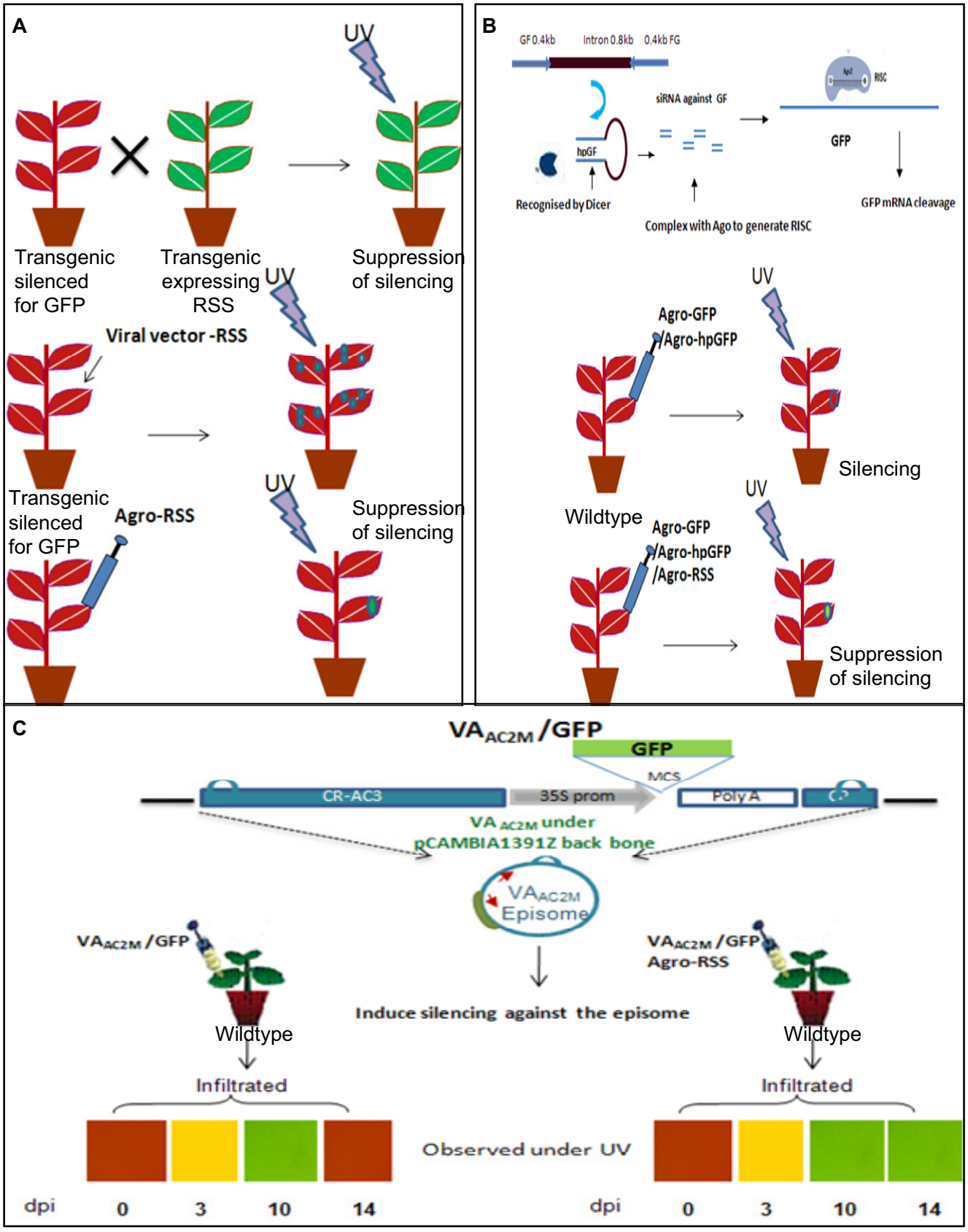

Fig. 3 RNA silencing suppressor screening assays. (**A**) Assay for reversal of established silencing. (**B**) Reversal of initiation of silencing. (**C**) Viral amplicon enhancement assay.

without purified DICER protein) reaction. The DICER extract is incubated with labeled dsRNA in presence or absence of the viral protein (to be tested for RSS activity) followed by monitoring the accumulation level of siRNA during the dicing reaction. Many RSSs, *viz.* HCV-core protein, FHV-B2, NS3 (Chao *et al.* 2005; Chen *et al.* 2008; Schnettler *et al.* 2008) have been analyzed by *in vitro* dicing assay. Interestingly, the assay not only helps in the screening of viral protein for the RSS activity but also provides a hint for the mechanism of suppression. However, we have shown that many RSSs, like MYMIV-AC2, do not interfere with dicing activity and thus cannot be screened by the method (Kumar *et al.* unpublished). Moreover, the process in-

volves multisteps and requires purified proteins.

(b) **Slicing assay:** The assay primarily comprises of reconstituted RISC (cellular extract enriched with purified Argonaute proteins) which is incubated with labeled target RNA and corresponding siRNA in presence or absence of the viral protein (to be tested for RSS activity) and subsequent monitoring for the cleaved product due to RISC activity. Many viral proteins with RSS activity have been characterized using this *in vitro* method. The p19 and NS3 proteins are two landmark examples where the assay has been used to characterize their mechanism of suppression. The assay also shares the advantages and disadvantages, and thus the identification of RSS is limited to the ones which affect the slicing activity. Interestingly, though the MYMIV-AC2 failed to respond to the dicing assay as mentioned above, it responded positively in the slicing assay. However, the *in vitro* assays coupled with other screening protocols would provide better identification and characterization of viral proteins.

MECHANISM OF SUPPRESSION BY RSS

A striking feature that emerges from the analysis of the various RSSs is the huge diversity in sequence and structure of these proteins. Such diversity can be explained, at least partly, by the fact that silencing suppressors evolved as additional features of unrelated proteins that already had diverse functions, an example of evolutionary convergence. Intriguingly, most of the RSS vary from each other in the mechanism by which it suppresses the RNA silencing pathway. Nonetheless, the mechanism of suppression by RSS can be broadly classified on the basis of RNA binding activity or interaction with the RNA silencing factors.

Double stranded (ds)RNA binding activity

dsRNA binding is a common strategy for many of the viral encoded suppressors (Merai *et al*. 2005). RSS encoded from phylogenetically and evolutionarily divergent viruses like tombusvirus p19, closterovirus p21, carmovirus CP, pecluvirus p15, hordeivirus γB, potyvirus HC-Pro etc. show dsRNA binding activity. dsRNA serves as a key mediator of the pathway, where long dsRNA serves as a major inducer and small dsRNA acts as a major effector of RNA silencing pathway. The binding of RSS to long viral dsRNA generated during its life cycle as replicative intermediate or transcription product, protects from the attack of RNA silencing component like DICER, which will subsequently process them into siRNA. Similarly, binding to siRNA either make it biologically inactive by demethylation or sequester it from getting associated with RISC complex, which in turn protects the viral RNA from sequence specific endonucleolytic cleavage of RNA silencing. Even though, a few RSS, like p14, FHV-B2, can bind to both long and short dsRNA, others, like p19, p21, γb, HC-Pro, show greater affinity only for short siRNA. The crystal structure of RSS further indicates the mechanism of RNA binding also varies with RSSs (Chen *et al.* 2008). TAV2b recognizes siRNA duplex by a pair of hook-like structures; while p19 protein uses an extended β-sheet surface and a small α-helix to form a caliper-like architecture for binding and measuring the characteristic length of siRNAs. On the other hand; B2 protein uses a four-helical bundle fold to bind to both siRNA duplex and long dsRNA in a length-independent mode.

Interaction with the RNA silencing factors

In addition to RNA binding strategy, many of the RSS have been found to interact and inhibit RNA silencing factors to suppress the pathway. One of the important components of RNA silencing pathway is DICER/DCLs, which has been found to be targeted by various virus encoded suppressors, like TCV (*Turnip crinkle virus*)-encoded P38 suppresses DCL4 (Deleris *et al.* 2006), FHV-B2 interacts with the PAZ domain of DICER to suppress siRNA biogenesis (Singh *et al.* 2009), *Hepatitis C virus* core protein also directly interact with DICER to antagonize RNA silencing (Chen *et al.* 2008). Interference with the DICER activity directly affects the siRNA biogenesis and hence the silencing of the target gene. The nuclear localized P6 protein of CMV suppresses the host nuclear DRB4 activity which is required to facilitate the activity of DCL4 enzyme (Hass *et al*. 2008). Another important protein of RNA silencing pathway is AGO, which has been targeted by various RSS to suppress RNA silencing. Polerovirus encoded P0 suppresses RNA silencing by destabilizing the AGO1 (Bortolamiol *et al.* 2008). CMV-2b and MYMIV-AC2 has also been found to be directly interacting with AGO to inhibits its slicing activity (Ruiz-Ferrer and Voinnet 2007; Kumar *et al*. unpublished data). RDR6 is another important component associated with plant RNA silencing pathway, and it involves in the sense gene mediated silencing as well as transitive siRNA biogenesis by generating dsRNA. In our lab, MYMIV-AC2 has been found to be interacting with RDR6 to interfere with RNA silencing (Kumar *et al.* unpublished data). Recently, the poleroviral P0 silencing suppressor protein was shown to target the AGO proteins for degradation which are the core component of the RISC. It does not interfere with the slicer activity of pre-programmed siRNA/miRNA containing AGO1, but prevents *de novo* formation of siRNA/miRNA containing AGO1 (Csorba *et al*. 2010). Beside these direct players, RSS interacts with host proteins which indirectly manifest its activity on RNA silencing pathway. For example, AC2 interaction with ADK leads to suppression of RNA silencing (Wang *et al.* 2005). Recently it was reported that two unrelated plant viral proteins, potyvirus HC-Pro and carmovirus P38, recruit ethylene-inducible transcription factor RAV2 for suppression of RNA silencing. Using a hairpin transgene silencing system, it was demonstrated that both RSS require RAV2 to block the activity of primary siRNAs, while suppression of transitive silencing was RAV2-independent (Endres *et al*. 2010). With the advent in the knowledge of RNA silencing components and characterization of more RSSs, probably many more such examples would be known for suppression by RSSs.

ACTIVITIES OF A FEW WELL KNOWN RSSS

P19

Initial experiments with several different tombusviruses, including *Cucumber necrosis virus* (CNV), *Cymbidium ring spot virus* (CRV) and *Tomato bushy stunt virus* (TBSV) showed the p19 gene was not essential for cell-to-cell movement but functioned to assist systemic spread and symptom development in host plants (Russo *et al.* 1994). Further studies showed that functional p19 was required for systemic invasion in some hosts of TBSV but not others (Schlthof *et al.* 1995), suggesting that this host-dependent requirement of p19 might be important in some as yet undefined antiviral defense of the host plants. It was initially recognized as a suppressor of RNA silencing based on its ability to reactivate expression of a silenced GFP transgene in the systemic leaves of plants infected with either TBSV or PVX carrying a p19 insert (Voinnet *et al.* 1999). Subsequently, several groups have independently demonstrated the potent silencing suppressor activity of p19 from a number of different tombusviruses using the agro-infiltration assay (Qiu *et al.* 2002; Qu and Morris 2002; Silhavy *et al.* 2002). Impressive progress recently on the structural and functional properties of the p19 makes it now the best characterized of the viral RSS proteins. Notably, it was the first protein demonstrated to directly bind siRNAs, functioning presumably to prevent the siRNAs from entering the RISC complex (Silhavy *et al.* 2002). Subsequently, the p19-siRNA complex was crystallized and the structure of the complex resolved (Vargason *et al.* 2003; Ye *et al.* 2003). This elegantly established a structural explanation for how

dimerization of p19 was essential for binding siRNA. Additional studies by several groups have now verified that the degree of p19-siRNA binding *in vivo* correlates with the severity of viral infection (Chapman *et al.* 2004; Dunoyer *et al.* 2004; Lakatos *et al.* 2004). Hence, p19 constitutes the first suppressor for which a target in the silencing pathway has been identified.

HC-Pro

Studies using potyvirus, TEV as the model system established P1/HCPro as one of the first viral RSSs. It was established as a multifunctional protein that affected aphid transmission, polyprotein processing, genome amplification and the long distance movement of the virus (Kassachu *et al.* 1997). It was also known to act as a broad range pathogenicity enhancer causing increased viral RNA accumulation and severe viral symptoms in a number of unrelated virus infections (Pruss *et al.* 1997). Later it was shown that P1/HCPro strongly suppresses RNA silencing, most likely by acting on a maintenance step affecting the assembly and/or targeting of the RISC complex (Mallory *et al.* 2001; Chapman *et al.* 2004). Recent studies have demonstrated that it also plays an important role in genome amplification and its long distance movement correlates with its silencing suppressor activity. The mutant PVY viruses that lack HC-Pro suppressor activity, while capable of initiating the replication process, are unable to sustain robust amplification and hence display debilitated long distance movement. This shows that suppression of the RNA silencing-based host defense is critical for vigorous viral RNA replication and efficient viral spread throughout infected plants (Kasschau and Carrington 2001). A breakthrough discovery regarding possible mechanism of silencing suppression was the demonstration of interaction between P1/HC-Pro of TEV and rgs-CaM, a tobacco calmodulin-like protein (Anandalakshmi *et al.* 2000). These authors showed that rgs-CaM suppresses RNA silencing itself upon over-expression in the plants suggesting that RNA silencing is tightly regulated in plants.

CMV-2b

The 2b protein of cucumoviruses was recognized as a silencing suppressor at about the same time as P1/HC-Pro of potyviruses (Brigneti *et al.* 1998). It was initially recognized as a small protein of about 100 amino acids encoded by a cryptic ORF in the viral genome (Ding *et al.* 1994). It was found to enhance the long distance movement of CMV in a host-dependent manner. CMV-2b mutants were capable of systemic invasion of tobacco but not cucumber plants (Ding *et al.* 1995). It was later shown that systemic infection of plants containing a silenced GFP transgene by either CMV or a PVX vector expressing 2b restored GFP expression in the leaves emerging after infection; but not in leaves where GFP silencing had already been established (Brigneti *et al.* 1998). This and additional studies suggest that CMV-2b functions to prevent the systemic spread of RNA silencing signals (Guo and Ding 2002). CMV-2b has been found to interfere with the slicing activity through direct interaction with Ago1 (Ruiz-Ferrer and Voinnet 2007). It has also been found to interact with siRNA, indicating that CMV-2b might suppress RNA silencing by binding directly to siRNAs (Goto *et al.* 2007). Recently its crystal structure has been solved with siRNA (Chen *et al.* 2008). From the structural studies it was evident that TAV-2b recognizes siRNA duplex by a pair of hook-like structures by fitting the protein backbones inside the major groove and wrapping around both faces of the dsRNA to recognize the major groove.

AC2

Geminivirus AC2 is one of the multifunctional proteins encoded by all members of the genus *Begomovirus* (formerly subgroup III). The protein is one of the earliest identified RSS (Voinnet *et al.* 1999), however, its precise mode of action is yet to be deciphered. AC2 does not bind to single or double stranded RNA, like most of the characterized suppressors. Some investigators consider suppression activity of AC2 is conjugated to its transactivation potential (Trinks *et al.* 2005). However, with MYMIV-AC2, we have found that the transactivation property can be uncoupled from the suppression activity of the protein. The host protein interaction profile of the AC2 throws much light on its mode of action like its interaction with SNF1 and adenosine kinases, enzymes which appear to be involved in defense response (Hao *et al.* 2003; Wang *et al.* 2003), and finally has implication as a suppressor of RNA silencing (Voinnet *et al.* 1999; van Wezel *et al.* 2001; Selth *et al.* 2004; Vanitharani *et al.* 2004; Trinks *et al.* 2005; Wang *et al.* 2005). Similarly, we found its direct interaction with RNA silencing pathway components like, RDR6 and AGO1, which indicates its dual action site on the pathway to make the suppression more strong and effective (Kumar *et al.* unpublished data). We have further found that like most of the RSS, its oligomeric status defines its suppressor activity (Kumar *et al.* unpublished data). Thus AC2 is different from many other RSSs but is quite efficient in its RNAi suppression activity.

APPLICATION OF RSS

Molecular probes for dissection of RNA silencing pathway

Studies in transgenic *N. benthamiana* revealed the existence of two distinct siRNAs species, 21 nt and 24 nt respectively. Using the co-infiltration procedures, it was found that the two siRNA species are differentially affected by some silencing suppressors, suggesting that these molecules could play different roles (Hamilton *et al.* 2002). Subsequent experiments showed that the absence of the 24 nt species, caused for instance specifically by the P1 protein, did not prevent intracellular silencing but inhibited its long-distance spread in the vasculature (Hamilton *et al.* 2002). Localized induction of silencing in plants not only triggers long-distance spread but also short-distance movement of the silenced state over a nearly constant number of 10–15 cells around the zone of initiation. Co-expression of the silencing suppressors also showed that this cell-to-cell movement is probably distinct from the long-distance spread because the two processes could be selectively inhibited by the P1 and AC2 proteins (Himber *et al.* 2003). Moreover, short-distance spread occurred around the P1-treated zones despite the absence of the 24 nt siRNA species. Therefore, occurrence of the 21 nt siRNA is probably sufficient for RNA degradation and cell-to-cell silencing movement but not for its long-distance spread, whereas the 24 nt siRNA seems to be correlated with long-distance movement in the phloem. Recent data obtained in wheat germ extracts indicate that the 21nt and 24 nt siRNAs are not only functionally but also biosynthetically distinct because separate DCL enzymes are probably involved in their processing (Tang *et al.* 2003). These results provide an example of how viral suppressors can be informative about the mechanism of RNA silencing (**Fig. 4**).

Biotechnological applications

1. Molecular farming

Many biotechnological applications in plants require high levels of protein expression from the transgene(s). However, transgene expression is often suboptimal due to a variety of reasons including the plant's perception of transgene as a foreign element. One attempt to increase expression levels was to exploit transgenic lines that encode a replicating RNA virus vector carrying a gene of interest, a technology coined 'amplicon' (Angell and Baulcombe 1997). The rationale was that transcription of the amplicon transgene

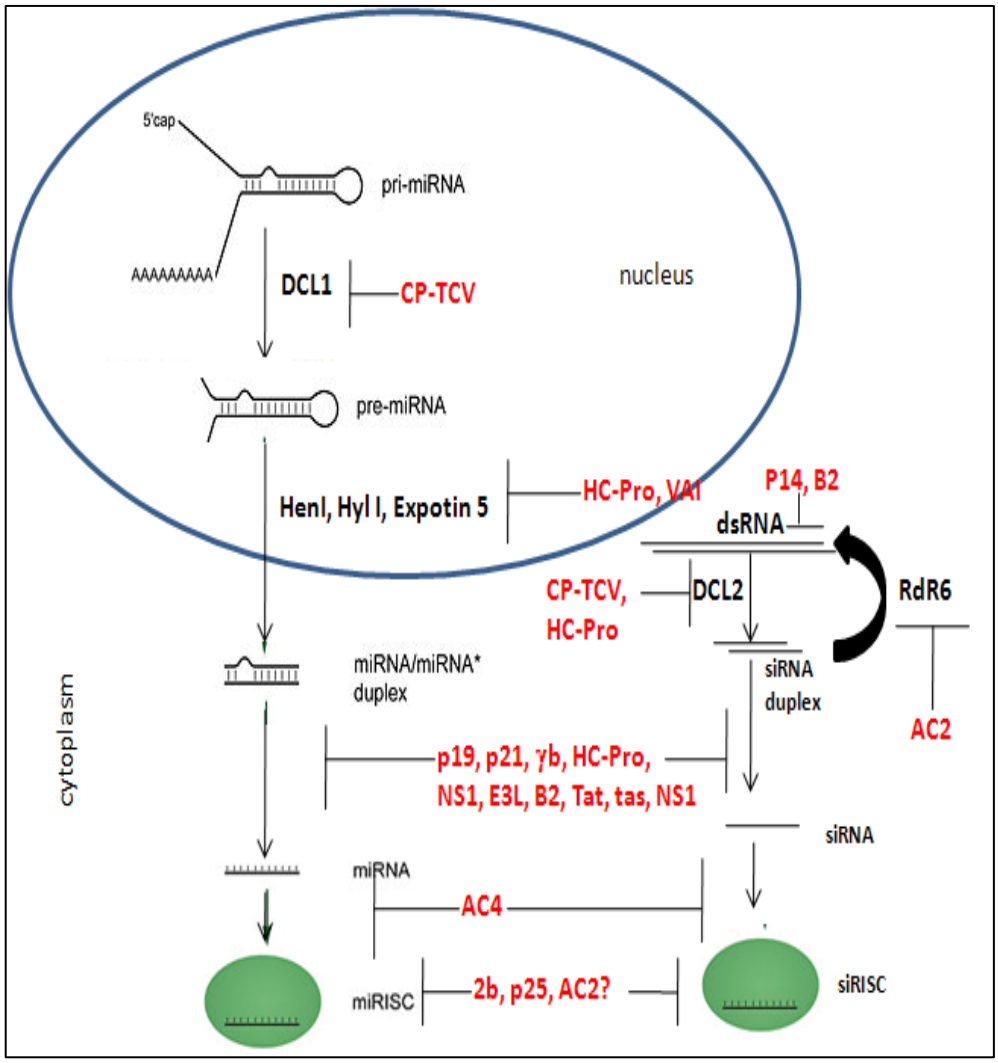

Fig. 4 Diagrammatic representation of various known RNA silencing suppressors and their possible interference site on RNA silencing pathway.

would initiate viral RNA replication and gene expression, and consequently, high accumulation of the product of interest. However, this strategy failed, as all of the transformants consistently exhibited RNA silencing of the amplicon transgene (Angell and Baulcombe 1997). Presumably, the viral dsRNA replication intermediates produced in every cell of the transgenic plants were recognized as potent triggers of the silencing-based defense mechanism normally elicited in the course of natural infections. Based on those findings, it was subsequently reasoned that co-expression of viral suppressors could possibly prevent this adverse response and allow the high levels of gene expression initially envisaged for amplicons. To test this idea, transgenic tobacco plants expressing the TEV HC-Pro were crossed with amplicon lines that had been designed to express a GUS reporter gene from the PVX genome (Mallory *et al.* 2002). Pairing the suppressor and the amplicon locus resulted in a dramatic increase in virus accumulation and gene expression, such that leaves of mature plants accumulated the GUS protein up to 3% of total soluble protein. Remarkably, in spite of high virus accumulation, those plants did not suffer from viral disease and remained symptomless (Mallory *et al.* 2002).

Enhanced *Agrobacterium*-mediated transient expression system, as opposed to stable, transgenic expression, is a fast, flexible and reproducible approach to expression of useful proteins. In plants, recombinant strains of *A. tumefaciens* can be used for transient expression of genes that have been inserted into the T-DNA region of the binary-Ti plasmid. A bacterial culture is vacuum-infiltrated into leaves, and upon T-DNA transfer, there is ectopic expression of the gene of interest in the plant cells. In principle, this system could allow high levels of gene expression; however, its utility has thus far been limited because the ectopic protein expression usually ceases after few days. RNA silencing is, in fact, a major cause for this lack of efficiency and it was therefore anticipated that co-delivery of *Agrobacterium* cultures with silencing suppressors would enhance expression levels of the genes of interest (Johansen and Carrington 2001; Voinnet *et al.* 2005). The best results were obtained with the P19 protein of TBSV. Indeed, expression of a range of proteins was enhanced 50-fold or more in the presence of this suppressor. The experiments with GFP indicated that the co infiltrated tissues accumulated the protein up to 7% of total soluble protein (Voinnet *et al.* 2003). Because of its simplicity and rapidity, the P19-enhanced expression system is currently used in industrial production as well as a research tool for isolation and biochemical characterization of a broad range of proteins without the need for the time-consuming regeneration of stably transformed plants. In our laboratory, we have successfully used MYMIV-AC2 for molecular farming. A GFP-silent tobacco line was genetically hybridized with another line expressing moderate amount of AC2 protein. About 20% of the F1 progeny lines expressed huge amount of GFP and the developmental abnormalities in these lines were minimal (Rahaman *et al.* unpublished).

2. Molecular probe to understand biological phenomenon: Ribozyme activity improvement

The hammerhead ribozyme, engineered to target the MYMIV-rep mRNA, showed 33% cleavage activity *in vitro* and almost 50% reduction *in vivo* in the surrogate host *Saccharomyces cerevisiae*. In both the cases, the catalytically inactive mutant ribozyme was used as a control that showed absolutely no reduction (Chilakamarthi *et al.* 2007). However, when the experiment was carried out *in planta*, similar degree of reduction in target mRNA was observed both in presence of the wild type as well as mutant ribozyme. As the ribozyme harbored 'rep'-complementary sequences on the hybridizing arms, we speculated that the observed reduction could be due to the RNAi activity which was masking the endonucleolytic activity of the ribozyme. However, upon co expressing the ribozyme with MYMIV-AC2, the reduction was observed in presence of the wild type ribozyme only and not with mutant ribozyme (Karjee *et al*. unpublished). Thus RSS can be used as an aid to ribozyme technology *in-planta*. Understanding of this phenomenon enabled us to further appreciate the ribozyme technology and its application in developing virus resistance transgenic.

VIGS efficiency enhancement

The VIGS phenomenon has been used to develop vectors to generate transient loss of host function assays as a more rapid alternative to stable transformation. However, the major problem encountered often with the engineered VIGS vector is incomplete silencing of target host gene. If the VIGS vector construct comprise an ORF that has RSS activity, the silencing efficiency of the host gene would be significantly low. For example the efficiency of MYMIV-based VIGS vector enhanced significantly when the RSS AC2 was mutated (Pandey *et al.* 2009). Thus, the knowledge of RSS can be used in enhancing the efficiency of VIGS vectors.

Drug target: antiviral agent

RNA silencing is a major antiviral defense response and RSS is the counter defense strategy from the virus. The virus mutant for the RSS show attenuated symptom development and mild growth. Thus mutation or inhibition of viral ORF with RSS activity can serve as an important target for the virus resistance strategy. The chemical compound(s) that could interfere with the suppression activity may constitute important virucide that could protect various agro-economically important crops devastated by the virus. Recently, it has been shown that a chemical library can be screened using protoplast based strategy with RNA silencing principle to identify inhibitors of RSS (Shimura *et al.* 2008). The inhibitor thus found has also been found to confer resistance to the corresponding viral disease.

CONCLUSIONS

RSSs are extremely important elements and factors for virus biology. Many viruses encode multiple RSSs from the same genome and it would be difficult to predict the number of RSSs the virus would require for successful multiplication within the host. For example, the CTV with its large genome of about 40 kb encodes three RSSs whereas the geminivirus with its only 2.7 kb DNA genome encodes at least three suppressors. Obviously the size of the genome is not an indicator of the number of RSSs, the virus might encode. As the suppressor do not have any signature motif(s) it is not easy to predict the mode of RSS activity of an uncharacterized suppressor. However we have put forward a model showing the RSS activities of a few of the known suppressors (**Fig. 4**). The suppressor might contribute to the virus functions (viz. replication, movement, coat, etc.) independent of the RNAi response or these could be present only for the encountering host RNAi. It would be interesting to know how and when in evolution the viruses have acquired suppression activity. Conversely, it is also important to understand how a mild virus becomes lethal just by changing few amino acids of its suppressor. Such understanding will help us either devise the robust antiviral strategy or build synthetic viruses as lethal weapons. Recently a viral suppressor is also reported with increased systemic silencing activity, a phenomenon quite incompatible with the concept of suppression of RNAi activity (Lacombe *et al*. 2010). Such reports highlight the yet-unknown characteristics of the viral RNAi suppressors. Hence the field of RNAi suppression holds the potential of throwing many unexpected surprises and thus is the object of intense investigations.

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