RNA रव–शामक के विषाण्विक निरोधकों का गुण–निर्धारण और पादप जीन अभिव्यक्ति में उनकी नियामक भूमिका

Characterization of viral suppressors of RNA silencing and their regulatory role in plant gene expression

SATENDRA KUMAR MANGRAUTHIA

DIVISION OF BIOCHEMISTRY
INDIAN AGRICULTURAL RESEARCH INSTITUTE
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Characterization of viral suppressors of RNA silencing and their regulatory role in plant gene expression

By

SATENDRA KUMAR MANGRAUTHIA

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Approved by:

Chairperson : _____
(Dr. Shelly Praveen)

Co-Chairperson : _____
(Dr. Archana Sachdeva)

Members : _____
(Dr. R.K. Jain)

_____
(Dr. T.R. Sharma)

_____
(Dr. A.K. Singh)

Division of Biochemistry
Indian Agricultural Research Institute
New Delhi-110 012

Dr. Shelly Praveen
Senior Scientist

CERTIFICATE

This is to certify that the thesis entitled **"Characterization of viral suppressors of RNA silencing and their regulatory role in plant gene expression"** submitted to the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the degree of **Doctor of Philosophy in Biochemistry**, embodies the results of *bonafide* research work carried out by **Mr. Satendra Kumar Mangrauthia** under my guidance and supervision. No part of the thesis has been submitted for any other degree or diploma.

All the assistance and help received during the course of the investigation have been duly acknowledged by him.

(Shelly Praveen)
Chairperson
Advisory Committee

New Delhi-110 012
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Dedicated to my beloved mother
(On her sudden departure to heavenly abode)

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1. INTRODUCTION

RNA silencing is a versatile, complex gene regulation and defense mechanism targeting parasitic or endogenous RNA in a highly sequence-specific manner. These evolutionarily conserved processes are now known to be operative in most, if not all eukaryotic organisms (Tomari and Zamore, 2005). RNA silencing operates through a set of core reactions that are triggered by dsRNA, which is processed into 21-24 nt RNA duplexes by the RNase III enzyme Dicer and its homologues (Bernstein *et al.,* 2001). These, in turn, mediate multiple regulatory and defense functions in cells (Brodersen and Voinnet, 2006). In plants, there are three RNA silencing pathways (Baulcombe, 2004). The first pathway is transcriptional gene silencing (TGS) that is associated with siRNA-directed epigenetic changes targeting *de novo* cytosine or histone methylation to their homologous DNA sequences to induce chromatin silencing (Xie *et al.,* 2004; Chan *et al.,* 2004, 2006). The second pathway involves a class of endogenous small RNAs, micro RNAs (miRNAs). Micro RNAs are generated by Dicer-like 1 (DCL1) from miRNA precursors that are transcribed from miRNA genes. Many transcription factors mediating the differentiation of multicellular organisms are regulated via these miRNAs, and severe developmental disturbances are associated with malfunctions of the miRNA pathways (Bartel 2004; Deleris *et al.,* 2006). The third pathway, primarily associated with defense functions, comprises 21 nt small interfering RNAs (siRNAs) that are processed from double-stranded RNAs (dsRNAs). The source of dsRNAs includes replication inter13mediates of plant RNA viruses, transgenic inverted repeats, and products of RNA-dependent RNA polymerases (RdRps) etc.

The RNA silencing pathway in plants presents a formidable defense against viral pathogens (Buchon and Vaury, 2006). To counteract this defense mechanism, viruses have evolved a wide range of mechanisms to overcome RNA silencing, providing yet another example of the continuing evolutionary molecular arms race between hosts and parasites (Voinnet, 2005). Many plant viruses encode for specific RNA silencing suppressor proteins which allow the viruses to proliferate in their specific hosts. The importance of these suppressors is reflected by the fact that many of them previously

have been identified as pathogenicity factors or viral cell-to-cell or long-distance movement proteins, essential for infectivity in indicated hosts. Viral suppressors interfere with the function of host miRNAs also and causes developmental abnormalities (Kasschau *et al.,* 2003; Chapman *et al.,* 2004). Although it might be a secondary consequence of the host defense and viral counter defense, as miRNAs share the structure and enzymatic machinery with siRNAs, more attention is needed to decipher the role of suppressors in miRNA regulated developmental disturbances leading to virus symptom development. Interestingly, some of the abiotic factors also like temperature have been shown to affect the population and symptoms of different viruses in various hosts (Goodman *et al.,* 1986; Lavina and Battle, 1993; White *et al.,* 1994; Llamas-Llamas *et al.,* 1998; Zheng *et al.,* 2005). Szittya *et al.,* (2003) reported that RNA silencing mediated antiviral defense is temperature dependent. Alternatively, one can assume that the activity of viral silencing suppressors varies over the temperature ranges and hence the symptoms. This seems to be more true for the suppressor that acts through the binding of the small RNAs (Lakatos *et al.,* 2006; Merai *et al.,* 2006) which is greatly affected by physiological parameters such as pH and salt concentration (Koukiekolo *et al.,* 2007). Silencing suppressor proteins encoded by unrelated RNA and DNA viruses bear no similarity to each other in either coding sequence or protein structure, suggesting separate origins and variable functional mechanisms for each suppressor type. In recent years, the interactions of different silencing suppressors with the RNA silencing pathways have been studied intensively. These includes HcPro of *potyviruses*, P19 of t*ombusviruses*, P15 of p*ecluviruses*, P21 of c*losteroviruses*, P25 of potexvirus*es*, P38 of *carmoviruses*, P50 of *trichoviruses,* 2b of c*ucumoviruses and AC4 of geminiviruses* (Kasschau *et al.,* 2003; Chapman *et al.,* 2004; Dunoyer *et al.,* 2004; Bayne *et al.,* 2005; Deleris *et al.,* 2006; Yaegashi *et al.,* 2007 ; Siddiqui *et al.,* 2008).

Geminiviruses have evolved to encode multiple suppressors (Voinnet *et al.,* 1999; Van Wezel *et al.,* 2002; Vanitharani *et al.,* 2004; Cui *et al.,* 2005) that are capable of overcoming RNA silencing mechanism (Brigneti *et al.,* 1998; Carrington *et al.,* 2001; Chapman *et al.,* 2004). The transcription activator protein (TrAp) AC2, AC4 and *βC1,* a gene of satellite molecule were shown to have suppressor activity in different geminiviruses. Among all the three suppressors, AC4 has been demonstrated as

suppressor of RNA silencing in two geminiviruses, *African cassava mosaic virus*, Cameroon strain (ACMV-Cam) and *Srilankan cassava mosaic virus* (SLCMV) (Vanitharani *et al.,* 2004). In most of the geminiviruses the AC4 protein is found to be quite variable in length, sequence and function (Vanitharani *et al.,* 2005). Small ORF present within the ORF of replication initiator protein in DNA A of begomovirus is being described to possess multiple roles in different viruses. Although till date it is difficult to assign universal role to this ORF in all the geminiviruses. In some of the viruses it has been shown as strong pathogenicity determinant (Krake *et al.,* 1998; Chellappan *et al.,* 2005). Since most of the viral suppressors are found to be pathogenicity determinants (Brigneti *et al.,* 1998; Chen *et al.,* 2004), based on similar pattern AC4 is characterized to decipher its role in RNA silencing. The variability in length, size and function of this AC4 ORF suggests that viral genomes are evolved with suppressor proteins to counteract the natural phenomenon of RNA silencing.

The *2b* gene of different cucumoviruses has been shown to have a major effect on systemic infection as well as on the degree of virulence (Ding *et al.,* 1995, 1996; Ji and Ding 2001; Shi *et al.,* 2002; Soards *et al.,* 2002).The approximately 11 to 13 kDa 2b protein was shown to be localized primarily in the nucleus and mutations affecting its nuclear localization also affected the ability of the 2b protein to suppress RNA silencing (Lucy *et al.,* 2000; Mayers *et al.,* 2000). The 2b protein may also be considered a determinant of host specificity (Ding *et al.,* 1995; Li *et al.,* 1999; Shi *et al.,* 2002). The *Cucumber mosaic virus* (CMV) 2b protein was shown not to affect virus replication in protoplasts (Ding *et al.,* 1996; Soards *et al.,* 2002), but did affect the extent and pattern of CMV movement in tobacco (Soards *et al.,* 2002) and cucumber (Ding *et al.,* 1995). These effects are believed to be due to the ability of the 2b protein to inhibit various defense reactions, such as by interference with the salicylic acid-mediated defense response (Ji and Ding, 2001) and by suppression of the RNA silencing mechanism (Brigneti *et al.,* 1998; Guo and Ding, 2002). 2b protein from different strains of the CMV behaves differentially in RNA silencing pathway by acting either at nucleus (Lucy *et al.,* 2000) or interacting directly with Argonaute protein (Zhang *et al.,* 2006) or by directly binding with small RNAs (Goto *et al.,* 2007). Moreover, strain specific differences between the

2b silencing proteins determine whether only siRNA (Chapman *et al.,* 2004) or both siRNA and miRNA pathways are disrupted (Zhang *et al.,* 2006; Lewsey *et al.,* 2007).

The helper component proteinase (HcPro) of potyviruses is a multifunctional plant virus protein that has been characterized in detail (Revers *et al.,* 1999; Urcuqui-Inchima *et al.,* 2001). HcPro intervenes at different steps of the virus replication cycle: maintenance of genome amplification (Kasschau *et al.,* 1997), long-distance movement (Klein *et al.,* 1994; Cronin *et al.,* 1995; Kasschau *et al.,* 1997), proteolytic processing (Carrington *et al.,* 1989a) and aphid transmission (Raccah *et al.,* 2001). In addition, HcPro exhibits nonspecific RNA binding activity (Merits *et al.,* 1998; Urcuqui-Inchima *et al.,* 2000). Over recent years, potyvirus HcPro has also been shown to be a highly effective suppressor of gene silencing in transient silencing-suppression assays (Llave *et al.,* 2000; Johansen & Carrington, 2001; Hamilton *et al.,* 2002; Silhavy and Burgyan, 2004) and in transgenic plants (Anandalakshmi *et al.,* 1998; Kasschau & Carrington, 1998; Llave *et al.,* 2000). Potyvirus HcPro prevents and reverses already established RNA silencing (Brigneti *et al.,* 1998). Moreover, expression of potyvirus HcPro protein in *Nicotiana tabacum* and *Arabidopsis thaliana* alters micro RNA (miRNA) accumulation, prevents cleavage of miRNA targets and induces developmental defects (Mallory *et al.,* 2002; Kasschau *et al.,* 2003; Dunoyer *et al.,* 2004). Different studies in Tobacco and Arabidopsis (Mallory *et al.,* 2002; Dunoyer *et al.,* 2004) have shown that transgenically expressed HcPro partially reduces double-stranded (ds) RNA processing by Dicer. In addition, HcPro inhibits activity of the RNA induced silencing complex (RISC). However, in a recent study, TEV-HcPro was shown to inhibit RNA silencing via binding to small RNAs size selectively (Lakatos *et al.,* 2006; Merai *et al.,* 2006). HcPro, encoded by *Sugarcane mosaic virus* (SCMV), regulates the accumulation of different siRNAs and has more than one target in the RNA silencing pathway (Zhang *et al.,* 2008). Taken together, these findings indicate that the prevention of accumulation of siRNAs by potyvirus HcPro depends on the experimental system and on the viral origin of the protein analyzed. In an interesting finding, Gonza´lez-Jara *et al,* (2005) pinpointed a link between silencing suppression by HcPro of *Plum pox virus* (PPV) and its capacity to induce synergism with *Potato virus* X (PVX).

Synergistic interactions between plant viruses can lead to increased disease in crops that are susceptible to the various virus combinations (Bennett, 1952; Pio-Ribeiro *et al.,* 1978; Poolpol & Inouye, 1986; Sano & Kojima, 1989; Palukaitis & Kaplan, 1997; Hunter *et al.,* 2002; Wang *et al.,* 2002). In addition, interviral synergy can lead to resistance breakage (Murphy & Kyle, 1995; Choi *et al.,* 2002; Wang *et al.,* 2004) or limited spread of another virus (Saenz *et al.,* 2002). In some cases, these synergistic interactions are mediated by proteins that have been shown to be suppressors of RNA silencing (Pruss *et al.,* 1997; Brigneti *et al.,* 1998; Saenz *et al.,* 2001; Qiu *et al.,* 2002; Selth *et al.,* 2004). Expression of such proteins from heterologous viral expression vectors results in increased disease and/or virus accumulation in some host species, but not in others (Pruss *et al.,* 1997; Brigneti *et al.,* 1998; Li *et al.,* 1999; Qiu *et al.,* 2002), as has been observed for interviral synergy (Garces-Orejuela & Pound, 1957; Fukumoto *et al.,* 2003; Gonza´lez-Jara *et al.,* 2004). HcPro of several potyviruses is known to enhance RNA accumulation and symptoms of heterologous viruses in double infections, i.e. with *Potato virus* X (PVX) (Vance, 1991; Vance *et al.,* 1995; Pruss *et al.,* 1997). Shi *et al.,* (1997) allocated this function to the central domain of the TEV-HcPro and showed that the N terminus was dispensable. Given the fact that most of the suppressor protein are pathogenicity factors and are involved in host specificity, the role of HcPro in evolution of host specific strains of *Papaya ringspot virus* (PRSV) is still lacking.

Papaya ringspot virus (PRSV), a species of the *Potyviridae,* is classified into two pathotypes which can be distinguished only by host range. The P type (papaya-infecting) pathotypes of PRSV cause extensive damage to papaya production throughout the tropics and subtropics, whereas the W type pathotypes (i.e. *Watermelon mosaic virus* 1, non-papaya-infecting) are of economic importance in cucurbits production world-wide (Yeh *et al.,* 1984; Purcifull *et al.,* 1984). The PRSV genome is well characterized, and complete genome sequence of eleven PRSV isolates, one each from Hawaii and Mexico, two each from Brazil (two W isolates) and Thailand (one P isolate and one W isolate), and five isolates from Taiwan (four P isolates and one W isolate) are available (Parameswari *et al.,* 2007). However, information on the complete genome sequence of PRSV from India is lacking. Previous reports of geographical origin of PRSV and emergence of host specific strains were based on either coat protein genes (Jain *et al.,*

1998; 2004; Bateson *et al.,* 2002) or lacked the adequate records from many countries when studied at full genome level (Noa-Carrazana *et al.,* 2007).

Keeping in mind the importance of RNA silencing pathways and the crucial role of viral suppressors in regulating these pathways, following objectives were proposed to study the viral suppressors from different viral origin, their synergistic role and to decipher full length genome of PRSV for determining host specificity.

1. Characterization of viral suppressors: potyviral HcPro (helper component proteinase), geminiviral AC4 and cucumoviral 2b.
2. Study the effect of HcPro and AC4 on plant development and their synergistic effect in gene regulation.
3. Characterization of HcPro for its role in binding small RNAs *in vitro.*
4. Characterizing genomic components of the viral genome of *Papaya ringspot virus* to study plant-virus interaction.

RNA interference (RNAi) is an evolutionarily conserved, homology dependent RNA silencing phenomenon found in all the eukaryotes. It operates through non-coding small RNA molecules, which recently gained wide spread attention, as molecular switches in complex gene regulatory networks. The journey from small non-coding RNAs towards their role in plant gene regulation and antiviral immunity to the recent updates of viral counter defense proteins and their role in reprogramming of plant gene regulation has been elucidated here in a systemic fashion.

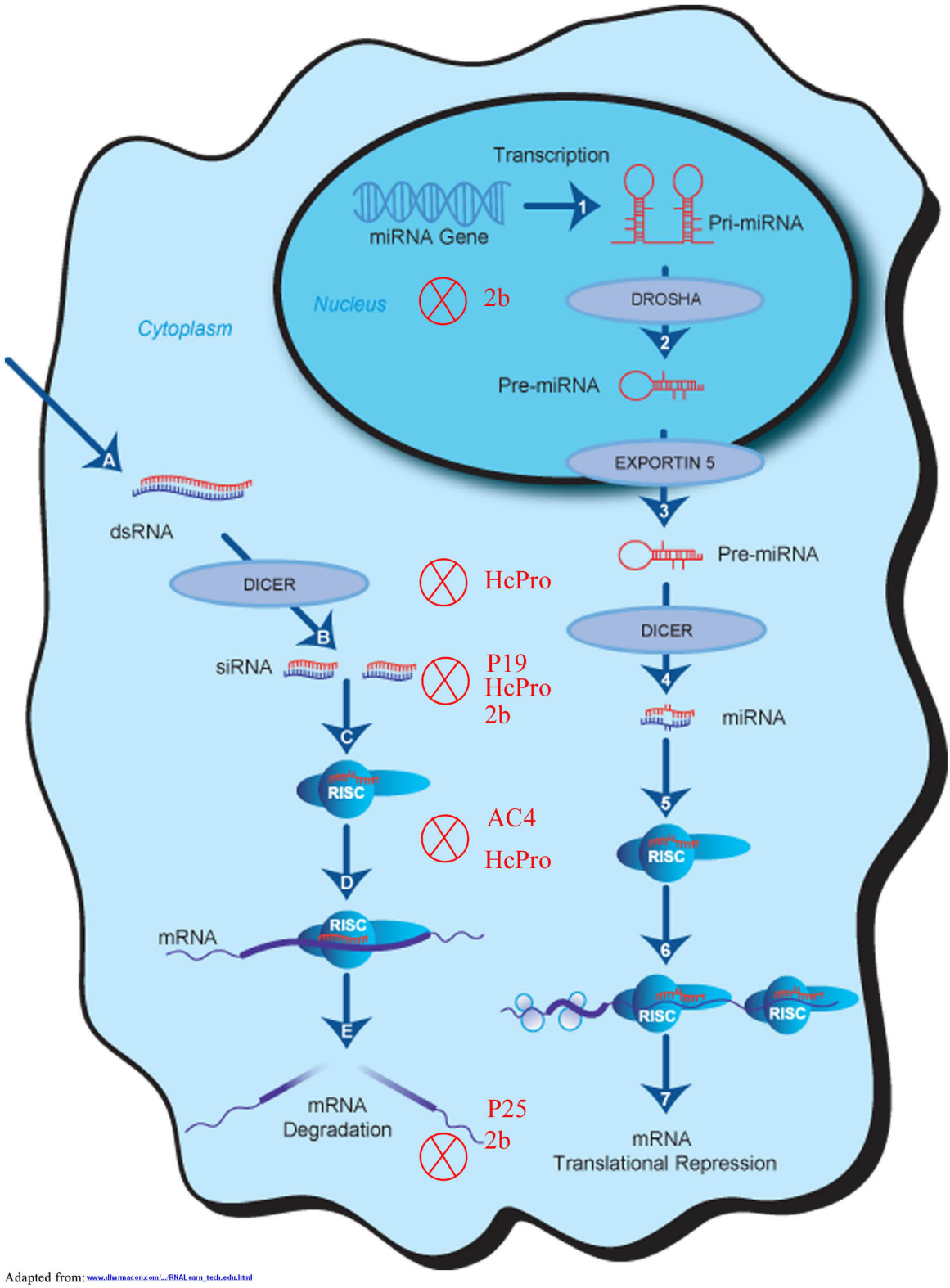
RNA interference - a brief account

RNA interference was first observed in plants and fungi when the expression of an additional copy of a gene inhibited the expression of both the original gene and transgene (Napoli *et al.*, 1990; Van der Krol *et al.,* 1990). In an attempt to generate violet petunias, they over expressed chalcone (CHS) synthase in petunias, which unexpectedly resulted in white petunias. The levels of endogenous as well as introduced CHS were 50-fold lower than in wild-type petunias, which led them to hypothesize that the introduced transgene was "cosuppressing" the endogenous *CHS* gene. In 1992, Romano and Macino reported a similar phenomenon in *Neurospora crassa*, noting that introduction of homologous RNA sequences caused "quelling" of the endogenous gene. RNA silencing was first documented in animals by Guo and Kemphues in 1995 where they observed that the introduction of sense or antisense RNA to par-1 mRNA resulted in degradation of the par-1 mRNA in *Caenorhabditis elegans*. Surprisingly, when Guo and colleagues performed control experiments using only the sense par-1 RNA, which would not hybridize with the endogenous par-1 transcript, the par-1 mRNA was still targeted for degradation. These finding caused investigators to rethink the current dogma. Anderw Fire and Craig Mello made the Nobel Prize winning observation in *Caenorhabditis elegans* worms that this interference affect is mediated by dsRNA (Fire *et al.,* 1998) and David Baulcombe provided evidence supporting a role for 21-25 RNA cleavage products as mediators of the RNAi effect in plants (Hamilton and Baulcombe, 1999). Introduction of synthetic versions of these 21 nt RNA duplexes with perfect homology to their target

sequences could recapitulate the RNAi effect in mammalian cells (Elbashir *et al.,* 2001a) and were termed small interfering RNAs (siRNAs). On the basis of the structural similarity between these cleavage products and 21-23 nt micro RNAs (miRNAs) originally described in worms that had less complete complementarity to their targets (Lee *et al.,* 1993, Wightman *et al.,* 1993), it was then shown that miRNAs could also cause RNA silencing (Olsen and Ambros, 1999; Reinhart *et al.,* 2000) and that siRNAs were co-opting this endogenous machinery for RNA silencing.

RNA interference – core machinery involved in dicing and slicing

Biochemical and genetic experiments have established a general mechanistic model for silencing pathways and identified factors that are required for RNA silencing in a variety of organisms **[Fig.1]**. The goal of the initiator step of RNAi is the generation of siRNAs from long dsRNAs, or mature micro RNAs from their primary transcript which is achieved by the action of two families of RNase III genes, Dicer and Drosha respectively. The dsRNA trigger is cleaved by a ribonuclease III (RNAse III)-like enzyme termed Dicer into 21–24 nucleotide duplexes termed short-interfering RNAs (siRNAs) (Hamilton and Baulcombe, 1999; Zamore *et al.,* 2000; Bernstein *et al.,* 2001). RNaseIII enzymes fall into three classes (Nicholson, 2003). Class I enzymes, found in bacteria and yeast, contain a single RNaseIII domain joined to a double stranded RNA binding domain (dsRBD). Class II and III enzymes contain two RNaseIII catalytic domains. Class III enzymes are further characterized by a helicase domain and a PAZ (Piwi/Argonaute/Zwille) domain. This last domain is also present in Argonaute family proteins, known to be essential for RNAi, (Tabara *et al.,* 1999; Bass, 2000). The production of siRNAs by Dicer is an ATP-dependent step (Zamore *et al*., 2000; Bernstein *et al.,* 2001) and probably involves interactions with other proteins (Tabara *et al.,* 2002). There are four *Dicer-like* (DCL) homologs in *Arabidopsis*; however, the specific enzyme responsible for siRNA production in plants has not yet been identified. The siRNAs produced from a fully double-stranded RNA substrate by Dicer have distinctive characteristics: they represent both polarities and have two nucleotide 3'overhangs with 5' phosphate and 3' hydroxyl groups (Elbashir *et al.,* 2001b,c). The link between the RNAi and the micro RNA pathways provided an exciting role for RNAi in the regulation of gene expression. Drosha is a Class II enzyme. This enzyme assumes a pseudo-dimer



Adapted from: www.dharmacon.com/.../RNALearn_tech.edu.html

Fig.1 Micro and small interfering RNA (miRNA and siRNA) pathways and the mechanism of suppression of these pathways by viral suppressors of RNA silencing. Viral suppressors have been shown to affect the initiation, maintenance and signaling step of small RNA pathways.

catalytic core similar to Dicer (Han *et al.,* 2004). The substrate of Drosha, micro RNA primary transcripts, is structurally distinct from Dicer substrates. Drosha does not process from a dsRNA terminus, rather, data suggests that the stem–loop structure is recognized. In particular, the loop size appears to be important for recognition (Zheng *et al.,* 2005). Drosha and dicer are associated with dsRBD cofactors. The first dsRBD that was identified, rde-4 (RNAi deficient-4), arose from a genetic screen in *C. elegans* (Tabara *et al.,* 1999; 2002). In Drosophila, Dicer-1, Dicer-2 and Drosha are associated with Loquacious, R2D2, and Pasha, respectively (Denli *et al.,* 2004; Gregory *et al.,* 2004; Landthaler *et al.,* 2004). In another ATP-dependent step (Nykaken *et al.,* 2001), the siRNAs and miRNAs are denatured and incorporated into a multi-subunit endonuclease silencing complex called RNA-induced silencing complex (RISC) (Hammond *et al.,* 2000). Chromatographic purification of RISC nuclease activity from Drosophila cells revealed several RISC components. The first identified component was Argonaute2 (Hammond *et al.,* 2001). This protein is a member of a gene family conserved in most eukaryotic and several prokaryotic genomes. The other components in this complex include the RNA binding protein VIG, the Drosophila homolog of the Fragile X protein, dFXR, helicase proteins, and Tudor-SN (Ishizuka *et al.,* 2002; Caudy *et al.,* 2002, 2003). Effector complexes called RISCs are assembled upon loading of one selected small RNA strand into one member of the Argonaute (Ago) protein family (Tolia and Joshua-Tor, 2007). Assembly of this RISC loading complex (RLC) may be a single step or may include multiple steps with different, uncharacterized accessory proteins. In a concerted manner, the siRNA and miRNAs are unwound and the guide strand is transferred from the RLC into RISC. Within the activated RISC, single-stranded siRNAs / miRNAs act as guides to bring the complex into contact with complementary mRNAs and thereby cause their degradation (Zamore *et al.,* 2000; Bernstein *et al.,* 2001; Elbashir *et al.,* 2001a; Hammond *et al.,* 2001). While the microRNA and siRNA pathway share the same core machinery, some specialization may exist. For example, in Drosophila Ago1 preferentially binds microRNAs and Ago2 siRNAs (Caudy *et al.,* 2002; Okamura *et al.,* 2004). Similarly, Dicer-1 is essential for microRNA processing (Lee *et al.,* 2004).

RNA interference – scenes of small RNAs

Mainly, two types of RNA molecules have potential to serve as trigger of RNA silencing. Small interfering RNAs (siRNAs) are implicated in variety of processes including defense against viruses, establishment of heterochromatin, silencing of transposons and transgenes and post transcriptional regulation of genes (Baulcombe, 2004). Micro RNAs (miRNAs) are small endogenous RNAs that regulate gene expression in plants and animals. Micro RNAs regulate important biological processes, and hence plants and animals with compromised miRNA functions display severe developmental defects (Bartel, 2004; Vaucheret, 2006).They are primarily associated with genes regulated during temporal and spatial development (Rhoades *et al.,* 2006). Though, mi and siRNAs have much in common, fundamental differences are there between the two classes of small RNAs. siRNAs are processed from long dsRNAs (Elbashir *et al.,* 2001b,c), whereas miRNAs are processed from single RNA molecule that include an imperfect stem loop secondary structure (Lagos *et al.,* 2001; Reinhart *et al.,* 2002; Laufs *et al.,* 2004). Many miRNAs are conserved between related organisms, whereas most exogenously expressed siRNAs are not (Reinhart *et al.,* 2002). Many (but not all) siRNAs targets the gene from which they are derived or very closely related genes. In contrast miRNA regulates gene unrelated to loci encoding the miRNAs. Finally, although most of the proteins required for siRNA and miRNA biogenesis are related and sometimes overlap, there are differences at the dicing and slicing components involved. In the fruit fly Drosophila, miRNAs and siRNAs are products of two distinct Dicers, Dicer-1 and Dicer-2, respectively (Hammond, 2005). On the other hand, worms and vertebrates have only one Dicer that produces both miRNAs and siRNAs. The model plant *Arabidopsis thaliana* encodes four Dicer-like proteins (DCL1 to DCL4): DCL1 primarily synthesizes miRNAs (Bartel, 2004), whereas DCL2, DCL3, and DCL4 process long dsRNA molecules of various cellular origins into siRNA populations that are 22, 24, and 21 nucleotides in length, respectively (Brodersen and Voinnet, 2006). Recently in addition to siRNAs and miRNAs, one more class of small RNAs, piRNAs—which are ~30 nucleotides in length and are found in the germline of flies and vertebrates—are Dicer independent (Zamore, 2007).

RNA interference – an antiviral immunity

Viruses are obligate intracellular pathogens that infect all forms of life. Organisms have diverse mechanisms for combating viral infections. One mechanism—discovered first in plants and subsequently in invertebrates—is through RNA silencing. Infact, the first biological function established for RNA silencing was as an antiviral mechanism in plants (Lindbo *et al.,* 1993; Ratcliff *et al.,* 1997; Anandalakshmi *et al.,* 1998; Hamilton and Baulcombe, 1999). Recovery and establishment of the virus-resistant states were correlated with a post-transcriptional breakdown of the mRNA. It was thus concluded that virus infection induces RNA silencing, which then targets the viral RNAs to confer virus resistance (Lindbo *et al.,* 1993). Furthermore, virus specific siRNAs of both positive and negative polarities accumulate in plants infected with viruses (Yoo *et al.,* 2004; Molnar *et al.,* 2005), demonstrating that viruses are both inducers and targets of RNA silencing in plants. The idea that RNA silencing is an antiviral mechanism in plants is further supported by two additional lines of evidence. Mutants carrying loss-of-function mutations in essential silencing pathway genes such as *rdr6*, *ago1*, and *dcl2* show enhanced disease susceptibility to virus infection (Mourrain *et al.,* 2000; Morel *et al.,* 2002; Xie *et al.,* 2004). Second, RNA silencing as an antiviral mechanism in plants is strongly supported by the demonstration that essential virulence factors of many plant RNA and DNA viruses are viral suppressors of RNA silencing (VSRs).

Viral suppressors of RNA silencing: anti- antiviral mechanism

Albeit the effective functioning of RNA silencing mechanism in various host system is to combat the virus infection, viruses are strong enough to establish in the system by suppressing the host defense mechanism. The direct involvement of viral suppressor proteins in interfering with the RNAi mechanism was demonstrated with HcPro protein (Anandalakshmi *et al.,* 1998; Brigneti *et al.,* 1998; Kasschau and Carrington, 1998). Different family of plant and animal viruses possesses wide repertoire of proteins to affect this counter defense mechanism and their mode of action in the RNAi pathway is depicted in **Fig.1, Table.1.** The viral

Table.1 Suppressors of RNA silencing from different viral origin

Virus gene	Virus name	VSR	Motif implicated	References
Positive- strand RNA viruses in plants				
Aureusvirus	**Pothos latent virus**	P14	dsRNA binding	Mallory *et al.*, 2001
Carmovirus	*Turnip crinkle virus*	CP		Qi *et al.*, 2004; Qu *et al.*, 2003
Closterovirus	*Beet yellows virus* *Citrus tristeza virus* *Grapevine leafroll-associated virus-2* *Beet yellow stunt virus*	P21 P20 P23 CP P24 P22	dsRNA binding	Chapman *et al.*, 2004; Chiba *et al.*, 2006; Lu *et al.*, 2004; Reed *et al.*, 2003
Crinivirus	*Sweet potato chlorotic stunt virus*	P22 RNase3	 RNaseIII	Kreuze *et al.*, 2005
Comovirus	*Cowpea mosaic virus*	Small CP		Liu *et al.*, 2004
Cucumovirus	*Cucumber mosaic virus* *Tomato aspermy virus*	2b	dsRNA binding	Brigneti *et al.*, 1998; Li *et al.*,1999; Qi *et al.*, 2004
Furovirus	*Soil borne wheat mosaic virus*	19K	Cysteine rich protein	Te *et al.*, 2005
Hordeivirus	*Barley stripe mosaic virus*	γb	Cysteine rich protein	Donald & Jackson, 1996; Yelina *et al.*, 2002
Pecluvirus	*Peanut clump virus*	P15	Cysteine rich protein	Dunoyer *et al.*, 2004; Dunoyer *et al.*, 2002
Polerovirus	*Beet western yellows virus* *Cucurbit aphid-born yellows virus*	P0		Pfeffer *et al.*, 2002
Potexvirus	*Potato virus X*	P25		Voinnet *et al.*, 2000
Potyvirus	*Tobacco etch virus* *Potato virus Y* *Turnip mosaic virus*	HcPro		Anandlakshami *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carington, 1998; Kasschau *et al.*, 2003
Sobemovirus	*Rice yellow mottle virus*	P1		Voinnet *et al.*, 1999
Tobamovirus	*Tobacco mosaic viruses*	P130		Kubota *et al.*, 2003

	Tomato mosaic viruses			
Tobravirus	*Tobacco rattle virus*	16K	Cysteine rich protein	Liu *et al.*, 2002
Tombusvirus	*Tomato bushy stunt virus* *Cymbidium ringspot virus*	P19	dsRNA binding[a]	Qi *et al.*, 2004; Silhavy *et al.*, 2002; Voinnet *et al.*, 1999
Tymovirus	*Turnip yellow mosaic virus*	P69		Chen *et al.*, 2004
Vitiviruses	*Grapevine virus-A*	P10		Chiba *et al.*, 2006
Negative-strand RNA viruses in plants				
Tenuivirus	*Rice hoja blanca virus*	NS3		Bucher *et al.*, 2003; Schnettler *et al.*, 2008
Tospovirus	*Tomato spotted wilt virus*	NSs		Bucher *et al.*, 2003; Takeda *et al.*, 2002
Double-stranded RNA viruses in plants				
Phytoreovirus	*Rice dwarf virus*	Pns10		Cao *et al.*, 2005
DNA viruses in plants				
Begomovirus	*Tomato leaf curl virus*	C2	DNA binding,NLS	Chellappan *et al.*, 2005; Cui *et al*, 2005; Trinks *et al.*, 2005; Van *et al.*, 2002; Vanitharani *et al.*,2004; Voinnet *et al.*, 1999; Wang *et al.*, 2005
	TYLCCNV-Y10Y10β	βC1	DNA binding,NLS	
	African cassava mosaic virus (KE) EACMCV, ICMV, TGMV *Mungbean yellow mosaic virus*	AC2	DNA binding, NLS, AD	
	African cassava mosaic virus (CM)	AC4	miRNA binding[b]	
Curtovirus	*Beet curly top virus*	L2	Protein binding	Wang *et al.*, 2005
Positive- strand RNA viruses in animals				
Nodavirus	*Flock house virus, Nodamura virus,*	B2	dsRNA binding	Fenner *et al.*, 2006; Iwamoto *et al.*,

	Striped jack nervous necrosis virus, Greasy grouper nervous necrosis virus			2005; Li *et al.*, 2002; Li *et al.*, 2004
Negative-strand RNA viruses in animals				
Orthomyxovirus	*Influenza virus A*	NS1	dsRNA binding	Li *et al.*, 2004
Orthobunyavirus	*La Crosse virus*	NSs		Soldan *et al.*, 2005
Double-stranded RNA viruses in animals				
Orthoreovirus		σ3	dsRNA binding[c]	Lichner *et al.*, 2003, Yue & Shatkin, 1997
Retroviruses in animals				
Lentivirus	*HIV-1*	Tat		Bennasser *et al.*, 2005
Spumavirus	*PFV-1*	Tas		Lecellier *et al.*, 2005
DNA viruses in animals				
Adenovirus	*Adenovirus*	VA1 RNA	Dicer binding	Lu & Cullen, 2004
Poxvirus	*Vaccinia virus*	E3L	dsRNA binding	Li *et al.*, 2004

suppressor proteins do not share any sequence or functional homology with each-other. They act at different steps of the RNA silencing pathway and thus suggest their independent evolution to counteract the plants defense mechanism.

Viral suppressors of RNA silencing from Plant Viruses

Majority of plant virus that has been studied to date possess a VSR, and these include viruses having positive, negative or double-strand RNA genomes as well as geminiviruses with a single-stranded circular DNA genome. However, the VSR encoded by each virus often targets only one of the step of RNA silencing. Nevertheless, *Citrus tristeza virus* (CTV) and geminiviruses encode multiple VSRs, each of which have a distinct mode of action (Vanitharani *et al.,* 2004, Lu *et al.,* 2004).

Viral suppressors of RNA silencing from Animal Viruses

Mammalian VSRs include NS1 of influenza A, B and C viruses (Li *et al.,* 2004), E3L of *vaccinia virus* (Li *et al.,* 2004), B2 of nodavirus (Sullivan and Ganem, 2005), NSs of *La Crosse virus* (Soldan *et al.,* 2005), VA1 of adenovirus (Lu and Culeen, 2004), Tas of PFV- 1 (Lecellier *et al.,* 2005), and Tat of *Human Immunodeficiency virus* (Bennasser *et al.,* 2005). B2 from two fish nodaviruses has been shown to suppress RNA silencing in other systems. In addition, The reovirus σ3 protein have been shown to suppress RNA silencing in plants (Lichner *et al.,* 2003), but its activity is not verified in animal cells. Infection of *Drosophila* with *Cricket paralysis virus* (CrPV) induces RNA silencing and CrPV encodes a VSR (Wang *et al.,* 2006). Thus, suppression of RNA silencing represents a conserved function of animal viruses, which are pathogenic.

Structured Viral RNAs as Viral suppressors of RNA silencing

In addition to well established suppressor proteins, the folded and structured RNA of adenovirus, VA1, inhibits RNA silencing (Anderson *et al.,* 2005). In addition, Takeda *et al.,* in 2005 have demonstrated suppression of RNA silencing by replicative intermediates of *Red clover necrotic mosaic virus* (RCNMV) but not by any of the viral encoded proteins.

Diverse mechanisms of viral suppression of RNA silencing

Various experiments have led to the rudimentary characterization of suppressor mechanism, but conflicting results from different assays have made it difficult to draw firm conclusions for many suppressors. Interestingly, the currently known suppressors share no obvious similarities at either the nucleic acid or the protein level, perhaps reflecting differences at the mechanistic level as well. At present, some of the known mechanisms of silencing suppression for diverse suppressors are explained here.

Suppression of siRNA Production

Inhibition of viral siRNA production in infected cells occurs possibly by preventing Dicer from access to the viral RNA trigger(s). Inhibition of the dicing of long dsRNA by B2 of FHV was first shown *in vitro* using the Dicer extracts from *Drosophila* cells (Lu *et al.,* 2005; Chao *et al.,* 2005). These findings thus established inhibition of siRNA production as a mechanism in B2 suppression of RNA silencing. VA1 appears to inhibit the RNA silencing by a mechanism distinct to B2 as it directly binds to Dicer and thus compete with input long dsRNA for Dicer binding (Lu *et al.,* 2004; Anderson *et al.,* 2005). A similar mechanism may be used by RCNMV (Takeda *et al.,* 2005). Expression of both, P25 of *Potato virus X* (PVX) and P1 of *Rice yellow mottle virus* specifically inhibited the accumulation of the 24-nt siRNA but had a less pronounced effect on the accumulation of the 21-nt siRNA (Hamilton *et al.,* 2002). Interestingly, it appears that only the shorter class of viral siRNAs accumulated in plants infected with PVX (Schwach *et al.,* 2005), suggesting that P25 expression may also inhibit the production of the longer class of viral siRNAs in infected plants.

Sequestration of small RNAs

It is now clear that a major class of VSRs is dsRNA-binding proteins, as revealed first for the tombusviral P19 (Silhavy *et al.,* 2002). However, dsRNA binding is unusual for P19 among the dsRNA binding proteins known so far because it specifically selects its substrates on the basis of the length of the duplex region of the RNA. Selective binding of the 21- to 22-nt class but not the 24-nt class of siRNAs by P19 suggests a unique mechanism of RNA-silencing suppression by sequestering siRNAs (Silhavy *et al.,* 2002; Vargason *et al.,* 2003; Ye *et al.,* 2003). The role of siRNA sequestering by P19 has been

examined in both in vitro *Drosophila* embryo extracts (Lakatos *et al.,* 2004) and infected plants (Szittya *et al.,* 2002; Havelda *et al.,* 2003). Similar to P19, TEV HcPro inhibits the RNA silencing via binding to small RNAs size selectively and was shown to bind 21 nt siRNA duplexes containing 2nt overhangs with higher affinity than to 19nt duplexes lacking overhangs or 24nt siRNA duplexes (Lakatos *et al.,* 2006 ; Merai *et al.,* 2006). Such a size selection in dsRNA binding has not been observed for influenza NS1 (Li *et al.,* 2004; Bucher *et al.,* 2004), nodaviral B2 (Lu *et al.,* 2005), closteroviral P21 (Ye and Patel, 2005), cucumoviral 2b (Goto *et al.,* 2007), or aureusviral P14 (Merai *et al.,* 2005). All these proteins bind duplex small RNAs and long dsRNA, and B2 in fact exhibits higher affinity to long dsRNA than to small RNAs (Lu *et al.,* 2005). *Cucumber vein yellowing ipomovirus* (CVYV) P1b resembles potyviral HCPro and other viral proteins in interfering RNA silencing by preventing siRNA loading into the RNA-induced silencing complex (Valli *et al*., 2008). Interestingly, vaccinia E3L (Li *et al.,* 2004) is the only known suppressor among the dsRNA binding VSRs that has sequence similarity to the dsRNA binding motif (DSRM) found in many cellular proteins, such as *Drosophila* Staufen protein, Dicer, and R2D2 (Nanduri *et al.,* 1998). By contrast, HcPro, 2b, NS1, P19, B2, and P21 share no similarities with the DSRM and each adopts a novel protein structure. The geminiviral suppressor AC4 binds the single-stranded mature siRNA or miRNA (Chellappan *et al.,* 2005). Thus, AC4 probably inhibits the RISC activity after its maturation. This provides further support at the structural level for independent origins of VSRs encoded by the novel overlapping gene.

Inhibition of Systemic Silencing

Suppression of the phloem-dependent signaling of RNA silencing represents another distinct viral strategy for evading the RNA-silencing in plants. This was first demonstrated for P25, as systemic silencing of a transgene did not occur in *Nicotiana benthamiana* unless P25 was inactivated (Voinnet *et al.,* 2000). A recent study further supported that P25 suppression of RNA silencing is required for the cell-to-cell movement of PVX (Bayne *et al.,* 2005). Systemic silencing suppression by the cucumoviral 2b might be due to the inactivation of the signal, as demonstrated by grafting experiments (Guo and Ding, 2002). Silencing suppressor protein 2b interfered with the transgene DNA methylation and prevented transgene silencing from spreading

into reporter scions. Phloem dependent suppression of silencing signals by 2b protein is in close agreement with previous studies that have shown a role for 2b in the long-distance movement of *Cucumber mosaic virus* (Ding *et al.,* 1995).

Other mechanisms of silencing Suppression

Suppressor proteins encoded by the family *Geminiviridae* include AC2 , AC4 and βC1 (Voinnet *et al.,* 1999; Vanitharani *et al.,* 2004; Cui *et al.,* 2005). The AC2 protein contains a zinc-finger domain and acts as a transcription factor. It also possess a C-terminal acidic type of activation domain and a nuclear localization signal. Mutations in any of the three domains abolished the silencing suppressor activity of AC2 which suggests that AC2 suppression of RNA silencing is transcription dependent (Dong, 2003; Trinks *et al.,* 2005; Bisaro, 2006). Inactivation of adenosine kinase (ADK) by AC2 may interfere with a general methylation pathway in plants (Rocha *et al.,* 2005). ADK catalyzes the synthesis of 5'AMP from adenosine and ATP and plays a key role in sustaining the methyl cycle. This suggests that the viral genome may be targeted for silencing through methylation triggered by the viral siRNAs (Li and Ding, 2005).

Viral suppression of RNA silencing and the miRNA pathway: **Altering the Host Function**

Micro RNAs (miRNAs) have been shown recently to be important in developmental gene regulation in both animal and plant systems (He and Hannon, 2004). In plants, most of the miRNA targets are genes that are critical for various developmental processes. A somewhat unexpected, but very interesting property of VSRs was observed when they were expressed constitutively in plants as transgenes (Kasschau *et al.,* 2003). Stable expression of VSRs in transgenic plants interferes with the function of host miRNAs, which may explain why these plants often exhibit developmental abnormalities (Kasschau *et al.,* 2003; Chapman *et al.,* 2004; Dunoyer *et al.,* 2004; Chellappan *et al.,* 2005). Altered accumulation of host miRNAs has been observed in plants expressing HcPro, P19, AC4, 2b, P21, and P69 (Chapman *et al.,* 2004; Chen *et al.,* 2004; Dunoyer *et al.,* 2004; Chellappan *et al.,* 2005; Zhang *et al.,* 2006). Although these observations can be explained as a consequence of the similarities between the siRNA and miRNA-mediated pathways, a better understanding of suppressor function will be needed to fully

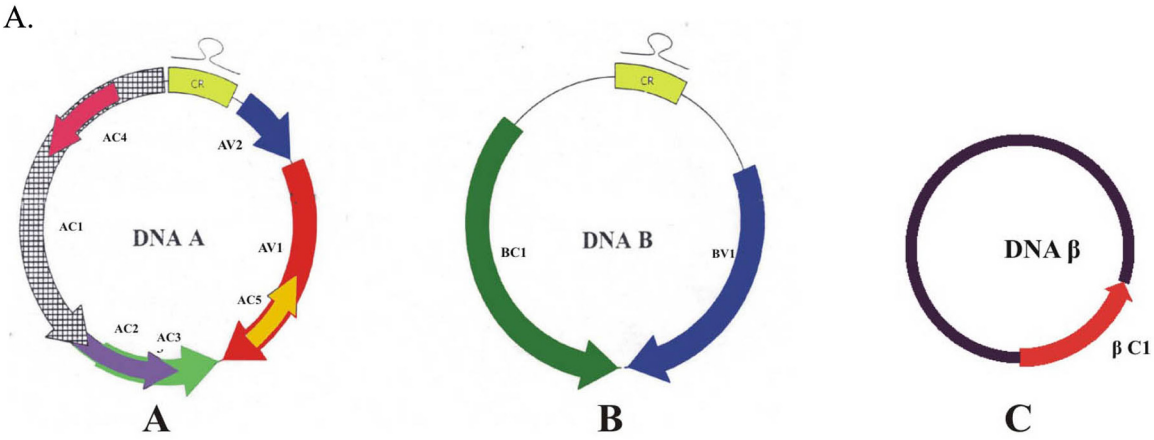
appreciate the role of suppressors in virus symptom development. Notably, these observations have provided an attractive model to explain viral pathogenesis as a result of viral suppression of shared steps in siRNA silencing as an antiviral defense and miRNA silencing required for development.

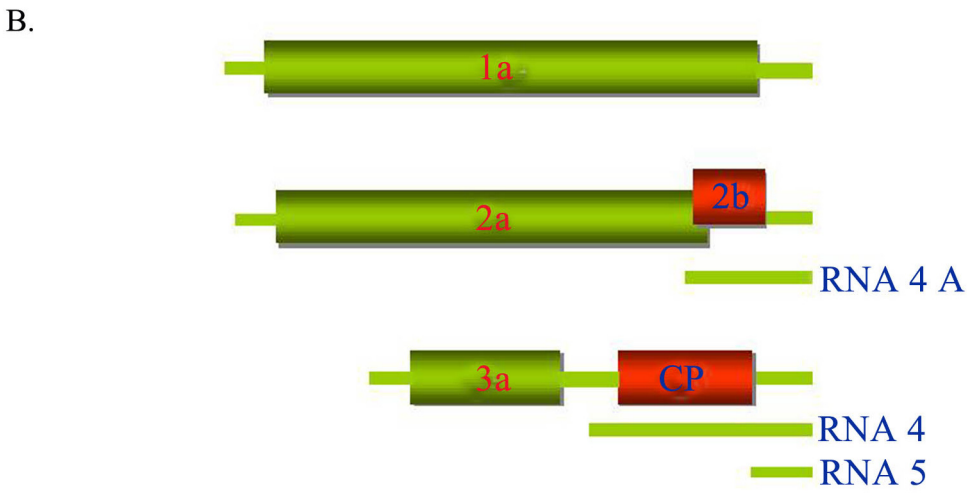
Viral suppressors of RNA silencing from geminivruses, cucumoviruses and potyviruses: emerging themes in molecular arm race

Viruses belonging to three different genera, gemini, cucumo and poty constitute major proportion of losses due to viral infection in plants world. Their genome is packaged into virions, comprise ssDNA or RNA **[Fig.2]**. All the three viruses are known to possess potent suppressors of RNA silencing, gaining attention as one of the most important and interesting emerging viral genomic components after the coat protein and replicase. Recent updates of suppressor proteins: geminiviral AC4, cucumoviral 2b and potyviral HcPro are discussed here.

Geminiviral AC4

Geminiviruses are inducers and targets of RNA silencing. Geminiviruses have circular ssDNA genome that replicates in nucleus by rolling circle mechanism, with a dsDNA intermediate and gene transcription is bidirectional from the common region. So, does not have a dsRNA phase in their replication cycle but can trigger RNA silencing in plants possibly by the bidirectional transcription of overlapping ORFs which leads to ds-RNA formation (Chellappan *et al.,* 2004; Vanitharani *et al.,* 2005). Against RNA silencing mechanism geminiviruses encodes three kinds of suppressors AC2 (Voinnet *et al.,* 1999), AC4 (Vanitharani *et al.,* 2004) and βC1 (Cui *et al.,* 2005). Although, the mechanism of silencing suppression for all the three suppressors is different, they share the common primary function. All the three suppressors are pathogenicity determinant, playing role in plant developmental processes and host virus interactions. ORF C4 (AC4 or AL4) is contained entirely within *rep* (AC1) coding region but in different reading frame. Studies on the function of AC4 ORF have led to differing conclusions. AC4 is least conserved ORF in geminivirus. In bipartite geminiviruses mutagenesis of the AC4-ORF of *Tomato golden mosaic virus* (Elmer *et al.,* 1988; Pooma and Pitty, 1996), *African cassava mosaic virus* (Etessami *et al.,* 1991), *Bean golden mosaic virus* (Hoogstraten *et*





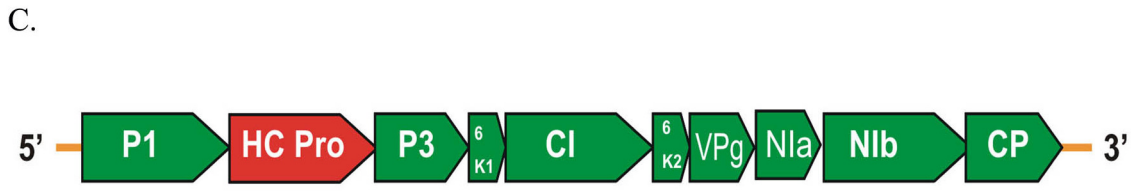


Fig. 2 Genomes of **(A)** Begomoviruses **(B)** Cucumoviruses and **(C)** Potyviruses showing different genetic components.

al., 1996), and *Potato yellow mosaic virus* (Sung and Coutts, 1995), did not demonstrate any effect on viral replication or symptom development. This indicated AC4 ORF of these geminiviruses was nonfunctional in the hosts tested. In contrast, the AC4 ORF of monopartite viruses appears to be multifunctional. *AC4* mutant of *Tomato yellow leaf curl virus* (TYLCV) capable of autonomous replication but was unable to systemically infect tomato, suggesting that the AC4 protein is involved in virus movements (Jupin *et al.,* 1994). Further, it was observed as determinant of viral disease symptoms. Mutagenesis studies on monopartite geminivirus *Beet curl top virus* (BCTV) proved that AC4 ORF plays a significant role in development of viral disease symptoms (Stanley and Latham, 1992). Krake *et al.,* (1998) observed that when AC4 was expressed in tobacco plants, severe virus like symptoms appeared, including rugosity, blistering, and curling of leaf margins, deformation of leaves, stunted growth etc. Pathogenicity of this protein was linked to suppressor of RNA silencing (Vanitharani *et al.,* 2004). AC4 of ACMV (CM) and SLCMV shows suppressor activity with increased accumulation of GFP mRNA and inhibition of GFP-specific siRNAs. Although the exact mechanism of AC4 as the suppressor of RNA silencing is not known but *in vitro* binding assay revealed that it binds single stranded form of miRNAs and siRNAs and hence affecting the accumulation of small RNAs. So, it appears to be a unique suppressor that binds and inactivate mature miRNAs thus blocking a miRNA and mediated regulation, resulting in developmental defects in *Arabidopsis* such as narrow rosette leaves, lack of reproductive tissue growth, stunted growth etc. (Chellappan *et al.,* 2005). So, this is a multifunctional protein having role in viral movement, pathogenicity, and suppression of silencing.

Cucumoviral 2b

Cucumoviral genome contains a single stranded positive sense RNA divided into three segments, which contain five genes encoding proteins designated as *1a, 2a, 2b, 3a* and *3b* (Shi *et al.,* 2002). Recently identified 2b protein 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). This gene is located at 3' end of RNA2 and overlaps, but is out of frame with *2a* gene. Like potyviral HcPro, 2b of cucumoviruses is a protein involved in various interlinked molecular events viz silencing suppression, virus movement and virulence. The difference in virulence of different strains of CMV was shown to be

mediated by differences in the respective 2b proteins (Shi *et al.,* 2002; Shi *et al.,* 2003; You Do *et al.,* 2007). There are many strains and isolates of CMV which can be grouped into two subgroups (Palukaitis *et al.,* 1992) and subgroup I strains may in general be more virulent than subgroup II strains (Wahyuni *et al.,* 1992; Zhang *et al.,* 1994). The CMV 2b protein localizes in the nucleus via an arginine rich nuclear localization signal (NLS). Functionality of arginine rich NLS in the 2b protein encoded by CMV have been demonstrated and provided evidence that nuclear targeting region is involved in suppression of RNA silencing (Lucy *et al.,* 2000). All of the 2b protein encoded by the cucumoviruses (Ding *et al.,* 1994; Shi *et al.,* 1997) contains the potential NLS in the N-terminal region. Later, two putative NLS motifs were predicted in CMV subgroup I strains, and interestingly both the NLSs sequences were found to be associated with symptom elicitation. The other NLS sequences were completely conserved in subgroup I CMV strains and varied by only one amino acid (E→A) in subgroup II CMV strains (Wang *et al.,* 2004). The CMV 2b protein affect virulence in host but does not affect the virus accumulation/replication (Soards *et al.,* 2002; Shi *et al.,* 2003). Insertion of an early translational termination sequence or complete deletion of the *2b* gene had the same effect on the accumulation of CMV in protoplasts and whole plants (Ding *et al.,* 1996), as well as on the ability of a chimaric CMV–umbravirus to promote cell-to-cell movement of the phloem-limited *Potato leafroll virus* (Ryabov *et al.,* 2001). It was found to enhance the long distance movement of CMV in a host-dependent manner as CMV *2b* mutants were capable of systemic invasion of tobacco but not cucumber plants (Ding *et al.,* 1995). The CMV 2b protein was one of the first identified suppressors of RNA silencing and also one of the best studied from a mechanistic standpoint. The initial indication that CMV 2b suppressed silencing came from the reversal of silencing assay in which 2b expressed from PVX could prevent the initiation of silencing but could not reverse silencing that was already established (Brigneti *et al.,* 1998). That early result raised the possibility that 2b might block systemic silencing. Subsequently, stable expression assays and grafting experiments provided an elegant demonstration that 2b blocks the movement of the systemic silencing signal (Guo and Ding, 2002). CMV 2b prevents transmission of the systemic silencing signal in a stable expression assay. Paradoxically, the same type of co-infiltration experiments in another laboratory produced a different result: CMV 2b delayed but did not block systemic silencing (Hamilton *et al.,* 2002). The ability of 2b

protein to prevent the transmission of the signal suggests that it either sequesters or inactivates the signal in the phloem stream. One possibility is that 2b acts directly by binding to the signal. However, the finding that 2b localizes to the nucleus (Lucy *et al.,* 2000) suggests that the suppressor acts indirectly, perhaps by activating one or more processes that subsequently affect the signal. In fact, it is suggested that 2b protein from different strains of the CMV behaves differentially in RNAi pathway by acting either at nucleus (Lucy *et al.,* 2000) or interacting directly with Argonaute protein (Zhang *et al.,* 2006) or by directly binding with small RNAs (Goto *et al.,* 2007). Moreover, strain specific differences between the 2b silencing proteins determine whether only siRNA (Chapman *et al.,* 2004) or both siRNA and mi RNA destruction pathways are disrupted (Zhang *et al.,* 2006; Lewsey *et al.,* 2007). The 2b protein interferes with miRNA pathway eliciting developmental anomalies (Lewsey *et al.,* 2007). Involvement of 2b protein in miRNA pathway has recently been demonstrated either its interaction with AGO1 (Zhang *et al.,* 2006) or through direct binding with small RNAs (Goto *et al.,* 2007). Interestingly, theses features of 2b protein are mostly present in severe strains of CMV.

Potyviral HcPro

The RNA genome of plant viruses in the genus Potyvirus is translated into a polyprotein that is further processed by three virus-encoded proteinases (Carrington *et al.,* 1989a; Carrington *et al.,* 1990). One of these proteinases, HcPro, is a multifunctional protein (Maia *et al.,* 1996, Varrelmann *et al.,*2007) and its journey from its role in viral transmission to the recent updates as a viral counter defense protein and its role in host gene regulation has been summarized in **Table.2**. As a strictly cis-acting proteinase, it is responsible for its self-cleavage from the polyprotein precursor (Carrington *et al.,* 1989b). It is also involved in a number of infectious processes as diverse as aphid transmission (Thornburry *et al.,* 1993), cell-to-cell (Rojas *et al.,* 1997) and long-distance movement (Cronin *et al.,* 1995), genome amplification (Cronin *et al.,* 1995) and suppression of RNA silencing (Anandlakshami *et al.,*1998). The first report giving an indication of TEV-HcPro involvement in antiviral defense pathway came from the

Table.2 A brief history of Potyviral multifunctional protein HcPro

Year	Reference	Discovery	Virus studied
Transmission and Proteinase			
1961	Kassanis	First indications of requirement of a specific protein in the potyvirus transmission.	PVY and PVA
1971	Kassanis & Govier	Helper factor is required to mediate aphid transmission.	
1977	Govier *et al*	➢ First attempt to purify the helper component (Hc) from infected plants. ➢ Proteinaceous nature of Hc.	
1985	Thornburry *et al*	Biologically active form of Hc is dimer	TEV
1986	Hellmann *et al*	Hc was mapped to the N terminus of potyvirus polyprotein (TVMV)	TVMV
1988	Harrison and Robinson	Interaction of Hc with DAG motif of virion CP	
1989	Robaglia *et al*	Zn finger like motif in N terminal of Hc	PVY
1989	Carrington *et al*	Proteinase activity at carboxy terminal of Hc. Termed as Helper component proteinase. Cleavage site Gly/Gly. Autocatalysis/cis acting	TEV
1989	Oh and carrington	Cystein type family protease/Papain like protease	TEV
1993	Thornburry *et al*	Lack of transmission activity of HcPro expressed in bacteria or insect.	TVMV
1993	Atreya & Prione	Point mutations in N terminal of HcPro to reveal its transmission function and emphasized importance of lysine residues in this domain.	TVMV
1994	Huet *et al*	Importance of C terminal in transmission (PTK box)	ZYMV
1996	Wang *et al*	Interaction of HcPro with insect stylet	
1998	Blanc *et al*	Involvement of KITC motif in HcPro-stylet interaction	TVMV
2002	Roudet Tavert *et al*	DAG (CP of Virion) and PTK (HcPro) interaction	LMV, PV & PVY
2003	Plisson *et al*	HcPro is dimer in solution	LMV
2004	Ruiz Ferrer *et al*	HcPro expressed in yeast cells can retain transmission function of HcPro	TEV

2005	Ruiz Ferrer *et al*	Support of bridge model hypothesis of transmission of HcPro by analytical centrifugation and single particle EM. Different oligomeric species of HcPro detected based on sedimentation equlibrium	TEV
2006	Goyita *et al*	Development of transient *Agrobacterium* mediated production of active HcPro for transmission function	PPV
2007	Dombrovsky *et al*	Interaction of KLSC motif of HcPro with cuticle proteins of *Myzus persicae*	ZYMV
RNA silencing suppressor			
1997	Pruss *et al*	➢ First report of synergistic role of HcPro. ➢ Involvement of HcPro in antiviral defense pathway as it enhanced accumulation of PVX, CMV and TMV.	TEV
1997	Shi *et al*	Central region of HcPro is involved in potyviral synergism and symptom expression.	TEV
1998	Anandlakshami *et al*& Kassachau & Carrington	Direct evidence showing RNAi suppression activity of HcPro	TEV
1998	Brigneti *et al*	HcPro act at the maintenance step of RNA silencing	PVY
1999	Voinnet *et al*	Suppression of gene silencing of HcPro in young and old leaves supporting its action at maintenance step.	PVY
2000	Llave *et al*	➢ Suppress a step upstream or at the point of production of small RNA. ➢ Role of HcPro in transgene methylation	TEV
2000	Marathe *et al*	Non-involvement of HcPro in TGS.	TEV
2000	Anandlakshami *et al*	Its interaction with calmodulin related protein.	TEV
2001	Mallory *et al*	HcPro is not involved in methylaion of transgene and targets Dicer or RISC steps	TEV
2001	Jansen and Carrington	➢ Grafting experiment to prove interference of HcPro in mobile silencing signal. ➢ It interacts with Ago I	TEV
2002	Hamilton *et al*	Suppression of both classes of siRNAs i.e short (21-22 nt) and long (24-26 nt) by HcPro.	PVY

2002	Mallory *et al*	➢ Differential regulation of siRNA and miRNAs by HcPro. ➢ Acts at Dicer step. ➢ Correlation between viral symptoms and silencing suppression.	TEV
2003	Kassachau & Carrington	➢ HcPro induced developmental abnormalities. ➢ Close resemblance with *dcl1* mutants. ➢ Interfered with miR171 functioning.	TuMV
2003	Plisson *et al*	Central region of HcPro is involved in silencing suppression with overlapping functions of genome amplification and viral movement.	LMV
2004	Pruss *et al*	Role of HcPro in multiple pathogen resistance through salicylic acid dependent mechanism.	
2004	Chapman *et al*	HcPro involvement in miRNA/miRNA* unwinding and RISC assembly.	TuMV
2004	Dunoyer *et al*	➢ Development defects of HcPro are secondary consequences of siRNA pathway blockage, a step shared with miRNA pathway. ➢ Acts at Dicer or RISC steps with a common factor required for mi and si RNA pathway viz rgs-CaM. ➢ Regulation of DCL-1 by miR162 and reasoned for the enhanced accumulation of miRNAs in response to HcPro expression.	TuMV
2004	Qui *et al*	TEV-HcPro acts downstream of siRNA production.	TEV
2005	Mlotshwa *et al*	TuMV HcPro induced severe developmental anomalies with defect in si & mi RNA pathways	TuMV
2005	Ballut *et al*	HcPro interacts with 20S proteasome and inhibits its RNase activity	LMV
2005	Ebhardt *et al*	HcPro decreased 3′ terminal modification/methylation of viral derived siRNAs without affecting miRNAs and 24 nt siRNAs	---
2006	Lakatos *et al*	HcPro inhibits programming of RISC or RISC assembly via small RNA binding	TEV
2006	Merai *et al*	HcPro binds ds small RNAs size selectively (21 nt)	TEV
2007	Varrelmann *et al*	➢ There is no defined motifs in the cental region of HcPro for silencing suppression ➢ C terminal of HcPro is also involved in PTGS suppression	----
2007	Shiboleth *et al*	FRNK motif of HcPro have primary role in small RNA binding	ZYMV

2007	Jin *et al*	N terminal (1-97) amino acids of HcPro interacts with 20S proteasome	-----
2008	Siddiqui *et al*	HcPro affects the leaf tissues of *Nicotiana benthamiana* and *N tabaccum* by causing hyperplasia. It also suppresses the spread of TMV	PVY
Replication, Movement and Symptom expression			
1992	Atreya *et al*	HcPro is involved in virus accumulation and symptoms expression	TVMV
1993	Atreya and Prione	HcPro is involved in virulence and symptom expression	TVMV
1994	Klein *et al*	First evidence of role of HcPro in potyvirus movement	TVMV
1995	Cronin *et al*	Central region of HcPro is involved in systemic movement (CC/SC motif) and replication (IGN motif)	TEV
1996	Maia and Bernardi	HcPro possess a sequence non specific RNA binding activity	PVY and PVA
1997	Rojas *et al*	C terminal of HcPro is involved in cell to cell movement	BCMNV & LMV
1999	Guo *et al*	N terminal and C terminal of HcPro are involved in self interaction	PVA
2000	Urcuqui-Inchima *et al*	Two independent RNA binding domains are located in the central region	PVY

studies of Pruss *et al.,* (1997) where HcPro was shown to enhance the pathogenecity and RNA accumulation of PVX, CMV and TMV. The direct evidences showing the silencing suppressor activity of TEV-HcPro was reported in reverse silencing assay where effect of HcPro was assessed on *Uid A* silenced transgenic tobacco. This study could explain the mechanism of synergistic effect of HcPro in disease cycle of heterologous viruses (Anandlakshami *et al.,* 1998). In the same year HcPro was demonstrated to act by blocking the maintenance step of PTGS where silencing has already been established. PVY-HcPro reversed the silencing of GFP transgene in *Nicotiana benthamiana* and suppressed the silencing in young and old leaves (Brigneti *et al.,* 1998; Kasschau and Carrington, 1998; Voinnet *et al.,* 1999). TEV-HcPro reversed the silencing of a preexisting GUS silenced transgene and reduced the silencing associated transgene methylation of cystine. This suggested that HcPro targets one or more steps of maintenance and DNA methylation. Since the introduction of HcPro reduced the level of small RNAs, it was interpreted that HcPro suppresses a step upstream of or at the point of production of small RNAs. Alternatively it could affect the point downstream of small RNA production by blocking the feedback amplification. At that time, it was suggested that transgene methylation might be guided by small RNAs or loss of small RNAs would lead to reduced methylation of transgene (Llave *et al.,* 2000). Further, expression of TEV-HcPro neither reversed the established transcriptional gene silencing (TGS) nor interfered with initiation of TGS and suggested that the TGS and PTGS operate through unlinked pathways (Marathe *et al.,* 2000). Mallory *et al.,* (2001) reported that TEV-HcPro does not interfere with the methylation of transgene which was in conflict with the report of Llave *et al.,* (2000). They also showed that HcPro interferes with the small RNA accumulation and suggested its target as dicer or RISC steps. Grafting experiments indicated that HcPro prevents the plant from responding to the mobile silencing signal but does not eliminate its ability to produce or send the signal. They also raised the possibility that HcPro may exert its effect via interaction with AgoI because of the similarity between *agoI* mutants and HcPro phenotypes. Using PVY-HcPro, Hamilton *et al.,* (2002) showed that it suppresses both the classes of short interfering RNAs (i.e. short (21-22nt) and long (24-26nt) siRNAs), long siRNAs were found to be associated with systemic silencing and methylation while short siRNA class correlates with mRNA

degradation. In an interesting study, TEV-HcPro was demonstrated in differential regulation of siRNA and miRNA. Introduction of HcPro in GUS silenced transgenic tobacco lines, carrying sense, inverted repeat and amplicon transgene led to the blockage of siRNA accumulation. The fact that HcPro caused the accumulation of dsRNA suggested that HcPro might be affecting the processing of dsRNA into siRNA by interfering with the Dicer. The accumulation of endogenous miRNAs was enhanced upon HcPro expression suggesting its differential action at the dicer or RISC steps. They also suggested that symptoms elicited by many viruses might be due to the developmental abnormalities caused by interference in miRNA metabolism (Mallory *et al.,* 2002). *Turnip mosaic virus* encoded HcPro induced developmental abnormalities in vegetative and reproductive organs which were in close resemblance with the miRNA deficient *dcl1* mutants. Later it was shown that HcPro interfered with the functioning of miR171 which is needed for down regulation of scarecrow like transcription factors. Basis of TuMV and other disease in plants and the contribution of suppressor to viral symptoms may be the manifestations of altered RNA regulatory pathways (Kasschau *et al.,* 2003; Silhavy and Burgyan, 2004). HcPro was also demonstrated to take part in resistance to multiple pathogens via salicylic acid dependent and independent mechanism. Tobacco lines expressing the HcPro were found to be resistant to *Tobacco Mosaic Virus*, *Tomato Blackring nepovirus* and *Oomycete pernospora tabacina* (Pruss *et al.,* 2004). HcPro from TuMV resulted in accumulation of miRNA*, suggesting the suppression of miRNA/miRNA* unwinding and RISC assembly. However, no evidence was found for its interaction with miRNA/miRNA* complex and siRNA *in vivo*. Based on these finding, an alternative model for HcPro functioning was proposed in which HcPro interferes with protein or complex associated with miRNA/miRNA* duplex or by suppressing the factor required for complex production. Therefore HcPro was suggested to act through interference in duplex unwinding and assembly of active RISC (Chapman *et al.,* 2004). Dunoyer *et al.,* (2004) demonstrated that developmental anomalies caused by TuMV-HcPro were very similar to the suppressors of two unrelated viruses (PCV and TBSV) and proposed that morphological defects are the secondary consequences of siRNA pathway blockage at steps shared with miRNA pathway. They proposed that in addition to its interference on dicer activity, HcPro also affects the activity of RISC.

Further, they suggested that HcPro might be interacting with a factor overlapping in both siRNA/miRNA programmed RISC and Dicer. They suggested that this factor may be similar to the rgs-CaM, a calcium binding protein known to interact with HcPro and mimic its effect in tobacco (Anandlakshami *et al.,* 2000). Experiments of RNAi pathways from protoplast showed that TRV-HcPro suppress the silencing but does not affect siRNA accumulation, which suggested that HcPro acts downstream of siRNA production (Qi *et al.,* 2004). HcPro of TuMV induced severe developmental anomalies with the defect in both siRNA and miRNA pathways. Developmental abnormalities arises due to the expression of HcPro were relieved by over expression of DCL but this did not affect the impairments in small RNA directed RNAi pathways. This suggested that Dicer might be playing an important role in developmental biology which is independent of RNA degradation mediated by small RNAs (Mlotshwa *et al.,* 2005). HcPro decreased the 3' terminal modification/methylation of viral derived siRNAs without affecting the endogenous miRNAs and 24 nt siRNAs. Viral siRNAs are processed by DCL-2 which functions in cytoplasm and HcPro is also known to be localized in the cytoplasm, suggesting its preferential action on viral derived siRNAs only (Ebhardt *et al.,* 2005). TEV-HcPro inhibits the programming of RISC or RISC assembly via binding to small RNAs. The inhibition of siRNA-DCR2-R2D2 intermediate complex formation is likely due to the silencing suppressor possessing higher affinity than DCR2-R2D2 complex for siRNA duplexes. HcPro was also shown to bind 21 nt siRNA duplexes containing 2nt overhangs with higher affinity than to 19nt duplexes lacking overhangs or 24nt siRNA duplexes (Merai *et al.,* 2006). Helper component proteinase of LMV showed an interaction with 20S proteasome and inhibited proteasome RNAase activity. Selective degradation of viral RNAs by the 20S proteasome RNAase which is dependent on secondary structures of RNA represents an alternative pathway parallel to RNA silencing which is sequence specific (Ballut *et al.,* 2005). In a biochemical study, the central region of HcPro was demonstrated to have suppressor activity and this region overlapped with other two functions of HcPro i.e. genome amplification and viral movement. silencing suppression, virus movement and genome amplification are associated with helix rich domain and the hinge domain (Kasschau and Carington, 2001; Plisson *et al.,* 2003). A link between silencing suppression by HcPro of PPV and its capacity to induce synergism

with the central region of the protein was established by introducing one single amino acid mutation at position 134 (Gonza´lez-Jara *et al.,* . 2005). In addition, Saenz *et al.,* (2001) found evidence that a single amino acid change at position 109 in the HcPro of *Plum pox virus* (PPV) influenced its synergism with PVX.

Plant viral synergism

Higher plants are oftenly subjected to multiple virus infections resulting in intensification of symptoms expression and virus accumulation a phenomenon known as synergism (Hull, 2002). In synergistic viral interaction, co-infection with two independent unrelated viruses results in a much more serious disease than either virus induces in a single infection (Matthews, 1991; Rochow & Ross, 1955). In the two major classes of synergism reported, one include the well characterized potyvirus associated synergism in which a notable increase in the virus accumulation and symptoms involve the combination of potyviruses as a synergistic pair and a virus belonging to other genera. In potyvirus associated synergism, the synergy include a potyvirus and a non potyvirus as a pair which may be of a broad range of unrelated viruses, including potexvirus, *Potato virus* X (PVX) in tobacco (Vance, 1991; Vance *et al.,* 1995), machlovirus, *Maize chlorotic mottle virus* in maize (Goldberg *et al.,* 1987), comoviruses, *Bean pod mottle virus* and *Cowpea mosaic virus* in soybean (Calvert *et al.,* 1983; Anjos *et al.,* 1992), polerovirus, *Potato leafroll virus* in *Nicotiana clevelandis*, cucumovirus, *Cucumber mosaic virus* (CMV) in cucurbits and radish (Poolpol *et al.,* 1986; Sano *et al.,* 1989; Wang *et al.,* 2002; Zeng *et al.,* 2007), crinivirus, *Sweet potato chlorotic stunt virus* in sweet potato (Untiveros *et al.,* 2007). In most of these interactions, the increase in symptoms is correlated with an increase in accumulation of non potyvirus of the synergistic pair, but the corresponding increase or decrease in the level of potyvirus, was not observed (Rochow and Ross, 1955; Calvert and Ghabrial, 1983; Goldberg and Brakke, 1987; Vance, 1991; Pruss *et al.,* 1997). The best characterized of the potyvirus associated synergism is the interaction of PVX with the number of potyviruses including PVY and TEV (Wang *et al.,* 2002). The PVX potyviral interaction in tobacco results not only the increase in host symptoms, but also showed increase in accumulation of PVX (-) strand RNA (Vance, 1991).

A number of cases of non potyviral synergism have been reported in which neither virus is a member of potyvirus group (Khan and Demski, 1982; Vanitharani *et al.,* 2004). However, in contrast to the potyvirus associated synergism, none of the non potyviral synergism has been well characterized at the molecular level. Probably the best studied non potyviral synergism is the interaction of TMV and PVX which causes a synergistic disease in tomato called double virus streak (Blood, 1928; Rochow and Ross, 1955).

The extent to which the synergistic viral interactions occur in higher plants and role they play in mediating plant disease is not really clear at this point. Moreover the basic background information about genes responsible for plant viral synergism is deficient. In some cases, synergistic interactions are mediated by proteins that have been shown to be suppressors of RNA silencing (Pruss *et al.,* 1997; Brigneti *et al.,* 1998; Saenz *et al.,* 2001; Qiu *et al.,* 2002; Selth *et al.,* 2004). Expression of such proteins from heterologous viral expression vectors results in increased disease and/or virus accumulation in some host species, but not in others (Pruss *et al.,* 1997; Brigneti *et al.,* 1998; Li *et al.,* 1999; Qiu *et al.,* 2002), as has been observed for interviral synergy (Garces-Orejuela & Pound, 1957; Fukumoto *et al.,* 2003; Gonza´lez-Jara *et al.,* 2004). Synergy of viruses was mimicked in transgenic plants expressing the 5' proximal region of the potyviral genome and infected singly with PVX (Vance *et al.,* 1995). Furthermore, mutations in the coding region of HcPro abolished the PVY-PVX synergism, indicating direct involvement of the potyviral HcPro in the synergistic response (Shi *et al.,* 1997; Gonzalez Jara *et al.,* 2005). It is interesting to mention that the functional map of central region of this protein include the synergy, silencing suppression, symptom expression, genome amplification and binding of nucleic acids (Gonzalez Jara *et al.,* 2005; Valli *et al.,* 2007; Shiboleth *et al.,* 2007;). The fact that the mutations in the central region of HcPro impair the protein for synergism as well as silencing suppression suggests that the synergistic effect and silencing suppression are the interlinked functions (Shi *et al.,* 1997). Therefore, the inhibition of plant defense mechanism by viral suppressor proteins would allow the virus to accumulate beyond the normal host-imposed limits and induce more severe disease. Alternatively, the synergism may also involve the interference of suppressor proteins with multiple RNA silencing pathways including miRNA besides the

virus induced RNA silencing (Chapman *et al.,* 2004; Ding and Voinnet, 2007). Since the well characterized synergy phenomenon between potyviral and non potyviral groups is host and virus specific, beside the silencing suppressors protein HcPro, other component of potyviral genome might be having crucial role in host virus interactions.

Genome organization: PRSV, a potyvirus

Papaya ringspot virus (PRSV), a member of the aphid transmitted genus potyvirus in the family *potyviridae* is the cause of destructive disease and a major limiting factor for papaya and cucurbit cultivation worldwide (Purcifull *et al*., 1984). PRSV is grouped into papaya infecting type-P (PRSV-P) and non papaya infecting type-W (PRSV-W). PRSV-P infection is typically characterized by the production of ringspot symptoms on fruits of infected papaya trees. In addition to ringspot, PRSV produces a range of other symptoms such as leaf mosaic and chlorosis, water soaked oily streaks on the petiole and upper part of the trunk, distortion of young leaves that sometimes results in shoestrings like symptoms that resemble mite damage, stunting of infected plants and flower abortion (Tripathi *et al.,* 2008). Virions are flexuous, filamentous, particles ranging in size from 700-900 nm in length and 12 nm wide; its genome is a single stranded RNA of positive polarity of around 10,000 nucleotides (Shukla *et al.,* 1994). Based on their genome organization and their strategy of expression, potyviruses have been included in the subgroup of picorna-like viruses. The RNA genome carries a VPg (viral protein genome linked) covalently bound to its 5' end, and a polyA tail at its 3'end. The genome contains a single long open reading frame (ORF) translated into polyprotein that is further cleaved to produce final protein products. Properties of the different proteins of potyviruses are given in **Table. 3**.

The complete genome of thirteen PRSV isolates one each from China (GenBank Acc. No: EF 183499), Mexico (Noa-Carrazana *et al.,* 2007, GenBank Acc. No: AY231130), and Hawaii (Yeh *et al.,* 1992, GenBank Acc. No: NC001785, EU126128), two each from, Brazil (W isolates, WC - GenBank Acc. No: DQ374152, W1 - DQ374153) and Thailand (one P isolate (AY162218) and one W isolate (AY010722) and five P isolates from Taiwan (four P isolates - X97251, DQ340769, DQ340770 **,** DQ340771 and two W isolate - AY027810, AY010722) are already available in GenBank. The PRSV genome has a similar organization to other members of the potyvirus genus (Yeh

et al., 1992; Wang and Yeh, 1997). There is a 5' UTR of 85 nucleotides, which is short compared to many other poty-

Table.3 Properties of the different Potyvirus proteins

P1	Trypsin Like serine proteinase, C-terminal autocleavage, Symptomatology
HcPro	Aphid transmission, self-interaction, systemic movement, suppression of gene silencing, synergism and symptom development, Papain like proteinase, C-terminal autocleavage
P3	Plant pathogenicity
6K1	?
CI	ATPase/RNA helicase, cell to cell movement
6K2	Anchoring the viral replication complex to membranes
NIa	Celluler localization, protein- protein interaction, trypsin-like serine proteinase, acts in cis and in trans
VPg	Genome replication
NIb	RNA dependent RNA polymerase (RdRp), involved in genome replication
CP	Aphid transmission, cell to cell and systemic movement, virus assembly.

(Source: Urcuqui-Inchima *et al.,* 2001)

virus and a 3'UTR of 209 nucleotides which is polyadenylated (Yeh *et al.,* 1992). PRSV has a relatively long P1 coding region compared with other potyviruses (Wang and Yeh, 1997). The P1 protein appears to be the most variable potyviral protein and shows a wide variation in size (from 29K-63K) among reported potyviruses (Yeh and Gonsalves, 1994). Between the P1 proteins of Taiwanese and Hawaiian PRSV isolates there is only 70.9% nucleotide identity and 66.7% amino acid identity (Wang and Yeh, 1997). Next to the P1 protein, the P3 is the least conserved between PRSV and other potyviruses suggesting that the function of these proteins may be more virus specific (Yeh and Gonsalves, 1994). Between the Hawaiian and Taiwanese PRSV-P isolates, amino acid

sequence identity between proteins (Excluding P1) ranged from 91.2% (6K1) to 97.6% (CI) (Wang and Yeh, 1997). Comparative sequence analysis of PRSV–YK (Taiwan isolate) and PRSV-HA (Hawaii) isolates revealed that except P1 protein which shared only 70.9% and 66.7% identity at nucleotide and amino acid levels respectively, the other proteins of the two isolates showed high degree of identity at both nucleotide (82.5-92.3%) and amino acid levels (91.2%-97.6%), (Yeh *et al.,* 1992; Wang and Yeh, 1997). Sequence analysis of viral genomes of a *Papaya ringspot virus* P and W pathotypes from Thailand is suggests that the P type arose locally from type W. However; differences between types P and W have not yet been discovered to account for host specificity (Charoensilp *et al.,* 2003).

Molecular biological procedures outlined in Sambrook and Russell, 2000 were followed. Details of commonly used protocols as modified are given in Appendix-I and the composition of buffers and reagents are provided in Appendix-II.

Experimental Material

Papaya (Carica papaya) infected with PLCV, tobacoo (N.tabacum) infected with CMV and ToLCV and Papaya infected with PRSV at three temperature ranges (15-20ºC, 26-32ºC and 35-40ºC) were maintained in glass house under insect free conditions.

Cloning and sequencing PLCV-*ac4*, CMV-*2b* and PRSV- *HcPro* genes

The detailed protocols for amplification and cloning of ***ac4, 2b* and *HcPro*** genes is given in the Appendix-I. Total RNA (PRSV and CMV) and DNA (PLCV) from infected leaves was isolated using RNeasy or DNeasy Plant Mini Kit provided by Qiagen as per manufacturer's protocol. Total RNA was reverse transcribed using gene specific reverse primer. Complementary DNA (PRSV and CMV) and DNA (PLCV) were subjected to PCR amplification using gene specific primers to amplify the PLCV-*ac4*, CMV-*2b* and PRSV-*HcPro* genes. Specific primers were commercially synthesized: sequences of the forward and reverse primers were based on the sequence of genes available in NCBI database. The amplicons were subsequently cloned in pGEM-T Easy vector (Promega Inc, USA) and transformed in *Escherichia coli* (strain DH5α). Sequencing was performed at the commercial facility using the primers from T7 and SP6 promoter present in the vector.

Sequence analysis of AC4, HcPro and 2b proteins

For sequence analysis, viral protein sequences from geminiviruses, CMV strains and isolates, PRSV and other potyviruses were collected from GenBank are shown in **Table 4, 5 & 6.** The analysis utilized the amino acid sequences of AC4 proteins from

Table.4 Sources of AC4 protein sequences from different geminiviruses used in the study.

Isolate	**Accession number**	**Length**
East African cassava mosaic virus **(EACMV)**	CAM59416.1	70
Papaya leaf curl virus **(PLCV)**	NP_955748.1	97
Cotton leaf curl virus **(CLCuV)**	NP_803142.1	100
Tomato yellow leaf curl virus **(ToYLCV)**	ABG26076.1	99
Tomato leaf curl virus **(ToLCV)**	ABG26094.1	97
Corchorus golden mosaic virus **(CoGMV)**	ABG26010.1	100
Pepper yellow vein Mali virus **(PepYVMV)**	CAM84562.1	85
Mungbean yellow mosaic virus **(MYMV)**	NP_077092.1	99
Watermelon chlorotic stunt virus **(WmCSV)**	ABM91592.1	47
Bhendi yellow vein mosaic virus **(BYVMV)**	NP_579977.1	102
Indian cassava mosaic virus **(ICMV)**	CAE05935.1	102
Tomato leaf curl virus **(ToLCV) Bangalore**	ABH10497.1	97
Tomato leaf curl virus **(ToLCV) -Papaya NDLS**	EU006071.1	66
Tomato leaf curl virus **(ToLCV) -Chilli NDLS**	EU006070.1	65
Tomato leaf curl virus **(ToLCV) -Tobacco NDLS**	EU006069.1	58
Tomato leaf curl virus **(ToLCV) -Cucumber NDLS**	EU006068.1	58
Tomato leaf curl virus **(ToLCV) - NDLS**	DQ365829.1	58

Table.5 Sources of 2b protein sequences from different strains and subgroups of CMV used in the study.

S.No.	Strain	Subgroup	Accession No.
1	Fny	I	NC_002035
2	NT9	I	D28779
3	MB-CMV	I	AF150731
4	MB8	I	D86613
5	Lily Asiatic	I	AJ866769
6	Ixora	I	U20218
7	Mf	I	AJ276480
8	Kor	I	U66287
9	Rs	I	AJ517801
10	Ns	I	AJ511989
11	Ly2	I	AJ535914
12	As	I	AF033667
13	Tfn	I	Y16925
14	Pf	I	AB096214
15	ALS-NAK	II	AJ304395
16	ALS-IPO	II	AJ304396
17	ALS-LBO	II	AJ304394
18	Q	II	Z21863
19	Trk7	II	AJ007934
20	LS	II	AF416900
21	LY	II	AF198102

Table.6 Source of HcPro sequences used in the study; from different potyviruses (A) and from different PRSV isolates (B)

A. Potyviruses source	**Accession No.**
Lettuce mosaic virus	**NP734154**
Turnip mosaic virus	**NP734214**
Plum pox virus	**NP734340**
Potato virus A	**CAA74553**
Sweet potato feathery mottle virus	**NP734310**
Japanese yam mosaic virus	**NP734224**
Lily mottle virus	**NP945137**
Scallion mosaic virus	**NP734124**
Potato virus Y	**AAC54827**
Tobacco vein mottling virus	**NP734329**
Peru tomato mosaic virus	**NP787939**
Konjak mosaic virus	**YP529491**
Yam mosaic virus	**YP022753**
B. PRSV isolates source	
New Delhi (from this study)	**DQ855428**
Brazil	**ABD23971**
Brazil	**ABD23970**
Taiwan	**NP_734234**
Taiwan	**NP_056758**
Taiwan	**X67673**
Thailand-P	**AAO16605**
Thailand-W	**AAG47346**

different geminiviruses, 2b proteins from CMV strains belonging to subgroup I and II and HcPro proteins from PRSV isolates and other members of potyviruses.

BioEdit sequence alignment editor version 5.09.04 (Hall, 1999) was used for the analysis of amino acid sequence data. Alignment of the proteins was generated by the package of Clustal X version 1.81 (Thompson *et al.,* 1997). Gonnet series was followed as protein weight matrix for amino acid alignment. Neighbor joining trees were generated using CLUSTAL X with the default values of multiple alignment parameters. Robustness of the phylogenetic tree was assessed from the bootstrap value for each internal node of N-J tree by calculating the 1000 random resampling (Felsenstein, 1985). Conserved domain protein architecture of HcPro protein was modeled using *All-IN-ONE SEQ-ANALYZER version 1.35* (http://www-personal.umich.edu/~ino/blast.html).

Generation of viral suppressor's binary vector constructs

The detailed protocol for the generation of the viral suppressors constructs is given in the Appendix-I.

Construction of HcPro sense vector:

The *HcPro* gene of PRSV (New Delhi isolate) corresponding to RNAi suppressor was amplified from the viral genome using RT-PCR with the gene specific primers. Total RNA from infected leaves was isolated using RNeasy kit (Qiagen) as per manufacturer's protocol. Total RNA was reverse transcribed using sequence specific reverse primer. Complementary DNA was used for PCR amplification with high fidelity Phusion Taq DNA polymerase (New England Biolabs, Beverly, MA). Two primers were used: one was complementary to the 3' proximal region of the HcPro coding region, but with an added translation stop codon (TGA) followed by a sequence specifying an *Xho* I restriction enzyme site. The other primer was complementary to the 5' proximal portion of the proteolytically processed HcPro, but with 12 added upstream nucleotides, GGG CCC AUG GCC encoding an *Apa* I restriction site for cloning purpose and a translation start site followed by an alanine codon to provide a good context for translational initiation in plants (Lutcke *et al.,* 1987, Pruss *et al.,* 1997). Thus, translation of the sequence encoding HcPro on the binary vector produces a protein with two extra amino terminal amino acids (Met and Ala). Approximately 1.4 kb *Apa* I / *Xho* I restricted

fragment was cloned in pUC118 in sense orientation under the control of constitutive CaMV 35S promoter and transcriptional termination sequences. Orientation of constructs was confirmed by PCR and sequencing. Cassette of ~2 kb containing the ~1.4 kb *HcPro* gene under the control of 35S promoter was cloned in binary vector pCAMBIA 2301 carrying the kanamycin as a selection marker at *Bam*HI and *Hind*III sites in MCS.

Construction of 2b sense and antisense vectors

The 2b ORF from CMV New Delhi isolate (CMV-NDLS) corresponding to RNAi suppressor was amplified with high fidelity Phusion Taq DNA polymerase (New England Biolabs, Beverly, MA) from the viral genome using RT-PCR with the gene specific primers. The amplicon was cloned in pUC118 in sense as well as in antisense orientation under the control of constitutive CaMV 35S promoter and transcriptional termination sequences. Orientation of constructs was confirmed by PCR and sequencing. Cassette of ~900 bp containing the 336 bp *2b* gene (in sense and antisense) under the control of 35S promoter was cloned in binary vector pCAMBIA 2301 carrying the kanamycin as a selection marker at *Bam*HI and *Hind*III sites in MCS. Restriction analysis was done to confirm the proper cloning of the *2b* gene during various steps of construct formation.

Construction of ac4 sense vector

The AC4 ORF from PLCV corresponding to RNAi suppressor was amplified with high fidelity Phusion Taq DNA polymerase (New England Biolabs, Beverly, MA) from the viral genome using PCR with the gene specific primers. The amplicon was cloned in pUC118 in sense orientation under the control of constitutive CaMV 35S promoter and transcriptional termination sequences. Orientation of constructs was confirmed by PCR and sequencing. Cassette of ~1Kb containing the ~250 bp *ac4* gene under the control of 35S promoter was cloned in binary vector pCAMBIA 2301 carrying the kanamycin as a selection marker at *Bam*HI and *Hind*III sites in MCS. Restriction analysis was done to confirm the proper cloning of the *ac4* gene during various steps of construct formation.

Mobilization of constructs into *Agrobacterium*

The recombinant binary vector pCAMBIA 2301 carrying *ac*4 (sense), HcPro (sense), and *2b* (sense and antisense) were mobilized into *Agrobacterium tumefaciens* strain LBA

4404 by freeze and thaw method (Hofgen and Willmitzer, 1988) as described in Appendix I.

Tomato transformation (*Solanum lycopersicon*)

The leaves collected from one week old tomato plants were surface sterilized with 0.1% $HgCl_2$ for 5 minutes and washed with sterile double distilled water to remove any adhering sterilizing agents. After surface sterilization, explants were blot dried using autoclaved 3 mm filter paper discs and cut into small pieces with the help of scalpel. Such leaf discs were used as explants. Before *Agrobacterium* infection, leaf discs were allowed to incubate on the regeneration medium for 2 days at 25ºC temperature in tissue culture room. Proper care was taken while placing leaf discs on the medium so that dorsal surface was always in contact with the medium. *Agrobacterium* containing constructs were grown in Luria broth (LB) with kanamycin (50µg/ml) and streptomycin (100 µg/ml) at 28ºC and 200 rpm overnight. 50 ml of overnight grown agro-culture was taken in centrifuge tubes and pelleted at 4°C temperature, 6000 rpm for 10 minutes. Approximately 20 ml autoclaved MS liquid (MS salt without agar) along with 200 µl of agroculture were taken and the leaf discs was transferred into it and kept for 20 minutes with occasional slow shaking of the plate for proper infection. Excess Agrobacterium was removed from leaf discs by 3 times washing with half MS solution. The leaf discs were then transferred in another plate for drying using sterile blotting paper. After blot drying, the leaf discs were transferred into regeneration medium for two days with complete darkness for proper transfer of T-DNA into plant genome. After 48 hours of co-cultivation the leaf discs were transferred into selection medium for callus formation. Further, they were transferred to shooting medium for shoot development. Once shootlets were formed, they were transferred into rooting medium for complete plantlet regeneration.

Transformation of tobacco (*N.benthamiana and N.tabacum*) plants

Young leaves were collected from two week old seedlings and surface sterilized with 0.1% $HgCl_2$ for 1 min and washed with double sterile water thrice for 1min each. Leaves were cut from both sides and used for co-cultivation with *A. tu*

mefaciens harboring suppressor gene constructs as described by McCormick, 1991. Co-cultivated explants were incubated in 90mm petriplates on callusing medium containing MS salts, B5 viatmins, 3% sucrose, 0.2 mg/l NAA, 1 mg/l BAP, 50 mg/l kanamycin and 200 mg/l cefotaxime (pH 5.8). The cultures were kept at 25ºC for 16 h photoperiod of light intensity 1500 lux. They were transferred to shooting medium within 8-10 days (MS salts, 2.5 mg/l BAP, 0.2 mg/l IBA) and after two weeks to rooting medium (Half strength MS, 0.2 mg/l NAA).

Molecular analysis of transformants

PCR detection

Total DNA isolated from the transformed plants was used as templates for detection of transgene integration by performing PCR. The primers employed were gene specific which detects the transgene (as described in Appendix I).

RT-PCR detection

Total RNA isolated from the transformed plants was reverse transcribed by transgene specific primers. Complementary DNA was used as templates for detection of transgene integration by performing PCR. The primers employed were gene specific which detects the transcribed form of transgene (as described in Appendix I).

Construction of pMal-HcPro expression vector

The sequences of the primers used in PCR amplification were based on 3' proximal region of the HcPro coding region, but with an added translation stop codon (TGA) followed by a sequence specifying a *Pst* I restriction enzyme site. The other primer was complementary to the 5' proximal portion of HcPro with *Eco* RI restriction site for cloning. Complementary DNA of *HcPro* gene was obtained by RT-PCR performed on total RNA extracted from papaya plant infected with PRSV, cDNA was used for PCR amplification with high –fidelity Phusion *Taq* DNA polymerase (New England Biolabs, Beverly, MA). Approximately 1.4 kb *Eco* RI/ *Pst* I restricted fragment was cloned in pMal-c2x under the control of tac promoter. Restriction analysis was done to confirm the proper cloning of the *HcPro* gene in pMAL-c2x. Orientation of constructs was confirmed by PCR and sequencing.

Expression and Purification of MBP:HcPro and MBP: β-gal α

Expression and purification of the fusion protein were essentially as described by Rodriguez & Carrasco, 1993) with minor modifications. *Escherichia coli* (strain TB1) cells were transformed with pMAL-c2x which encodes an MBP-β galactosidase-α peptide fusion (MBP: β-galα) used as control and pMAL-HcPro to express the MBP:HcPro fusion protein and were grown in the LB rich medium containing ampicilin and glucose to an OD_{600} followed by addition of IPTG to different concentrations (0.2 mM, 0.3 mM and 0.4 mM) and induction for different time interval (1 hr, 2 hr, 3hr and 4 hr) at 28^{o}C and 37ºC , all subsequent steps were performed at 4^{o}C. Cells were collected by low speed centrifugation, resuspended in column buffer and stored at -20º C. After thawing, cells were sonicated with the 3 min impulses of 15 sec each. After centrifugation at 9000 ×g for 30 min, the supernatant was diluted to 50 ml with column buffer. Affinity chromatography was performed as recommended by the manufacturer (New England Biolabs). The purified fusion proteins were analyzed by SDS-PAGE (0.1% SDS, 12 % polyacrylamide). Owing to the presence of some breakdown products, the concentration of intact fusion protein was estimated by staining with Coomassie blue and comparing its intensity with known concentrations of BSA. Immunoblot assays were performed according to standard protocols using anti-MBP antibodies.

Protein quantification

A quartz cuvette was obtained and filled with column buffer. This was used as Blank to calibrate the spectrophotometric measurement. The cuvette was then put into the sample holder of the Ultra spectrophotometer (Eppendorf). The Blank was analyzed at 280nm wavelength. The cuvette was then cleaned with sterile distilled water and then with column buffer and filled with a 1:10 dilution of the fused protein and reanalyzed as before. This analysis was then repeated with a 1:100 dilution of the protein sample.

Preparation of labeled dsRNA probes:

The random primer labeling reaction was done in 50 µl reaction mixture. 100 ng of synthetic single stranded miR171 template and 10ng of hexamer primer based on sequences of miR171 was added into an microfuge tube containing 10 X labelling buffer, 10µm dNTPs without dCTP, 10 mM DTT, 2 µl BSA, 1 µl [α-^{32}P] dCTP (10 µCi/µl, 3000

Ci/mmol) and 5U Klenow enzyme were added to the microfuge tube and incubated at 37°C for 1-2 h. The labelled miR171 duplexes probe was stored at -20°C till further use.

Gel Mobility Shift Assay

In a binding reaction, labeled small RNAs (3ng) was incubated with the purified recombinant HcPro protein for 30min at room temperature, stopped by adding dyes and loaded on to 6% PAGE. Gels were dried and autoradigraphed. Binding reactions were performed in 83mM Tris-HCl (pH 7.5), 0.8 mM $MgCl_2$, 66mM KCl, 100mM NaCl, 10mM DTT and 0.02% Tween 20. Each sample contained 5unit RNasin. Maltose binding protein was expressed and purified according to manufacturer's protocol (New England Biolabs). In a direct competition assay, varying amount of protein (0.5 µg, 1.0 µg, 2.0 µg, 5.0 µg, 10.0 µg, 20.0 µg and 50.0 µg) was incubated with the fixed amount of double stranded small RNA duplexes (3ng). In a temperature dependent assay, 20µg of recombinant HcPro fusion protein was incubated with 3ng miR171 labeled duplexes at three temperature profiles, 15ºC, 25ºC and 35ºC.

Autoradiography

The gel was dried and placed within fold of saranwrap, placed in a lead cassette and exposed to X-ray film (KODAK) for 18h at -70^0C. Autoradiograph was then developed as per X-ray films manufacturer's instructions.

Virus quantification

DAC-ELISA

Virus titer in transgenic lines and virus infected plants was quantified by indirect ELISA. The ELISA plate wells were coated with 200µl of extracts prepared by grinding plant tissues (1:10 w: v) in 0.2 M sodium carbonate buffer (pH 9.6). After incubation at 37ºC for 1h and 3 time washing with wash buffer (PBS-T), 200µl of commercially available virus specific antibodies were added and incubated for 1.5 h at 37ºC. Finally plate was loaded with goat anti rabbit alkaline phosphatase conjugate (Sigma) (1:30,000) and incubated at 37ºC for 1.5 h. After washing, 200µl of the enzyme substrate p-nitrophenylphosphate (0.5 mg/ml) was added and absorbance was recorded at A_{405nm}.

Negative-positive thresholds were set at two times the mean of healthy control sample absorbance.

Quantitative PCR

Probe and Primer Designing

The oligonucleotide primers and the taqman florescent probe used to perform both quantitative real time PCR and conventional PCR were designed from ToLCV (IARI) *ac4* gene sequence available in GenBank, Accession no. DQ365829. The PCR primers i.e. forward 5' TCCGCATATCCATGTTCTCATCC 3' and reverse 5' AGAACGTCTCCGTCTTTGTCG 3', and taqman probe 5'FAM-TGGTTTCCCCAAGTCGGTCAGCACA-TAMRA3' for 200 bp amplicon were designed with the help of bioinformatic tool 'Primer3' programme (http://frodo.wi.mit.edu/) by considering all the basic parameters required for efficient working of taqman probe (Landt, 2001). The taqman probes for *ac4* gene were labeled with FAM (6-carboxy-flouroscein) as reporter dye at 5' site and TAMRA (6-carboxy-N, N, N', N'-tetramethyl-rhodamine) as quenching agent at 3' site **[Fig.3].**

Standardization of real time PCR for ToLCV-ac4

The standard curve can be produced by cloning target DNA in a plasmid vector or by using known amount of nucleic acid. Here we used the cloning method for standard curve generation.

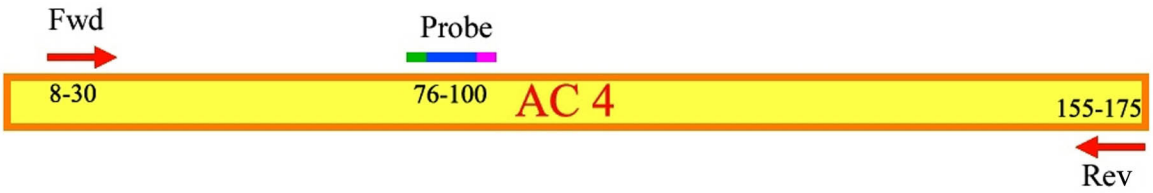
Standard Curve

Real time PCR reaction was performed with the four independent serial dilutions taking *ac4* plasmid DNA as a template in duplicates, together with positive and negative control for generation of standard curve. The standard curve method produced a linear plot for approximate copy number for the above standard samples versus Ct value **[Fig.3]**.

Genomic DNA extraction for Real time PCR

The DNA was extracted from 100mg of leaf sample by using DNAeasy Plant mini Kit as per manufacturer's instruction (Qiagen). Each sample was dissolved in 100µl of ddH_2O, and 1µl of DNA solution was used for each real time PCR reaction.

A.



B.

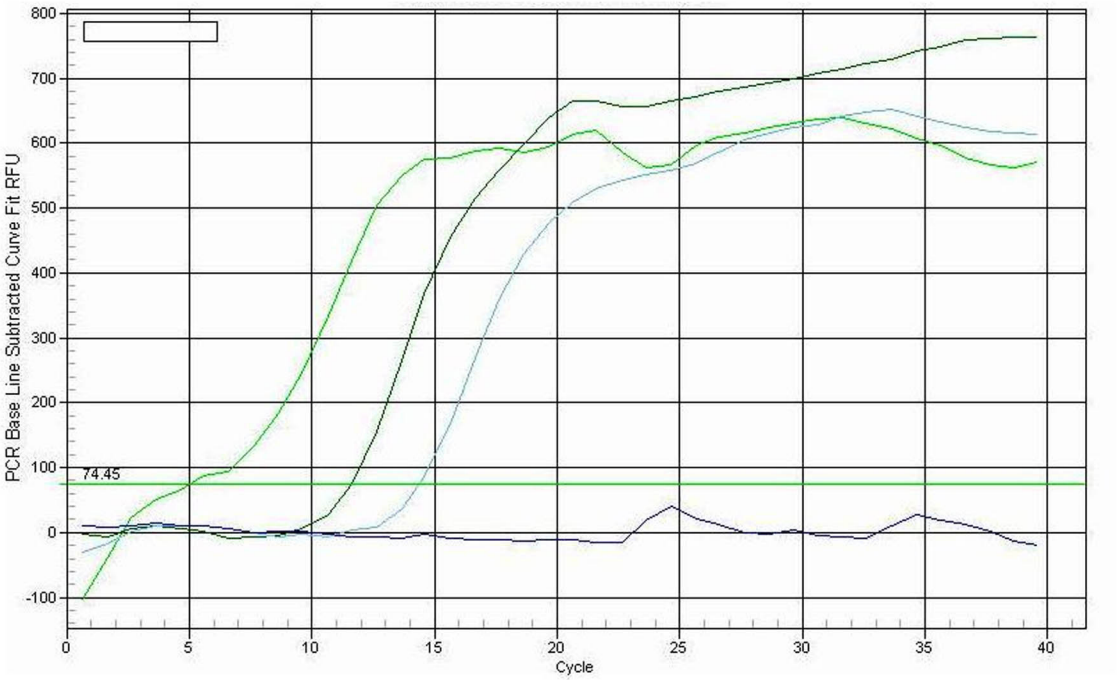


Fig.3 (A) Binding site for primers and bi-labeled fluroscent probe in *ac4* gene. Red arrows indicate the positions of forward and reverse primers in the *ac4* gene and the bar line indicate the Taqman probe with FAM reporter and TAMRA quencher dyes. **(B)** Real- Time amplification plot for ToLCV-*ac4* gene at three different dilutions.

Quantification by Real Time PCR

For the quantification of real time PCR there are three quantitative methods: the absolute standard curve method, the relative standard curve, and the comparative cycle time methods. Here we selected relative standard curve method, in which with sample DNA, the serial dilution of cloned *ac4* gene is run as standard in independent real time reactions. The Ct value of sample DNA is compared with the relative standard curve obtained by standard sample. Real time PCR reactions were performed in 200µl stripes with BioRad real time PCR machine. For each reaction 1µl of template DNA, 2 µl of 10X Hot start PCR reaction buffer (Genex), 20 pmol of *ac4* gene primers and 100nm of dual labeled probe were added, and reaction were brought to a final volume of 20µl with ddH_2O. PCR profile includes one cycle of 10 min at 95ºC, 40 cycles of 1 min at 95ºC, and 40 sec at 56ºC, the amplification of all the samples were confirmed by observing the amplification plot **[Fig.3]**. The Ct value was determine by using instrument's software and adjusted manually as necessary.

Cloning and sequencing of genomic components of PRSV

Viral RNA was extracted from PRSV infected *Carica papaya* leaves using RNeasy plant mini kit (Qiagen). The RNA was reverse transcribed and amplified using high fidelity Taq DNA polymerase (New England Biolabs). Purified amplicons were cloned into pGEM-T Easy (Promega, Inc, USA). Plasmids were maintained in *Escherichia coli* (DH5α). Sequences from each clone were determined using overlapping cloned plasmids to cover the complete genome. The sequences of cloned fragments of adjacent regions of the genome overlapped by 100-200 bp were generated to ensure that they were from same component of genome and in proper orientation. Sequence data were assembled using the BioEdit software version 5.09.04 (Hall, 1999). Sequence was submitted in GenBank (Accession number EF017707) and was published as annotated sequence record of first report of full PRSV genome sequence from India (Parameswari *et al.,* 2007).

Virus isolates and sequence data

Details of the PRSV isolates were collected from GenBank (http://www.ncbi.nlm.nih.gov) are shown in **Table**.**7**. The analysis utilized the complete

genome sequences in the form of nucleotide sequences and translated amino acid sequences. Whole genomes were further splitted for gene wise analysis.

Sequence analysis

Amino acid sequences representing the full genome and their cistrons were aligned using the CLUSTAL W (Thompson *et al.,* 1997). Neighbor joining trees were generated using CLUSTAL X with the default values of multiple alignment parameters. Robustness of the phylogenetic trees was assessed from the bootstrap value for each internal node of N-J tree by calculating the 1000 random resampling (Felsenstein, 1985). Sequence similarity matrix between polyproteins and specific cistrons was calculated using the BioEdit software version 5.09.04 (Hall, 1999).

Recombination analysis

In the present study, Sawyer's run test was used to detect the recombination as implemented in GENECONV. In simulations, it has been established as a robust method to identify the recombination (Possada *et al.,* 2002; Chare and Holmes, 2006). It works by analyzing the longer fragments within an alignment over which a pair of sequences are similar or identical. Analysis was done by using the default settings in GENECONV and a global permutation P value (with cut-off of $P< 0.05$).

Symptoms evaluation in papaya plants infected with PRSV-P

Data on symptom development and incidence (% of plants with symptoms) were taken for 25 days after inoculation (DAI). Symptom severity was evaluated 25 DAI with an arbitrary scale. Rating 1 was assigned to plants without mosaic symptoms and leaf deformations with good development; rating 5 was assigned to those exhibiting severe blisters, shoe string symptoms, severe leaf deformations and stunting; rating 2, 3 and 4 were assigned to intermediate symptom between the two extremes (Rezende & Pacheco, 1998).

Table.7 Sources of full genome sequences of PRSV isolates from different geographical locations used in this study for comparison

Name of the virus	Country	Accession number	Length (nt)
American isolates			
PRSV-W-C	Brazil	DQ374152	10,326
PRSV-P	Mexico	AY231130	10,320
PRSV-W-1	Brazil	DQ374153	10,332
PRSV-P	Hawaii	X67673	10,326
Asian isolates			
PRSV-P	China	EF183499	10,323
PRSV-P	India	EF017707	10,317
PRSV-P	Taiwan	X97251	10,326
PRSV-P1	Taiwan	DQ340769	10,326
PRSV-P2	Taiwan	DQ340770	10,326
PRSV-P3	Taiwan	DQ340771	10,326
PRSV-W	Thailand	AY010722	10,323
PRSV-P	Thailand	AY162218	10,323
PRSV-W	Taiwan	AY027810	10,334

RNA silencing occurring in broad range of eukaryotic organisms is an evolutionary conserved defense mechanism against the molecular parasites and regulates many processes including development, maintenance of genome stability and antiviral response. It is activated by small non coding RNAs to target the degradation of homologous transcript or to arrest their translation. These processes also mediate the effective defense mechanisms against invading genetic elements such as transposons, transgenes and viruses. Successful adaptation of viruses in plants requires expression of suppressor proteins to counteract an antiviral RNA silencing response. Most of the suppressor proteins are involved in regulation of small RNA molecules affecting both, developmental processes and the natural defense mechanism existing in plants. Silencing suppressors are strikingly diverse within and across the kingdom as there acquisition is through fast evolutionary convergence, confining silencing suppressors within tight lineages in virus phylogenies. Consequently, similar silencing suppression strategies may have evolved independently several times such that unrelated silencing suppressors might share analogous biochemical property. Since, suppressors from the diverse origin have been shown to interfere with distinct steps of small RNA pathways affecting both, developmental and antiviral response, a very complex view is emerging. To clarify the various silencing suppressor mechanisms, it is needed to study the structural and functional genomics of viral suppressors from different genera in relation to host gene regulation.

Characterization of viral suppressors

Silencing suppressor proteins are well established to redirect the host gene regulation to facilitate the virus multiplication by modulating the molecular switches known as small noncoding RNAs. As a secondary consequences of this phenomena, symptoms are produced due to the interference of suppressor proteins with microRNA (miRNA) metabolism that mimics the enzymatic machinery and most of the features of viral induced small interfering RNAs (siRNAs). Silencing suppressors derived from three different viral genera, HcPro of *Papaya ringspot virus* (potyvirus), 2b of *Cucmber*

mosaic virus (cucumovirus) and AC4 of *Papaya leaf curl virus* (geminivirus) were focused to study the silencing suppressor effects on the small RNA pathways. These identified silencing suppressor proteins may act at different steps in RNA silencing pathways. The potyvirus HcPro from different viral origins have been shown to affects the small RNA pathways at initiation, maintenance and signaling steps by interfering with Dicer, RISC or via direct binding with small RNAs. The 2b protein of cucumoviruses prevents the various steps of RNA silencing and regulates various classes of small RNAs differentially, in a strain specific manner. In geminiviruses, AC4 have been shown to be the most heterogenous protein which is highly variable in sequence and length and is not present in all the geminiviruses. Interestingly the activity of some of the geminiviral AC4 protein in RNA silencing indicates its uniqueness in each geminivirus.

To elucidate such a complex view, three suppressor proteins of diverse origin were studied here to characterize their individual and synergistic role in plant gene regulation. Various experiments were carried out to establish their role in RNA silencing pathways.

Geminiviral AC4

AC4 proteins of some of the begomoviruses have been reported to have potent suppressor activity, hence it is important to characterize AC4 of *Papaya leaf curl virus* (PLCV), which is an important begomovirus affecting papaya production in India and worldwide. Papaya plants infected with PLCV, showed symptoms that varied from mild to severe leaf curling, swelling of leaves, vein clearing, stunting, yellowing and profuse branching. It is a whitefly transmitted disease and the infected leaves under electron microscope revealed the association of twinned icosahedral particles characteristic of geminiviruses. The genome of these viruses is divided into two components defined as DNA-A and DNA-B, and both are required for infectivity of plants. The DNA- A component of bipartite genome contains six genes, AC1, AC2, AC3, AC4, AV1 and AV2. The suppressor protein AC4 is encoded by out of frame overlapping gene which is thought to be created by overprinting, in which an existing coding sequence of AC1 is translated in a different reading frame **[Fig.4]**.

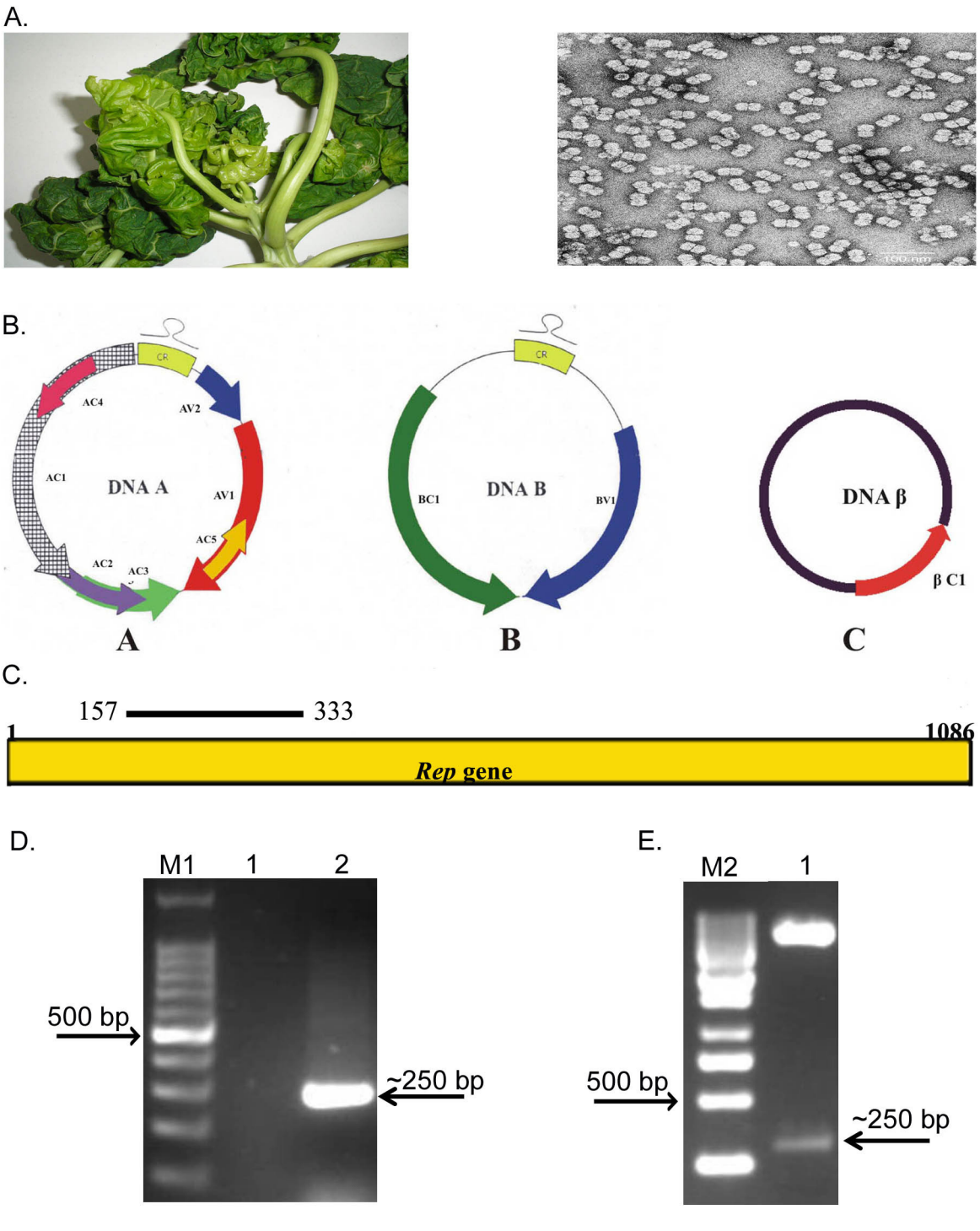


Fig.4 (A) *Papaya leafcurl virus* (PLCV) infected papaya leaf and its particles under Electron microscope. **(B)** Genome of begomoviruses. **(C)** *rep* gene (AC1) showing embeded ORF AC4 encoding putative viral suppressor protein. Numbers indicate the region of ac4 gene (157-333) within the AC1 ORF (1-1086). **(D)** Electrophoresis of an amplicon using specific primers for *ac4* gene from, Lane 1: healthy and Lane 2:PLCV infected papaya leaf against 100 bp DNA marker (M1). **(E)** *Not*I digested product from pGEMT clone containing *ac4* gene (Lane 1), Lane M2: 1Kb DNA marker.

Amplification and cloning of PLCV-ac4

An amplicon of ~250 bp corresponding to *ac4* region was obtained with DNA template of ToLCV infected plant while the healthy plant did not show any amplification indicating the viral origin of the amplicon **[Fig.4]**. The PCR purified amplicon (~ 250 bp) was then cloned in pGEM-T Easy vector. Sixty white colonies were found in X-gal, IPTG, ampicillin plate. All these colonies were streaked on master plates separately. From the master plates, ten colonies were subjected to colony PCR using the specific primers described earlier. On the basis of the colony PCR results, pGEMT-*ac4* clone no. 1, 2, 7 and 10 were found to be positive for the presence of ~250 bp band. Among these clones clone no. 1 was picked from the master plate and inoculated for recombinant plasmid isolation. The viral insert was released from the recombinant plasmid using *Not* I restriction enzyme. Recombinant plasmid DNA from positive colony was digested with *Not* I, and then subjected to electrophoresis. Positive clone released ~250 bp band, the expected length of *ac4* gene on restriction **[Fig.4]**.

Sequence analysis of PLCV-AC4

One of the clone (pGEMT-*ac4*-1) was sequenced to get the nucleotide sequences of *ac4*. The raw sequence obtained was analyzed using BioEdit Sequence Alignment Editor (version 5.0.9) to get the open reading frame of the gene, which was found to contain 177 nucleotides with G+C content of 45.20%, starting from ATG and terminated by TAG coding for 58 amino acid starting from methionine and ending at phenyalanine. On the BLAST analysis it showed maximum homology with AC4 of *Tomato leaf curl* New Delhi *virus*. The AC4 ORF sequence was submitted to GenBank (Accession number: EU006071). Protein was found to be rich in serine and threonine amino acids. We compiled a set of sequences of the AC4 protein of different geminiviruses from NCBI database and were analyzed using CLUSTAL W. AC4 protein was found to be highly variable in terms of sequence as well as its length [**Table 4]**, although some of the signature sequences like ^{3}MG, ^{6}L, ^{11}S, ^{14}S, ^{26}SS, ^{31}P, ^{35}QHIS, ^{40}I, ^{42}T and ^{46}L [**Fig.5]** are found to be conserved in AC4 from different geminiviral origin. On the other hand the AC4 protein from PLCV showed merely 28.5-30% amino acid similarity with the AC4 protein of leaf curl infected various hosts cucumber, chilly and tobacco.

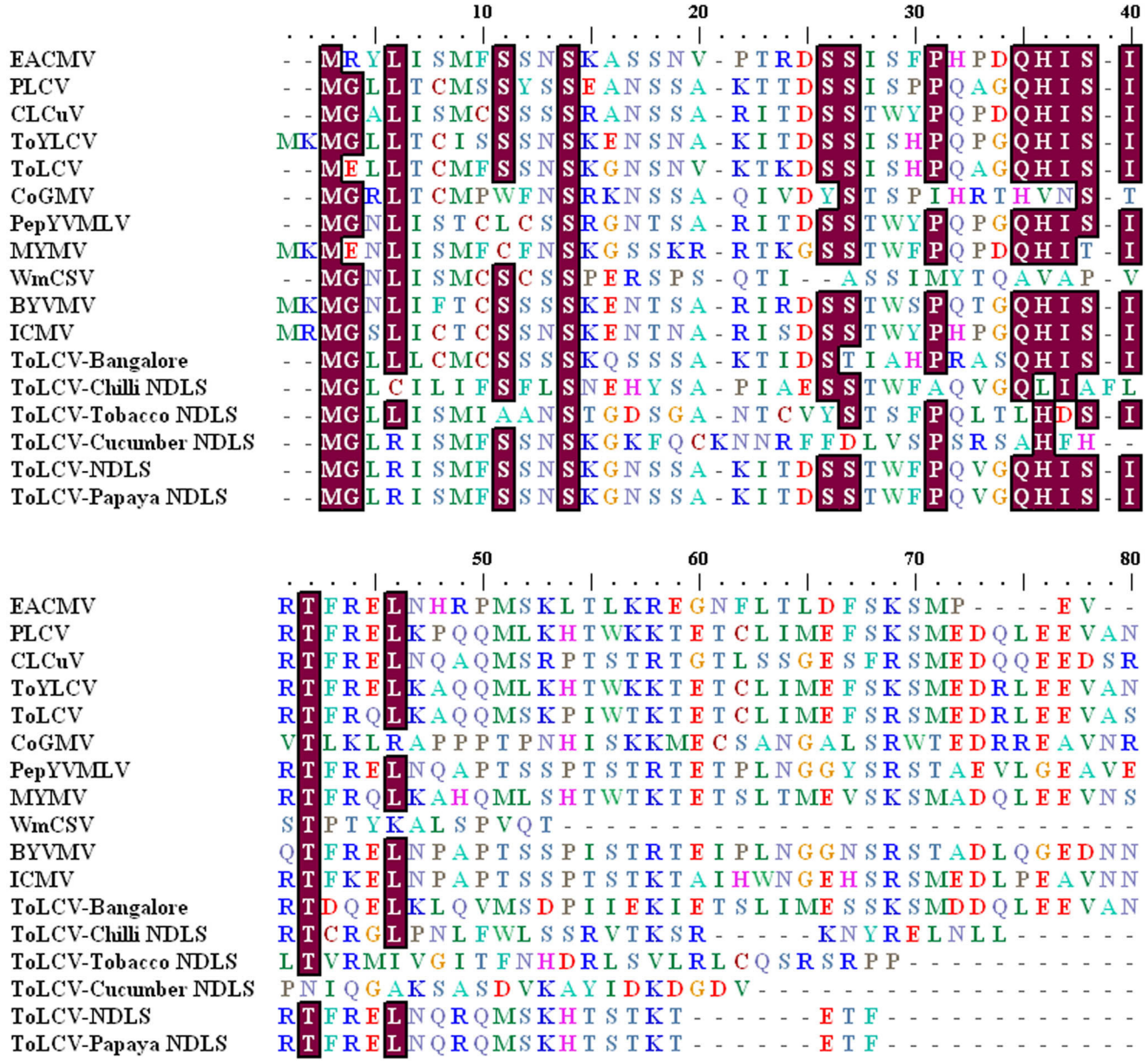


Fig.5 Amino acid sequence alignment of AC4 protein of different geminiviruses, conserved amino acid residues are highlighted . N terminal of protein showed more conservedness than the C terminal.

Construction of ac4 binary vector

To establish the role of PLCV-AC4 in small RNA metabolism, it was constitutively expressed *in planta*. For this a binary construct of PLCV-*ac4* was developed. The pGEMT-*ac4* clone was restricted with *Not*1 enzyme to release the gene. The fragment released having size of ~250 nucleotides was gel purified. The pUC118 vector was linearized with *Not* 1 restriction enzyme. The gel purified fragment containing the *ac4* gene released from the pGEMT-*ac4* clone was cloned in the pUC118 vector. Ten colonies were randomly picked for colony PCR. Out of ten colonies only four colonies were found to be positive showing an amplicon of ~250 bp. Plasmid DNA from the positive colonies designated as pUC118-*ac4* was restricted with *Not* 1 enzyme to release the ~250 bp. It was again reconfirmed by restriction with *Bam*H1 and *Hin*dIII enzymes, to release the whole cassette (CaMV 35S P + *ac4* gene + CaMV 35S ter), as one fragment of ~ 1 kb (the expected size). The presence as well as orientation in the above clone was confirmed by sequencing. The released ~ 1 kb fragment carrying the cassette was gel eluted and cloned in binary vector pCAMBIA 2301 which was linearised by double digestion with *Bam*HI and *Hin*dIII enzymes. The clones obtained in kanamycin selection plate were then screened for recombinants by colony PCR using specific primers. Plasmid DNA from positive colonies were then restricted with *Bam*HI and *Hin*dIII to confirm the release of a cassette **[Fig. 6]**.

Mobilization of ac4 construct into Agrobacterium

Recombinant plasmid carrying the *ac4* gene construct was mobilized into *Agrobacterium* strain LBA4404 by freeze and thaw method. Ten colonies, obtained on kanamycin and streptomycin selection plate were screened by colony PCR using specific primers. Four out of ten colonies screened were found to be positive in colony PCR. These positive clones were used for *Agrobacterium* mediated plant transformation.

Transformation of ac4 gene constructs in three different Solanaceous hosts

A total of 80 cotyledonary leaf explants of each, *Nicotiana tabacum, Nicotiana benthamiana* and *Solanum lycopersicon* were co-cultivated with *Agrobacterium* carrying *ac4* construct. Also 25 cotyledonary leaf explants were kept for regeneration as a negative control 52, 45 and 48 co-cultivated explants were selected on kanamycin

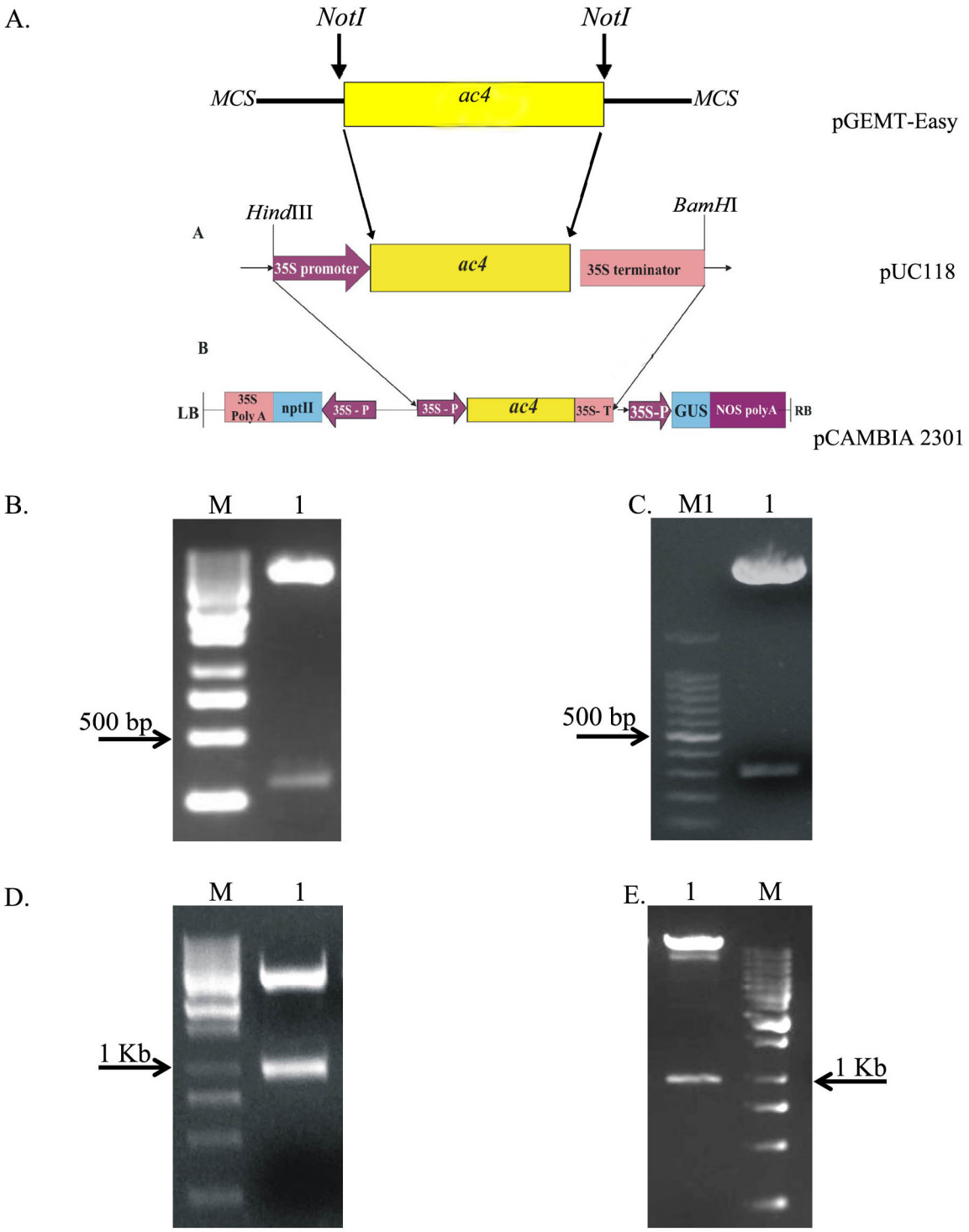


Fig.6 (A) Schematic picture showing the development of *ac4* construct.**(B)**Electrophoresis of the *Not*I digested pGEMT clone having *ac4* gene (Lane1).**(C)** pUC118 clone having *ac4* gene (Lane 1). **(D)** *BamH*I and *Hind*III digested pUC118 clone showing released cassette (~1Kb) containing 35S promoter, terminator and *ac4* gene (Lane 1). **(E)** *BamH*I and *Hind*III digested pCAMBIA 2301 clone showing released cassette containing 35S promoter, terminator and *ac4* gene (Lane 1). Lane M: 1 Kb DNA marker and Lane M1: 100 bp DNA marker.

leading to the formation of 20, 22 and 18 calli for each *N. tabacum, N. benthamiana and S. Lycopersicon* respectively, ready for organogenesis. Shoot induction was observed after 10-15 days on shooting medium with kanamycin followed by rooting for another 10-15 days in rooting medium. Molecular analysis of transformants was done using RT-PCR to confirm the transgene insertion and its expression. The transformants selected in kanamycin (50mg/l), were established by RT-PCR and transgenic lines were designated as AC-1 to AC-4 (*S. lycopersicon*) and AC-benth (*N. benthamiana*) and AC-tob (*N. tabacum*) **[Fig.7]**.

The phenotypes observed in the three different transgenic plants expressing the AC4 varied between the plant species. In the *S. lycopersicon* lines, the stems were strongly malformed causing a bending and twisting. Leaves were strongly narrow and needle shaped. Apical apoptosis were the most severe effects observed in *Solanum lycopersicon* due to expression of AC4 **[Fig.7]**. All transgenic lines in *N. benthamiana* exhibited very similar phenotypes causing to unregulated differentiation, severe stunting and leaves malformations. Similarly, *N. tabacum* lines also showed very similar phenotypes though different from *N. benthamiana*. AC4 expressing lines of *N. tabacum* showed stunting and malformed leaves with blisters. Representative AC4 expressing lines of both the *Nicotiana* spp are shown in **Fig.7.**

Cucumoviral 2b

2b proteins of the **cucumoviruses** have been reported to have potent suppressor activity in a strain specific manner, hence it is important to characterize 2b of *Cucumber mosaic virus* (CMV), which is an important **cucumovirus** affecting broad range of hosts including cucurbits and solanaceous crops. Tobacco plants infected with CMV, showed symptoms that varied from mild to severe leaf mosaic and yellowing. It is an aphid transmitted disease and the infected leaves under electron microscope revealed the association of icosahedral particles characteristic of **cucumoviruses**. The genome of these viruses is multipartite defined as RNA1-5. The RNA-2 component of multipartite genome contains two genes, *2a* and *2b*. The suppressor protein 2b is encoded by out of frame overlapping gene which is thought to be created by overprinting, in which an

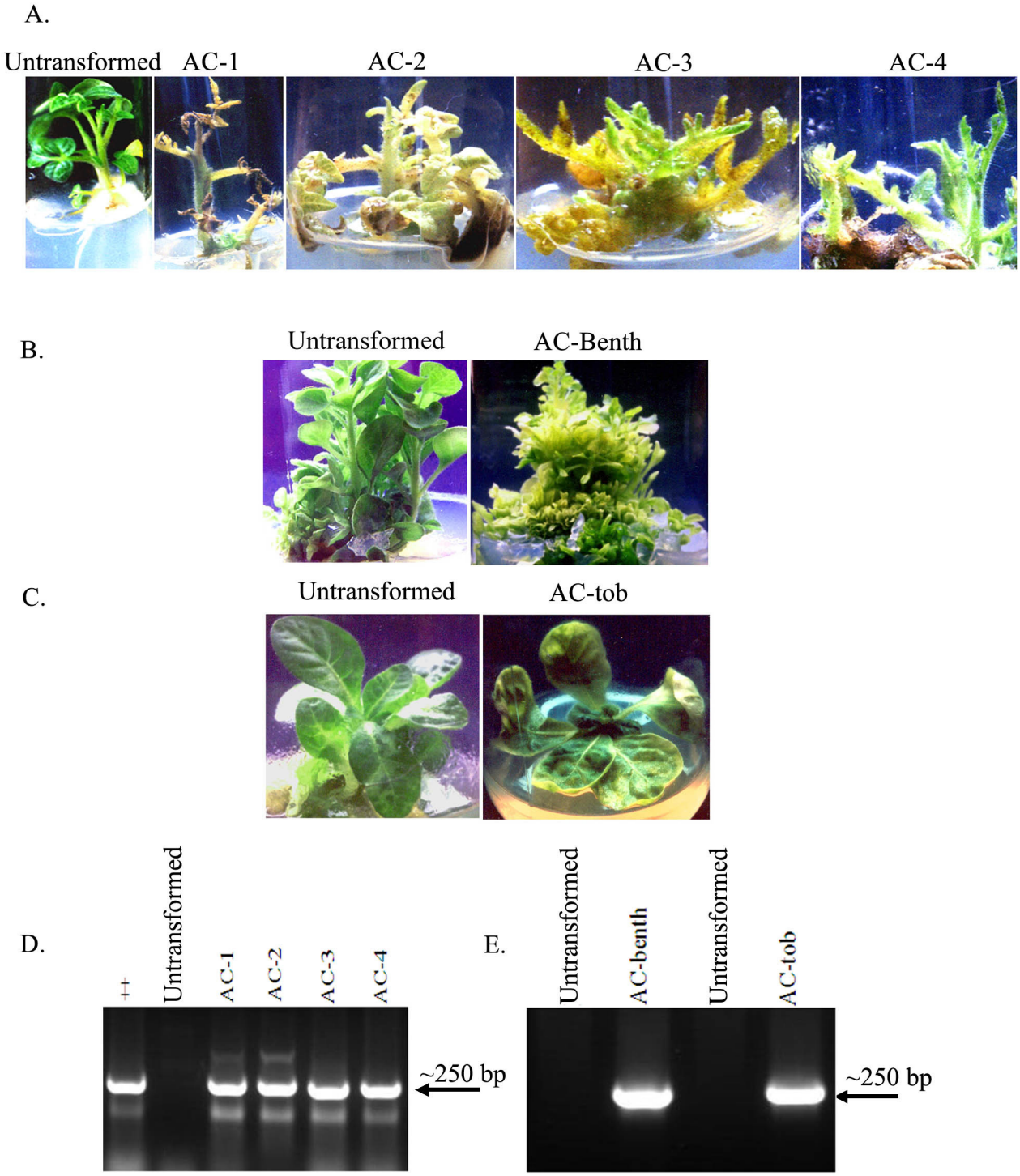


Fig.7 Phenotypic aberrations induced by constitutive expression of viral suppressor of RNA silencing AC4 in **(A)** *S.lycopersicon*, **(B)** *N.benthamiana* and **(C)** *N.tabacum* showing its interference in small RNA pathways. **(D & E)** Transgene confirmation of *ac4* transgenic lines through RT-PCR using gene specific primers showing the expression of gene in plant system.

existing coding sequence of 2a is translated in a different reading frame, having role in movement **[Fig.8]**.

***Amplification and cloning of** CMV-**2b** New Delhi isolate (CMV-NDLS)*

An amplicon of ~350 bp corresponding to *2b* gene was obtained with RNA template of CMV infected tobacco plant while the healthy plant did not show any amplification indicating the viral origin of the amplicon **[Fig.8]**. The PCR purified amplicon (~350 bp) was then cloned in pGEM-T Easy vector. Successful clones of the *2b* insert in the pGEM-T Easy vectors was identified by colour screening on indicator plates containing ampicillin, X-gal and IPTG. Around eighty white colonies were found in X-gal, IPTG, and ampicillin plate. They were streaked on master plates separately and ten representative colonies among them were screened for the presence of insert of ~350 bp by performing colony PCR using specific primers described in Appendix I. On the basis of the colony PCR results, pGEMT-*2b* clone no. 4,6,7,9 and 10 were found to be positive for the presence of ~350 bp *2b* insert. Among them, clone no. 6 (pGEMT –*2b*-6 clone) was picked from the master plate and subjected for restriction enzyme digestion to confirm the presence of the *2b* insert. Electrophoresis of the restriction enzyme digested mixture found that pGEMT-T-*2b*-6-clone released ~350 bp insert, the expected length of *2b* **[Fig.8]**.

Sequence analysis of** CMV-**2b

One of the clone (pGEMT-*2b*-6) was sequenced to get the nucleotide sequences of *2b*. The raw sequence obtained was analyzed using BioEdit Sequence Alignment Editor (version 5.0.9) to get the open reading frame of the gene, which was found to contain 336 nucleotides with G+C content of 50%, starting from ATG and terminated by TAG coding for 111 amino acid starting from methionine and ending at phenyalanine. On the BLAST analysis it showed homology with 2b protein of different strains of *Cucumber mosaic vius.* The *2b* gene sequence was submitted to GenBank (Accession number: **EU006067**). Protein was found to be rich in glutamate and arginine amino acids. We compiled a set of 2b protein sequences of different CMV strains covering both the subgroups (I & II), from NCBI database. There are many strains and isolates of CMV which can be grouped into two subgroups and subgroup I strains may in general be more

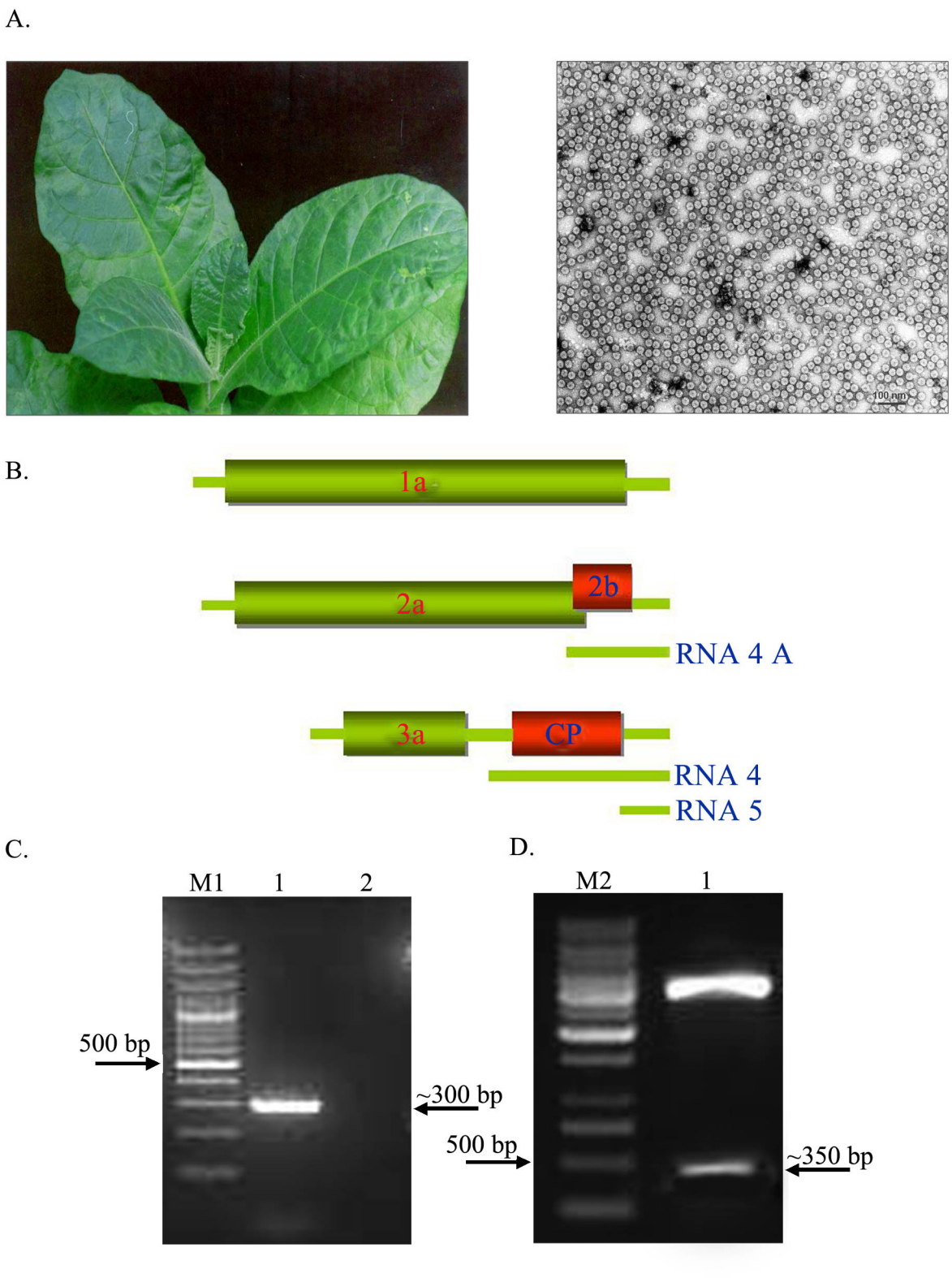


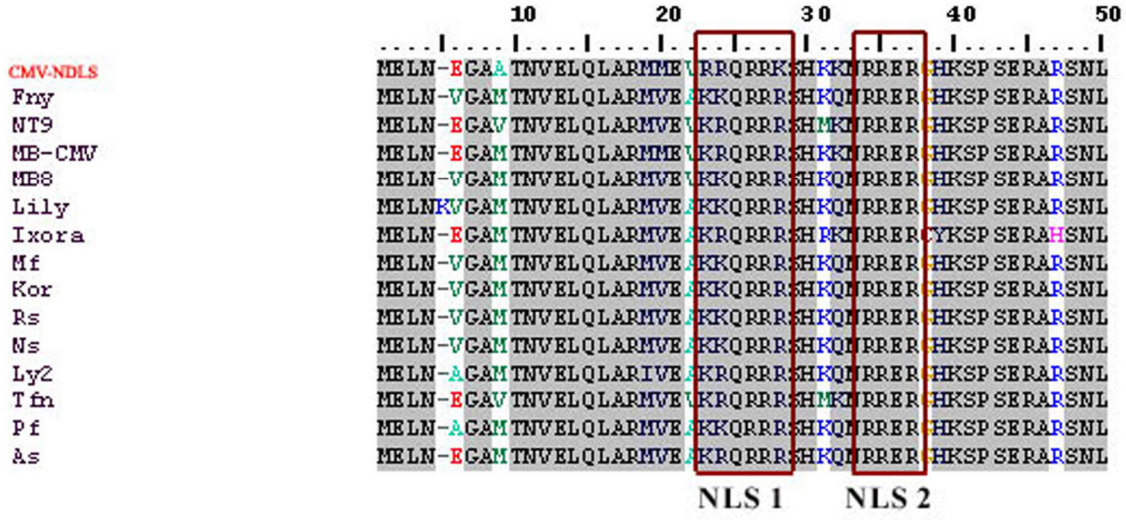
Fig.8 (A) *Cucumber mosaic virus* (CMV) infected tobacco leaf showing mosaic and its particles under Electron microscope. **(B)** Genome of CMV showing different genes organization. **(C)** Electrophoresis of an amplicon using specific primers for *2b* gene from healthy (Lane 2) and CMV infected tobacco leaf (Lane1) against 100 bp DNA marker (M1). **(D)** *NotI* digested product from pGEMT clone containing *2b* gene (Lane 1), Lane M2: 1Kb DNA marker.

virulent than subgroup II strains. A nuclear localizing signal ^{22}RRQRRK^{27} was found to be present in 2b protein from CMV New Delhi isolate (CMV-NDLS) when multiple alignments were performed with the subgroup I and subgroup II strains of CMV **[Fig.9]**. A second putative nuclear localizing signal motif (NLS 2) ^{33}RRER^{36} was predicted in Delhi CMV 2b protein with the help of online predict NLS server (http://cubic.bioc.columbia.edu/services/predictNLS) and clustal analysis with subgroup I strains **[Fig.9]**. The NJ tree for the aligned 2b protein sequences showed that there are two distinct grouping: one included the subgroup II strains of CMV and the other included CMV (New Delhi isolate) 2b along with other subgroup I CMV strains **[Fig.10]**. Presence of two NLS sequences and its clustering with strains belonging to subgroup I suggested that CMV (New Delhi isolate) is a severe strain of CMV belonging to subgroup I.

CMV-2b binary vector constructs

Sense and antisense constructs were designed by cloning the *2b* ORF sequences in to binary vector pCAMBIA 2301, which is shown schematically in **Fig.11**. The gel purified fragment containing the *2b* insert released from the pGEMT- *2b*-6 clone was cloned in the pUC118 vector in sense and antisense orientation. The gel purified fragment containing the *2b* insert released from the pGEMT-*2b*-6 with *Apa*I/*Xho*I and *Not*I was cloned in the pUC118 vector to get the sense and antisense constructs separately. Twenty five colonies were randomly picked for colony PCR from ampicillin plates as the vector carries ampicillin resistance marker gene. Out of these colonies seven colonies were found to be positive showing an amplicon of ~336Kb. Plasmid DNA from the positive colony designated as pUC118-*2b* was restricted with *Apa*I/*Xho*I and *Not*I enzymes to release the ~336Kb. It was again reconfirmed by restriction with *Bam*H1 and *Hin*dIII enzymes, as the positive clone released the whole cassette (CaMV 35S Promoter + *2b* gene + CaMV 35S terminator), as one fragment of ~ 900 bp. The presence as well as orientation of the *2b* gene in the above clone was confirmed by sequencing. The released ~ 900 bp fragment carrying the cassette was gel eluted and sub-cloned in binary vector pCAMBIA 2301 which was linearised by double digestion with *Bam*HI and *Hin*dIII enzymes. The 18 colonies obtained in kanamycin selection plate were then screened for recombinants by colony PCR using specific primers. Eight

A.



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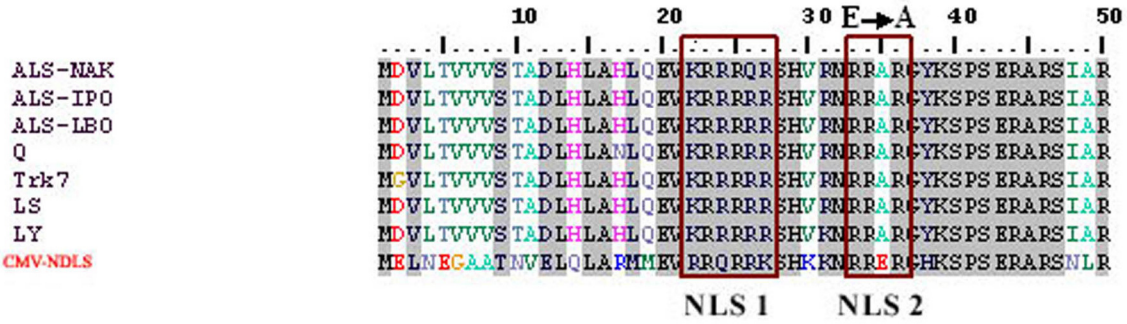


Fig.9 Amino acid sequence alignment of 2b protein of CMV strains of **(A)** Subgroup I (B) Subgroup II. Consensus amino acid residues of two putative NLSs are highlighted in rectangle boxes.

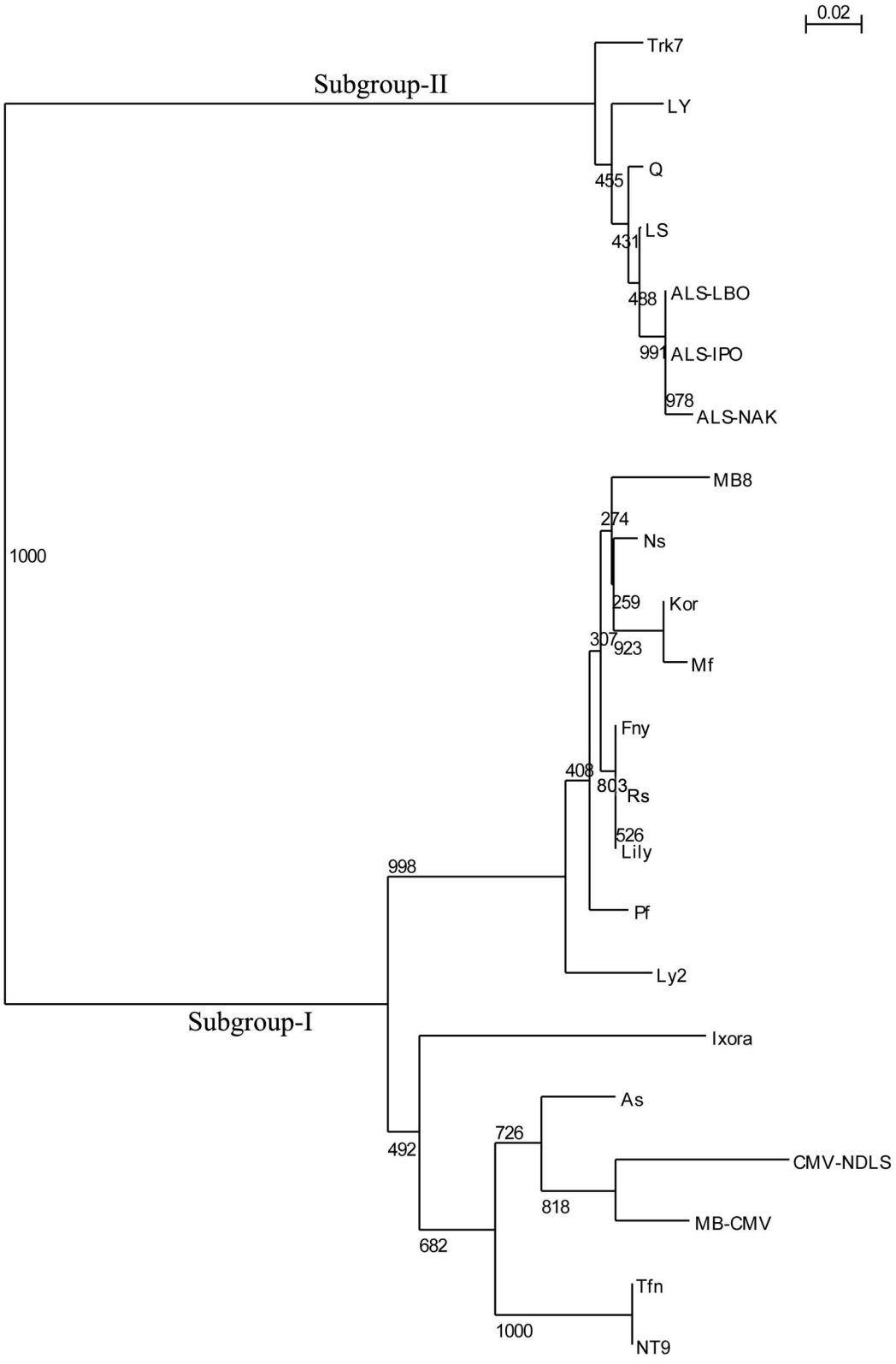


Fig.10 Phylogenetic tree for the aligned 2b protein sequences of the CMV strains of both the subgroups.CMV-NDLS clustered with CMV strains belonging to subgroup-I.

colonies were positive for the presence of *2b* insert as shown by the amplicon of ~900 bp from 35S promoter and terminator primers. Out of eight colonies, three were in sense (pCAMBIA 2301-*2b*) and five in antisense (pCAMBIA 2301-anti*2b*) orientation. Plasmid DNA from positive colonies was then restricted with *Bam*HI and *Hin*dIII to confirm the release of a cassette. The positive colonies (pCAMBIA 2301-*2b* and pCAMBIA 2301-anti*2b*) released ~ 900 bp fragment on restriction. In these constructs expression of CMV-2b is under the control of the CaMV 35S constitutive promoter. The vectors pCAMBIA 2301-*2b* and pCAMBIA 2301-anti*2b* carries only the region encoding 2b **[Fig.11]**.

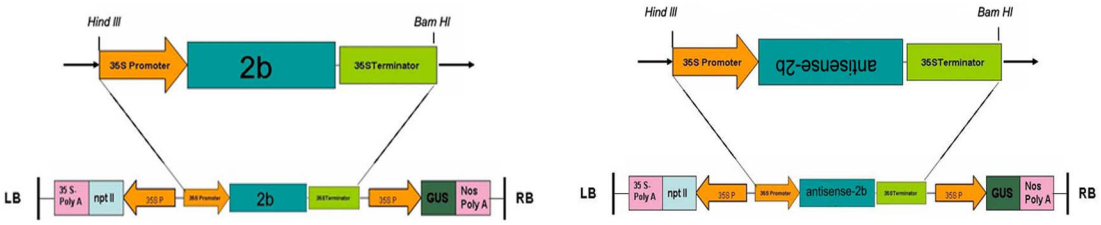
Mobilization of 2b constructs into Agrobacterium

Recombinant binary plasmids carrying *2b* sense and antisense gene constructs were mobilized into *Agrobacterium tumefaciens* strain LBA 4404 by freeze and thaw method. Number of colonies obtained on kanamycin and streptomycin selection plate were 11 for plasmid pCAMBIA 2301-*2b* and nine for pCAMBIA 2301-anti*2b*. Upon screening by colony PCR 1, 3, 8, and 11 *Agrobacterium* colonies were positive for the presence of pCAMBIA 2301-*2b,* while 7 and 9 *Agrobacterium* colonies were positive for the presence of pCAMBIA 2301-anti*2b* by showing the respective size amplicons.

Behaviour of CMV-2b over expression and silencing in absence and presence of virus

The 2b ORF from **CMV-NDLS** genome was expressed under the transcriptional control of CaMV 35S promoter in healthy *N. tabacum*. Molecular analysis of transformants was done using RT-PCR to confirm the transgene insertion. Out of the 24 transformants selected in kanamycin (50mg/L), about 4 putative transgenics were established using RT-PCR and these were designated as 2b-1 to 2b-4. Regenerated non transformed healthy tobacco plants of the same age were taken as 'Healthy' to compare the effects of constitutive expression of *2b* gene in plant phenotypes. The transgenic tobacco lines exacerbated abnormal morphological characteristics such as unregulated differentiation, chlorosis, apical apoptosis and underdeveloped leaf lamina**.** Minimum 10 non transformed healthy tobacco plants were analyzed to rule out the possibility of effect of somaclonal variation or tissue culture media in plant phenotypes. Here it is demonstrated that the phenotypic aberrations are observed on constitutive expression of

A.



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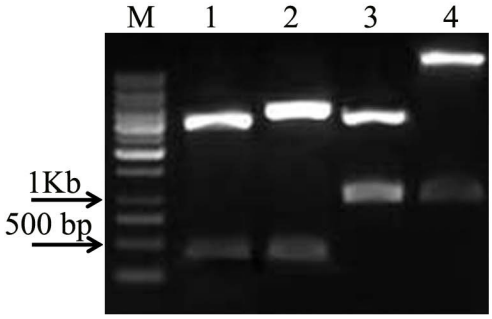


Fig.11 (A) Diagramatic representation of binary vector 2b sense and antisense constructs. Sense and antisense 2b genes were cloned under the 35 S promoter for constitutive expression. **(B)** Electrophoresis of *Not*I digested pGEMT-2b clone (Lane 1), *Not*I digested pUC118-2b clone (Lane 2), *Bam*HI & *Hind*III digested pUC118-2b clone releasing cassette containing 35S Promoter, terminator and *2b* gene (Lane 3), *Bam*HI & *Hind*III digested pCAMBIA 2301-2b clone releasing cassette containing 35S Promoter, terminator and *2b* gene (Lane 4)Lane M:1Kb DNA marker.

silencing suppressor 2b, which might be diverting its role in plant miRNA metabolism in the absence of virus.

To determine the role of CMV-NDLS, *2b* gene was over expressed in *N. tabacum* in presence of virus. In this approach, virus infected *N. tabacum* calli were transformed with *2b* sense constructs under the 35S constitutive promoter. The transgene insertion was confirmed by PCR from genomic DNA in order to rule out the possibility of *2b* amplification from CMV genome which is an RNA molecule. PCR established transgenics were designated as 2b-I. Non transformed tobacco plants regenerated from the CMV infected explants of the same age were taken as 'Control' to observe the effects of over expression of *2b* gene in plant phenotypes in the presence of virus. Ten lines from each, control and transgenic were analyzed to study the effect of 2b over expression in phenotypes of CMV infected tobacco. In contrast to the phenotypic aberrations observed on constitutive expression of 2b in healthy tobacco, phenotypic aberrations were found to be negligible in *2b* transformed CMV infected tobacco as shown in their representative lines. Here it is demonstrated that the phenotypic aberrations in CMV infected tobacco are not severe upon constitutive expression of silencing suppressor 2b, which might be due to its primary role in suppression of siRNAs derived from viral genome **[Fig.12]**.

Effect of up and down regulation of 2b in CMV accumulation

To understand the role of CMV-NDLS in virus accumulation, 2b protein was constitutively expressed and silenced (in a separate experiment) in presence of virus. In this approach, virus infected *N. tabacum* calli were transformed with *2b* sense and antisense gene constructs separately. The transgene insertion was confirmed by PCR from genomic DNA. Antisense transgenics were designated as anti 2b-I. Generation of sense transgenics have already been discussed. Regenerated plants from CMV infected explants of the same age were taken as 'Control' to study the effect of 2b over and under expression in change in CMV accumulation. Minimum 10 transgenic lines from each, sense and antisense, and similar number of regenerated control plants were analyzed and morphological characteristics of each representative line is shown in **Fig.12**. No significant difference was observed in virus titer as determined by ELISA**.** The mean

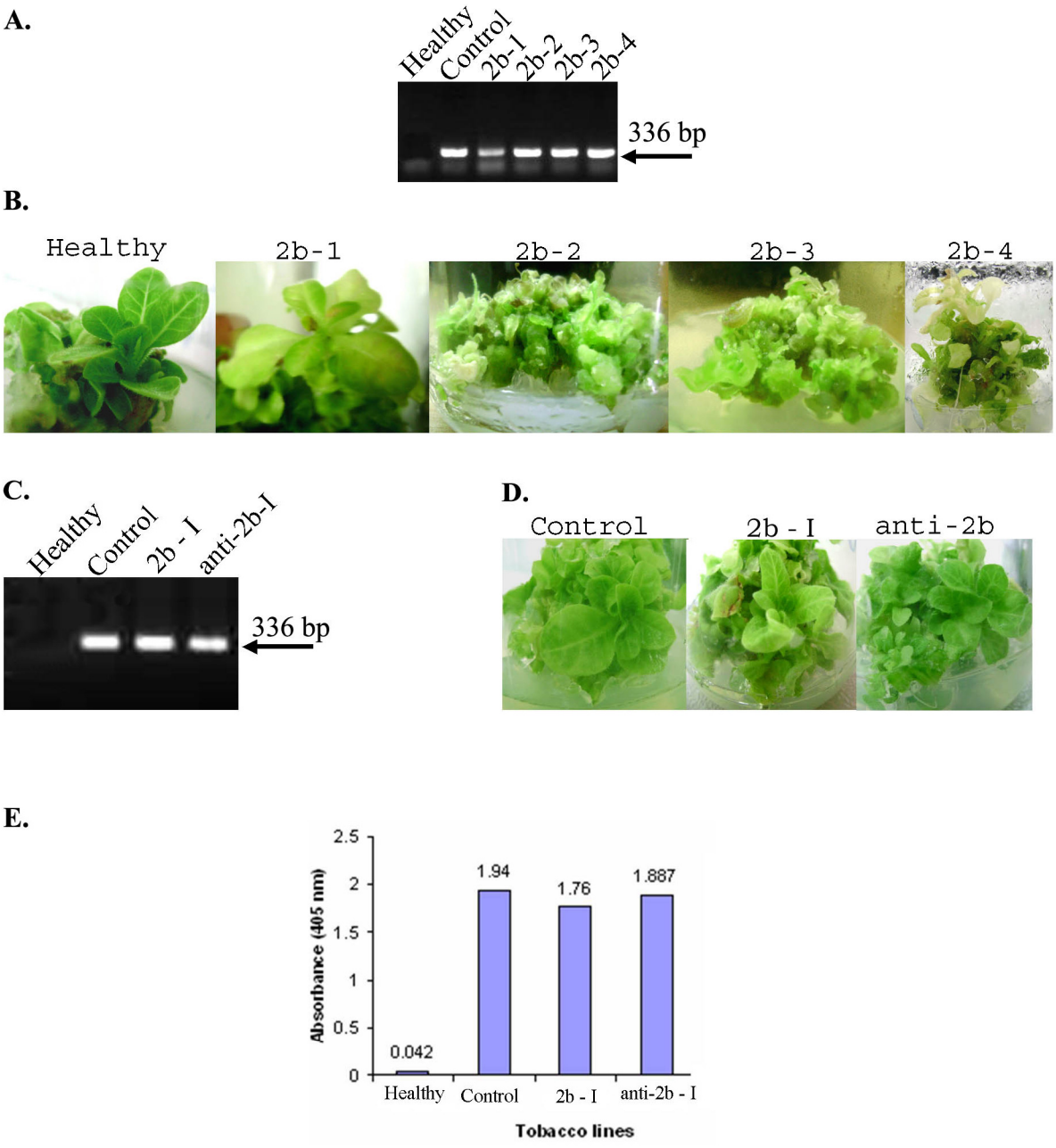


Fig.12 (A) Transgene confirmation of healthy tobacco *2b* transgenic lines through RT-PCR using gene specific primers. **(B)** Morphological phenotypes induced by CMV-2b in healthy tobacco transgenic lines. **(C)** Transgene confirmation of CMV infected tobacco *2b* (sense and antisense) transgenic lines through PCR using gene specific primers. **(D)** Morphological phenotypes induced by 2b over expression and under expression in CMV infected tobacco transgenic lines. **(E)** CMV quantification of CMV infected 2b (sense and antisense) transgenic lines by DAC-ELISA.

absorbance value of all the 10 plants was employed to study the effect of 2b suppressor in CMV accumulation **[Fig.12]**.

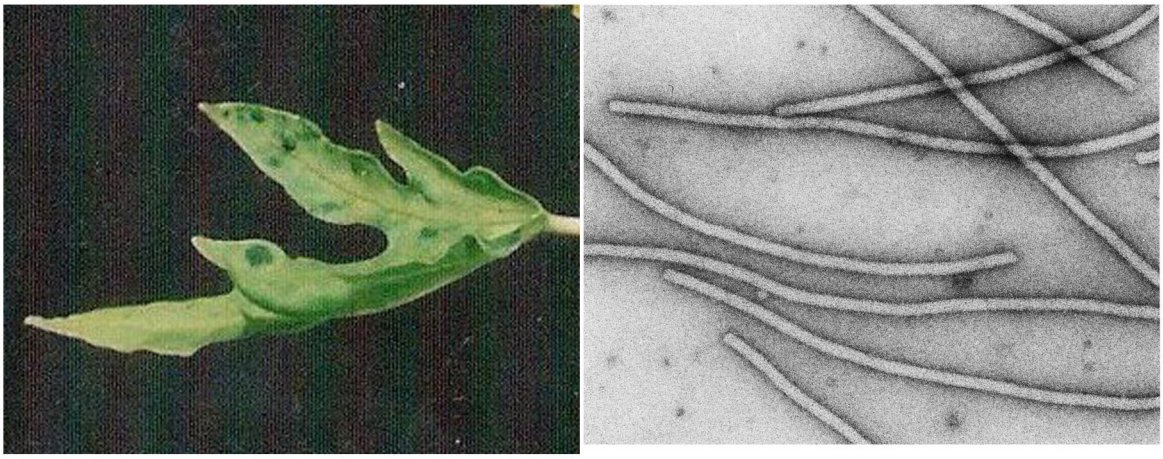
Potyviral HcPro

HcPro protein of the potyviruses have been reported to have potent suppressor activity, hence it is important to characterize HcPro of *Papaya ringspot virus* (PRSV), which is an important potyvirus affecting to papaya production in India and worldwide. Papaya plants infected with PRSV, showed symptoms that varied from mild to severe mosaic, oily and water soaked streaks on petiole, ring spots on fruit, shoe string, blistering. It is an aphid transmitted disease and the infected leaves under electron microscope revealed the association of flexuous particles characteristic of potyviruses. The genome of these viruses is a single stranded RNA of positive polarity containing a single ORF translated into large polyprotein which is further cleaved to produce individual protein products **[Fig.13].** HcPro is one of the important genomic component of potyviruses. It is the first identified and most studied suppressor of RNA silencing. It is a multifunctional protein: as a strictly cis-acting proteinase, it is responsible for its self-cleavage from the polyprotein precursor. It is also involved in a number of infectious processes as diverse as aphid transmission, cell-to-cell and long-distance movement and genome amplification. The highly orchestrated processes accomplished by HcPro may be regulated at different levels. HcPro also interacts with various host proteins, including a calmodulin related protein involved in gene silencing. HcPro protein of potyviruses has been reported to have potent RNA silencing suppressor activity also; hence it is important to characterize HcPro of *Papaya ringspot virus* (PRSV), its expression *in planta* and its role in small RNA binding.

Amplification and cloning of PRSV-HcPro

To obtain the complete nucleotide sequence of the PRSV-*HcPro*, molecular cloning of the cDNA by RT-PCR was carried out. An amplicon of ~1.4Kb corresponding to HcPro region was obtained with cDNA template of PRSV infected plant while the healthy plant did not show any amplification indicating the viral origin of the amplicon **[Fig.13]**. The *HcPro* gene was cloned by adopting A-T cloning strategy in pGEM-T Easy (Promega) cloning vector using T4 DNA ligase. Successful clones of the *HcPro* insert in

A.



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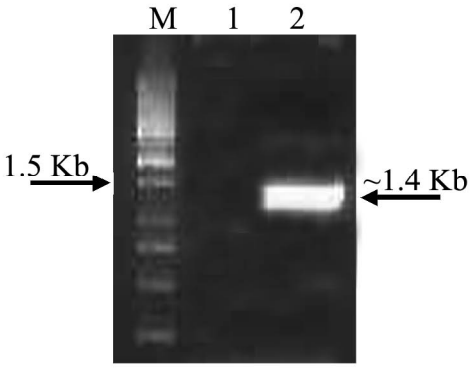
C.



D.

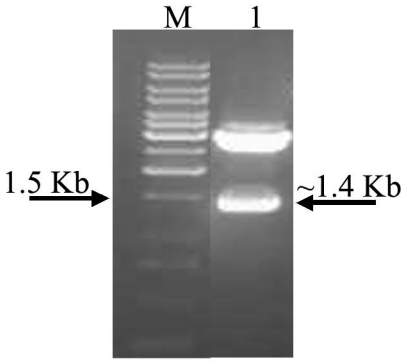


Fig.13 (A) Papaya leaf infected with *Papaya ringspot virus* (PRSV) showing blistering symptoms and its particles under Electron microscope. **(B)** Genome of PRSV showing its genes organization. **(C).** Electrophoresis of *HcPro* PCR product from healthy (Lane 1) and PRSV infected sample (Lane 2) against 1 Kb DNA marker (Lane M) **(D)** Electrophoresis of *Not*I digested pGEMT clone containing *HcPro* (Lane 1) gene against 1 Kb DNA marker (Lane M).

the pGEM-T Easy vectors was identified by colour screening on indicator plates containing ampicillin, X-gal and IPTG, as the vector contains the ampicillin resistance marker gene. Around 50 white colonies were found in X-gal, IPTG, and ampicillin plate. They were streaked on master plates separately and ten representative colonies among them were screened for the presence of insert of ~1.4Kb by performing colony PCR. On the basis of the colony PCR results, pGEMT-*HcPro* clone no. 1,2,4,6,7,8,9 and 10 were found to be positive for the presence of ~1.4Kb *HcPro* insert. Among them, clone no. 7 (pGEMT –*HcPro*-7 clone) was picked from the master plate and subjected for restriction enzyme digestion to confirm the presence of the *HcPro* insert. Electrophoresis of the restriction enzyme digested mixture found that pGEMT –*HcPro*-7 clone released ~1.4Kb insert, the expected length of *HcPro* **[Fig 13]**.

Sequence analysis of PRSV-HcPro

One of the clone (pGEMT-*HcPro*-7) was sequenced to get the nucleotide sequences of HcPro. The raw sequence obtained was analyzed using BioEdit Sequence Alignment Editor (version 5.0.9) to get the correct size of the gene, which was found to contain 1371 nucleotides, with G+C content of 42.67% coding for 457 amino acid starting from asparagine and ending at glycine having deduced MW of 52 kDa and PI of 8.23. On the BLAST analysis it showed close homology with HcPro of various potyviruses. The *HcPro* sequence was submitted to GenBank (Accession number: DQ855428). Protein was found to be rich in leucine, lysine and arginine amino acids. We compiled a set of sequences from the 5' terminal of the potyviral genome taken from NCBI database and restricted analysis to the region from 1724 to 3094 bps relative to the annotated sequences of HcPro. Different regions of the HcPro were analyzed for sequence-function relationship using CLUSTAL W **[Fig.14]**. The 1371 nucleotide of the 5' upstream region of the PRSV-HcPro was analyzed for the presence of various known and unknown regulatory sequence motifs by searching against different databases. The motifs were analyzed separately in the different domains of the helper component proteinase which are represented as follows:

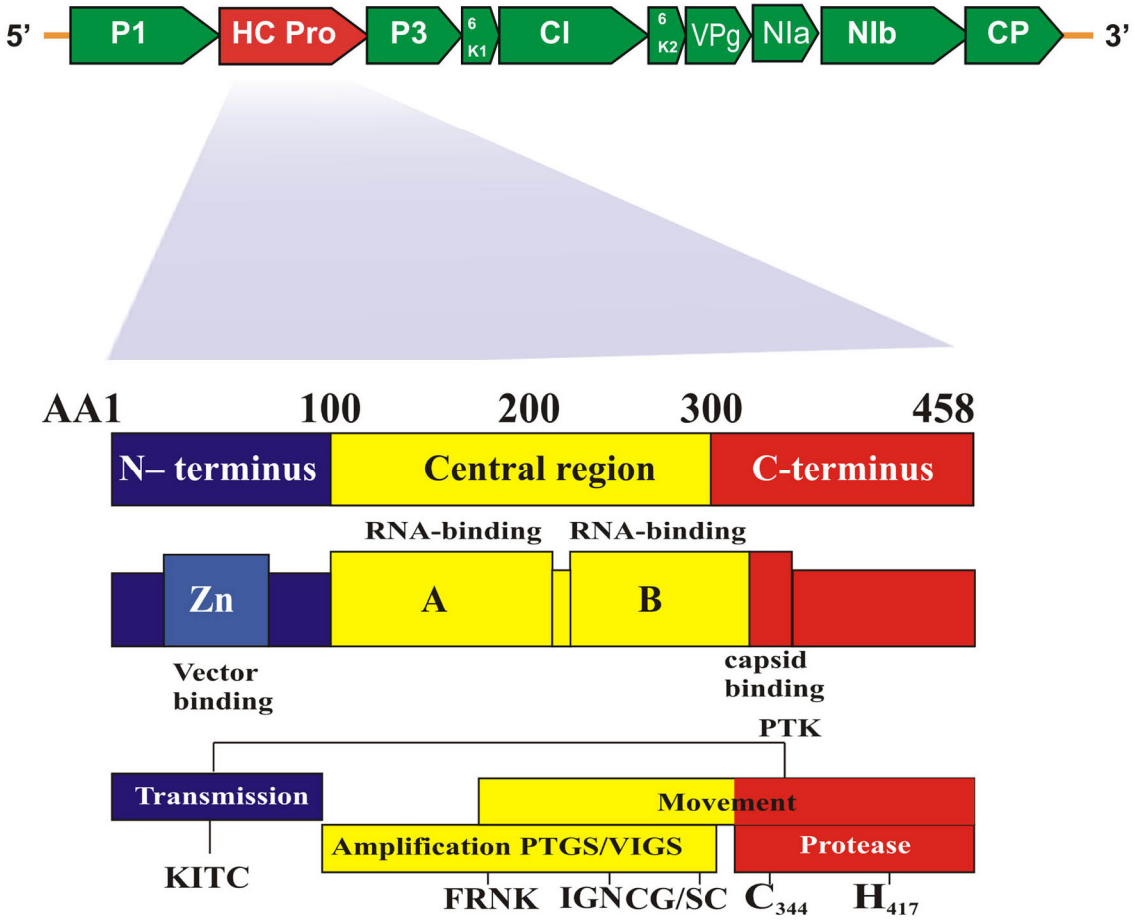


Fig.14 Functional regions of HcPro. HcPro can be divided into three regions; N-terminal is known for its function in transmission, central region for genome amplification and silencing suppression and C-terminal possessing protease activity. Some of the motifs shown are conserved in HcPro protein of different potyviruses.

N terminal – transmission domain

The transmission domain was nearly 100 % conserved among all PRSV isolates whereas only ~18% conserved in comparison with other potyviruses from different hosts **[Fig.15]**. The functional motif $KITC^{54}$ is evolutionary conserved in all the potyviruses having binding affinity to the aphid vector stylets. The other conserved motifs like CG^{36} and $VAAL^{41}$ **[Table.8]** in all potyviruses may have similar function. Beside these, some of the amino acids like H^{24}, C^{26}, C^{57}, F^{91}, H^{93} and L^{98} have shown identical positions in all the potyviruses indicating their probable role in metal binding which is supposed to be a key factor in virus transmission **[Fig.15]**. The presence of putative zinc finger motifs in this region probably has affinity for vector binding. Cross protein conservation in the N-terminal region indicates the presence of domains having affinity for metal binding like Nif D protein having affinity for Mo and Fe, Cytochrome peroxidase having affinity for Fe **[Fig.16]**.

Central region- RNA binding domain

Central region consists of two RNA binding domains. The first RNA binding domain responsible for genome amplification consists of some conserved motifs among all the potyviruses like $FRNK^{183}$, KG^{143} and $CDNQLD^{201}$ **[Fig.17, Table. 8]**. One interesting motif KRT^{169} is found to be conserved in all the PRSVs whereas K is replaced by N in all other potyviruses. This RNA binding domain is comparatively less conserved, indicating its interaction with the diverse potyviral genomes. Conserved domain architectures among different proteins showed its homology with two important protein domains; RP041 domain having role in the activity of RNA polymerase and Nrap domain, which is found to be evolutionary, conserved from yeast to human, playing crucial role in ribosome biogenesis by interacting with pre rRNA primary transcript **[Fig.16].**

The second RNA binding domain-having role in silencing suppression is found to be 60% conserved. The conserved character of this domain can be explained on the basis of the mechanism of viral suppression, indicating binding of small RNA by silencing suppressor is size dependent but not the sequence dependent, as reported for another

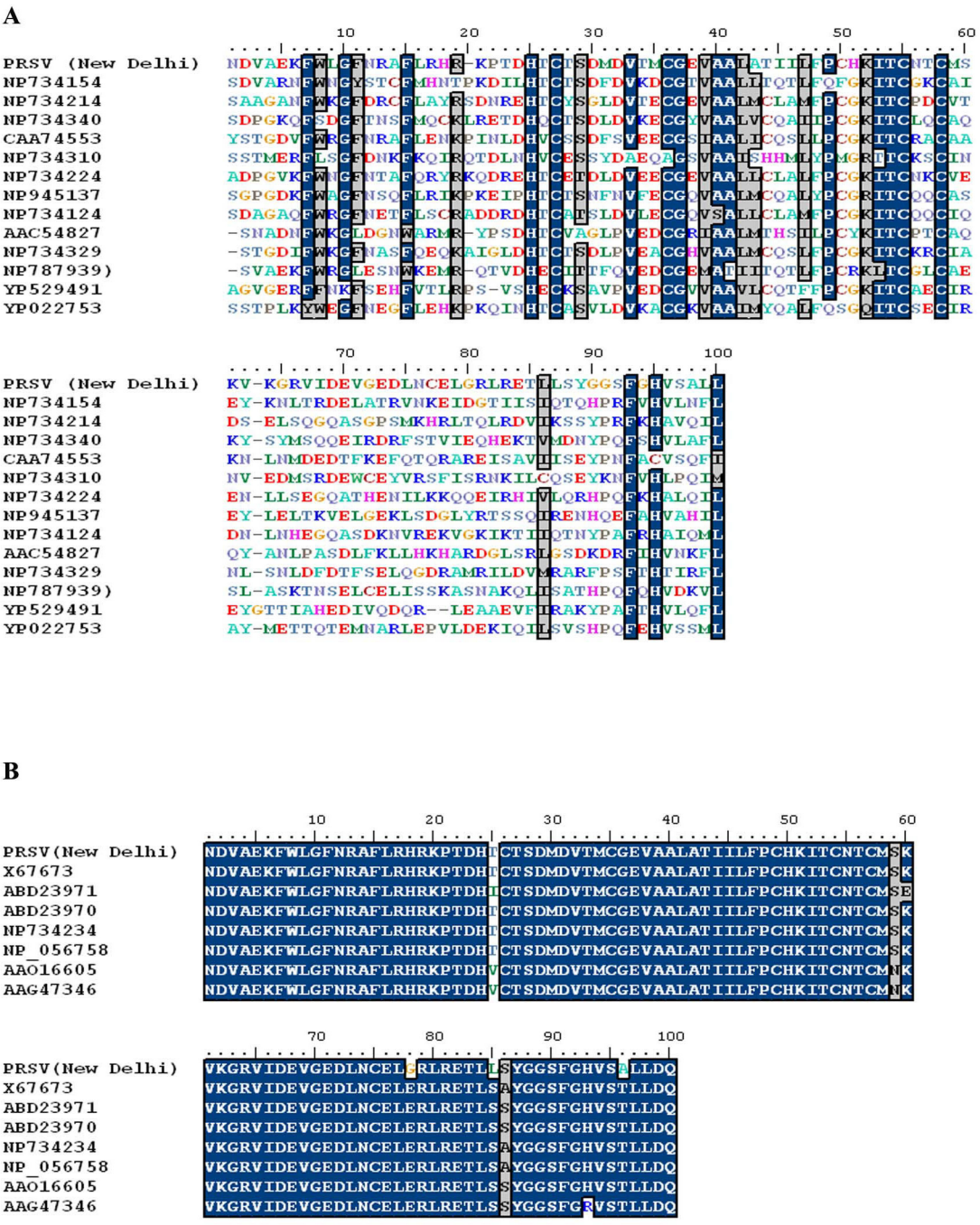
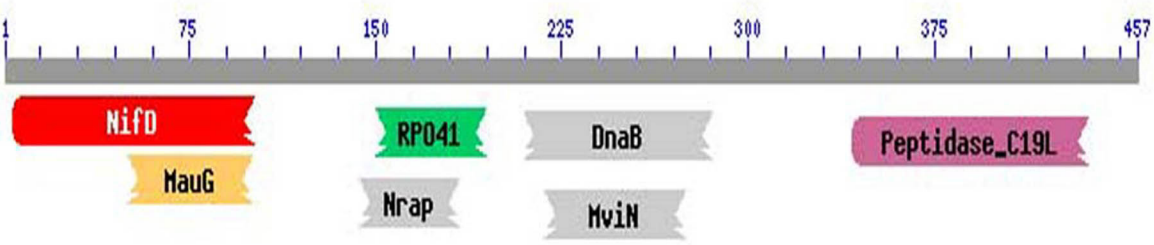


Fig. 15 Comparison of the specific sequences at N-terminal of HcPro. **(A)** Sequence analysis at the N-terminal (1-100 amino acids) of HcPro from different potyviruses. **(B)** Sequence analysis at the N-terminal (1-100 amino acids) of HcPro from different PRSV isolates.

A.



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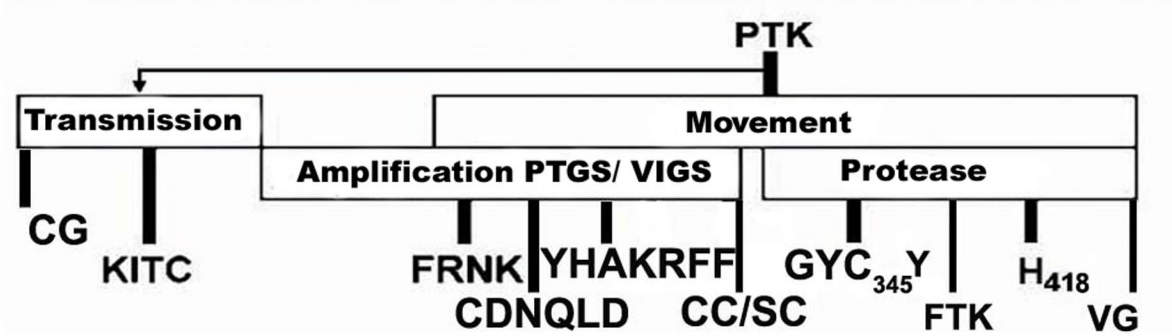


Fig.16 Cross species protein conservation at the three functional regions of HcPro having transmission, genome amplification, PTGS suppression, movement and proteolytic activity. **(A)** The image is generated at the ALL-IN-ONE-SEQ-ANALYZER version 1. 35 browser. Structural relatives of different domains of PRSV-HcPro are shown by cross protein conservation analysis **(B)** Specific conserved sequence motifs in three functional regions of HcPro responsible for crucial functions of the multifunctional protein.

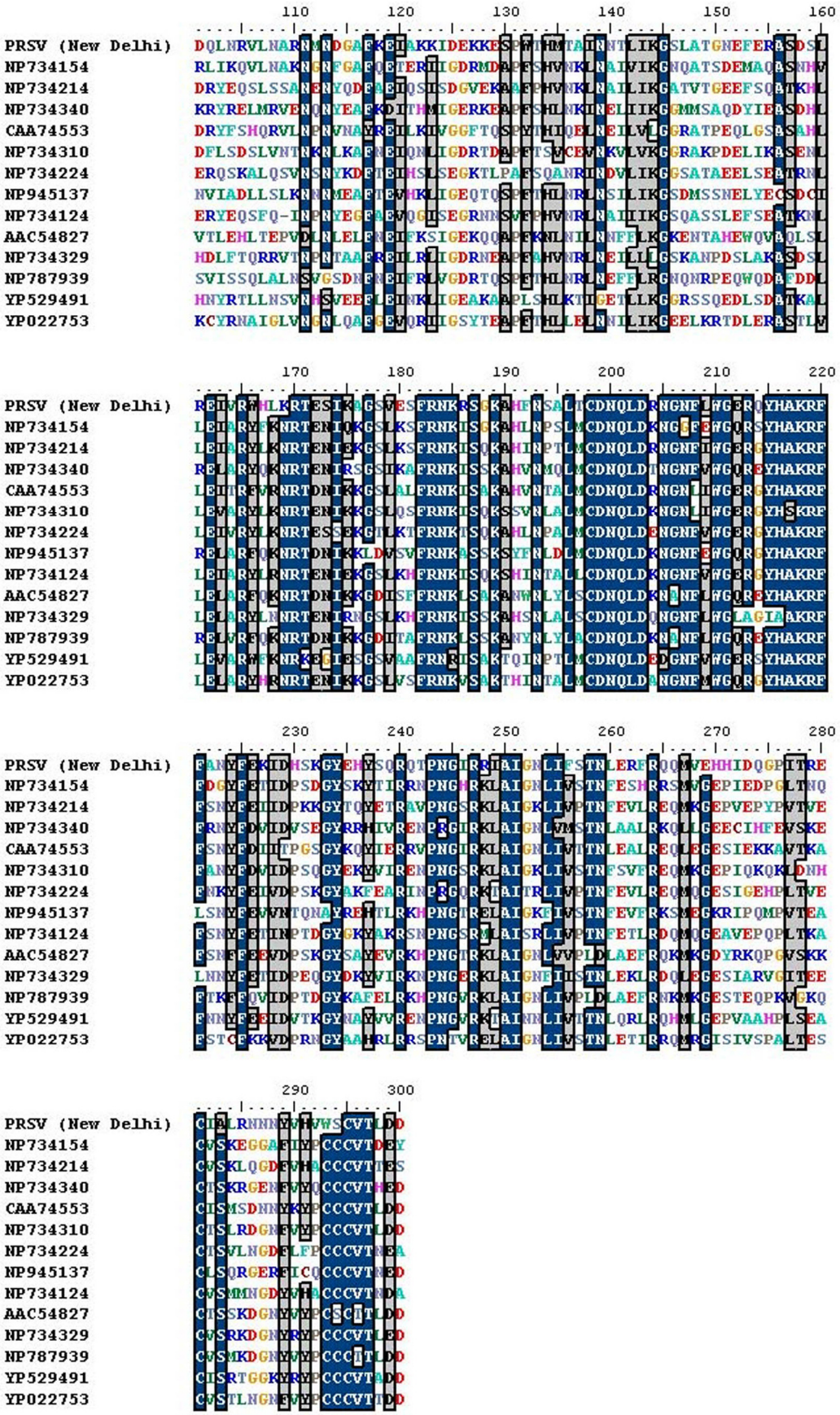


Fig.17 Comparison of the sequence motifs at the central region of HcPro from different potyviruses. First half of this region responsible for genome amplificaion is found to be less conserved than the next half responsible for silencing suppression.

suppressor tombuviral P19. Analysis predicts less variability in this domain, which also supports its non-sequence specific function.

Table.8 The identified sequence motifs in helper component proteinase of *Papaya ring spot virus* (New Delhi isolate)

Motifs	Width	Sequences	Location (N-terminal)	Domain	
M1	2	CG	35-36	Nif D	
M2	4	VAAL	38-41	Nif D	Transmission
M3	4	KITC	51-54	Nif D	
M4	4	LIKG	140-143	RP041 and Nrap	
M5	3	NRT	167-169	RP041 and Nrap	
M5	4	FRNK	180-183	RP041 and Nrap	
M5	6	CDNQLD	196-201	--	
M6	4	NGNF	203-206	--	
M7	2	WG	208-209	--	
M8	7	YHAKRFF	213-219	Dna B	RNA binding
M9	3	YFE	222-224	Dna B	
M10	2	GY	231-232	Dna B	
M11	3	PNG	241-243	Dna B	
M12	2	AI	248-249	Dna B	
M13	2	TN	256-257	Dna B	
M14	5	CCCVT	292-295	--	
M15	3	PTK	309-311	--	
M16	2	GN	317-318	--	
M17	2	GD	320-321	--	
M18	4	GYCY	341-344	Peptidase C19	
M19	7	NIFLAML	346--352	Peptidase C19	
M20	2	AK	360-361	Peptidase C19	
M21	3	FTK	363-365	Peptidase C19	
M21	3	VRD	367-369	Peptidase C19	
M22	2	LG	375-376	Peptidase C19	
M23	2	WP	378-379	Peptidase C19	Protease
M24	2	AT	385-387	Peptidase C19	
M25	10	AELPRILVDH	401-410	Peptidase C19	
M26	2	HV	416-417	Peptidase C19	
M27	2	DS	419-420	Peptidase C19	
M28	2	GS	422-423	Peptidase C19	
M29	4	TGYH	426-429	Peptidase C19	
M30	6	LKANTV	431-436	Peptidase C19	
M31	2	QL	438-439	--	
M32	2	VG	456-457	--	

Some of the conserved motifs in this domain are YHAKRFF[219], GY[232], PNG[243] and AIG [250] **[Fig.17]**. This RNA binding region shares an overlapping functional domain responsible for cell-to-cell movement of the virus. Conserved domain protein architecture reflects homology of this region with the domain of DnaB having role in membrane attachment and Mvi N, which is a membrane protein and a putative virulence factor suggesting its probable role in cell to cell movement **[Fig.16]**.

C terminal- proteolytic domain

The proteinase domain of HcPro has been mapped to the C terminal 157 amino acids and characterized as a cystein protease like activity. The presence of two conserved amino acid Cys [343] and His [416] at the active site of the protease in all the potyviruses confirmed its probable function uniformly. Beside these two amino acids, other conserved motifs are NIFLAML[352], AELPRILVDH[410], LKANTV[436] and VG[457] **[Fig.18]**. The other interesting motif in the C terminal region is PTK[311] that is found to be evolutionary conserved in all the potyviruses, probably contributes to binding of HcPro to the viral capsid protein's N terminal DAG motif. The presence of many conserved motifs in this region confirms its role as proteolytic enzyme in all the potyviruses irrespective of the host. This region shows strong homology with the other peptidases when compared with cross protein conserved domain architecture. The protease activity of HcPro is mainly responsible for autocleavage of the viral polyprotein expressed in host cell, which is a conserved phenomenon among all the potyviruses. Its close homology with the peptidase C19 L, a subfamily of peptidase C19, reflects an additional role of this protease beside autocleavage **[Fig.16]**. Proteases of this family are involved in intracellular proteolytic activity that removes ubiquitin molecule from polyubiquinated peptides, hence affecting the protein turnover through the proteosome system. Present analysis proposes a model for the probable function of this protease is deubiquitinylation of viral protein which is in close agreement of SARS coronaviral PLpro and rescuing them from the degradation in the host proteosome, suggesting one more level of virus counter defense at the protein level.

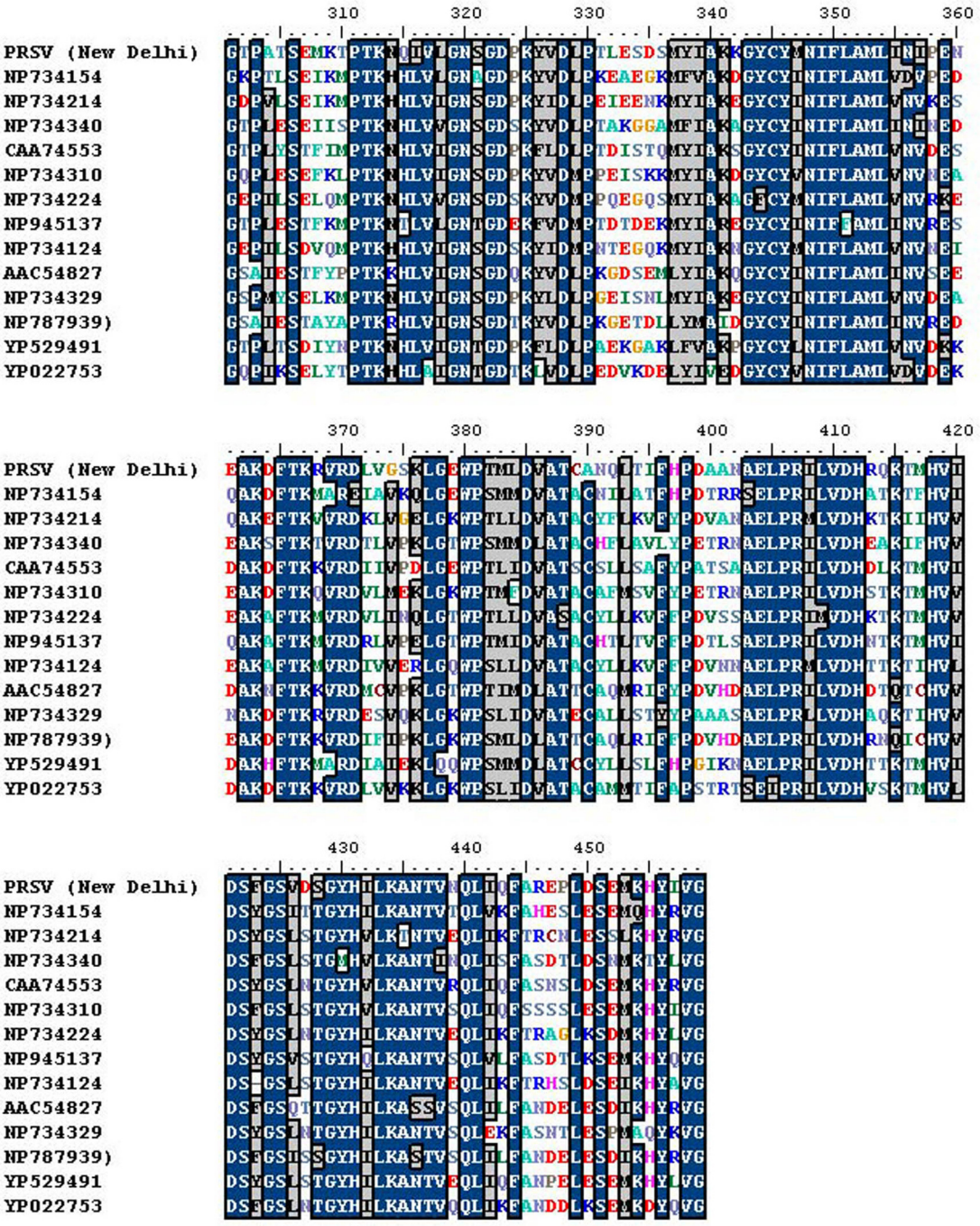
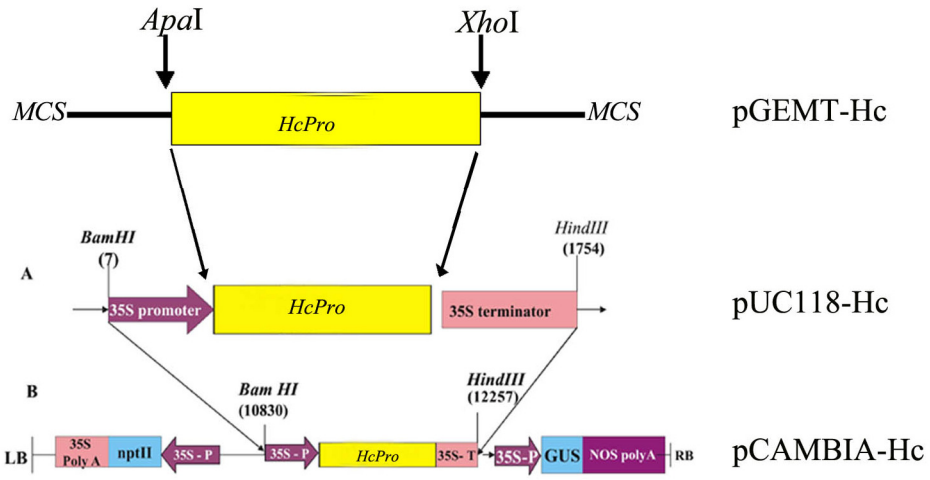


Fig.18 Comparison of sequences at the C-terminal of HcPro having functional role in protease activity from different potyviruses. Proteinase domain was shown to be more conserved than N terminal and Central domain.

PRSV-HcPro binary vector construct

Studies concerning the interactions of plants miRNAs with potyviral HcPro have been focused on the phenotypic effects of the suppressor protein in *Arabidposis* and *Nicotiana*. Inspite of extensively studied suppressor, HcPro from various potyviral origins revealed great degree of differences in small RNA metabolism in plants. To elucidate the effects of PRSV-HcPro in miRNAs controlled plant development, it was constitutively expressed in *Nicotiana benthamiana.* For this, **w**e designed a construct by cloning the *HcPro* gene in to binary vector pCAMBIA 2301, which is shown schematically in **Fig.19**. Primers based on the nucleotide sequence of PRSV-P *HcPro* with the embeded *Apa*I site and Met and Ala codons at the 5' end and stop codon and *XhoI* site at the 3' end were synthesized. An amplicon of ~1.4kb corresponding to *HcPro* region was obtained with cDNA template of PRSV infected papaya plants using high fidelity DNA polymerase. The pGEMT-*HcPro* clone was then subjected for restriction enzyme *Apa*I/*Xho*I digestion (restriction sites present on forward and reverse primers) and was cloned in the pUC118 vector. Twenty colonies were randomly picked for colony PCR from ampicillin plates as the vector carries ampicillin resistance marker gene. Out of these colonies only five colonies were found to be positive showing an amplicon of ~1.4Kb. Plasmid DNA from the positive colony designated as pUC118-*HcPro* was restricted with *Apa*I/*Xho*I enzyme to release the ~1.4Kb. It was again reconfirmed by restriction with *Bam*H1 and *Hin*dIII enzymes, as the positive clone released the whole cassette (CaMV 35S Promoter + *HcPro* gene + CaMV 35S terminator), as one fragment of ~ 2.0 kb. The presence as well as orientation of the *HcPro* gene in the above clone was confirmed by sequencing. The released ~ 2.0 kb fragment carrying the whole cassette was gel eluted and sub-cloned in binary vector pCAMBIA 2301 which was linearised by double digestion with *Bam*HI and *Hin*dIII enzymes. The 16 colonies obtained in kanamycin selection plate were then screened for recombinants by colony PCR using specific primers. Seven colonies (pCAMBIA 2301-*HcPro*) were positive for the presence of *HcPro* insert as shown by the amplicon of 2.0 Kb from 35S promoter and terminator primers. Plasmid DNA from positive colonies was then restricted with *Bam*HI and *Hin*dIII to confirm the release of a ~ 2.0 kb cassette. In this construct expression of PRSV-*HcPro* is under the control of the CaMV 35S constitutive promoter **[Fig.19]**. The

A.



B.

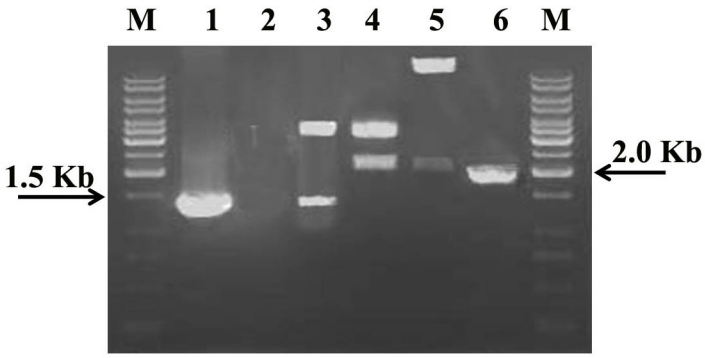


Fig.19 (A) Schematic diagram showing different stages of *HcPro* binary vector construct preparation. *HcPro* gene was cloned under the control of constitutive 35 S promoter. **(B)** Electrophoresis of PCR amplified product using primers for *HcPro* from PRSV infected (Lane 1) and healthy papaya leaf (Lane 2), *Apa*I and *Xho*I digested pGEMT clone having *HcPro* gene (Lane 3), *Bam*HI and *Hind*III digested pUC118 clone releasing cassette containing 35S promoter, terminator and *HcPro* gene (Lane 4), *Bam*HI and *Hind*III digested pCAMBIA 2301 clone releasing cassette containing 35S promoter, terminator and *HcPro* gene (Lane 5), PCR amplified product with 35S P-T primers (Lane 6), Lane M: 1Kb DNA marker.

vector pCAMBIA 2301-*HcPro* carries only the region encoding HcPro (1724 To 3094), with an additional start codon followed by GCC added at the 5' end and a stop codon at the 3' end of the insert so that the mature protein HcPro (with two additional N terminal residues, Met and Ala) is made without proteolytic processing.

Mobilization of HcPro construct into Agrobacterium

Recombinant binary plasmid carrying *HcPro* gene construct was mobilized into *Agrobacterium tumefaciens* strain LBA 4404 by freeze and thaw method. Number of colonies obtained on kanamycin and streptomycin selection plate were 13 for plasmid pCAMBIA 2301-*HcPro*. Upon screening by colony PCR 2, 8, 11 and 13 *Agrobacterium* colonies were positive for the presence of pCAMBIA 2301-*HcPro*, by showing the respective size amplicons. These positive clones were used for *Agrobacterium* mediated plant transformation.

Transformation of HcPro gene construct in Nicotiana benthamiana

Development abnormalities have been reported previously in both tobacco and Arabidopsis transgenic lines expressing HcPro from various sources and these have been attributed to defects in micro RNA pathways. To further investigate the nature of the defects caused by the PRSV-HcPro, we generated *Nicotiana benthamiana* transgenic lines expressing the 5' region of the PRSV genome encoding the HcPro protein. Molecular analysis of transformants was done using RT-PCR to confirm the transgene insertion and its expression. Out of the 31 transformants selected in kanamycin (50mg/L), about 6 putative transgenics were established using RT-PCR and these were designated as HC1 to HC6. We compared the transgenic tobacco lines expressing constitutively HcPro protein from healthy control. The transgenic tobacco lines exacerbated abnormal morphological characteristics such as unregulated differentiation, chlorosis, apical apoptosis and underdeveloped leaf lamina. Here it is demonstrated that the phenotypic aberrations are observed on over expression of PTGS suppressor protein HcPro, which might be affecting miRNAs metabolism through small RNA binding thereby affecting plant gene regulation **[Fig.20]**.

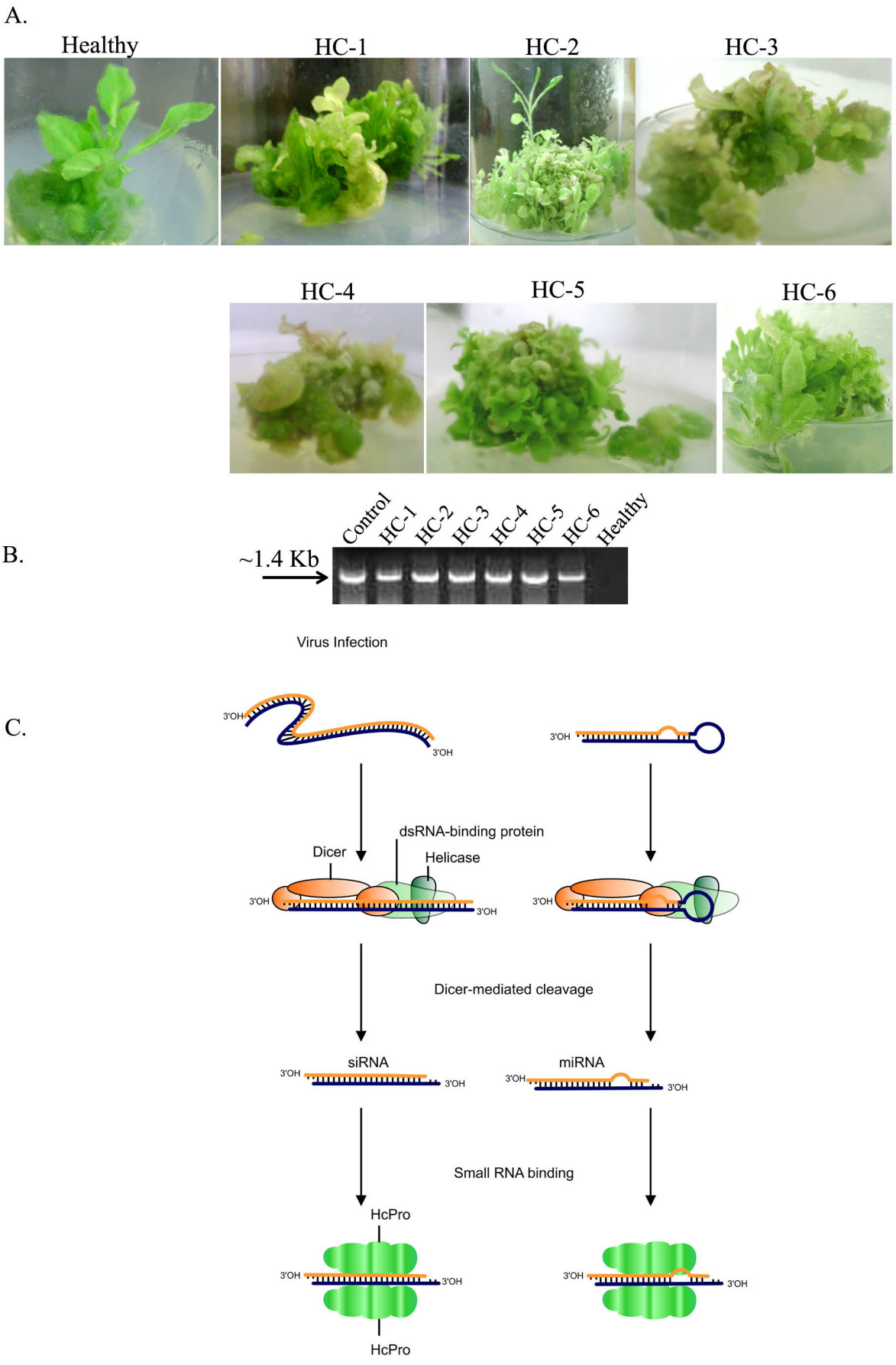


Fig. 20 (A) Phenotypic aberrations induced by HcPro in *N. benthamiana* transgenic lines. **(B)** Molecular analysis of transformants through RT-PCR using gene specific primers. **(C)** Proposed mechanism of action for PRSV-HcPro in small RNA regulation.

PRSV HcPro and its small RNA binding

Helper component proteinase (HcPro) gene amplicon of ~1.4Kb was obtained with cDNA template of PRSV infected plant using high fidelity DNA polymerase for HcPro expression. The *HcPro* gene was cloned into a 'tac' promoter driven fusion protein expression vector pMal-c2x to get pMal-MBP-HcPro. The pGEMT-*HcPro* clone was subjected for restriction enzyme *Eco*R*I/PstI* digestion (restriction sites present on forward and reverse primers) and was cloned in the pMAL c2x vector linearised with *Eco*RI and *Pst*I restriction enzymes. Successful clones of the *HcPro* insert in the pMAL c2x vectors was identified by colour screening on indicator plates containing ampicillin, X-gal and IPTG, as the vector contains the ampicillin resistance marker gene. Ten colonies were randomly picked for colony PCR. Out of ten colonies only three colonies were found to be positive showing an amplicon of ~1.4Kb. Plasmid DNA from the positive colonies designated as pMal-*HcPro* was restricted with *Eco*RI and *Pst*I restriction enzymes to release the 1371 bp insert of *HcPro* **[Fig 21]**. The presence as well as orientation in the above clone was further confirmed by sequencing.

Initial experiment establishes that the fusion protein with approximate molecular weight 94 Kd was inducibly expressed in *E. coli* (strain TB1) clones containing the pMal c2X- *HcPro* whereas 50 Kd maltose binding protein (MBP) fused with α-gal was expressed in clones containing pMal-c2X treated as control. Most of the MBP-HcPro existed in insoluble fraction when expressed at 37°C. To improve the production of soluble, biologically active recombinant MBP-HcPro, low temperature growth was employed. When solely expressed at 28ºC, the soluble MBP-HcPro increased significantly **[Fig.22]**. However further lowering in temperature for instance 20ºC, can not remarkably improve the solubility. The optimum time for harvesting the cells was 2hr after induction as the intensity of fusion protein band did not appear to increase after this time. We concluded that 0.4 mM IPTG induction and 2hr incubation time is required to get the maximum amount of recombinant protein and lower temperature cultivation improve the yield of soluble, biologically active MBP-HcPro. So we chose expression at 28ºC for large scale MBP-HcPro expression in soluble fraction. While doing Western hybridization, antisera of MBP had shown to hybridize within western blot 83 Kd and

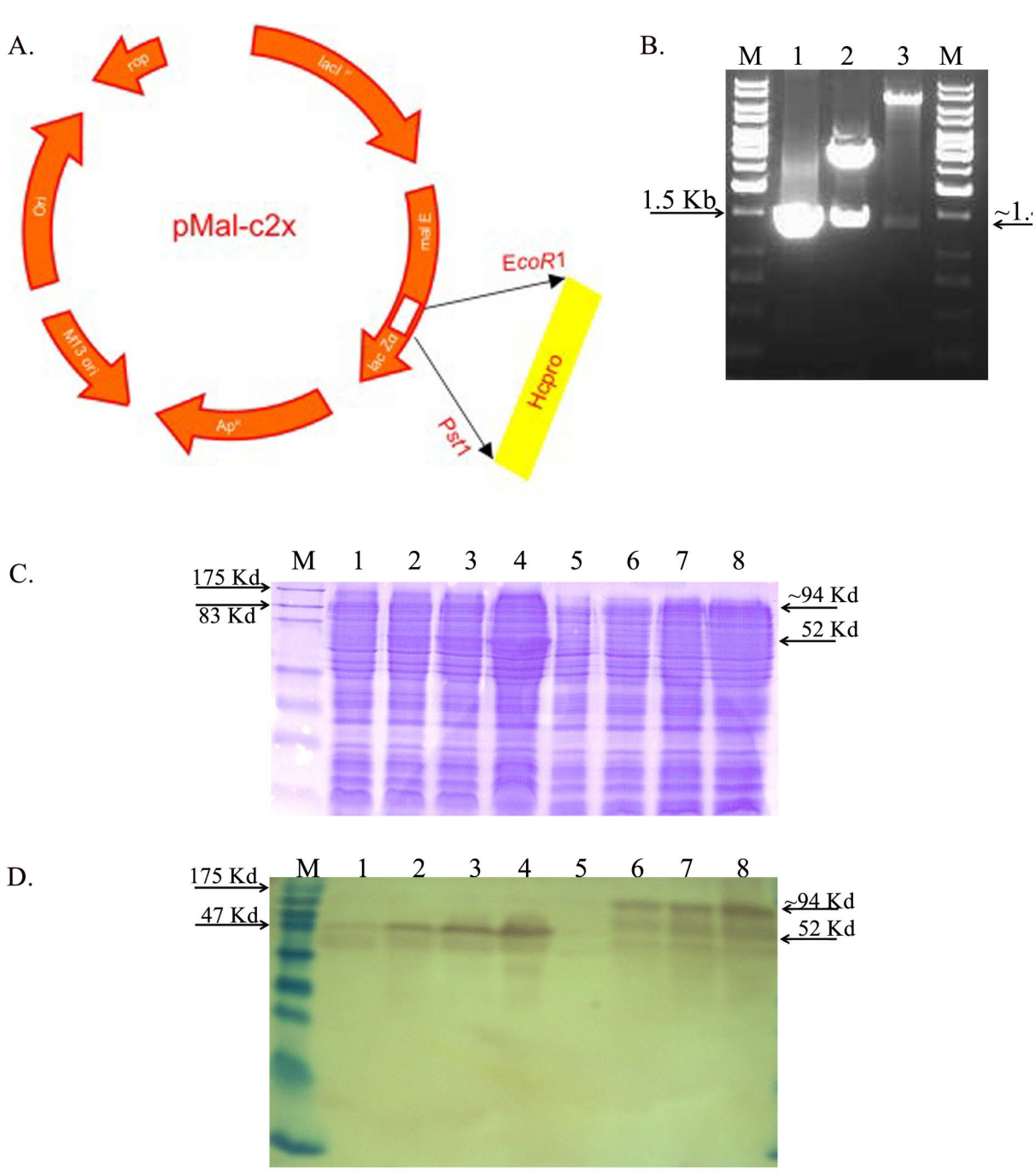


Fig.21 (A) Diagramatic presentation of pMAL-HcPro expression vector construct **(B)** Electrophoresis of PCR amplified *HcPro* gene (Lane 1), *EcoR*I and *Pst*I released pGEMT-*HcPro* clone (Lane 2), *EcoR*I & *Pst*I released pMAL-HcPro clone (Lane 3), Lane M: 1Kb DNA marker. **(C)** SDS-PAGE analysis of fused HcPro protein at different IPTG concentrations. 20 microlitre sample was loaded in each well. Lane 1: uninduced MBP clone, Lane 2-4: induced MBP with 0.2 to 0.4 mM IPTG, Lane 5: unin duced pMAL-HcPro clone, Lane 6-8: induced pMal-HcPro clone with 0.2 to 0.4 mM IPTG. Lane M: Prestained protein marker broad range. The marker contained fragments of 175 Kd, 83 Kd, 62 Kd, 47.5 Kd, 32.5 Kd, 25 Kd, 16.5 Kd and 6.5 Kd **(D)** Western Blot of the above mentioned SDS-PAGE.

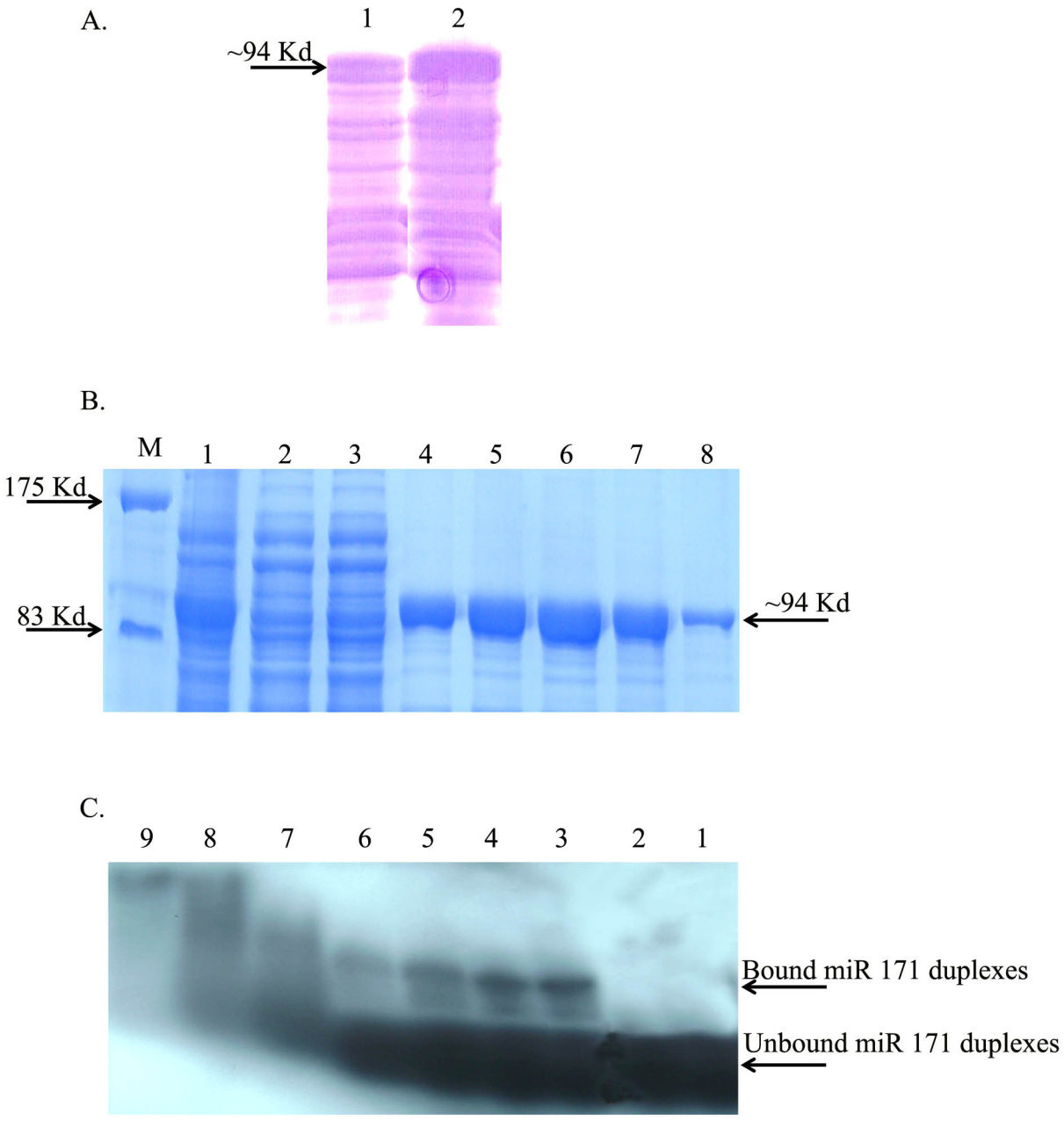


Fig.22 (A) Proportion of HcPro fusion protein in soluble fraction isolated from cells grown a 37^{O}C (Lane 1) and 28^{O}C (Lane 2) for two hours. **(B)** Purification of fused HcPro protein, Lane 1: protein present in soluble fraction, Lane 2&3: flowthrough and wash during purification, Lane 4-8: eluted protein from affinity chromatography using amylose resin (fraction 5,9,12,15 & 18). 20 microlitre volume was loaded in each well.**(C)** Electrophoretic mobility shift assay for miR171 with fused HcPro protein, Lane 1: miR171, Lane 2: miR171 + MBP, Lane 3-9: miR171 + fused HcPro protein at increasing concentrations. 3 ng small RNA was used for binding with purified HcPro.

175 Kd proteins in lysate from bacteria with pMal c2x- *HcPro*, also detection of some minor bands suggested that some degradation of fusion protein had occurred **[Fig.21]**.

Recombinant MBP-HcPro protein was purified by one step MBP affinity chromatography. From 1 litre flask culture, about 395 mg total proteins was measured in supernatant which yielded ~ 45 mg MBP-HcPro on purification. The purified protein was elecrtophoresed under 12% SDS-PAGE and a 94 kd fusion protein comprising 42 kd MBP and ~52 kd HcPro was detected **[Fig.22]**.

Invitro binding assay of MBP-HcPro with small RNA duplexes

Small RNA binding ability of viral suppressors from diverse sources would explain the independent evolutions of silencing suppression mechanism. To this aim, gel mobility shift experiment was carried out to test the small noncoding RNA binding ability of PRSV-HcPro as a MBP fused protein (expressed in *E. coli*). In a direct competition assay, PRSV-HcPro binds 21 nt ds labeled miR171 duplexes that were incubated with the increasing concentrations of purified recombinant protein. Mobility shift of small RNAs was not detected when small RNA duplexes were incubated with MBP alone. This ruled out the possibility of MBP interference in mobility of small RNAs. Incubation of small RNA duplexes with varying concentrations of MBP-HcPro (0.5µg, 1.0µg, 2.0µg, 5.0µg, 10.0µg, 20.0µg and 50.0µg) suggested the concentration dependent binding of small RNAs with HcPro. At the two highest concentrations of MBP-HcPro (20 µg and 50µg), there was disappearance of small RNA band which suggested that minimum 20 µg protein was required for complete binding of 3.0 ng small RNA duplexes **[Fig.22]**.

Synergistic effects of viral suppressor proteins

Higher plants are oftenly subjected to multiple virus infections resulting in the intensification of symptom expression and virus accumulation, a phenomenon known as synergism. The extent to which the synergistic viral interactions occur in higher plants and the role it plays in mediating plant disease is not really clear at this point. Moreover, the basic background information about the viral synergism and the involved viral genes is not very much clear. In most of cases, pathogenecity factor and suppressor proteins are found to play an important role in synergistic effect. Viral suppressors enhance the

accumulation of diverse virus types suggesting the suppressor proteins as an important candidate in establishing the virus synergisms. Hence, it is important to characterize the molecular basis of viral synergisms in relation to silencing suppressor proteins from diverse origin.

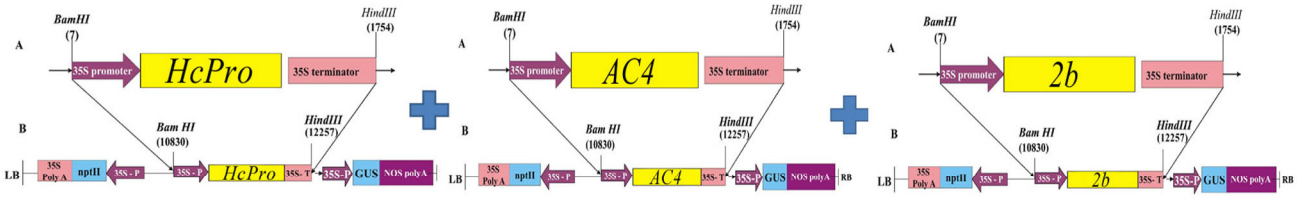
Synergistic effects of AC4, 2b and HcPro in CMV accumulation profiles

Four set of experiments with three independent replications were planned and analyzed to evaluate the synergistic effects of divergent suppressors in the accumulation of CMV population. CMV infected tobacco explants were transformed with HcPro, 2b, AC4 and with the combination of all the three RNAi suppressors. Non transformed regenerated CMV infected tobacco plants of the same age were taken as 'control' to study the effect of suppressor proteins in change in CMV accumulation. Minimum 12 transgenic lines (4 from each replica) from each set of experiment and similar number of regenerated control plants were analyzed and the mean absorbance value of all the 12 plants was employed to study the synergistic effect of suppressor proteins in CMV accumulation. Among the three suppressors from the three diverse origin tested here, only HcPro showed an appreciable increase in the level of CMV accumulation. However, there was no significant change in the level of CMV accumulation when transformed with 2b and AC4 as confirmed by ELISA **[Fig.23]**. Interestingly, the difference in accumulation of CMV in case of combination of all the three suppressors was very similar as in case HcPro transformation alone.

Synergistic effects of HcPro and 2b in ToLCV accumulation

Two sets of experiment were planned to identify the synergistic interaction of 2b and HcPro with ToLCV accumulation. ToLCV infected tobacco explants, transformed with HcPro and 2b, and non transformed regenerated ToLCV infected tobacco plants of same age group were considered as a control. For each sets of experiment three replica of each transformed line were taken with similar number of regenerated control plants. The relative amounts of ToLCV AC4 from transformed/non transformed regenerated ToLCV infected tobacco line were quantified by qPCR **[Fig.24]**. The mean Ct value of regenerated ToLCV infected tobacco plants was 12.11 while it was 9.30 and 1.67 for *2b* and *HcPro* transformed ToLCV infected tobacco plants respectively. HcPro seems to be

A.



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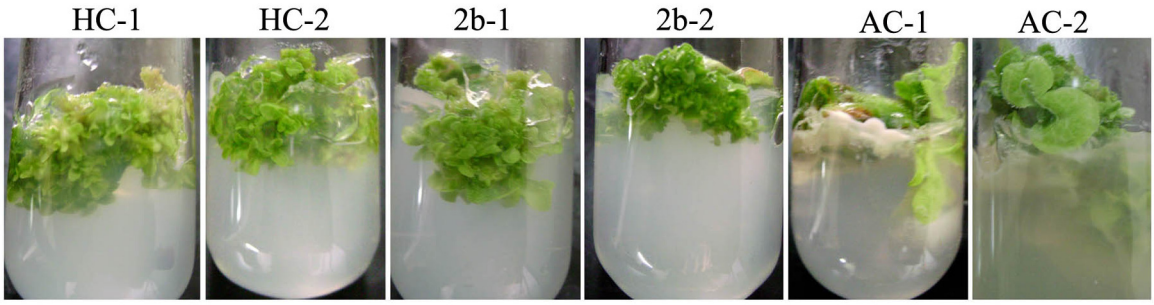

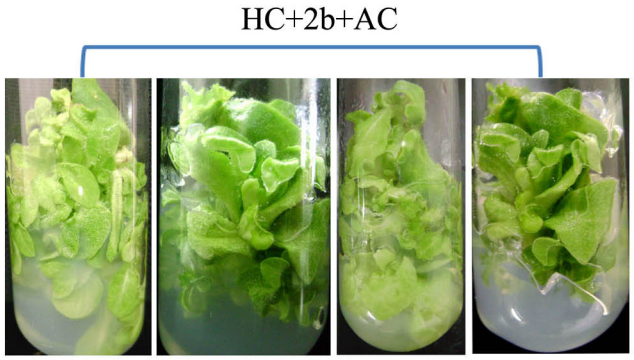

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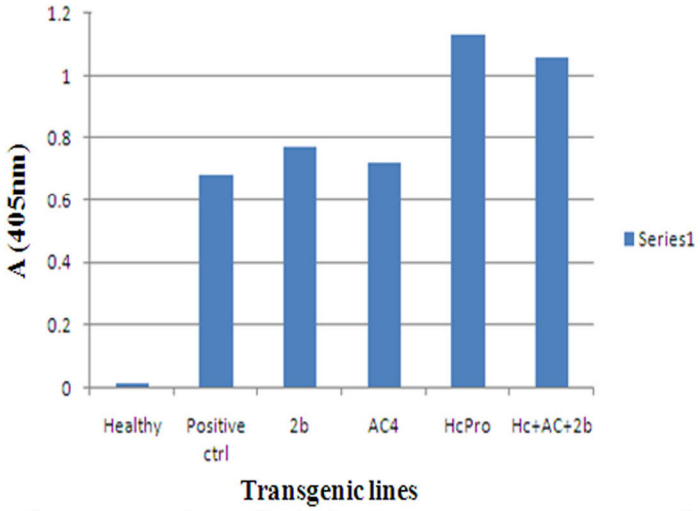
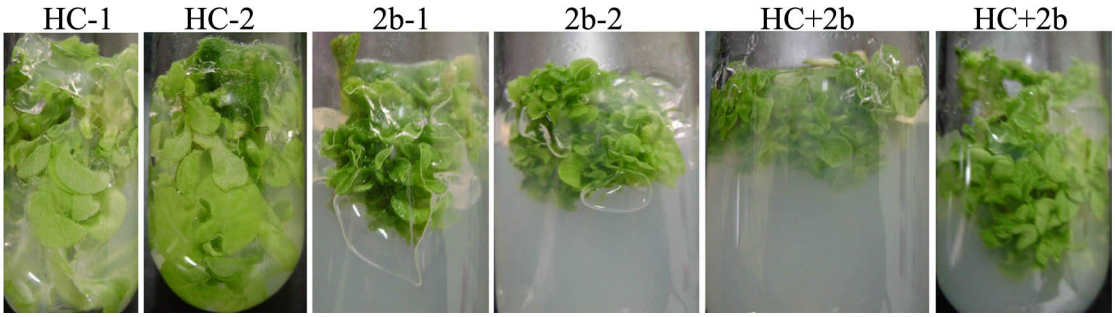


Fig.23 (A) Schematic presentation of viral suppressor constructs under the control of constitutive 35S promoter used in synergy experiments . **(B)** HcPro, AC4 and 2b representative transformants of CMV infected *N. tabacum.* **(C)** Changes in the accumulation of CMV due to expression of silencing suppressors from different viral origin evaluated by DAC-ELISA.

A.

B.



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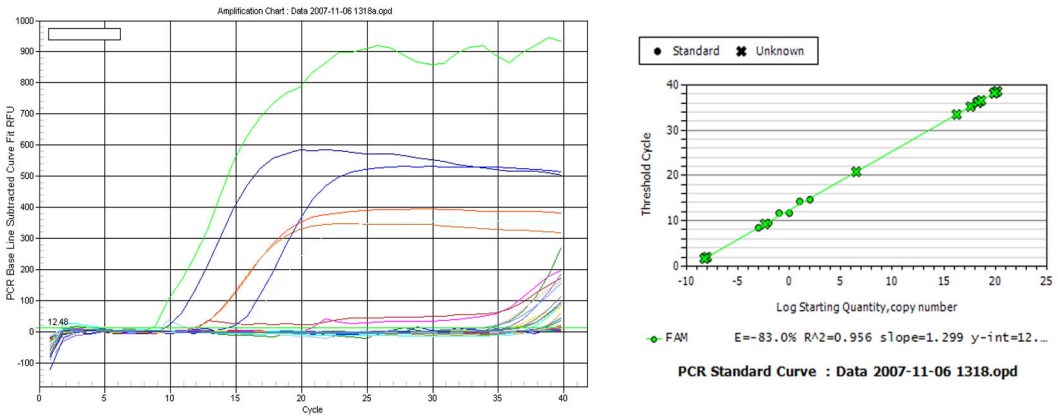

Fig.24 (A) Schematic diagram of silencing suppressor (HcPro and 2b) constructs under the control of constitutive 35S promoter used for synergistic study. **(B)** Potyviral HcPro and Cucumoviral 2b transformed ToLCV infected *N.tabacum* representative lines. **(C)** Graph showing quantification of ToLCV in HcPro and 2b transgenic lines by Real Time PCR using TaqMan probe for AC4 gene.

an important suppressor protein in viral synergistic studies as it provides synergism with both CMV and ToLCV. Besides RNAi suppression, it has multifunctional attributes, hence it is important to study HcPro in context with other viral component. For this full length genome of PRSV had been characterized.

Characterizing genomic components of the viral genome of *Papaya ring spot virus* to study plant-virus interaction

Papaya ringspot virus (PRSV) is a member of the genus potyvirus in the family potyviridae, with flexuous, filamentous, particles ranging from 700-900 nm in length and 12 nm wide; its genome is a single stranded RNA of positive polarity of around 10,000 nucleotides containing a single open reading frame translated into large polyprotein that is co-and/or post-translationally cleaved to produce final protein products. It affects the cultivation of papaya and cucurbits worldwide and two biotypes (P & W) have been recognized on the basis of their ability to infect papaya. The PRSV-DEL isolate was obtained from a severely mosaic-affected papaya (*Carica papaya* L.) plant from the experimental fields of IARI, New Delhi, and was maintained on papaya (cv. Pusa Nanha) by a series of sap inoculations under glasshouse conditions. The strategy employed for the elucidation of the complete nucleotide sequence of PRSV RNA is summarized in **Fig.25.** The genome fragments were PCR-amplified using total RNA. The full genome sequence was determined from nine overlapping cDNA clones and compared with eleven already available sequences. PCR fragments were cloned in pGEMT-Easy vector in order to obtain the sequence data. The overlapping regions of the amplified clones ranged from 50-200 nucleotides. The complete genome of the PRSV-DEL isolate from India is 10317 nucleotides long excluding the 3' terminal poly (A) tail and has a G+C content of 42%. The sequence has been deposited in GenBank with the accession no. EF017707.

Size and complexity of PRSV genome

The viral genome from India represent the shortest sequence in terms of length **[Table.7]**. The genome consists of a single large open reading frame (ORF) of 10023 nucleotides commencing at position 86 and terminates with UGA at position 10109-11, followed by a 3' untranslated region (UTR) of 206 nucleotides. The ORF potentially encodes a polyprotein of 3341 amino acids, possessing nine potential cleavage sites

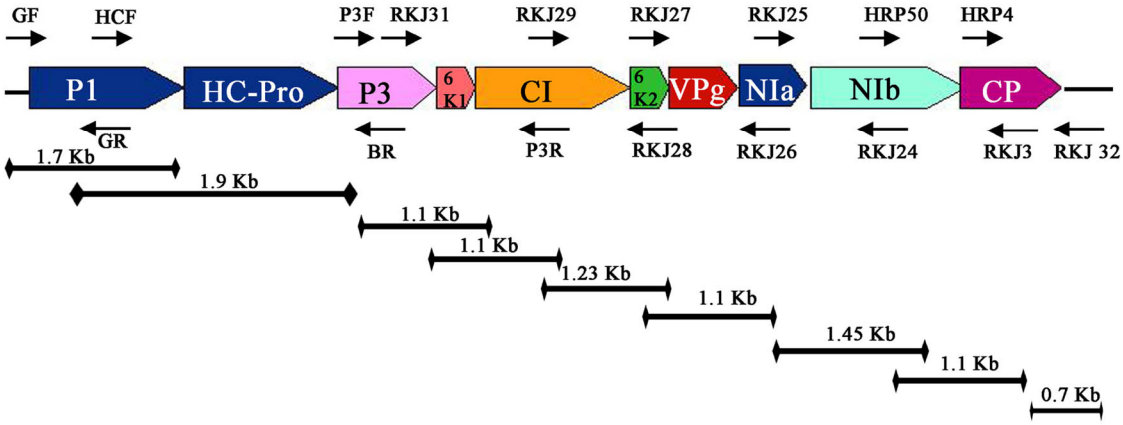

Fig. 25 Schematic strategy and primer map for cloning the complete PRSV genome. Size of amplicon obtained from each primer pair is shown.

[**Table.9**]. Comparative sequence analyses revealed that the PRSV-DEL isolate from India shared 83–89% and 90–92% overall sequence identities at the nucleotide and amino acid levels respectively, with other PRSV isolates **[Table.10]**. The regions encoding the protein 1 (P1), helper component proteinase (HcPro), protein 3(P3), 6kDa 1 protein (6K1), Cylindrical inclusion protein (CI), 6kDa 2 protein (6K2), genome-linked viral protein (VPg), nuclear inclusion proteinase 'a' protein (NIa-Pro), nuclear inclusion protein 'b'(NIb) and coat protein (CP) genes were 1638, 1371, 1032, 159, 1905, 171, 567, 714, 1508 and 861 nt respectively.

The amino acid distances for all the available 13 full length PRSV genomes reported from different part of the world including PRSV-PIndia from this study were used to generate the phylogenetic trees. The N-J tree for the aligned full genome sequences at the amino acid level showed that there are two distinct lineages: one included the sequences of 8 isolates of Asian origin and the other included four American isolates along with the one Indian isolate **[Fig.26]**. Those from the Asia (China, Taiwan & Thailand), different biotypes from one region clustered together. Within the American lineage, isolates from the Mexican origin showed clustering with Hawaii isolates, while isolates from Brazil were clustered together. The most unique and interesting feature of this lineage is grouping of Indian isolate with American sequences suggesting that it might probably be imported from Indian subcontinent. In the case of sequences of five proteins P1, CP, NIa, NIb and VPg, playing a major role in virus establishment, replication and movement, the major groupings that were detected mimicked the clustering pattern of the PRSV polyprotein **[Fig.27]**. When the sequence identity matrices for PRSV genome sequences from different geographical locations were closely studied, ~ 90% similarity at full genome polyprotein level was found. Most of the viral encoded proteins except P1 followed the identity matrix shown by full genome polyprotein. The most divergent P1 protein showed only ~71% mean amino acid sequence similarity. The two biotypes of PRSV (P&W) from the same geographical location were also found to be more divergent at the P1 protein level as compare to the other proteins and full genome polyprotein level. The amino acid sequence divergence in PRSV-Thai biotypes at the P1 protein was 18% with a maximum of 22% in PRSV-India biotypes **[Table.10]**.

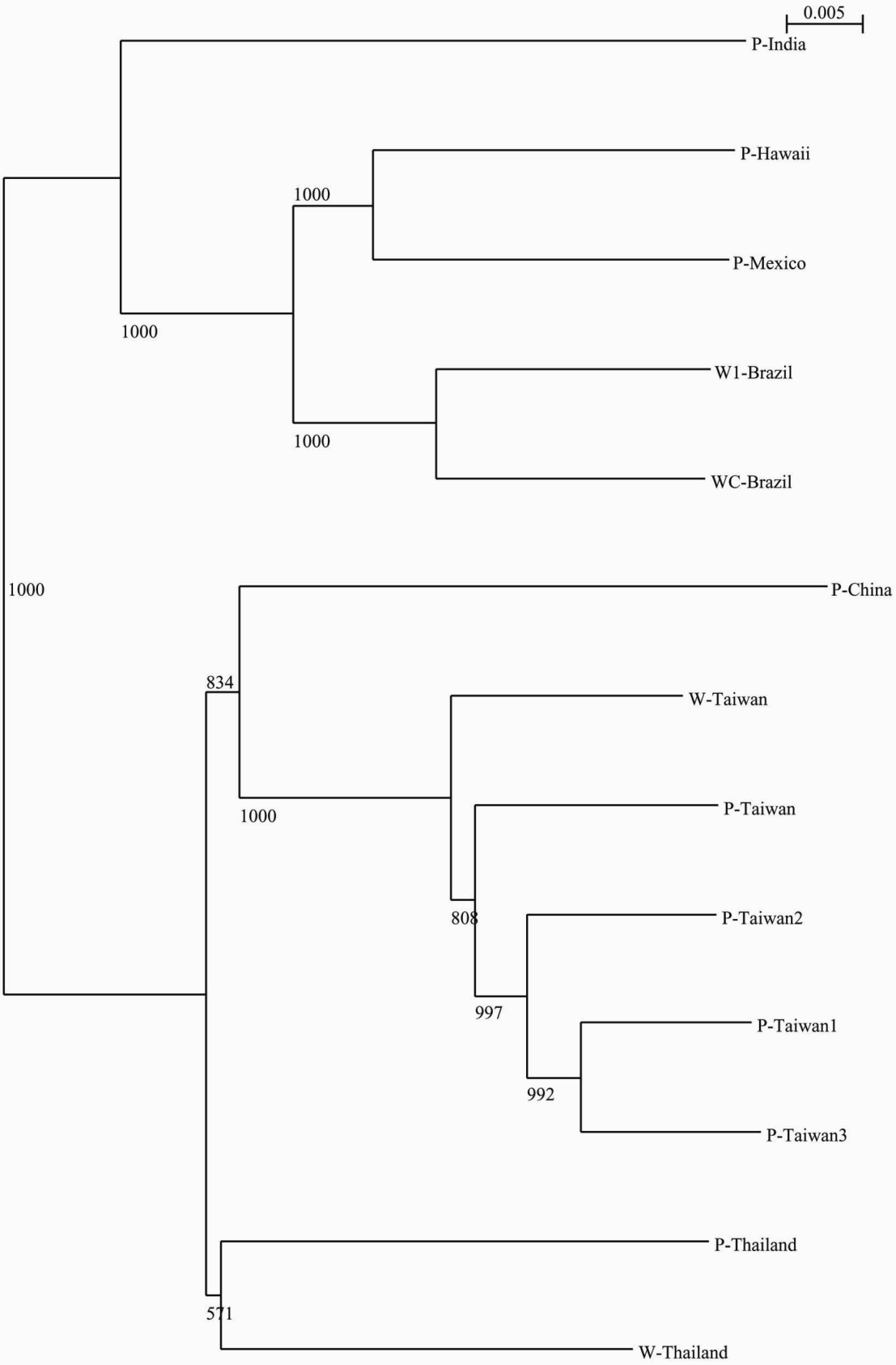

Fig.26 N-J tree of the aligned full genome polyprotein sequences of the thirteen PRSV isolates. Two clusters representing American and Asian lineages were drawn on the basis of phylogeny. Indian isolate, despite being the Asian, clustered with American isolates.

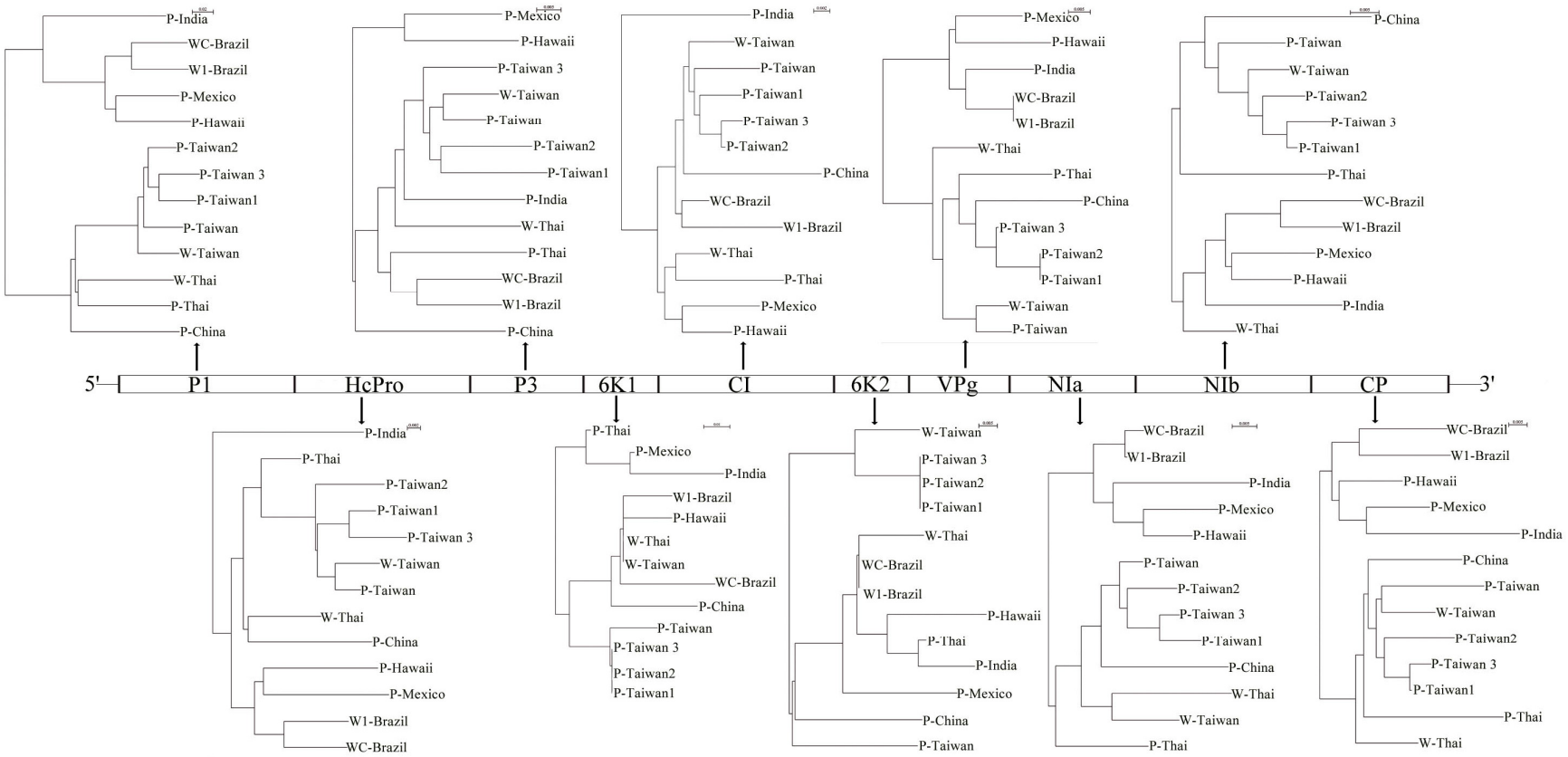

Fig.27 N-J tree of aligned PRSV proteins of the thirteen PRSV isolates.

Table.9 Functional genome map of PRSV-P India.

Untranslated regions and functional regions of polyprotein	**Size* (nt)**	**Predicted cleavage site**	**% Sequence identity with isolates from**	
			Americas	**Asia**
5'UTR	1-85 (85)	-	64-68 (-)	79-84 (-)
P1	86-1723(1638)	Y/ N	77-78 (74-76)	70-71 (67-70)
Hc-Pro	1724-3094 (1371)	G/G	89-91 (95)	85-87 (95-96)
P3	3095-4126 (1032)	H/Q	89-91 (94-95)	85-87 (94-96)
6K1	4127-4285 (159)	Q/S	89-90 (89-96)	87-91 (91-94)
CI	4286-6190 (1905)	Q/G	90-92 (97)	86-88 (96-98)
6K2	6191-6361 (171)	Q/G	88-92 (93-96)	83-89 (91-98)
VPg	6362-6928 (567)	E/G	90-93 (96-97)	82-84 (93-94)
NIa-Pro	6929-7642 (714)	Q/S	90-91 (95-96)	80-82 (92-95)
NIb	7643-9250 (1508)	H/Q	89-91 (95-96)	82-83 (94-96)
CP	9251-10111 (861)	-	92-94 (91-94)	88-90 (90-92)
3'UTR	10112-10317 (206)	-	94-95 (-)	92-92 (-)

*Position on the genome shown in parentheses

Table.10 Percent amino acid identity between full genomes and their individual cistrons of 12 *Papaya ringspot virus* isolates with respect to PRSV-P-India.

Genetic components	Percent similarity
Full genome	89.7-92
P1	67.2-75.6
HcPro	94.7-96.2
P3	93.6-95.9
6K1	86.7-96.2
CI	96.2-97.4
6K2	91.2-98.2
VPg	92-97.3
NIa	92.4-96.2
Nib	93.8-96.1
CP	88.9-94.1

The frequent occurrence of natural recombination in plant RNA viruses has recently been documented (Chare and Holmes, 2006). In order to determine whether similar recombinant isolates are established in PRSV potyvirus, the nucleotide sequence of the PRSV-P-India (Delhi isolate) was determined, as well as 12 other sequences obtained from the NCBI database were analyzed. In the present study, the recombination sites were detected throughout in the genome sequences of PRSV isolates. In total, 12 of the 13 *Papaya ringspot* viruses analyzed here showed the evidence for recombination under the Sawyer's run test, especially Indian isolate being the most prone. The nucleotide sequences of 10 ORFs and UTR regions were examined. Comparative recombination analyses detected 25 potential breakpoints which were ranked region wise as P1>P3 and CI> HcPro and CP>5'UTR, 6K2, VPg, NIa, NIb and 3'UTR. Out of the 25 putative recombination sites observed by GENECONV, 12 lies in the 5'UTR and P1

regions. When looked for hot spots of recombination, interestingly first 600 nucleotides of PRSV comprising of 5'UTR and P1 gene, contained maximum number of recombination sites (12 sites with 36 frequency) suggesting 5'region of PRSV contributes maximum, in shaping of the PRSV genomes **[Fig.28]**. Gene wise recombination sites and frequency covering both, Asian and American the lineages are summarized in **[Table. 11]**. In general, the genes located at the 5'end of the PRSV genome showed inter and intra lineage recombination while genes at 3' end showed only intra lineage recombination. One recombination site with 8 pairwise recombination frequency was detected in 5' UTR representing inter and intra lineage recombination. There were at least 11 recombination sites with 28 pairwise recombination frequencies in the *P1* gene at various positions mostly confined to the 5'end of the gene. Some of the recombination sites were from the parents from same lineage (intra lineage recombination site) other from different lineage (inter lineage). There were two recombination sites in *HcPro* gene and they represent both inter and intra lineage. Three recombination sites were detected in *P3* (5 recombination frequency) gene representing both inter and intra lineage. No recombination site was detected in *6K1* gene. There were 4 recombination sites (6 recombination frequency) in the *CI* gene showing inter and intra lineage recombination. There is only one intra lineage recombination site each in 6K2, VPg, NIa, NIb and 3'UTR. Two intra lineage sites were observed in CP gene. In summary a total of 25 recombination sites with 53 recombination frequency were found and in general inter lineage recombination was more prone than intra lineage recombination. P1 is the most variable PRSV protein and 6K1 showed high degree of conserved nature. It seemed that there might be some correlation between recombination, sequence pattern and deciphering the origin of PRSV. PRSV-P Indian isolate being an Asian, fall in the American lineage and shows both inter and intra lineage recombination as common parent **[Fig.29]** suggesting its probable import to American subcontinent. The recombination sites were detected in whole genome sequence of known 12 PRSV isolates from different geographical locations, PRSV-P India being the recent addition. W-Taiwan does not show any recombination with any of the isolate. Out of 12 recombination sites detected *in-silico* in 5'UTR and P1, PRSV- India appear to be the common parent that has undergone for recombination events with other 11 isolates

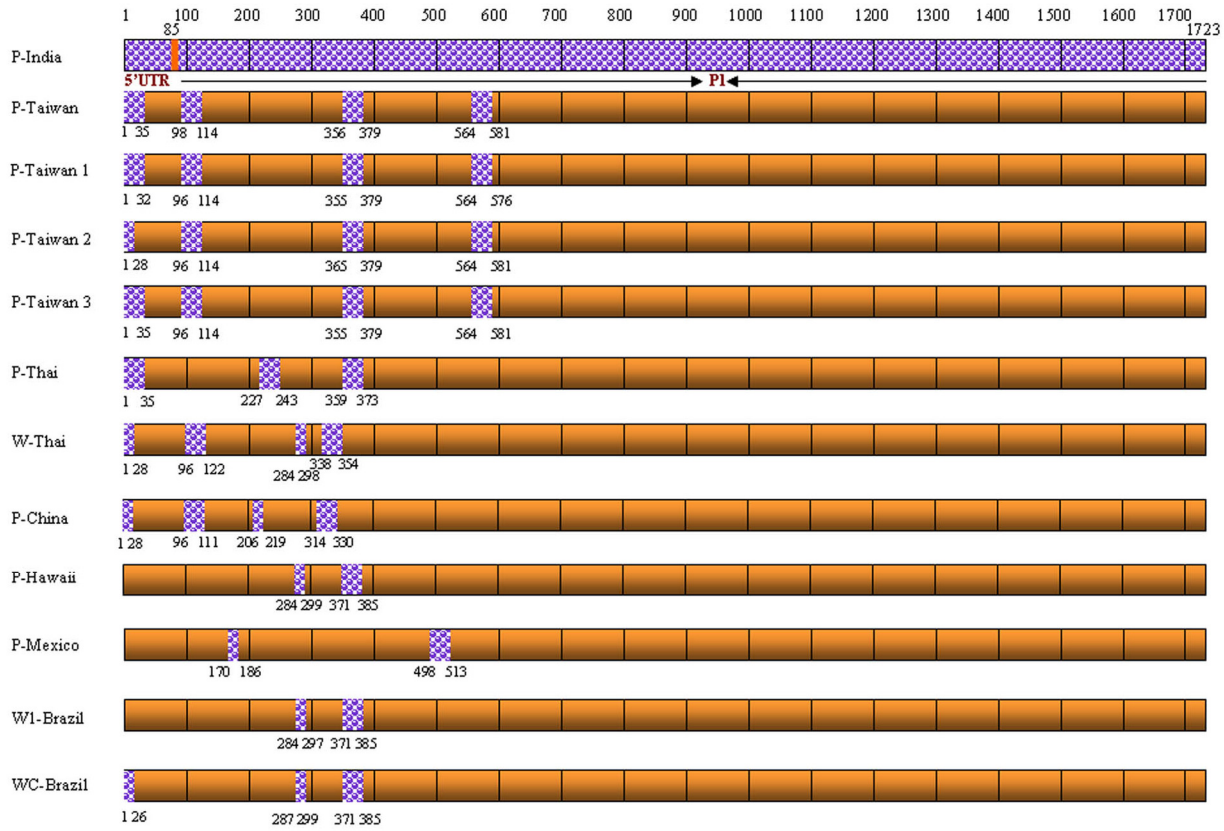

Fig. 28 Recombination hotspots detected in 5'UTR and P1 gene of PRSV genomes. Atleast 36 recombination events were found between PRSV-P India and 11 other PRSV genomes from rest of world. The ball filled boxes are the recombination hotspots assessed by Recombination Detecting Programme (GenConv).

A.

Isolates	Region/Gene	Recombination frequency	Putative recombination span regions in the viral genome detected
P India-W1 Brazil	P1	2	284-297, 371-385
P India- WC Brazil	5'UTR and P1	3	1-26, 287-299, 371-385
P-India-P-Hawaii	P1	2	284-299, 371-385
P-India-P-Mexico	P1	2	170-186, 498-513
P India-P-China	5'UTR and P1	4	1-28, 96-111,206-219, 314-330
P-India-P-Taiwan	5'UTR and P1	4	1-35, 98-114, 356-379, 564-581
P-India-P-Taiwan1	5'UTR and P1	4	1-32, 96-114, 355-379, 564-576
P-India-P-Taiwan2	5'UTR and P1	4	1-28, 96-114,365-379, 564-581
P-India-P-Taiwan3	5'UTR and P1	4	1-35, 96-114, 355-379, 564-581
P-India-P-Thai	5'UTR and P1	3	1-35,227-243, 359-373
P-India-W-Thai	5'UTR and P1	4	1-28, 96-122, 284-298, 338-354
P-China- P-Taiwan1	CI	1	5993-6075
P-China- P-Taiwan2	CI	1	5993-6075
P-China- P-Taiwan3	CI	1	5993-6075
P-China- P-Thai	CP	1	10003-10110
P-China- W1Brazil	HCPro	1	1906-1980
P-Taiwan1- P-Taiwan2	Vpg+CI+6K2	1	6137-7029
P-Taiwan1- P-Taiwan2	CP+3'UTR	1	9848-10,334
P-Taiwan2- P-Thai	P3	1	3641-3739
P-Taiwan- P-Thai	HCPro and CI	2	2633-2709, 5072-5147
P-Taiwan1- WC Brazil	P3	1	3632-3714
P-Taiwan- WC Brazil	P3	1	3632-3714
P-Thai- WC Brazil	CI	1	4773-4827
P-Mexico -W1 Brazil	NIb	1	8801-8922
P-Hawaii- P-Mexico	P3	2	3455-3573, 3575-3714
W1 Brazil- WC Brazil	NIa	1	7346-7461

B.

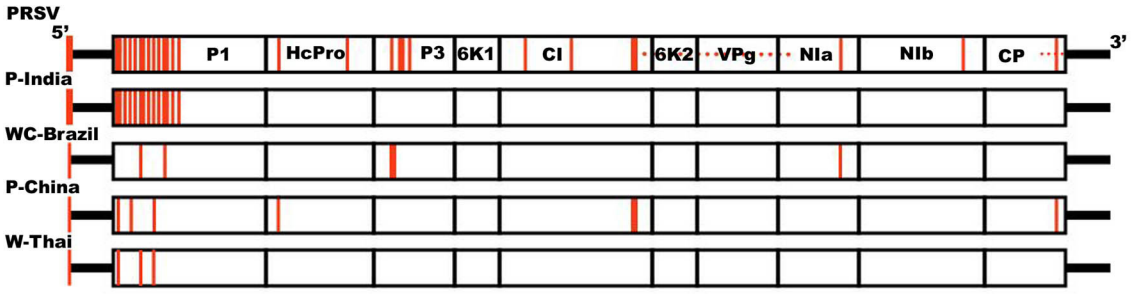

Fig.29 (A) The frequency of recombination between the isolates of PRSV belonging to American and Asian lineages. Recombination frequency is the number of recombination events between two isolates. **(B)** Recombination sites were detected throughout in the PRSV genome except 6K1. PRSV-P India representing all the recombination sites of 5' UTR and P1 shows recombination with American and Asian PRSV lineages. Recombination sites at 5' region of PRSV were also detected between P & W isolates. Lines in bold represents the sites showing multiple recombination events.

reported from rest of the world with as much frequency as 36 **[Fig.28]**. When the biotypes of PRSV (P&W) were analyzed for recombination sites, 7 pairs (both inter and intra lineage) were showing sites mostly confined to 5'end of the genome representing 5'UTR, P1, HcPro, P3 and CI **[Fig.29].**

From this study *HcPro* emerged as a potential viral gene involved in host determination, synergism, antiviral silencing and symptoms expressions. Hence it is important to study the effect of physiological parameters such as temperature in symptoms expression and virus accumulation in relation to HcPro function.

Role of HcPro in symptom development and viral accumulation

In order to understand the role of HcPro in symptoms expression and virus accumulation during PRSV infection in papaya, *invitro* binding assay of HcPro with small RNAs (miRNA) were studied at different temperatures. Symptoms of infection by PRSV on papaya plants include leaf mosaic, blistering, shoe string, vein clearing and necrosis etc. Symptom expression induced by viruses in the host is greatly affected by temperature. Also, high or low temperature has been shown to affect the virus population of different viruses in various hosts. However, the definitions of 'high' or 'low' temperature vary from host to host as the perception of ambient temperature varies in plants. While the literature is vast in information regarding the effect of temperature on virus accumulation and symptoms expression, little is known about the effect of temperature on PRSV symptoms and accumulation and the molecular basis for this temperature dependent mechanism. Hence, keeping in view the climatic changes in India, it is important to characterize the temperature regulated PRSV infection cycle with respect to RNA silencing pathways regulated by HcPro.

Four plants of PRSV inoculated papaya were subjected to each temperature regime i.e., high, ambient and low. The high temperature regime (Day time 35±5°C. night time 30±5°C) and low temperature (Day time 15±5°C. night time 10±5°C) caused suppression in both incidence and rate of development of symptoms on *Carica papaya*, when compared with ambient temperature regime (Day time 26±5°C. night time 21±5°C). At high and low temperature, all the 8 plants showed negligible symptoms and particularly at high temperature there was complete loss of symptoms in all the 4 plants.

Table.11 Recombination sites and frequency in inter and intra lineage PRSV genomes and in each gene detected by Recombination Detecting Programme.

Region/gene	Recombination sites	Inter lineage*	Intra lineage*	Total recombination sites	Recombination frequency**
5′UTR	1-35	++	++	1	8
P1	96-122, 170-186, 206-219, 227-243, 284-299, 314-330, 338-354, 355-379, 371-385, 498-573, 564-581	++	++	11	28
HcPro	1906-1980, 2633- 2709	++	++	2	2
P3	3455-3573, 3575-3714, 3641-3739	++	++	3	5
CI	4773-4827, 5072-5147, 5993-6075	++	++	3	5
VPg+ CI+6K2	6137-7029		++	1	1
NIa	7346-7461		++	1	1
NIb	8801-8922		++	1	1
CP	10003-10110		++	1	1
CP+3′UTR	9848-10334		++	1	1

* + &- signifies the presence and absence of recombination
** recombination frequency denotes the number of recombination events at the same site

However at low temperature, 3 out of 4 plants showed minor mosaic symptoms. On all the plants tested at ambient temperature severe symptoms appeared as blistering, shoestring in leaves **[Fig.30]**. Rate and incidence of symptoms severity is summarized in **[Table.12]**.

Table.12 Incidence and severity of PRSV infection at three different temperature ranges

Temp^{0}C Day/Night	**Incidence**	**Severity**	**Days**	
			First	**Max Expression**
35±5 - 30±5	0	1	-	-
26±5 - 21±5	100	5.0	5	25
15±5 - 10±5	75	2.1	14	25

Virion accumulation as determined by ELISA studies in the plant leaves was affected differently by the three temperature profiles studied. In papaya, accumulation of PRSV was higher at ambient temperature regime. In contrast at high temperature, the virion concentration in *C. papaya* leaves was generally negligible in all plants and no significant differences between healthy and inoculated plants were detected. Although, virion accumulation at low temperature is comparatively higher than the high temperature treatment but not significant as compared to ambient temperature **[Fig.30]**.

Symptoms elicited by many viruses might be due to the developmental abnormalities caused by interference in miRNA metabolism. HcPro has been shown to interfere with activity of miR171 which directs the cleavage of several mRNA coding for scare crow transcription factors. In the earlier experiment, we have established that PRSV-HcPro regulates the host gene expression by direct binding with 21 nt miR171 duplexes. To correlate the effect of temperature and binding of miR171 by HcPro with the symptom development, gel mobility shift assay experiment was carried out at different temperature profiles. In a temperature dependent assay, the purified PRSV-HcPro protein binds 21 nt. ds miR171 duplexes more efficiently at ambient temperature (25ºC) than the high (35-40ºC) or low temperature (15ºC) tested **[Fig.30]**. Mobility shift of small RNAs was not detected when small RNA duplexes were incubated with PRSV

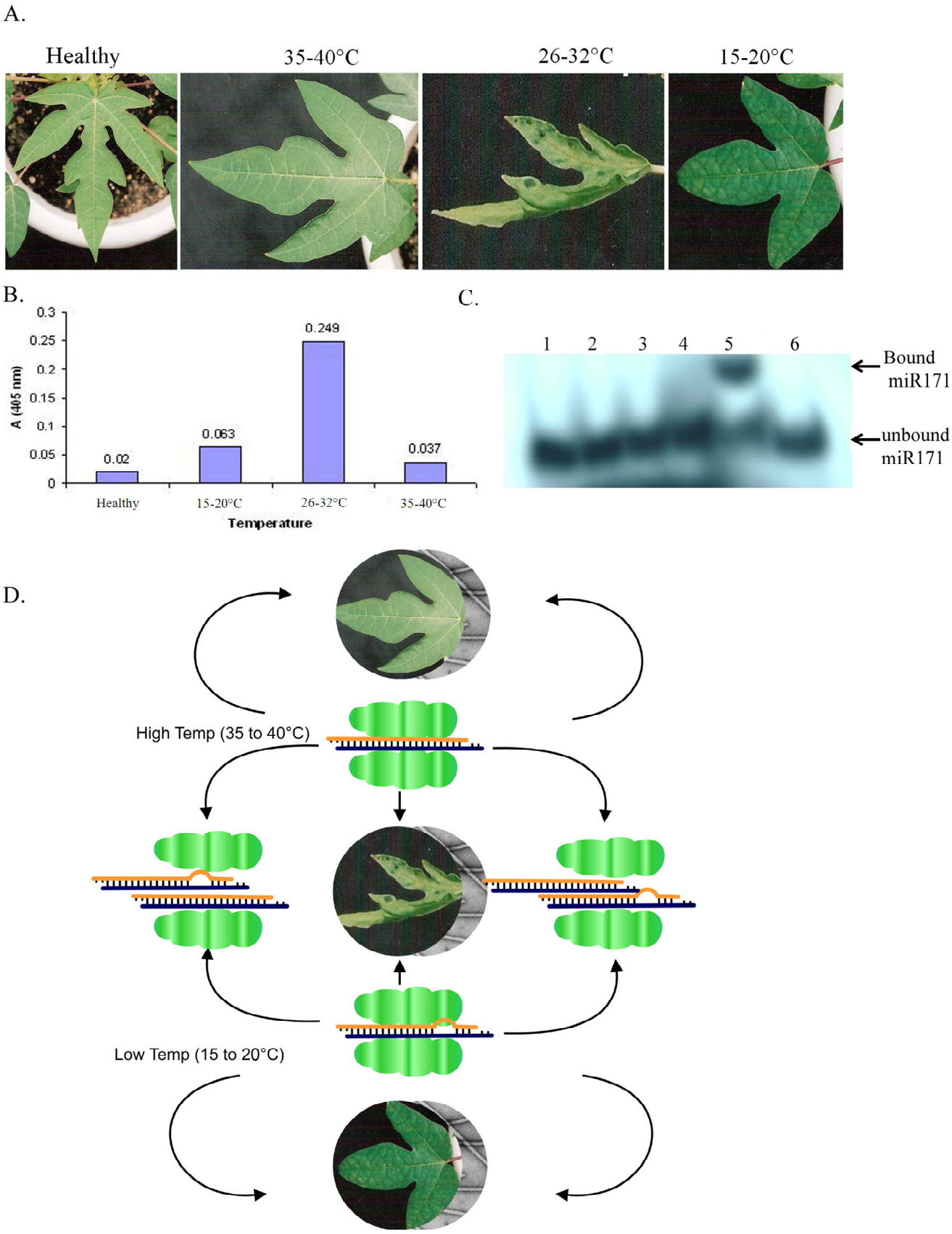

Fig.30 (A) Symptom development of PRSV at different temperature profile. **(B)** Level of vrius accumulation at three temperature regime. **(C)** Temperature dependent binding assay of small RNAs with HcPro protein, Lane 1: miR171 duplexes, Lane 2: miR171+MBP, Lanc 3: miR171+HcPro (45 ^{0}C), Lanc 4: miR171+HcPro (35 ^{0}C), Lanc 5: miR171+HcPro (25 ^{0}C), Lane 6: miR171+HcPro (15 ^{0}C). 20 microgram purified HcPro protein and 3ng small RNAs was used for binding assay **(D)** The proposed model for temperature dependent small RNA binding of HcPro in relation to symptom development.

HcPro at high or low temperature regimes. In summary, the small RNA binding ability of PRSV-HcPro was hampered at high or low temperature while the ambient temperature was shown to a necessary factor for small RNA binding. Small RNAs binding by viral suppressors has been shown to be affected by other physiological parameters such as pH and salt concentration.

Viruses are obligate intracellular parasites and use the silencing machinery for their survival and replication. The success of the virus essentially depends on its ability to efficiently and effectively use the host machinery to propagate itself. This dependence on the host also makes it susceptible to the host gene regulatory mechanisms. Though, the gene regulatory mechanisms involving both, host and viral proteins have extensively studied, data on small RNA mediated gene regulation in viral infection is just emerging. Small RNA molecule regulates gene expression by RNA interference in a sequence specific manner. One of the major roles of RNA silencing in plants is to provide a defense system against viruses. Therefore, viruses are under strong selection pressure to develop ways of evading or counter-acting the silencing machinery. Many, if not all, plant viruses and at least some animal viruses consequently encode proteins that suppress RNA silencing. Viral suppressor proteins have apparently evolved independently of each other because they exhibit a broad spectrum of activities and interactions with the host silencing machinery. The ability of viral silencing suppressors to interfere with different steps of RNA silencing pathways make them ideal tools to dissect these pathways. Among the important suppressor proteins involved in interfering host gene regulation are: the Potyvirus HcPro, Tombusvirus P19, Cucumovirus 2b and Geminivirus AC4. The present investigation sought to understand the role of suppressor proteins, Geminiviral AC4, Cucumoviral 2b and Potyviral HcPro on plant gene expression and regulation.

Geminiviral AC4: Genomics and functional attributes

Geminiviruses are inducers and targets of RNA silencing. In most of the cases geminiviruses encodes three pathogenicity determinants AC2, AC4 and βC1. Recent studies indicate their secondary role as a silencing suppressor (Voinnet *et al.,* 1999; Vanitharani *et al.,* 2004; Cui *et al.,* 2005). In two of geminiviruses, *African cassava mosaic virus* - Cameroon strain (ACMV-Cam) and *Srilankan cassava virus* (SLCMV), AC4 is characterized as a suppressor of RNA silencing (Vanitharani *et al.,* 2004; Chellappan *et al.,* 2005). Hence it is essential at this juncture to have information about this protein from *Papaya leaf curl New Delhi virus* (PLCV*)* causing a major loss in

papaya production. To explore the possibility of PLCV-AC4 involvement in host gene regulation, *Papaya leaf curl New Delhi virus*-AC4, has been characterized in terms of primary sequence data and was constitutively expressed in *N. tabacum*, *N. benthamiana* and *Solanum lycopersicon*.

ORF *ac4* is a small, embedded ORF within the replicase protein (AC1) coding ORF but with different reading frame (Vanitharani *et al.,* 2004). *ac4* gene of PLCV consists of 177 bp encoding a 58 amino acid long protein. Comparison of AC4 protein from different geminiviruses and leaf curl viruses affecting various hosts revealed some interesting facts. It showed close resemblance with AC4 protein of *Tomato leaf curl virus* while 28.5-30% similarity with AC4 of leaf curl infecting cucumber, chilly and tobacco suggesting its possible role in host specificity. Its length varied from 47-102 amino acids in different geminiviruses studied here indicating its possible evolution as a suppressor protein by overprinting, in which an existing coding sequence of AC1 is translated in a different reading frame (Li and Ding, 2006). The overprinting varied in different geminiviruses as per the need of selection pressure giving rise to highly heterogeneous protein that does not possess the suppressor activity in all the geminiviruses (Vanitharani *et al.,* 2004). In spite of its highly heterogeneous nature some of the conserved signature sequences in all the geminiviruses like ^{3}MG, ^{6}L, ^{11}S, ^{14}S, ^{26}SS, ^{31}P, ^{35}QHIS, ^{40}I, ^{42}T and ^{46}L suggests their involvement in regulation of small RNAs. Differential role of AC4 protein as an RNAi suppressor and having role in binding of small RNAs that leads to developmental defects (Chellappan *et al.,* 2005) provided insight to study the AC4 protein of PLCV in small non coding RNAs regulation and plant development.

For characterizing PLCV-AC4 role in plant development, it was constitutively expressed in three different hosts. All the three hosts, *N. tabacum, N. benthamiana* and *S. lycopersicon* harboring the PLCV-*ac4* transgene exhibited altered phenotypes which might be due to the interference of AC4 in host gene regulation through molecular switches. Severe developmental abnormalities in the form of blistering and curling of leaves and underdeveloped leaf lamina were observed in *N. tabacum*. Similar results were obtained in *Arabidopsis* by expression of ACMV-AC4 (Chellappan *et al.,* 2005). Various other suppressors like geminiviral AC2 and βC1 when expressed in transgenic plants like *Arabidopsis*, tobacco, showed similar developmental defects, reflecting their

probable role in binding the micro RNAs, which play a role in plant development (Sunter *et al.,* 2001; Cui *et al.,* 2004; Dunoyer *et al.,* 2004; Saeed *et al.,* 2005). AC4 caused severe stunting, unregulated differentiation and leaf malformations in the transgenic *N. benthamiana* but the abnormalities was dissimilar to *N. tabacum.* On the other hand PLCV-AC4 caused various observable phenotypic aberrations in S. lycopersicon on the growth and leaf development like needle shaped leaves, stunting, apical apoptosis, and aberrant organogenesis were the major aberrations noticed during somatic embryogenesis.

The disturbed phenotypes in the silencing suppressor expressing transgenic plants likely are due to the interference of these suppressors with the endogenous RNA silencing pathways. Previously, it has been shown that viral suppressors interfere with miRNA biosynthesis in *Arabidopsis* and alters their target genes regulation during plant development (Ray *et al.,* 1996; Jacobsen *et al.,* 1999; Llave *et al.,* 2002; Park *et al.,* 2002; Mallory *et al.,* 2002, 2004; Kasschau *et al.,* 2003; Vazquez *et al.,* 2004; Chapman *et al.,* 2004; Dunoyer *et al.,* 2004; Millar and Gubler 2005; Alvarez *et al.,* 2006). Such interference, targeted at an early step in the silencing pathways, would impair the regulation of multiple miRNA-regulated target genes, such as *SCL6,* targeted by miR171; NAC-domain proteins (*CUC1* and *CUC2*), targeted by miR164 (Rhoades *et al.,* 2002; Mallory *et al.,* 2004); *AP2*, and *ARF8* and *ARF 10*, coding for transcription factors, expressed specifically in inflorescence and leaves, respectively; and regulating their differentiation (Mallory *et al.,* 2002; Park *et al.,* 2002; Kasschau *et al.,* 2003; Chapman *et al.,* 2004; Dunoyer *et al.,* 2004). Constitutive expression of PLCV-AC4 in three different host caused severe phenotypic aberrations mostly related to leaf development, which might be due to the altered gene expression of various gene families like *GAMYB* like genes (e.g., *MYB33*) resulting in stunting, spindly growth, sterility, and reduced petiole lengths in *Arabidopsis* (Millar and Gubler 2005), and the *PHABULOSA* gene, causing leaves with upward curling (Mallory *et al.,* 2004).

Cucumoviral 2b: Genomics and functional attributes

CMV 2b was among the first suppressor identified that interferes with RNA silencing (Brigneti *et al.,* 1998). CMV 2b is the most differentially behaved RNAi

suppressor. 2b protein of two different subgroups of CMV regulates miRNA and siRNA driven pathways differentially. It has been shown that the *2b* gene from a severe strain causes more severe developmental abnormalities in *Arabidopsis* by altering the miRNA driven RNA silencing pathways (Zhang *et al.,* 2006; Lewsey *et al.,* 2007). On the other hand the *2b* gene from a mild strain of CMV (Kin) caused no developmental abnormalities, when constitutively expressed in *Nicotiana* spp (Siddiqui *et al.,* 2008), same results with another mild strain CMV-Q when expressed in *N. tabacum* (Ji and Ding, 2001) and *Arabidopsis* (Zhang *et al.,* 2006; Lewsey *et al.,* 2007) *were observed*. Considering that 2b suppressor is involved in different steps of RNA silencing and also playing important role in miRNA pathways, in strain specific manner, we investigated the behaviour of 2b protein from CMV- Indian isolate (CMV-NDLS).

Here we show that that CMV-NDLS possess two nuclear localization signals (NLSs) (^{22}RRQRRK^{27} and ^{33}RRER^{36}) similar to the severe strains (subgroup I). Phylogenetic analysis on the basis of 2b protein sequences also suggested its close resemblance with 2b from CMV subgroup I. Subgroup I strains of CMV are more virulent on tobacco than subgroup II strains (Palukaitis *et al.,* 1992; Zhang *et al.,* 1994).

2b of CMV-Fny (severe strain, Subgroup I) has recently been shown to affect the plant development by interference with Argonaute1 (Zhang *et al.,* 2006). Clustering of CMV-NDLS in subgroup I along with the CMV-Fny and the presence of two NLSs in CMV-NDLS similar to the CMV-Fny suggested that 2b protein from CMV-NDLS might be involved in regulating miRNA controlled developmental pathways. Since interference with miRNA pathway is a common feature of many other Subgroup-I 2b suppressors (Dunoyer *et al.,* 2004; Mlotshwa *et al.,* 2005), CMV-NDLS 2b was constitutively expressed *in planta* for deciphering its role in miRNA regulated pathways. The constitutive expression of CMV-NDLS 2b gene in *N. tabacum* displayed different developmental defects similar to 2b of CMV-Fny in Arabidopsis (Zhang *et al.,* 2006), mostly governed by a group of miRNAs regulating various transcription factors associated with developmental regulation of host plant **[Table.13]**.

Table.13 Plant developmental aberrations and their corresponding micro RNA regulated specific targets.

S.No.	miRNA	Consequences of altered expression	Target Gene family	References
1	miR 319	Un even leaf shape and curvature	TCP transcription factors	Palatnik *et al.,* 2003
2	miR 164	Aberrant leaf shape	CUC-1/CUC-2	Laufs *et al.,* 2004 ; Mallory *et al.,* 2004
3	Put-miR3	Early apical apoptosis, decreased apical dominance, uneven leaf shape/ curvature, agravitropic roots, decreased lateral rooting, shortened petiole, reduced stature and aberrant leaf shape	Not known	Pilcher *et al.,* 2007

Notably many of the defects in 2b over expressing lines like altered shoot meristem, narrow and serrated leaves, apical apoptosis, reduced leaf lamina and chlorosis might be due to interference with miRNAs like miR156, 159, 160 and 164. The significance of the 2b over expression in healthy tobacco and its role in miRNA metabolism is not entirely cleared but data on phenotypic aberrations in 2b transformed *N. tabacum* suggests the diversion of viral suppressor protein towards miRNA pathway in the absence of virus induced siRNA pathway. It is interesting to note that the phenotypic aberrations in *N. tabacum* due over expression of 2b protein in the presence and absence of virus greatly varied. The over expression of 2b gene in CMV infected N. tabacum displayed negligible phenotypic changes. Hence based on data it is proposed as

hypothesis that the 2b protein expression in the absence of virus may be diverted towards the miRNA metabolism and affects the host phenotypes severely. On the contrary, in the presence of virus, the suppressor protein function is more confined to the viral induced siRNA pathway and to other functions like movement of virion particle.

Silencing and over expression of *2b* gene in CMV infected tobacco did not show any significant difference in the existing virus accumulation. The 2b concentration did not show a direct relationship with virus population suggesting its interference at the initiation step of RNA silencing and once virus induced RNA silencing is established, increase or decrease in the concentration of 2b protein does not have any effect in RNA silencing and thereby the virus accumulation.

Potyviral HcPro: Genomics and functional attributes

The RNA genome of *Papaya ringspot virus*, a potyvirus (family Potyviridae) is translated into a polyprotein that is further processed by three virus-encoded proteinases (Carrington *et al.,* 1989a). One of these proteinases, HcPro, is a multifunctional protein (Maia *et al.,* 1996, Varrelman *et al.,* 2007): as a strictly cis-acting proteinase, it is responsible for its self-cleavage from the polyprotein precursor (Carrington *et al.,* 1989b) and besides, it is also involved in a number of infectious processes as diverse as aphid transmission (Govier *et al.,* 1977), cell-to-cell and long-distance movement (Rojas *et al.,* 1997; Saenz *et al.,* 2001), genome amplification and suppression of RNAi (Anandlakshami *et al.,* 1998). Many models of the mechanism of RNAi suppression by HcPro have been proposed since it came in to the light as a small RNA counteracting protein. HcPro protein of various potyviruses was demonstrated to act by blocking the step upstream or downstream or at the point of production of small RNAs by interfering the Dicer or RISC steps (Brigneti *et al.,* 1998; Kasschau and Carrington, 1998; Mallory *et al.,* 2001). Further, in a recent study, TEV-HcPro was shown to inhibit RNAi via binding to small RNAs size selectively and was shown to bind 21 nt siRNA duplexes with higher affinity (Lakatos *et al.,* 2006; Merai *et al.,* 2006). Hence it is important to characterize the role of PRSV-HcPro in small RNA regulation and various other molecular events. Sequence analysis of PRSV-HcPro and its comparison with other helper component proteinases derived from different potyviruses was carried out. Protein was

systematically compared for its domains involved in different functional attributes viz; transmission, silencing suppression and polyproteolytic activity.

In each functional module, all motifs were examined to explore evolutionarily conserved and divergent sequences. In addition, related motifs for inter and intra-species functional profiling of this multifunctional protein have also been examined. Proteins were analyzed by annotation through conserved domain protein architecture for each functional module and obtained a great discriminating power in the functional profiling. Despite the fact that only PRSV-*HcPro* gene was used in this study, a number of functional modules were identified that were conserved and thus predicted to be essential for performing multiple functions. Thus it generated a global conserved domain protein architecture and comprehensive functional portrait of HcPro, featured by conserved and divergent landscapes emphasizing fundamental and species-specific mechanisms. Sequence motifs comparison is a powerful predictor of functional mechanisms that are fundamental for multiple roles played by HcPro. Much of current knowledge of functional domains of HcPro is supported or reaffirmed by this correlation analysis, justifying the prediction. The most interesting in N-terminal domain is the first 100 amino acids having a putative Zn finger motif (Robaglia *et al.,* 1989) involved in virus transmission. Sequence analysis of this region strongly suggests that variability at the N terminal is due to host-virus interaction in different potyviruses. The N terminal region of the potyviruses (PRSV) from the same host papaya is found to be universally conserved reflecting its direct relationship with the host. Although the presence of conserved motif KITC having interaction with aphid stylets is found universally conserved in all potyviruses transmitted through aphids. Central region of HcPro from 101-300 amino acids is assumed to be important in genome amplification as well as PTGS suppression (Vargason *et al.,* 2003). It has two RNA binding domains, which is evident from high lysine, arginine and asparagine content in this region. Probably one is playing a role in viral RNA binding for genome amplification, which is found to be variable, as evident from its role in binding with diverse viral genomes. While other may be involved in binding with small RNAs to inhibit intermediate step of RNAi, which is size specific rather than sequence specific, hence more conserved. The highly conserved proteinase domain of HcPro has been mapped to the C terminal 157 amino acids and characterized

as a cystein protease like activity. The annotation of this domain with peptidase C-19L having unique property of deubiquitylation suggests its role in rescuing viral proteins from proteolytic cleavage with host proteosome (Ballut *et al.,* 2005).

Many double stranded RNA binding suppressors are evolutionarily unrelated, suggesting that the suppressor proteins have evolved independently. TEV-HcPro, a size selective dsRNA binding protein while HcPro of PVY showing no affinity for dsRNAs suggests the independent evolution of RNAi suppression mechanism of helper component proteinase from different potyviruses (Merai *et al.,* 2006). To characterize the small RNA binding ability of PRSV-HcPro, it was expressed and purified from *E. coli*. Generally, the biochemical functions of the potyviral HcPro has long been hampered by the difficulties encountered in obtaining the proper folded protein. Expression systems other than plants such as *E. coli* and insects cells infected with baculovirus produced HcPro protein that remained inactive when tested in aphid transmission assays (Thornburry *et al.,* 1993). However, biologically active HcPro protein was expressed and purified in the heterologous system, methylotrophic yeast *Pichia pastoris*, although the degree of transmission process was weaker compared to HcPro from plants (Ruiz-Ferrer *et al.,* 2004). Therefore, plant produced HcPro proteins seems to be more adequate to perform structural studies that could be correlated with its activity (Ruiz-Ferrer *et al.,* 2005). To date many *in vitro* functions has been ascribed to bacterially expressed HcPro (Carrington *et al.,* 1989; Maia and Bernardi, 1996; Urcuqui-Inchima *et al.,* 2000). Small RNA binding ability of TEV-HcPro extracted from plant systems have recently been tested (Lakatos *et al.,* 2006; Merai *et al.,* 2006). This study provides the first evidence of production of PRSV HcPro in *E. coli* expression system which is capable of small RNA binding. This suggests that PRSV-HcPro inhibits the intermediate step of RNA silencing via binding of small RNA produced by the Dicer enzyme. The strong affinity of PRSV-HcPro towards miR171 duplexes indicate that it interfere with both, mi and siRNA driven pathways by competing from RISC for small RNA duplexes. Therefore, PRSV-HcPro might be operating through sequestration model for suppressor function which is essentially a competitive inhibition model. Viral suppressor proteins like P19, P21, P15 and γB interfere in RNA silencing pathway by binding of small dsRNAs (Merai *et al.,* 2006). Similarly, highly conserved FRNK box in the HcPro of the potyviruses is a

probable of contact with siRNA and miRNA duplex (Shiboleth *et al.,* 2007). PRSV-HcPro binds 21 nt small RNA duplexes in a dose dependent manner which might be due to the requirement of host cellular factor that are involved in small RNA binding affinity as shown in case of TEV-HcPro (Silhavy *et al.,* 2002; Lakatos *et al.,* 2004; Merai *et al.,* 2006; Lakatos *et al.,* 2006) . In plants silencing generates only 21nt double stranded small RNAs from RNA viruses (Silhavy and Burgyan, 2004; Molnar *et al.,* 2005) and hence the expression of the protein that preferentially binds theses molecules appears to be an ideal viral counter defensive strategy. Silencing inhibition through small RNA binding seems to be advantageous as production of siRNAs is a conserved element of the antiviral silencing serve as primers to an RdRp and required to formation of active mobile complex.

The PRSV-HcPro has also shown dramatic effects on miRNA pathways and the normal progression through development due to the micro RNA sequestration. The effect of HcPro on miRNA pathways is manifest as an increase in the level of target mRNAs in some plant tissues (Mallory *et al.,* 2002; Kasschau *et al.,* 2003; Chapmann *et al.,* 2004; Dunoyer *et al.,* 2004). It is of interest to compare these observed phenotypes with other silencing suppressor transgenic plants described in the various studies. *HcPro* gene has been extensively studied, and transgenic plants harbouring either *HcPro* gene alone or together with *P1* gene from different potyviruses have been produced in *A. thaliana*, *N. benthamiana*, and *N. tabacum* species (Carrington *et al.,* 1990; Mallory *et al.,* 2001; Savenkov and Valkonen 2002; Mlotshwa *et al.,* 2002, 2005; Kasschau *et al.,* 2003; Pruss *et al.,* 2004; Shams-Bakhsh *et al.,* 2007). The *N. benthamiana* transformed in this work with the *HcPro* gene of PRSV displayed a clear phenotype, including curled leaves with changed vein patterns, soft stems, and strongly malformed leaves, stunting and unregulated differentiation. This is in contrast with PVY-HcPro and PVA-HcPro transgenic *N. benthamiana* and *N. tabacum* cv. Samsun NN plants where constitutive expression did not cause any phenotypic aberrations (Mlotshwa *et al.,* 2002; Savenkov and Valkonen 2002; Shams-Bakhsh *et al.,* 2007). On the other hand, severe malformations were reported in transgenic tobacco and Arabidopsis plants expressing TEV-HcPro and TuMV-*HcPro* (Anandalakshmi *et al.,* 2000; Kasschau *et al.,* 2003; Chapman *et al.,* 2004; Pruss *et al.,* 2004; Mlotshwa *et al.,* 2005). Behavior of HcPro from

different potyviruses and in different hosts may be due to specific binding of miRNAs in a host dependent manner.

Synergistic effects of viral suppressors

Synergistic viral diseases of higher plants are caused by the interaction of two or more independent viruses in the same host are characterized by dramatic increases in symptoms and in accumulation of one of the co-infecting viruses. The extent to which the synergistic viral interactions occur in higher plants and role they play in mediating plant disease is not really clear at this point. Moreover the basic background information about genes responsible for plant viral synergism is deficient. In some cases, synergistic interactions are mediated by proteins that have been shown to be suppressors of RNA silencing (Pruss *et al.,* 1997; Brigneti *et al.,* 1998; Saenz *et al.,* 2001; Qiu *et al.,* 2002; Selth *et al.,* 2004). Expression of such proteins from heterologous virus results in increased disease and/or virus accumulation in some host species, but not in others (Pruss *et al.,* 1997; Brigneti *et al.,* 1998; Qiu *et al.,* 2002; Fukumoto *et al.,* 2003; Gonza´lez-Jara *et al.,* 2004). Synergy of viruses was mimicked in transgenic plants expressing the 5' proximal region of the potyviral genome, showing synergistic effect on infection with PVX (Vance *et al.,* 1995). Furthermore, mutations in the coding central region of HcPro abolished the PVY-PVX synergism, indicating direct involvement of the potyviral HcPro in the synergistic response (Shi *et al.,* 1997; Gonzalez Fara *et al.,* 2005). Functional map of central region of this protein include the RNA silencing, symptom expression, and genome amplification (Valli *et al.,* 2007; Shiboleth *et al.,* 2007; Gonzalez Fara *et al.,* 2005). The fact that the mutations in the central region of HcPro impair the protein for synergism as well as RNA silencing suppression suggests that the synergistic effect and RNA silencing are the interlinked functions (Shi *et al.,* 1997). Similarly, 2b protein from cucumovirus played important role in synergistic viral disease development as the CMV 2b protein enhanced the pathogenecity when expressed from PVX vector (Lucy *et al.,* 2000). In addition, synergy among the suppressors were also studied by Vanitarani *et al* (2004) by demonstrating that the two gene, geminiviral EACMCV *ac2* and ACMV *ac4* are involved in enhanced viral DNA accumulation and are able to suppress locally induced silencing. In present study using the suppressor protein mediated synergistic effect, it was demonstrated that HcPro is involved in the enhanced accumulation of CMV

and ToLCV while AC4 and 2b expression had no effect on the accumulation of CMV. Also the 2b expression did not influenced ToLCV population significantly. Molecular basis of viral synergism in relation to silencing suppressor proteins from diverse origin has been established during present investigation, but the synergism is a highly specific phenomenon and dependent on various other factors such as host specificity, interaction between different viral components and their replication strategy.

Thus the basic fact in involvement of viral suppressor proteins in synergistic viral disease development will be complemented with other features of host virus interactions

Structural genomics of PRSV components

In order to understand the more clear mechanisms of HcPro in relation to RNA silencing, small RNA regulation and synergistic effects, it is important to study other PRSV genomic components.

Full length sequence of PRSV-P Indian isolate has been generated to study the variability and recombinational events in different viral genes/cistrons in order to establish host specificity and geographical (Bateson *et al.,* 2002). Molecular variability of PRSV genomes and the recombinational events shaping the PRSV genome dynamics were studied carefully. The phylogenetic analysis of PRSV isolates supports and extends the earlier results of constituting two separate lineages i.e, Asian and American isolates (Nao Carrazana *et al.,* 2007). But unique finding in the present study includes Indian isolate despite being the Asian isolate, cluster with American lineage. Not only whole genomes polyprotein, rather majority of cistrons based clusters followed the same pattern. The grouping of Indian isolate with those of Americans (Brazil, Mexico and Hawaii) and the level of divergence seen in coat protein gene among the Indian isolates (Jain *et al.,* 1998; Bag *et al.,* 2007) certainly implicates Indian subcontinent in the spread of PRSV to these countries. Close sequence similarity between two pathotypes of PRSV (P&W) from same geographical region suggests that they may be evolved by mutations (Bateson *et al.,* 1994). Sequence similarity matrix generated from whole genome sequences of PRSV isolates reported from different geographical locations and their individual genetic components revealed that all the cistrons follow the divergence exhibited by the whole genome except the P1 which is much more variable as compared

to full genome. When we looked for the divergence between the two pathotypes from the same geographical location (P-Thai and W-Thai, P-India and W-India), P1 shows the maximum divergence suggesting its involvement in host specificity.

The present study of recombination in 13 complete genomes of PRSV supports that the recombination is a dominant feature of RNA virus evolution (Lai, 1992; Worobey and Holmes 1999). More number of complete genome sequences available in GenBank, particularly from India, allowed to identify the more robust occurrence of recombination sites and hotspots which was a major constraint in previous studies (Noa Carrazana *et al.,* 2007). Molecular architecture of the PRSV genomes based on recombination provides better understanding in terms of evolution, host specificity and geographical distribution. Recombination sites detected *in silico* suggest that the whole PRSV genome is prone to recombination though the more hotspots are confined to the 5' end of the genome which is in consistent with other potyviruses (Oshima *et al.,* 2007). Most of the recombination sites are present in the *P1* gene followed by, *P3, CI* and *HcPro* genes, this suggests that 5' end of the genome has played a vital role in the genome dynamics of PRSV. No recombination sites in the *6K1* gene and 3' end of the genome shows the important role of these proteins in virus survival and therefore the genetic changes are intolerable. In this study the clusters of recombination sites were found in the 5' UTR and *P1* gene suggesting they are the vulnerable generic regions in shaping of the PRSV genomes. It is noteworthy that all the recombination sites detected in this P1 region contained PRSV India as a common parent, strengthening the concept of PRSV origin in Indian subcontinent (Bateson *et al.,* 2002). Based on the coat protein gene analysis, Indian isolates were shown to be the most heterogeneous (Jain *et al.,* 1998; Bag *et al.,* 2007), suggesting that this was the population from which the other isolates originated. It is however, not possible to provide a definite proof to this hypothesis, unless the same conclusion will have been drawn with more number of full PRSV genomes from Indian subcontinent. Moreover, the available number of full genome sequences does not cover the PRSV isolates from other Asian, American, Australian and particularly African countries. PRSV-P evolved on many occasions from PRSV-W, and it was speculated based on *cp* gene analysis only that recombination is much less significant than mutation in the molecular evolution of PRSV (Bateson *et al.,* 2002). In

contrast to this, based on full genome recombination analysis it is demonstrated here that the recombination is significant event between two pathotypes. 8 recombination sites with 15 recombination frequencies were detected between P and W pathotypes of PRSV, which are scattered throughout the genome, though majority in 5' UTR and P1 regions. Given the fact that very few full genomes of PRSV-W are available till date, more data on PRSV-W will add and strengthen to this concept. The greatest variability as well as maximum number of recombinational sites occurred in the N terminus of the P1 protein; however there was still considerable conservation towards C terminus of the protein. C terminus of the P1 does not show any recombination site which posses the serine protease cleavage activity necessary for viability of virus (Reichmann *et al.,* 1992; Klein *et al.,* 1994; Verchot and Carrington, 1995a, b; Yang *et al.,* 1998; Urcuqui-Ichima *et al.,* 2001). These results suggest that the primary function of the N terminal region of P1 protein is the molecular remodeling of the PRSV genome under the various selection pressures as this region is the facilitator in amplification and movement but is not essential for viability. Similar results are reported from other potyviruses where recombination is a major event in N terminal region of the P1 protein (Valli *et al.,* 2007).

HcPro: a key player involved in symptom development

The interaction between papaya host and PRSV is complex because of different PRSV strains with different virulence and multiple papaya genotypes conditioning the host response. Results in the present investigation indicate that environmental factor such as temperature plays an important role in the PRSV-Papaya interaction. Temperature had a marked effect on symptom expression in PRSV infected papaya plants. The optimum temperature for symptom development in PRSV infected papaya appeared to be 26-32ºC. Lower temperature (15-20ºC) tended to slow down the plant growth and delayed viral symptoms expression. Similar results have been reported in other host virus system where low temperature affected symptoms development. Zheng *et al,* (2005) reported that low temperature (10-15ºC) inhibited *Soybean mosaic virus* (SMV) replication and movement and had a great influence on symptom development. High temperature (35-40ºC) also delayed symptom development in PRSV infected papaya which have been reported in other host-virus systems. *Banana streak badnavirus* showed more severe symptoms and had significantly higher virus titre at 22 ºC than the

plants that grew at 28-35 ºC (Dahal *et al.,* 1998). The present work has also shown that accumulation of virus is also affected by the temperature to which the plants are exposed. Though, the anatomy and the physiology of the host as well as the environmental factors such as temperature and water stress affect virus mobility. The differences among the hosts in accumulation of viruses during disease development may not be explained exclusively by host physiology and anatomy (Gilbertson and Lucas, 1996; Llamas-Llamas *et al.,* 1998). A recent report revealed that the RNA silencing mediated defense response is temperature dependent by the fact that *Cymbidium ringspot virus* induced symptom severity was found to be higher at low temperature and decreased with rising temperature and elevated the levels of virus derived siRNAs (Szittya *et al.,* 2003). The similar results were drawn by Chellappan *et al,* (2005) where they observed that Cassava geminivirus induced RNA silencing is also increased with rising temperature. However the role of temperature in symptom development and virus accumulation can not be generalized as the definition of high and low temperature varies from host to host. Zheng *et al,* (2005) reported the 25-32ºC temperature requirement for the symptom development of *Soyabean mosaic virus* in soyabean while the 15ºC temperature was reported for the symptom development of *Cymbidium ringspot virus* in *Nicotiana benthamiana* (Szittya *et al.,* 2003). Therefore, the symptom development and virus accumulation varies from host to host and virus to virus at different temperature regimes. Since the replication of is viruses is known to be inhibited by high or low temperature, one can assume that the activity of viral silencing suppressors varies over the temperature ranges that interfere the viral systemic infection. Conversely, it can be predicted that at ambient temperature of plant, the RNA silencing would be more readily overcome by the viral silencing suppressors. Given the fact that the suppressors acts through the binding of the small RNAs (Lakatos *et al.,* 2006; Merai *et al.,* 2006) which is greatly affected by physiological parameters such as pH and salt concentration (Koukiekolo *et al.,* 2007), temperature dependent binding of small RNAs by PRSV-HcPro suggests that along with the pH and salt concentration, temperature is also an important physiological parameter to affect the small RNA binding of viral suppressors. These proteins might be adapted according to the host physiology and thereby the effect of temperature in symptom development and virus accumulation varies host to host.

The reprogramming of host gene regulation by viral suppressors of RNA silencing has been an established fact. The present study has highlighted basic understanding of the role of these suppressor proteins in small RNA metabolism. The over expression of the viral suppressor proteins from three different origins in different host causes phenotypic aberrations which is a reflection of altered plant gene regulation. Co-expression of viral suppressors in a common host helps in synergistic development of viral diseases which may vary from virus to virus and host to host. Viral symptom development in the plant may also be linked to affinity of these suppressor proteins for small RNAs. Beside, suppressor proteins, different viral component and host factors are playing a crucial role in host viral interactions.

6.SUMMARY

RNA interference (RNAi) is a general phenomenon in eukaryotic organisms and plays important roles in diverse biological processes including developmental regulation, chromatin remodeling and antiviral defense. As a counter-defense, viruses are evolved with the diverse strategies for evading RNA silencing immunity. Besides being an area of intense, upfront basic research, the process is gaining importance in better understanding of co-evolution of molecular arm race between virus and host. Given the virus specific suppression mechanisms with which the phenomenon works, it is conceivable that more and more studies in this arena will help in elucidating the virus induced altered host gene regulation and their various secondary consequences in the form of virus and host specific symptoms. *To explore the molecular mechanism of viral synergism, an important threat of severe diseases and emergence of new viruses, RNA silencing pathways and suppressor proteins are the areas of attention. Beside the suppressor proteins, role of other components of genome in the virus host interaction and in the evolution of host specific strains is equally important. Among the various viruses causing severe losses in plant products, viral suppressors from three viruses belonging to different genera; Papaya ringspot virus (Potyvirus), Papaya leaf curl virus (Geminivirus) and Cucumber mosaic virus (Cucumovirus) were studied to explore their role in host gene regulation.*

The following are the major findings of present study:

- For characterizing AC4, the geminiviral suppressor of RNAi , *Papaya leaf curl virus* (PLCV)-*ac4* was sequenced and characterized.

    1. AC4 protein was highly variable among different geminiviruses in terms of amino acids sequence (70-71.5%) and the length (47-102).

    2. Its expression *in planta* showed malformed stems with bending and twisting, narrow & needle shaped leaves and apical apoptosis in *S. lycopersicon*, while major phenotypic aberrations observed in *N. benthamiana* were unregulated differentiation, severe stunting & malformed leaves. Similarly, stunting &

malformed leaves with blisters were observed in *N. tabacum* suggesting its probable role in host specific gene regulation.

- For characterizing 2b, the cucumoviral suppressor of RNAi , *Cucumber mosaic virus* (CMV)-*2b* was sequenced and characterized.

3. Based on phylogenetic analysis and presence of two putative nuclear localization signal sequences (NLSs) in the 2b protein suggested the resemblance of CMV-NDLS with subgroup I.
4. Constitutive expression of *2b* gene in healthy causes unregulated differentiation, chlorosis, apical apoptosis and underdeveloped leaf lamina, while, its over expression in CMV infected *N.* tabacum produced negligible phenotypic aberrations.
5. 2b over expression and silencing in CMV infected *N. tabacum* plants do not alter existing viral titer.
6. This study demonstrates that constitutive expression of RNAi suppressor 2b in a common host differentially regulates the small RNA metabolism in the presence and absence of virus.

- For characterizing HcPro, the potyviral suppressor of RNAi , *Papaya ringspot virus* (PRSV)-*HcPro* was sequenced and characterized.

7. The cross-protein conservation revealed the close resemblance of protease domain of HcPro with the peptidase C19L suggesting the deubiquitinylation role of this proteinase in rescuing the viral proteins from degradation.
8. PRSV-HcPro, an RNAi suppressor was shown to have affinity for 21 nt dsRNAs (miR171) in a dose dependent manner.
9. Its expression in prokaryotic system did not hamper its binding activity of small RNAs.
10. The phenotypic aberrations observed in *Nicotiana benthamiana* due to its over expression were unregulated differentiation, chlorosis, apical apoptosis and

underdeveloped leaf lamina suggesting its interference in miRNA turnover in host.

11. High (35-40ºC) and low (15-20ºC) temperature caused suppression in both, viral symptoms and multiplication, while, at ambient temperature (26-32ºC) typical PRSV symptoms such as blistering and shoestring in leaves and high multiplication of virus was observed. The small RNA binding ability of PRSV-HcPro only at ambient temperature suggested the possible role of viral suppressor of RNA silencing in temperature dependent viral symptoms and multiplication.

- Molecular basis of viral synergism in relation to silencing suppressor proteins from diverse origin was studied.

12. *HcPro* over expression caused increased population of CMV and ToLCV in *N. tabacum* suggesting its positive synergism with the two heterologous viruses CMV and ToLCV.

13. *2b* and *ac4* over expression did not show a significant change in population of CMV and ToLCV in *N. tabacum*.

- **Complete** genome sequence of *Papaya ringspot virus* (PRSV), generated in this study, was analyzed.

14. Phylogenetic analysis based on full genome polyprotein and individual cistron suggested close resemblance of PRSV-P India with PRSV isolates of America lineages strengthening the concept of origin of PRSV in Indian subcontinent.

15. In recombination analysis PRSV-P India appeared as a common parent showing recombination with both, PRSV from American and Asian lineages, providing another proof for origin of PRSV in Indian Subcontinent.

16. High degree of recombination and sequence variability at the 5' end of the PRSV genome between two pathotypes of PRSV suggested the probable evolution of host specific pathotypes of PRSV through recombination.

Many questions concerning the mechanism of viral suppressors of RNA silencing remain to be addressed. Regulation of miRNA by viral suppressors is an important key to decipher the altered host gene expression during viral infection leading to host and virus specific symptom development.

Name	:	Satendra Kumar Mangrauthia
Roll No.	:	9079
Degree for which thesis submitted	:	Ph.D.
Discipline	:	Biochemistry
Date of joining the P.G. School	:	August 6, 2004
Date of thesis Seminar	:	January 5, 2008
Date of submission of Thesis	:	March , 2008
Major field	:	Biochemistry
Minor field (s)	:	• Molecular Biology and Biotechnology • Genetics
Name of the Chairperson	:	Dr. Shelly Praveen
Advisory Committee Members		
Co-chairperson	**:**	Dr. Archana Sachdeva
Members	:	Dr. R.K. Jain Dr. T.R. Sharma Dr. A.K. Singh
Title of Thesis	**:**	"Characterization of viral suppressors of RNA silencing and their regulatory role in plant gene expression"

ABSTRACT

The RNA silencing pathway in plants presents a formidable defense against molecular parasites and regulates many processes including development, maintenance of genome stability and antiviral response. Many, viruses consequently encode proteins that

suppress RNA silencing which allow the viruses to proliferate in their specific hosts. Viral suppressor proteins from different viral origin were studied for their individual and synergistic role in host gene regulation. RNA silencing suppressors from *Papaya leaf curl virus* (PLCV), *Cucumber mosaic virus* (CMV) and *Papaya ringspot virus* (PRSV) were sequenced and characterized. AC4 suppressor protein of PLCV was highly variable among different geminiviruses in terms of amino acids sequence and the length. Its expression *in planta* showed different phenotypic aberrations in three different solanaceous hosts suggesting its probable role in host specific gene regulation. 2b suppressor protein of CMV possesses two putative nuclear localization signal sequences (NLSs) and its phylogenetic analysis suggested the resemblance of CMV-New Delhi Isolate with subgroup I of CMV. Constitutive expression of *2b* gene in healthy and CMV infected *Nicotiana tabacum* caused differential phenotypic changes. Moreover, its over expression and silencing in CMV infected *N. tabacum* plants did not alter existing viral titer. This study demonstrated that constitutive expression of RNAi suppressor 2b in a common host in the presence and absence of virus differentially regulates the small RNA metabolism. HcPro suppressor protein of PRSV exhibited close homology with the Peptidase C19L that suggests the deubiquitinylation role of HcPro in rescuing the viral proteins from degradation. PRSV-HcPro was shown to have affinity for 21 nt dsRNAs (miR171) in a dose dependent manner. Recombinant PRSV-HcPro had shown similar affinity for small dsRNAs. The phenotypic aberrations observed in *Nicotiana benthamiana* due to its over expression suggested its interference in miRNA turnover in host. Its interference in miRNA metabolism, might be correlated with PRSV symptoms development and viral population in a temperature dependent manner. The viral

synergism in relation to silencing suppressor proteins from diverse origin was also studied. *HcPro* over expression showed positive synergism with the two heterologous viruses CMV and ToLCV, while *2b* and *ac4* over expression did not show a significant change in population of CMV and ToLCV showing no role in synergistic interactions. Full length genome of PRSV was deciphered for determining the viral components and their role in different pathotypes. Phylogenetic and recombination analysis suggested origin of PRSV in Indian subcontinent. The divergence of two biotypes of PRSV was studied based on recombination and sequence similarity analysis suggesting 5' end of the genome playing most important role in PRSV genome architecture.

RNA रव–शामक के विषाण्विक निरोधकों का गुण–निर्धारण और पादप जीन अभिव्यक्ति में उनकी नियामक भूमिका

सारांश

पौधों में RNA रव–शामक पाथवे आण्विक परजीवियों के विरुद्ध उल्लेखनीय प्रतिरक्षा प्रदर्शित करता है और विकास, जीनोम स्थिरता के अनुरक्षण एवं प्रतिविषाण्विक प्रत्युत्तर जैसी अनेक प्रक्रियाओं को विनियमित करता है। परिणामस्वरूप अनेक विषाणु उन प्रोटीनों को इनकोड करते हैं जो RNA रव–शमन का निरोध करते हैं जिससे विषाणु अपने विशिष्ट परपोषियों में संक्रमण उत्पन्न करते हैं। विभिन्न विषाण्विक मूल की विषाण्विक शामक प्रोटीनों का अध्ययन उनके विशिष्ट गुणों और पोषक जीन विनियमन में उनकी संश्लेषी भूमिका के लिए किया गया। इसके लिए पपीते के पत्ती मोड़क विषाणु या *पपाया लीफ कर्ल वाइरस* (पीएलसीवी), खीरा–ककड़ी चित्ती विषाणु या *कुकम्बर मोज़ेइक वाइरस* (सीएमवी) और पपीते का छल्ला धब्बा विषाणु या *पपाया रिंगस्पॉट वाइरस* (पीआरएसवी) का क्रमण करके उनका गुण–निर्धारण किया गया। पीएलसीवी की AC4 निरोधक प्रोटीन, एमीनो अम्लों के क्रमण और लम्बाई के संदर्भ में विभिन्न जेमिनीविषाणुओं में अत्यधिक विविधतापूर्ण थी। पौधे में इसकी अभिव्यक्ति के दौरान तीन विभिन्न सोलेनेसी कुल के पोषकों में विभिन्न गुणप्ररूपी विपथन देखा गया जिससे पोषक विशिष्ट जीन विनियमन में इसकी संभावित भूमिका का संकेत मिलता है। CMV की 2b निरोधक प्रोटीन में दो तथाकल्पित नाभिकीय स्थानिक संकेत क्रम या न्यूक्लियर लोकलाइज़ेशन सिग्नल सिक्वेंसिस (NLSs) होते हैं और इसके जातिवृत्तीय विश्लेषण से CMV-New Delhi आइसोलेट की CMV के उपसमूह I के साथ समानता देखी गई। स्वस्थ तथा CMV संक्रमित *निकोटीना टबेकम* में *2b* जीन की रचनात्मक अभिव्यक्ति के कारण विभिन्न प्रकार के गुणप्ररूपी परिवर्तन उत्पन्न हुए। तथापि, CMV से संक्रमित *एन.टबेकम* के पौधों में इसकी अति अभिव्यक्ति और इसके शमन से विद्यमान विषाण्विक टाइटर में कोई परिवर्तन नहीं हुआ। इस अध्ययन से यह प्रदर्शित हुआ कि विषाणु की उपस्थिति या अनुपस्थिति में किसी सामान्य पोषक में RNAi निरोधक की रचनात्मक अभिव्यक्ति, लघु RNA चयापचयन को विभिन्न प्रकार से विनियमित करती है। PRSV के HcPro निरोधक प्रोटीन ने पेप्टाइडेज़ C19L के साथ घनिष्ठ समांगता प्रदर्शित की जिससे विषाण्विक प्रोटीनों के बचाव में

HcPro की डियूबिक्यूटीनाइलेशन भूमिका का संकेत मिलता है और इससे विषाण्विक प्रोटीनें अपघटित नहीं होती हैं। PRSV-HcPro में 21 nt dsRNAs (miR171) के लिए सहजननता थी जिस पर औषध की खुराक के कारण कोई प्रभाव नहीं पाया गया। *निकोटीना बेंथामियाना* में इसकी अति अभिव्यक्ति के कारण गुण–प्ररूपी विपथन देखा गया जिससे पोषक में miRNA टर्नओवर की दृष्टि से हस्तक्षेप का संकेत मिलता है। miRNA चयापचयन में HcPro के हस्तक्षेप का संबंध PRSV लक्षणों के विकास और तापमान पर आधारित विषाण्विक जनसंख्या की वृद्धि से हो सकता है। विभिन्न स्रोतों से प्राप्त रव–शमनकारी निरोधक प्रोटीनों के संदर्भ में विषाण्विक सहजनन का अध्ययन भी किया गया। *HcPro* की अति अभिव्यक्ति से दो विषमांगी विषाणुओं CMV और ToLCV में सकारात्मक सहजननता पाई गई, जबकि *2b* और *AC4* की अभिव्यक्ति से CMV तथा ToLCV जनसंख्या में कोई महत्वपूर्ण परिवर्तन नहीं पाया गया जिससे सहजनन अंतरक्रियाओं में इनकी किसी भी प्रकार की भूमिका के न होने का संकेत मिलता है। विषाण्विक घटकों तथा विभिन्न रोगप्ररूपों में उनकी भूमिका का पता लगाने के लिए PRSV का सम्पूर्ण लंबाई वाला जीनोम डेसिफर किया गया। जातिवृत्तीय और पुनर्संयोगी विश्लेषण से भारतीय उपमहाद्वीप में PRSV के मूल उद्भव का संकेत मिलता है। PRSV के दो जैवप्ररूपों में अपसारण का अध्ययन किया गया जो पुनर्संयोग तथा क्रम समानता विश्लेषण पर आधारित था जिससे यह संकेत मिला कि जीनोम का 5′ छोर PRSV जीनोम आर्कीटैक्चर में महत्वपूर्ण भूमिका निभाता है।

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APPENDIX-I

1. Test Plants

Papaya (*Carica papaya*) infected with PLCV, tobacoo (*N.tabacum*) infected with CMV and ToLCV and papaya infected with PRSV at three temperature ranges (15-20ºC, 26-32ºC and 35-40ºC) were maintained in glass house and phytotron under insect free conditions.

2. Isolation of Total DNA/RNA from Infected Leaf Sample

Total DNA/RNA from virus infected leaf samples, showing symptoms, were isolated by DNeasy/ RNeasy Plant Mini Kit provided by Qiagen.

- 100 mg of washed and dried leaf samples were ground in liquid nitrogen to a fine powder using a sterile mortar and pestle. Sample powder and liquid nitrogen were transferred to an appropriate size sterile eppendorf tube and the liquid nitrogen was allowed to evaporate but care was taken so that sample does not thaw.
- Subsequent steps were followed as per manufacturer's instructions.

3. Polymerase Chain Reaction (PCR) and RT-PCR Amplification

Specific primers were synthesized from Microsynth Pvt. Ltd. Sequences of *ac4* gene primers were based on sequence of ToLCNDV (Sinha *et al.,* 2004). Sequences of 2b gene primers were based on sequence of CMV available in NCBI database. Sequences of HcPro gene primers and primers for the other PRSV genomic components were based on sequence of PRSV available in NCBI database. Details of the primers used in this study are given below in Table.

Table. Details of primers and PCR profile used in the present study

S.No.	Primer name	Primer sequence (5'-3')	PCR Programme	Region	Amplicon size
1	GF GR	AAATAAAACATCTCAACACAACACAAT CCAGTTTTGATCTACCATCAAATC	94---5min 94---30 sec 64---40 sec 72- 1.5 min 72--10 min	5' UTR and P1 0f PRSV	~1.6 Kb
2	HC-PRO Forward- BR	TGA TGG TAG ATC AAA ACT GGC GCGTAGGTTTTTTCCACAGCCTCACG	94---5min 94---30 sec 50---40 sec 72- 1.5 min 72--10 min	HcPro and part of P3 of PRSV	~1.8 Kb
3	P3F P3R	CTA TTG AGT GTG TAT GCG AAT TC TTC TCA CAA AGA GGT CTG GTG	94---5min 94---30 sec 59---40 sec 72- 1.0 min 72--10 min	Part of P3 and Part of 6K1 of PRSV	~1.2 Kb
4	RKJ31 RKJ30	TGTGAT/AGGAGCGTGAA/GCCAAG GCCACTAGTG/TGAA/GATTTCAGT	42---45 min 94---30 sec 58---1 min 72---1.0 min 72---10min	6K1 and part of CI of PRSV	1.1Kb
5	RKJ29	ACTGAAATT/CTCC/AACTAGTGGC	42---45 min	Part of CI and	1.23Kb

	RKJ28	CTGT/CGC/GGCA/GGAGAAACCTTG	94---30 sec 58---1 min 72---1.0 min 72---10min	6K2 of PRSV	
6	RKJ27 RKJ26	GTG/ATTTCAC/TCAAGGTTTCTC TAG/ACTCCAGTGTTTG/ACTCC	42---45 min 94---30 sec 51---1 min 72---1.0 min 72---10min	6 K2, VPg and part of NIa of PRSV	1.1Kb
7	RKJ25 RKJ24	TTA/GACT/ATGGAGC/TAAACACTGG CCCATGAC/TTCTATCATTGC	42---45 min 94---30 sec 51---1 min 72---1.0 min 72---10min	Part of NIa and NIb of PRSV	1.45 Kb
8	HRP 50 RKJ3	ATG ATA GAG TCA TGG GG GTTGCGCATACTCAGAG	42---45 min 94---30 sec 46---2 min 72---1.0 min 72---10min	Part of NIb and CP of PRSV	1.1Kb
9	HRP 4 RKJ32	TGGTGCATA/T/CGAA/C/G/T AATGG CTCTCATTCTAAGAGGC	42---45 min 94---30 sec 48---1 min 72---1.0 min 72---10min	Part of CP and 3' UTR of PRSV	700bp
10	SP1F SP1R	GGA GGG CCC ATG GCC AAT GAC GTG GCT GAA AAA TTC TG CCG CTC GAG TCA GCC GAC AAT GTA GTG CTT CAT T	94---5min 94---30 sec 60---40 sec 72- 1.5 min 72--10 min	HcPro with 5' *Apa* 1, Met and Ala codons and 3' stop codon and *Xho*1 site	~1.4 Kb used for binary vector construct preparation
11	SP2F	CCG GAA TTC AAT GAC GTG GCT GAA	94---5min	HcPro with 5'	~1.4KB used for

	SP2R	AAA TTC TG CCG CTG CAG TCA GCC GAC AAT GTA GTG CTT CAT T	94---30 sec 60---40 sec 72- 1.5 min 72--10 min	*Eco*R1, and 3' stop codon and *Pst*1 site	Protein expression in pMAL vector
12	2B FORWARD 2B REVERSE	5'ATG GAA TTG AAC GAA GGC GCA G 3' 5'TCA GAA CGA CCC TTC CGC CC3'	94--2.5min 94---30 sec 51---40 sec 72- 1.0 min 72--10 min	2b ORF	~336 bps
13	AC4 forward AC4 Reverse	CACGAGCAGATCGTCCATC CCAACTGCGAAGAAATTCATC	94^{o}C ---30 sec 56^{o}C ---40 72^{o}C --- 30 sec 72^{o}C --- 10 min	AC 4 region	~250 bps

3.1. ac*4, 2b, HcPro* and other genomic components of PRSV

Using these specific primers, *ac4, 2b, HcPro* and other genomic components of PRSV were amplified from total DNA or RNA isolated from all the infected leaf samples mentioned above.

cDNA synthesis

For cDNA synthesis, RT (NEB) was used. cDNA was synthesized using the reverse primer and the reaction mix was prepared in 200µl microfuge tube. The reaction was carried out at 42ºC for 60min. The protocol followed for cDNA synthesis is given below.

10µl template RNA was mixed with 1µl (100 pM/µl) reverse primer. The mixture was heated for 70ºC at 5 min, followed by a short spin for 10 sec after chilling on ice. The reaction mixture for RT was prepared and added template mixture to it and mixed well. The reaction mix was heated to 25ºC for 5 min followed by 42ºC for 60 min for reverse transcription. Heated the reaction mix at 70ºC for 15 min.

The PCR reaction mixtures were prepared as follows

Reagents	**Volume required (µl)**
RT buffer (10 X)	4
25 mM $MgCl_2$	3
10 mM dNTPs	1
Reverse primer100pM (100pM/µl)	1
RNase inhibitor	1
Reverse Transcriptase (5 units/µl)	1
RNA template	10
RNase free water	19
Total	40

Reagents	**Volume required (µl)**
Total DNA/cDNA (25 ng/µl)	2
Forward primer (200 ng/µl)	1
Reverse primer (200 ng/µl)	1
10 X PCR buffer	2
10 mM dNTPs	1

Taq DNA polymerase	1
Nuclease free water	12
Total	20

After PCR reaction was over, 1 µl of amplified product was subjected to electrophoresis in 1% agarose gel to observe the DNA fragment of predicted size.

4. PCR Purification of Amplified Product

The remaining PCR product left after checking the amplified product by agarose gel electrophoresis was purified by QIAquick PCR purification kit following the manufacturer's protocol. This protocol is designed to purify single or double stranded DNA fragments from primers, nucleotides, polymerase and salts present in PCR products mixture. The purified PCR product was subjected to electrophoresis in 1% agarose gel, to check its purity. Then product was thereafter used for cloning purposes.

5. Cloning of amplified *ac4, 2b, HcPro* and other genomic components of PRSV in pGEM-T Easy Cloning Vector

pGEM-T Easy vector (3015 bp) from Promega is convenient system for the cloning of PCR products. Successful clones of the above mentioned inserts in the pGEM-T Easy vectors is identified by colour screening on indicator plates containing ampicillin, X-gal and IPTG, as the vector contains the ampicillin resistance marker gene and the insert interrupts the coding sequence of β-galactosidase thus producing white colonies upon overnight incubation at 37°C temperature.

5.1. Optimization of Insert: Vector Molar Ratios

1:3 ratio of the vector to DNA insert provided good result. The concentration of PCR product was estimated on comparison to DNA mass standards on agarose gel.

5.2. Ligation of PCR Product to pGEM-T Easy Cloning Vector DNA

Ligation reactions were carried out between vector and PCR product. The reaction mix prepared for the purpose was as follows:

pGEM-T Easy vector (50 ng/µl)	1 µl
PCR amplified product (50 ng/µl)	3 µl
10X Ligation buffer	1 µl
10 mM ATP	1 µl
T_4 DNA ligase (3U/µl)	1 µl
Sterile distilled water	3 µl
Total	10 µl

The ligation mixtures were incubated at 4ºC for 18 hours.

5.3 Preparation of Competent Cells

The competent cells were prepared by $CaCl_2$ method described by Sambrook and Russel, 2000.

- 50 ml Luria Broth (LB) was inoculated with overnight grown culture of DH5α strain of *Escherichia coli* and incubated at 37ºC for 1 h and 15 min. with constant shaking at 200 rpm in a shaker incubator till the
- Bacterial growth as measured by optical density reached 0.3 O.D. at 600 nm.
- The culture was then aseptically transferred to 40 ml sterile screw capped tubes and kept on ice for 10 min.
- The culture was centrifuged at 5000 rpm for 10 min. at 4^{O}C in a Sigma 3K30 centrifuge to obtain the cells as pellet.
- The cells were resuspended gently in 10 ml ice cold 0.1 M $MgCl_2$ solution and centrifuged at 5000 rpm for 10 min. at 4ºC.

- The pellet was resuspended in 10 ml ice cold 0.1 M $CaCl_2$ solution and kept on ice for 1 h.
- The cells were recovered by centrifuging at 5000 rpm for 10 min. at 4ºC and the pellets were resuspended in 1 ml of chilled 0.1 M $CaCl_2$ and used for transformation after keeping on ice for 1 h.

5.4 Transformation of Competent Cells

- 200 µl competent cells were added to 20 µl of each of the ligation mixtures in two separate sterile microfuge tubes and were gently mixed and kept on ice for 1 h.
- The competent cells were given heat shock at 42ºC for 90 sec. 1 ml of LB medium was then added and the transformants were allowed to grow at 37ºC for 1 h in shaker incubator at 200 rpm.
- Two sets of 200 µl of serially diluted cell suspensions were asceptically plated on Luria Agar (LA) plates separately containing ampicillin, X-gal and IPTG (50 µl of 50 µg/ml ampicillin, 100 µl of 2 per cent X-gal and 10 µl of 0.1 M IPTG in 50 ml LA).
- The plates were incubated overnight at 37ºC.

5.5 Selection of Transformants

The transformants were selected on the basis of blue/white colonies. The white colonies were selected and subsequently streaked on LA Plates (master plates) containing IPTG, X-gal and ampicillin.

6. Rapid Screening for the Recombinant Clones by Colony PCR Method

From the master plates colonies were picked up randomly and screened by polymerase chain reaction, using the gene specific primers. In this case of colony PCR, a single colony was taken in each reaction mix in lieu of DNA sample

Following was the colony PCR reaction master mix:

Forward primer (200 ng/µl)	20 µl
Reverse primer (200 ng/µl)	20 µl
10X PCR buffer	40 µl
10 mM dNTPs	10 µl
Taq DNA polymerase (5U/µl)	10 µl
Sterile distilled water	300 µl
Total	400 µl

Aliquot of 40 µl of master mix was taken in ten different PCR tubes and the white colonies were taken one in each tube. The tubes were then placed in the same thermal cycler. The temperature profile and cycle were same as used in amplification of gene.

7 Isolation of Recombinant Plasmid DNA by Miniprep Method

The Recombinant plasmid from the positive clones was isolated following the modified alkaline lysis method (Sambrook and Russel, 2000).

- Selected white colonies of positive clones, found positive in colony PCR reaction were individually inoculated in 2 ml of LB medium containing ampicillin (50 µg/ml) in sterile capped culture tubes.
- Tubes were then incubated overnight at 37^{o}C at 200 rpm in a shaker incubator.
- The overnight grown bacterial cells were then transferred to 1.5 ml sterile eppendorf tube and cells were harvested by centrifuging in a table top centrifuge for 1 min. Care was taken to remove the medium adhering to the cell pellet.
- The pellet was resuspended in 100 µl of solution I and mixed vigorously by vortexing.
- The 200 µl of freshly prepared lysis solution (solution II) was then added and mixed gently.
- 150 µl of ice cold solution III was added next and mixed gently with lysed cell suspension and the mixture was kept on ice for 15 min.

- The chromosomal DNA and the bacterial cell debris were removed by centrifuging at 10,000 rpm for 20 min, at 4°C in a table top centrifuge (Sigma 112).
- The supernatant was again centrifuged for another 20 min at 10,000 rpm at 4°C to remove any unwanted bacterial debris as pellet.
- The supernatant was collected and equal volume of phenol: chloroform: isoamyl alcohol mixture (25:24:1) was added. It was vortexed well, centrifuged in a tabletop centrifuge for 15 min. at room temperature. The clear aqueous phase was transferred to fresh eppendorf tube.
- The DNA in aqueous phase was precipitated by adding 0.8 volume of isopropanol and kept on ice for 10 min.
- The mixture was then centrifuged at 15000 rpm for 20 min at 4°C.
- To the pellet 200 µl of 70% ethanol was added. The tube was rotated well so that the pellet from the wall gets suspended in 70% alcohol. This ensures removal of adhering salts by 70% alcohol. DNA was then pelletized by centrifuging at 15000 rpm for 5 min.
- The pellet was finally suspended in 30 µl sterile double distilled water.

8. Release of Inserts with Restriction Enzyme

Recombinant plasmids from positive clones were subjected to digestion with *Not*I restriction enzyme. This enzyme was so chosen because the restriction site of this enzyme is present in both side of insertion site of vector but not present in the insert. Restriction mix was incubated at 37°C for overnight for complete digestion. Restriction was done to release the insert and also to know the insert size. The reaction mixtures were prepared for each of the restriction digestion as follows:

Recombinant plasmid DNA (2 mg/ml)	10.0 µl
10X reaction buffer	2.5 µl
Restriction enzyme (*Not I*) (10U/µl)	0.5 µl
Sterile double distilled water	12.0 µl

Total	25.0 µl

After restriction digestion, the products were electrophoresed on 1% agarose gel. Fragment size was assessed in comparison with 1 kb DNA ladder loaded as molecular weight marker on to the same gel along with the DNA samples.

9. Cloning of AC-4, 2b, and HcPro in pUC 118-35 S-P-T vector

The AC-4, 2b, and HcPro region was cloned in pUC118-35S P-T vector.

***9.*1 Isolation of Recombinant Plasmid from pGEMT–HcPro, pGEMT–AC4, pGEMT-2b clone.**

Plasmid isolation was done by the modified alkaline lysis method (Sambrook and russel, 2000) as stated earlier. Isolated plasmid was checked on 1% agarose gel.

9.2 Release of AC-4, 2b, and HcPro region from the pGEM-T- clones

Recombinant plasmids from pGEMT AC4, 2b, and HcPro were subjected to digestion with *Not*I or *Apa*I/ *Xho*I restriction enzyme as above.

9.3 Gel Purification of Restricted Product

Released insert were excised from the agarose gel and purified using Qiagen Gel Purification kit following manufacturer's instructions.

9.4 Linearization of Vector pUC118-35S-P-T

The vector pUC118 was linearized with enzyme *Not* or *Apa/XhoI* to produce cohesive ends on the vector. The reaction mixture was prepared as follows

Vector DNA (0.5 mg/ml)	2 µl
Enzyme (5 U/ml)	1 µl

10X reaction buffer	2 µl
Sterile distilled water	15 µl
Total	20 µl

The reaction mixture was incubated at 37°C overnight.

9.5 Ligation of Inserts with pUC118 -35S-P-T Vector DNA

Ligation reaction between gel purified DNA and vector DNA was carried out using T4 DNA ligase by following cohesive end ligation method. The reaction mix was prepared as follows

Gel purified fragment (~100 ng/ml)	10µl
Vector (~100 ng/ml)	2 µl
Ligase (high concentration) (200 U/ml)	1 µl
10X reaction buffer	2 µl
10 mM ATP	1 µl
Sterile distilled water	4 µl
Total	20 µl

In addition to standard reaction mix, positive and negative control mixes were also prepared by adding control insert and no DNA in ligation mix respectively. The ligation mixtures were incubated at 16^{O}C overnight. Transformation of the *E. coli* (DH5α) competent cells was done. The cell suspensions after transformation was asceptically plated on Luria Agar (LA) plates separately containing ampicillin (50 µl of 50 µg/ml ampicillin in 50 ml LA). The plates were incubated overnight at 37^{O}C. The colonies obtained were screened for the presence of the insert by restriction analysis.

9.6 Selection of Transformants

The transformants were selected on the basis of restriction. The recombinant DNA from the pUC118 clones were isolated and restricted with restriction enzymes.After

restriction digestion, the products were electrophoresed in 1% agarose gel. Fragment size was assessed in comparison with 1 kb/or 100bp DNA molecular weight marker.

10. Cloning of constructs into Binary Vector

Besides pUC118-AC-4 (Sense), pUC118-2b (Sense and antisense) and pUC118-HcPro(Sense) were sub cloned in pCAMBIA 2301, a plant transformation compatible binary vector as follows.

10.1 Isolation of Recombinant Plasmid from pUC118-recombinant Clone:

Plasmid isolation was done by the modified alkaline lysis method (Sambrook and Russel, 2000) as stated earlier. Isolated plasmid was checked on 1% agarose gel.

10.2 Release of Inserts with Restriction Enzyme

Gene construct (CaMV 35S P + *ac4/2b/HcPro*+ CaMV 35S ter) were released from the vector by double digestion with the enzymes *Bam*H1 and *Hin*dIII. These enzymes were used because they have sites in both pUC118-35S-P-T and pCAMB1A 2301 vector and these sites were absent in insert.

Recombinant plasmid (0.5 mg/ml)	10 µl
Bam HI (10U/ml)	0.5 µl
*Hin*d III	0.5 µl
10X reaction buffer	2.0 µl
Sterile distilled water	7.0 µl
Total	20 µl

The mixture was incubated at 37°C overnight, and the restricted product was then subjected to 1% agarose electrophoresis.

10.3 Gel Purification of Restricted Product

Released insert were excised from the agarose gel and the DNA was purified from the gel using Qiagen Gel Purification kit by following manufacturer's instructions.

10.4 Linearization of Vector pCAMBIA 2301

The *Agrobacterium tumefaciens* Ti plasmid derived binary vector pCAMBIA 2301 was linearised using *Bam* HI and *Hin*dIII enzyme, producing cohesive ends on the vector. The reaction mixture was prepared as follows.

Vector DNA (0.5 mg/ml)	2 µl
Bam HI	0.5 µl
*Hin*d III	0.5 µl
10X reaction buffer	2 µl
Sterile distilled water	15 µl
Total	20 µl

The reaction mixture was incubated at 37°C overnight.

10.5 Ligation of Insert with pCAMBIA Vector DNA

Ligation reaction between gel purified insert and vector DNA was carried out using T_4 DNA ligase by following cohesive end ligation method as above. The ligation mixture was prepared as follows.

Gel purified fragment (~100 ng/ml)	12µl
Vector (~100 ng/ml)	3 µl
T_4 DNA Ligase (high concentration) (200 U/ml)	2 µl
10X reaction buffer	2 µl
10 mM ATP	1 µl
Total	20 µl

In addition to standard reaction mix, positive and negative control mixes were also prepared by adding control insert and no DNA in ligation mix respectively. The ligation mixtures were incubated at 16^{O} C overnight. Transformation of the *E.coli* (DH5α) competent cells was done. The cell suspension after transformation was

aseptically plated on Luria Agar (LA) plates separately containing kanamycin, (25 µl of 50 µg/ml kanamycin in 50 ml LA). The plates were incubated overnight at 37^{O} C.

10.6 Selection of Transformants

The colonies obtained were screened for the presence of the insert by colony PCR and restriction analysis with *Bam*H1 and *Hin*dIII. The recombinant plasmid DNA from pCAMBIA 2301 clones were isolated and restricted with *Bam*H1 and *Hin*dIII. After restriction digestion, the products were electrophoresed in 1% agarose gel. Fragment size was assessed in comparison with 1 kb or 100 bp DNA marker.

11. Mobilization of the constructs into *Agrobacterium tumefaciens* LBA 4404

11.1 Preparation of competent cells

An overnight grown culture of *Agrobacterium* was diluted in 50 ml Luria broth (LB) and incubated at 28° C for 3-4 hours. Logarithmically growing cells were centrifuged at 5500 rpm for 20 minutes at 4° C. The pellet was washed once in 10 ml precooled TE (10mM Tris-HCl, pH 7.5 ; 1 mM EDTA) and resuspended in 20 ml LB medium. Aliquots of 500 µl were used directly for transformation.

11.2 Transformation

Stored cells were thawed on ice prior to transformation. Competent *Agrobacterium* cells are mixed with 0.5 - 1.0 µg recombinant pCAMBIA-2301 DNA. The cells were subjected successively to 5 minutes ice, 5 minutes liquid nitrogen and 5 minutes 37° C treatment. After dilution in 1 ml LB medium the cells were shaken 2-4 hr at 28° C. Aliquots of 200 µl were plated on LA plate containing Kanamycin (50mg/l) and streptomycin (100 mg/ml) and incubated for two days at 28° C.

11.3 Screening for recombinant clones by colony PCR

The colonies obtained were screened for the presence of the insert by colony PCR with gene specific primers or using 35 S P-T primers to screen for the presence of entire cassette.

11.4. 35 S P-T primers

Specific primers were synthesized from Microsynth Pvt for 35 S Promoter and terminator region flanking the MCS in pUC 118 vector hence it can be used for the screening of constructs .

Primer sequences were

Forward primer (35 S -P) : 5' ATTGCGATAAAGGGAAGGCC 3'

Reverse primer (35 S-T) : 5' CCTGCAGGTACCACTGGATT 3'

The PCR reaction mixtures were prepared as follows:

Total DNA (25 ng/µl) (plasmid or Plant genomic DNA)	2 µl
Forward primer (200 ng/µl)	1 µl
Reverse primer (200 ng/µl)	1 µl
10X PCR buffer	2 µl
10 mM dNTPs	1 µl
Taq DNA polymerase (5 U/µl)	1 µl
Sterile distilled water	12 µl
Total	20 µl

The mixtures were then placed in thermal cycler separately (ERICOMP Power Block II System). Following were the temperature profile and cycles performed

Steps	**Temperature**	**Time**	**Cycle**
Denaturation of DNA	94°C	4 min	1
Denaturation	94°C	30 sec	

Annealing	58°C	40 sec	
Primer extension	72°C	30 sec	
Final primer extension	72°C	5 min	1

After PCR reaction was over, 1 µl of amplified product was subjected to electrophoresis in 1% agarose gel to observe the DNA fragment of predicted size.

12. SDS-PAGE analysis

- Inoculated 50 ml LB containing 25g/ml ampicilin with 2.5 ml overnight culture of *E.coli* (strain TB1) harboring recombinant expression vector.
- Incubated culture flask at 37°C at 200 rpm till the OD value at 600nm reached to 5-7.
- Pipetted out 2 ml non induced fraction and dissolved in 50µl 1X SDS-PAGE sample buffer after pelleting.
- Added IPTG to a final concentration of 1mM for protein induction and incubated at 37°C at 200 rpm for 5 hrs.
- Collected induced and non-induced culture (2 ml each), pelleted and dissolved in 50 µl 1X SDS-PAGE sample buffer.
- Harvested cells by centrifugation at 4000 rpm at 4°C for 20 min and stored at –20°C.
- Dissolved the pellet in 5 ml lysis buffer and stored at –20°C.
- Thawed the cell pellet in lysis buffer and stored at –20°C.
- Added lysozyme (1 mg/ml) and incubated on ice for 30 min.
- Sonicated 6x10s with 10s pauses and kept the lysate on ice at all times.
- Centrifuged the lysate at 10,000Xg at 4°C for 20-30 min and stored the supernanatant separately at –20°C (soluble fraction).
- The pellet was resuspended in 5 ml lysis buffer and was stored at –20°C (insoluble fraction).
- SDS page of non induced and induced *E. coli* cells with insert as well soluble and insoluble fractions of their cell lysate was carried out in 12% resolving gel and 5% stacking gel.

12.1. Sample preparation and loading

20 µl sample mixed with dye were denatured by boiling at 95-100°C for 5 min and loaded in gel along with known molecular weight markers (prestained protein marker). The gel was electrophoresed at constant voltage of 50V in stacking gel and at 90V in resolving gel. Gel was stained with 0.1 % coomassive blue overnight and destained in destaining solution till the background became clear at room temperature.

13. Western blot

Western blot was performed to check the specificity of the expressed protein. After electrophoresis one gel was used for staining and destaining and other one was used for western bloting as per protocol mentioned.

- After electrophoresis, trimmed the gel to the desired size and soaked in transfer buffer for 30 min.
- Cut a set of Whatman sheets no.1 (3mm) to the size of the gel and soaked in the transfer buffer.
- Placed the gel above the soaked sheets.
- Kept NCM and other set of wetted Whatman sheets above the gel and align gently with the glass rod.
- Removed excess of running buffer and covered the apparatus gently and electrophoresed at 15V for overnight.
- Dried the membrane for sometime over a tissue paper and transferred to a blocking solution and incubated at 4°C overnight.
- Incubated NCM with primary antibody (1:10,000) diluted in TBS-BSA for 1 h at room temperature.
- Rinsed NCM thrice in TBS (10 min each).
- Transferred NCM to antirabbit immunoglobulin alkaline phosphatase (sigma, USA) diluted in TBA-BSA (1:30, 000) for 1 h at room temperature.
- Rinsed NCM thrice in TBS (10 min each).
- Incubated NCM in substrate solution at room temperature and washed for purple colour development.

- Rinsed NCM twice in TBS (10 min each) in fixing solution and air dried.

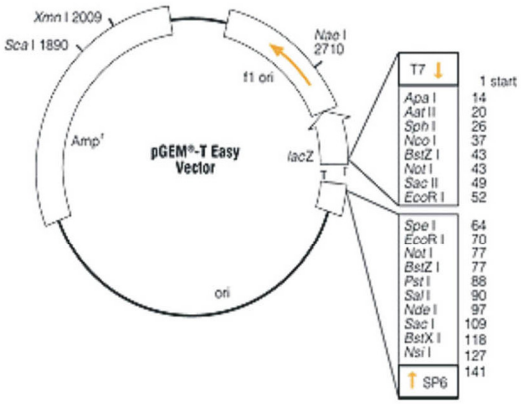
14. Direct antigen-coated enzyme linked immuno sorbent assay (DAC-ELISA)

DAC-ELISA test was performed to detect the CMV and PRSV. Commercially available polyclonal antiserum directed against coat protein gene of *Papaya ringspot virus* and *Cucumber mosaic virus* was used. The assay was performed in 96 well polystyrene microtitre plates (Costar, Sigma, USA). The composition of various buffers and reagents used is given in Appendix II.

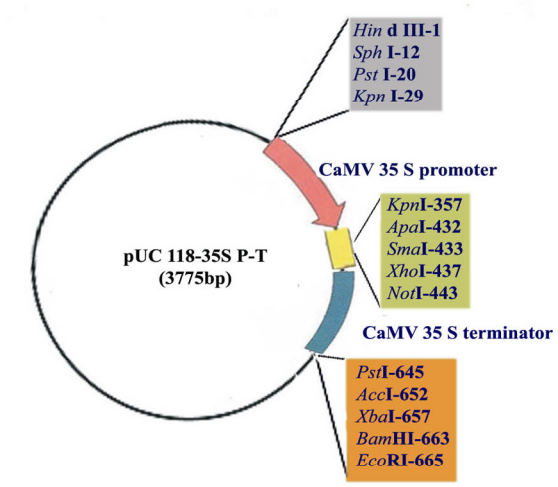
- Dispensed 200µl extract from symptomatic as well as healthy leaves in coating buffer containing 2% polyvinyl pyrollidone (PVP, MW 40,000) (1:1 dilution, w/v) to each well of the microtitre plate. Covered the plate and incubated at 37ºC for 1h
- Emptied and washed the plate by flooding the wells with PBS-T for 3 min. Repeated washing and soaking operation thrice, and shook out residue liquid draining on a paper towel
- Dispensed 200 µl blocking solution (Bovine Serum Albumin, 1%, w/v) to each well. Incubated at 37ºC for 1h to block polystyrene well reactive surfaces
- Washed the plate three times as above.
- Dispensed 200µl crude antiserum (Primary antibody) diluted (1:4000) in PBS-TPO to each well. Incubated at 37˚C for 2h.
- Washed the plate three times as above.
- Dispensed 200µl anti-rabbit immunoglobulin alkaline phosphatase (universal conjugate, Sigma, USA) diluted in PBS-TPO (1:20,000) to each well. Incubated at 37ºC for 2h.
- Washed the plate three times as above.

- Dispensed 200µl freshly prepared substrate (p-nitrophenyl phosphate-PNPP, Sigma, USA) (0.5mg/ml) solution in substrate buffer to each well. Incubated at 37ºC for 2h.
- Measured the intensity of color in each well at 405nm by using a Tecan Sunrise (version 1.2) ELISA reader. Compared the absorbance values of the test samples with healthy control.
- Samples showing absorbance (OD_{405}) values more than two times of healthy control were considered positive.

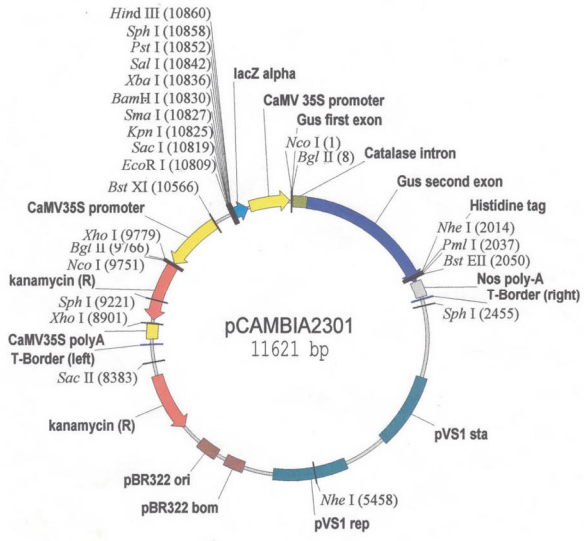
(a)



(b)



(c)



Maps of the plasmids employed in generation of the RNAi constructs
(a) pGEM-T-Easy (b) pUC-118-35S-P-T (c) pCAMBIA 2301

APPENDIX II

Common Reagents, Buffers and Media Used

Antibiotics

Ampicillin Stock solution (50 mg/ml) of the antibiotic was made in double distilled water, filter sterilized (through 0.22 micron filter) and distributed into 200 µl aliquots and stored at -20^{o} C. It was used at a concentration of 50 µg/ml.

Kanamycin Stock solution (50 mg/ml) prepared similarly and stored at -20ºC. It was used at a concentration of 25 µg/ml.

Streptomycin Stock solution (100 mg/ml) prepared similarly and stored at -20^{o} C. It was used at a concentration of 100 µg/ml.

Plasmid Isolation Buffers

Solution I (Resuspension buffers)	Tris HCl pH (8.0)	25 mM
	Glucose	50 mM
	EDTA	10 mM
Solution II (Lysis buffer)	NaOH	0.2 N
	SDS	1%
Solution III (Neutralization buffer) pH 4.8	Sodium acetate	3 M

Agarose gel Electrophoresis Reagents

50X TAE	Tris base	242.0 g
	Glacial acetic acid	57.1 ml
	0.5 M EDTA (pH 8.0)	100ml
	Distilled water to	1 litre

Loading dye	1% Bromophenol blue	200 µl
	Glycerol	200 µl
	10% SDS	60µl
	0.5 M EDTA	50µl
	10X TAE	60µl
	Distilled water	30µl
Ethidium bromide (10mg/ml)	1 g ethidium bromide was added to 100 ml distilled water and stirred on magnetic stirrer for several hours to ensure that the dye has dissolved. The solution was transferred to dark bottle and stored at room temperature.	

DNA Molecular Weight Marker

One kilobase (1 kb) DNA ladder of MBI Fermentas was used as marker. The ladder is formed by fourteen DNA fragments of 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3.5 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb, 0.75 kb, 0.5 kb and 0.25 kb. 100bp DNA ladder of pro mega consists of 11 fragments of 100 bp to 1 kb and 1.5 kb.

Buffer

Ligation buffer 10X	Tris HCl (pH 7.8)	0.5 M
	$MgCl_2$	0.5 M
	Dithiothreitol	0.1 M
	Bovine serum albumin	500 µg ml^{-1}

Direct antigen coated-enzyme linked immunosorbent assay (DAC-ELISA)

Coating buffer (pH 9.6)	Na_2CO_3	1.59 g
	$NaHCO_3$	2.93 g
	Distilled water	Up to 1000 ml

Phosphate buffer saline (10X PBS, pH 7.4)	NaCl	8.0 g
	KH_2PO_4	0.2 g
	$Na_2HPO_4.12\ H_2O$	2.9 g or
	Na_2HPO_4	1.5 g or
	$Na_2HPO_4.2\ H_2O$	1.44 g
	KCl	0.2 g
	Distilled water	Up to 1000 ml
Washing buffer (PBS-T)	Add .5 ml of Tween 20 (0.05 %) to 1000 ml of 1XPBS	
Blocking solution	BSA	1 g
	PBS-T	100ml
Antibody/conjugate buffer (PBS-TPO)	PBS-T	100 ml
	Polyvinyl pyrolidone (2% MW 40,000)	2 g
	Ovalbumin (0.2%)/ egg albumin	0.2 g
Substrate buffer (pH 9.8)	Diethanolamine	97 ml
	Distilled water	Upto 800 ml
	PH adjusted to 9.8 with 1N HCl (about 67 ml) and made upto 1000 ml with distilled water	
Stop solution	NaOH	3M

Protein expression and Purification

Rich broth	Tryptone	10 g
	Yeast extract	5 g
	NaCl	5 g
	Glucose	2 g

pH was adjusted to 7.0 with 5N NaOH and volume made up to 1 L with deionized water. The medium was aliquoted into 50 ml in 250 ml flasks and sterilized by autoclaving for 20 min at 15 psi.

Column buffer	Tris HCl (pH 7.4)	20 mM
	NaCl	200 mM
	EDTA	1 mM
	Sodium azide	1 mM
	β-mercaptoethanol	10 mM or
	DTT	1 mM
	Distilled water	Upto 1000 ml

SDS-PAGE analysis

Acrylamide and N,N'-methylene bis acrylamide	Acrylamide	30.0 g
	N,N'-methylene bis acrylamide	0.8 g
	Distilled water	Upto 100 ml

Filtered through Whatman paper No.1 and used

1.5 M Tris HCl (pH 8.8)	Tris base	18.5 g
	Distilled water	Upto 100 ml
1.0 M Tris HCl (pH 6.8)	Tris base	12.0 g
	Distilled water	Upto 100 ml
10 % SDS solution	Sodium Dodecyl Sulphate	10 g
	Distilled water	Upto 100 ml
10 % APS	Ammonium per sulpahte	0.1 g
	Distilled water	1ml
	(Should be prepared just before use)	
TEMED	Electrophoresis grade TEMED was used	
1X Loading dye	Tris-HCl	50 mM
	DTT	100mM
	SDS	2% (w/v)
	Bromophenol blue	0.1%
	Glycerol	10%
Tris glycine buffer (pH 8.3) 5X	Tris base	15 g
	Glycine	72 g
	SDS	5 g
	Distilled water	Upto 1000 ml
Staining solution	Co-omassive brilliant blue	100 mg
	Methanol	50 ml
	Dissolved and then added	10 ml
	Acetic acid	40 ml
	Distilled water	

Destaining solution	Methanol	40 ml
	Acetic acid	10 ml
	Distilled water	50 ml

Western blotting

Western transfer buffer	Tris base	3.02 g
	Glycine	14.4 g
	Methanol	200ml
	Distilled water	Upto 1000ml
Tris buffer saline (pH 7.5) (TBS)	Tris	0.02 M
	NaCl	0.5 M
	Distilled water	Upto 1000ml
Blocking solution (TBS-BSA)	BSA	5.0 g
	TBS	100ml
Antibody/conjugate buffer	Same as blocking solution	
TSB-T (Washing buffer)	1X TBS containing 0.05% Tween 20	
Substrate buffer (pH 9.5)	Tris base	0.1 M
	NaCl	0.1 M
	$MgCl_2$. $6H_2O$	5 mM
	Distilled water	Upto 1000 ml
Substrate solution	Solution A. dissolve 75 mg of nitro blue tetrazolium (NBT) in 1 ml of dimethylformamide (DMFA)	
	Solution B. dissolve 50 mg of 5-bromo 3-chloro indolyl phosphate (BCIP) in 1 ml if dimethylformamide (DMFA)	
Fixing solution (pH 7.5)	Tris	10 mM
	EDTA	1 mM
	Distilled water	Upto 1000ml

Electrophoretic mobility shift assay

Tris-HCl (pH 7.5)	83mM
$MgCl_2$	0.8mM
KCl	66mM
NaCl	100mM
DTT	10mM
Tween-20	0.02%

Medium and solutions for tissue culture

Composition of MS Medium	**Concentration of ingredients in the medium (mg/L)**	
Macronutrients	NH_4NO_3	1650
	KNO_3	1900
	$MgSO_4.7H_2O$	370
	KH_2PO_4	170
	$CaCl_2.2H_2O$	440
Micronutrients	$MnSO_4.4H_2O$	22.3
	$NZSO_4.7H_2O$	8.6
	H_3BO_3	6.2
	KI	0.25
	$Na_2MoO_4.2H_2O$	0.83
Cu Compounds	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
Fe-EDTA	Na_2 EDTA.$2H_2O$	37.30
	$FeSO_4.7H_2O$	27.80
Organic compounds	Glycine	2.0
	Nicotinic acid	0.5
	Pyridoxin-HCl	0.5
	Thiamin-HCL	0.1
	Myo-inositol	100
Other	Agar-2	8 g/L
	Sucrose	30 g/L

Regeneration medium and Cocultivation medium	MS salts	4.4 g/L
	BAP	1.0 mg/L
	IBA	0.4 mg/L
	Agar-2	8 g/L
	$CaCl_2$	440 mg/L
Selection medium	MS-salt	4.2 g/L
	BAP	1 mg/L
	IBA	0.5 mg/L
	Agar-2	8 g/L
	$CaCl_2$	440 mg/L
	Sucrose	30 g/L
	Kanamycin	100 mg/L
	Augmantine	250 mg/L
Shooting medium	MS salt	4.2 g/L
	BAP	2.5 mg/L
	IBA	0.2 mg/L
	Kinetin	1 mg/L
	$CaCl_2$	440 mg/L
	Sucrose	30 mg/L
	Kanazmycin	100 mg/L
	Augmantine	250 mg/L
	Agar-2	8 g/L
Rooting medium	MS salt	2.1 gm/L
	$CaCl_2$	600 mg/L
	IAA	0.5 mg/L
	Sucrose	30 mg/L
	Agar	6 g/L

Commonly used stock solution

0.1 M Adenosine triphosphate (ATP)	60.0 mg of ATP was dissolved in 0.8 ml of distilled water. The pH was adjusted to 7.0 with 0.1 N NaOH and volume made up to 1 ml with distilled water. The solution was dispensed into small aliquots and stored at -70^{o} C.
1 M $CaCl_2$	54.0 g of $CaCl_2.2H_2O$ was dissolved in 200 ml of pure water. The solution was sterilized by passing through a 0.22 micron filter and stored in 1 ml aliquots at 4° C.
0.5 M EDTA (pH 8.0)	186.1 g of ethylenediamine tetra acetic acid disodium salt $2H_2O$ was added to 800 ml of distilled water, stirred vigorously on a magnetic stirrer, pH was adjusted to 8.0 with NaOH (20.0 g of NaOH pellets). Volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
Ethidium bromide (10 mg ml^{-1})	1.0 g of ethidium bromide was added to 100 ml of distilled water and stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The solution was transferred to a dark bottle and stored at room temperature
Phenol : chloroform : isoamyl alcohol	Buffer saturated phenol, chloroform and isoamyl alcohol were mixed in the ratio of 25:24:1. The equilibrated mixture was stored under a layer of 0.01 M Tris-HCl (pH 7.6) at 4^{o} C in dark glass bottle.
IPTG (Isopropyl--b-D-thiogalacto- pyranoside)	A solution of IPTG was made by dissolving 2.0 g of IPTGin 8 ml of distilled water. Volume was made upto 10 ml with distilled water and sterilized by filtration through a 0.22 µ disposable filter. The solution was dispensed into 1 ml of aliquots and stored at -20^{o} C.
1M $MgCl_2$	203.3 g of $MgCl_2.6H_2O$ was dissolved in 800 ml of distilled water. The volume was made upto 1L, dispensed into aliquots and sterilized by autoclaving.
3M Sodium acetate (pH 4.8)	408.1 g of $NaOAc.3H_2O$ was dissolved in 800 ml of distilled water. The pH was adjusted to 4.8 with glacial acetic acid. Volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
5M NaCl	233.8 g of NaCl was dissolved in 800 ml of distilled water, volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.

10 N NaOH	Dissolve 400 g of NaOH in 800 ml of distilled water and make up the volume to 1 L with distilled water.
1M Tris-HCl	121.1 g of Tris base was dissolved in 800 ml of distilled water. pH was adjusted to the desired value by adding concentrated HCl (for pH 7.4, HCl 70 ml; for pH 8.0, HCl 42 ml). The solution was allowed to cool down to room temperature before making final adjustment to the pH. The volume was made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
X-gal (5-bromo-4- chloro-3-indolyl-b- D-galactopyranoside)	The stock solution was made by dissolving X-gal in dimethyl formamide to make a 20 mg/ml solution and stored at -20° C.