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YEB SWARNALOK DE
Interactions of Potyviral Protein HCPro with Host Methionine Cycle Enzymes and Scaffolding Protein VARICOSE in Potato Virus A Infection

DEPARTMENT OF MICROBIOLOGY
FACULTY OF AGRICULTURE AND FORESTRY
DOCTORAL PROGRAMME IN PLANT SCIENCES
UNIVERSITY OF HELSINKI
Interactions of potyviral protein HCPro with host methionine cycle enzymes and scaffolding protein VARICOSE in potato virus A infection

Swarnalok De

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LIST OF ORIGINAL PUBLICATIONS:


II. De, S., Pollari, M., Varjosalo, M. and Mäkinen, K. ”Interaction between HCPro and host protein VARICOSE affects RNA silencing suppression, translation, encapsidation and long-distance movement in potato virus A infection” Submitted.


CONTRIBUTION:

I. Swarnalok De cloned SAMS, SAHH and HEN1 silencing constructs and carried out the gene silencing experiments. He contributed to the western analyses of the high molecular weight complexes.

II. Swarnalok De discovered the HCProWD mutant, which was instrumental for understanding the role of HCPro-VARICOSE interaction during PVA infection. He participated in cloning of the constructs used in this study. He carried out most of the experiments. He prepared the figures and wrote the first draft of the MS. He proposed the hypothesis of HCPro-VCS containing core complex, which is proposed to guide PVA RNA through the different stages of infection in the model figure.

III. Swarnalok De studied mixed infection by PVA and PVX. He took part into cloning of the constructs used in this study. He did most of the experiments. He generated the hypotheses of the possible connection of mixed infection to glutathione biosynthesis pathway and with the method developed together with M.W. showed glutathione reduction in N. benthamiana samples. He prepared the figures and wrote the first draft of the MS.
ABBREVIATIONS

(-)-strand – antisense RNA strand
(+) - strand – sense RNA strand
AGO1, 2, 10 – argonaute 1, 2 and 10
CFP – cerulean fluorescent protein
CHIP – C-terminus of Hsc70 interacting protein
CI – PVA cylindrical (cytoplasmic) inclusion protein
CMV – cucumber mosaic virus
CP – coat protein
CPIP – coat protein-binding protein
DCL – Dicer-like endoribonucleases
dpi – days post infiltration
dsRNA – double-stranded RNA
eEF1A – eukaryotic translation elongation factor 1A
eIF4A – eukaryotic translation initiation factor 4A
eIF4E/eIF(iso)4E – eukaryotic translation initiation factor 4E/(iso)4E
eIF4G – eukaryotic translation initiation factor 4G
EM – electron microscopy
FLUC – firefly luciferase
ER – endoplasmic reticulum
Ex/Em – excitation/emission
GFP – green fluorescent protein
gRNA – genomic RNA
GSH – glutathione (reduced form)
GSSG – glutathione (oxidised form)
HCPro – helper component proteinase
HCY – homocysteine
HEN1 – HUA enhancer 1
HMW – high molecular weight
HSP70 – heat shock protein 70
icDNA – infectious complementary DNA
ic-qRT-PCR – immunocapture-qRT-PCR
IRES – internal ribosome entry site
kDa – kiloDalton
LC-MS/MS – liquid chromatography tandem-mass spectrometry
LMV – Lettuce mosaic virus
MET – methionine
MT – methyltransferase
MS – methionine synthase
NIa – nuclear inclusion protein a
NIb – nuclear inclusion protein b
ORF – open reading frame
P0 – acidic ribosomal protein P0
P19 – tombusvirus protein 19
P21 – closteroviruses protein 21
P25 – potexviral protein 25
PABP – polyadenylate binding protein
PapMV – Papaya mosaic virus
PB – processing body
PD – plasmodesma
PG – PVA induced granule
PIPO – pretty interesting potyvirus ORF
PLRV – potato leafroll virus
PPV – plum pox virus
PRSV – Papaya Ringspot virus
PVA – potato virus A
PVS – potato virus S
PVX – potato virus X
PVY – potato virus Y
RDR6 – RNA-dependent RNA polymerase 6
RDRP – RNA-dependent RNA polymerase
RFP – red fluorescent protein
RISC – RNA-induced silencing complex
RLUC – renilla luciferase
RNP – ribonucleoprotein
RPL18B – ribosomal protein L18B Arabidopsis
SABP3 – salicylic acid binding protein 3
SAH – S-adenosyl-L-homocysteine
SAHH – S-adenosyl-L-homocysteine hydrolase
SAM – S-adenosyl-L-methionine
SAMS – S-adenosyl-L-methionine synthase
SG – stress granule
sgRNA – subgenomic RNA
siRNA – small interfering RNA
sRNA – small RNA
ssRNA – single-stranded RNA
TBSV – tomato bushy stunt virus
TEV – tobacco etch virus
TuMV – turnip mosaic virus
UBP1 – oligouridylate binding protein 1
UPR – unfolded protein response
UTR – untranslated region
VCS – varicose protein
VPg – viral protein genome-linked
VRC – viral replication complex
vRNA – viral RNA
WT – wild type
YFP – yellow fluorescent protein
ZYMV – zucchini yellow mosaic virus

The standard abbreviations for nucleotides and amino acids are used.
ABSTRACT
Potyviral helper component proteinase (HCPro) is a quintessential example of a multifunctional viral protein. Its name comes from two of its earliest identified functions—Helper Component—involved in aphid-mediated plant-plant transmission of the virus, and cysteine proteinase responsible for its self-cleavage from the rest of the viral polyprotein. HCPro’s best-studied function is its ability to suppress RNA silencing. One of the factors underlying the multifunctionality of HCPro is its ability to interact with a wide range of host factors causing perturbations in several cellular pathways. In this study, interaction of HCPro with the host proteins S-adenosyl-L-methionine synthase (SAMS), S-adenosyl-L-homocysteine hydrolase (SAHH), ARGONAUTE 1 (AGO1) and VARICOSE (VCS) was addressed and implications of these interactions in potato virus A (PVA; genus Potyvirus) infection, and potato virus X (PVX)-PVA mixed infection were studied.

In this study, HCPro was found to interact with the host methionine cycle enzymes SAMS and SAHH and to inhibit SAMS activity. Disruption of the methionine cycle promoted PVA infection. Methionine cycle plays a crucial role in the smooth running of RNA silencing by providing S-adenosyl methionine (SAM) for methyltransferase hua enhancer 1 (HEN1). Small RNA (sRNA) duplexes, which are methylated by HEN1, are stable and capable to act in RNA silencing, whereas, the unmethylated sRNAs are polyuridylated and targeted to degradation. A blockage in sRNA methylation via HCPro-mediated methionine cycle disruption was proposed to act as a circuit breaker of RNA silencing pathway for the benefit of PVA infection. SAHH was also found to be involved in PVX-PVA synergism. Blockage of the methionine cycle at SAHH, coupled with synergism-specific downregulation of closely associated glutathione (GSH) biosynthesis pathway enhanced PVX genomic RNA accumulation and subgenomic RNA expression. Moreover, depletion of cellular antioxidant GSH was suggested to be the reason behind induction of severe oxidative stress during potex–potyvirus mixed infection.

In another line of this study, formation of HCPro-associated high molecular weight (HMW) complexes and their functions were studied. Interaction between HCPro and a WD40 domain containing scaffold protein VCS was shown to be crucial for formation and stability of HCPro-associated HMW complexes. Importance of HCPro-VCS interaction in governing the assembly of PVA-induced granules (PGs) was demonstrated. This study reinforced the correlation between the PGs and RNA silencing suppression. Interestingly, HCPro, AGO1, VPg and CI were detected in the ribosome-associated HMW-complexes. Association of AGO1 with ribosomes may indicate occurrence of RISC-mediated translational repression as an additional defense mechanism against PVA infection. While, presence of HCPro, VPg and CI therein suggested a putative mechanism by which HCPro-derived ribosome-associated HMW complexes might participate in relieving PVA translational repression. Accordingly, co-operation between HCPro, VCS and VPg was shown to act in favor of active PVA translation. Intriguingly, importance of HCPro-VCS interaction was also found to be important in PVA encapsidation. In conclusion this study provides evidence for interaction between HCPro and host proteins SAMS, SAHH, VCS and AGO1 in planta. Furthermore, importance of these interactions are demonstrated to play crucial role in governing various viral processes during PVA infection.
1. INTRODUCTION

1.1 Potyviruses

Potyviruses belong to the family *Potyviridae*, which comprises eight genera in total to form the largest family of plant RNA viruses and accounts for approximately 30% of known plant viruses (Wei et al., 2010; Wylie et al., 2017). In addition to agricultural crops, metagenomics study revealed their widespread distribution in wild plants also (Roossinck, 2012). Genus *Potyvirus* is the largest one in the family and it alone includes 186 member species and 36 related, unclassified viruses (Wylie et al., 2017). Potyviruses possess a single-stranded positive-sense (ss)RNA genome to which viral protein genome-linked (VPg) is attached at the 5’ end. The 3’ end is polyadenylated. They form flexuous filament-like virions approximately 680–900 nm in length and 11–13 nm in width. Potyviruses are generally transmitted in a non-persistent manner by approximately 40 aphid species, while transmission via seeds or mechanical inoculation is also possible.

Potyviruses cumulatively infect a wide range of hosts and are a menace to many economically important food crops. Brunt et al. (1996) listed more than 500 plant species belonging to 59 families as their potential hosts. Though the individual species are known to be quite narrow in their host range, a few of them can infect plant species belonging to as high as 30 families. Type species potato virus Y (PVY) is among the most devastating ones and is accounted for up to 70% loss in crop yield (Bartels, 1971; Nolte et al., 2004). Risk to global food security from potyviruses is aggravated further due to their involvement in a phenomenon commonly known to as potyviral synergism. Under field conditions, interactions between viruses and development of mixed infections thereby are quite common. Encounter between unrelated viruses can lead to alteration in their individual pathogenicity (Syller, 2012). Broadly, these interactions are categorised into two types - antagonistic and synergistic (Garcia-Cano et al., 2006; Untiveros et al., 2007; Renteria-Canett et al., 2011). During antagonistic response, infection by one virus leads to suppression of pathogenicity of a subsequently infecting virus (Gal-On and Shiboleth 2005; Gonzalez-Jara et al., 2009; DaPalma et al., 2010). On the other hand, during synergistic interaction one or both of the viruses enhances accumulation and infectivity of the other, resulting in unprecedented severity in symptom development (Pruss et al., 1997; Zhang et al., 2001; Gonzalez-Jara et al., 2004, 2005; Untiveros et al., 2007; Wang et al., 2009; Senanayake and Mandal, 2014). Potyviruses in many occasions have been reported to be involved in enhancement of pathogenicity of unrelated viruses like cucumber mosaic virus (CMV; genus *Cucumovirus*), potato leafroll virus (PLRV; genus *Polerovirus*) and potato virus X (PVX; genus *Potexvirus*) (Vance, 1991; Pruss et al., 1997; Srinivasan and Alvarez, 2007; Mascia et al., 2010). Synergistic relation of potexviruses with other members of potyvirus group has been of immense interest to the scientific community for many decades now. The
enhancement of pathogenicity can be so drastic that even the relatively mild strains like potato virus A (PVA; genus *Potyvirus*) can cause up to 40% yield loss upon co-infection with PVX and potato virus S (PVS; genus *Carlavirus*) (Dedic, 1975; Hameed et al., 2014). Suppression of host’s defence response, enhanced movement and replication are considered among causes underlying potex-potyviral synergism (Untiveros et al., 2007). Interestingly, antagonistic relationship between potex- and potyviruses has also been reported (Ross, 1950). Mixed infection by papaya mosaic virus (PapMV, a potexvirus) and papaya ringspot virus (PRSV, a potyvirus) evokes either synergistic or antagonistic response based on their order of entry in to the host (Chavez-Calvillo et al., 2016). Synergism occurs when the host is simultaneously infected with PRSV and PapMV, or PRSV is inoculated before PapMV. However, infection in a reversal order, PapMV first followed by PRSV, leads to antagonism. The order- dependent either synergistic or antagonistic response reveals complex host-virus and virus-virus interactions during potex-potyviral mixed infection.

Based on the scientific, economic, food security and overall impact on humankind, two independent reviews published ‘top-ten’ list of plant viruses. Three members (PVY, Maize dwarf mosaic / Sugarcane mosaic virus, Sweet potato feathery mottle virus) of genus *Potyvirus* were on the list (Scholthof et al., 2011; Rybicki, 2015). Despite being studied for several decades, many aspects of potyvirus infection still remain elusive. In order to establish infection successfully viral proteins need to interact with a wide array of host proteins at each step. Majority of the resistance genes against potyviruses are recessive in nature and resistance comes from the incompatibility between the viral proteins and their host targets. Most important host factor identified so far belongs to the family of translation initiation proteins, more specifically eukaryotic initiation factors 4E and (iso)4E ((eIF4E/eIF(iso)4E); Robaglia and Caranta, 2006). However, researchers and plant breeders worldwide are constantly trying to identify novel target proteins to achieve virus resistance in cultivated plants.

1.2 Potyviral genome organization and proteins

Potyviral virions carry a positive-sense ssRNA of approximately 10 kb in size. PVA is the model potyviral strain that was used in this study. Its genome contains two open reading frames (ORFs) (Fig. 1).
Figure 1. Schematic representation of the PVA genome organization and proteins. Potyviral genome comprises one positive sense ssRNA covalently-linked to VPg at the 5’ end. The 3’ end is polyadenylated. The genome hosts 2 ORFs, which are directly translated to produce a long and a short polyprotein, which are subsequently cleaved to produce 11 individual proteins. Cleavage sites are shown with arrows in the figure. Notably the long polyprotein is processed to produce ten potyviral proteins. The N-terminal sequence of P3 protein followed by the PIPO sequence (P3N-PIPO) is the 11th potyviral protein. P3N-PIPO is processed apart from the short polyprotein.

The first and the major ORF is translated to produce a 3059 amino acids long polyprotein, which is subsequently cleaved to generate 10 individual proteins namely- P1, helper component proteinase (HCPro), P3, 6K1, cylindrical inclusion protein (CI), 6K2, nuclear inclusion protein a (NIa) consisting of NIa-pro and VPg, nuclear inclusion protein b (NIb) and coat protein (CP). The second and much shorter ORF called pretty interesting potyvirus ORF (PIPO) is a rather recent discovery (Chung et al., 2008). This ORF is embedded within P3 cistron, and comes to the picture during viral replication due to a frameshift caused by polymerase slippage on a GA₆ motif (Olspert et al., 2015). The resultant 11th potyviral protein is called P3N-PIPO as it carries N-terminal P3 sequence followed by PIPO sequence at its C-terminus. Decades of functional studies revealed basic features of the individual proteins and the roles they play during infection (Table 1). However, it has to be kept in mind that compared to the hosts they infect, viruses have a tiny genome with only a few proteins in their repertoire. In order to successfully hijack host cellular machineries to serve for their propagation, many of their proteins are multifunctional in nature. Therefore, in addition to their primary functions, many of the viral proteins interact with myriads of host proteins and carry out multiple essential roles throughout the viral infection cycle.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Brief description and functions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>a serine protease responsible for self-cleavage from the rest of the polyprotein</td>
<td>Verchot et al., 1991</td>
</tr>
<tr>
<td></td>
<td>involved in genome amplification</td>
<td>Verchot and Carrington, 1995</td>
</tr>
<tr>
<td></td>
<td>involved in a silencing suppression-independent mechanism to enhance infectivity of plum pox virus (PPV)</td>
<td>Pasin et al., 2014</td>
</tr>
<tr>
<td>HCPro*</td>
<td>cysteine protease responsible for self-cleavage from the rest of the polyprotein</td>
<td>Carrington et al., 1989</td>
</tr>
<tr>
<td></td>
<td>essential factor for aphid mediated plant-plant transmission</td>
<td>Govier et al., 1988</td>
</tr>
<tr>
<td></td>
<td>silencing suppression</td>
<td>Anandalakshmi et al., 1998</td>
</tr>
<tr>
<td></td>
<td>facilitates viral particle encapsidation</td>
<td>Kasschau and Carrington, 1998</td>
</tr>
<tr>
<td>P3</td>
<td>involved in viral replication</td>
<td>Klein et al., 1994</td>
</tr>
<tr>
<td></td>
<td>symptom and avirulence determinant</td>
<td>Jenner et al., 2003</td>
</tr>
<tr>
<td></td>
<td>endoplasmic reticulum (ER) stress, unfolded protein response and viral pathogenicity</td>
<td>Luan, 2016</td>
</tr>
<tr>
<td>PIPO</td>
<td>newly discovered potyviral protein translated from the second potyviral ORF embedded within the P3 cistron.</td>
<td>Chung et al., 2008</td>
</tr>
<tr>
<td></td>
<td>involved in virus movement</td>
<td>Vijayapalani et al., 2012</td>
</tr>
<tr>
<td>6K1</td>
<td>one of the smallest potyviral proteins, involved in viral replication</td>
<td>Cui and Wang, 2016</td>
</tr>
<tr>
<td></td>
<td>potyviral protein involved in formation of characteristic pinwheel structures in cytoplasm (cylindrical inclusion)</td>
<td>Roberts et al., 1998</td>
</tr>
<tr>
<td></td>
<td>possesses ATPase and RNA helicase activity and is involved in viral replication</td>
<td>Lain et al., 1990, 1991; Eagles et al., 1994; Fernandez et al., 1997</td>
</tr>
<tr>
<td>CI</td>
<td>involved in viral movement along with P3N-PIPO</td>
<td>Rodriguez-Cerezo et al., 1997; Wei et al., 2010</td>
</tr>
<tr>
<td></td>
<td>interacts with several of host and viral factors and also found to be associated with 5' tip structure of potyviral virions.</td>
<td>Bilgin et al., 2003; Jimenez et al., 2006; Gabrenaitie-Verkhovskaya et al., 2008; Tavert-Roudet et al., 2012; Elena and Rodrigo, 2012; Bosque et al., 2014</td>
</tr>
<tr>
<td>6K2</td>
<td>another small protein membrane-associated protein involved in cellular endomembrane remodelling to produce virus replication vesicles.</td>
<td>Schaad et al., 1997a; Cotton et al., 2009</td>
</tr>
<tr>
<td></td>
<td>involved in long distance movement and symptom development</td>
<td>Septz and Valkonen, 2004</td>
</tr>
<tr>
<td>Protein</td>
<td>Description</td>
<td>References</td>
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<tr>
<td>VPg</td>
<td>Partially disordered protein interacting with a wide range of host and viral proteins and thought to play a critical role throughout the stages of virus infection. Remains covalently attached to the 5’ end of potyviral genome and acts as a primer to initiate replication.</td>
<td>Oruetxebarria et al., 2001; Rantalainen et al., 2008, 2011; Anindya et al., 2005; Puustinen &amp; Mäkinen, 2004</td>
</tr>
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<td></td>
<td>Involved in interactions with eIF4E/(iso)4E, polyadenylate-binding protein (PABP), eukaryotic elongation factor 1A (eEF1A), ribosomal protein P0 and HCPro to facilitate potyviral replication / translation. Suppresses sense-mediated RNA silencing by interacting with SGS3.</td>
<td>Wittmann et al., 1997; Leonard et al., 2000, 2004; Beauchemin et al., 2007; Eskellin et al., 2011; Hafren et al., 2013, 2015; Rajamäki et al., 2014</td>
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<tr>
<td>Nla-Pro</td>
<td>Carries a protease domain and cleaves individual proteins from the central to the C-terminal region of potyviral polyprotein (see Fig. 1). Internal cleavage site between VPg and Nla-Pro is often poorly utilised leading to substantial accumulation of conjoined form of VPg and Nla-Pro (Nla) during infection. This inefficient internal processing is proposed to have regulatory function. Nla localised in nucleus is proposed to have regulatory role in host gene expression. Interaction network of tobacco etch virus (TEV) Nla with host proteins from Arabidopsis shows that Nla target a wide range of host proteins regulating biotic and abiotic stress, photosynthesis, metabolism and ethylene-mediated defense response.</td>
<td>Adams et al., 2005; Schaad; et al., 1996; Anindya and Savithri, 2004; Rajamäki et al., 2009; Martinez et al., 2016</td>
</tr>
<tr>
<td>Nlb</td>
<td>Carries out uridylylation of VPg enabling it to act as a primer in replication. Interacts with eEF1A, PABP, heat shock protein Hsc70-3 to facilitate formation of replication complexes.</td>
<td>Hong and Hunt, 1996; Anindya et al., 2005; Puustinen &amp; Mäkinen, 2004; Dufrense et al., 2008a, b; Thivierge et al., 2008</td>
</tr>
<tr>
<td>CP</td>
<td>Primary function is to encapsidate potyviral RNA. Is known to strongly inhibit vRNA expression and must be kept away from interacting with vRNA during initial phase of infection. Is required at very high concentration during later stage for vRNA encapsidation. Is a phosphoprotein phosphorylated by protein kinase CK2, and its stability is regulated by CP-interacting protein (CPIP), heat shock protein 70 (HSP70) and C-terminus of Hsc70 interacting protein (CHIP).</td>
<td>Jagadish et al., 1991; Besong-Ndika, 2015; Lohmus et al., 2016; Ivanov et al., 2001, 2003; Hafren et al., 2010; Lohmus et al., 2016</td>
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</tbody>
</table>

*Functions of HCPro are discussed in details in the subsequent sections.*
1.3 Potyvirus infection cycle

Propagation of potyviral infection involves several distinct stages. First, the viruses enter to a host via mechanical inoculation or from the aphid’s stylet during their act of feeding. Uncoating of the virions to release vRNA into the host’s cytoplasm follows viral entry. The initial round of translation takes place directly from the released vRNAs to produce a set of viral proteins. This is followed by replication of the vRNAs by viral replicase Nib. Being a positive-sense RNA virus, replication occurs via synthesis of intermediate minus-strands, which are then used as templates to produce millions of copies of plus-strands. Post-translational uridylylation of VPg by Nib is a crucial step, which enables it to act as a primer for vRNA replication (Anindya et al., 2005; Puustinen & Mäkinen, 2004). Potyviral multiplication takes place within vesicle-like structures produced by modification of host endomembrane. 6K2 is the viral protein shown to be involved in the host membrane remodelling (Schaad et al., 1997a; Cotton et al., 2009). In addition, HCPro, P3, CI and Nia have also been proposed to be involved in viral replication (Hong and Hunt, 1996; Li et al., 1997; Merits et al., 1999, 2002; Cui et al., 2010; Ala-Poikela et al., 2011). CP, is mostly considered antagonistic to replication. Its overexpression has been shown to inhibit PVA gene expression possibly by promoting premature particle encapsidation (Hafren et al., 2010; Besong-Ndika et al., 2015). However, interaction of tobacco vein mottling virus CP specifically with functional Nib indicated its possible association with potyviral replication. Upon replication nascent vRNAs can take one of these three routes. 1. Vesicles containing viral replication complexes (VRCs) moves intracellularly along actin filaments towards plasmodesmata (PD), where viral movement associated proteins- P3N-PIPO, CI, VPg, CP enables them to pass to next cells (Schaad et al., 1997b; Carrington et al., 1998; Dolja et al., 1994; Wei et al., 2010). 2. vRNAs can serve as a trigger to activate plants RNA silencing machinery, which then targets back vRNAs to degrade them (reviewed by Carrington et al., 2001). 3. Viral RNA silencing suppressor HCPro subdues host defence response and safeguards it within potyvirus-induced granules (PGs) until it is ready for translation (Hafren et al., 2015). VPg is thought to be the major factor governing translation of the vRNAs (Eskellin et al., 2011; Hafren et al., 2013, 2015). Involvement of HCPro, eIF4E/eIF(iso)4E, and ribosomal protein P0 has also been predicted to function in translation (Hafren et al., 2013). Post translation, viral RNA is either taken back for new rounds of replication or encapsidated to form progeny virions. Involvement of both CP and HCPro in proper packaging of vRNAs has been proposed (Valli et al., 2014). A schematic diagram of major events in potyviral infection cycle is presented in Fig. 2.
Figure 2. A schematic representation of the major events of potyviral infection within a single cell. Potyviruses enter a cell through the stylet of an aphid during their feeding process or through mechanical inoculation. The virus then goes through uncoating and an initial round of translation to produce the viral proteins required for formation of viral replication complexes (VRCs). Many copies of vRNAs are produced within VRCs. HCPro, being a silencing suppressor, protects the vRNAs from host's RNA silencing machinery using multiple strategies. Upon replication it has been proposed that vRNA-containing PGs serve as the sites for RNA silencing suppression, wherefrom VPg aids the transfer of vRNAs to polysomes for translation of viral proteins. Translation is either followed by encapsidation of the vRNAs to form progeny virions or the vRNAs can re-enter into new rounds of replication. A fraction of vRNAs also travel to the subsequent cells either in form of particles or membrane bound vesicles to infect the plants systemically.

1.4 HCPro

HCPro is among the most studied potyviral proteins and perhaps one of the best examples of how a single viral protein can perform multiple functions (reviewed in Reevers and Garcia, 2015). Its name comes from its first identified function i.e., being a ‘Helper Component’ involved in plant-to-plant transmission of potyviruses by aphids. Apart from this, it also carries a cysteine proteinase domain in its C-terminus responsible for its self-
cleavage from the rest of the polyprotein. Subsequent researches identified many of its other properties, of which the best-known one is its ability to suppress antiviral RNA silencing.

1.4.1 Sequence, structure and domains

Potyviral HCPro is approximately 450-460 amino acids long (457 amino acids for PVA HCPro). Based on structural and functional features studied in HCPro of other potyviruses like lettuce mosaic virus (LMV), TEV and turnip mosaic virus (TuMV) (Plisson et al., 2003; Guo et al., 2011; reviewed in Valli et al., 2018; Fig. 3), PVA HCPro sequence can be demarcated into three regions (Fig. 3) - N-terminal (amino acids 1-100), central (amino acids 101-299) and C-terminal region (amino acids 300-457). N-terminal region of HCPro is characterised by a putative cysteine rich zinc-finger like motif (Robaglia et al., 1989). This region is predicted to be structurally isolated and less likely to interfere with the functions of the rest of the molecule (Plisson et al., 2003). This domain harbours crucial KITC motif (amino acids 51-54 in PVA HCPro) responsible for interaction of HCPro with aphid vector’s stylet (Thornbury et al., 1990; Fig. 3). Central region of HCPro is probably its most functionally rich segment and contains several motifs contributing to its diverse properties (Fig. 3). Overall, central region has further been characterized to have domains A and B. Both of these domains are shown to bind RNA independently (Urcuqui-Inchima et al., 2000; Plisson et al., 2003; Shiboleth et al., 2007). FRNK (amino acids 180-183 in PVA HCPro) motif present within the domain A has been correlated with its interaction with small interfering (si)RNAs (Shiboleth et al., 2007), which in turn links to its silencing suppression property. On the other hand, domain B has certain homology to ribonucleoproteins, and it harbours IGN motif (amino acids 249-251 in PVA HCPro) within a conserved LAIGNL box (amino acids 247-252 in PVA HCPro) responsible for genome amplification (Cronin et al., 1995; Urcuqui-Inchima et al., 2000). Apart from this, central domain contains several other amino acid stretches corresponding to functional properties like systemic movement (Cronin et al., 1995; Kasschau et al., 1997), synergistic interaction with other viruses (Shi et al., 1997) and virion formation (Valli et al., 2014). Intriguingly, several of the mutations carried out in the central region of HCPro resulted in debilitation or complete loss of its silencing suppression property (Kasschau and Carrington, 2001). C-terminal region is best known for carrying the cysteine proteinase domain (Fig. 3). It stretches though last 155 amino acids in the C-terminus, while, cysteine and histidine (residues 343 and 416 in PVA HCPro) forms the active site. Furthermore, PTK motif (amino acids 309 to 311 in PVA HCPro) is also present in this region. PTK motif of HCPro binds to DAG motif present in CP (Huet et al., 1994; Atreya et al., 1995; Peng et al., 1998). This motif along with KITC is proposed to form the ‘bridge’ between aphid’s stylets and virions leading to their plant-to-plant transmission (Govier and Kassanis, 1974; Huet et al., 1994; Peng et al., 1998; Blanc et al., 1998). Apart from this the C-terminal region of HCPro has also been implied to be involved in cell-to-cell movement (Rojas et al., 1997) and self-interaction of HCPro molecules to form oligomers (Guo et al., 1999; Plisson et al., 2003).
Figure 3. Annotation of the major domains and interaction motifs of PVA HCPro.
Demarcation of the domains and characterization of the motifs within PVA HCPro are based on the available reports on LMV, TEV and TuMV HCPro. The N-terminal region of HCPro comprises approximately 100 first amino acids. It is predicted to be structurally isolated from the other two regions. It harbours a putative zinc-finger motif and a KITC motif responsible for interaction with aphid’s stylet. The central region runs approximately through the next 200 amino acids, and is the segment of HCPro that is rich in functional motifs. Primarily it carries two RNA binding domains (A and B), and within them multiple motifs, responsible for many functions including RNA silencing suppression, genome amplification and systemic movement. The C-terminal region starts from approximately 300th amino acid and comprises rest of the molecule. This region mainly harbours the autocatalytic cysteine protease domain. Moreover, it contains the PTK motif responsible for CP interaction and together with the KITC motif it aids in plant-to-plant movement.

Plisson et al. (2003) did a 2D crystal structure analysis of HCPro of LMV using electron microscopy (EM). The study revealed that HCPro structure contains two helix-rich regions formed by amino acids from 40 to 235 and from 330 to 458. A hinge-like structure of about 95 amino acids connects these two domains. This region is highly resistant to trypsin digestion and predicted to be mainly consisting of β-sheets. It could be seen that each of these domains has some sort of self-contained functions, like the proteinase activity of the C-terminal domain, as well as cross-domain functions, like interdependence between the KITC motif in the N-terminal domain and the PTK motif in the hinge region. It has been hypothesised that the hinge structure could play a role in regulating accessibility of certain
motifs by either masking or exposing them via movement of the C-terminal helix-rich domain. The C-terminal cysteine proteinase domain has been further characterized by crystallography (Guo et al., 2011). Interestingly, their study revealed that the cysteine proteinase domain of TuMV HCPro bears low degree of homology with the standard papain-like cysteine proteases. Rather it shared similar topology with another cysteine proteinase, Venezuelan equine encephalitis virus nsP2 (genus *Alphavirus*; Guo et al., 2011).

### 1.4.2 HCPro- one of the major players in host-virus interaction

Several studies conducted in last few years have shown involvement of HCPro in almost all stages of infection. Recently compositional analysis of viral replication vesicles was carried out via mass spectrometry (MS). 6K2 being the potyviral protein responsible for endomembrane remodelling and viral replication complex (VRC) formation (Schaad et al., 1997a; Cotton et al., 2009), was fused to a Twin-Strep tag from its N-terminus and used as a bait to purify membrane bound potyviral replication complexes (Lohmus et al., 2016). Intriguingly, HCPro is the third most abundant protein present in the purified PVA replication factories. Viral RNA remains under constant threat from host’s RNA silencing machinery. Especially the double-stranded replication intermediates or the inherent stretches of double-stranded secondary structures present in vRNA triggers RNA silencing response in the host. HCPro being a suppressor of antiviral RNA silencing plays a major role in protecting vRNAs from host’s RNA silencing machinery (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). Pathway from replication to translation of the vRNAs, as well as molecular cues governing this transition are poorly understood. A recent study in this context proposed that vRNAs after replication goes to translation via the route of PVA induced granules (PGs) (Hafren et al., 2015). PGs are suggested as the site for protection of vRNA from host’s silencing machinery and this intermediate step has been proposed to be essential for efficient infection (see Fig. 2). Interestingly, HCPro is the sole potyviral component responsible for the granule induction (Hafren et al., 2015). VPg on the other hand prevents the formation of or dissolves PGs to release vRNAs and transport them to the polysomes. HCPro in this context enhances PVA translation in coordination with VPg (Hafren et al., 2015). Finally, Valli et al. (2014) produced strong evidence in support of the role of PPV HCPro in proper formation of viral particles. Apart from these, roles of HCPro in local and systemic movement as well as in aphid-mediated transmission have been known for decades (Huet et al., 1994; Kasschau et al., 1997; Peng et al., 1998; Kasschau and Carrington, 2001). Intriguingly, HCPro is also found to be present in the tip structure of potyviral particles (Torrance et al., 2006). This tip complex is further hypothesized to be involved in particle assembly / disassembly, translation initiation, directional cell-to-cell movement and aphid transmission (Torrance et al., 2006).
An integrated protein-protein interaction model compiled from information available on the potyvirus- *Arabidopsis* pathosystem indicated interaction of HCPro with a large number of host proteins (Elena and Rodrigo, 2012). This could be a reason behind its multifunctional nature. Many of these interactions were studied to some extent using both in vivo and in vitro systems, however comprehensive understanding about their biological relevance is still lacking. Among different interactors of HCPro there are e.g. 20S proteosome subunits-α5, PAA, PBB and PBE. HCPro interactions is proposed to inhibit their endonuclease / protease activity (Ballut et al., 2005; Sahana et al., 2012). Its silencing suppression activity is linked to its ability to interact with calmodulin-related protein rgs-CaM (Anandalakshmi et al. 2000), ethylene-inducible transcription factor RAV2 (Endres et al. 2010) and HUA ENHANCER 1 (HEN1) methyl transferase responsible for small (s)RNA methylation (Jamous et al., 2011). Also translation initiation factors eIF4E/iso4E (Ala-Poikela et al. 2011), RING-finger protein HIP1 (Guo et al. 2003), microtubule-associated protein HIP2 (Haikonen et al. 2013), calreticulin (Shen et al., 2010), chloroplast division related protein NtMinD, and chloroplast precursor ferredoxin-5 (Cheng et al., 2008) was shown to interact with HCPro. A recent study done by yeast-two-hybrid and in planta co-localization methods, demonstrated that HCPro interacts with AtCA1, an *Arabidopsis* homolog of salicylic acid binding protein 3 (SABP3). HCPro-mediated downregulation of AtCA1 has further been shown to be responsible for weakening of salicylic acid-mediated defence response (Poque et al., 2018). In planta outcomes of these interactions are not fully characterised. However, keeping in mind that most of these components are pivotal factors in cellular processes like translation, protein / RNA quality control, energy synthesis, defence signalling etc. it is possible to assume that cumulatively these events can lead to major perturbations in many cellular pathways during potyviral infection.

In this work, four novel interacting partners of PVA HCPro- S-adenosyl-L-methionine synthase (SAMS), S-adenosyl-L-homocysteine hydrolase (SAHH), Argonaute 1 (AGO1) and Varicose (VCS) have been identified in planta. Importance of these interactions in silencing suppression, synergistic interaction with PVX, vRNA protection, relieving translational repression and particle encapsidation has been investigated. Therefore, in the subsequent sections these aspects of potyviral HCPro will be discussed in details.

1.4.3 RNA silencing and its suppression

RNA silencing is an evolutionary conserved mechanism present in eukaryotes (reviewed in Guo et al., 2016) and possibly the strongest line of defence plants have against RNA viruses. Double-stranded RNA (dsRNA) triggers this response. Plants use this pathway to regulate their own gene expression through endogenously generated sRNAs produced from imperfect hairpins in the transcripts of non-coding micro RNA (miRNA) genes. A similar mechanism is activated when a plant is infected with an RNA virus (reviewed in Incarbone and Dunoyer, 2013). In this case the stem-loop structures present in vRNA or viral dsRNA replication
intermediates acts as a trigger (Ding and Voinnet 2007; Szittya et al., 2010). Dicer-like (DCL) endoribonucleases, a member of the ribonuclease III family of enzymes, recognize and cleave them into sRNA duplexes typically of 21-24 nucleotides in length. The sRNAs are then stabilized / protected from degradation through the 2′-O-methylation of their 3′ ends by the methyltransferase HEN1 (Yu et al., 2005; Yang et al., 2006). These DCL-processed sRNAs are at the heart of antiviral RNA silencing. Upon stabilization sRNA duplexes are recognized by one of the several Argonaute (AGO) proteins, which catalyzes unwinding of the sRNA duplexes followed by discarding one of the duplex strands. The other strand that is retained together (guide strand) with AGO forms the functional RNA-induced silencing complex (RISC; reviewed in Feng and Qi, 2016). RISC then uses the guide strand to scan partially or fully complementary target regions within vRNAs and to down-regulate its expression majorly via endonucleolytic cleavage (Jones-Rhoades et al., 2006). In order to amplify the silencing response host-encoded RNA dependent RNA polymerases (RDRPs) generate dsRNAs from the newly sliced RNA fragments (Vaucheret, 2006). The sRNAs can take part in intensifying the silencing process by supplying substrates to AGOs for RISC formation, or they can travel along the vascular system to spread silencing signals to the systemic leaves ahead of the spread of viral infection (reviewed in Melnyk et al., 2011).

In this never-ending molecular arms race between hosts and viruses, the latter have evolved an arsenal of proteins called RNA silencing suppressors that inhibit various stages of the silencing pathway. Although almost two decades have passed since HCPro was identified to have vRNA silencing suppression property (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998), yet its molecular mechanism could not be delineated precisely. Interactions between HCPro and many components of RNA silencing machinery have been detected so far, and based on them several working hypotheses were made. It appears that HCPro is probably using an overlapping yet multipronged approach to deal with plant’s RNA silencing system. In the following sections some of the major hypotheses will be described.

1.4.3.1 Sequestration of sRNAs

According to the most prevalent hypothesis, silencing suppression property of HCPro is attributed to its ability to sequester siRNAs (Lakatos et al., 2006; Shibolet et al., 2007; Garcia-Ruiz et al., 2015). This strategy is also common for some viral suppressors of RNA silencing, like P19 of tombusviruses and P21 of closteroviruses (Lakatos et al., 2006). In the case of HCPro, highly conserved FRNK motif in its central domain has been shown to have the propensity to bind siRNA (Shibolet et al., 2007). Since HCPro has not been shown to disrupt fully assembled RISC complex (Lakatos et al., 2006), it is prudent to assume that its interference with RNA silencing pathway is based upstream of RISC formation. Therefore, preventing loading of AGOs with virus-derived siRNAs seemed highly plausible theory in this context. Garcia-Ruiz et al. (2015), further pinpointed the importance of *Arabidopsis*
AGO2 in carrying out antiviral defense in TuMV infected leaves, while the same is taken care of by AGO1 and AGO10 in the inflorescence tissues. Furthermore, AGO-associated viral siRNA profiling in the presence of wild type / silencing suppression-deficient HCPro provided clear evidence supporting HCPro-mediated sequestration of viral siRNAs away from AGO1, AGO2 and AGO10 (Garcia-Ruiz et al., 2015). A rather recent study in this line has shown HCPro to have greater selectivity towards virus derived 21 and 22 nucleotide siRNAs. Interestingly, the study also showed that silencing suppression deficient PVY HCPro, an equivalent to TuMV HCPro AS9 mutant reported by Kasschau and Carrington (1998), completely lacked any bias towards virus derived siRNAs (del Toro et al., 2017). Cumulatively these studies have shown direct correlation between siRNA binding and silencing suppression property of HCPro.

1.4.3.2 Inhibition of sRNA methylation

According to another major hypothesis, HCPro-mediated inhibition of siRNA methylation contributes to its silencing suppression property. Availability of mature siRNAs is crucial for sustained RNA silencing response. DCL processed sRNA duplexes are not directly used for RISC formation, rather they are targeted to polyuridylation and degradation (Yu et al. 2005). In order to stabilize the sRNAs and protect them from degradation, biogenesis of sRNAs requires an additional step of 2’-O-methylation on the 3’ terminal ribose of sRNAs (Li et al., 2005; Ramachandran and Chen, 2008). This is a function of HEN1. In support of this hypothesis, HCPro of Zucchini yellow mosaic virus (ZYMV) has been reported to physically interact with HEN1 and inhibit its methyltransferase activity in vitro (Jamous et al., 2011). In the downside direct interaction model between HCPro and HEN1 could not be established, as pull-down assays from transgenic plants expressing P1/HCPro of TuMV could not identify HEN1 as an in vivo binding partner of HCPro. However, this did not exclude the possibility that HCPro might influence its subcellular localization or interact with some of the factors, which HEN1 might require for its proper functioning (Yang et al., 2006). In this line, HCPro was shown to interact with SAHH in planta by bimolecular fluorescence complementation (BiFC) assay (Cañizares et al., 2013). Understanding the relationship between SAHH and HEN1 functionality requires knowledge about the methionine cycle, an important metabolic pathway in the hosts (Fig. 4). HEN1 is essentially a methyltransferase that uses universal methyl group donor molecule S-adenosyl-L-methionine (SAM) as its substrate to methylate sRNAs (Yu et al. 2005). Therefore, in order to function properly HEN1 needs a constant supply of SAM from a smoothly running methionine cycle. S-adenosylmethionine synthase (SAMS) and S-adenosyl-L-homocysteine hydrolase (SAHH) are at the heart of the methionine cycle. SAMS uses methionine as its substrate to produce SAM. Methyltransferases, HEN1 in this particular case, uses SAM to methylate their targets, sRNAs in this case. When doing so, S-adenosyl-L-homocysteine (SAH) is produced as a byproduct. SAH being a potential feedback inhibitor of most of the methyl transferases including HEN1, is therefore rapidly broken down to adenosine and L-
homocysteine (HCY) by SAHH. Methionine synthase (MS) converts HCY back to methionine to complete the cycle. As can be seen SAHH holds an important position in ensuring sustained functioning of HEN1 and as a support to this hypothesis SAHH has been shown essential for local RNA silencing (Cañizares et al., 2013).

**Figure 4. A simplified representation of the host methionine cycle in the context of sRNA methylation and RNA silencing pathway.** Double stranded intermediates from vRNA replication are targeted by DICER like endoribonucleases (DCLs) to cleave into siRNAs 21-24 nucleotides long. sRNAs are methylated at their 3' end by methyltransferase HEN1. This step is important as it protects siRNA from polyuridylation and degradation. Therefore, siRNA methylation is an essential step in stabilizing siRNAs prior to their loading onto RISC complex and further degradation of targeted vRNAs downstream. siRNA methylation marks crucial overlap between RNA silencing pathway and methionine cycle. Methionine cycle provides HEN1 its substrate SAM, the universal methyl group donor, to methylate siRNAs. Methionine cycle is also responsible for removing the byproduct SAH, which is a feedback inhibitor of methyltransferases including HEN1. Therefore, smooth running of the methionine cycle is essential for the RNA silencing pathway.

1.4.3.3 **Rearrangements on host’s gene expression profile**

In addition to the aforementioned theories on silencing suppression, several other lines of studies have proposed many overlapping yet alternative strategies for HCPro-mediated
downregulation of host’s RNA silencing pathway. For example, HCPro-mediated regulation of miR168 has shown to reduce AGO1 level in planta and proposed as a mean to control potential AGO1-RISC formation (Varallyay and Havelda, 2013). Another strategy HCPro might use to tackle RNA silencing is to downregulate host RNA-dependent RNA polymerase 6 (RDR6) by reducing its mRNA level (Zhang et al., 2008). RDR6 plays crucial role in amplification stage of the silencing pathway. Therefore, controlling RDR6 transcription has been proposed to be an effective mean to control antiviral RNA silencing response. It can be a matter of debate, whether these events are independent approaches to handle antiviral RNA silencing or conjoined part of a master-regulation strategy that causes genome-wide alteration of proteome profile by altering transcript levels. In support of the latter one, HCPro has been shown to grossly perturb miRNA-mediated endogene regulation in tobacco (Soitamo et al., 2011). Microarray based analysis of the transcript levels in tobacco plants expressing PVY HCPro constitutively have been carried out and compared to wild type tobacco plants. Differential regulation of genes related to defense, hormone response, stress response as well as vital processes like photosynthesis, sugar metabolism etc. has been noticed. Intriguingly, transcript level of SAMS, methionine cycle component discussed in the previous section, was also found to reduce in HCPro expressing plants causing a reduction in methyltransferase reactions (Soitamo et al., 2011). Although the authors of the article believed that downregulation of SAM level in the cell would have affected processes like chloroplast biogenesis, pectin synthesis etc., however, it does not exclude the possibility that this could be equally important in reducing transmethylation capacity of HEN1.

1.4.4 HCPro in potex-potyviral synergism

One of the intriguing aspects of potex-potyviral synergism is that only potexvirus gets advantage from the synergistic interactions. For example, PVX accumulates to several folds higher titre than usual during a synergistic infection in tobacco, while no such variation in potyvirus titre could be seen (Vance 1991, Vance et al., 1995). According to the working hypothesis on potex-potyviral synergism, HCPro being a suppressor of RNA silencing, is thought to breakdown plants antiviral defense system, the advantage of which is taken by PVX to accumulate beyond normal host limits. In support of this idea it has been noticed that participation of whole potyviral genome is not necessary for synergistic interaction to happen, rather expression of P1-HCPro segment is enough to achieve similar degree of effect (Pruss et al., 1997). Furthermore, mutations in the central domains of HCPro impairing its ability to suppress RNA silencing, resulted in complete loss of its ability to induce synergism (Shi et al., 1997, Kasschau and Carrington, 1998, Marathe et al., 2000, Kasschau and Carrington, 2001, Voïnnet, 2001, Gonzalez-Jara et al., 2005). However, molecular events leading to induction of potex-potyviral synergism and the role of HCPro therein are not as straightforward as it looks from this perspective. In Nicotiana benthamiana based study system, synergistic interaction did not cause significantly higher accumulation in virus titre,
however symptom development was drastically elevated leading to plant death (González-Jara et al., 2004). A recent study in this context has shown PVX pathogenicity determinant P25 to induce cell death upon co-expression with HCPro. Acute ER stress and unfolded protein response has been proposed to be the cause underlying induction of programmed cell death (Aguilar et al., 2018). In another line of study, transcriptional profiling has shown that in PVX–PVY–benthamiana based pathosystem, synergism leads to heavily increased oxidative stress in the host which is not common to single infection by either of the virus (Garcia-Marcos et al., 2009). Redox balance in cells is predominantly maintained by the antioxidant glutathione (GSH) by quenching oxidative radicals. Depletion of GSH and accumulation of its oxidized from GSSG are common biomarkers for oxidative stress (Mytilineou et al., 2002). Intriguingly, GSH biosynthesis and methionine cycle are closely associated biochemical pathways with the transsulfuration pathway as a bridge linking cysteine and homocysteine, respective the intermediates of these pathways (Wilson et al., 1976). Proximity of the methionine cycle with both the RNA silencing pathway and the GSH biosynthesis pathway leads to the question, whether HCPro-mediated disruption of methionine cycle can be a major event in development of synergistic response during potex-potyvirus mixed infection.

1.4.5 PVA-induced RNA granules

PGs are the amorphous cytoplasmic bodies, characteristic to potyviral infection and are induced by the action of HCPro. The proposed function of these structures is to protect vRNAs from host’s antiviral defense agents. Since, silencing suppression deficient variants of HCPro also failed to induce PGs, an obvious inter-connection between these two properties of HCPro has been proposed (Hafren et al., 2015). However, PGs deserve a special mention outside standard silencing suppression strategies of HCPro. They are co-localization sites for many host and viral factors. Involvement of all these components is essential in PVA infection, and a hairpin-mediated downregulation of the individual components have been shown to damage PVA infectivity drastically. PGs bear similarity to two of the visual aggregates of ribonucleoprotein (RNP) complexes involved in mRNA metabolism- stress granules (SGs) and processing bodies (PBs). SGs are the RNP complexes formed around stalled translation initiation complexes. They mean to protect RNAs from cellular threats upon stress conditions. On the other hand, PBs are the constitutively present housekeeping entities, primarily dealing with mRNA decay. Additionally, both SGs and PBs can be distinguished based on many of their mutually exclusive constituents. Several studies have emphasized importance of SGs and PBs in viral infection cycle in either positive or negative ways (reviewed in Beckham and Parker, 2008). Interestingly, PGs does not conform to the canonical models of either of them. Rather they contain unique combination of components both from SGs and PBs plus viral protein HCPro. Hallmark components of SGs present in PGs include oligouridylate-binding protein 1 (UBP1), eIF4E and PABP. Distinctive components from PBs present in PGs include VCS and AGO1 (Hafren et al.,
An interesting explanation for the unique composition of PGs has been provided recently while studying antiviral autophagy. Markers for SGs and PBs were proposed to co-aggregate in RNA granules during a selective pathway of autophagy termed granulophagy. VPg is the potyviral protein that has been proposed to both rescue PGs from autophagic degradation and transport vRNAs from PGs to polysomes (Hafren et al., 2015, 2018). However, molecular cues governing formation as well as dissolution of PGs are still unknown.

1.5 Translational repression- an overlooked aspect in potyviral infection

In addition to the canonical model of endonucleolytic cleavage by RISC, mRNA expression can be downregulated by translational repression. The degree of complementarity between sRNAs and the target mRNA is thought to govern whether slicing or translational repression will prevail (Hutvagner and Zamore, 2002). According to the perceived notion, in animal system, where imperfect base pairing is common, translational repression is believed to be the predominant silencing mechanism, whereas in plant system, sRNAs being highly complementary to their targets, slicing is considered to be the default one. However, this idea has started to change and translational repression is getting acceptance as equally widespread mechanism in plants as they are in animals (Jones-Rhoades et al., 2006; Goeres et al., 2007; Brodersen et al., 2008; Lanet et al., 2009; Xu and Chua, 2009). On the same line, the results of a recent study pinpoints that translational repression could be an antiviral strategy employed by plants against potyviruses (Iwakawa and Tomari, 2013). In this work, a heterologous Renilla luciferase (RLUC) mRNA was fused to TEV 5’UTR, which contains an internal ribosome entry site (IRES; Iwakawa and Tomari, 2013). The outcome was an mRNA poorly compatible with cap-dependent translation. AGO1-RISC complex programmed with sRNAs fully complementary to TEV 5’UTR as well as the RLUC ORF was shown to strongly repress cap-independent translation. However, in contrast to canonical animal model, translational repression did not happen for the 3’ UTR target sites. Based on these observations two plant specific repression models for cap-independent translation were suggested. In the first model, AGO1-RISC bound to the 5’UTR was thought to physically block ribosome recruitment, while in the alternative model AGO1-RISC bound to the ORF was proposed to sterically hinder forward movement of the ribosomes resulting in stalled translation (Iwakawa and Tomari, 2013).

Any of the host or viral factors responsible for relieving potyviral translational repression has not been identified hitherto. However, close association of HCPro with AGO1 and VCS, two of the host factors previously linked with translational repression, plus established role of HCPro in enhancing PVA translation in co-ordination with VPg, arises the question-whether HCPro is the potyviral factor responsible for relieving translational repression? Moreover, role of VCS in potyvirus infection cycle has not yet been explored in depth. Earlier results from our lab suggested involvement of VCS in multiple levels of PVA
infection. VCS is a WD40-repeat protein associated with PBs. Multiple lines of evidence indicates its involvement in RNA metabolism (mRNA decapping and translational repression) and in regulating post-embryonic development in Arabidopsis (Xu et al., 2006; Goeres et al., 2007; Xu and Chua, 2009; reviewed in Ma et al., 2013). Similar to its human analogue Ge-1/Hedls, VCS is also predicted to act as a scaffold for protein-protein interactions. Ge-1 has earlier been identified as a pro-viral factor and its depletion has been shown to reduce Hepatitis C virus protein and RNA accumulation significantly (Fenger-Gron et al., 2005; Pager et al., 2013). Likely enough, VCS also has a positive impact in PVA infectivity. It is an integral component of PGs, and its downregulation affects PVA expression and coat protein accumulation adversely (Hafren et al., 2015). Moreover, seminal work on translational regulation by sRNAs in Arabidopsis showed increased protein accumulation from some mRNAs in VCS mutant lines (Brodersen et al., 2008). This further consolidated importance of VCS in translational repression (Xu et al., 2006; Goeres et al., 2007; Brodersen et al., 2008; Xu and Chua, 2009).
2. AIMS OF THE STUDY

The aim of this study was to elucidate the molecular mechanisms underlying various functions HCPro performs during PVA infection. We set to look into HCPro interacting partners via conducting an HCPro pull-down study for samples derived from PVA infected plants. First, we aimed to address the role of HCPro in RNA silencing suppression. HCPro had been predicted to interfere with HEN1-mediated siRNA methylation, but direct interaction between HCPro and HEN1 had not been established (Yu et al., 2006; Jamous et al., 2011). On the other hand, interaction between HCPro and the methionine cycle component SAHH had been proposed (Cañizares et al., 2013). Therefore, our objective was to decipher the mechanism by which HCPro inhibits the host methionine cycle and to study how this affects PVA infection.

Silencing suppression property of HCPro is a factor responsible for induction of synergism during potex-potyviral mixed infection (Gonzalez-Jara et al., 2005). Inhibition of siRNA methylation in turn is considered as a possible strategy HCPro employs to achieve silencing suppression (Jamous et al., 2011, Cañizares et al., 2013). Therefore, as the second aim of this study we decided to probe into the role of methionine cycle in the context of mixed PVX-PVA infection.

HCPro induce PGs, which in turn were suggested as sites of vRNA protection from the host’s RNA silencing machinery (Hafren et al., 2015). The combined effort of both VPg and HCPro enhances PVA translation (Eskelin et al., 2011; Hafren et al., 2015). In this context VPg was proposed to transport vRNAs from PGs to polysomes (Hafren et al., 2015). As the third aim of this study, we decided to look into the molecular cues governing assembly of PGs and subsequent transition of vRNAs to translation. Evidence suggesting AGO1-RISC-mediated translational repression targeting TEV 5’UTR-IRES (Iwakawa and Tomari, 2013), prompted us also to investigate, if translational repression is active during PVA infection.
3. MATERIALS AND METHODS

3.1 Plants

Most of the experiments were conducted on *Nicotiana benthamiana* plants (NCBI: txid4100). Plants were grown at 22 °C under 16 h light / 8 h dark photoperiod in environmentally controlled greenhouses. Draft genome sequence could be found at Sol Genomics Network (https://solgenomics.net; Bombarely et al., 2012). For FLAG-tag-mediated ribosome purification studies transgenic *N. benthamiana* constitutively expressing *Arabidopsis thaliana* RPL18B kindly provided by Peter Moffett was used. Young *N. benthamiana* plants at 4-6 leaf stage were chosen for agroinfiltration.

3.2 Viruses

All the experiments involved with potyvirus were carried out using with genetically engineered versions of PVA strain B11 (GenBank accession number AJ296311). PVX-PVA mixed infection studies were conducted using binary vector pGreen carrying PVX icDNA (pgR106/7; http://www.plantsci.cam.ac.uk/research/davidbaulcombe/methods/vigs).

3.3 Other methods and list of constructs

All the methods used in this study are described in the publications as indicated in Table 2. Additionally, this study required the use of many different viral constructs, both wild type and mutated ones, as well as various expression and silencing constructs. A detailed list of all the constructs along with brief description of each of them are presented in Table 3.

### Table 2. Methods used in the studies

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<tr>
<th>Methods</th>
<th>Publication</th>
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<td><em>Agrobacterium</em> infiltration</td>
<td>I, II, III</td>
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<tr>
<td>Confocal microscopy</td>
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</tr>
<tr>
<td>Dual luciferase assay</td>
<td>I, II, III</td>
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<td>Electron microscopy</td>
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<td>Epifluorescence microscopy</td>
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<td>FLAG-affinity purification of ribosomes</td>
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<td>Fluorescence intensity quantification</td>
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<tr>
<td>Immunocapture-RT-PCR (ic-RT-PCR)</td>
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<tr>
<td>LC-MS/MS</td>
<td>I, II, III</td>
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</table>
Molecular cloning  I, II, III
Quantitative RT-PCR (qRT-PCR)  II, III
Reverse transcription PCR (RT-PCR)  I, II, III
SAMS activity assay  I
Silver staining  I, II
Stability assay of HCPro derived HMW complexes  II
Strep-tag affinity purification of HCPro  I, II
Ultracentrifugation to fractionate ribosomes  I
Western blot analysis  I, II

<p>| Table 3. Molecular constructs used in this study along with brief description |
|-----------------|-----------------|---------|-----------------|--------|
| Construct name  | Gene Cassette   | Vector  | Description                                              | Ref    |
| PVA&lt;sup&gt;(RFP/YFP/CFP)&lt;/sup&gt; | 35S-PVA&lt;sup&gt;wt&lt;/sup&gt;::RFP/YFP/CFP-nos | pRD400 | Full length wild type PVA icDNA tagged with either RFP / YFP or CFP | Ivanov et al., 2003; III |
| PVA&lt;sup&gt;WD&lt;/sup&gt; | 35S-PVA&lt;sup&gt;WD&lt;/sup&gt;:RLUC&lt;sup&gt;int&lt;/sup&gt;-nos | pRD400 | RLUC-tagged full-length infectious cDNA clone of PVA with mutation in WD domain interacting motif of HCPro | II |
| PVA&lt;sup&gt;WD&lt;/sup&gt;-Strep-RFP | 35S-PVA-[(2xStrep)-RFP-HCPro&lt;sup&gt;WD&lt;/sup&gt;]:RLUC&lt;sup&gt;int&lt;/sup&gt;-nos | pRD400 | RLUC-tagged PVA expressing HCPro&lt;sup&gt;WD&lt;/sup&gt; fused to the red fluorescent protein (RFP) and two copies of the Strep-tag II | II |
| PVA&lt;sup&gt;WT&lt;/sup&gt; | 35S-PVA&lt;sup&gt;WT&lt;/sup&gt;:RLUC&lt;sup&gt;int&lt;/sup&gt;-nos | pRD400 | RLUC-tagged full-length infectious cDNA clone of PVA | Eskelin et al., 2010 |
| PVA&lt;sup&gt;WT&lt;/sup&gt;-Strep-RFP | 35S-PVA-[(2xStrep)-RFP-HCPro&lt;sup&gt;WT&lt;/sup&gt;]:RLUC&lt;sup&gt;int&lt;/sup&gt;-nos | pRD400 | RLUC-tagged PVA expressing HCPro&lt;sup&gt;WT&lt;/sup&gt; fused to the red fluorescent protein (RFP) and two copies of the Strep-tag II | Hafrén et al., 2015 |</p>
<table>
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<tr>
<th>Construct name</th>
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<tr>
<td>GUS</td>
<td>35S-GUS-nos</td>
<td>pRD400</td>
<td>Plasmid expressing uidA gene encoding β-glucuronidase (GUS)</td>
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<td>HCPro&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>35S-HCPro-nos</td>
<td>pRD400</td>
<td>Plasmid expressing PVA HCPro</td>
<td>Hafrén et al., 2015</td>
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<tr>
<td>HCPro&lt;sup&gt;4EBM&lt;/sup&gt;</td>
<td>35S-HCPro&lt;sub&gt;4EBM&lt;/sub&gt;-RFP-nos</td>
<td>pSITEII6C1</td>
<td>Plasmid expressing eIF4E binding deficient mutant of HCPro fused to RFP</td>
<td>Hafrén et al., 2015</td>
</tr>
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</table>

**Protein expression constructs**

PVA<sup>AGDD-HCPro<sub>WD</sub></sup> 35S-PVA-[HCPro<sub>WD-Nib<sup>AGDD</sup></sub>]<sup>::</sup>RLUC<sup>int</sup>-nos pRD400 RLUC-tagged non-replicating variant of PVA with mutation in GDD motif of its replicase. HCPro expressed has mutation in its WD domain interacting motif Hafrén et al., 2015

PVA<sup>AGDD-HCPro<sub>WT</sub></sup> 35S-PVA-[HCPro<sub>WT-Nib<sup>AGDD</sup></sub>]<sup>::</sup>RLUC<sup>int</sup>-nos pRD400 RLUC-tagged non-replicating variant of PVA with mutation in GDD motif of its replicase. HCPro expressed is wild type Hafrén et al., 2015

PVA<sup>AGDD-HCPro<sub>ΔHCPro</sub></sup> 35S-PVA<sup>ΔHCPro<sub>Δ</sub></sup>:[::RLUC<sup>int</sup>-nos pRD400 RLUC-tagged non-replicating variant of PVA with mutation in GDD motif of its replicase. This PVA construct lacks HCPro Hafrén et al., 2015

PVA<sup>ΔHCPro</sup> 35S-PVA<sup>ΔHCPro</sup>:[::RLUC<sup>int</sup>-nos pRD400 PVA lacking HCPro tagged with RLUC Hafrén et al., 2015

PVX<sup>GFP/RLUC</sup> 35S-PVX::GFP/RLUC<sup>int</sup>-nos pGreen Full length wild type PVX icDNA tagged with GFP or RLUC Hafrén et al., 2015
<table>
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<th>Construct</th>
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<td>pSITEII6 C1</td>
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<td>HCP{\textsuperscript{SDM}}</td>
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<td>Plasmid expressing silencing suppression deficient mutant of HCP{\textsuperscript{RFP}} fused to RFP</td>
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<td>HCP{\textsuperscript{WD}}</td>
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<td>Plasmid expressing \textit{N. benthamiana} VCS-A</td>
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<td>VCS-A{\textsuperscript{YFP}}</td>
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<td>pRD400</td>
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<td>Plasmid expressing \textit{N. benthamiana} VCS-C</td>
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<td>VPg</td>
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Hafrén et al., 2015

Eskelin et al., 2011
<table>
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<tr>
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<th>Description</th>
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<tr>
<td>pHG-CTRL</td>
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<td>pHG12</td>
<td>Plasmid expressing no hairpin</td>
<td>Hafrén et al., 2013</td>
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<tr>
<td>pHG-GSHS</td>
<td>35S-GSHS(hp)-ocs</td>
<td>pHG8</td>
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<tr>
<td>pHG-HEN1</td>
<td>35S-HEN1(hp)-ocs</td>
<td>pHG12</td>
<td>Plasmid expressing hairpin RNA targeting the HEN1 gene family</td>
<td>I</td>
</tr>
<tr>
<td>pHG-SAHH</td>
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<td>Plasmid expressing hairpin RNA targeting the SAHH gene family</td>
<td>I</td>
</tr>
<tr>
<td>pHG-SAMS</td>
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<td>pHG12</td>
<td>Plasmid expressing hairpin RNA targeting the SAMS gene family</td>
<td>I</td>
</tr>
<tr>
<td>pHG-RLUC</td>
<td>35S-RLUC(hp)-ocs</td>
<td>pHG12</td>
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<td>Hafrén et al., 2015</td>
</tr>
<tr>
<td>pHG-VCS</td>
<td>35S-VCS(hp)-ocs</td>
<td>pHG12</td>
<td>Plasmid expressing hairpin RNA targeting the VCS gene family</td>
<td>Hafrén et al., 2015</td>
</tr>
</tbody>
</table>
4. RESULTS AND DISCUSSION

4.1 Detection of HCPro interaction partners during PVA infection

4.1.1 HCPro purification strategy

HCPro has to carry out multiple roles during a successful PVA infection. To probe into the functions of HCPro in a comprehensive way, the approach undertaken herein was to look into its binding partners. In order to do so, a genetically engineered PVA construct with its HCPro fused to RFP and twin Strep-tag at its N-terminus (PVAWT-Strep-RFP) was allowed to spread and infect young Nicotiana benthamiana plants. Systemically infected leaves were harvested and HCPro along with its binding partners was co-purified via Strep-affinity purification (I, Fig. 1a).

4.1.2 Analysis of the co-purified proteins

HCPro and HCPro-bound host / viral proteins were purified from PVA infected leaves followed by their identification via liquid chromatography tandem-mass spectrometry (LC-MS/MS). Nonspecific binding of the host and viral proteins with the Strep-Tactin matrix poses chance of having false candidates in to the list of detected proteins. In order to counter that issue PVA with untagged HCPro (PVAWT) has been used as the negative control. Exclusion of the proteins found in negative control from the list of proteins detected in the PVAWT-Strep-RFP purified samples eliminated the risk of having those candidates, which are not specifically purified due to the binding of strep-tagged HCPro to the matrix, in the proteome. Moreover, to account for biological variation between the samples, each set of purification and LC-MS/MS detection was carried out in triplicates. Quality of purification was assessed by silver staining (I, Supplementary Fig. 1). All the control samples visually looked distinctly different from that of the actual Strep-purified samples. This ensured the validity of the purification approach applied herein. Moreover, multiple bands below and above ~80 kDa range (corresponding to monomeric HCProWT-Strep-RFP) and specific enrichment of the high molecular weight (HMW) region (>250 kDa) suggested purification of HCPro-bound HMW-complexes and possible co-purification of other host and viral proteins also. Presence of faint bands in the control samples of course indicated the possibility of some non-specific interactions, however total absence of bands in the HMW region was a good sign for enrichment of virus specific complexes in the purified samples. Following this, the next step was to detect the host and viral proteins co-purified with HCPro. Many host and viral components including CI, VPg, SAMS, SAHH and several ribosomal proteins were detected specifically in HCProWT-Strep-RFP purified sets. The ones, which are important for this study, have been enlisted in (I, Table 1; Fig. 1a). Presence of SAMS, VPg and CI was validated subsequently via western blotting (I, Fig. 1b, c). Although not shown in this particular experiment, the presence of SAHH was also validated later from a relatively
similar experimental setup in the second study (II, Fig. 3E). Moreover, SDS-PAGE with single-wide lane loaded with strep purified samples was cut into multiple strips and probed with individual antibodies. A distinct band at the HMW region with approximately similar electrophoretic mobility, suggested that all of the tested proteins were part of one or more large multiprotein complexes in which HCPro is the common denominator (I, Fig. 2). However, it is unknown at the moment, to how many distinct complexes these proteins belong.

4.1.3 Significance of SAMS and SAHH in the HCPro interactome

4.1.3.1 HCPro inhibits SAMS activity during PVA infection

As described already, a few independent studies in recent years have provided fragments of information connecting HCPro with the methionine cycle (Jamous et al., 2011; Soitamo et al., 2011; Cañizares et al., 2013). In this work, SAMS was shown for the first time to be associated with HCPro during PVA infection. Moreover, a pull-down study validated the ability of HCPro to form stable HMW complexes with both SAMS and SAHH. SAMS uses methionine to produce SAM, the universal methyl donor used in most of the transmethylation reactions. Hence, it is considered as a key player in regulating methylation in the host cells. In order to assess the practical implications of this finding, in the next step a study was carried out to determine if HCPro-SAMS interaction led to reduction in SAMS activity during PVA infection. SAMS activity was determined by incubating plant sap in an appropriate buffer supplemented with $^{35}$S-labeled methionine and ATP. Afterwards, quantification of radioactivity incorporated into newly formed $^{35}$S-SAM due to the SAMS activity, was carried out by liquid scintillation counting technique (Shen et al., 2002). Since, the idea was to determine if PVA infection reduces SAMS activity in HCPro-dependent manner, several controls were included into the experimental design. Firstly, plants infiltrated with Agrobacterium carrying 35S-β-glucuronidase gene (GUS) served as the non-infected control. Samples from 35S-HCPro overexpressing plants were used as a control to show if HCPro can affect SAMS activity alone or it needs an authentic infection as an addendum to do so. HCPro-less PVA (PVA$^{ΔHCPro}$) was the control to determine if SAMS activity in the infected cells is specifically HCPro dependent or other viral factors also have an impact in SAMS activity. Finally, plants infected with wild type PVA (PVA$^{WT}$) served as the sample for assessing the effect of HCPro on SAMS activity in the context of bona fide PVA infection. Background radioactivity coming from the nonspecific interactions was removed by subtracting the residual activity retained in the membrane calculated from SAMS-silenced plants (I, Supplementary Fig. 2). The study revealed that HCPro can reduce SAMS activity during PVA infection. However, a co-operative effect between HCPro and other PVA protein(s) in this context seemed necessary, as neither HCPro alone nor PVA$^{ΔHCPro}$ could significantly affect SAMS-activity (I, Fig. 3a; Supplementary Fig. 3). Moreover, to show that the decrease in SAMS activity in PVA infected plants is not because
of reduced accumulation of SAMS, western blot from control and PVA infected plants was carried out against SAMS antibody (I, Fig. 3b). Similar level of SAMS was found in both infected and non-infected samples, which reinforced the fact that inhibition of SAMS activity during PVA infection is a causality of HCPro-SAMS interaction.

4.1.3.2 Silencing SAMS and SAHH partially rescues expression of PVA$^{\Delta}$HCPro

Both SAMS and SAHH being shown to be associated with HCPro and partial characterization of both of the interactions being done (from this study and Cañizares et al., 2013), the next imminent question was to find out the implications of these interactions in PVA infection. The approach undertaken herein was to verify if knockdown of SAMS (pHG-SAMS) and SAHH (pHG-SAHH) could rescue deficiency of HCPro during PVA infection. Therefore, N. benthamiana plants were infected with PVA$^{\Delta}$HCPro virus with simultaneous downregulation of SAMS or SAHH followed by quantification of viral RNA expression and accumulation therein. Hairpin constructs targeting all known members of SAMS (4 members) or SAHH (7 members) of N. benthamiana were designed (pHG-SAMS and pHG-SAHH) to silence the corresponding genes locally (I, Supplementary Methods). The experimental setup also included sets infiltrated with an empty silencing vector as a control (pHG-CTRL). Moreover, simultaneous downregulation of both SAMS and SAHH was also conducted in another sample set to achieve stronger downregulation of the methionine cycle. Agrobacterium carrying 35S-firefly luciferase (FLUC) has been added in all of the infiltrations to account for the non-specific effects of silencing on overall gene expression in the host. HCPro being a vital factor in multiple stages of PVA infection cycle its absence leads to a huge reduction in PVA infectivity. However transient knockdown of SAMS and SAHH both led to partial rescue of RLUC expression from PVA$^{\Delta}$HCPro. Furthermore, simultaneous silencing of SAMS and SAHH, which supposedly resulted in stronger downregulation of the methionine cycle and led to even higher degree of rescue than individual knockdowns, a result being in favour of direct relevance of the methionine cycle in PVA infection (I, Fig. 4). However, methionine cycle being responsible for maintenance of a multitude of methylation-dependent cellular processes, its downregulation inevitably had some non-specific effects. Its effect in translation of unrelated mRNAs was demonstrated by quantification of the internal standard FLUC. Simultaneous silencing of SAMS and SAHH led to reduced expression of FLUC. However, RLUC derived from PVA RNA was not reduced like-wise, showing evidence for virus specific effect of the methionine cycle to PVA expression (I, Supplementary Fig. 4). Therefore, to demonstrate the virus specific effect of SAMS and SAHH silencing on PVA expression, results are presented in terms of the ratio between RLUC/FLUC activities (I, Fig. 4a). Moreover, to demonstrate that the effect of methionine cycle downregulation is not merely limited to enhancement of viral expression, PVA RNA accumulation level was also quantified. This further validated PVA expression results by showing commensurate increase in PVA RNA level in the
corresponding samples (I, Fig. 4). Moreover, this in turn also suggested that downregulation of SAMS and SAHH resulted in stabilization of viral RNA, possibly by disrupting its degradation process. Idea behind the hypothesis in practical terms is- HCPo mediates breakdown of methionine cycle causing disruption in sRNA methylation by HEN1, a crucial step of RNA silencing pathway, all the way leading to silencing suppression during PVA infection. The hypothesis if true, should demonstrate similar pattern of outcomes upon HEN1 silencing also. Therefore, in order to validate the hypothesis, other experiments were conducted where RLUC expressed from PVA$^{\text{AHCPo}}$ was monitored in HEN1 silenced background (pHG-HEN1). Interestingly, HEN1 downregulation also resulted in partial rescue of HCPo-less PVA expression, advocating in favour of the hypothesis (I, Fig. 4). However, the term ‘partial rescue’ needed a special emphasis, since RLUC expression from PVA$^{\text{WT}}$ still remained several fold higher than PVA$^{\text{AHCPo}}$ even upon simultaneous silencing of SAMS and SAHH (I, Supplementary Fig. S5). This reinforced the fact that HCPo contributes to PVA amplification in multiple stages of infection and likely with several independent mechanisms. Nevertheless, these results also establish that HCPo-mediated disruption of the methionine cycle could be one of the putative mechanisms by which it suppresses the antiviral RNA silencing during PVA infection.

4.1.3.3 Hypothetical model on molecular mechanism underlying HCPo-mediated RNA silencing suppression

Methionine cycle plays a crucial role in ensuring smooth running of RNA silencing machinery in the cells. Based on the above findings it has been hypothesized that during PVA infection HCPo along with other viral proteins breaks down methionine cycle in the host by interacting with its two key components SAMS and SAHH (I, Fig. 7b). HCPo in the presence of other PVA factors inhibits SAMS activity resulting in decreased conversion of methionine to cellular methyl group donor SAM. Like most of the methyltransferases, HEN1 also uses SAM as its substrate to methylate target sRNAs. Therefore, HCPo mediated deactivation of SAMS leads to scarcity of substrate for HEN1, which in turn is reflected into reduced sRNA methylation. Unmethylated sRNA are polyuridylated and degraded causing disruption in RNA silencing pathway (Li et al., 2005; Ramachandran and Chen, 2008). From the other direction, inhibition of SAHH by HCPo leads to accumulation of SAH in the system. SAH being the feedback inhibitor of many methyltransferases including HEN1, also potentially inhibits sRNA methylation, further intensifying their cumulative effect. HEN1 deactivation via HCPo mediated disruption of methionine cycle upstream acts as a circuit breaker in RNA silencing pathway leading to its suppression. This hypothesis is in accordance with the observations of Yang et al. (2006), where P1-HCPo segment from TuMV caused reduction in sRNA methylation in transgenic plants constitutively expressing it. However, it is unknown whether breakdown of the methionine cycle during PVA infection is a global phenomenon or a locally controlled event, for say
within virus-induced cytoplasmic compartments. In support of the latter one transgenic lines expressing TEV HCPro were reported not to interfere with DNA methylation in the nucleus (Reytor et al., 2009; Lee et al., 2012).

4.2 Significance of the methionine cycle disruption in PVX-PVA synergism

Earlier studies have established HCPro as the main architect behind development of potex-potyviral synergism (Vance et al., 1995; Pruss et al., 1997). Moreover, silencing suppression property of HCPro has been highlighted as the major factor underlying synergistic enhancement in PVX infectivity during mixed infection (Shi et al., 1997; Kasschau and Carrington, 1998; Marathe et al., 2000; Kasschau and Carrington, 2001; Voinnet, 2001). Earlier in this study, we have suggested that, one of the mechanisms by which HCPro suppresses RNA silencing is by inhibiting SAMS and SAHH leading to disruption of methionine cycle and impaired sRNA methylation. Whether it is also this same mechanism that plays part in development of synergistic response was not known hitherto. Therefore, in the following sections attempt has been made to probe into the molecular events underlying PVX-PVA synergism.

4.2.1 Establishment of PVX, PVA and *Nicotiana benthamiana* as a model host-virus pathosystem for studying potex-potyviral synergism

Most of the earlier studies on potex-potyviral synergism were based on PVX- PVY/TEV mixed infection in the host *Nicotiana tabacum* (Vance 1991; Vance et al., 1995; Pruss et al., 1997). However, further investigations revealed synergistic response as a host-dependent phenomenon. Certain parameters associated with mixed-infection, like PVX accumulation level and severity of the symptoms vary significantly between the hosts (González-Jara et al., 2004). While simultaneous potex-potyvirus infection is manifested by greater accumulation of PVX in *N. tabacum*, there is no significant increase in *N. benthamiana*. In spite of this, the development of symptoms is more severe in mixed infection than in single infection in *N. benthamiana* (Yang and Ravelonandro, 2002; González-Jara et al., 2004; García-Marcos et al., 2009). In this study, *N. benthamiana* infection by both PVX (a potexvirus) and PVA (a potyvirus) was explored as a novel pathosystem for studying potex-potyviral synergism.

4.2.1.1 Development of a method for simultaneous detection of PVX and PVA during mixed infection

Conventional methods of studying infection dynamics of multiple viruses in a mixed infection involve extraction and quantification of RNA / proteins from individual strains. These include methods like qPCR, western blot and northern blot. However, most of these techniques are tedious and time consuming. Advent of fluorescent marker based techniques on the other hand, opened avenues for development of novel methods for imaging and
quantification of viral infection from intact leaf tissues. Fluorescent proteins are stable and easy to detect, and they can be inserted within viral genome and their expression within the host cell can reliably represent viral expression level (Richards et al., 2003; Dhillon et al., 2009; Stephan et al., 2011). Recently, a fast and easy method to quantify fluorescent proteins expressed from potyviral RNA was developed. This was done directly from leaf discs using a 96-well monochromator-based plate reader set at appropriate excitation/emission (Ex/Em) wavelengths for the concerned markers (Pasin et al., 2014). In this study, the same approach was employed to develop a method to quantify PVX and PVA expression levels simultaneously in a mixed infection by tagging their infectious complementary DNA (icDNA) with different fluorescent markers having non-overlapping Ex/Em spectra. The idea was to select a pair of markers, which would be sensitive enough to reliably quantify expression level of their corresponding viruses, while having well separated Ex/Em spectra so that signal from one marker does not interfere with that of the other. In order to screen for a suitable pair for green fluorescent protein (GFP)-tagged PVX (PVX\textsuperscript{GFP}), PVA was tagged with red fluorescent protein (RFP), cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) (PVA\textsuperscript{RFP} / PVA\textsuperscript{CFP} / PVA\textsuperscript{YFP}). Agrobacterium harbouring tagged icDNA of the viruses were locally expressed at OD\textsubscript{600} = 0.5. Agrobacterium carrying GUS (OD\textsubscript{600} = 0.5) served as negative control. Leaf discs were collected from locally infected regions at 4 days post infection (dpi) and fluorescence intensities were measured from those samples. Fluorescence signals obtained from the virus-infected samples were compared to the negative control and the results were presented in terms of fold increase over the background signal level coming from the negative control. Table 2 (III) compiles the highest fold difference obtained for each marker and the corresponding Ex/Em wavelength. Although YFP and RFP showed maximally 263-fold higher fluorescence than background, yet very close proximity between their Ex/Em spectra restricted their use as a pair. Also CFP being the weakest among the tested ones, was not considered to qualify. GFP on the other hand had a reasonable 13-fold difference in fluorescence signal over the background, while its Ex/Em wavelength ranged quite far from that of RFP. Therefore, GFP and RFP were selected as the apt pair for simultaneous quantification of their expression levels from PVX and PVA RNAs during a mixed infection.

In the next step PVX\textsuperscript{GFP} and PVA\textsuperscript{RFP} were agroinfiltrated in a series of increasing dilutions. Leaf discs from local infiltration spots were collected at 3 dpi and GFP / RFP fluorescence from the respective sets were measured. Fluorescence signal from both the markers commensurately increased with the sequential increase in the infiltrated Agrobacterium amount (III, Fig. 1 A, B). The idea was to estimate the sensitivity of the developed method. Therefore, as another independent way of validation, correlation between the RNA accumulation level of PVX\textsuperscript{GFP} and PVA\textsuperscript{RFP} and the GFP / RFP fluorescence intensity of the corresponding samples were plotted (III, Supplementary Fig. 2D, F). Both markers demonstrated satisfactory level of coefficient of determination in linear regression ($r^2$) in
both the trials suggesting their suitability in assessing the expression level of the viruses reliably. Finally, during mixed infection there remains a possibility that fluorescence signal from one marker might interfere with that of the other. To address that, a study was conducted to show the extent of signal leakage from PVX\textsuperscript{GFP} and PVA\textsuperscript{RFP} under non-cognate filters i.e., RFP and GFP filters respectively. A small degree of leakage in fluorescence signal was noticed in the case of both GFP and RFP, however when compared to the true signal detected under their cognate filters, the amount of interference seemed virtually insignificant (III, Supplementary Fig. 2A, B, C, E). Cumulatively, the results affirmed suitability of the method to measure PVX\textsuperscript{GFP} and PVA\textsuperscript{RFP} levels from a mixed infection simultaneously.

4.2.1.2 Accumulation and expression pattern of PVX and PVA during synergistic interaction

One aim of this study was to understand the infection dynamics of PVX and PVA during co-infection. In this context, expression pattern in both local and systemic leaves were taken into account. Moreover, development of symptoms during synergistic interaction was also compared side-by-side with singly infected plants. From the visual examination of the infected plants at 14 dpi, it is evident that the symptom development in PVX\textsuperscript{GFP}-PVA\textsuperscript{RFP} co-infection was remarkably greater than in any of the singly infected plants (III, Supplementary Fig. 1). Both PVX\textsuperscript{GFP} and PVA\textsuperscript{RFP} when infected alone showed mild symptoms in the leaves with slight retardation in growth compared to the mock plants infiltrated with GUS. However, their synergistic interaction led to a stunted phenotype along with systemic necrosis symptoms during the mixed infection. Samples from the local leaves were collected at 3, 5, 7 and 10 dpi, while systemic samples were collected at 5, 7, 10 and 14 dpi. Expression levels of PVX\textsuperscript{GFP} and PVA\textsuperscript{RFP} in mixed infection were compared to their expression levels during single infection. As an independent measure of infectivity, accumulation of genomic (g)RNA of the individual viruses were also quantified from the same sets. GFP expression from PVX\textsuperscript{GFP} subgenomic (sg)RNA and accumulation levels of its gRNA during single and mixed infection are presented in Fig. 2A (local), 2B (systemic) and Fig. 2E (local), 2F (systemic) respectively in (III). Similarly, RFP fluorescence intensity derived from PVA\textsuperscript{RFP} in single and mixed infection, and accumulation levels of its RNA therein, are presented in Fig. 2C (local), 2D (systemic) and Fig. 2G (local), 2H (systemic) respectively in (III).

RFP expression from PVA RNA in single and mixed infection followed more or less similar pattern in both local and systemic leaves. However, from both RFP fluorescence as well as RNA levels it is evident that PVA\textsuperscript{RFP} accumulated less in mixed infection than in plants infected only by PVA\textsuperscript{RFP}. Biological significance of this is not known, however increase in the total viral load and the extreme stress conditions in the host created by synergistic response might be a reason underlying the slight reduction in PVA accumulation. Nevertheless, in systemic leaves PVA\textsuperscript{RFP} level kept increasing until 10 dpi in both of the
cases. After attaining the peak value on 10\textsuperscript{th} day, a decreasing trend in the RFP level was noticed at 14 dpi. In local leaves however, PVA\textsuperscript{RFP} reached a plateau by 7\textsuperscript{th} day in mixed infection, while it still showed marginal increasing trend between 7\textsuperscript{th} and 10\textsuperscript{th} day in single infection. PVX\textsuperscript{GFP} on the other hand accumulated clearly in a different manner during mixed infection than single infection. In local leaves GFP expression reached a plateau by 5\textsuperscript{th} day and subsequently started to drop in single infection by 10\textsuperscript{th} day. However, GFP levels in the mixed infection continued to show an increasing trend between 7\textsuperscript{th} and 10\textsuperscript{th} day. Similar to the local leaves, in the systemic ones also comparable levels of PVX-derived GFP was detected on 5\textsuperscript{th} day in both synergistic and single infections. Intriguingly, in single PVX\textsuperscript{GFP} infection, the GFP level started to decrease drastically after 7\textsuperscript{th} day and the systemic leaves were more-or-less recovered from PVX\textsuperscript{GFP} infection by 14 dpi. However, synergistic interaction with PVA allowed PVX\textsuperscript{GFP} to keep on accumulating throughout the whole period of experiment.

One of the pioneering work on potex-potyviral interactions established that during synergism prolonged accumulation of PVX RNA as well as protein expression thereof is achieved (Pruss et al., 1997). Investigations conducted in N. tabacum protoplasts demonstrated that PVX, when infected alone, increases similarly as in PVX + TEV infection. However, this rate of increase gradually decreases followed by a subsequent fall in the RNA and protein expression levels. On the other hand, expression of P1-HCPro sequence from TEV, which induces synergistic response, allows PVX and its proteins to accumulate at a higher rate for a longer time (Pruss et al., 1997). Outcomes of the current study conducted in N. benthamiana plants, especially concerning the systemic infection results, corroborated well with their observations. Moreover, the similar pattern of PVX gRNA accumulation and GFP expression from PVX sgRNA further consolidated validity of PVX-PVA-N. benthamiana pathosystem to study potex-potyviral synergism.

4.2.2 Involvement of methionine cycle in PVX-PVA mixed infection

4.2.2.1 Silencing suppression property of HCPro is necessary for synergistic response

Earlier studies have indicated correlation between the central domain of HCPro and its silencing suppression property with its ability to induce synergism. Mutations made in to the HCPro central domain compromises this ability (Shi et al., 1997; Marathe et al., 2000; Kasschau and Carrington, 2001). In order to further pinpoint the molecular function of HCPro involved in PVX-PVA synergism, two genetically re-engineered HCPro with mutation in its central domain (amino acids 238, 239, 245, 246) and C-terminal domain (amino acids 344, 349) were tested for their ability to induce synergism with PVX. The former one is a silencing suppression deficient mutant of HCPro (HCPro\textsuperscript{SDM}), equivalent to AS9 mutant of TEV (Kasschau et al., 1997). Latter one is an eIF4E binding deficient mutant of HCPro (HCPro\textsuperscript{4EBM}) (Ala-Poikila et al., 2011). Interestingly, HCPro\textsuperscript{4EBM} is also deficient
in silencing suppression (Hafren et al., 2015). The experimental design also included wild type HCPpro (HCPpro\(^{WT}\)) and GUS as positive and negative controls for synergistic interaction respectively. Both HCPpro\(^{SDM}\) and HCPpro\(^{4EBM}\) failed to enhance PVX\(^{RLUC}\) expression synergistically (III, Fig. 3A). Though, the mutated regions are located in separate domains and have different biological relevance, the lack of silencing suppression is common to both of them. This in turn reinforced the idea that silencing suppression by HCPpro is an essential requirement for synergistic response.

4.2.2.2 Role of methionine cycle in boosting PVX expression

As described in the earlier sections, silencing suppression property of HCPpro to some extent could be attributed to its ability to disrupt methionine cycle. Inhibition of SAMS and SAHH are proposed to be the key events involved therein. To test if a similar mechanism also underlies PVX-PVA synergism, PVX\(^{RLUC}\) expression was monitored under SAMS, SAHH and HEN1 silenced background. PVX\(^{RLUC}\) expression level in both the local and systemic infection contexts was taken into account. Interestingly, the effect of the methionine cycle on PVX\(^{RLUC}\) infectivity did not follow the same pattern as it had in case of PVA\(^{AHCpro}\) (see I, Fig. 4). Several points were observed, which suggested an alternative relationship between the methionine cycle and PVX accumulation. First, only SAHH silencing led to a significant increase in PVX\(^{RLUC}\) expression level in both local and systemically infected leaves. Second, in contrast to the relationship of the methionine cycle with PVA infection, SAMS silencing in none of the experiments was able to enhance PVX\(^{RLUC}\) expression. Moreover, silencing of HEN1 also did not increase PVX\(^{RLUC}\) derived RLUC level in local leaves. Intriguingly, in contrast to local infection, HEN1 and SAMS+SAHH silencing in systemic leaves enhanced PVX\(^{RLUC}\) expression significantly (III, Fig. 3C, D). This observation was further validated visually by examining GFP fluorescence in the leaves, which were systemically infected by PVX\(^{GFP}\) and had SAMS and SAHH simultaneously silenced. Control silencing construct (pHG-CTRL) and HCPpro\(^{WT}\) overexpression were used as negative and positive controls, respectively. Comparable levels of GFP accumulation in both HCPpro\(^{WT}\) overexpressed and SAMS+SAHH silenced plants reinforced the possibility of having some kind of interconnection between the methionine cycle and synergism (III, Fig. 3B).

The differences in PVX accumulation between local and systemic leaves were not very surprising, as earlier also in Fig. 2 (III) we saw quite drastic variation between PVX accumulation in the local and systemic leaves. However, the complete absence of any effect from SAMS silencing in either of the cases was unexpected, and, even more so because SAHH boosted PVX expression in both the cases. In order to understand the system better, PVX\(^{RLUC}\) infection dynamics was studied between 3-9 dpi in SAMS, SAHH, HEN1 and SAMS+SAHH silenced backgrounds. Moreover, both RLUC expression pattern from PVX\(^{RLUC}\) sgRNA and accumulation pattern of PVX\(^{RLUC}\) gRNA were followed. The study revealed that, only downregulation of SAHH consistently boosted PVX\(^{RLUC}\) sgRNA
expression. SAMS and HEN1 knockdown showed marginal enhancement in PVX<sub>RLUC</sub> expression at few time points, but in most of the instances neither of them affected PVX<sub>RLUC</sub> infection significantly. Finally, simultaneous silencing of SAMS and SAHH in the local leaves did not additively increase RLUC expressed from PVX<sub>RLUC</sub> sgRNA. The pattern of PVX<sub>RLUC</sub> expression upon SAMS+SAHH downregulation appeared similar to that of SAHH silencing alone (III, Fig. 4). PVX being a cap-dependent virus might essentially require SAMS for its cap methylation purpose. Since SAMS silencing does not only affect RNA silencing pathway, rather downregulates SAM production globally, it might have a grossly negative impact on PVX infection cycle. This could provide a possible explanation why SAMS silencing did not result in enhancement of PVX<sub>RLUC</sub> expression.

4.2.2.3 Disruption of methionine cycle prolongs PVX-RNA accumulation

As disruption of the methionine cycle is proposed here to interfere with the RNA silencing pathway, it should also stabilize PVX RNA by reducing its degradation. Prolonged accumulation of PVX RNA and disproportionate increase in its negative strand are two hallmarks of potex-potyviral synergism (Vance et al., 1995; Pruss et al., 1997; Gonzalez-Jara et al., 2004; Andika et al., 2012). Therefore, in this study, accumulation dynamics of both the positive (+) and negative (-) strand PVX<sub>RLUC</sub> RNA was studied. From the results it is evident that with all of the silencing constructs accumulation of PVX<sub>RLUC</sub> gRNA is lower than the control sets during the earlier phase of infection (3 dpi). However, this difference gradually diminishes as the infection proceeds and by 9 dpi PVX RNA level in the silenced sets outstripped the control sets (III, Fig. 5). This could be seen as an outcome of prolonged accumulation of PVX<sub>RLUC</sub> RNA due to reduction in its degradation. Since, SAMS, SAHH and HEN1 has been earlier indicated to be instrumental for the smooth running of the RNA silencing pathway, reduced degradation of PVX RNA upon their silencing becomes self-explanatory. Moreover, knockdown of both SAMS and SAHH showed significantly higher accumulation in the PVX<sub>RLUC</sub> gRNA (-)-strand with respect to that of the CTRL on 9 dpi.

4.2.2.4 Dual role of SAHH in enhancement of PVX infection

Cumulatively, the results from these experiments, especially the ones pertaining to SAHH-silencing agreed to most of the characteristic features of synergistic response. However, there are few points, which deserves emphasis. First, all the silencing constructs displayed similar effect on PVX<sub>RLUC</sub> RNA accumulation. Due to high biological variations in the samples, statistical significance could not be attained in some of the cases, nevertheless, accumulation pattern was consistent in all of them showing a relationship between methionine cycle disruption and enhanced PVX<sub>RLUC</sub> gRNA accumulation. Second, among all the candidates silenced, only SAHH could convincingly enhance RLUC expression from PVX<sub>RLUC</sub> sgRNA, while the rest barely had any effect on PVX<sub>RLUC</sub> translation from sgRNA. Moreover, the RLUC expression pattern did not overlap with that of RNA accumulation.
pattern. The highest fold difference in RLUC expression in SAHH silenced plants were observed during the initial phase of infection (3 dpi), while PVX$_{RLUC}$ gRNA accumulation was maximum during the later phase of infection (9 dpi). Taken together, these observations indicate that PVX accumulation and translation might be influenced by two distinct mechanisms. sRNA destabilization triggered by inhibition of SAMS, SAHH or HEN1 could contribute to the former one, while SAHH alone was found to be involved in a yet to be identified mechanism, which boosted translation from PVX$_{RLUC}$ sgRNA. Obvious involvement of SAMS in cap-dependent translation of PVX, obscured assessment of its role in synergism. HCPro$^{WT}$ overexpression on the other hand led to significant upregulation in PVX$_{RLUC}$ gRNA accumulation and RLUC expression from corresponding sgRNA throughout all the time points. In contrast to global silencing of SAMS and SAHH, which might have non-specific effects on overall cellular processes, HCPro might operate locally within specialized cytoplasmic compartments to tightly regulate methionine cycle activity and specifically enhance PVX translation and accumulation. Moreover, hairpin-mediated silencing technique does not ensure complete downregulation of the targets, which can be seen in the Supplementary Fig. 3 (III). Collectively these could be the reasons why the effect of SAMS or SAHH silencing on PVX infectivity did not appear to be as pronounced as it was in the case of HCPro overexpression. Additionally, HCPro being a multifunctional protein, might be involved in simultaneous perturbation of several other cellular networks beside the methionine cycle. A cumulative effect might be needed for the enhancement of PVX infectivity during synergism. In support of this, genome-wide transcriptomic analysis confirmed alterations in the level of genes involved in chloroplast function, carbohydrate and protein metabolism, stress response etc. during PVX-PVY synergism (García-Marcos et al., 2009). Furthermore, during mixed infection in N. benthamiana, severe symptom development with necrosis of the emerging leaves was noted. Although, PVX accumulation in such cases did not increase significantly, molecular events happening during synergistic interaction led to plant death (Yang and Ravelonandro, 2002; González-Jara et al., 2004; García-Marcos et al., 2009). This clearly indicates that enhanced accumulation of PVX does not solely explain the story behind synergism. Rather a deep rooted perturbation of cellular networks synchronized by HCPro and PVX could be proposed, which is manifested in extreme symptom development commonly observed during synergism.

4.2.3 Co-regulation of the methionine cycle and the GSH biosynthesis pathway during PVX-PVA synergism

Coming to the disturbances in cellular metabolic pathways during synergism, potex-potyviral dual infection has been reported to alter redox homeostasis in the cells. During PVX-PVY mixed infection in N. benthamiana, severe oxidative stress has been noted, which is uncommon to infection by either of the virus alone (García-Marcos et al., 2009). One of the ubiquitous molecule involved in maintaining redox balance in the cells is GSH. It is non-protein tri-peptide molecule comprising three amino acids- cysteine, glutamic acid and
glycine. Several studies have linked GSH to defense related functions in plants against viral, bacterial and fungal pathogens (Ball et al., 2004; Kuźniak and Sklodowska, 2005; Parisy et al., 2007; Höller et al., 2010). Furthermore, evidence for cross-talk between GSH and other defense related cell signaling pathways (salicylic acid, jasmonic acid, abscisic acid, ethylene, reactive oxygen species) consolidates its role in combating pathogen invasion (Gullner and Kômives, 2006; Ghanta and Chattopadhyay, 2011; Dubreuil-Maurizi and Poinssot, 2012). Alteration in GSH level during viral infection is not an uncommon phenomenon either. Reduction in symptom development or induction of sulfur-enhanced-defense during Zucchini yellow mosaic virus (ZYMV) and Tobacco mosaic virus (TMV) infection has been linked with elevated level of GSH (Gulner et al., 1999; Zechman et al., 2007b; Höller et al., 2010). Similarly, ZYMV, which is also a potyvirus has been reported to reduce GSH accumulation by altering levels of its precursors in different cellular compartments (Zechman et al., 2007a).

4.2.3.1 PVX-PVA mixed infection leads to GSH depletion via interference with methionine cycle

Induction of severe oxidative stress response during potex-potyviral mixed infection, prompted us to form the hypothesis of the synergism-specific involvement of GSH biosynthesis pathway therein. GSH biosynthesis broadly occurs in two steps. In the first step cysteine and glutamate is combined to produce γ-glutamylcysteine. This reaction is catalyzed by γ-glutamylcysteine synthase. In Arabidopsis, this has been shown to be strictly localized within plastids (Wachter et al., 2005). γ-glutamylcysteine is then transported to cytoplasm, where glutathione synthetase (GSHS) more efficiently carries out addition of glycine to the C-terminal end of γ-glutamylcysteine to produce GSH (Pasternak et al., 2008). Cysteine is considered as one of the rate limiting precursors of GSH biosynthesis (Gullner et al., 1999; Harms et al., 2000; Bloem et al., 2004; Zechmann et al., 2007, 2008b, 2014). Interestingly, within plastids methionine is also produced directly from cysteine via the transsulfuration pathway with homocysteine as the shared intermediate with methionine cycle (Ravanel et al., 2004). Roughly 80 % methionine is used for SAM synthesis and approximately 90 % of SAM therefrom is used in transmethylation reactions and recycled back to methionine (reviewed in Gigolashvili and Kopriva, 2014; Giovanelli et al., 1985). Therefore it can be presumed that ~70% of total methionine demand is replenished from methionine cycle and the remaining ~30% is contributed from de novo methionine biosynthesis via transsulfuration pathway. In this work, it was hypothesized that downregulation of SAHH would partially block SAH to homocysteine conversion leading to lower recovery of methionine from the methionine cycle. Reduction in the methionine pool is thought to be compensated by de novo methionine synthesis within plastids using the same pool of cysteine, which would otherwise be used for GSH biosynthesis. In order to test this hypothesis, percentage of variation in the GSH level between 2 dpi and 9 dpi was
measured from PVX<sup>RLUC</sup> infected samples with simultaneous silencing of SAMS, SAHH, HEN1 and SAMS+SAHH. PVX<sup>RLUC</sup> plus HCPro<sup>WT</sup> co-expression served as the set reflecting authentic synergism scenario. GSHS silencing (pHG-GSHS) was used as a positive control for LC-MS detection. While, plants infiltrated with the induction buffer only, PVX<sup>RLUC</sup> only, PVX<sup>RLUC</sup>+GUS, PVX<sup>RLUC</sup>+CTRL, PVA<sup>WT</sup> only and HCPro<sup>WT</sup> only served as negative controls to account for all other effects, which are not pertinent to synergism specifically. Interestingly, in all of the negative controls GSH level either stayed the same or increased within the studied period. However, silencing of either SAMS or SAHH with simultaneous PVX infection caused approximately 10 % reduction in the GSH level. Further reduction in GSH level (~25 %) was noticed when PVX<sup>RLUC</sup> infection was accompanied with stronger inhibition of the methionine cycle (SAMS+SAHH silenced together). Positive control, i.e., GSHS silenced set, showed a ~50 % reduction in the GSH level, while synergism induced by PVX<sup>RLUC</sup>-HCPro<sup>WT</sup> co-expression was also accounted for a similar degree of GSH depletion (III, Fig. 6A). Intriguingly, HEN1, which is not a component of the methionine cycle, when silenced behaved similarly to the negative controls. No reduction in the GSH level was noticed when PVX<sup>RLUC</sup>, PVA<sup>WT</sup> or HCPro<sup>WT</sup> alone were infiltrated. This clearly indicates that the increased GSH depletion is purely a synergism specific event and only HCPro-mediated disruption of the methionine cycle is not sufficient for this to happen. In order to investigate if GSH depletion has any pro-PVX effect, RLUC expression from PVX<sup>RLUC</sup> sgRNA was measured upon GSHS silencing. Interestingly, RLUC measured from all the time points (2, 5, 7 dpi) unanimously supported significant enhancement of PVX<sup>RLUC</sup> translation in the GSHS silenced background (III, Fig. 6B, C, D).

**4.2.3.2 Hypothetical model to describe the mechanism underlying HCPro-mediated enhancement of PVX**

Based on these observations, a model for PVX-PVA synergism specific co-regulation of the methionine cycle and GSH biosynthesis has been proposed (III, Fig. 7). According to this model during synergistic interaction HCPro-mediated inhibition of SAHH and a yet to be identified action from PVX counterpart lead to enhancement of expression and accumulation of PVX RNA. Inhibition of SAHH has a two pronged effect on PVX infection. Firstly, SAHH downregulation leads to increased accumulation of SAH in the system. SAH being a potential inhibitor of many methyltransferases including HEN1, inhibits its activity and thereby destabilizes sRNAs. We are proposing this to be beneficial for the prolonged accumulation of PVX RNA. This part of the hypothesis is quite similar to the HCPro-mediated methionine cycle disruption model proposed for PVA. However, the role of SAMS herein remained unclear as its activity is also required for 5’ cap methylation of PVX genome. Moreover, this study and many earlier ones have shown that HCPro alone can synergistically enhance PVX infectivity, the other potyviral proteins are not strictly necessary. On the other hand, inhibition of SAMS activity, though mediated by HCPro,
required expression of other PVA proteins also. Keeping these two arguments in mind SAMS deactivation did not seem very important in synergism context. Coming to the second line of effect originating from SAHH downregulation, as SAH is not effectively hydrolyzed a reduction in homocysteine pool is predicted. This would lead to lower recycling of methionine via this route. Methionine is a ubiquitously required sulfur containing amino acid. Apart from this, its primary product SAM is the second most used co-factor in the enzymatic reactions, after ATP (reviewed in Ducker and Rabinowitz, 2017). Additionally, SAM also acts as a precursor for several other metabolic pathways downstream (Giovanelli et al., 1985; Hesse et al., 2004; Gigolashvili and Kopriva, 2014). Therefore, methionine biosynthesis is a tightly controlled process, and SAM level acts as a feedback regulator of de novo methionine biosynthesis via posttranscriptional autoregulation of cystathionine γ-synthase, the first unique enzyme in methionine biosynthesis pathway (Chiba et al., 2003). Methionine synthase (MS), catalyzing the terminal step of methionine synthesis, is distributed both in cytoplasm and plastids. Cytoplasmic MS mediates methionine recovery via the methionine cycle, while plastidial MS is responsible for the methionine synthesis from cysteine (Ravanel et al., 2004). Lower recovery of methionine via the methionine cycle due to blockage in SAH to homocysteine conversion is expected to cause scarcity of methionine in cytosol. To maintain the supply-demand balance de novo methionine synthesis from cysteine within plastids via transsulfuration pathway is proposed to prevail. SAM level in the cell being a rate-limiting factor for this pathway (Ravanel et al., 1998; Chiba et al., 2003), is thought to kick-start this route via feedback regulation as its level in cytosol goes down. GSH, which would otherwise be acting as a cysteine reservoir, is therefore deprived of its fair share as the same plastidial pool of cysteine now gets channeled towards the methionine synthesis. This hypothesis is also supported by the fact that, the variation in GSH level is more dependent on the flux of cysteine towards GSH / methionine biosynthesis pathways, rather than the transcript level of the genes involved therein (Matityahu et al., 2013). The outcome of this is manifested in acute oxidative stress and depletion of GSH level during synergistic interaction. Moreover, in this study reduction in GSH level has been correlated to enhanced expression of PVX sgRNA. However, inhibition of SAHH by HCPro alone is not enough to downregulate GSH biosynthesis and therefore, this is a truly synergism specific event. A yet to be identified role of PVX protein(s) in this context is proposed to be necessary to induce GSH depletion.

4.3 Role of HCPro-associated HMW complexes in PVA infection cycle

4.3.1 Polysome association of HCPro

HCPro WT and VPg enhance PVA translation in an interdependent manner. Their co-expression enhanced RLUC expression from a replication-deficient variant of HCPro-less PVA RNA (PVA ΔGDD-ΔHCPro) (Hafren et al., 2015). Moreover, detection of several ribosomal proteins from HCPro WT-Strep-RFP purified samples (see Section 4.1.2) further strengthened the
idea of involvement of HCPro in PVA translation (I, Fig. 1a). To study the polysome association of HCPro, transgenic *N. benthamiana* plants constitutively expressing *Arabidopsis* FLAG-tagged ribosomal protein L18B (RPL18B), were infected with PVA\textsuperscript{WT-Step-RFP}. Subsequently, FLAG-tagged ribosomes along with associated host and viral factors were isolated via FLAG affinity purification and their identification was carried out using LC-MS/MS (I, Fig. 5a). Interestingly, apart from the ribosomal proteins, HCPro\textsuperscript{WT-Step-RFP}, CI and VPg were also detected from the FLAG purified samples. Quality of purification and enrichment of the ribosomal RNAs and FLAG-tagged RPL18B were validated via silver staining, western blotting and agarose gel electrophoresis (I, Fig. 5b, c). Furthermore, presence of HCPro\textsuperscript{WT-Step-RFP} and CI in the purified samples were validated via western blotting (I, Supplementary Fig. 8). Low abundance of VPg in the purified fraction could be a reason for it being not detected in the western blots. Intriguingly, similar to HCPro\textsuperscript{WT-Step-RFP} purified samples (I, Fig. 2), ribosome purified samples (I, Supplementary Fig. 8) also demonstrated the presence of HMW complexes containing HCPro and other host and viral factors relevant to PVA infection. It is not known whether some of these complexes are common in samples purified from either the total protein or the ribosome fraction. However, a feature common to all of them is their incredible stability. All of these complexes are able to withstand harsh SDS-\(\beta\)-mercaptoethanol treatment prior to SDS-PAGE. Previously HCPro has also been shown to induce PGs which are also large multiprotein complexes harboring several host factors essential of PVA infection (Hafren et al., 2015). Although PGs are predicted to protect vRNAs from host’s RNA silencing machinery, functions of the ribosome-associated and possible other HCPro-containing HMW complexes remains to be elucidated. Moreover, molecular cues governing their assembly are not known either.

### 4.3.2 HCPro and WD40 domain containing protein VCS are binding partners

WD40 repeat domain containing proteins are ubiquitously present in eukaryotes (Neer et al., 1994; Wang et al., 2013). These proteins contain multiple repeats of 40-60 amino acid stretches beginning with G, H and ending with W, D (Wang et al., 2013; van Nocker and Ludwig, 2003). These domains fold to form multiple beta propeller structure, where various protein complexes can assemble. WD40 repeat domain containing proteins are immensely diverse group of proteins carrying out a wide-range of regulatory roles in the cells. However, many of them are interaction hubs within cellular networks and act as scaffolding elements for multiprotein complex assembly (van Nocker and Ludwig, 2003; reviewed in Stirnimann et al., 2010; Jain and Pandey, 2018). VCS is one such WD40 repeat protein present in PBs and RNP complexes, associated with mRNA metabolism (Xu et al., 2006). Moreover, it has been reported to be a PG-associated host factor and an indispensable infectivity determinant for PVA (Hafren et al., 2015). WD40 proteins are highly versatile in regard to substrate binding and can interact with multiple proteins using all sides of its surface. The same WD40 protein can recruit different binding partners in a similar or distinct binding modes (reviewed in Xu and Min, 2011). VCS could be a WD40 domain protein hijacked by HCPro to work
in favour of PVA infection. Although predicted as an integral part of PGs (Hafren et al., 2015), direct interaction between HCPro and VCS was not demonstrated yet. Taken together, the idea to test, if VCS acts in coordination with HCPro to assemble HMW complexes associated with PVA infection seemed reasonable. Interestingly, bioinformatics analysis of HCPro sequence revealed the presence of several WD40 domain interacting motifs. Based on factors like conservation, secondary structure and surface accessibility the most probable one was selected to be studied in detail. This motif comprises five highly conserved amino acids A, E, L, P and R and resides within the C-terminal domain of HCPro (amino acids 401-405). The sequence conforms to the WD domain interacting motif [EDSTY].{0,4}[VIPLA][TSDEKR][ILVA] and is thoroughly conserved among many versions of HCPro throughout the genus Potyvirus (II, Fig. 1A; Supplementary Fig. 1). Moreover, crystallographic structure of the C-terminal domain of TuMV HCPro revealed disordered nature and surface accessibility of this motif. In this study, the two charged residues present in PVA HCProWT sequence ‘E’ and ‘R’ were replaced with two ‘A’s. We wanted to study if this mutation in HCPro (HCProWD), compromise its interaction with WD40 domain containing host protein VCS. Apart from this, it also seemed interesting to investigate the repercussions of debilitated interaction between HCPro and WD40 repeat protein upon assembly and stability of HMW complexes associated with PVA infection. As this motif resides within the C-terminal domain of HCPro and lies in close vicinity to its cysteine protease domain, it was necessary to confirm that this mutation does not hamper the autocatalytic activity of HCPro. α-HCPro western blot showing the presence of monomeric HCPro from PVAWD infected plants (II, Fig. 1G) ensured retention of potyprotein processing ability of HCProWD. Additionally, a western blot analysis of plant samples expressing HCProWD and HCProWT (II, Fig. 1H) revealed that both variants of HCPro were expressed on a similar level when the expression was initiated with an equal amount of Agrobacterium in the infiltrate.

As the first step of this study, co-localization of HCPro and VCS has been checked in planta. Strep-RFP tagged HCProWT or its mutated version HCProWD (HCProWD-Strep-RFP / HCProWT-Strep-RFP at OD600 = 0.1) was co-expressed along with all known members of VCS in N. benthamiana, tagged with YFP (3 members in total- VCS-A\textsuperscript{YFP}, VCS-B\textsuperscript{YFP}, VCS-C\textsuperscript{YFP}, all expressed at OD600 = 0.1 each, and cumulatively referred to as VCS\textsuperscript{YFP} in the text and figures). Localization pattern of the fluorescent marker-tagged proteins were visually examined at 3 dpi via confocal laser scanning microscopy in a sequential scanning mode in order to avoid crossover of fluorescence emission. The extent of co-localization between VCS and HCPro variants were calculated from the confocal images using Fiji (ImageJ) image analysis software package (with colocalization threshold plugin), and selecting HCPro-containing granules as the regions of interest (ROI). More than 80 % co-localization between HCPro\textsuperscript{WT-Strep-RFP} and VCS\textsuperscript{YFP} was observed, as both of them were found to aggregate within few cytoplasmic foci. However, due to the mutated interaction motif
between HCPro and VCS approximately 30 % reduction in the degree of co-localization was noticed. From the images this impairment seemed more apparent. While HCPro\textsuperscript{WD-Strep-RFP} still localised within few cellular foci, VCS\textsuperscript{YFP} was found to be distributed randomly throughout the cytoplasm (II, Fig. 2A-G). Reference images for VCS and HCPro\textsuperscript{WT/WD}, when expressed along with control 35S-RFP and 35S-YFP respectively are presented in Supplementary Fig. 3A-I (II). Having shown that HCPro\textsuperscript{WT-Strep-RFP} and VCS co-localizes in planta and that the extent of co-localization is significantly reduced in the case of HCPro\textsuperscript{WD-Strep-RFP}, the same mutation has been incorporated in to the full length PVA icDNA (PVA\textsuperscript{WD-Strep-RFP}). Following, a similar purification technique as in Section 4.1.1, both HCPro\textsuperscript{WT-Strep-RFP} and HCPro\textsuperscript{WD-Strep-RFP} were purified from the PVA\textsuperscript{WT-Strep-RFP} / PVA\textsuperscript{WD-Strep-RFP} infected leaves, along with their binding partners. Western blot carried out with anti-HCPro and - VCS antibodies revealed that, VCS is either in direct or indirect interaction with HCPro. Both the samples demonstrated HMW protein complexes (>250 kDa), which contained both HCPro and VCS in bands having similar electrophoretic mobility (marked with asterisks in II, Fig. 2H). Similar to our earlier observation (I, Fig. 1, 2), here also we saw multiple bands in the HMW range. However, when compared between HCPro\textsuperscript{WT-Strep-RFP} and HCPro\textsuperscript{WD-Strep-RFP}, band pattern of the HMW complexes appeared different (II, Fig. 2H; Supplementary Fig. 4A). While analysing the western blots (II, Fig. 2H), it has been taken into account that the overall signal from HCPro\textsuperscript{WD} is lower than HCPro\textsuperscript{WT}. Since the plants were infected with PVA\textsuperscript{WD-Strep-RFP}, which usually has ~2-5 fold lower amount of viral gene expression than PVA\textsuperscript{WT-Strep-RFP} at equal OD\textsubscript{600} of infiltration (II, Fig. 1G; Supplementary Fig. 2; also discussed later in section 4.3.4), it seemed logical that the signal intensity of HCPro\textsuperscript{WD-Strep-RFP} expressed therefrom, will also be commensurately lower in the western blot. However, it is interesting to note that the reduction pattern of all the three bands is not uniform. There is disproportionate reduction in the uppermost band as compared to the two lower bands (both in HCPro and VCS blot), which cannot be explained solely by lower expression level of HCPro\textsuperscript{WD} (II, Supplementary Fig. 4A, C, D). Since, it is tough to achieve enough high resolution between bands at >250 kDa range, the number of HMW complexes present might be higher than the number of bands visible in the blot. Rather, it is prudent to think these bands as a possible overlap of multiple HMW complexes. If, HCPro-VCS interaction is necessary for the assembly of certain complexes therein, disappearance / severe reduction of the corresponding bands from both α-HCPro and α-VCS blots seemed logical in the case of HCPro\textsuperscript{WD-Strep-RFP} samples.

Cumulatively, these observations indicate that HCPro either directly or indirectly interacts with VCS, during PVA infection. They co-localize within the cells to form HMW complexes, however, the extent of their co-localization as well as their ability to form HMW complexes got reduced as the interaction motif between HCPro and VCS is mutated. Furthermore, co-purification of VCS along with HCPro\textsuperscript{WD-Strep-RFP} despite their impaired interaction suggests that not all HMW complexes formed during PVA infection strictly
require HCPro and VCS to interact directly. Image analysis data from confocal microscopy also support the same argument as the co-localization between HCPro and VCS was not completely abolished. There could also be an alternative explanation for this. HCPro sequence contained several other stretches of amino acids, which conferred to WD-domain interaction motif. The particular site mutated in this study seemed most probable (based on its conservation) and might well be the predominant one in mediating HCPro-VCS interaction. However, week interaction with VCS using one or more of those redundant sites could not be ignored.

4.3.3 Effect of HCPro-VCS interaction in assembly and stability of RNA-protein complexes during PVA infection

As VCS belongs to the WD-repeat protein family, its role as a scaffolding platform in multiprotein complex assembly during PVA infection seemed possible. The fact that VCS is an integral part of PGs, encouraged the idea to investigate if HCPro-VCS interaction is important for PGs to assemble. Following the PG visualization technique described in Hafren et al. (2015), HCPro\(^{WT}\) / HCPro\(^{WD}\) (at OD\(_{600}\) = 0.1) were co-expressed with P0\(^{YFP}\) (at OD\(_{600}\) = 0.1). Number of granules formed was calculated under epi-fluorescence microscope at 3 dpi (II, Fig. 2I-K). Relevant control images are presented in Supplementary Fig. 3J, K (II). Interestingly, HCPro\(^{WD}\) induced ~5 fold less PGs compared to HCPro\(^{WT}\). Since, both versions of HCPro express in similar amounts, it could be argued that, PG induction property of HCPro is grossly affected due to the mutation in its WD interacting domain. HCPro\(^{WD}\) being less efficient in sequestering VCS from the cytoplasm, in addition, both of them being components of PGs, impairment in HCPro-VCS interaction might be deemed responsible for the reduced assembly of PGs.

Next, the stability of HCPro-associated HMW complexes purified from PVA\(^{WT-Strep-RFP}\) / PVA\(^{WD-Strep-RFP}\) infected plants were tested against DNase, Proteinase K and RNase A. Corresponding products after each of the treatments were ran in SDS-PAGE and subsequently silver stained to visualise their band pattern. Equivalent amount of untreated eluate was used as a control. HMW complexes from both PVA\(^{WT-Strep-RFP}\) and PVA\(^{WD-Strep-RFP}\) infected sets remained visually unchanged after DNase treatment. On the other hand, Proteinase K treatment led to disruption of the complexes in both of the cases. Intriguing difference was seen in the case of RNase A treatment as HMW complexes formed by HCPro\(^{WD-Strep-RFP}\) during PVA\(^{WD-Strep-RFP}\) infection were largely degraded by RNase A, while, those formed by wild type HCPro during PVA\(^{WT-Strep-RFP}\) infection stayed unaffected (II, Fig. 3A). This observation sheds light on two important aspects of HCPro-derived HMW complexes. First, these tightly bound complexes comprise both RNA and proteins (RNP complexes). One of the possible roles these complexes may play is to protect vRNAs from host’s RNA degrading agents. This in turn leads to the second point- as the HMW complexes formed by HCPro\(^{WD-Strep-RFP}\) were degraded selectively by RNase A, it could be postulated
that HCPro-VCS interaction might be crucial in maintaining stability of these HMW complexes. Interestingly, silver staining can stain both RNA and proteins in SDS-PAGE (Blum et al., 1987; Paleologue et al., 1988), therefore from this gels, it is not possible to speculate about the relative RNA / protein content of these RNP complexes. However, bulk reduction and smearing of the HMW complexes upon RNase treatment (II, Supplementary Fig. 4B) indicate the presence of a substantial portion of RNA in the complexes. Moreover, smearing instead of distinct low molecular weight bands upon disruption of the complexes, indicate the presence of multiple proteins in loosely bound complexes in addition to HCPro and VCS.

Having shown that impairment of HCPro-VCS interaction affects assembly of multiprotein complexes during PVA infection both quantitatively and qualitatively, further compositional differences between HMW complexes formed by HCPro\textsuperscript{WT} and HCPro\textsuperscript{WD} were assessed via LC-MS/MS. Interestingly, several of the previously identified PVA infection associated candidates were not detected in HCPro\textsuperscript{WD-Strep-RFP} interactome (II, Supplementary Table 2). Subsequently, western blotting against respective antibodies were carried out, followed by both visual and densitometry analysis of the bands to demarcate between reduced and non-reduced components (II, Fig. 3B-E; Supplementary Fig. 4C, D). As discussed for Fig. 2H (II), lower expression level of PVA\textsuperscript{WD-Strep-RFP} could lead to some degree of reduction in the signal intensity of the proteins in HCPro\textsuperscript{WD-Strep-RFP} samples. This was taken into account. Only those proteins which both by densitometry and LC-MS analysis showed disproportionate reduction were considered to be in the category of the reduced proteins (II, Supplementary Fig. 4C, D; Supplementary Table 2). In conclusion, the absence or drastic reduction of CI, SAMS and VPg in HCPro\textsuperscript{WD-Strep-RFP}-associated HMW complexes was noted, whereas SAHH level seemed unchanged between the two variants of HCPro (II, Fig. 3B). Although AGO1 was reduced to some degree according to densitometry (supported by LC-MS also), yet the bands in the western blot were not very distinct. \(\alpha\)-AGO1 antibody used herein is from Arabidopsis, and it was already shown to be less sensitive towards \(N\). benthamiana AGO1 (I, Supplementary Fig. S7). Due to lack of distinct AGO1 bands, it was considered not to be reduced. In a nutshell these results indicate that, in spite of having weakened interaction with VCS, HCPro\textsuperscript{WD} was still able to generate some RNP complexes. However, structurally they seemed unstable as the RNA was accessible to RNase A. Impaired functioning of scaffolding element VCS, leading loosely bound complex formation could explain these observations. Moreover, gross reduction in vRNA-associated proteins like CI and VPg also could explain compositional change in HMW complexes leading to destabilization of their structure.
4.3.4 Impact of impaired HCPro-VCS interaction on PVA RNA accumulation and protein expression

To study the effect of HCPro-VCS interaction on PVA gene expression two complementary approaches have combined together. In the first one RLUC expression from locally infected PVA\textsuperscript{WD} was monitored and compared to expression level of PVA\textsuperscript{AHPro} and PVA\textsuperscript{WT}. The RLUC activity measurements indicated that the expression level of PVA\textsuperscript{WD} is much higher than that of PVA\textsuperscript{AHPro}. However, when compared to the expression level of PVA\textsuperscript{WT}, it fared consistently lower. The level of RLUC expressions is in concordance with the corresponding RNA levels showing approximately a 5-fold reduction in the accumulation of PVA\textsuperscript{WD} compared to PVA\textsuperscript{WT} (II, Fig. 4A, B). As the second way of validating the importance of VCS in PVA infectivity, PVA\textsuperscript{AHPro}, PVA\textsuperscript{WD} and PVA\textsuperscript{WT} were infiltrated similarly in to the local leaves, however, in this case a simultaneous knockdown of VCS was conducted therein. Interestingly, VCS downregulation brought down PVA\textsuperscript{WT} to the level of PVA\textsuperscript{WD}, further reinforcing the importance of HCPro-VCS interaction. Intriguingly, VCS downregulation increased the RNA and RLUC expression levels from PVA\textsuperscript{AHPro}. These results implied a possible dual role of VCS in PVA infection. In presence of a functional HCPro, it acts in favour of PVA infection (PVA\textsuperscript{WT}), however, in complete absence of HCPro its role reverts (PVA\textsuperscript{AHPro}). Partial rescue of PVA\textsuperscript{AHPro} gene expression upon VCS silencing might indicate its association with RNA silencing. Replication-deficient variants of all the viruses did not show any significant difference in their RLUC expression even in the VCS-silenced background, suggesting that the expression of the non-replicating PVA RNA was not affected by the mutation or silencing (II, Fig. 4C). Most intriguing difference between PVA\textsuperscript{WT} and PVA\textsuperscript{WD} was however seen when systemic infection was taken into account. Almost a 1000-fold reduction in the RLUC expression compared to PVA\textsuperscript{WT} was seen in case of PVA\textsuperscript{WD}, although both of them consistently reached systemic leaves within a comparable timeframe (II, Fig. 4D).

4.3.5 The capacity of HCPro to assist in virion formation and function in silencing suppression is compromised in HCPro\textsuperscript{WD}

Systemic infection of several plant viruses has been correlated to their stable virion formation (Vaewhongs and Lommel, 1995; Tenllado and Bol; 2000; Hipper et al., 2014; Solovyev and Savenkov, 2014; Zhao et al., 2015). In case of potyvirus PPV, HCPro has been established to assist particle formation (Valli et al., 2014). Moreover, significant reduction in PVA CP accumulation has also been shown to happen during VCS silencing (Hafren et al., 2015). Combining these pieces of evidence along with the defective systemic movement of PVA\textsuperscript{WD}, impelled the need to investigate particle formation efficiency of PVA\textsuperscript{WD}. Samples from both locally and systemically infected leaves were tested for the presence of particles via immunocapture-RT-qPCR (ic-RT-qPCR) and electron microscopy (EM). Interestingly, neither of the techniques could detect authentic PVA particles from local or systemic leaves.
EM images from the systemic leaves in certain occasions showed extremely few undersized filament-like structures, however, it could not be concluded whether they are deformed virions or mere artifacts (II, Fig. 4E, F). It is evident from the RLUC values (local infection results in II, Supplementary Fig. 5), that the expression level of PVA<sub>WD</sub> was ~6.5-fold lower than that of PVA<sub>WT</sub>. As RLUC and CP are both part of the same large potyviral polyprotein, equivalent amount of CP is supposed to be expressed therein. Therefore, a commensurate reduction in the particle count in PVA<sub>WD</sub> infected plants could be expected. However, neither ic-RT-qPCR nor EM studies revealed any signs of PVA particles. Consequently, it is predicted that the mutation in WD interacting domain of HCPro grossly affected, if not completely abolished, the particle formation capacity of PVA<sub>WD</sub>,

In addition to particle formation, another function of HCPro that has been linked to systemic spread of potyviruses is its ability to suppress RNA silencing (Kasschau et al., 1996; Kasschau and Carrington, 2001). Reduced efficiency of HCPro<sub>WD</sub> to induce PG formation, disappearance of SAMS from the HCPro<sub>WD</sub> interactome and vulnerability of HCPro<sub>WD</sub> derived RNP complexes towards degrading agents urged the question whether HCPro<sub>WD</sub> is able to suppress RNA silencing. To assess the silencing suppression efficiency of HCPro<sub>WD</sub>, a hairpin-targeted RNA silencing assay was used in three different contexts. In two of the cases, a transgene was expressed along with the hairpin targeting it. In the first set of experiments HCPro<sub>WD</sub> alone was expressed to restore transgene expression, while in the other set HCPro<sub>WD</sub> along with PVA<sup>AHCPro</sup> was expressed to account for any additional effects that might come from other potyviral proteins. In the third case, PVA<sup>AHCPro</sup> plus a hairpin (pHG-RLUC) targeting RLUC gene within PVA genome were co-expressed. In all the cases, HCPro<sub>WT</sub> and GUS overexpression constructs were used as the positive and negative controls, respectively. Interestingly, HCPro<sub>WT</sub> restored expression of the transgenes as well as PVA<sup>AHCP</sup> by suppressing the dsRNA-triggered RNA silencing, while HCPro<sub>WD</sub> summarily failed to rescue gene expression in all of the cases (II, Fig. 5B-D). Cumulatively, these results provided unequivocal evidence for confirming HCPro<sub>WD</sub>’s defect in suppression of dsRNA-triggered RNA silencing. Subsequently another set of experiments was carried out to compare HCPro<sub>WD</sub> with two pre-established silencing suppression-deficient mutants of HCPro- HCPro<sup>SDM</sup> and HCPro<sup>4EBM</sup>. In these experiments PVA<sup>AHCPro</sup> was complemented with ectopically expressed HCPro<sup>SDM</sup>, HCPro<sup>4EBM</sup>, HCPro<sub>WD</sub> and HCPro<sub>WT</sub>. The RLUC activity levels from PVA<sup>AHCPro</sup> plus GUS overexpression samples (the negative control) was similar to that in the samples from HCPro<sup>SDM</sup> / HCPro<sup>4EBM</sup> overexpression, suggesting neither of them could complement the absence of HCPro in PVA<sup>AHCPro</sup>. On the other hand, HCPro<sub>WD</sub> overexpression partially elevated gene expression from PVA<sup>AHCPro</sup> (II, Fig. 6A). The fold-difference in RLUC activities between HCPro<sub>WD</sub> and the positive control HCPro<sub>WT</sub> followed a similar trend to that of PVA<sub>WD</sub> and PVA<sub>WT</sub> infection (II, Fig. 3A and 6A). This essentially indicates that HCPro<sub>WD</sub>, though defective in silencing suppression does not behave similarly to the other silencing suppression-deficient mutants. Moreover, it also suggests that certain
functions of HCPro, which are not dependent on its ability to suppress RNA silencing, might be preserved in this mutant allowing it to partially complement PVA\textsuperscript{ΔHCPro} expression.

In a nutshell, these results highlight several aspects of HCPro-VCS interaction during PVA infection. Firstly, during PVA\textsuperscript{WD} infection, the mutation incorporated in WD-domain interaction motif of HCPro prevents it from fully interacting with VCS. As an outcome of this weakened interaction PVA\textsuperscript{WD} accumulation got \(\sim 5\) fold reduced in local leaves. Debilitated silencing suppression capacity of HCPro could be the reason behind this, which in turn could also be attributed to its inefficient granule formation ability. PGs are proposed to be the sites for RNA silencing suppression and a prerequisite for efficient infection (Hafren et al., 2015). Striking correlation between reduction in the granule count and reduction in the level of RLUC expression/RNA accumulation (both approximately 5-folds) advocates a direct relationship between these two aspects of PVA infection. Regarding the drastic reduction in systemic infectivity, it is tough to pinpoint whether lack or particle formation or debilitated silencing suppression could be held responsible for this defect. Moreover, both being \textit{bona fide} functions of HCPro, they might also have an overlapping role in systemic spread of PVA. One point that could be emphasized here is the absence of SAMS from the HCPro\textsuperscript{WD} interactome. If this indicates HCPro\textsuperscript{WD} cannot disrupt the methionine cycle, lack of its silencing suppression becomes self-explanatory. Moreover, siRNAs can travel systemically to spread silencing (Fagard and Vaucheret, 2000; Melnyk et al., 2011) and several silencing suppressors have been predicted to interfere with this process (Voinnet et al., 1999; Voinnet, 2005; Csorba et al., 2015). HEN1-stabilized siRNAs in case of PVA\textsuperscript{WD} infection, can act as mobile signals to activate systemic silencing response, and prepare the newly emerging leaves to combat against systemically moving viral RNP complexes. Finally, susceptibility of HCPro\textsuperscript{WD} derived HMW RNP-complexes against RNA degradation agents could be an additional challenge faced by PVA\textsuperscript{WD} while moving to systemic leaves already primed with siRNA signals.

4.3.6 Role of HCPro in PVA translation

Enhancement in PVA translation coordinated by HCPro and VPg has already been demonstrated by Eskelin et al. (2011) and Hafern et al. (2015). However, it was not deciphered whether this effect was due to stabilization of vRNA from host’s RNA silencing machinery or there exists a specific translation related function of HCPro that causes enhanced expression of RLUC from the replication-deficient PVA (PVA\textsuperscript{AGDD}). In this study it was investigated whether HCPro\textsuperscript{WD}, which is deficient in RNA silencing suppression, is capable of upregulating PVA gene expression. The experiment consisted of complementation of PVA\textsuperscript{AGDD-ΔHCPro} with HCPro\textsuperscript{SDM} / HCPro\textsuperscript{4EBM} / HCPro\textsuperscript{WD} / HCPro\textsuperscript{WT} accompanied with simultaneous supplementation of VPg therein. Interestingly, HCPro\textsuperscript{WD} complemented VPg-mediated translational enhancement similar to HCPro\textsuperscript{WT}, while none of the other silencing suppression deficient mutants of HCPro could do so (II, Fig. 6B). This
clearly indicated that unlike HCP\textsubscript{SDM} and HCP\textsubscript{4EBM} translation associated functions of HCP\textsubscript{Pro} might be partially preserved in HCP\textsubscript{WD}. Moreover, it also confirms silencing suppression and VPg-mediated translational enhancements are two mutually exclusive functions of HCP\textsubscript{Pro}.

4.3.6.1 Evidence for translational repression in PVA infection

Association of HCP\textsubscript{Pro} and VPg with ribosomes (Section 4.3.1) and involvement of both of them in enhancement of PVA\textsuperscript{AGDD-\textsubscript{HCP}Pro} gene expression impelled the need to investigate whether this function of HCP\textsubscript{Pro} somehow leads to relieving of translational repression during PVA infection. To start with, PVA infection derived HMW complexes (section 4.1.2) were analysed for the presence of AGO1 via a western blot using anti-AGO1 antibody. Interestingly, AGO1 was found with HCP\textsubscript{Pro} from the HMW complexes of similar electrophoretic mobility as were VPg and CI (I, Fig. 6b; Supplementary Fig. 8B). In another experiment AGO1\textsubscript{CFP} was transiently expressed with either PVA\textsubscript{WT} or HCP\textsubscript{Pro\textsubscript{WT}} and shown to form similar stable HMW complexes. Absence of any such complexes in plants overexpressing AGO1\textsubscript{CFP} plus GUS / PVA\textsubscript{AHCP\textsubscript{Pro}} confirmed that these complexes are specifically associated with HCP\textsubscript{Pro}.

Association of AGO1 with ribosomes is considered as a hallmark of translational repression (Lanet et al., 2009). Furthermore, we have demonstrated also that AGO1 associates with HCP\textsubscript{Pro} derived HMW complexes. In the same line our next objective was to verify if both of them are associated with ribosomes during PVA infection. Therefore, AGO1\textsubscript{CFP} along with HCP\textsubscript{Pro\textsubscript{WT-RFP}} / PVA\textsubscript{WT-Strep-RFP} were expressed locally in transgenic N. benthamiana expressing FLAG-tagged RPL18B. Subsequently, a two-step purification strategy was adopted to isolate ribosomes from the transiently infected local leaves. In the first step ribosomes were fractionated via sucrose gradient ultracentrifugation, and subsequently the ribosome-enriched fraction was subjected to a second round of FLAG-affinity based purification. Western blot analysis of the samples confirmed the presence of both AGO1 and HCP\textsubscript{Pro} among the purified ribosomes (I, Fig. 6c). Considering this observation as a possible evidence for the existence of translational repression as a defence mechanism against PVA infection, a working hypothesis for HCP\textsubscript{Pro}-mediated relieving of translational repression was made. According to the model for translational repression proposed by Iwakawa and Tomari (2013), a putative mechanism by which AGO1-RISC can induce translational repression is by dissociating eIF4A from the 5’UTR of target mRNA. Based on our observations we postulate that, a virus-induced multiprotein complex at the 5’UTR of the vRNA prevents AGO1-RISC-mediated dissociation of eIF4A. The idea is further strengthened by the fact that eIF4A was detected as one of the interacting partners of HCP\textsubscript{Pro\textsubscript{WT-Strep-RFP}} during PVA infection (II, Supplementary Table 2). The complex therefore is predicted to aid in initiation of translation by recruiting preinitiation complex to IRES bound eIF4F complex.
4.3.6.2 Role of VCS in regulating PVA translation

Envisioning VCS as the scaffolding protein in formation of the viral 5’UTR associated HMW complex, its role in PVA translation has been tested. The experimental setup comprised transient expression of VCS and VPg separately as well as in combination together with PVA<sup>AGDD-HCPro</sup> / PVA<sup>AGDD-HCProWD</sup> / PVA<sup>AGDD-HCProWT</sup>. Experiments were restricted to the replication-deficient variants of PVA to specifically highlight the impact of VCS overexpression on PVA translation. PVA<sup>AGDD-HCProWT</sup> was responsive to individual overexpression of both VCS and VPg. Furthermore, simultaneous over-expression of VCS and VPg additively upregulated gene expression from PVA<sup>AGDD-HCProWT</sup>. Interestingly, PVA<sup>AGDD-HCProWD</sup> only responded to VPg-mediated translational enhancement, while, overexpression of VCS was unable to elevate RLUC expression thereof. Gene expression from PVA<sup>AGDD-HCPro</sup> virtually stayed unchanged from either of the overexpression. Moreover, PVA<sup>AGDD-HCProWD</sup> and PVA<sup>AGDD-HCPro</sup> showed similar expression level upon VCS plus VPg overexpression (II, Fig. 7A). Cumulatively these results indicated the ability of VCS to enhance PVA gene expression. However, this effect was most prominent when VCS was aided by VPg and a fully functional HCPro. HCPro<sup>WD</sup>, which is deficient in VCS interaction could not contribute fully to VPg-VCS-HCPro-mediated translational enhancement.

The ratio between the RNA accumulation level and the corresponding protein expression level can be used to detect possible occurrence of translational repression or its release (Brodersen et al., 2008). To verify if VPg-VCS-HCPro mediated translational enhancement is actually a case of relieving translational repression, conventional approach of monitoring virus derived protein / vRNA ratio during infection was adopted (Iwakawa and Tomari, 2013). The overall idea of this experiment was to assess the effect of VCS overexpression and downregulation on RLUC/RNA ratio during PVA infection. Moreover, the role of HCPro and VPg was explored therein. To accommodate all these factors, a comprehensive experiment was planned. The experimental design comprised infection by following replicative variants of PVA- PVA<sup>HCPro</sup> / PVA<sup>WD</sup> / PVA<sup>WT</sup>. Each of the viral constructs were co-infiltrated with VCS overexpression / silencing constructs in two sets. One set was further supplemented with VPg while in the other set the cell count of agroinfiltration was balanced using Agrobacterium carrying the GUS construct. Empty Hellsgate vector (pHG-CTRL) and GUS overexpression constructs were used as negative controls in VCS silencing and overexpression experiments, respectively. In most of the cases RLUC and RNA levels varied proportionately. Out of 24 sets, the only instance where significant increase in RLUC/RNA ratio could be seen was when PVA<sup>WT</sup> was co-expressed with VPg and VCS (II, Fig.7B-E). Based on these observations it could be predicted that VPg and VCS together with HCPro acts in favour of PVA translation. PVA<sup>WD</sup>, where HCPro is unable to form certain HMW complexes with VCS, no such upregulation for RLUC/RNA ratio was noticed.
Translational repression mediated by RNA-protein interaction at the 5’UTR is a well-known phenomenon (Nie and Htun, 2006). Ever since translational repression was established as a widespread mechanism in plants (Brodersen et al., 2008; Lanet et al., 2009), an increasing body of evidence evidences supporting translational repression as a \textit{bona fide} antiviral defence mechanism in plants has been obtained (reviewed by Calil and Fontes, 2017; Machado et al., 2017). Antiviral translational repression can be a versatile mechanism leading to global downregulation of translation. NIK1, a transmembrane immune receptor has been shown to adopt a strategy of inhibiting both host and viral translation by suppressing translation related genes as a defense measure against a begomovirus (Zorzatto et al., 2015). Or as shown in case of RLUC fused with the 5’UTR-IRES of TEV, translation can be suppressed from a target RNA by AGO1-RISC in a sequence specific manner (Iwakawa and Tomari, 2013). VCS is shown to be involved in translational repression in two different \textit{Arabidopsis} mutant lines (Goeres et al., 2007; Brodersen et al., 2008; Lanet et al., 2009; Xu and Chua, 2009). The studies mostly concerned developmental aspect of the plants, role of VCS in antiviral translational repression has not been established yet. According to the current understanding AGO1-RISC-bound mRNAs are transported to rough ER or P-bodies, both of which are reported to be sites of translational repression. VCS-mediated translational repression is thought to be associated with P-bodies, however the mechanism involved remains to be elucidated (reviewed in Ma et al., 2013). In this study, we approached the question of possible RNA silencing based translational repression during PVA infection. Keeping in mind ribosomal association of AGO1, HCPro, Vpg and CI plus the necessity of having HCPro-VCS interaction in this process, a PVA specific mechanism to overcome host imposed translational repression could be proposed. Formation of a virus specific complex at the 5’UTR of PVA RNA using VCS as the central scaffolding element could be proposed to have a role in releasing AGO1-RISC induced translational repression. Form another perspective, subverting AGO1 to a host factor promoting PVA infection via its binding to HCPro seems also plausible. Pro-PVA role of AGO1 predicted by Hafren et al. (2015) supports this argument.

4.3.6.3 Hypothetical model depicting the role of HCPro-VCS derived HMW complexes in different stages of PVA infection cycle

Combining the major outcomes of this study, a holistic model is proposed for PVA infection cycle emphasizing the role of HCPro-VCS derived multiprotein complexes therein (Fig. 5). During steady state virus infection, progeny vRNAs are generated within VRCs. Upon exit from VRCs, the newly synthesized vRNAs are targeted by host’s RNA silencing machinery. AGO2 predominantly executes degradation of vRNAs by inducing AGO2-RISC mediated endonucleolytic cleavage (Carbonell et al., 2012). RNA-silencing suppressor HCPro protects vRNAs from degradation using multiple interdependent approaches. One such approach discovered in this study is disruption of methionine cycle to destabilize virus derived siRNAs. This has been proposed to degrade siRNAs prior to their loading onto RISC
complex, all the way leading to silencing suppression downstream. Binding SAMS within HCPRO-VCS derived HMW complexes to inhibit its activity is something to be emphasized here. Loss of SAMS binding as well as the silencing suppression property as a result of weakened HCPRO-VCS interaction in case of HCPRO<sup>WD</sup> supports this argument. In order to temporarily safeguard nascent vRNAs from antiviral defense machinery, HCPRO recruits VCS and several other host factors (P0, AGO1, eIF4E etc.) to form large multiprotein complexes around the vRNAs. These large multiprotein complexes likely serve as seeds for larger aggregates that may become macroscopically visible PGs. HCPRO-VCS interaction is proposed to play a crucial role in the assembly and stability of PGs. This statement is supported by the fact that PVA<sup>WD</sup> was unable to sequester VCS within PGs, and loss of interaction between HCPRO and VCS within HCPRO derived RNP complexes rendered them vulnerable to degradation by RNase A. Sufficient accumulation of VPg has been proposed to shift the momentum towards translation by dissolving the PGs and transporting vRNA to the polysomes. AGO1-RISC-induced translational repression is another host imposed hurdle, which PVA may need to overcome in order to infect efficiently. AGO1-RISC is proposed to inhibit translation initiation by binding to the potyviral 5’UTR-IRES followed by dissociation of DExD/H-box helicase eIF4A from the target mRNA (Iwakawa and Tomari, 2013). To overcome this, VPg is predicted to recruit HCPRO-VCS complex along with several host and viral factors- CI, eIF4E / eIF(iso)4E etc. Using VCS as the scaffolding element, the HMW complex around the 5’UTR of PVA is proposed to form, which would prevent the AGO1-RISC-induced dissociation of eIF4A (Fukao et al., 2014, Fukaya et al., 2014) and further allow assembly of the pre-initiation complex around the IRES-bound eIF4G complex. CI being the virus-encoded helicase is proposed to aid in dissociation of AGO1-RISC from the vRNAs. In support of this postulation, VPg, CI and AGO1 has been shown to be a part of the ribosome-associated as well as HCPRO-VCS-associated HMW complexes. Combined expression of VCS and VPg in case of PVA<sup>WT</sup> has been shown to enhance the RLUC/RNA ratio, thereby providing another piece of evidence for the release of translational repression. However, in case of PVA<sup>WD</sup>, impaired interaction between HCPRO and VCS led to absence of certain complexes from the HMW region. Interestingly, VPg, and CI were strikingly reduced in HCPRO<sup>WD</sup> interactome, and in similar line overexpression of VCS and VPg also could not elevate the RLUC/RNA ratio. Taken together, these data demonstrate that HCPRO-VCS interaction is necessary for formation of the HMW complexes, which in turn may be essential for relieving translational repression. Finally, weakened interaction between HCPRO and VCS deteriorated PVA encapsidation. The connection between HCPRO-VCS interaction and its role in virion formation is not yet well understood. However, PVA encapsidation has been proposed to happen during later stage of translation when CP from <i>trans</i> interacts with CP expressed in <i>cis</i> to initiate the assembly process (Besong-Ndika et al., 2015). In another study, encapsidation of potyvirus tobacco vein mottling virus (TVMV) (Wu and Shaw, 1998) has been shown to initiate from the 5’ terminus. In canonical model of eukaryotic translation, 5’ and 3’ ends interact with
each other via poly-A binding protein and 7-methyl-guanosine-cap (Machado et al., 2017; Graber and Holcik, 2007). A similar model of vRNA circularization due to interaction between PVA genome linked Vpg and its 3’UTR could be assumed where HCPPro-VCS mediated HMW complex at the 5’UTR could stabilize vRNAs and allow CP-CP mediated initiation of encapsidation.

Figure 5. A hypothetical model depicting the role of HCPPro in different stages of PVA infection. HCPPro and other viral components assists in efficient vRNA amplification within VRCs. Replicated vRNAs are under a constant threat from host’s RNA silencing machinery. HCPPro suppresses RNA silencing with a multipronged approach. One such established approach is sequestration of siRNAs, keeping it away from AGOs, thus preventing RISC assembly. The other approach hypothesized in this study, is via disruption of methionine cycle, which is proposed to prevent methylation of siRNA by HEN1, leading to their degradation prior to RISC assembly. Further in this study, HCPPro-VCS interaction is suggested to be important for assembly of the PGs, a dynamic multiprotein complex designed to protect vRNAs from degradation. VPG enables transition of vRNAs from PGs to active translation. In this stage of infection, co-ordinated action from VPG, HCPPro and VCS is proposed to relieve AGO1-RISC mediated translational repression targeting the 5’UTR of vRNAs. Potyviral helicase CI is suggested to aid in relieve of translational
repression. Finally, HCPro-VCS interaction is proposed to be necessary for efficient encapsidation of progeny virions.

In a nutshell during PVA infection formation of a vRNA-associated core-complex is envisioned. HCPro and VCS are two of its identified central components, while many of the other remains to be discovered. In each infection stage this complex might selectively recruit or release certain viral and host factors to form virus specific RNP complexes like PGs and the 5’UTR- associated HMW complexes. Purpose of this complex is thought to guide vRNA through different stages of viral infection, while at the same time protecting them from host imposed threats.
5. CONCLUSIONS AND FUTURE PERSPECTIVES

Inhibition of siRNA methylation is one of the widely accepted mechanisms by which HCPro suppresses antiviral RNA silencing (Jamous et al., 2011). However, the mechanism underlying deactivation of HEN1-mediated siRNA methylation was unknown. In this study we show that HCPro interacts with SAMS and SAHH in planta and downregulates SAMS activity. This act of disrupting methionine cycle could be a strategy adopted by HCPro to inhibit HEN1-mediated siRNA methylation. Accordingly, downregulation of SAMS, SAHH and HEN1 was shown to rescue PVA^{AHPro} expression, further consolidating relevance of this strategy in context of PVA infection. Interestingly, disruption of the methionine cycle was also found to be one of the instrumental factors underlying induction of synergism during a mixed PVX-PVA infection. In addition to siRNA destabilization, we also observed synergism-specific downregulation of GSH biosynthesis. Since, this phenomenon was observed only when methionine cycle disruption / HCPro overexpression was carried out in conjunction to PVX infection, we proposed involvement of a yet to be identified PVX component in this process. Moreover, the mode of action of this PVX-component remains to be studied. A recent finding on potex-potyviral synergism indicated that PVX pathogenicity determinant P25 along with HCPro, induces ER stress and UPR ultimately leading to PCD (Aguilar et al., 2018). Multiple lines of evidence suggesting correlation between oxidative stress, ER stress and UPR (reviewed in Malhotra and Kaufman, 2007; Eletto et al., 2014; Cao and Kaufman 2014; Amodio et al., 2018) makes it reasonable to hypothesize that, these two independent observations during synergism might somehow be interconnected. If so, P25 could well be the missing PVX-factor responsible for synergism specific GSH depletion, we proposed in this study. Therefore, implication of P25-HCPro interaction in induction of oxidative stress, could be a potential point to be studied in future. Molecular cues involved in perturbation of these pathways and possible involvement of other PVX proteins therein could also be investigated. In another line of study, tissue / cell overlapping of PVX^{GFP} and PVA^{RFP} could be studied. This would be convenient to address as both the viruses are already tagged with fluorescent markers. However, it could reveal a lot of information on PVX-PVA mixed infection like the temporal and spatial distribution of the individual viruses and the extent of their overlap during the period of infection.

In this study presence of a core complex composed of several host and viral components has been envisioned. This complex has been proposed to guide vRNAs from replication to encapsidation. During different stages of infection, the complex is predicted to dynamically release or recruit host and viral factors to generate specialized bodies like PGs and translation associated HMW complexes. HCPro and VCS are currently thought to be two essential components of this core complex. In the future, attempt could be made to identify other factors required to form this complex. Also, in planta association of HCPro and VCS is demonstrated in this work, however, whether they interact directly or not is not known yet. In future, development of a method to elucidate their nature of interaction, might be worth
exploring. This study also proposes the presence of a 5’UTR associated HMW complex promoting PVA translation and encapsidation, and provided evidence for increased RLUC/RNA ratio as a possible sign of translational enhancement. One of the questions unresolved in this study is the mechanism behind HCPro-VCS-VPg co-ordinated active translation of PVA RNA from lower amount of total RNA. This could well be a case of release in translational repression, however, with methods available at the moment it is difficult to predict the actual reason. During active infection in the cells the total vRNA pool can be divided into many fractions: within VRCs, PGs, polysome bound and stored in virions. Since we do not know the actual amount of vRNA in active translation, any estimation of the relief of translational repression could be biased due to misinterpretation of the polysome-bound vRNA amount. In the future, a method to segregate and quantify polysome-bound vRNAs contributing to active translation could be developed. With this information the questions about the regulatory roles of 5’UTR associated HMW complexes in PVA translation could be easier to address. Role of another host factor, which we did not address in this study, is AGO1. Evidences exist to suggest that it could have both anti- and pro-viral roles (Iwakawa and Tomari 2013; Garcia-Ruiz et al., 2015; Hafren et al., 2015). We found AGO1 to be present in HCPro-derived ribosome-associated HMW complexes. It could be possible that AGO1, though an innately anti-viral host protein, is hijacked to serve in a pro-viral purpose. An in depth study of HCPro-AGO1 interaction is proposed as a prospective future work.
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7. REFERENCES


Virol, 89, 4237-4248.


58
S-adenosyl-L-methionine is an effector in the posttranscriptional autoregulation of the cystathionine gamma-synthase gene in Arabidopsis. *Proc Natl Acad Sci U S A*, 100, 10225-10230.


Dufresne, P. J., Thivierge, K., Cotton, S., Beauchemin, C., Ide, C., Ubalijoro, E., et al. (2008a) Heat shock 70 protein interaction with Turnip mosaic virus RNA-


synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. The Plant Cell, 9, 859-868.


Rodríguez-Cerezo, E., Findlay, K., Shaw, J. G., Lomonossoff, G. P., Qiu, S. G., Linstead,


