The viral coat protein is regulated by HSP70 and HSP40 in *Potato virus A* infection

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ACADEMIC DISSERTATION

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REPRINTS OF ORIGINAL PUBLICATIONS

List of Original Publications

This thesis is based on the following original publications that are referred to using the roman numerals throughout the text, and on unpublished results.

I **Anders Hafrén** and Kristiina Mäkinen. 2008. Purification of viral genome-linked protein VPg from potato virus A-infected plants reveals several post-translationally modified forms of the protein. Journal of General Virology 89:1509-1518.

II Katri Eskelin, Taina Suntio, Satu Hyvärinen, **Anders Hafrén** and Kristiina Mäkinen. 2010. *Renilla* luciferase –based quantitation of *Potato virus A* infection initiated with *Agrobacterium* infiltration of *N. benthamiana* leaves. Journal of Virological Methods 164:101-110.

III **Anders Hafrén**, Daniel Hofius, Gunilla Rönnholm, Uwe Sonnewald and Kristiina Mäkinen. 2010. HSP70 and its co-chaperone CPIP promote potyvirus infection in *N. benthamiana* by regulating viral coat protein functions. The Plant Cell 22:523-535.

ABBREVIATIONS

ATP	adenosine triphosphate
BMV	*Brome mosaic virus*
ChiVMV	*Chilli vein mottle virus*
CI	cylindrical inclusion protein
CK2	casein kinase II
CMV	*Cucumber mosaic virus*
CP	coat protein
CPIP	coat protein interacting protein
DAI	days after infiltration
eIF	eukaryotic Initiation Factor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FLUC	firefly luciferase
HA-tag	haemagglutinin-tag
His-tag	6 X histidine-tag
HC-pro	helper component proteinase
HMW	high-molecular-weight
HSP70	heat shock protein 70
icDNA	infectious complementary DNA
IRES	internal ribosome entry site
JDP	J-domain protein
mRNA	messenger RNA
NIa	nuclear inclusion protein A
NIa-pro	NIa-proteinase
NIb	nuclear inclusion protein B
ORF	open reading frame
PABP	poly (A)-binding protein
PVA	*Potato virus A*
PPV	*Plum pox virus*
PVIP	potyvirus VPg-interacting protein
RAT	replication-associated translation
RC	replication complex
RdRp	RNA-dependent RNA polymerase
RLUC	*Renilla* luciferase
RNP	ribonucleoprotein
RT-PCR	reverse transcription polymerase chain reaction
SFV	*Semliki forest virus*
SIII-tag	strep-tag III
SDS-PAGE	sodiumdodecylsulfate-polyacrylamide gel electrophoresis
sqPCR	semi-quantitative polymerase chain reaction
TBSV	*Tomato bushy stunt virus*
TEV	*Tobacco etch virus*
TMV	*Tobacco mosaic virus*

TVMV	*Tobacco vein mottling virus*
UTR	untranslated region
VIGS	virus-induced gene silencing
VPg	viral genome-linked protein
wt	wild type
2D-BN-PAGE	two-dimensional blue native-polyacrylamide gel electrophoresis

ABSTRACT

Plus-stranded (plus) RNA viruses multiply within a cellular environment as tightly integrated units and rely on the genetic information carried within their genomes for multiplication and, hence, persistence. The minimal genomes of plus RNA viruses are unable to encode the molecular machineries that are required for virus multiplication. This sets requisites for the virus, which must form compatible interactions with host components during multiplication to successfully utilize primary metabolites as building blocks or metabolic energy, and to divert the protein synthesis machinery for production of viral proteins. In fact, the emerging picture of a virus-infected cell displays tight integration with the virus, from simple host and virus protein interactions through to major changes in the physiological state of the host cell.

This study set out to develop a method for the identification of host components, mainly host proteins, that interact with proteins of *Potato virus A* (PVA; Potyvirus) during infection. This goal was approached by developing affinity-tag–based methods for the purification of viral proteins complexed with associated host proteins from infected plants. Using this method, host membrane-associated viral ribonucleoprotein (RNP) complexes were obtained, and several host and viral proteins could be identified as components of these complexes. One of the host proteins identified using this strategy was a member of the heat shock protein 70 (HSP70) family, and this protein was chosen for further analysis. To enable the analysis of viral gene expression, a second method was developed based on *Agrobacterium*-mediated virus genome delivery into plant cells, and detection of virally expressed *Renilla* luciferase (RLUC) as a quantitative measure of viral gene expression. Using this method, it was observed that down-regulation of HSP70 caused a PVA coat protein (CP)-mediated defect associated with replication. Further experimentation suggested that CP can inhibit viral gene expression and that a distinct translational activity coupled to replication, referred to as replication-associated translation (RAT), exists. Unlike translation of replication-deficient viral RNA, RAT was dependent on HSP70 and its co-chaperone CPIP. HSP70 and CPIP together regulated CP turnover by promoting its modification by ubiquitin. Based on these results, an HSP70 and CPIP-driven mechanism that functions to regulate CP during viral RNA replication and/or translation is proposed, possibly to prevent premature particle assembly caused by CP association with viral RNA.

INTRODUCTION

Viral infection cycles are complicated processes when one considers the multiple tasks that need to be carried out successfully by a virus in order to persist. The virus must establish the means to spread between hosts and to multiply within a host. During infection, plant viruses produce large amounts of progeny virus to increase the likelihood of interplant movement, simultaneously causing damage to the host. To counteract a plant virus, the host species will allocate resources to suppress viral infection by a variety of mechanisms, including gene silencing and innate immune responses such as the hypersensitive response. In a compatible virus-host interaction, a balance between these measures is established in order for both virus and host to survive. Comparing genome sizes, plus RNA viruses are small in comparison to their hosts. The small genome size and limited coding capacity of plus RNA viruses sets definite constraints on life style; they rely on the host to synthesize all the metabolites that viruses consist of, i.e., nucleotides and amino acids, and also metabolic energy to drive any required process. In addition to exploiting the pre-existing molecular synthesis machinery of the host, viruses have co-opted host components for selfish needs like RNA-dependent RNA synthesis. Traditional core metabolic enzymes like glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Wang and Nagy, 2008) or translational components like eukaryotic initiation factor 3 (eIF-3) (Quadt et al., 1993; Osman and Buck, 1997) can be subverted to play a role in virus RNA transcription. Of course, multifunctional knowledge of host proteins is by no means complete, and functioning of host proteins in virus infection may well elaborate on previous unknown mechanisms that are operating also in the absence of virus infection, as exemplified by the discovery of RNA silencing and the use of viral movement proteins in plasmodesmata research. The dependence of a virus on the molecular components of its host predicts tight integration and a requirement for co-evolution to maintain successful recognition between host and virus, resulting in a limited host range. An example of this is provided by the reported co-evolution between the viral genome-linked protein (VPg) of potyviruses and eukaryotic initiation factor 4E (eIF4E) (Charron et al., 2008). In contrast, some plant viruses like *Cucumber mosaic virus* (CMV) have a broad host range and can infect as many as 1600 different plant species from 100 different families. In addition, the ability of some animal and plant viruses to replicate successfully in yeast (Janda and Ahlquist, 1993; Panavas and Nagy, 2003; Weeks and Miller, 2008) suggests that many features required for virus multiplication are well conserved in hosts. One might think that it is a safer strategy for the virus to exploit evolutionarily stable host components in order to reduce the risk of a compatible host becoming incompatible, and also to increase the possibility of obtaining a broad host range for a virus, both important parameters in virus sustainability.

1. Potyviruses

Two plant virus genera, *Potyvirus* and *Begomovirus* are especially species-rich and encompass one third of all plant virus species (Fauquet et al., 2005). Potyviruses belong to the picornavirus supergroup of plus RNA viruses (Koonin and Dolja, 1993) and are grouped accordingly due to similarities in their RNA-dependent RNA-polymerase (RdRp) and genome organization, but their helicase localize within supergroup II. The genome is of messenger RNA polarity and is non-segmented with a size around 10 000 nucleotides. Potyviruses contain a 5´-terminal viral genome-linked protein (VPg) and a 3´-terminal poly (A)-tail and, when encapsidated, form a filamentous type of virus particle that is commonly transmitted by aphid vectors in a non-persistent manner. The genome is polycistronic and encodes 10 proteins in one single open reading frame (ORF) that is initially translated into a polyprotein (Figure 1). Co- and post-translational maturation catalyzed by three viral proteases occurs, giving rise to the different protein species (Riechmann et al., 1992). An additional ORF was recently discovered in the potyviral genome termed *pipo* (Chung et al., 2008).



Figure 1. Schematic map of the PVA genome, including the *pipo* ORF. The position of individual viral cistrons are indicated.

This section introduces three potyviral proteins, VPg, nuclear inclusion protein b (NIb) and coat protein (CP), each of which has a central role in the present study. Reviews exist that cover potyviral protein functions in general (Rajamäki et al., 2004; Urcuqui-Inchima et al., 2001). The different potyviral proteins have been associated with a range of functions and can be referred to as multifunctional. The multiple functions could partially be enabled via different polyprotein intermediates, and VPg for example, exists abundantly as a part of the nuclear inclusion protein a (NIa) precursor protein (Carrington et al., 1993). Both NIa and VPg accumulate in the nucleus, due to nuclear localization signals present in VPg, and here VPg has been reported to attribute to suppression of gene-silencing (Carrington et al., 1991; Rajamaki and Valkonen, 2009). In the polyprotein, VPg is preceded by the 6K2 protein that targets to the endoplasmic reticulum (ER) (Schaad et al., 1997a), and hence could also function to target VPg to the ER when fused to it (Leonard et al., 2004). The replication of potyviruses involves ER-association (Martin et al., 1995; Schaad et al., 1997a), and one function of VPg is to act as a protein primer for viral RNA-synthesis via its uridylylation by NIb

(Puustinen and Makinen, 2004; Anindya et al., 2005). VPg can interact with and stimulate the RdRp activity of NIb (Hong et al., 1995; Li et al., 1997; Fellers et al., 1998; Daros et al., 1999). Recently, VPg was shown to possess membrane-modifying properties that could potentially play a role in virus replication (Rantalainen et al., 2009). VPg has also been shown to affect viral movement within plants (Schaad et al., 1997b; Rajamaki and Valkonen, 2002), and two VPg-interacting host proteins, potyvirus VPg-interacting protein (PVIP) and eIF4E, could mediate this function of VPg (Dunoyer et al., 2004; Gao et al., 2004). The most studied host and potyvirus protein interaction appears to be that formed between VPg and eIF4E, a critical interaction in determining host-virus compatibility (Wittmann et al., 1997; Charron et al., 2008). Hypotheses have been put forward in which the interaction between eIF4E and VPg contributes to viral translation initiation, genome stability, or virus movement (Lellis et al., 2002), but the exact function of this critical interaction is still obscure.

The NIb protein is the catalytic subunit of the potyviral replicase, which belongs to the picornavirus-like, RNA-dependent RNA polymerases (Koonin and Dolja, 1993). NIb accumulates in the nucleus (Restrepo et al., 1990; Li et al., 1997), and during replication it also localizes to virus-induced vesicles (Dufresne et al., 2008a; Cotton et al., 2009). Unlike NIa and VPg, NIb is not preceded by a membrane-targeting protein. However, NIb interacts with NIa or VPg (Hong et al., 1995; Li et al., 1997; Fellers et al., 1998; Daros et al., 1999), and this has prompted the idea that targeting of NIb to the replication-associated membrane environment occurs via a 6K2-NIa interaction (Li et al., 1997). Also, heterologously expressed NIb can localize to virus-induced membrane-structures during infection. In uninfected cells however, it accumulates in the nucleus (Wei et al., 2010) and is also able to complement certain NIb-defective mutant viruses (Li and Carrington, 1995; Li et al., 1997).

An obvious function of CP is to encapsidate the viral genome. The potyvirus particle is filamentous and is mainly composed of CP and genomic RNA (Shukla et al., 1994). PVA particles can also possess a 5´ structure that contains both surface-exposed VPg and helper-component protease (HC-pro) (Puustinen et al., 2002; Torrance et al., 2006). The cylindrical inclusion protein (CI) can also associate with the PVA particle (Gabrenaite-Verkhovskaya et al., 2008). CP has been shown to function in virus cell-to-cell movement (Rojas et al., 1997) and can associate with plasmodesmata (Rodriguez-Cerezo et al., 1997). Furthermore, the specific functions of CP in assembly, cell-to-cell movement and systemic movement can be differentiated by introducing various mutations into the CP (Dolja et al., 1994; Dolja et al., 1995). Phosphorylation of CP by casein kinase II (CKII) was required for PVA infection (Ivanov et al., 2003). Phosphorylation and glycosylation of CP has been demonstrated also for *Plum pox virus* (PPV) (Fernandez-Fernandez et al., 2002), but its role in infection was unclear (de Jesus Perez et al., 2006). No direct role for CP in potyvirus replication has yet been presented. Although successful replication does not require CP, translation has to proceed at least half way into the CP cistron to get

productive replication (Mahajan et al., 1996). Poly (A)-binding protein (PABP) localizes to virus-induced vesicles (Beauchemin and Laliberte, 2007), affects virus accumulation (Dufresne et al., 2008b), and can interact with CP (Seo et al., 2007), VPg (Leonard et al., 2004) and NIb (Wang et al., 2000). CP can also interact with NIb (Hong et al., 1995), and together an interesting interaction network can readily be imagined. Following sections of the introduction will address translation and replication of the viral genome during infection of a host cell, and therefore a brief illustration of the infection cycle is given (Figure 2).

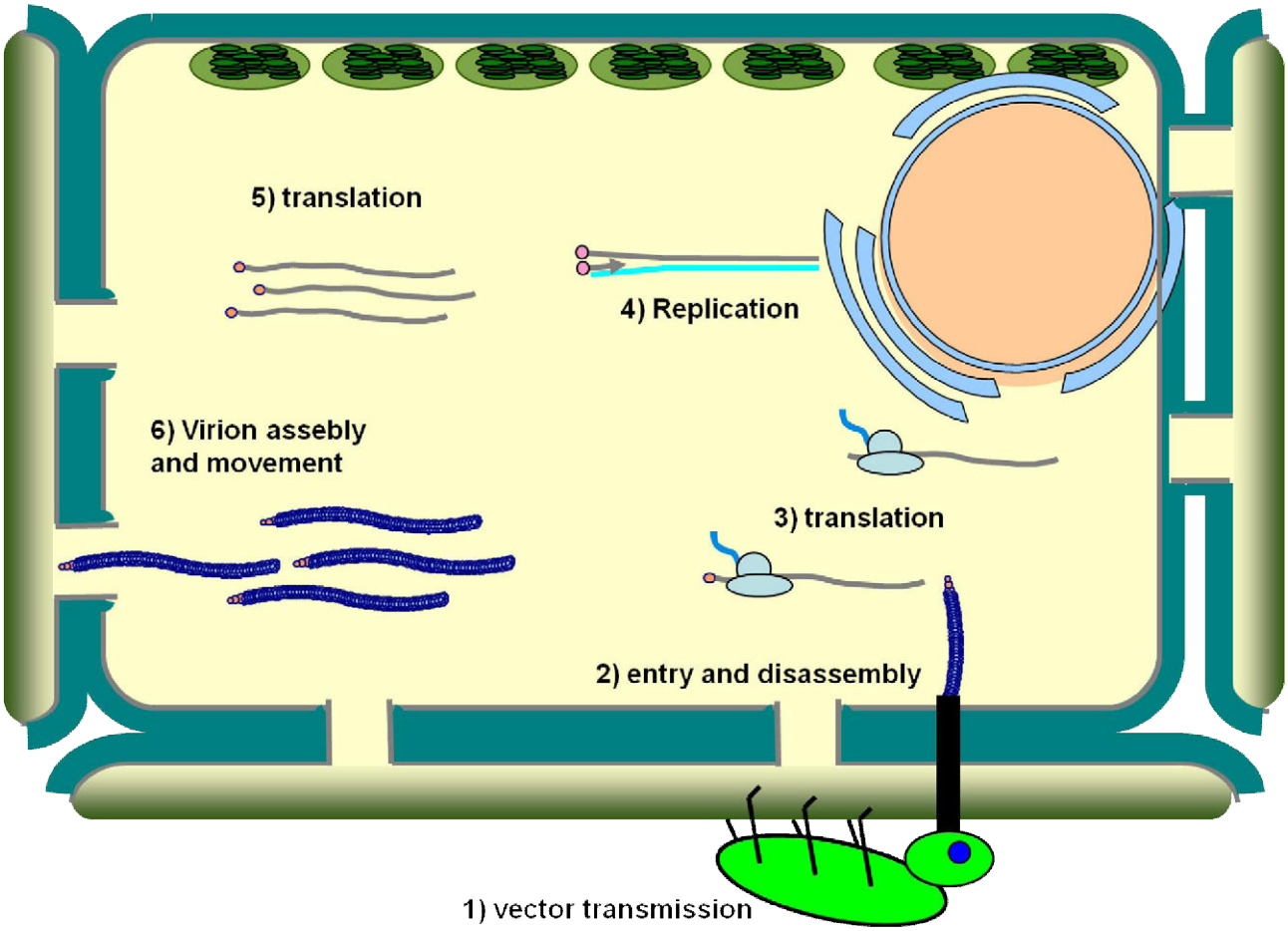


Figure 2. A simplified illustration of the infection cycle of potyviruses within the host cell. The potyvirus virion enters the plant cell commonly via aphid transmission (1). After cell entry, the viral genome will access the host translational machinery, and this involves removal of the protein coat shielding the genome (2, 3). Virus genome translation results in accumulation of viral replication proteins that mediate a shift from genome translation to RNA replication in association with host membranes (4). For the assembly of newly synthesized genomes into virions (6), translation of capsid protein is required (5).

1.2. Translation of the potyviral genome

The genomes of plus RNA viruses serve as both messengers for translation and templates for replication during infection. These viruses do not encapsidate viral proteins required for replication, and, hence, the infection cycle after cell entry proceeds with genome translation. If the genome is enclosed by a protein capsid, removal of the capsid protein is required prior to translation as it shields the RNA genome from translating ribosomes. For *Tobacco mosaic virus* (TMV), capsid removal is thought to take place co-translationally, with the ribosomes removing CP from viral RNA as translation progresses (Wilson, 1984; Shaw et al., 1986). Although the plus-stranded genome functions as a template for both translation and minus-strand synthesis, these processes cannot operate simultaneously on one particular genome (Gamarnik and Andino, 1998), and the template genome usage shifts from translation to minus-strand synthesis. How this is achieved in detail is not well known, but it can be anticipated to involve the association of newly synthesized viral replication proteins with specific RNA elements in the genome.

Cellular messenger RNAs (mRNAs) most commonly contain a 5´-terminal cap-structure, which is also present in several viral genomes. Potyviral genomes, however, do not possess a 5´-cap, but instead the viral protein VPg or NIa are covalently linked to the 5´-end (Murphy et al., 1990; Murphy et al., 1991; Oruetxebarria et al., 2001). Canonical translation in the cell is cap-dependent; the assembly of translation initiation factors prior to ribosome association involves the cap-structure and its interaction with eIF4Es (Browning, 1996). Various potyviral VPgs can interact with eIF4Es, and this interaction is required to establish infection (Wittmann et al., 1997; Schaad et al., 2000; Duprat et al., 2002; Lellis et al., 2002; Charron et al., 2008). The interaction between eIF4E and VPg suggests that VPg could play a similar role to the cap-structure in assembly of the translation complex. In contrast to this view, several reports propose that a cap-independent translational mechanism operates during potyvirus translation, mediated by an internal ribosomal entry site (IRES) (Levis and Astier-Manifacier, 1993; Niepel and Gallie, 1999). IRES-driven translation is a common strategy amongst viruses (Kieft, 2008), and a fraction of cellular mRNAs also possesses IRES-elements that mediate their translation (Baird et al., 2006). While viruses have adapted several strategies to efficiently translate their genomes in host cells (Thivierge et al., 2005; Dreher and Miller, 2006; Kneller et al., 2006), they may also simultaneously repress translation of host mRNAs by a variety of mechanisms (Bushell and Sarnow, 2002; Thompson and Sarnow, 2003; Lloyd, 2006). Potyviral VPgs have been implicated in the inhibition of cap-dependent, but not cap-independent, translation (Grzela et al., 2006; Khan et al., 2008). This suggests that potyviruses might interfere with canonical translation via an eIF4E interaction, and would therefore be anticipated to utilize an alternative mechanism to translate their own genome, possibly via an IRES-based, eIF4G-dependent and PABP-favoured mechanism (Gallie, 2001). Seminal studies demonstrated that host transcription is selectively repressed or induced during potyvirus infection (Wang and Maule, 1995; Aranda et al., 1996). Furthermore,

the transcription and translation of host proteins HSP70 and polyubiquitin were increased. More extensive transcriptional profiling subsequently demonstrated that extensive changes occur as a consequence of infection (Babu et al., 2008), but no comparable studies exist yet on changes in the host proteome due to potyvirus infection.

1.3. Replication of the potyviral genome

Mutational analysis by genomic insertions into *Tobacco vein mottling virus* (TVMV) indicated that the potyviral proteins P3, CI, 6K2, NIa and NIb are required for efficient virus replication (Klein et al., 1994). Extensive transposition-based insertions have been generated in the PVA genome, and this study implied that essential sites for virus propagation are located throughout the genome, ranging from the 5´- to the 3´-untranslated region (UTR) (Kekarainen et al., 2002), thereby suggesting that all potyviral proteins are potentially involved in virus replication. Biochemical analyses have also shown that most PVA proteins can bind RNA (Merits et al., 1998), and that a multifaceted viral protein interaction network exists (Merits et al., 1999; Guo et al., 2001).

Plus RNA viruses generally assemble their replication complexes (RCs) at host membranes, a process involving the induction of diverse virus-specific membrane structures (Mackenzie, 2005; Salonen et al., 2005; Denison, 2008; Miller and Krijnse-Locker, 2008). Why viruses induce these membrane-structures is not clear, but possible reasons include the generation of a protected and structured environment where viral and host factors concentrate and organize the process of viral genome multiplication. Depending on the virus, membranes derived from a variety of organelles are targets for the assembly of RCs, including those from the ER, vacuoles, peroxisomes, mitochondria and chloroplasts. For potyviruses, early work demonstrated that replication of both *Tobacco etch virus* (TEV) and *Plum pox virus* (PPV) are membrane-associated (Martin and Garcia, 1991; Martin et al., 1995; Schaad et al., 1997a). The 6K2 viral protein is involved in targeting viral components to membranes (Restrepo-Hartwig and Carrington, 1994) originating from the ER (Martin et al., 1995; Schaad et al., 1997a). Subsequent studies on the origin and nature of potyvirus vesicles have revealed that they originate from ER exit sites (Wei and Wang, 2008) and later target to the chloroplasts (Wei et al., 2010). VPg is adjacent to 6K2 in the polyprotein and is capable of modifying membranes *in vitro* (Rantalainen et al., 2009). P3 is yet another viral membrane-protein that localizes to ER exit sites and the early secretory pathway (Cui et al., 2010). During infection, these proteins may co-operate to achieve specific membrane-modification.

Initial translation of the viral genome will produce replication-associated viral proteins that initiate minus-strand genome synthesis via interactions with the minus-strand promoter, which appears to be located in the 3´- UTR of the potyvirus genome (Teycheney et al., 2000). From the synthesized minus-strand genomes, plus-strand progeny genomes are produced, in a process that is believed to take place within the virus-induced vesicles, since they are known to harbour double stranded RNA (Cotton et al., 2009; Wei et al., 2010). Progeny plus-strand genomes can in turn

be allocated to translation, a further round of minus-strand synthesis, or virus particle formation. The regulation of genome allocation to these different fates and the spatial, sequential and temporal co-ordination of these processes during virus multiplication are not well understood. The picture emerging point towards a tight coupling of all processes throughout virus multiplication, including translation, RNA synthesis and particle assembly.

1.4. Virus-host interactions

As outlined above, the genomes of plus RNA viruses are small and encode only a minimum number of proteins to successfully orchestrate virus multiplication within hosts. One apparent adaptation to the restricted coding capacity is the multifunctional nature of these viral proteins (Rajamäki et al., 2004; Urcuqui-Inchima et al., 2001). Another adaptation is the integration of host factors in viral processes, thereby avoiding the need to carry the required genetic information for these factors in the viral genome.

1.5. Host factors and their identification

Identification of host proteins that are required by viruses for multiplication has been approached in several different ways. Forward genetic screens in yeast have revealed numerous mutations in yeast genes that affect replication of both *Brome mosaic virus* (BMV) and *Tomato bushy stunt virus* (TBSV) (Ishikawa et al., 1997; Serviene et al., 2005; Jiang et al., 2006; Nagy, 2008; Panavas et al., 2008). Several follow-up studies on individual host candidates that were identified in these high-throughput screens have proven the value of this experimental approach (e.g. Noueiry et al., 2003; Barajas et al., 2009). Especially in the case of TBSV, where yeast is used as a model organism to study plus RNA virus replication and recombination, the approach is at the level of systems biology. Forward genetic screens have also been applied in plants for the analysis of host factors that affect virus multiplication, with studies involving mutant screening of *Arabidopsis thaliana* (Yamanaka et al., 2000; Lellis et al., 2002) and virus-induced gene-silencing (VIGS) (Lu et al., 2003; Burch-Smith et al., 2004). Host proteins that interact with viral proteins have been screened using *in vitro* proteomics screens (Li et al., 2008) and the yeast two- and three-hybrid systems (Bilgin et al., 2003; Jimenez et al., 2006; Hofius et al., 2007), yielding a number of candidate host proteins for characterisation. Finally, purification of protein complexes containing viral proteins has revealed host factors in plants that associate with viral proteins during infection. Purification of viral components from infected tissue can be approached, for example, by selecting for viral RNA synthesis activity throughout purification (Quadt et al., 1993; Osman and Buck, 1997), or affinity tag-based purification of viral components (Serva and Nagy, 2006).

1.6. Analysing virus multiplication

Methods to quantitate virus infection are important when different host factors are analysed for their role in virus infection, and a number of different approaches have been taken to achieve this. The enzyme-linked immunosorbent assay (ELISA) is commonly applied for the detection of viral CP, but may lack the sensitivity

needed, especially for studying the early events of the cellular infection cycle. Quantitation of *Potato virus Y* (PVY) infection by quantitative polymerase chain reaction (qPCR) was demonstrated to be several orders of magnitude more sensitive than ELISA (Kogovsek et al., 2008). Cell-free assays that use radioactive labelling of viral RNA are sensitive, but these systems are relatively difficult to set up (Osman and Buck, 1997; Pogany et al., 2008). The use of reporter genes inserted in viral infectious complementary DNAs (icDNA) can facilitate quantitation of infection. For example, potyvirus replication and movement has been analysed and quantitated using β-glucuronidase (GUS) (Dolja et al., 1994) or GFP (Ivanov et al., 2003; Hofius et al., 2007). At present, *Renilla* luciferase (RLUC) is amongst the most sensitive reporter genes (Mayerhof et al., 1995), and has for example been used to quantitate gene expression of *Semliki forest virus* (SFV) (Pohjala et al., 2008). Once a sensitive quantitation method has been established, analysis of the impact of different host factor manipulations on virus infection can be made.

1.7. HSP70 and its co-chaperone network

Members of the HSP70 protein family have been identified as host factors involved in infection by numerous different viruses (Sullivan and Pipas, 2001; Mayer, 2005). HSP70 and a co-chaperone are central factors in this study and hence this subject is introduced more thoroughly.

HSP70s are chaperones that are conserved across multiple biological kingdoms and are involved in an astonishing variety of cellular processes, including the folding of newly synthesized proteins, refolding of mis-folded or aggregated proteins, translocation of organellar and secretory proteins, protein complex assembly and disassembly, protein degradation, and receptor signalling (Hartl and Hayer-Hartl, 2002; Young et al., 2004; Mayer and Bukau, 2005; Mayer and Bukau, 2005). Consistent with the wide variety of processes HSP70s take part in, and therefore the versatile substrates they need to regulate, is that protein sequences capable of an HSP70 substrate-interaction occur on average every 36 residues (Rudiger et al., 1997). This means that most polypeptides are potential HSP70-substrates. HSP70s function by an ATP hydrolysis-driven conformational change that regulates substrate binding and release (Szabo et al., 1994; Bukau and Horwich, 1998; Zhu et al., 2007). The intrinsic ATP-hydrolysis activity of HSP70 is weak (Russell et al., 1998), but can be dramatically enhanced by the HSP70 co-chaperone DnaJ (Russell et al., 1999). There are several reviews on how J-domain proteins (JDPs), with the type member being DnaJ, stimulate the ATP-hydrolysis activity of HSP70s through an interaction between the J-domain and HSP70 (Kelley, 1999; Fan et al., 2003; Hennessy et al., 2005; Qiu et al., 2006). Because HSP70 function is dependent on ATP-hydrolysis, JDPs play an important role in the regulation of HSP70s. In addition to stimulating ATP-hydrolysis of HSP70, JDPs also mediate delivery and release of client proteins to HSP70 (Summers et al., 2009).

To date, several HSP70 co-chaperones have been described. JDPs comprise a fairly abundant group of

HSP70 co-chaperones, being especially numerous in plants. Using an *in silico* approach, Miernyk (2001) identified a total of 89 different JDPs in *Arabidopsis*, suggesting that the relative amount of JDPs is particularly high in plants in comparison to bacteria, yeast, worm, fly and mouse. Another study identified a total of 120 JDPs from *Arabidopsis*, of which the vast majority were type III JDPs (Rajan and D'Silva, 2009). Whereas type I and II JDPs share domains additional to the J-domain, only the J-domain is shared by type III JDPs, to which also CPIP belongs. The reason plants have such a high number of JDPs is not clear, but it may be related to abiotic stress management required by sessile organisms like plants (Rajan and D'Silva, 2009). Biotic stress caused by pathogens is also dealt with via different mechanisms in plants than, for example, mammals. Besides JDPs, there are additional HSP70 co-chaperones. Hip is a co-chaperone that interacts with HSP70s and stabilizes the high-affinity state of HSP70s for its substrates (Hohfeld et al., 1995). There are two orthologs of Hip in *Arabidopsis* (Webb et al., 2001). Another protein signature that is commonly involved in HSP70 interactions is the tetratricopeptide repeat (TPR) (Liu et al., 1999). The TPR is found in a wide variety of proteins including HSP70 and HSP90 co-chaperones (Lamb et al., 1995; Chen and Smith, 1998; Blatch and Lassle, 1999). Hop is a TPR protein that can function to mediate integration of the HSP70 and HSP90 chaperone machineries (Chen and Smith, 1998). Another protein domain mediating HSP70 interactions is the BAG-domain, which can regulate ATP-hydrolysis in HSP70s by binding to the ATPase domain (Takayama et al., 1997; Takayama et al., 1999). BAG proteins are found in yeast, human and plants, and are involved in modulating a range of processes, including stress responses (Kabbage and Dickman, 2008). In addition to their roles in protein folding and functional regulation of substrate proteins, chaperones also co-operate with the protein degradation machinery to remove undesired proteins (Esser et al., 2004). CHIP is a TPR-containing protein that can associate with HSP70 and inhibit JDP-stimulated ATP-hydrolysis. CHIP also possesses an ubiquitin ligase domain that mediates ubiquitination of HSP70 and HSP90 substrates, thereby promoting their degradation via the proteasome (Ballinger et al., 1999; Connell et al., 2001; Jiang et al., 2001). BAG-1 can also facilitate delivery of HSP70 substrates to the proteasome in co-operation with CHIP (Luders et al., 2000; Demand et al., 2001; Alberti et al., 2002). These studies point to a highly complex and regulated chaperone network, in which the fate of client proteins of this network, substrates, is determined by the composition of HSP70-associated co-factors. The system is involved in ‘protein triage’, in which proteins undergo either productive folding or degradation (Arndt et al., 2007). One illustrative example of the integrated cellular chaperone network is p53, which is proposed to interact with HSP40, HSP70, HSP90, BAG-1, Hop (King et al., 2001), and CHIP (Esser et al., 2005).

Seminal work linking HSP70 to potyvirus infection showed that transcription of HSP70 was induced at the infection front of *Pea seed borne mosaic virus* (PSbMV) (Aranda et al., 1996). Subsequent studies have demonstrated that HSP70s are commonly induced during infection by different plant viruses (Escaler

et al., 2000; Whitham et al., 2003; Chen et al., 2008). Recently, the transcriptional changes in *Arabidopsis* following PPV infection were analysed, and numerous HSPs were induced in systemically infected leaves, including HSP70s (Babu et al., 2008). In contrast, only one HSP transcript, a JDP, was induced in protoplasts by the approximate time of maximal viral RNA synthesis (Raghupathy et al., 2006; Babu et al., 2008). This suggests that induction of HSP70 is not directly linked to potyvirus RNA replication, keeping in mind the possible stress caused by protoplast preparation. That induction of HSP70 would not be directly coupled to replication is further supported by the observation that strong ectopic expression of individual potyviral proteins and a variety host proteins are able to induce HSP70 via a cytoplasmic unfolded protein response (Aparicio et al., 2005). Instead, it appears reasonable that HSP70 is induced as a general response to the production of high amounts of aggregation-prone viral proteins occurring at later times during infection. For example, during TMV infection, the induction of HSP70 correlates with the amount of aggregated viral CP (Jockusch et al., 2001). Even though induction of HSP70 would be a late response to potyvirus infection, with a role in managing cellular stress and protein homeostasis, this does not exclude the exploitation of HSP70 by plant viruses to carry out specific tasks relating to virus multiplication.

AIMS OF THE STUDY

The purpose of the present study was to gain further insights into molecular mechanisms operating in virus multiplication during plant infection and, more specifically, to understand the role of host proteins in this process. Understanding the interactions between an infecting virus and its host is essential for understanding the outcome of viral infection, which is essential for the development of antiviral strategies. The undertaking of this study is justified because infections by several potyviruses cause economically important losses in crop yield. For example PVA infection can reduce the yield of potato by 40% (Bartels, 1971).

In order to identify candidate host proteins, methods were developed for the purification of viral protein complexes from infected plants and to identify associated host proteins. This involved the insertion of affinity tags into viral proteins in the PVA genome. The host protein candidates were analysed to determine how their manipulation affected virus multiplication. This approach required the development of a method for the quantitation of virus multiplication. Once the required tools were established, the final aim was to describe the interactions that take place during infection between host and viral proteins, and what functions these interactions play in regulating virus multiplication.

MATERIALS AND METHODS

Table 1. Viruses used in this study. All PVA-based viruses are based on the PVA B11 isolate. Viruses are referred to the corresponding publication numerically.

Virus	**Publication**
PVA-*gfp*	I, III
PVA-*gfp* (VPg^{HisHA})	I
PVA- *gfp* ($NIb^{Strep\text{-}III}$)	III
PVA- *gfp* ($VPg^{Strep\text{-}III}$)	III
PVA-*rluc* (wild type; PVA^{wt})	II, III
PVA-*rluc* (CP-mutant; movement deficient; PVA^{CPmut})	II, III
PVA-*rluc* (ΔGDD; replication deficient; $PVA^{\Delta GDD}$)	II, III
PVA-rluc (ΔGDD and CP-mutant; $PVA^{CPmut_\Delta GDD}$)	III
PVA-*rluc* (CP-mutant; CK2-phophorylation-deficient; PVA^{CPAAA})	unpublished

Table 2. Methods used in the present study and the location of their detailed description.

Method	**Publication**
Affinity purification of viral proteins from plants;	
His-tag–dependent (in denaturing and native conditions)	I
HA-tag–dependent	I
Strep-tag–dependent	III
agroinfiltration	II, III
BN-PAGE, performed essentially as in Wittig et al. (2006)	III
luciferase assay	II, III
ELISA	II, III
Immunoprecipitation	I, III
LC-MS/MS	III
Protein phosphatase treatment	I
qPCR	II
RT-PCR	I, II, III
SDS-PAGE	I, III
Western blotting	I, III
Virus-induced gene-silencing	III
2D-PAGE	I

RESULTS

4.1. *In-genome* affinity tag fusions to viral proteins

To enable the purification of viral proteins from infected plant tissues, the genome of PVA was engineered to express two of its replication-associated proteins, NIb and VPg, as affinity tag fusions (I, III). Strep III affinity tags (SIII-tag) were fused to both NIb and VPg, and a six-histidine haemagglutinin (HisHA-tag) was also fused to VPg. The resulting mutant viruses were able to accumulate in systemic non-inoculated plant leaves of *Nicotiana benthamiana* as judged by the presence of virus-encoded GFP fluorescence. This indicates that replication and movement within plants were both still functional despite these genetic insertions. A triple HisHA-tag and GFP was also fused to VPg, but this caused loss of viral systemic movement and, hence, these recombinant viruses were not used for further studies (I).

Because RNA viruses exhibit high mutation rates and frequent recombination, the stability of the HisHA-tag fused to VPg was analysed by RT-PCR from upper non-inoculated leaves (I). Sequence analysis of the HisHA insertion site showed that the dominant sequence remained intact for at least three passages in *N. benthamiana*. Because both the HisHA-tag and the SIII-tags were functional in affinity-purification, these insertions appeared to be genetically stable (I, III).

4.2. Purification of viral proteins from infected plants

Chronologically, the HisHA-tag based purification system was established prior to the SIII-tag system. This tag was designed to allow for affinity-purification using denaturing conditions via the His-tag, and also to use the His- and HA-tags sequentially for the tandem purification of protein complexes in native conditions. By utilizing the His-tag to purify VPg from systemically infected plants under denaturing conditions, several isoelectric isoforms of the protein were observed, which were characterized by an acidic shift from the theoretical isoelectric point (pI) (I; Figure 5). Using phosphatase treatment, several of these isoforms were shown to arise from protein phosphorylation (I; Figure 6). Only a minor proportion of the total VPg protein was found at its theoretical pI.

To purify and characterize viral protein complexes, tandem-purification of HisHA-tagged VPg from systemically infected plants was carried out. Plant lysates were subjected to His-tag–dependent, followed by HA-tag–dependent, affinity-purification. This strategy was successful in enriching VPg protein, as detected by western blot analysis (I; Figure 7). Total protein staining of the purified samples showed that several proteins were enriched in the tagged sample. Some protein bands were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), but failed to give identifications of high confidence. By western blot analysis, NIb,

CI and HC-pro all co-purified with VPg, whereas CP did not (I; Figure 8). The main focus of the study was the purification of protein complexes formed during viral replication. Hence, heavy-membrane fractions that had been shown to harbour the RNA replication activity for two potyviruses (Martin and Garcia, 1991; Schaad et al., 1997a), and for other plus RNA viruses, were prepared and subjected to tandem purification. However, the method appeared to lack the sensitivity required to enrich VPg from these samples (data not shown). Because tandem-purification was both time-consuming and expensive, no further effort was invested to optimize the method and alternative affinity tags were considered instead.

The strep III tag (SIII-tag) was chosen because it can achieve high purity in a single-step protocol (Junttila et al., 2005), it is time and cost efficient, and previous studies had shown that the strep II tag resulted in quite good purity when used to purify recombinant protein from plants (Witte et al., 2004). An additional benefit was that the charge on the SIII-tag is relatively neutral compared to several other tags, e.g., c-myc and HA, and hence should be less likely to interfere with the function of the fused protein. Strep-tag–dependent purification was carried out from detergent-extracted, heavy-membrane fractions prepared from plants infected with PVA encoding either SIII-tagged VPg or NIb, or with wild-type (wt) PVA as a control (Figure 3 and III; Figure 1). Total protein detection by silver staining indicated that several proteins were enriched in both tagged samples. Protein bands were analysed by LC-MS/MS, and both NIb and NIa were identified. HSP70 and PABP were among the identified host proteins. The specificity of the affinity-purification was analysed by western blotting, which showed that NIb, NIa, VPg, NIa-pro, HC-pro, CP, HSP70 and PABP were enriched in SIII-tagged samples. Two proteins were detected by the anti-CI serum, one at 60 kDa that was also present in the control, and one at ~65 kDa that was enriched in the SIII-tagged samples. RT-PCR analysis demonstrated that viral RNA was found specifically in the tagged samples. Two-dimensional blue-native PAGE (2D-BN-PAGE) was also used to analyse the purified native complexes. The migration patterns of HSP70, NIb and NIa overlapped and were smear-like, suggesting that the size of the complex was not defined, ranging from mega–kDa size. An alternative interpretation is that the complex was in the mega–kDa size range, but was slowly disassociating during electrophoresis and therefore giving rise to a smeared pattern. Overall, the composition of the samples derived from SIII-tag purification indicated that the same components, and therefore the same complexes, were co-purified regardless of purified via VPg or NIb. One difference observed between the samples purified by tags on NIb and VPg samples was the specific co-purification of NIa-pro, the proteinase domain of NIa, with NIb. Also, the smaller CP form was only enriched in the SIII-tagged VPg sample.

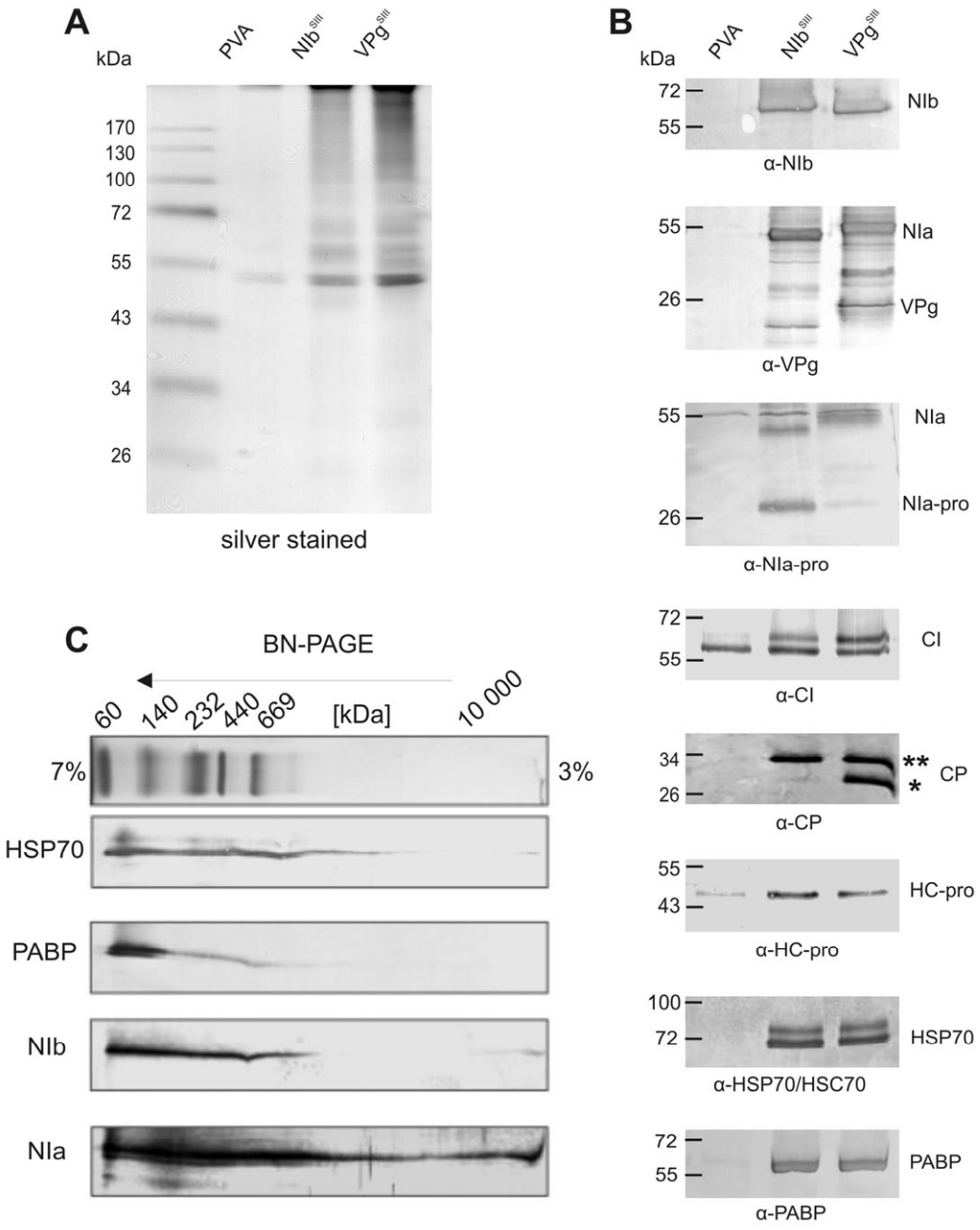


Figure 3. Affinity-purification of viral proteins from infected plants utilizing the SIII-tag. Control (PVA-*gfp*) and SIII-tagged samples were analysed by SDS-PAGE followed by silver staining (A) and LC-MS/MS identification or western blotting using indicated antibodies (B). 2D BN-PAGE western blot analysis was carried out for the NIb^{SIII}-sample (C). In (B), the different CP forms are indicated with the smaller (*) corresponding to virion sized CP. Adapted partially from publication III.

4.3. Analysis of viral gene expression

To quantitate viral infection, and with a special interest to be able to discriminate between viral translation, replication and movement, an agro-infection based sensitive assay was developed (II). Here, PVA gene expression is initiated by infiltrating *Agrobacterium* carrying PVA-*rluc* icDNA as a transgene into plant leaves and quantitated by measuring virally expressed RLUC. *Agrobacterium* carrying

firefly luciferase (FLUC) is co-infiltrated as an internal control and used to normalize viral RLUC data. Three different RLUC-tagged viral constructs were made: first, wild-type PVA (PVA^{wt}); second, a virus carrying a similar mutation in the CP (PVA^{CPmut}) to that shown to debilitate cell-to-cell movement in TEV (Dolja et al., 1994; Varrelmann and Maiss, 2000); and third, a virus carrying a deletion in the RdRp GDD-motif leading to impaired replication ($PVA^{\Delta GDD}$). The rationale for using these 3 viruses was as stated, to be able to discriminate between the fundamental events related to virus translation, RNA synthesis and movement. In order to emphasize differences in gene expression derived from PVA^{wt}, PVA^{CPmut} and $PVA^{\Delta GDD}$, several parameters were optimized. First, non-saturating optical densities of *Agrobacterium* were determined (II; Figure 3), meaning that not all cells were transformed within the infiltration area. This allowed for PVA^{wt} to enhance its gene expression via cell-to-cell movement compared to PVA^{CPmut}. Second, both plant size and the particular leaf used for *Agrobacterium* infiltration affected the level of T-DNA derived gene expression (II; Figure 4). Experimental variation was minimized in this regard by using plants and leaves of a similar size within a particular experiment. By further adjusting the sampling technique so that each parallel sample represented a pool derived from multiple different *Agrobacterium* infiltration areas and plants, gene expression in the different viruses were clearly separable and variations in gene expression between replicate samples was minimized (Figure 4 and II; Figure 7).

When quantitation of RLUC activity and CP accumulation were compared, an increase in the amount of CP correlated with increased RLUC expression (II; Figure 10). However, RLUC-based detection of viral gene expression was substantially more sensitive; the positive signal threshold was reached much earlier with this assay. RLUC-based detection of viral gene expression was also compared to sqPCR-based detection of viral genomic RNA (II; Figure 8). Accumulation of viral RNA analysed by sqPCR coincided with increased RLUC expression. Time course analysis showed that gene expression of both PVA^{wt} and PVA^{CPmut} started to diverge from $PVA^{\Delta GDD}$ at 2 days after infiltration (DAI). Because RLUC activities were used as the measure for viral gene expression and these are derived via translation, viral RNA replication had occurred already at this time point. By 3 DAI, there was further amplification of the differences between gene expression derived from non-replicating and replicating viral genomes. At this time point PVA^{wt} was already 10-fold above PVA^{CPmut}, indicating amplified gene expression via cell-to-cell movement. The time points illustrated are not absolute in that sense, that different treatments like co-expressions and also plant physiology can affect the temporal appearance of detectable gene expression. $PVA^{\Delta GDD}$ was initially constructed to estimate non-replication associated viral gene-expression in the infection assay and this level of gene expression was expected to always occur regardless of any defects related to viral replication. This is however not the case and gene expression of PVA^{wt} can be below that of $PVA^{\Delta GDD}$ as exemplified by CP expression *in trans* (Figure 4B). This was also observed for PVA^{wt} during *HSP70*-silencing (III; Figure

2F) and PVA^{CPAAA} (Figure 6A), as explained later. However, this suggests that translation of these viruses is regulated differentially to that of PVA$^{\Delta GDD}$ due to replication at an early stage of infection, and that gene expression from PVA$^{\Delta GDD}$ cannot be regarded as a background translation level that would be present in the replicating viruses equally. There are differences between replicated and non-replicated viral genomes to be emphasized. Initially the transcripts for PVA^{wt}, PVA^{CPmut} and PVA$^{\Delta GDD}$ are all nuclear derived via T-DNA expression and contain a 5´-terminal cap structure. The replicated genomes however do not have a 5´-cap and instead should have a 5´-terminal VPg. Also, the cytoplasmic localization can be expected to be different for replicated genomes.

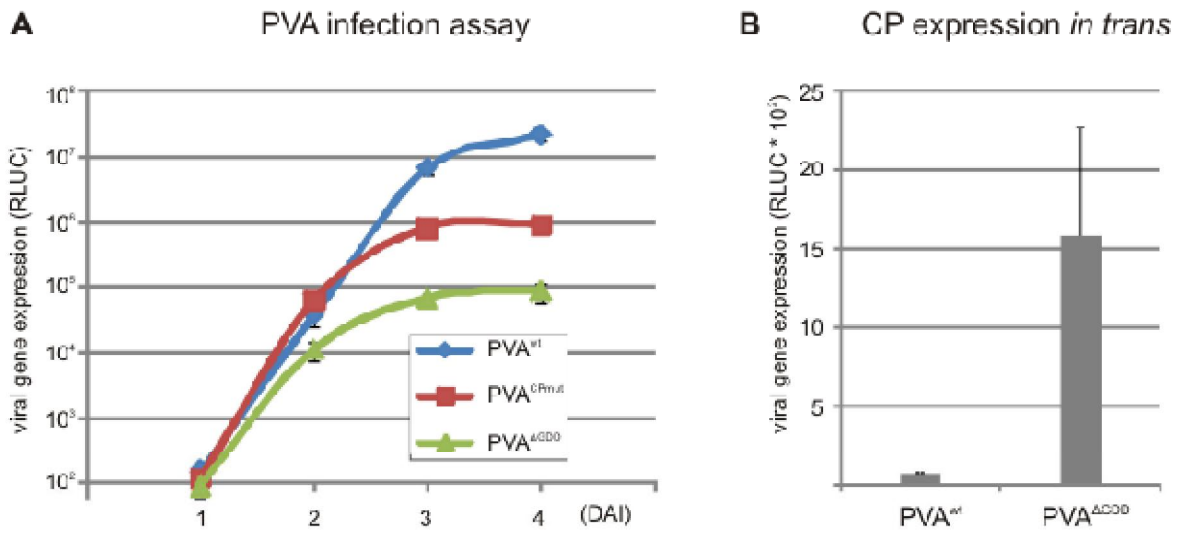


Figure 4. PVA infection assay. A) Time course of gene expression derived from PVA^{wt}, PVA^{CPmut} and PVA$^{\Delta GDD}$. Gene expression of viruses became detectable between 1 and 2 days after infiltrations (DAI) and the replicating viruses (PVA^{wt} and PVA^{CPmut}) had diverged from non-replicating PVA$^{\Delta GDD}$ at 2 DAI. By 3 DAI, viral cell-to-cell movement had occurred as gene expression PVA^{wt} was 10-fold above that from PVA^{CPmut}. B) Gene expression from replicating PVA^{wt} was 10-fold reduced compared to non-replicating PVA$^{\Delta GDD}$ during CP co-expression. A) is adapted partially from publication II.

4.4. HSP70 is a functional component in PVA infection

From the protein candidates identified via step-purification and LC-MS/MS analysis, HSP70 was among the proteins selected for further study. To examine whether HSP70 has a role in PVA infection, VIGS vectors were used to down-regulate *HSP70* during PVA infection (III; Figure 2). First, down-regulation of HSP70 had a negative impact on systemic movement of PVA. Second, the initiation of gene expression from PVA^{wt} was delayed compared to both PVA^{CPmut} and PVA$^{\Delta GDD}$ due to *HSP70*-silencing. The early delay of PVA^{wt} compared to that of PVA$^{\Delta GDD}$, suggests that HSP70 is involved in early replication events, while the early delay of PVA^{wt} compared to PVA^{CPmut}, suggests a

requirement for HSP70 in a CP-related phenomenon. A reduction in the gene expression of both the internal control FLUC and the different viruses was observed due to *HSP70*-silencing.

4.5. CP-mediated inhibition of viral gene expression

Because down-regulation of *HSP70* suggested that CP, but not CP^{mut}, required HSP70 during replication, further experimentation was initiated to analyse this relationship. First, wt $CP^{(wt)}$ and CP^{mut} were expressed *in trans* in the infection assay (III; Figure 3). Co-expression of CP^{wt}, but not CP^{mut}, efficiently inhibited viral gene expression in a virus-specific manner, an effect that was mediated via the CP protein and not by gene-mediated RNA-silencing. Additionally, CP^{mut} appeared to interfere with virus movement *in trans*. Viral gene expression was inhibited by expressing CP^{wt} *in trans*, but the level of inhibition varied between different viruses; PVA^{CPmut} showed a 10- to 50-fold higher gene expression than $PVA^{\Delta GDD}$ and PVA^{wt}. The elevated gene expression of PVA^{CPmut} was possibly attributable to the mutated endogenous CP, and it supports the hypothesis generated from the experiment were down-regulation of HSP70 indicated the potential of CP^{wt}, but not CP^{mut} to inhibit replication. To further support this, CP^{mut} was transferred to $PVA^{\Delta GDD}$, which is replication-deficient. The level of gene expression from the resulting $PVA^{\Delta GDD_CPmut}$ was the same as that from $PVA^{\Delta GDD}$ during expression of CP^{wt} *in trans*. This demonstrated that CP can influence gene expression *in cis* mainly in connection to viral replication.

4.6. CPIP-mediated regulation of CP

An HSP70 co-chaperone, CPIP, was previously described and shown to interact with the CP of *Potato virus Y* (PVY) (Hofius et al., 2007). In that study, the authors constructed transgenic plants expressing a J-domain–deficient version of CPIP ($CPIP^{\Delta 66}$), which showed reduced virus accumulation and rate of cell-to-cell movement following infection, an effect that was probably due to the inability of $CPIP^{\Delta 66}$ to interact with HSP70. The results from down-regulating HSP70 and the expression of exogenous CP in the PVA infection assay together suggested that CP^{wt} can inhibit viral gene expression related to replication, and that HSP70 is a functional component working against this inhibition.

The potential role of CPIP in mediating the link between HSP70 and CP in connection to virus replication was addressed next by co-expressing CPIP or $CPIP^{\Delta 66}$ together with PVA^{wt} (Figure 5A and III; Figure 4). Co-expression of $CPIP^{\Delta 66}$ with PVA^{wt} reduced viral gene expression to approximately 20% of the level obtained with CPIP co-expression at days 2 and 3 after infection. Also, systemic accumulation of PVA, tobacco vein mottling virus (TVMV) and TEV was similarly reduced in $CPIP^{\Delta 66}$-transgenic plants (III; Figure 4C). Next, the capacity of CPIP to counteract CP-mediated inhibition of viral gene expression was analysed by co-expressing CPIPs and CP^{wt} with viruses (Figure 5B, C, D and III; Figure 4). Gene expression of non-

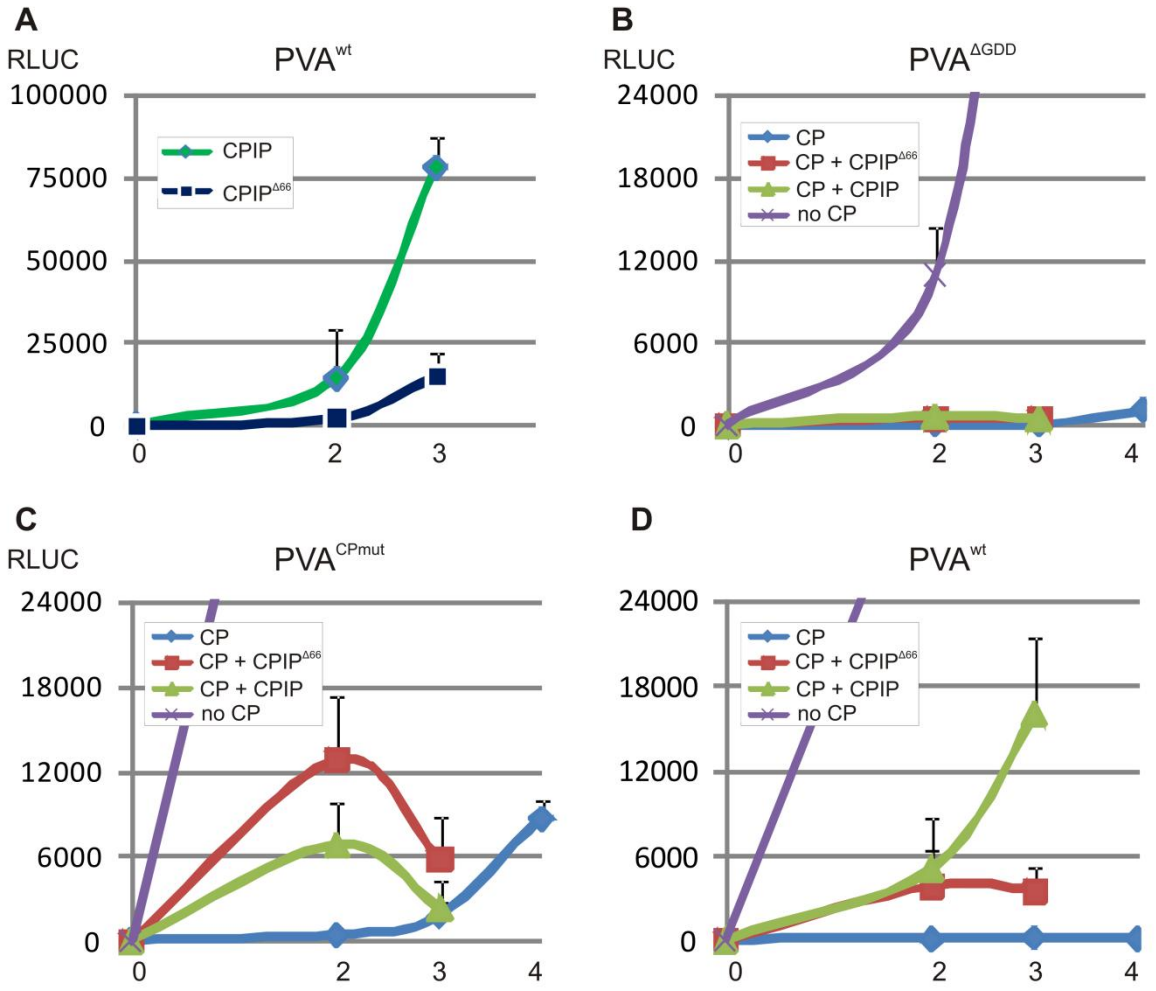


Figure 5. Gene expression was analysed by RLUC activity when PVA^{wt} was co-expressed with CPIP or $CPIP^{\Delta 66}$ (A), and when viruses were expressed alone and co-expressed with CP alone or together with either CPIP or $CPIP^{\Delta 66}$ (B, C, D). In B, C, and D, the RLUC activity from viruses expressed alone is out of scale in order to more clearly show the CPIP-mediated response in viral gene expression. The time points of analysis are indicated on the X-axis as days after infection. In 5A, the actual RLUC activities are 10 times higher at 3 days after infection but presented as such in order to more clearly visualize the differences found at both time points in the same graph. Data adapted from publication III.

replicating $PVA^{\Delta GDD}$ was not elevated due to co-expression of either CPIPs compared to the replicating viruses. Consistent with the appearance that PVA^{CPmut} does not require HSP70 as suggested by the *HSP70*-silencing experiment, PVA^{CPmut} did not require functional CPIP because co-expression of $CPIP^{\Delta 66}$ elevated its gene expression to a greater extent than did CPIP. CP^{mut} does not interact with CPIP (result presented in next section) and hence, the effect of co-expressing CPIPs are through their impact on *in trans* expressed CP^{wt} in the case of PVA^{CPmut}. Interestingly, gene expression of PVA^{CPmut} peaked at day 2 and declined by day 3 after infection due to over-expression of CPIPs, whereas during co-expression of CP^{wt} only, gene expression of PVA^{CPmut} was highest at day 4 post infection. Also, movement

deficiency of PVA^{CPmut} did not appear to be complemented by co-expression of CPIP and *in trans* CP. In contrast, PVA^{wt} required functional CPIP to reach the same level of gene expression as PVA^{CPmut}, and when co-expressed with CP^{wt} and $CPIP^{\Delta 66}$, gene expression of PVA^{wt} was substantially lower compared to PVA^{CPmut} (Figure 5C and D).

Co-precipitation analysis showed that CPIP could interact with PVA CP^{wt} but not CP^{mut} (III; Figure 5). Therefore CP^{mut} is unable to sequester endogenous CPIP when expressed *in trans*, which is consistent with the inability of CP^{mut} to inhibit viral gene expression from PVA^{wt}. CP was co-precipitated only with $CPIP^{\Delta 66}$ suggesting that the interaction between CPIP and CP^{wt} was transient due to delivery of CP by CPIP to HSP70. CP^{wt} also interacted with HSP70, and this interaction could be disassociated using ATP (III; Figure 5), as common for HSP70 and their substrates (Szabo et al., 1994). These results confirmed a link between HSP70 and CP, and suggested that CPIP is only required by a replicating virus for the delivery of its CP to HSP70.

4.7. CPIP promotes CP degradation and ubiquitination

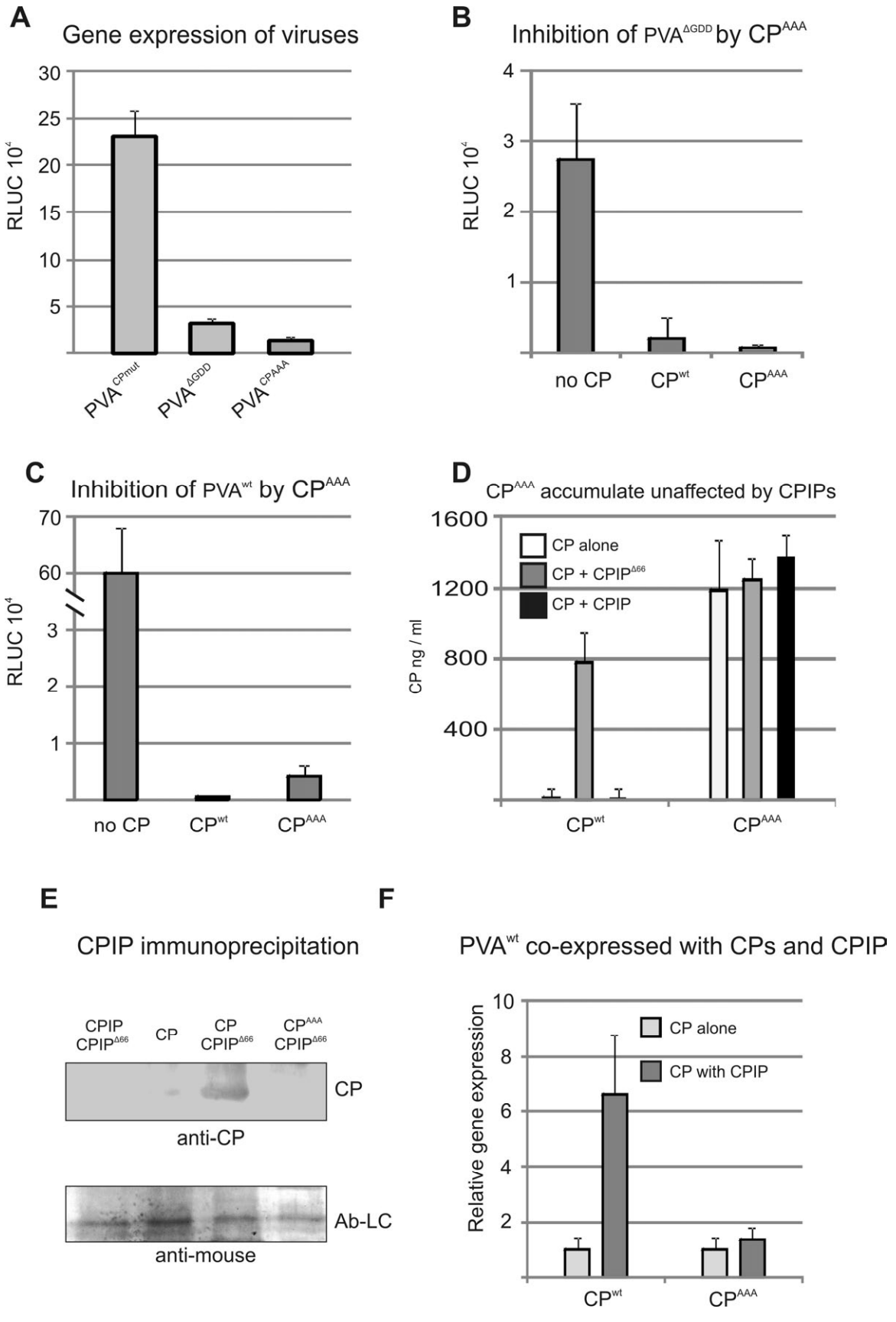
As outlined in the Introduction, the fates of HSP70 client proteins are many, including degradation (Esser et al., 2004). To test whether the interaction of HSP70 and CPIP with CP affected its accumulation, quantitation of CP^{wt}, expressed alone or together with CPIP or $CPIP^{\Delta 66}$, was carried out in uninfected cells (III; Figure 6). When co-expressed with CPIP, CP accumulation decreased. In contrast, substantial accumulation of CP was observed when co-expressed with $CPIP^{\Delta 66}$, demonstrating that a functional CPIP/HSP70 association promotes degradation of CP, and that disruption of this system results in aberrant CP accumulation. Additionally, CPIP-mediated delivery of CP to HSP70 promoted CP modification by ubiquitin, and high-molecular weight (HMW) CP and ubiquitin were both detected in CP-immunoprecipitates, and the intensity of the ubiquitin signal correlated with functional CPIP expression. Modification by ubiquitin conjugation is a common mechanism to target proteins for degradation (Glickman and Ciechanover, 2002) and, hence, CPIP-mediated CP ubiquitination may explain the negative effect of CPIP on CP accumulation. In the context of infection, HMW ubiquitin and CP could also be detected from the strep-tag–purified samples derived from infected plant membranes that also harboured HSP70 and CPIP. These samples were, however, derived via purification of NIb^{SIII} and VPg^{SIII}, and hence, the presence of an ubiquitin signal is not CP-specific. Therefore, ubiquitination of CP during infection remains to be fully verified. Of interest, a higher MW form of CP (~35 kDa) was the main form found in the purified membrane samples (Figure 1).

4.8. CK2-dependent CP phosphorylation and CPIP interaction

All results presented in this section are unpublished. Previously, PVA infection was shown to require CP phosphorylation by CK2 for normal infection (Ivanov et al., 2003). In that study, PVA viruses with mutations in the CK2 phosphorylation

consensus-site were unable to spread the infection from initially inoculated single cells. One of the mutant viruses used in that study had the CK2-site threonines and serine substituted for alanines (CP^{AAA}). In this study, gene expression in a virus carrying this mutation (PVA^{CPAAA}) was analysed using the RLUC-based assay (Figure 6A). By this it was found that gene expression from PVA^{CPAAA} was even below that from $PVA^{\Delta GDD}$ (Figure 6A). This implied that the negative effect of this mutation acted already at the level of replication, prior to cell-to-cell movement. Next, the capacity of CP^{AAA} to inhibit viral gene expression when expressed *in trans* was analysed. Expression of CP^{AAA} was capable of inhibiting gene expression in $PVA^{\Delta GDD}$ even more efficiently than CP^{wt} (Figure 6B). CP^{AAA} also inhibited gene expression in PVA^{wt}, but in this case less than CP^{wt} did (Figure 6C). When the accumulation of CPwt and CP^{AAA} when co-expressed with CPIPs analysed by ELISA and compared, CPAAA accumulated to high levels and this was furthermore unaffected by CPIPs (Figure 6D). One possibility was that CP phosphorylation mediates the CP and CPIP interaction and to test this, CP^{wt} or CP^{AAA} were co-expressed with $CPIP^{\Delta 66}$, and co-precipitation of the CPs with $CPIP^{\Delta 66}$ was analysed (Figure 6E). Whereas CP^{wt} co-precipitated with $CPIP^{\Delta 66}$ as before (also III; Figure 5C), CP^{AAA} did not. If CP^{AAA} is incapable of interacting with CPIP, it does not sequester CPIP when expressed *in trans.* This is in agreement with the reduced capacity of *in trans* expressed CP^{AAA} to inhibit gene expression in PVA^{wt} compared to CP^{wt} (Figure 6C). Also, gene expression in PVA^{wt} could only be elevated by CPIP during CP^{wt}, and not CP^{AAA} expression *in trans* (Figure 6 F). This would be expected if CPIP and CP^{AAA} do not interact. Together, the results suggest first, that CK2-mediated phosphorylation of CP is important in connection to virus replication and second, that this phosphorylation event affects to the interaction between CP and CPIP.

Figure 6. CK2-mediated CP phosphorylation affects the interaction between CP and CPIP. A) Gene expression of PVA^{CPmut}, $PVA^{\Delta GDD}$ and PVA^{CPAAA} at 3 DAI. CP^{AAA} is able to inhibit gene expression of $PVA^{\Delta GDD}$ (B) and PVA^{wt} (C) when expressed *in trans*. In C), the y-axis is discontinuous in order to more clearly resolve the differences due to CP^{wt} and CP^{AAA} co-expression with PVA^{wt}. D) Accumulation of CP^{wt} and CP^{AAA} when co-expressed co-expressed with CPIPs and analysed by ELISA 4 days after infiltration. E) Analysis of CP^{AAA} co-precipitation with $CPIP^{\Delta 66}$. Expressed proteins are indicated at the top of the western blot to corresponding lanes. Immunoprecipitation was by anti-myc monoclonal Ab and detection with anti-CP polyclonal Ab. Anti-mouse HRP-conjugated secondary antibody was used to detect the anti-myc antibody present in the immunoprecipitates and the signal from the antibody light-chain (Ab-LC) is shown as a loading reference. F) Effect of CPIP co-expression with CP^{wt} or CP^{AAA} on gene expression in PVA^{wt} at 3 DAI. The y-axis indicates the relative increase in gene expression due to CPIP co-expression where gene expression that is present without CPIP co-expression is adjusted to the value 1.


A
Gene expression of viruses
RLUC 10⁴
30
25
20
15
10
5
PVA CPmut
PVA ΔGDD
PVA CPAAA
B
Inhibition of PVA ΔGDD by CP AAA
RLUC 10⁴
4
3
2
1
no CP
CP wt
CP AAA
C
Inhibition of PVA wt by CP AAA
RLUC 10⁴
70
60
3
2
1
no CP
CP wt
CP AAA
D
CP AAA accumulate unaffected by CPIPs
CP ng / ml
1600
1200
800
400
CP alone
CP + CPIP Δ66
CP + CPIP
CP wt
CP AAA
E
CPIP immunoprecipitation
CPIP CPIP Δ66
CP
CP CPIP Δ66
CP AAA CPIP Δ66
CP
anti-CP
Ab-LC
anti-mouse
F
PVA wt co-expressed with CPs and CPIP
Relative gene expression
10
8
6
4
2
CP alone
CP with CPIP
CP wt
CP AAA

DISCUSSION

This study aimed to identify host factors that participate in the infection cycle of PVA, and to elucidate the function these components play in virus multiplication. The identification of host factors was undertaken by developing an affinity-purification method for the isolation of viral RNP complexes. Components of these RNP complexes were then identified. For functional analysis of the host proteins, a sensitive quantitation method was developed and used to analyse changes in viral gene expression arising from a range of manipulations related to the specific host protein. HSP70 was identified as part of a membrane-associated viral RNP complex, and a function is proposed for HSP70, together with the co-chaperone CPIP, in regulating the activity of potyviral CP during virus replication. In addition, evidence supporting a role for CK2 in regulating the CPIP and CP interaction is presented.

5.1. Identification of host factors – an affinity-purification approach

The identification of host factors that affect and associate with viruses during infection has been approached in several ways. In the present study, purification of viral RNP complexes was accomplished by manipulating the viral genome to express some of its cistrons as affinity tag fusions. This approach has been previously adopted for potyviruses, and HC-pro, 6K1, 6K2 and CP have all been successfully expressed from their native position in the genome as affinity tag–fused versions without disrupting virus infectivity (Restrepo-Hartwig and Carrington, 1994; Blanc et al., 1999; Arazi et al., 2001; Waltermann and Maiss, 2006). These studies, however, did not report on the purification of viral protein complexes. Affinity tags have also been applied for viral protein complex purification by fusing tags to individual viral proteins in different heterologous systems (Mayer et al., 2005; Serva and Nagy, 2006). Manipulation of sequences encoding for tags directly in the genome has several advantages. One important aspect to consider is that the potyviral genome is polycistronic, with individual proteins being produced as part of a polyprotein. Regulated processing of the polyprotein will give rise to products of different composition that may be crucial in determining the fate and function of the polypeptide. This is clearly exemplified by the 6K2 protein that targets VPg, which otherwise localizes to the nucleus, to the ER. Therefore, direct manipulation of the viral genome introduces a tagged version of the protein into the correct polyprotein environment. The existence and function of different polyprotein intermediates is common, and for potyviruses, several polyprotein intermediates are believed to exist and carry out specific tasks during infection (Restrepo-Hartwig and Carrington, 1992; Restrepo-Hartwig and Carrington, 1994; Martin et al., 1995; Schaad et al., 1997a; Merits et al., 2002). In this study, VPg was detected from both plant total protein, and when purified from either total protein or membrane-enriched samples, all derived from infected plants.

The presence and abundance of differently sized VPg proteins varied between the different samples, and numerous VPg forms were detected in the membrane-derived sample, indicating that there is spatial specificity for different polyprotein intermediates.

A genome-wide screen was carried out for PVA where the effect of 15 bases random insertions into the genome on replication was assessed (Kekarainen et al., 2002). This showed that over 70% of a total 1125 random insertions did not disrupt replication, indicating that small genomic insertions are generally well tolerated, at least by PVA. The present study, together with the references mentioned about genomic insertions for potyviruses, indicates that small affinity tags can readily be inserted into the genome of potyviruses and subsequently used for viral protein isolation from infected plants. The purified samples can be used to analyse the tagged protein itself, including its possible post-translational modifications, and also for the analysis of protein complexes, both exemplified by this study. At present, numerous affinity tag based systems are available for protein purification, and the choice of a particular system can depend on the specific application (Terpe, 2003). One important aspect to bear in mind is that when virus-infected plant material is used for the purification of viral proteins and complexes, the infected cells are not in synchrony. This will result in the detection of proteins and protein complexes derived from cells that are in early to late stages of the infection cycle, and also from cells recovering from infection. Therefore, this type of approach will result in a highly complex sample, in which specific complexes are possibly only minor constituents. To reduce this complexity and improve the yield of specific complexes, cell culture approaches like that described for TMV, in which transgenic cell lines expressing the viral genome under an inducible promoter are used to initiate more synchronised infections (Dohi et al., 2006), could prove fruitful.

5.2. Characterization of viral gene expression

After identifying candidate host proteins that possibly affect virus infection, characterization of their function in virus infection presents major challenges. Several commonly applied methods exist for regulating the expression of candidate host factors, including RNA-silencing and over-expression using strong promoters, but to analyse the impact of these manipulations on virus infection, a quantitative measure of viral activity is required. There are essentially two viral parameters that can be used to quantitate viral infection, either viral genomic RNA or viral proteins. Here, a sensitive and easy-to-use method for the quantitation of viral gene expression was established. *Agrobacterium*-mediated transformation of plants was reported over two decades ago as a method to initiate viral infections, a process described as agroinfection (Grimsley et al., 1986). As a routine method, infiltration of *Agrobacterium* is relatively easy and cost efficient. RLUC is a sensitive reporter gene (Mayerhofer et al., 1995), and has been used previously to quantitate virus replication, at least for *Semliki forest virus* (SFV) (Pohjala et al., 2008). The infection assay presented in this

study was able to discriminate between replicating viruses and a non-replicating mutant. Gene expression of both replicating viruses and the non-replicating mutant was in the detectable range during early infection (2 DAI). By 2 DAI, RNA multiplication was occurring because both PVA^{wt} and PVA^{CPmut} began to diverge from $PVA^{\Delta GDD}$, and by 3 DAI, cell-to-cell movement had taken place as indicated by further divergence of PVA^{wt} from PVA^{CPmut}. Including non-replicating $PVA^{\Delta GDD}$ in the assay has benefits; in addition to setting a reference point for replicating viruses, its translation can still respond in a virus-specific manner as exemplified by co-expression of viral CP. Despite that $PVA^{\Delta GDD}$ does not replicate and hence differs from replicated genomes at least by carrying a 5´-terminal cap structure instead of VPg it still possesses RNA elements that regulate translation and replication. The sensitivity of this system allows these aspects to be addressed. To summarize, this infection assay allows quantitation of gene expression associated with replicating viruses at early infection, and also that of a non-replicating viral genome undergoing only translation. Both these advantages of the technique were crucial in assessing the role of HSP70 and CPIP in PVA infection. Because this assay does not discriminate between changes in translation and transcription for replicating viruses, combining methods for the analysis of viral genome accumulation, e.g. qPCR can be used.

5.3. HSP70 and virus infections

In this project, HSP70 was identified as a component of a purified membrane-associated viral protein complex together with several viral proteins, PABP and viral RNA. HSP70 has previously been shown to localize with membrane structures that are induced during infection by the potyvirus TuMV (Dufresne et al., 2008a). These membrane structures also co-localize with PABP, eIF4E, eEF1A and double-stranded viral RNA (Beauchemin and Laliberte, 2007; Beauchemin et al., 2007; Thivierge et al., 2008; Cotton et al., 2009). In addition, TuMV NIb interacts with HSP70 (Dufresne et al., 2008a). The similarity in sample composition suggests that the purified membrane-derived viral complex originates from similar structures to those observed during TuMV infection. HSP70 also associates with membrane-localized replication proteins of *Tomato mosaic virus* (ToMV) (Nishikiori et al., 2006), is a component of the membrane-associated *Cucumber necrosis virus* (CNV) replicase (Serva and Nagy, 2006), is required for the assembly of the *Tomato bushy stunt virus* (TBSV) replicase at membrane sites (Wang et al., 2009), and is also required for *in vitro* assembly of the TBSV replicase (Pogany et al., 2008). During replication of *Flock house virus* (FHV), different HSP70s have opposite roles in that they may either promote or reduce virus replication (Weeks et al., 2010). Furthermore, FHV replication was unaffected in most of the single-chaperone deletion yeast strains used in the study except for those belonging to the JDP family. In particular, the JDP YDJ1 is required for FHV replication and replication complex assembly at membranes (Weeks and Miller, 2008). During infection with the plant virus BMV, YDJ1 also functions in the assembly of the replication complex at membranes that carry out minus-strand synthesis (Tomita et al., 2003). Hence, HSP70 and co-

chaperones are frequently associated with the formation of replication complexes at membranes. JDPs have also been found to interact with several viral movement proteins (MPs), including NSm of *Tomato spotted wilt virus* (TSWV) (Soellick et al., 2000), CP of PVY (Hofius et al., 2007), and Pc4 of *Rice stripe virus* (RSV) (Lu et al., 2009), and a role for HSP70 in virus movement processes has previously been discussed (Boevink and Oparka, 2005). Indeed, down-regulation of *HSP70* impaired systemic movement of PVA in this study. Both heat shock and HSP70 over-expression facilitate infection and systemic movement of a range of plant viruses, whereas down-regulation of HSP70 causes the opposite (Chen et al., 2008), supporting a role for HSP70 in virus movement. Even though the impairment of systemic movement of PVA due to HSP70 down-regulation, and the interaction of CPIP with CP, suggests the involvement of HSP70 in potyvirus movement, CPIP is proposed to function in regulating CP in a process primarily related to replication, but not necessarily excluding movement. The multiple roles of HSP70, and the extensive co-chaperone network present within the cell, suggests that chaperones are likely to be involved in multiple viral processes, as exemplified by the targeted study on chaperone-requirement during FHV infection (Weeks et al., 2010).

5.4. CP-mediated inhibition of viral gene expression

Plant viral genomes are involved in four different activities during infection; translation, replication, particle formation and movement. By expressing CP *in trans*, viral gene expression was efficiently suppressed in both replicating and non-replicating PVA. Furthermore, gene expression from replicating PVA was also influenced by CP *in cis*. During infection, CP is produced *in cis* from a particular viral genome, but may act *in trans* on other viral genomic RNAs present within the same cell or RC. Because CPs function to provide a protective shell for the viral genome, it is reasonable to postulate that the presence of CP influences viral processes like genome translation and replication. CPs can also inhibit translation in other plus RNA viruses (Shimoike et al., 1999; Karpova et al., 2006; Yi et al., 2009a; Yi et al., 2009b). BMV, one of the best known plant viruses, displays versatile regulation of viral processes via its CP (Yi et al., 2009a; Yi et al., 2009b). CP can bind to different RNA elements active in both translation and replication, and thereby suppresses their function. BMV CP is produced from a subgenomic RNA at a later stage of infection, which is a common strategy amongst viruses for CP production. Hence, CP presumably functions to shut down translation and replication, with an associated shift towards virus particle assembly. Production of CP via subgenomic RNAs ensures temporal control of CP appearance, but potyviruses appear not to employ this strategy and instead are believed to synthesize their proteins in equimolar amounts as part of a large polyprotein. This results in the presence of CP during the early stages of cellular infection, and it is therefore likely that regulatory mechanisms exist to prevent premature CP-mediated interference with viral translation and replication. One such mechanism could be CP degradation via chaperone-mediated ubiquitination as suggested by this study.

5.5. CK2 regulates CP association with CPIP

The results presented in this study suggest that CK2-mediated phosphorylation regulates the association of CP with CPIP and hence, that CK2 would function in the CPIP-mediated HSP70-pathway. A study of the virus particle indicated that the CK2-site is not surface-exposed, and instead this site is believed to reside in close proximity to the genomic RNA within the particle (Baratova et al., 2001). CK2-mediated phosphorylation was shown to decrease the affinity between CP and RNA, and it was postulated that CK2-mediated phosphorylation regulates the association of CP with viral RNA, where phosphorylation of CP prevents RNA-association, possibly during replication (Ivanov et al., 2003). Mutant CP^{AAA} can be assumed to retain a relatively native protein structure because it is capable of forming virus like particles and structural comparison of CP^{AAA} to that of native CP using CD-spectroscopy revealed no apparent changes in the secondary protein structure (Ivanov et al., 2003). Because the CK2 target-domain appears critical for CP to interact with CPIP, it could be that this is the actual domain through which CP interacts with CPIP. Also, this suggests that CPIP interacts with CP that is not associated with viral RNA, supported by the ability of also $CPIP^{\Delta 66}$ to enhance viral gene expression during expression of CP *in trans*. It is hard to imagine that dysfunctional $CPIP^{\Delta 66}$ would be able to do this by acting on CP already associated with the viral genome, and still be affected by the non-surface exposed CP^{AAA} mutation. Instead, a working hypothesis has been generated in which phosphorylation of CP by CK2 occurs during replication. Whether CP phosphorylation functions up- or down-stream of CPIP, and if this event promotes or antagonizes CPIP association remains to be determined.

5.6. Coupled viral translation and replication

In this study, a translational activity connected to replication was observed, referred to as RAT. This translational activity was observed because it was prone to inhibition by CP *in cis*, an effect that could be reversed by CPIP. Furthermore, *in cis* inhibition of viral gene expression by CP and its reversal by CPIP was only observed for replicating virus. Hence, the term replication-associated translation (RAT) was used. It has been suggested that translation and replication are coupled during plus RNA virus multiplication, based on the differential translation of viral RNAs depending on whether they are delivered *in cis* or *in trans* (Mizumoto et al., 2006; Sanz et al., 2007; Sanz et al., 2009), and the selective *in trans* complementation of different mutant viruses with functional components (Novak and Kirkegaard, 1994; Schaad et al., 1996; Yi and Kao, 2008). For *Poliovirus* (PV), translation, replication and membrane-modification are all required to maintain RCs (Egger et al., 2000). These reports collectively suggest a protected replication environment (or RCs) to which factors have limited *in trans* access. RAT was apparently also protected from CP-mediated suppression *in trans*, but not *in cis*. This supports the idea of coupled translation and replication, and replication protected from factors supplied *in trans*. Because some replication-

associated mutations, like those in the conserved GDD-motif, can be complemented *in trans* (Li and Carrington, 1995; Wang and Gillam, 2001; Hafren and Makinen), successive complementation could depend on what process is defective, and whether or not the endogenous mutation act in a dominant-negative fashion by for example preventing access of a trans factor to a protein complex. Also, the accessibility of *in trans* supplied factors to RCs can alter in temporality. Recent studies have indicated that individual replication-associated vesicles (RCs) of potyviruses arise mainly from single viral genomes, and that vesicle-associated translation occurs (Cotton et al., 2009). Taken together, these studies suggest a model whereby translation products of plus RNA viral genomes initiate assembly of RCs *in cis*. On the other hand, a replication-assay based on heterologous expression of viral proteins that multiply a viral replicon is fully functional *in trans* (Panavas and Nagy, 2003), and also minus-strand RNA synthesis by potyviruses can occur *in trans* (Teycheney et al., 2000). The RAT described in this study is proposed to represent vesicle-associated translation, and because RAT is absent in the replication-deficient $PVA^{\Delta GDD}$ it possibly requires, and occurs only after, RNA synthesis.

5.7. CPIP promotes replication by regulating CP

Experiments to distinguish between the involvement of CPIP in viral translation, replication, or assembly/movement are complicated by the fact that these processes are closely coupled to each other during plus RNA virus infection as discussed above (Nugent et al., 1999; Khromykh et al., 2001; Venter et al., 2005; Annamalai and Rao, 2007). However, a model is proposed in which CPIP and HSP70 function during virus replication to regulate CP, and thereby prevent CP from causing early cessation of replication (III; Figure 7). In addition, CK2 is proposed to affect CP association with CPIP. A number of arguments support a role for CPIP in potyvirus replication, as described below.

1) Down-regulation of HSP70 inhibited early gene expression in replicating PVA^{wt} that carried the native CP, but not PVA^{CPmut}. The inhibition was apparent at the earliest times of detectable viral gene expression. Functional CPIP is only required by PVA^{wt}, because CP^{mut} is unable to inhibit viral gene expression.

2) Co-expression of $CPIP^{\Delta 66}$ caused a major reduction in viral gene expression at the earliest time points of detectable gene expression in the infection assay, a time point at which movement-related gene expression would not be expected. In $CPIP^{\Delta 66}$-transgenic plants, systemic potyvirus accumulation in leaves was reduced to a similar extent, and no striking delay in systemic appearance was apparent.

3) During *in trans* expression of CP, RAT was enhanced by CPIP co-expression. However, RAT of PVA^{wt} was substantially lower than that of the movement-defective PVA^{CPmut} when co-expressed with $CPIP^{\Delta 66}$, and the difference in gene expression between PVA^{wt} and PVA^{CPmut} was similar to that caused by the presence of $CPIP^{\Delta 66}$ during virus infection, as described in 2) above. CP^{mut} does not interact with CPIP, nor does it inhibit viral gene expression of replicating or non-replicating viruses when

expressed *in trans*. This suggests that PVA^{CPmut}, does not require CPIP and the effects observed in gene expression due to co-expression of CPIPs relates to their action on *in trans* CP only. CPIP was required for PVA^{wt} to reach the same level of gene expression as PVA^{CPmut}.

4) CPIP promoted CP modification by ubiquitin followed by its degradation, suggesting its involvement in a process opposite to virion assembly and movement.

5) A CP mutation affecting the ability of CK2 to phosphorylate CP (PVA^{CPAAA}) resulted in impaired replication. The same mutation also disrupted the ability of CPIP and CP to interact.

Potyvirus-induced cytoplasmic vesicles were initially reported for TEV (Schaad et al., 1997a). Subsequently, studies mainly using TuMV have contributed to our knowledge about the nature of these vesicles. In a recent study, viral translation was proposed to occur in the virus-induced structures that are thought to represent RCs (Cotton et al., 2009). It appears plausible that RAT occurs within these RCs, as it appears to be protected from external interference. Indeed, several proteins that play a role in translation were localized to the presumed RCs, including eIF4E, eEF1A and PABP, possibly supporting the occurrence of translation within RCs. CP was absent from the RCs and, instead, was localized adjacent to them (Cotton et al., 2009). This does not contradict the idea that CP is regulated within the RCs via degradation as this would cause its absence in RCs. If CP is degraded in RCs, production of CP needs to be accomplished separately. Alternatively CP could be transferred from within the RC and distributed to adjacent sites where virus particle assembly is taking place. The latter scenario could be considered as a more economic use of CP, and would assign a role for CPIP in both RAT and movement.

5.8. Temporal regulation of viral gene expression

Many plant plus RNA viruses employ subgenomic (sg) RNAs for the production of both coat and movement proteins that are required in high quantities at later stages of infection (Miller and Koev, 2000). By this, they avoid a competition for genomic RNA between the proteins engaged in replication and encapsidation or movement. However, viruses of the picorna-like supergroup, including the family *Potyviridae* do not produce sgRNAs and instead commonly express their proteins as part of a polyprotein. This results in the appearance of also structural proteins during early infection stages, which can associate with the genomic RNA and thereby counteract both translation and replication. Therefore, if CPIP and HSP70 down-regulate CP in connection to replication, as can be speculated based on the presented results, this represent a different strategy by a virus to regulate temporal CP accumulation. Evidently CP is needed, at later stages of infection for particle assembly, and multiple ways to avoid CP degradation by this system can be imagined, including spatial separation of CP-production and the regulator CPIP.

5.9. Mechanisms of virus resistance

Studies on the mechanisms of plant infection with viruses and other pathogens aim to broaden our fundamental understanding of pathogen infection and thereby discover mechanisms that can be engineered to produce pathogen resistance. This study identified three different mechanisms of resistance. First, *in trans* expression of viral CP efficiently suppressed viral gene expression of PVA. Plants transgenic for potyviral CP sequences resist infection with the parent virus, and this resistance appears to be mediated through RNA silencing, e.g., (Sivamani et al., 2002; Higgins et al., 2004; Hily et al., 2004; Lindbo and Dougherty, 2005). The mechanism behind the CP-mediated repression of viral gene expression in this study was based on the CP protein because CP and CP^{mut} induced very different responses despite their almost identical RNA sequence. Also, the *in trans* expressed CPs accumulated in plants, and over-expression of CPIP suppressed this CP-mediated inhibition of viral gene expression. Probably the most studied plant virus is TMV, and here CP-mediated resistance also operates via the CP protein (Beachy, 1999). Similarly, CP-mediated resistance against PVX is not considered to be attributable to gene silencing (Bazzini et al., 2006). In some studies, CP-transgenic plants show expression of CP and offer a broader range of resistance against potyviruses than plants transgenic for other viral proteins (Maiti et al., 1993). This could reflect resistance operating at the protein level, which is supported by the present study. *In trans* expression of CP had at least two consequences: it suppressed translation of the viral genome and sequestered the CP-interacting host protein CPIP that is required by the virus for normal infection. In the case of TMV CP-mediated resistance, the transgenic CP has also been speculated to sequester a host component required for infection (Beachy, 1999). Second, the presence of CP^{mut} interfered with PVA infection, and CP^{mut} may exhibit a dominant-negative effect on virus movement *in trans*. Similarly mutated CP of PPV expressed in transgenic plants has been reported to confer resistance towards infection of PVY and *Chilli veinal mottle virus* (ChiVMV) (Varrelmann and Maiss, 2000). In this study, the transgenic CPs accumulated, but it was not reported whether resistance was due to CP protein or RNA silencing. Third, infection with PVY was attenuated in plants transgenic for $CPIP^{\Delta 66}$ (Hofius et al., 2007). The present study broadened the spectrum of this resistance to PVA, TVMV and TEV, and provided insight into the mechanism by which $CPIP^{\Delta 66}$-mediated resistance operates. $CPIP^{\Delta 66}$ and CP^{mut} do not interact and mediate resistance due to interference on two different aspects of infection, i.e., replication and movement. Therefore, transgenic plants where both $CPIP^{\Delta 66}$ and CP^{mut} are combined could possibly result in broad resistance against potyviruses, because interference by CP^{mut} appears not to be virus strain-specific (Varrelmann and Maiss, 2000).

CONCLUSIONS AND FUTURE PERSPECTIVES

The knowledge obtained, and questions that have arisen from this study are summarized in Figure 7. One of the interesting questions is how potyviruses produce their CP for virus particle assembly. If CP is degraded via an HSP70-dependent pathway during infection and RAT, this would mean that a second, separable translational activity is needed for producing virus particle CP or that the degrading system is shut down or inadequate. And how is the inhibitory effect of CP on virus translation regulated here? Several studies exist that report virus particle assembly occurring in connection, i.e., *in cis*, with replication (Nugent et al., 1999; Khromykh et al., 2001; Venter et al., 2005; Annamalai and Rao, 2007). CPIP is proposed to be required during replication to counteract inhibition of viral gene expression by CP. One possibility, apart from CP degradation, is the translocation of CP from the vicinity of replication to sites of assembly. This site could reside outside the RC as suggested by the study by Cotton et al., (2009). It could also be that the assembly process requires organization within RCs, where RNA replication, translation and virus particle assembly occur simultaneously. If so, it could be expected that CP would require coordinated allocation to assembly-destined genomes in such an environment. It would not be surprising to find that a number of viral proteins require similar regulation to CP in order to maintain the multiple functions within the RC if translation is occurring at this site. Also, CK2-mediated phosphorylation of CP appears to regulate its association with CPIP and hence, its degradation. Whether CP phosphorylation promotes or diverts a CP and CPIP association remains to be established. Because mutant CP^{AAA} is clearly unavailable for CPIP-mediated degradation, it possibly represents a powerful mutant to study this degradation pathway in more detail.

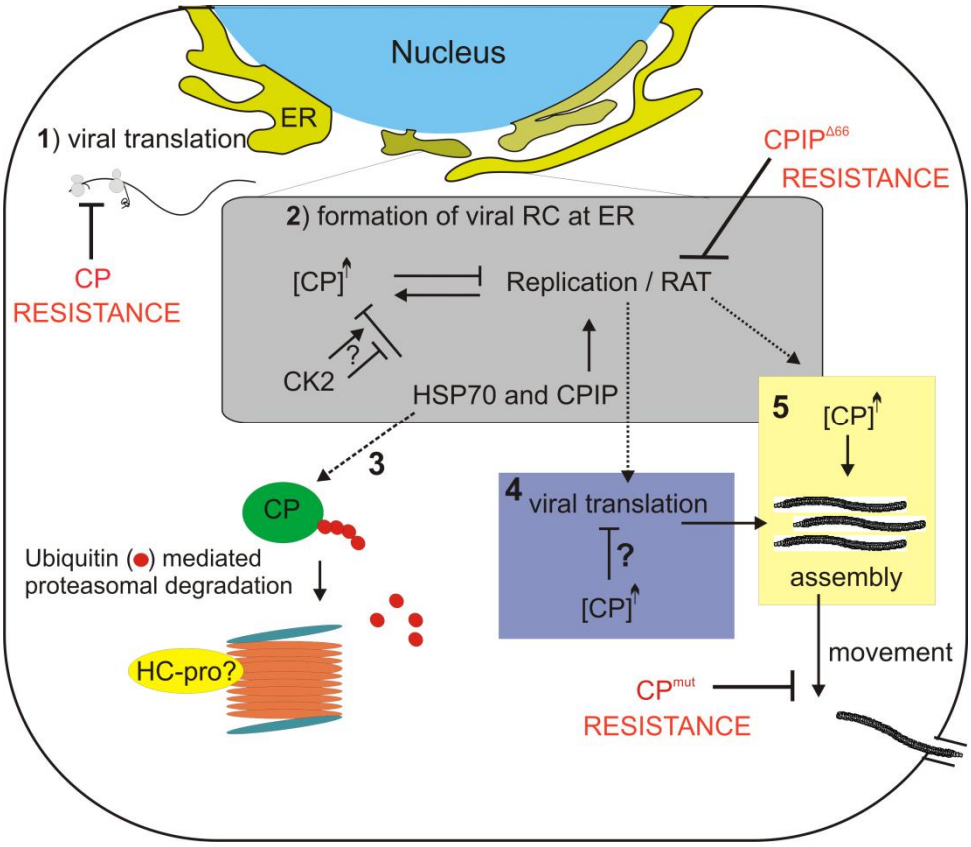


Figure 7. CP during plant infection by potyviruses. Initial translation of the viral genome is required to produce viral proteins used for RNA replication (1), leading to the formation of replication complexes (RCs) (2). RAT is proposed to reside within the RC. RAT or replication can be inhibited by CP, and HSP70, together with CPIP, regulates the fate of CP, suppressing its potential to inhibit viral gene expression. CK2 potentially affects the interaction between CP and CPIP. What is the destiny of CP after association with HSP70? CP may be degraded via HSP70-mediated ubiquitin modification (McDonough and Patterson, 2003) and the proteasome pathway (3). Another potyviral protein, HC-pro, is known to associate with proteasomes during infection, and the morphology of the proteasome is altered during infection (Ballut et al., 2005). Is there a connection between these events? If CP produced during RAT is degraded, alternative mechanisms for virus particle CP production are required, possibly spatially distinct (4). How is this organized to avoid premature feedback inhibition by CP? Also, CPIP could possibly mediate translocation of CP to sites of virus particle assembly (5). The aspects of CP-related resistance are pointed out at the level of translation, RAT and/or replication and movement.

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