HSP70 and Its Cochaperone CPIP Promote Potyvirus Infection in *Nicotiana benthamiana* by Regulating Viral Coat Protein Functions

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This study demonstrates that heat shock protein 70 (HSP70) together with its cochaperone CPIP regulates the function of a potyviral coat protein (CP), which in turn can interfere with viral gene expression. HSP70 was copurified as a component of a membrane-associated viral ribonucleoprotein complex from *Potato virus A*-infected plants. Downregulation of HSP70 caused a CP-mediated defect associated with replication. When PVA CP was expressed in trans, it interfered with viral gene expression and replication-associated translation (RAT). However, CP produced in cis interfered specifically with RAT. CPIP binds to potyviral CP, and overexpression of CPIP was sufficient to restore RAT inhibited by expression of CP in trans. Restoration of RAT was dependent on the ability of CPIP to interact with HSP70 since expression of a J-domain mutant, CPIP<sup>ΔJ</sup>, had only a minor effect on RAT. CPIP-mediated delivery of CP to HSP70 promoted CP degradation by increasing its ubiquitination when assayed in the absence of virus infection. In conclusion, CPIP and HSP70 are crucial components of a distinct translation activity that is associated with potyvirus replication.

INTRODUCTION

Plus-stranded RNA viruses induce host membrane rearrangements to assemble viral replication complexes (RCs) (Miller and Krijnse-Locker, 2008). Within these membrane-associated RCs, viral proteins, together with host factors, direct viral RNA synthesis. The requirement for host factors in viral multiplication has been firmly established and extensively reviewed (Ahlquist et al., 2003). Because host factors involved in viral multiplication represent potential targets for virus control, their identification and functional characterization is one of the major frontiers in virus research. Different approaches have been taken to identify host proteins important for viral replication. Studies based on forward genetic screens have demonstrated that a large number of host factors affect viral replication (Lellis et al., 2002; Lu et al., 2003; Panavas et al., 2005). The yeast two- and three-hybrid systems have identified direct protein interactions between host and viral factors (Bliggin et al., 2003), and purification of viral protein complexes from infected cells has also been used to identify associated host factors (Serva and Nagy, 2006).

HSP70s represent a conserved family of cellular chaperones. These proteins function as core components in the cellular chaperone network and participate in a wide variety of processes, including the folding of newly synthesized proteins, refolding of misfolded or aggregated proteins, translocation of organellar and secretory proteins, protein complex assembly or disassembly, and protein degradation (Mayer and Bukau, 2005). The mechanisms by which HSP70s operate contribute to their functional diversity. First, almost all proteins have the potential to be HSP70 substrates because the amino acid sequence recognized by HSP70 is statistically very frequent (Rudiger et al., 1997). Second, ATP hydrolysis is required for HSP70s to interact with their substrates (Szabo et al., 1994). The intrinsic ATPase activity of HSP70s is weak, but association with cochaperones, mainly J-domain proteins (JDPs), stimulates the ATPase activity and enables HSP70 to interact with substrate proteins (Fan et al., 2003). Thereby, JDPs regulate the function of specific substrate proteins by delivering them to HSP70s.

Considering the fundamental role of HSP70s in cell biology, it is not surprising that they are tightly associated with viral infections (Mayer, 2005). The functions of HSP70s during viral infection extend beyond that of merely folding viral proteins; HSP70s appear to be involved in regulating both the viral infection cycle and host physiology. HSP70s together with their JDP partners have been connected with various viruses and with several processes in the viral infection cycle. These include virus replication (Tomita et al., 2003; Serva and Nagy, 2006; Weeks and Miller, 2008), assembly or disassembly of the viral capsid (Satyanarayana et al., 2000; Ivanovic et al., 2007; Li et al., 2009), and protein translocation or membrane association of viral proteins (Lambert and Prange, 2003; Wang et al., 2009).

Infections of different plant viruses have been reported to induce the expression of HSP70 (Aranda et al., 1996; Whitham et al., 2003). Potyvirus infection appears to upregulate HSP70...
through a cytoplasmic unfolded protein response (Aparicio et al., 2005; Sugio et al., 2009). Additionally, induction of HSP70 by Tobacco mosaic virus (TMV) is associated with an increased amount of aggregated viral coat protein (CP) (Jockusch et al., 2001). HSP70 colocalizes with virus-induced membrane structures and interacts with the RNA-dependent RNA polymerase of Turnip mosaic virus (TuMV) (Dufresne et al., 2008), suggesting that HSP70 plays a role in potyvirus replication. Two JDPs have, to our knowledge, been reported to function during potyvirus infections. P58IPK interacts with the helicases of both Tobacco etch virus (TEV) and TMV and appears to play a role in plant survival during infection (Bilgin et al., 2003). The JDP CPIP interacts with the CP of Potato virus Y (PVY), and the disruption of CPIP’s ability to interact with HSP70 results in effective resistance to the virus (Hofius et al., 2007).

In this study, we aimed at identifying host factors involved in Potato virus A (PVA) infection. Using an affinity purification approach, we identified HSP70 as a component of a membrane-associated viral ribonucleoprotein (RNP) complex. When the function of HSP70 was further examined, we were able to demonstrate a role for HSP70, together with the cochaperone CPIP, in regulating functions of the viral CP. At the same time, we showed that replication and translation are coupled during PVA infection.

RESULTS

HSP70 as a Component of a Purified Viral Protein Complex

Because we were interested in finding host proteins involved in virus replication, we set out to purify viral RNP complexes from infected plant tissues and identify any associated host proteins. To that end, the genome of PVA was engineered to express the polymerase NiB (PVANIb-SIII) and the VPg (PVAVPg-SIII) as strep-III affinity tag (SIII-tag) fusion proteins in separate infectious cDNAs (icDNA) (Figure 1A). These viral clones infected plants similar to wild-type PVA (wt) as judged by the expression of virus-encoded green fluorescent protein (GFP). To isolate the RNP complexes, we prepared heavy membrane fractions, which harbor the RCs of potyviruses (Martin and Garcia, 1991; Schaad et al., 1997), from Nicotiana benthamiana plants infected with either PVA wt, PVANIb-SIII, or PVAVPg-SIII, followed by affinity purification of SIII-tagged proteins. When the purified samples were analyzed by SDS-PAGE and silver staining, several protein bands were detected in the SIII-tagged samples but not in the PVA wt control (Figure 1B). Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used to identify the proteins. Among these were two viral proteins, NiB and Nla, and the host protein HSP70. The 17 identified peptides covered 31% of the Nicotiana tabacum HSP70-3 amino acid sequence. We used immunodetection with antisera to HSP70 and viral components to verify the specificity of the purification protocol (Figure 1C). Three specific bands were detected by the HSP70/HSC70 antibody in both tagged

Figure 1. HSP70 as a Component of a Purified Viral Protein Complex.  
(A) Diagram of the reporter- and SIII-tagged PVA. The position of the SIII-tag in the sequence of wild-type PVA VPg and NiB is shown.  
(B) Affinity purification of membrane-associated viral RNP complexes. A silver-stained SDS-PAGE gel of column eluates from strep-tactin sepharose is shown. Numbers indicate the positions of proteins identified by LC-MS/MS analysis as follows: (1) HSP70-3, (2) NiB, and (3) Nla. The masses of the molecular mass markers are indicated on the left.  
(C) The specific presence of LC-MS/MS identified proteins in the column eluates of NiB SIII and VPg SIII samples was verified by immunoblot analysis. Protein species are indicated on the right and molecular mass markers on the left.  
(D) The presence of viral RNA in the column eluates of NiB SIII and VPg SIII samples was analyzed by RT-PCR with the expected product being 350 bp. RNA samples from SIII purifications were incubated prior to PCR with (+) or without (−) reverse transcriptase (Moloney murine leukemia virus [M-MLV]-RT) either in the presence of a (−)-strand or a (+)-strand-specific primer. PVA icDNA was used as a positive control and water as a negative control in the PCR reactions.
samples. The doublet band at 70 kD migrated similarly to HSP70-3 identified by LC-MS/MS analysis. In addition to Nb and Nla, Vpg was also present in both SIII-tagged samples. Several additional proteins migrating between Nla and Vpg were detected by the anti-Vpg antibody. These proteins showed an apparent SIII-tag-dependent shift in SDS-PAGE migration, verifying that they indeed were Vpg derivatives. Some of these Vpg species were not observed when Vpg was purified from plant total protein (Hafren and Makinen, 2008) and could represent membrane-associated polyprotein intermediates or posttranslationally modified forms of the protein.

RT-PCR analysis was used to detect viral RNA in the purified samples (Figure 1D). Viral RNA was present in both SIII-tagged samples. The analysis also indicated that viral RNA of both positive and negative polarity was present. Because all detected components (i.e., HSP70, Nb, Nla, Vpg, and viral RNA) were represented in the NbSIII and VpgSIII samples, it seems probable that the same molecular complex was purified regardless of which of the two viral proteins was tagged.

**Downregulation of HSP70 Delays Initiation of PVA Infection**

To investigate the role of HSP70 in PVA infection, we used virus-induced gene silencing (VIGS) to downregulate HSP70 in *N. benthamiana*. Immunodetection showed that the level of HSP70 protein was reduced in silenced plants (Figure 2A). The plants showed a similar dwarfed and crinkled phenotype as previously demonstrated in *HSP70* silenced plants (Wang et al., 2009). Systemic infection of PVA was impaired in HSP70-silenced plants (TRV:HSP70) compared with empty VIGS vector plants (TRV:00) (Figure 2B). By 5 d after infection (DAI), all TRV:00 plants (n = 12) exhibited a systemic PVA infection as detected by the *Renilla* luciferase (RLUC) marker gene expressed from the viral genome. By contrast, by 5 and 6 DAI, only three out of 11 TRV:HSP70 plants had supported systemic accumulation of PVA.

Next, local PVA infection in HSP70-silenced plants was studied using a recently developed infection assay (Eskelin et al., 2009). The assay is based on quantitation of PVA gene expression by measuring virally expressed RLUC and normalizing the value with an internal firefly luciferase (FLUC) control. Viral gene expression was initiated by infiltrating *Agrobacterium tumefaciens* carrying PVA:RLUC icDNA as a transgene into plant leaves. Three different viral constructs were used: PVAwt, PVA{\textsuperscript{CPmut}}, which replicates but cannot undergo cell-to-cell movement, and PVA{\textsuperscript{AGDD}}, which translates the viral genome but is incapable of replication. When CP of TEV was mutated correspondingly as in PVA{\textsuperscript{CPmut}}, TEV could replicate similarly as the wild-type virus but it was deficient in cell-to-cell movement (Dolja et al., 1994; Varrelmann and Maiss, 2000). TEV carrying a GDD-to-VNN mutation in Nb was used in these experiments to report for gene expression from a replication-deficient viral genome. PVAwt and PVA{\textsuperscript{CPmut}} are replicating viruses and consequently exhibit amplified RLUC expression in initially infected cells through viral genome replication. PVAwt can further amplify RLUC expression via cell-to-cell movement since infection is not initiated uniformly from all cells with the nonsaturating concentration of *Agrobacterium* used. Using this assay, we observed that expression of both PVA-derived RLUC and virus-independent FLUC was reduced by ~90% at 4 DAI in TRV:HSP70 plants compared with TRV:00 plants (Figures 2C to 2E). Interestingly, initiation of PVAwt gene expression showed a transient delay compared with PVA{\textsuperscript{CPmut}} and PVA{\textsuperscript{AGDD}} in TRV:HSP70 plants (Figure 2F). Compared with PVA{\textsuperscript{AGDD}}, the PVAwt delay implied a role for HSP70 in early replication events. Because replicating PVA{\textsuperscript{CPmut}} was unaffected, a hypothesis wherein CP can interfere with replication-associated gene expression was put forward. Previously, an HSP70 cochaperone, CPIP, was reported to interact with the CP of PVY (Hofius et al., 2007). Whereas CPIP was proposed to function in movement, this coupling of HSP70, CP, and replication raised the possibility that CPIP could function in the context of viral replication.

Quercetin is a flavonoid that can be used to inhibit HSP70 gene expression (Hosokawa et al., 1990) and has been applied to downregulate HSP70 in *N. benthamiana* to study the role of HSP70 in the multiplication of a plant virus (Wang et al., 2009). We used quercetin as an alternative method to downregulate HSP70 in the infection assay (Figures 2G and 2H). Quercetin inhibited viral gene expression by ~50% for all viruses at 2 DAI compared with control plants, whereas expression of FLUC was unaffected (Figure 2I). This level of inhibition remained for PVA{\textsuperscript{CPmut}} and PVA{\textsuperscript{AGDD}} at 4 DAI. By contrast, gene expression of PVAwt was reduced ~90% by 4 DAI. Collectively, these results demonstrated that HSP70 is a functional host component of PVA infection.

**Exogenous CP Expression Inhibits Both Viral Translation and Replication**

The results from downregulating HSP70 suggested that CP interferes with viral replication in a HSP70-associated manner. To investigate further the role of PVA CP in replication, we first expressed CPwt (pro35S:CPwt) with PVAwt, PVA{\textsuperscript{CPmut}}, and PVA{\textsuperscript{AGDD}} in the infection assay. Coexpression of CPwt reduced viral gene expression from all viruses (Figures 3A to 3C). Because coexpression of CPwt inhibited gene expression of nonreplicating PVA{\textsuperscript{AGDD}}, expression of exogenous CP inhibited viral gene expression independent of replication. By contrast, coexpression of PVA{\textsuperscript{CPmut}}, the CP mutant that is the same as that expressed from PVA{\textsuperscript{CPmut}}, interfered less with viral gene expression. Expression of CPwt and CPmut was verified by immunodetection (Figure 3D). Since the protein products were present and CPmut differs from CPwt by only six nucleotides, we concluded that the mechanism of inhibition was based on the native CP protein and not CP gene-mediated RNA silencing. The expression of FLUC was unaffected by CP (Figure 3E), indicating that CP-mediated inhibition of gene expression was virus specific. When CPmut was coexpressed with PVAwt, viral gene expression was initiated normally but repressed at later times appearing similar to that of PVA{\textsuperscript{CPmut}}, whereas expression continued to develop in control plants (Figures 3A and 3B). This indicated that CPmut could interfere with wild-type infection possibly by debilitating cell-to-cell movement when expressed in trans.

Although gene expression of all viruses was inhibited by exogenous CPwt expression, the RLUC activity was comparably 10- to 50-fold higher for PVA{\textsuperscript{CPmut}} than that for PVA{\textsuperscript{AGDD}} and PVAwt, respectively (Figure 3F). There are two possible
explanations: (1) endogenous CPmut originating from PVA CPmut could interfere with inhibition mediated by exogenous CP, or (2) endogenous CP of PVA wt could inhibit gene expression in cis, a process that PVA CPmut would be unable to undergo. To discriminate between these two possibilities, the CP mutant was introduced into PVA DGG, producing PVA DGG + CPmut. When PVA DGG + CPmut was coexpressed with CP wt, its expression was not enhanced compared with PVA DGG (Figure 3F). This showed that elevated gene expression of PVA CPmut was coupled to replication. Taken together, these experiments demonstrated distinct gene expression that was coupled to replication, which will be referred to from now on as replication-associated translation (RAT).

CPIP Antagonizes CP-Mediated Inhibition of RAT

Because PVA wt RAT could be inhibited by both exogenous CP expression and transiently with HSP70 silencing, either HSP70 or a functionally linked host factor appeared to be involved in CP-mediated events during PVA replication. We reasoned that this
host factor was sequestered from replication by expression of exogenous CPwt, but not CPmut, and inhibited during HSP70 silencing, both resulting in the selective repression of PVAwt carrying the native CP. Next, we wanted to test if the HSP70 cochaperone and CP-interacting CPIP was functioning in RAT. First, we examined how expression of the CPIP J-domain mutant (CPIP<sub>D66</sub>) affected PVA wt gene expression in the infection assay. CPIP<sub>D66</sub> is unable to interact with HSP70, but its binding to PVY CP is unaltered (Hofius et al., 2007). Expression of CPIP<sub>D66</sub> had a negative impact on PVA infection at 4 DAI, reducing viral gene expression by 80% compared with control and CPIP-expressing plants (Figure 4A). Overexpression of CPIP did not enhance virus infection compared with control plants. Immuno-detection was used to verify the expression of CPIP and CPIP<sub>D66</sub> (Figure 4B). Systemic accumulation of PVA as well as TEV and tobacco vein mottling virus (TVMV) was evaluated in transgenic CPIP<sub>3466</sub> expressing plants by ELISA (Figure 4C). All viruses showed reduced accumulation similar to that previously observed for PVY (Hofius et al., 2007), demonstrating that CPIP<sub>D66</sub> was able to interfere with viral infection of potyviruses in general.

We asked whether CPIP could reverse CP-mediated inhibition of viral gene expression. CP alone or together with either CPIP or CPIP<sub>D66</sub> was coexpressed with viral constructs (Figures 4D and 4E). As before, expression of CP alone severely inhibited viral gene expression of all PVA viruses. Gene expression of the replicating viruses, PVAwt and PVA<sub>CPmut</sub>, was more enhanced when coexpressed with either CPIP or CPIP<sub>D66</sub> than that of PVA<sub>DGDD</sub>. Since the elevated gene expression was connected to the replicating viruses, it was mainly attributable to RAT and required that RAT was released from inhibition by exogenous CP.
Coexpression of CPIP or CPIP\textsuperscript{D66} affected viral gene expression differentially. CPIP\textsuperscript{D66} was able to increase gene expression of both PVA\textsubscript{CPmut} and PVA\textsubscript{wt}, being comparably fivefold higher for PVA\textsubscript{CPmut} at 2 DAI. Gene expression of PVA\textsubscript{CPmut} declined by 3 DAI (Figure 4E). This could represent cessation of RAT for a virus impaired in cell-to-cell movement. When PVA\textsubscript{wt} was coexpressed with CPIP, viral gene expression continued to develop by 3 DAI in contrast with when coexpressed with CPIP\textsuperscript{D66}. This showed that functional CPIP–HSP70 interaction was required for development of PVA\textsubscript{wt} infection. These results were in agreement with the HSP70-silencing experiment that argued for a process, where HSP70 is needed for replication in the presence of CP\textsubscript{wt} but not CP\textsubscript{mut} (Figures 2E and 2F) and with the finding that expression of CP\textsubscript{wt} from the viral genome inhibits RAT when CPIP has been depleted by an excess of exogenous CP (Figure 3F).

**CP Interacts with CPIP and HSP70**

The results presented thus far show that HSP70 and CPIP function to suppress CP-mediated inhibition of RAT. To provide additional evidence to support the link between CP and HSP70, we immunoprecipitated CP with anti-PVA CP from leaves expressing CP alone or CP together with either CPIP or CPIP\textsuperscript{D66}. Detection of HSP70 showed that it coprecipitated with the viral CP (Figure 5A). The interaction between HSP70 proteins and their substrates can be disassociated by ATP (Szabo et al., 1994). Therefore, we tested whether coprecipitation of HSP70 with CP was sensitive to ATP. CP was expressed in plant leaves, immunoprecipitated either in the absence or presence of ATP followed by immunodetection of both HSP70 and CP (Figure 5B). HSP70 coprecipitation was ATP sensitive, whereas CP precipitation was not. This indicated that the CP–HSP70 interaction represented a functional HSP70 and substrate interaction.

Next, we performed immunoprecipitations for CPIP and CPIP\textsuperscript{D66} coexpressed with either CP or CP\textsubscript{mut} (Figure 5C). CP was found to coprecipitate with CPIP\textsuperscript{D66}, providing direct evidence for the interaction between PVA CP and CPIP. Importantly, CP\textsubscript{mut} did not coprecipitate with either CPIP or CPIP\textsuperscript{D66}, showing that it did not interact with CPIP. Therefore, CP\textsubscript{mut} is unable to sequester endogenous CPIP and hence to inhibit RAT when expressed in trans (Figures 3A to 3C). A similar mutation to that in CP\textsubscript{mut} also disrupted CPIP interaction with PVY CP (Hofius et al., 2007). Neither HSP70 nor CP was enriched in the CPIP immunoprecipitate (Figure 5C). This indicated the transient nature of the interaction between CP and CPIP, probably reflecting on CP delivery and release to HSP70. Because HSP70 was initially identified from purifications of Nib\textsubscript{SIII} and VPg\textsubscript{SIII}, we analyzed these samples for the presence of endogenous CPIP.

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**Figure 4.** CPIP Can Regulate CP-Mediated Inhibition of Viral Gene Expression.

(A) Gene expression of PVA\textsubscript{wt} in pRD400 control plants and when coexpressed with CPIP and CPIP\textsuperscript{D66} at 2 and 3 DAI.

(B) Expression of CPIP and CPIP\textsuperscript{D66} was detected by immunoblot analysis using anti-c-myc. Plant total protein was used as negative control (−).

(C) General inhibition of potyvirus infection by constitutive CPIP\textsuperscript{D66} expression. PVA, TEV, and TVMV CP levels in systemic leaves of control and transgenic plants expressing CPIP\textsuperscript{D66} (line CPIP-39; Hofius et al., 2007) at 13 (PVA) and 14 (TEV and TVMV) DAI. Values represent means (n = 20) ± SE and are given as percentage of wild-type level. Plants had developed six to eight leaves prior to virus infection and were sampled in leaves 5 to 7 above the inoculated leaf.

(D) and (E) Coexpression of CPIP or CPIP\textsuperscript{D66} antagonizes CP-mediated inhibition of viral gene expression. Viral gene expression was analyzed in plants expressing CP\textsubscript{wt} alone or together with CPIP or CPIP\textsuperscript{D66} at 3 (D) and 4 (E) DAI. Asterisks indicate the low viral gene expression during CP\textsubscript{wt} coexpression only. The error bars indicate the SD.
A protein that corresponded in size to CPIP was detected specifically in the tagged samples by anti-CPIP pAbs. Immunoprecipitations suggested that both the CPIP–CP and CPIP–HSP70 interactions were transient, possibly the reason for the weak CPIP signal in the NibSIII and VPgSIII membrane-derived samples.

**CPIP-Mediated HSP70 Delivery Affects CP Stability**

We used ELISA to quantitate CP in plants expressing CP alone or CP together with CPIP or CPIP<sup>D66</sup> (Figure 6A). The amount of CP was reduced in CPIP and elevated in CPIP<sup>D66</sup> coexpressing plants compared with plants expressing CP alone. The amount of CP increased more than fivefold due to CPIP<sup>D66</sup> expression between the two time points, whereas the amount remained the same in CP alone or further decreased due to CPIP coexpression. This indicated that delivery of CP to HSP70 increased its degradation. HSP70-mediated protein degradation can involve protein ubiquitination (McDonough and Patterson, 2003). Therefore, the ubiquitination status of CP was analyzed in connection to CPIP or CPIP<sup>D66</sup>. For this, we used ELISA where anti-CP mAbs were used for coating as before but detection was with anti-ubiquitin (Figure 6B). The relative level of ubiquitin to CP was markedly increased by CPIP coexpression, supporting the HSP70-dependent targeting of CP for degradation. Next, CP and ubiquitin were detected from CP immunoprecipitates (Figure 6C). High molecular weight (HMW) CP forms appeared most abundant in CPIP and the least in CPIP<sup>D66</sup> expressing plants and seemed to correlate with the ubiquitin signals. We also analyzed CP and ubiquitin in the strep-purified samples from which HSP70 was originally identified (Figure 6D). Indeed, HMW CP and ubiquitin were also observed in these samples. However, the ubiquitin signal is not CP specific and may originate partially from other proteins. Only the VPg<sup>SIII</sup> sample contained virion forming CP (CP*), and both contained a higher CP form (CP**) that was clearly enriched in the CP and CPIP sample (Figure 6C). Possibly, CP** was modified by a process involving HSP70.

**DISCUSSION**

We assayed virus mutants impaired either in replication (PVA<sup>DGDD</sup>) or cell-to-cell movement (PVA<sup>CPmut</sup>) in parallel with the wild-type virus (PVA<sup>wt</sup>) using a simple and sensitive quantitation method. Using this assay, we were able to determine a role of the host chaperones HSP70 and CPIP in regulating the function of a potyviral CP, which can interfere with viral translation and replication. During this study, we noticed that virus-derived RLUC expression by the replicating viruses was...
differentially affected, compared with that of the nonreplicating viruses, under the experimental conditions used. RAT referring to viral gene expression from replicating viruses (PVA\textsuperscript{wt} and PVA\textsuperscript{CPmut}) is distinct from PVA\textsuperscript{DGGD}-derived nonreplication-associated translation. The observed changes in RAT-derived RLUC activity can include both changes in replication (i.e., genome-associated transcription activity) and changes in translational efficiency of replication-associated viral RNA. Based on the results presented, we have drawn the following conclusions. (1) PVA CP has the capacity to interfere with viral gene expression. (2) RAT, translation coupled to replication, exists and needs to be protected from inhibition by CP. (3) HSP70, together with its cochaperone CPIP, functions to antagonize CP-mediated inhibition of viral gene expression. (4) CPIP-mediated delivery of CP to HSP70 promotes ubiquitination and degradation of CP. We propose a model in which CPIP delivers CP to HSP70 during RAT. This promotes modification of CP by ubiquitin, leading to CP degradation and prevention of the CP–RNA interaction that would cause premature cessation of RAT (Figure 7).

**CP Can Inhibit Viral Translation**

Expression of exogenous CP repressed PVA gene expression efficiently (>90%). Capsid proteins of other plus-stranded RNA viruses can inhibit viral gene expression specifically through their association with RNA elements within the viral 5' untranslated region (UTR) (Shimoike et al., 1999; Karpova et al., 2006; Yi et al., 2009a). Viral genomes commonly possess RNA elements involved in the regulation of both translation and replication (Wu et al., 2009). BMV CP can regulate both viral translation and replication through different RNA interactions (Yi et al., 2009a, 2009b). Because inhibition of PVA gene expression occurred both for replicating and nonreplicating viruses, the inhibition was uncoupled from replication and therefore most probably CP functioned to repress translation. Gene expression of the virus-independent reporter was unaffected by CP, arguing for specific RNA elements within viral transcripts through which CP can regulate viral gene expression. CP\textsuperscript{mut} was unable to inhibit viral gene expression. The mutation in CP\textsuperscript{mut} interferes with virion assembly (Dolja et al., 1994; Varrelmann and Maiss, 2000); hence, inhibition of viral gene expression by CP could be related to virion assembly. The potyviral 5'-genomic region contains an RNA element from which assembly of the virion initiates, the putative origin-of-assembly (Wu and Shaw, 1998). Genome translation of *Potato virus X* is inhibited either when assembled in virion form or when CP is associated with the 5'-UTR (Atabekov et al., 2001; Karpova et al., 2006). Subsequent phosphorylation of CP enables *Potato virus X* translation. Similarly, phosphorylation of PVA CP decreases its affinity for the 5'-UTR and is required for virus infection (Ivanov et al., 2001, 2003). The question whether virion assembly or other CP-RNA interactions causes inhibition of viral genome translation remains interesting.

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**Figure 6.** CPIP-Mediated HSP70 Delivery of CP Promotes Modification by Ubiquitin and CP Degradation.

(A) CP amounts detected by ELISA when expressed alone or together with CPIP or CPIP\textsuperscript{D66} given as ng of CP/mL at 3 and 4 DAI.

(B) Ubiquitination level of CP when expressed alone or together with CPIP or CPIP\textsuperscript{D66} at 4 DAI given as the relative amount of ubiquitin per CP.

(C) Presence of ubiquitinated high molecular weight CP in CP immunoprecipitates. The proteins expressed in each sample are indicated at the top. IgG heavy chain detected with anti-mouse Ab was used as loading control. The masses of the molecular mass markers are indicated on the left ([C] and [D]).

(D) Presence of high molecular weight ubiquitinated proteins and CP in the column eluates of Nb\textsuperscript{SIII} and VPg\textsuperscript{SIII} samples.
induced membrane environment. We find it likely that RAT resides within a protected virus-

translation that is spatially protected from in trans inhibition by inhibition. This is mainly based on different responses of replicating and nonreplicating viruses to in trans replication. This is based on the following: First, PVACPmut gene expression was required to restore RAT for PVA wt. Finally, the impaired systemic accumulation of PVA upon silencing inhibited early gene expression specifically for PVA wt. HSP70 and CPIP Regulate CP during RAT

In this study, HSP70 was copurified with viral replication proteins from similar membrane fractions that have been shown to contain the functional replicase of two other potyviruses, TEV and plum pox virus (Martin and Garcia, 1991; Schaad et al., 1997). Because HSP70, VPg, Nla, Nlb, a modified CP (CPmut), CPIP, and viral RNA were copurified with either VPgSIII or NlbSIII tagged proteins, these components could be part of a higher-order entity. It probably originates from viral replication, consistent with the presence of both positive- and negative-strand viral RNAs. HSP70 has been shown to colocalize with virus-induced membrane structures and to interact with TuMV Nlb (Dufresne et al., 2008). HSP70 associates with membrane-localized replication proteins of Tomato mosaic virus (Nishikiori et al., 2006), is a component of the Cucumber necrosis virus replicase (Serva and Nagy, 2006), and is recruited by TBSV during assembly of its replicase at host membrane sites (Wang et al., 2009). In addition, downregulation of HSP70 using VIGS and/or quercetin inhibits TBSV, TMV, and Turnip crinkle virus infection to an extent similar to what we report here (Wang et al., 2009). These studies indicate general functionality of HSP70 in plant virus replication. This is not surprising, since replication involves the assembly of different RNP complexes that regulate events such as minus- or plus-strand synthesis, translation, and virion assembly, which are processes that clearly would benefit from chaperone assistance.

CPIP was initially reported to interact with PVY CP and to dominantly inhibit virus infection if expressed as a J-domain–deficient version (CPIPΔ66) (Hofius et al., 2007). In this study, we showed a similar inhibition of PVA, TEV, and TVMV accumulation in systemic leaves in CPIPΔ66 transgenic plants, indicating a general role for the HSP70–CPIP system in potyvirus infection and thereby the potential to use CPIPΔ66 to engineer potyvirus resistance. Previously it was proposed that CPIP functioned in virus movement (Hofius et al., 2007), which could also be supported by the impaired systemic accumulation of PVA upon HSP70 silencing. Nevertheless, this study shows that CPIP function during replication to prevent CP from interfering with viral gene expression.

This is based on the following: First, PVA(CPmut) gene expression was 10–50-fold elevated compared with that of PVAΔGDD and PVAΔGDD and exogenous CP expression. The mutation in the CP gene could only enhance gene expression of a replicating (PVA(CPmut)) but not that of a nonreplicating (PVAΔGDD, CPmut) virus. Second, coexpression of CPIP with CP enhanced RAT. Gene expression of PVA(CPmut) and PVAΔGDD were both elevated by CPIPΔ66 during CP expression. We hypothesize that both CPIP and CPIPΔ66 interaction can block CP from binding viral RNA independent of HSP70 and thereby increase gene expression. However, CPIP, and not CPIPΔ66, was selectively required by replicating PVAwt that carries the native CP to continue infection. CPmut expressed in trans did not interact with CPIP and it did not inhibit early replication and translation of PVAwt. This supports the idea that expression of CP in trans sequestered CPIP, causing inhibition of RAT for PVAwt, but not PVA(CPmut), and CPIP overexpression was required to restore RAT for PVAwt. Finally, HSP70 silencing inhibited early gene expression specifically for PVAwt. Together, these results show that CP is regulated by HSP70 and CPIP during RAT.
According to the common transfer mechanism of substrates from JDPS to HSP70s (Summers et al., 2009), we believe that CPIP delivers CP to HSP70 in a successive manner. Simultaneously, HSP70 inactivates, either temporally or irreversibly, the capacity of CP to inhibit RAT. Our results indicate that CP is a HSP70 substrate and that this association promotes CP modification by ubiquitin and degradation, at least in the absence of virus infection. We consider this as functional evidence for CPIP-mediated CP delivery to HSP70 since HSP70 substrates are commonly subjected to degradation (McDonough and Patterson, 2003; Esser et al., 2004). Because both CPIP and corresponding HMW ubiquitin and CP are found from the membrane-derived purifications, altogether this hypothesis appears plausible. However, it remains to be demonstrated in the context of viral infection. Commonly, several virus groups regulate timing and quantity of CP by subgenomic RNAs and thereby allocation of genomes from replication or translation to assembly. Potyviruses, however, do not encode subgenomic RNAs, and CP is produced as a part of the polyprotein. The CPIP-HSP70 system may therefore have been co-opted as an alternative mechanism to subgenomic RNA for downregulation of CP during early replication events. When CP is abundant at a later infection stage, CPIP becomes limiting and, hence, RAT is inhibited. By this mechanism, CP could function to negatively backregulate replication, and thereby a controlled level of viral multiplication would be achieved.

METHODS

Plants, and Virus and Plant Expression Constructs

Nicotiana benthamiana plants were kept under greenhouse conditions with 22°C 18 h light and 18°C 6 h dark and infected at the four to six leaf stage. Nicotiana tabacum cv Samsun NN plants were kept under greenhouse conditions with 22°C 16 h light and 18°C 8 h dark and infected at the six to eight leaf stage. Transgenic tobacco plants constitutively expressing CPIPHis6 (line 39) were described previously (Hofius et al., 2007).

Viral constructs used in the Agrobacterium tumefaciens-mediated infection experiments of this study were based on the full-length infectious DNA copies of PVA strain B11 (GenBank accession number AJ296311) tagged with rLucC18SS - PVA-rLuc (Gabrenaite-Verkhovskaya et al., 2006). Replication-deficient PVA (PVAICP0) was constructed by deleting both aspartic acids of the catalytically active GDD motif of PVA Nib, and movement-deficient PVA (PVACPmut) was constructed by substituting R159 with D, and Q160 with V within the PVA CP-encoding sequence (Eskenel et al., 2009). An intron was inserted into RLuc within PVA icDNA, and the gene cassettes pr0SSS - PVAwPVAICP0SSS or PVAICP0SSS - rLuc-intron-5SS and pr0SSS-Fluc-5SS into pRD400 (Datla et al., 1992) were cloned for Agrobacterium-mediated initiation of viral infection (Eskenel et al., 2009). A strep-tag III sequence (Junttila et al., 2004) was fused to the C terminus of VPg and the N terminus of Nib in infectious cDNA (icDNA) clones of PVA (Figure 1A), similarly as for the previously described HisHA tag insertion within PVA:GFP (Hafren and Makinen, 2008). The viruses that were generated were named PVAICPVSB3 III and PVAICP0SSS III, and the respective proteins were named NibPS3 and VPgPS3. The gene cassette containing pr0SSS - PVAICPVSB3 III/PVAICP0SSS III/GFP- nos was cloned into the binary vector pRD400 for Agrobacterium infiltration. Expression constructs for CPIP and CPmut were based on the sequence of PVA strain B11 (AJ296311) and cloned into pRD400 driven by the pr0SSS. CPmut contained the same mutations in the CP sequence as PVAICP0SSS. Expression constructs for CPIP and CPmutHis6 were previously described by Hofius et al. (2007). All constructs were used to transform Agrobacterium strain C58C1 pGV2260. Agrobacterium transformed with an empty pRD400 was used as a control in the expression experiments.

Infiltration of Agrobacterium

Agrobacterium was grown in the presence of kanamycin and carbenicillin (50 µg/mL each) in Luria-Bertani broth to OD600 = 0.5 to 1 with shaking (200 rpm) at 30°C. Cultures were diluted 1:10 in the same medium supplemented with 10 mM MES, pH 6.3, and 10 µM acetylsoygnine. After reaching OD600 ~ 0.5, cells were harvested by centrifugation at 3000g for 5 min, washed once with double distilled water, and harvested again by centrifugation. Finally, Agrobacterium pellets were resuspended in infiltration buffer (10 mM MES, pH 6.3, 10 mM MgCl2, and 150 µM acetylsoygnine) and incubated for 2 h at room temperature before infiltration of plant leaves. A syringe was used for infiltration.

SII Tag–Dependent Protein Purification from Plant Membranes

All procedures were performed at 4°C using prechilled buffers. Systemically infected leaves of N. benthamiana were harvested 8 d after infiltration with Agrobacterium carrying either PVA, PVAICP0SSS III, or PVAICP0SSS III icDNA and homogenized in 2 mL buffer (100 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM PMFS, and 13% sucrose, pH 8) per gram of leaf tissue using a blender. The lysate was cleared by centrifugation at 4000g for 10 min. A membrane fraction was prepared by centrifugation of the cleared lysate at 30,000g for 20 min. The resulting membrane pellet was resuspended in buffer with protease inhibitors (Roche EDTA-free complete protease inhibitor cocktail, one tablet in 10 mL, no PMFS) at a concentration of 1 mL buffer per 4 g initial leaf tissue, using a dounce homogenizer, and extracted with 1% Triton X-100 for 30 min on ice. After extraction, samples were cleared by centrifugation at 30,000g for 20 min. The supernatant was supplemented with 100 µg/mL aprotinin and subjected to purification using strep-tactin sepharose (IBA) by gravity flow (1 mL matrix per 100 g of initial leaf tissue). After binding, the matrix was washed twice with 25 mL wash buffer (100 mM Tris-HCl, pH 8, 150 mM NaCl, and 5 mM EDTA) on a rotator for 1 min. The beads were pelleted by centrifugation at 1000g for 1 min. Bound proteins were eluted with wash buffer containing 2.5 mM desthiobiotin.

Protein Concentrations, SDS-PAGE, and Immunoblotting

Total protein concentrations were determined using BCA (Pierce). Proteins were separated by SDS-PAGE (Laemmli, 1970) and detected with mass spectrometry-compatible silver staining (O’Connell and Stults, 1997). Immunodetection was performed as described previously (Hafren and Makinen, 2008). A commercial HSP70/HSC70 polyclonal antibody (Stressgen; catalog number SPA-757) was used in immunodetection of HSP70 and monoclonal anti-C-myc (9E10; Santa Cruz Biotechnology) or polyclonal anti-CPIP (Hofius et al., 2007) to detect CPIPs.

LC-MS/MS

Proteins were in-gel digested with trypsin, and the resulting peptides were analyzed by LC-MS/MS using an Ultimate 3000 nano-LC (Dionex) and a QSTAR Elite hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex) with nano-electrospray ionization. The LC-MS/MS samples were first loaded on a ProteCol C18 trap column (10 mm × 150 µm, 3 µm, 120 Å) and subsequently separated on a PepMap100 C18 analytical column (15 cm × 75 µm, 3 µm, 100 Å) (LC Packings/Dionex) at a flow rate of 200 nL/min. A separation gradient from 0 to 50% B in 50 min (buffer A, 0.1% formic acid; buffer B, 0.8% formic acid in 80% acetonitrile) was applied. MS/MS data were acquired automatically using the Analyst QS 2.0 software. Information-dependent acquisition consisted of a 0.5-s time-of-flight-MS survey scan of m/z 400 to 1000.
Quercetin was dissolved in DMSO to prepare a 200 mM stock solution.

**Protein Identification by LC-MS/MS**

Database searches using the LC-MS/MS data were conducted using the publicly available Mascot search engine and the SwissProt database (http://www.matrixscience.com). The search criteria were (1) Viridiplan-tae or viruses taxonomy, (2) trypsin digestion with one missed cleavage allowed, and (3) carbamidomethyl modification of Cys as a fixed modification and oxidation of Met as a variable modification. For the LC-MS/MS spectra, both the maximum precursor ion mass tolerance and MS/MS fragment ion mass tolerance were 100 ppm, and a peptide charge state of +1, +2, or +3 was used. All of the reported protein identifications were statistically significant (P < 0.05).

**RT-PCR**

RNA was extracted from strep-purified samples using Trizol (Invitrogen). RT was performed with Moloney murine leukemia virus reverse transcriptase (Promega) using PVA (-) (5'-CATGGTATGGTGCAATGAGAATGG-3') and (+) (GACATTTCGTCAGTCCAACAGT) strand-specific primers. For PCR, the same primers were used to amplify a 3'-terminal region of the (+) genome.

**HSP70 Silencing and Systemic Infection**

For silencing, we used TRV vectors (Ratcliff et al., 2001). A HSP70 sequence (GU575116) was amplified from an N. benthamiana cDNA library and cloned into the pTV:00 vector. The resulting pTV: Nb HSP70 cDNA fragment for VIGS.

For silencing experiment, each time point (Figures 2D and 2E) represents a 405 nm with a microplate reader (Bio-Rad model 680). Different amounts of purified virions and recombinant ubiquitin (Millipore) were used as reference samples to estimate CP and ubiquitin concentrations, respectively.

**Double Antibody Sandwich ELISA**

Samples lysates were prepared as for immunoprecipitations. Samples were diluted 1:1 in buffer supplemented with 3% skimmed milk and incubated on ELISA microtiter plates (Costar 3590, high binding) coated with a PVA-CP–specific MAb 58/0 (Adgen). Detection was conducted using anti-ubiquitin pAb (Agrisera) followed by alkaline phosphatase–conjugated goat anti-rabbit secondary antibody or alkaline phosphatase–conjugated PVA-CP–specific pAb (Adgen). Absorbances were recorded at 405 nm with a microplate reader (Bio-Rad model 680). Different amounts of purified virions and recombinant ubiquitin (Millipore) were used as reference samples to estimate CP and ubiquitin concentrations, respectively.

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