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The HCPro from the *Potyviridae* family: an enviable multitasking Helper Component that every virus would like to have.

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SUMMARY

RNA viruses have very compact genomes, so that they provide a unique opportunity to study how the evolution works to optimize the use of very limited genomic information. A widespread viral strategy to solve this issue concerning the coding space relies on the expression of proteins with multiple functions. Members of the family *Potyviridae*, the most abundant group of RNA viruses in plants, certainly offer several attractive examples of viral factors playing roles in diverse infection-related pathways. The Helper Component Proteinase (HCPro) is an essential and well-characterized multitasking protein for which three independent functions, at least, have been described: (i) viral plant-to-plant transmission, (ii) polyprotein maturation, (iii) RNA silencing suppression. Moreover, multitudes of host factors have been found to interact with HCPro. Intriguingly, most of these partners have not been ascribed to any of the HCPro roles during the infectious cycle, supporting the idea that this protein might play even more roles than those already established. In this comprehensive review, we attempt to summarise our current knowledge about HCPro and its already attributed and putative novel roles, to then finally discuss about similarities and differences regarding this factor in members of this important viral family.

INTRODUCTION

Members of the family *Potyviridae* are the most abundant and socio-economically relevant RNA viruses infecting plants (Scholthof *et al.*, 2011; Valli *et al.*, 2015) and, because of that, they have been subject of intense studies worldwide. This family is formed by eight genera (*Brambyvirus*, *Bymovirus*, *Ipomovirus*, *Macluravirus*, *Poacevirus*, *Potyvirus*, *Rymovirus* and *Tritimovirus*) that are differentiated by their genome composition and structure, RNA sequence and transmission vectors (Revers and García, 2015). Most potyvirids (i.e. viruses belonging to the *Potyviridae* family) have monopartite, single-stranded and positive-sense genomes of around 10000 nucleotides that are encapsidated by multiple units of a single coat protein (CP) in flexuous and filamentous virus particles of 680 to 900 nm in length and 11 to 14 nm in diameter (Kendall *et al.*, 2008). Exceptionally, bymoviruses are peculiar in these regards, as they have a bipartite genome that is

encapsidated separately. Inside the infected cells, the viral RNA of potyvirids is uncoated and translated into polyproteins that are proteolytically processed by viral-encoded proteinases producing, in most of the cases, the following mature viral gene products: P1, the helper component proteinase (HCPro), P3, 6K1, CI, 6K2, NIa (VPg + Pro), NIb and CP. As mentioned, bymoviruses have two genomic RNA segments that are independently translated. In addition to the large polyproteins, transframe products named P3N-PIPO and P3N-ALT, which share the N-terminal region of P3, are produced from RNA variants generated via transcriptional slippage during viral replication (Hagiwara-Komoda *et al.*, 2016; Olspert *et al.*, 2015; Rodamilans *et al.*, 2015). Furthermore, the same mechanism is also used during the replication of some sweet potato potyviruses to produce an additional transframe product, termed P1N-PISPO, which overlaps with the P1 cistron (Mingot *et al.*, 2016; Untiveros *et al.*, 2016).

RNA viruses in general are known to have small and condensed genomes, which might be, at least in part, due (i) to intrinsic structural restrictions (e.g. topology and stability) of the RNA molecule (Gorbalenya *et al.*, 2006), (ii) to the need of minimizing the negative impact of the error-prone viral replication (Holmes, 2003), or even (iii) to protect themselves from the action of antiviral host defence mechanisms (Eusebio-Cope and Suzuki, 2015). As a consequence, RNA viruses are under intense selective pressures to optimize the use of their genomic information. To cope with this restriction, they exploit diverse strategies in order to produce/recruit all the required components ensuring the infection success (Ahlquist *et al.*, 2003; Atkins *et al.*, 2016; Firth and Brierley, 2012; Sztuba-Solinska *et al.*, 2011). One of these strategies relies on the expression of viral proteins with several functions. In particular, the well-characterized RNA viruses of the family *Potyviridae* provide fascinating examples of multitasking proteins (e.g. (Sorel *et al.*, 2014; Weber and Bujarski, 2015)). Here we present a comprehensive review concerning the potyvirid HCPro, with particular emphasis on members of the genus *Potyvirus*, in which at least three clearly independent functions have been described.

TRANSMISSION – *a historical overview of HCPro discovery*

In the particular case of potyviruses, they are transmitted by aphids, by a mode of transmission that is described as non-persistent, since it occurs rapidly, with the duration of acquisition and inoculation phases in the range of seconds to minutes without retention periods (Bradley, 1952; Day and Irzykiewicz, 1954; Kassanis, 1941). This fast, and usually efficient, mode of transmission was recognized as a serious caveat in the adoption of control measures against pathogenic virus dissemination, because it leaves virtually no time available for effective insecticide treatment aimed to target their vectors. Therefore, intense research efforts took place to better understand potyviral transmission. In this context, the role of HCPro in this process was found even before knowing that it was a viral protein. The name "Helper Component" was coined to describe the existence of a "component" of unknown source, but present in infected plants, which "helped" the transmission of potyviruses mediated by aphid vectors. How this function was identified is an extraordinary story that reveals the resources, skills and imagination of those researchers involved in the discovery (Pirone and Thornbury, 1984). Chronologically, the finding of certain natural virus isolates with altered transmission properties was the first indication that this function was genetically regulated (Kamm, 1969; Simons, 1976). The use of aphid artificial feeding systems, based on stretched plastic paraffin films, was instrumental to verify that insects often failed to transmit the disease when purified virions were used for the transmission assay (Pirone and Megahed, 1966). Hence, this result indicated that the viral particle alone is not enough for efficient transmission. Taking advantage of UV-radiation treatments to inactivate viral RNAs, it was shown that a UV-resistant component (likely a protein) should be acquired by aphids simultaneously (or prior) to virions in order to transmit the virus (Govier and Kassanis, 1974a, b; Kassanis and Govier, 1971a, b). Later on, equipped with very simple experimental tools, the purification of the active factor was achieved and allowed the generation of specific antisera (Govier *et al.*, 1977; Thornbury *et al.*, 1985), which was certainly crucial to establish its origin as part of the viral polyprotein (Carrington *et al.*, 1989a; Dougherty and Hiebert, 1980; Hiebert *et al.*, 1984). Indeed, antibodies against HCPro have been very useful to establish the presence of this viral factor in amorphous inclusions of cells infected with some

potyviruses (De Mejia *et al.*, 1985), as well as to unravel other aspects of HCPro that will be described in diverse sections of this article.

Based on results from the experiments described above, a molecular mechanism by which HCPro participates in the transmission process was suggested long ago by Govier and Kassanis (1974b). The so-called "bridge hypothesis" proposes that the helper component acts as a reversible link between the viral particle and the vector mouthparts (Fig. 1). Over the years, accumulative evidences have provided ample support to this hypothesis, while alternative models, such as that proposing a direct interaction between CP and aphid receptors with the HCPro acting to expose CP binding sites (Salomon and Bernardi, 1995), failed to reach generalization. Among the most remarkable outcomes of these efforts were the identification and validation of conserved domains in CP and HCPro that are involved in vector transmission. In the CP, a highly conserved "DAG" motif had been earlier predicted to play a role in transmission (Harrison and Robinson, 1988; Laín *et al.*, 1988), which was further confirmed by mutagenesis analyses (Atreya *et al.*, 1990; Atreya *et al.*, 1991; Atreya *et al.*, 1995). Regarding the identification of relevant domains in HCPro, the characterization of transmission-defective isolates in different viruses (Huet *et al.*, 1994; Peng *et al.*, 1998; Thornbury *et al.*, 1990) lead to identify at least two separate motifs required for the bridge hypothesis to occur: a PTK amino acid triad that interacts with the CP (Huet *et al.*, 1994; Peng *et al.*, 1998) and a KITC motif that participates in retention to an unknown structure in the aphid mouthparts (Blanc *et al.*, 1998; Huet *et al.*, 1994) (Fig. 1). The presence of these amino acids might be not sufficient for the function, and indeed other regions in HCPro have been later proposed to affect transmissibility (Canto *et al.*, 1995; Llave *et al.*, 2002; Seo *et al.*, 2010). Importantly, predictions based on the bridge hypothesis have been confirmed, and they include: (i) the identification of HCPro retention sites in aphid stylets (Moreno *et al.*, 2012; Wang *et al.*, 1998), (ii) the direct interaction between CP and HCPro (Blanc *et al.*, 1997; Roudet-Tavert *et al.*, 2002; Seo *et al.*, 2010), and (iii) the location of HCPro in a protruding tip at one end of the viral particle (Torrance *et al.*, 2006).

An intriguing observation linked to the discovery of HCPro is the unusual aphid-mediated transmission of the potexvirus *Potato aucuba mosaic virus* (PaMV),

which only takes place when PaMV infected plants are co-infected with a potyvirus (Kassanis, 1961). The further finding of an equivalent DAG motif at the N-terminus of the PaMV CP provided a putative explanation for the observed trans-complementation. In the same study, an elegant demonstration of the relevance of the DAG amino acid triad was obtained by engineering this motif in the CP of *Potato virus X*, a non-DAG, non aphid-borne, potexvirus, as this modification rendered the aphid transmission of this virus HCPro-dependent (Baulcombe *et al.*, 1993). It is worth mentioning that compatibility of different HCPro to support transmission of other potyviruses has been also confirmed (Flasinski and Cassidy, 1998; Lecoq and Pitrat, 1985; López-Moya *et al.*, 1995; Sako and Ogata, 1981). Indeed, this trans-complementation property of HCPro is believed to play an important ecological role by driving the evolution of the helper strategy as a way to avoid the negative impact of genetic bottlenecks associated with nonpersistent virus transmission (Pirone and Blanc, 1996).

The purification of an HCPro still active during transmission was useful for the study of diverse features of this protein. Even though the insertion of a 6xHis tag facilitated the HCPro purification in the context of a viral infection by using a Ni^{+2}-charged resin (Blanc *et al.*, 1999), the same purification protocol was successfully applied in other viruses without attaching the 6xHis tag to HCPro (Wang and Pirone, 1999). These results suggest that intrinsic biochemical properties of the protein require the interactions with metallic ions, an observation that agrees with previous studies mentioning the relevance of divalent cations in the buffer (in particular Mg^{+2}) during transmission assays (Thornbury *et al.*, 1985; Thornbury and Pirone, 1983).

The expression of functional HCPro in heterologous systems has provided a useful methodology to speed up research on potyvirus transmission. Hence, the proper activity of HCPro was maintained when the protein was expressed in transgenic plants (Berger *et al.*, 1989), in insect cells using a baculovirus-based system (Thornbury *et al.*, 1993), or in yeast (Ruiz-Ferrer *et al.*, 2004). In addition, transient expression systems in plants, using either viral vectors (Sasaya *et al.*, 2000) or agro-infiltration (Goytia *et al.*, 2006), also succeeded in producing transmission-active HCPro.

Remarkably, it was also shown that HCPro plays a key role in the semi-persistent dispersion of *Wheat streak mosaic virus,* a member of the *Tritimovirus* genus transmitted by eriophyid mites (Stenger *et al.*, 2005b). Moreover, despite the low overall sequence similarity between the tritimovirus and the potyvirus HCPros, mutations in conserved Cys residues affected the transmission process in viruses belonging to these two genera (Atreya and Pirone, 1993; Llave *et al.*, 1999; Young *et al.*, 2007). As a detailed characterization of the HCPro role in transmission mediated by vectors others than aphids awaits to be addressed, it is not currently clear whether this function has been acquired independently in different *Potyviridae* genera (convergent evolution), or it has derived from a common ancestral virus that was transmitted by an ancestral arthropod (adaptation).

Finally, other aspects that remain to be determined in order to better understand the role of HCPro in transmission include the stoichiometry and geometry of the reversible interactions virion//HCPro//vector, which seems to involve multimers of HCPro (Plisson *et al.*, 2003; Ruiz-Ferrer *et al.*, 2005) and the location of this factor at one end of the viral particles (Torrance *et al.*, 2006). Curiously, the visualization of virions within insect stylets has only been attempted and achieved with potyviruses in a very reduced number of studies (Wang *et al.*, 1996), and just a few attempts to discover the vector receptors have been pursued and communicated (Dombrovsky *et al.*, 2007; Fernández-Calvino *et al.*, 2010). Thus, at this point, it is still uncertain if the potyvirus-specific aphid receptor co-localizes or shares properties with the putative receptors of viruses from other families (Blanc *et al.*, 2014; Uzest *et al.*, 2007).

RNA SILENCING SUPPRESSION – *fight for survival*

RNA silencing is a highly conserved, sequence specific, regulatory mechanism that shuts the expression of target genes down at the transcriptional and post-transcriptional level. The entire silencing machinery is formed by partially overlapping modules, which are accordingly activated in the presence of diverse double-stranded (ds) RNA molecules and have different roles during development (some nice reviews about RNA silencing were recently published: Bologna and Voinnet, 2014; Castel and Martienssen, 2013; Chang *et al.*, 2012). As part of its many tasks, RNA silencing plays a key antiviral role in organisms from different

kingdoms (Bronkhorst and van Rij, 2014; Chang *et al.*, 2012; Ding, 2010; Huang *et al.*, 2014; Li *et al.*, 2002; Szittya and Burgyan, 2013; Zhang *et al.*, 2015). In the case of plants, for instance, it is well established that viruses generate viral-derived dsRNAs as a consequence of (i) viral replication, (ii) RNA tendency to fold in hairpin-like structures, and/or (iii) transcription of bidirectional mRNAs. These dsRNAs are first recognized and processed by RNase III-like enzymes belonging to the Dicer family, which cut them in viral-derived short interfering (vsi)RNA duplexes 21-to-24 nucleotides in length. Analogously, another batch of these vsiRNAs derives from newly synthesised dsRNAs generated by the action of RNA-dependent RNA polymerases (RDRs). After stabilization via HEN1-mediated methylation of their 3' ends, vsiRNA duplexes are recruited by Argonaute (AGO)-containing complexes, where only the so-called "guide strand" is retained to further direct the complex towards complementary RNA/DNA sequences in order to promote silencing (Zhang *et al.*, 2015). A basic description of the antiviral silencing pathway against plant RNA viruses is illustrated in Figure 2.

During their evolution, viruses had to develop ways to fight back against RNA silencing in order to survive. The most effective strategy appears to be that based on the expression of viral proteins, called RNA silencing suppressors (RSSs) with the capacity to block or interfere with the antiviral silencing. The HCPro protein from members of the genus *Potyvirus* was indeed the first ever-described RSS (Anandalakshmi *et al.*, 1998; Kasschau and Carrington, 1998). Many studies since then have revealed that HCPro can counteract the silencing-based defensive barrier by targeting multiple steps of the cascade (Fig. 2 and Table 1). Interestingly, some of these studies have also shown that only HCPro from members of *Potyvirus* and *Rymovirus* genera appears to have RNA silencing suppression activity, whereas this function relies on another protein in members of the remaining genera (Giner *et al.*, 2010; Mingot *et al.*, 2016; Tatineni *et al.*, 2012; Untiveros *et al.*, 2016; Young *et al.*, 2012).

The molecular mechanism by which HCPro interferes with the antiviral silencing remained elusive until 2006, when Lakatos and co-workers found that, similarly to the well-characterized tombusviral RSS P19, the *Tobacco etch virus* (TEV) HCPro prevents the loading of vsiRNAs into the silencing effector complexes by direct binding to these molecules in a size-specific manner (Lakatos *et al.*,

2006). Although vsiRNA sequestration seems to be a quite common antisilencing mechanism for the HCPro of diverse potyviruses, other non-mutually exclusive alternatives were proposed (Table 1). For instances, HCPro was found to interfere with methylation of vsiRNA 3' end either by inhibiting the production of methyl group through disturbing the methionine cycle (Ivanov *et al.*, 2016; Soitamo *et al.*, 2011), or by direct interaction with and inhibition of HEN1 (Jamous *et al.*, 2011). Interference with AGO-containing effector complexes was also described for the HCPro expressed by TEV and *Potato virus A* (PVA). In the first case, TEV HCPro takes advantage of the homeostatic self-regulation properties of the host RNA silencing pathway (Mallory and Vaucheret, 2010) and enhances the expression of miRNA168 with the consequent down-regulation of its endogenous targets, which include the mRNA of the antiviral AGO1 (Varallyay and Havelda, 2013). In the second case, PVA HCPro directly interacts with the AGO1 in ribosomal complexes, supporting the idea that this RSS is able to somehow alleviate the putative translational repression of the potyviral genome mediated by RNA silencing (Ivanov *et al.*, 2016). Furthermore, HCPro can interfere with the RDR-mediated amplification step, as in the case of the *Sugarcane mosaic virus* (SCMV) HCPro, which down-regulates RDR6 by interfering with the transcription of *RDR6* mRNA (Zhang *et al.*, 2008). Finally, it has been also observed that HCPro blocks a long-distance silencing signal that moves ahead of the viral infection (Delgadillo *et al.*, 2004; Hamilton *et al.*, 2002; Pfeffer *et al.*, 2002). Based on previous results (Lewsey *et al.*, 2016an; Melnyk *et al.*, 2011; Molnar *et al.*, 2010), it is reasonable to hypothesise that vsiRNAs move through the whole plant via the vascular system, and that HCPro-mediated blockage of the long-distance silencing signal relies on direct vsiRNA interaction and sequestration at the infected tissues.

Host factors are also relevant for the HCPro-mediated silencing suppression. Such is the case of the tobacco rgs-CaM, a calmodulin-related protein that directly interacts with TEV HCPro and works as an endogenous (e)RSS (Anandalakshmi *et al.*, 2000). On the other hand, Endres and co-workers found that the RAV2 ethylene-induced transcription factor from *Arabidopsis thaliana* is required for the antisilencing activity of the *Turnip mosaic virus* (TuMV) HCPro. They observed that HCPro interacts with RAV2 and induces the transcription of some putative eRSSs, including the calmodulin-related protein CML38, which seems to be the *A. thaliana*

homolog of the above-mentioned tobacco rgs-CaM (Endres *et al.*, 2010). Altogether, these results raise the possibility that HCPro recruits eRSSs, in a direct (protein-protein interaction) and/or indirect (by RAV2-mediated transcriptional activation) fashion in order to interfere with host defence mechanisms mediated by RNA silencing. Intriguingly, results from other experiments, which are discussed below, indicate that HCPro/rgs-CaM interaction certainly targets this viral protein for degradation (Nakahara *et al.*, 2012).

As the different RNA silencing modules in plants partially overlap, viral RSSs usually interfere not only with the antiviral part, but also with those modules controlling plant developmental programs. Indeed, the presence of pleiotropic developmental defects, associated to disturbances in miRNA function, in transgenic plants constitutively expressing HCPro supports this assumption (Chapman *et al.*, 2004; Kasschau *et al.*, 2003; Mallory *et al.*, 2002) and makes reasonable the idea that the silencing suppression activity of HCPro causes some of the observed potyviral-induced disease symptoms in infected plants. Mlotshwa *et al.* (2005) observed that overexpression of Dicer-like protein 1, the enzyme responsible for miRNA synthesis, rescued the developmental anomalies caused by HCPro but did not correct defects in miRNA pathways. This suggests that disturbance in one or a few miRNA-controlled factors, rather than general impairments in miRNA function, underlies the HCPro-associated developmental disorders. In agreement with this suggestion, misregulation of *AUXIN RESPONSE FACTOR 8* by miR167 was concluded to be the main cause of developmental abnormalities induced by HCPro and other viral silencing suppressors (Jay *et al.*, 2011). However, more recent results challenge this conclusion (Mlotshwa *et al.*, 2016).

Whether HCPro interference with diverse RNA silencing modules is either a collateral effect of silencing suppression or a deliberated viral strategy to favour the infection process, is still a matter of debate. In this regard, synthetic evolution experiments offer an attractive opportunity to analyse these two options. Torres-Barceló and collaborators, for instance, introduced several mutations on TEV HCPro and tested not only the effects of these changes on RNA silencing suppression activity (Torres-Barceló *et al.*, 2008), but also on the infection of tobacco plants (Torres-Barceló *et al.*, 2010), the natural TEV host. Hence, they found that HCPro hypersuppressor variants rapidly evolve toward variants with

moderate, wild type-like, antisilencing capacity, suggesting that this HCPro activity is indeed fine-tuned during TEV infection to minimize the unwanted side-effects of silencing blockage on normal plant development patterns (Torres-Barceló *et al.*, 2010).

STRUCTURE VERSUS FUNCTION – *HCPro is a multidomain viral protein*

After the discovery of its contribution to the aphid-mediated plant-to-plant transmission, another function was ascribed to HCPro: maturation of viral factors by releasing itself from the rest of the polyprotein. Bacterial and *in vitro* studies provided evidence that HCPro is a *cis*-acting proteinase that functions co-translationally and independently of a plant factor, with the cleavage site between a glycine dipeptide at its C-terminus (Carrington *et al.*, 1989a; Carrington *et al.*, 1989b). Genetic analyses by site-directed mutagenesis further characterized two residues, one cysteine and one histidine as the catalytic diad for proteolytic activity, categorizing HCPro in the cysteine-type proteinase family (Oh and Carrington, 1989). Further analyses defined the consensus cleavage sequence surrounding the glycine dipeptide at the HCPro C-terminus to be Y*X*VGG (positions P4 to P1′) (Carrington and Herndon, 1992). HCPro is currently classified in the C6 peptidase superfamily (Rawlings *et al.*, 2016).

Along with the characterization of the protease domain, amino acids and motifs relevant for aphid transmission, movement, RNA binding and RNA silencing suppression were also examined. Schematically, HCPro can be divided into three domains (indicated positions correspond to TEV HCPro): an N-terminal part (amino acids 1-100) required for aphid transmission; a central region (amino acids 101-299) in charge of RNA silencing suppression and other functions; and a C-terminal domain (amino acids 300-459) harboring the proteolytic activity of HCPro (Hasiów-Jaroszewska *et al.*, 2014) (Fig. 3A). As mentioned above in this review, a zinc finger-like domain located at the N-terminus of HCPro, which includes the KITC motif, is associated with potyviral aphid-mediated transmission (Atreya *et al.*, 1992; Atreya and Pirone, 1993) (Fig. 3A). The specific involvement in helping transmission of the N-terminal part was also supported by the emergence of spontaneous TEV, *Lettuce mosaic virus* (LMV) and *Onion yellow dwarf virus* deletion mutants, which even lacking the first 89, 108 or 92 amino acids of HCPro,

respectively, were able to complete the whole viral infection cycle, except propagation by aphids (Dolja *et al.*, 1993; German-Retana *et al.*, 2000; Takaki *et al.*, 2006).

In 1995, Cronin *et al.* described two motifs in the central region of HCPro relevant for viral movement. Two years later, using a series of alanine-scanning mutants built in a TEV-GUS chimeric virus background, Kasschau *et al.* (1997) described several amino acids relevant for genome amplification and long-distance movement that were located mainly in the central region of HCPro. In 2001, and after HCPro was characterized as an RSS, the same group found a strong correlation between silencing suppression and the genome amplification and movement defects that they had observed in the alanine-scanning mutants (Kasschau and Carrington, 2001). They also showed that proteinase and anti-silencing activities worked independently in most studied cases. This indicates that the proteinase function *per se* is not needed for RNA silencing suppression. However, there was a mutation located at the C-terminal part of the protein which disturbed both proteolytic activity and RNA silencing suppression, which demonstrate that the protease domain is also required for silencing suppression activity, for instance, to provide the protein with a proper folding. Furthermore, experiments of scanning mutagenesis via pentapeptide-insertions in the *Plum pox virus* (PPV) HCPro (Varrelmann *et al.*, 2007) or point amino acid substitutions in TEV HCPro (Torres-Barceló *et al.*, 2008) also support a key role of the protein central domain for RNA silencing suppression, and corroborated the idea of inter-domain interactions. On the other hand, a study on *Papaya ringspot virus* (PRSV) showed that the amino terminal part of HCPro is involved in the systemic infection of zucchini (Yap *et al.*, 2009) (Fig. 3A), which is in agreement with the results previously obtained by Atreya *et al.* (1993) in *Tobacco vein mottling virus*. All these findings suggest that HCPro from distinct viruses might have different inter-domain interactions and such interplay between domains might be relevant from structural and functional points of view.

Some early studies attributed to HCPro the ability to bind nucleic acids in a sequence non-specific manner (Maia and Bernardi, 1996; Merits *et al.*, 1998). The involvement of the central region of HCPro in RNA binding was further described by using different deletion mutants (Urcuqui-Inchima *et al.*, 2000). This study

divided the central region of the *Potato virus Y* (PVY) HCPro into domains A and B, which bind RNA *in vitro* independently (Fig. 3A). Remarkably, Lakatos *et al*. showed by 2006 that the RNA silencing suppression activity of TEV HCPro involved siRNA binding (see above), and later on the conserved FRNK motif, which overlaps with the RNA binding domain A, was shown to be relevant for HCPro/siRNA interaction (Shiboleth *et al.*, 2007; Wu *et al.*, 2010). On the other hand, a study based on the HCPro from PPV described that this protein also works as an enhancer of viral particle yield (see below). Mutational analyses located the relevant amino acids for this novel activity also in the central region of HCPro (Valli *et al.*, 2014) (Fig. 3A).

Even from early reports about HCPro, it was proposed that this viral protein normally adopts a complex quaternary structure (Thornbury *et al.*, 1985). This idea was later supported by diverse works on the self-interaction of the HCPro from PVA, PVY and LMV, in which crucial motifs for oligomerization were found by yeast two-hybrid at both the N-terminal and the C-terminal parts of the protein (Guo *et al.*, 1999; Urcuqui-Inchima *et al.*, 1999a; Urcuqui-Inchima *et al.*, 1999b). Similar results were obtained years later for TuMV HCPro by using bimolecular fluorescence complementation assays (Zheng *et al.*, 2011). Plisson *et al*. (2003) studied this matter more precisely via protein purification from infected plants and characterization of both wild type and an N-terminal deletion mutant of LMV HCPro, which lacks its first 100 amino acids. Since full-length protein and the shorter version were observed in size exclusion analysis to behave as dimer or trimer in solution, the authors concluded that the N-terminus of LMV HCPro is not involved in self-interaction. Furthermore, chemical crosslinking confirmed the presence of dimers, tetramers and higher order oligomers in solution, whereas the observation of 2D crystals by electron microscopy showed the appearance of dimers that bound to form tetramers. In agreement with earlier observations regarding the role of cations in HCPro stabilization, crystal formation occurred only in the presence of Mg^{2+}. Additional structural studies, which were conducted with TEV HCPro purified from infected plants and observed by electron microscopy, confirmed the oligomerization states mentioned above. Although dimers, tetramers and hexamers of HCPro were indeed observed in solution, an adjusted model proposed that, at least in the particular case of TEV, the self-

interaction between monomers occurs on a V-shape conformation with HCPro located in an antiparallel orientation (Ruiz-Ferrer *et al.*, 2005).

The most recent data regarding structural features of HCPro comes from a 3D crystal structure solved by Guo *et al.* (2011) corresponding to 158 amino acids, including the protease domain, from TuMV HCPro (Fig. 3B). This peptide was produced in bacteria, and formation of oligomers was actively avoided in order to facilitate crystal formation, so that structural questions regarding dimerization are still unanswered. In any case, the atomic structure of amino acids 336-458 showed several features of high interest. First, it confirmed the identity of the previously proposed protease catalytic diad and established the presence of the C-terminal glycine tightly bound to the enzymatic cleft. This observation might indeed explain the exclusive *cis*-acting mode of HCPro, since the terminal glycine would occupy the space needed for the catalytic site to remain active. Unfortunately, the attempts of Guo *et al*. to remove this amino acid in order to make the proteinase active *in trans*, as was later done for the CP serine proteinase of alphaviruses (Aggarwal *et al.*, 2014), were unsuccessful. Second, the overall structure of this domain allowed for accurate comparisons with existing structures of other cysteine-like proteinases, such as papain, indicating that HCPro atomic arrangement differs significantly from the distinctive papain-like folding. It presents a highly reduced 4-helical domain that harbors the catalytic cysteine and in which helices α1-α3 roughly covers the L domain of papain (Fig. 3B, in green), and it has a small ß-barrel that carries the catalytic histidine in which strands ß1-ß2 would match the R domain of papain (Fig. 3B, in orange). Intriguingly, comparison with other cysteine proteinases revealed clear similarities between HCPro and the alphavirus nsP2 protein, as both have a compact fold with similar secondary structure topology. All in all, the atomic model of this domain represents the perfect opportunity to get more fully acquainted with its proteinase activity. Previous studies using high doses of human cystatin C (García *et al.*, 1993) and phytocystatins and human stefin A (Wen *et al.*, 2004) showed inhibition of the HCPro proteolytic activity *in vitro,* and genetically modified plants expressing oryzacystatin I proved to be resistant to TEV and PVY infection (Gutierrez-Campos *et al.*, 1999). Now, with a molecular structure of the protease domain available, it

should be possible to design novel chemicals aiming to disturb HCPro self-cleavage as an effective antiviral strategy.

Bacterially expressed HCPro was also useful to raise antisera allowing the study of protein subcellular localization. For instance, antiserum to PPV HCPro recognized not only this protein, but also the HCPro from ten other potyviral species, and was able to (i) label amorphous inclusions in the cytoplasm of plant cells infected with PPV, PRSV, *Pepper mottle virus* and *Tobacco vein mottling virus*, (ii) label pinwheels in cells infected with *Bean yellow mosaic virus* and *Clover yellow vein virus* (ClYVV), (iii) gave scattered signals in the cytoplasm of cells infected with *Bidens mottle virus*, and (iv) highlight nuclear inclusions in cells infected with TEV and *Beet mosaic virus* (Riedel *et al.*, 1998). Similarly, HCPro from *Cowpea aphid-borne mosaic virus* was used to prepare antiserum for immunofluorescence assays, which showed diffuse distribution of the protein in the cytoplasm of naturally infected cells (Mlotshwa *et al.*, 2002). Bimolecular fluorescence complementation assays located transiently expressed TuMV HCPro oligomers diffused in the cytoplasm of plant cells and/or associated in granules along the endoplasmic reticulum (Zheng *et al.*, 2011; Zilian and Maiss, 2011). The most recent and thorough examination of subcellular localization comes from a study performed by del Toro *et al*. (2014) with the PVY HCPro fused to diverse fluorophores. In addition to a diffuse presence of this viral protein in the cytoplasm, they also observed distinct protein distributions (e.g. amorphous cytoplasm inclusions containing α-tubulin, dot-like inclusions distributing regularly throughout the cytoplasm and associated to the endoplasmic reticulum and the microtubule cytoskeleton, all over the microtubules) that are influenced by the environmental conditions. Altogether, these results suggest that HCPro might be not attached to one single place inside infected cells; instead its location may change during the infection cycle in order to cope with its multiple functions and/or as a response to external changes. The spatial/temporal distribution of HCPro, as well as the putative link between this potentially dynamic subcellular localization and diverse HCPro functions, indeed deserves further studies.

ADDITIONAL ROLES OF HCPro - *the advantage of being promiscuous*

HCPro interacts with several host and viral proteins, and because most of these interactions appear to be unrelated to the three well-known roles of this viral factor - namely aphid transmission, viral polyprotein processing (see below) and suppression of host antiviral RNA silencing - it has been proposed that such interactions are part of additional, much less characterized, functions of HCPro during potyvirid infections (Table 2 summarizes these interactions and the hypothetical role that they play during the infection cycle). For instance, it has been shown that HCPro from several potyviruses interacts and modulates the activity of the host proteasome. Ballut *et al.*, who proposed this role for the first time by 2005, found that LMV HCPro binds to and inhibits the activity of the 20S proteasome. Surprisingly, the presence of HCPro just inhibited the RNase activity of this multi-catalytic complex, which targets *in vitro* the viral RNA genome for degradation, whereas the proteolytic activity of the 20S proteasome was either unchanged or even slightly stimulated (Ballut *et al.*, 2005). Further on, it was described that PVY HCPro interacts with the PAA, PBB and PBE subunits of the *A. thaliana* 20S proteasome, but not with the PAE subunit, which certainly carries the ribonuclease activity (Jin *et al.*, 2007a). However, Dielen *et al.* (2011) were later able to detect the interaction between LMV HCPro and PAE in diverse systems, even in the context of a LMV infection in lettuce. Similar studies with PRSV proved that the proteasome inhibitor MG132 has a positive effect on PRSV accumulation in papaya, and that PRSV HCPro, similarly to PVY HCPro, interacts with the PAA, but not with the PAE subunit of the papaya 20S proteasome (Sahana *et al.*, 2012). Moreover, additional experiments of Sahana *et al.* indicated that PAA and PAE subunits interact with each other. Thus, these authors mitigated discrepancies with the HCPro-PAE interaction and its consequences by proposing that (i) binding between HCPro and PAA may either be sufficient to disturb the RNase activity of PAE or prevent the interaction of the PAA and PAE subunits, and (ii) HCPro from different potyviruses might interact with different components of the 20S proteasome, depending on the specific plant/virus combination (Sahana *et al.*, 2012). All in all, results from the above-mentioned studies suggest that the 20S proteasome works as another defence layer against members of the *Potyviridae* family, and that HCPro interferes with the proteasome activity as a viral counteractive measure.

Potyvirid infections frequently alter the chloroplast number and morphology, leading to decreased level of photosynthesis in the infected tissue (Pompe-Novak *et al.*, 2001). Indeed, HCPro was earlier found to accumulate in chloroplasts of PVY-infected tobacco cells (Gunasinghe and Berger, 1991), and further analyses reported an interaction between a chloroplast protein, NtMinD, and PVY HCPro (Jin *et al.*, 2007b). Given that homodimers of NtMinD participate in chloroplast division, PVY HCPro might prevent the NtMinD self-interaction with the consequent alteration in the chloroplast number (Jin *et al.*, 2007b). Moreover, a recent work not only confirmed the presence of PVY HCPro in the chloroplast, but also showed that the ATPase activity of NtMinD is reduced in the presence of this viral protein (Tu *et al.*, 2015b). Such observations allowed these authors to provide an explanation for the commonly observed abnormal morphology of chloroplasts in the presence of PVY. In a parallel study, Tu *et al.* (2015a) also found that PVY HCPro interacts in tobacco with the CF1β–subunit of the chloroplast ATP synthase. Such interaction leads to a decreased number of active enzymatic complexes, with the consequent overall reduction of the ATP synthesis in the chloroplast of both HCPro transgenic and PVY-infected tobacco plants, which in the end reduces the net photosynthetic rate. The interaction between HCPro and the tobacco chloroplast protein 1-deoxy-D-xylulose-5-phosphate synthase (NtDXS) has been recently described (Li *et al.*, 2015). Since NtDXS is a limiting enzyme for plastidic isoprenoid biosynthesis in plants (Estévez *et al.*, 2001), an effect of this interaction in the production of diverse isoprenoids, such as chlorophylls, tocopherols, carotenoids or abscisic acid (ABA), is expected. Certainly, PVY HCPro enhances the activity of NtDXS, thereby boosting the isoprenoid biosynthesis pathway with the consequent increase in the level of certain pigments, ABA and ABA-responsive genes (Li *et al.*, 2015). On the other hand, Cheng *et al* (2008). showed that SCMV HCPro interacts with the maize chloroplast precursor, but not the mature form, of ferrodoxin-5. Therefore, this interaction might disturb the post-translational import of ferrodoxin-5 into maize chloroplasts, which would then lead to the perturbation of chloroplast structure and function. However, even though evidences for the implication of HCPro in chloroplast distortion, photosynthesis reduction and alteration of isoprenoid metabolism in infected plants are very

strong, the meanings of these HCPro-mediated effects for the virus infection remain unclear.

PRSV HCPro binds to the papaya calreticulin (PaCRT) protein, in particular with its calcium-binding domain located at the protein C-terminus, whereas PRSV infection enhances *PaCRT* transcription at the early days post-infection (Shen *et al.*, 2010). Given that Ca^{+2} is considered an essential second messenger that participates in many plant signal pathways, including defence signalling (Zhang *et al.*, 2014), HCPro might be disturbing the calcium-binding capacity of PaCRT and thereby mitigating the activation of downstream pathways (Shen *et al.*, 2010).

PVA HCPro was found to interact with the HCPro interacting protein 2 (HIP2) from *Solanum tuberosum* and *Nicotiana tabacum,* two natural hosts of PVA (Haikonen *et al.*, 2013b). Moreover, as a positive interaction was also observed for HCPro from PVY and TEV, which have a similar host range than PVA, but not for HCPro from *Pea seed-borne mosaic virus* PSbMV, which infect just a few species in the *Solanaceae* family, a role of this interaction in virus/host specificity was proposed (Haikonen *et al.*, 2013a). HIP2 is a microtubule-associated protein similar to *A. thaliana* SPR2 and, as evidence of the HCPro/HIP2 importance for viral infection, depletion of this host factor or mutations in HCPro abolishing HIP2 binding reduced PVA titre in different hosts. Although the precise functional role of this interaction is currently unknown, SPR2 interacts with (i) many receptor-like kinases associated with plant innate immunity and (ii) two transcription factors related to immune responses (Mukhtar *et al.*, 2011). This led Haikonen *et al.* (2013a; 2013b) to hypothesize that HIP2 controls some signalling networks of defence responses, and that HCPro might subvert this controller, via protein/protein interaction, to the benefit of the virus.

PVA induces the formation of small aggregates containing the acidic ribosomal protein P0 in the cytoplasm of infected cells referred to as PVA-induced granules (PGs) (Hafrén *et al.*, 2015). The formation of PGs was specifically triggered by HCPro and, besides P0, they contain HCPro, the RNA silencing effector AGO1, the oligouridylate-binding protein 1, varicose, an isoform of translation initiation factor 4E [eIF(iso)4E], and even the viral RNA genome (Hafrén *et al.*, 2015). Notably, only anti-silencing proficient HCPro variants were shown to promote the formation of PGs, as observed by direct mutagenesis. Based on these

results, and the known link between host proteins located in PGs and the viral VPg, the authors proposed that the formation of these granules are required to overcome RNA silencing-based defences via relocation of AGO1 towards PGs, and to achieve optimal viral expression mediated by VPg (Hafrén *et al.*, 2015).

Ala-Poikela and co-workers found clear evidences of direct interaction between the HCPro from three different potyviruses (PVA, PVY and TEV) and the translation initiation factors eIF(iso)4E and eIF4E from potato and tobacco (Ala-Poikela *et al.*, 2011). Moreover, a putative eIF4E-binding motif was identified at the C-terminal part of PVA HCPro, which showed a high degree of conservation among other potyviruses. Certainly, the disruption of this motif by direct mutagenesis had a negative impact on HCPro/eIF4E binding and was detrimental to the virulence of PVA, supporting the idea that such interaction plays an important, yet unknown, role during viral infection (Ala-Poikela *et al.*, 2011). However, this inference should be taken with some caution, as a further study showed that this mutation strongly reduced the RNA silencing suppression activity of PVA HCPro (Hafrén *et al.*, 2015).

HCPro interacts with itself (discussed above) and with some of the other viral proteins. Physical interaction between HCPro and VPg has been described for different potyviruses (Ivanov *et al.*, 2016; Roudet-Tavert *et al.*, 2007; Yambao *et al.*, 2003), suggesting that joint action of these two proteins might play a general role during potyviral infections. Intriguingly, as already mentioned, Torrance *et al.* (2006) showed the presence of a protruding tip at one end in a fraction of potyviral virions, which was suggested to be formed by HCPro in association with VPg. The authors discussed that this interaction might play a role in aphid-mediated plant-to-plant transmission or even in cell-to-cell movement. On the other hand, different lines of evidence showed that interaction of HCPro with VPg involves the same central domain of the latter protein that interacts with eIF4E (Roudet-Tavert *et al.*, 2007; Yambao *et al.*, 2003) Indeed, HCPro and eIF4E from LMV and lettuce, respectively, compete for VPg binding (Roudet-Tavert *et al.*, 2007). The outstanding relevance of the VPg/eIF4E (in its two isoforms) interaction for potyvirid infections has been extensively studied as a model of plant recessive resistant (Robaglia and Caranta, 2006; Truniger and Aranda, 2009; Wang and Krishnaswamy, 2012). The most accepted, but not yet demonstrated, model proposes that VPg works as a pseudo cap structure that recruits translation

complexes for the viral use. As already mentioned, HCPro and eIF4E also interact with each other (Ala-Poikela *et al.*, 2011), so that deducing the actual role of HCPro in this protein trio seems complicated. HCPro might be part of the translational complex that is recruited by VPg at the 5' end of the viral genome to either carry out an unknown function or, in line with the silencing suppression activities of both viral proteins (Rajamäki and Valkonen, 2009), interfere with the hypothetical inhibition of virus translation mediated by host-deployed RNA silencing defences, as recently proposed (Ivanov *et al.*, 2016).

Interaction of HCPro with the CI protein of a quite large number of potyvirids has also been detected using different experimental systems (Choi *et al.*, 2000; Guo *et al.*, 2001; Ivanov *et al.*, 2016; Zilian and Maiss, 2011). CI is a multifunctional RNA helicase that participates in viral replication and cell-to-cell movement (Sorel *et al.*, 2014) and, as for HCPro, it is attached to the tip at one end of a fraction of viral particles, at least in the case of PVA (Gabrenaite-Verkhovskaya *et al.*, 2008). It is possible to envisage a scenario in which HCPro somehow collaborates with CI in virus cell-to-cell movement or even that HCPro moves between adjacent cells, as part of a ribonucleic complex, to exert any of its multiple functions in a newly infected neighbour cell, as previously suggested (Rojas *et al.*, 1997).

Given the well-established role of HCPro in viral plant-to-plant transmission, at least for members of *Potyvirus* and *Tritimovirus* genera, the interaction between HCPro and CP is the most evident among potyvirid proteins. As expected, such binding has been detected in diverse viruses by different methods (Blanc *et al.*, 1997; Guo *et al.*, 2001; Kang *et al.*, 2004; Lin *et al.*, 2009; Peng *et al.*, 1998; Roudet-Tavert *et al.*, 2002; Seo *et al.*, 2010). Intriguingly, the HCPro/CP interaction has been also detected in aphid non-transmissible potyviruses (Manoussopoulos *et al.*, 2000; Roudet-Tavert *et al.*, 2002), suggesting the existence of a functional role for this protein/protein complex different from aphid-mediated transmission. In agreement with this hypothesis, Valli *et al.* (2014) found that HCPro plays a key role in PPV infection by enhancing the yield of full-length viral particles. This novel function of HCPro is not linked to its other main activities, as observed by direct mutagenesis. Furthermore, this activity appears to be highly specific, meaning that HCPro would act only upon its cognate CP. Even though the exact molecular mechanism by which HCPro enhances the yield of intact virions is currently

unknown, authors proposed two non-mutually exclusive possibilities, both agreeing with known localization of HCPro at the end of the viral particles: (i) HCPro is involved in initial steps of the assembly of CP subunits, and/or (ii) HCPro stabilizes viral particles once they are fully assembled. They also speculated about how the spatiotemporal availability of HCPro might function as a device that coordinates different stages of the viral cycle, namely translation, replication and encapsidation, in the infected cell (Valli *et al.*, 2014). As a matter of fact, a recent report has located HCPro in 6K2-induced replication vesicles in PVA infected plants (Lõhmus *et al.*, 2016).

HCPro AS TRIGGER AND TARGET OF PLANT DEFENCE RESPONSES – *defence, counter-defence, counter-counter-defence*

Given the outstanding importance of HCPro in multiple steps of the viral infection, it is not surprising that its recognition by the host might induce mechanisms to counteract its action and trigger other defence responses. And, as the proviral activities of HCPro do, the antiviral reactions elicited by HCPro can also contribute to the development of disease symptoms (García and Pallás, 2015).

Defence responses triggered by HCPro can be non-specific. For instance, Pruss *et al.* (2004) showed that, whereas TEV HCPro suppresses RNA silencing-related antiviral defences, it confers enhanced broad-spectrum resistance against multiple pathogens, including heterologous viruses, via both salicylic acid (SA)-dependent and SA-independent mechanisms. Evidence for alteration of SA-mediated defences as a consequence of TEV HCPro expression in transgenic lines was also provided by Alamillo *et al.* (2006). Enhancement of host defence responses induced by potyviral HCPro appears to be temperature dependent (Shams-Bakhsh *et al.*, 2007). More recent results suggested that HCPro might enhance the expression of defence-related genes in the SA pathway by reducing the DNA methylation at their promoter regions, which is associated with a drastic reduction of siRNAs deriving from these sequences (Yang *et al.*, 2016).

HCPro also induces more specific defence responses. Namely, this viral protein can act as elicitor of *R* gene-driven effector-triggered immunity. This is the case of some strains of PVY, which induce a hypersensitive response (HR) that restricts the virus in necrotic local lesions in potato cultivars harboring the

dominant resistance genes Nc_{tbr} and Ny_{tbr} (Moury *et al.*, 2011; Tian and Valkonen, 2015). These resistance genes appear to recognize similar structural determinants in the central region of HCPro of PVY^{O} (Ny_{tbr}) and PVY^{C} (Nc_{tbr}) strains (Tian and Valkonen, 2013, 2015). PVY isolates overcoming Ny_{tbr} often cause veinal necrosis in tobacco, and some determinants of this phenotype have been identified in HCPro (Faurez *et al.*, 2012; Tribodet *et al.*, 2005). However, avirulence determinants of Ny_{tbr} are different from those responsible for veinal necrosis induction (Tian and Valkonen, 2015).

Some PVY isolates induce necrotic symptoms in potato tubers and a mutation in HCPro linked to the ability to induce tuber necrosis is also involved in induction of veinal necrosis in tobacco (Glais *et al.*, 2015; Tribodet *et al.*, 2005). There is no evidence that veinal necrosis in tobacco and potato tuber necrosis are HR-like responses to specific interactions between avirulence factors. The fitness decrease caused by point mutations associated with the acquisition of necrosis properties in tobacco may suggest that the necrotic reaction was connected with a defensive response (Rolland *et al.*, 2009). However, the fact that these mutations also had a fitness cost in a host that does not show necrotic symptoms questions this conclusion.

Necrotic symptoms were also observed in tobacco plants infected with PVA modified by mutations in a highly variable region of the central part of the HCPro protein (Haikonen *et al.*, 2013a). These mutations, which affect interactions with a microtubule-associated protein (see above for HCPro/HIP2 interaction) and were suggested to cause conformational changes in adjacent regions of the protein, were associated with reduction of viral accumulation and induction of many defence-related genes including ethylene- and jasmonic acid-inducible genes, at necrosis onset (Haikonen *et al.*, 2013a). Taking together all these data, a scenario has been proposed in which alterations of HCPro conformation by mutations that overcome *R* gene-mediated specific resistance affect functional interactions with other host factors and induce alternative defence responses (Tian and Valkonen, 2015).

Another example of a resistance gene elicited by the HCPro of a potyvirus is a gene located at the complex *Rsv1* locus of soybean, likely belonging to the NB-LRR class, which recognizes the HCPro of *Soybean mosaic virus* (SMV) (Eggenberger *et al.*, 2008; Hajimorad *et al.*, 2008; Wen *et al.*, 2013). The precise mechanism

involved in induction of resistance by SMV HCPro is still unclear. The HCPro-responsive resistance gene alone allows limited replication at the inoculation site. However, the complete *Rsv1* cluster, which includes at least one additional, P3-responsive, SMV resistance gene, confers extreme resistance against avirulent SMV variants (Wen *et al.*, 2013). The first identified SMV isolate able to overcome the resistance conferred by the *Rsv1* locus caused a lethal systemic HR phenotype probably due to a weak interaction of the viral avirulence factors and the host resistance genes (Hajimorad *et al.*, 2005). HCPro is likely contributing to this phenotype, since some SMV isolates carrying mutations at HCPro also provoked systemic HR in soybean plants only containing the HCPro-responsive gene of the *Rsv1* cluster (Wen *et al.*, 2013), and a single amino acid substitution in this viral protein allowed virulent SMV to cause severe rugosity and local necrotic lesions, instead of lethal systemic HR, in soybean expressing the complete *Rsv1* cluster (Seo *et al.*, 2011). Interestingly, gain of virulence of SMV on the *Rsv1* soybean genotype had a fitness penalty in susceptible *rsv1* plants, and this trade-off was a consequence of the mutations introduced in HCPro, during the adaptation to the resistance selective pressure (Khatabi *et al.*, 2013). This observation emphasizes the convenience for the host of triggering antiviral defences against important multifunctional proteins, as this strategy might cause a high global fitness cost, even extinction, for the escaping viruses.

Some of the HCPro contributions in the induction of host defence responses may be indirect. It is reported that ClYVV activates SA signaling and HR-related pathways causing systemic necrosis and plant death in pea containing *Cyn1*, a gene mapped in a genomic region that corresponds to an *R*-gene-analog gene cluster in the genome of *Medicago truncatula* (Ravelo *et al.*, 2007). Point mutations in ClYVV HCPro that attenuate RNA silencing suppression activity and symptom expression in broad bean (Yambao *et al.*, 2008), indeed, reduced the ability of ClYVV to activate the SA signaling pathway and to induce cell death in the *Cyn1*-containing plants (Atsumi *et al.*, 2009). Although these results might suggest that ClYVV HCPro itself is the elicitor of the *Cyn1*-controlled response, the authors consider it is more likely that the reduced activity of the mutated HCPro limits viral amplification and, subsequently, the accumulation of the host factor(s) triggering the defence response (Atsumi *et al.*, 2009). A similar scenario, in which reduced HCPro activity

maintains viral amplification below levels inducing host detrimental effects, has been proposed to explain why a PPV mutant with unrestricted P1/HCPro processing causes more severe symptoms with lower accumulation levels than the wild type virus (Pasin *et al.*, 2014).

Destroying HCPro is another defence response that the plant deploys to counteract RNA silencing suppression and other activities of this important virulence factor. As mentioned above, the calmodulin-related protein rgs-CaM from tobacco was identified as a host factor that interacts with TEV HCPro and contributes by itself to suppress RNA silencing (Anandalakshmi *et al.*, 2000). More recently, it was observed that binding of rgs-CaM to the dsRNA binding domains of different viral RSSs, including HCPro from ClYVV, directs them to the autophagy-like pathway for degradation (Nakahara *et al.*, 2012). Therefore, whereas HCPro/rgs-CaM interaction is soundly supported by all experimental evidence available, the integration of both positive and negative effects of this interaction on suppression of RNA silencing in a comprehensive model is still missing.

THE DIVERSITY OF HCPro AND HCPro-LIKE PROTEINS – *Similar but different*

The *Potyviridae* family comprises viruses from eight different genera. Most of the studies presented here have been carried out with the HCPro from species of the *Potyvirus* genus, which is by far the most abundant one. In members of this genus, the N-terminal part of viral polyproteins follows the same pattern: a P1 leader serine proteinase that processes itself to separate from HCPro, which in turn cleaves at its C-terminus to be released from the rest of the polyprotein (Fig. 4). In potyviruses, as well as in members of the genus *Rymovirus*, HCPro has a molecular weight of around 50KDa, and those motifs described in this review are predominantly conserved. As described above, the most outstanding feature of the HCPro from poty- and rymoviruses is its ability to suppress RNA silencing. To date, the only discovered exceptions to this rule are the sweet potato-infecting potyviruses, which express an apparently normal HCPro variant that has no evident anti-silencing activity. Even more surprising is the fact that all of these viruses express an atypically long P1 with a viral polymerase slippage site that generates an extra ORF, termed PISPO. This new ORF gives rise to a transframe

protein, named P1N-PISPO, with RNA silencing suppression activity (Clark *et al.*, 2012; Li *et al.*, 2012; Mingot *et al.*, 2016; Untiveros *et al.*, 2016).

Poace- and tritimoviruses are two related genera bearing HCPro of similar or slightly reduced size compared to poty- and rymoviruses. Although these four genera share the same genome organization, the RNA silencing suppression activity is exerted by P1, instead of HCPro, in poace- and tritimoviruses, and the HCPro of the tritimovirus *Wheat streak mosaic virus* is not needed for virus viability (Stenger *et al.*, 2005a; Stenger *et al.*, 2007; Tatineni *et al.*, 2012; Young *et al.*, 2012). These observations are in perfect agreement with sequence comparison data showing that strong similarities among HCPro variants from viruses of these four genera are just displayed at the protease domain (C-terminal region) (Guo *et al.*, 2011). In contrast, the central region of poace- and tritimoviral HCPros, where the anti-silencing activity mainly maps in potyviruses, is highly different and does not have the typical FRNK motif.

The most diverse potyvirids regarding genome organization at the 5' end are those belonging to the *Ipomovirus* genus, which can be even divided into two groups based on the presence or absence of HCPro. The first ipomovirus species to be described was *Sweet potato mild mottle virus* (SPMMV) (Colinet *et al.*, 1998), a virus that encodes an unusually large P1 protein that works as a RSS (Giner *et al.*, 2010). Interestingly, this virus codes for an HCPro that is similar in size to that of potyviruses, but contains no RNA silencing suppression activity. Phylogenetic analyses aligned SPMMV closer to tritimoviruses than to other potyvirids (Stenger *et al.*, 1998) and, as expected, SPMMV HCPro lacks sequence similarity with potyviral HCPros outside the protease domain. Ipomoviruses without HCPro have one P1 copy (Mbanzibwa *et al.*, 2009) or two divergent P1 copies in tandem (Desbiez *et al.*, 2016; Janssen *et al.*, 2005; Li *et al.*, 2008; Valli *et al.*, 2006) at the N-terminal part of the viral polyprotein. Remarkably, like in the case of SPMMV, all ipomoviruses lacking HCPro use P1 as an RSS.

In 2008, Susaimuthu *et al.* identified and fully sequenced *Blackberry virus Y*, which was classified as the founder member of a new potyvirid genus, named *Brambyvirus*. Downstream of an unusual P1, the *Blackberry virus Y* genome codes for an also atypical HCPro, reduced in size (36 KDa) and bearing in common with HCPro from other potyvirids only the cysteine protease domain. It is still unknown

what protein from this virus, if any, blocks the RNA silencing-based defences deployed by the host.

Bymovirus is the only bipartite genus of the *Potyviridae* family. Bymovirus RNA1 codes for a polyprotein that starts at a protein homologous to the potyviral P3 and follows the *Potyviridae* genomic pattern until the 3' UTR (Kashiwazaki *et al.*, 1990). The bymovirus RNA2 codes for two proteins, the second one is not related to any of the potyvirid proteins, but the first one is described as HCPro-like because of its cysteine proteinase domain (Kashiwazaki *et al.*, 1991). This protein (P2-1) is very small (28 kDa) and has no other motifs that relate it to other potyvirid HCPro.

The first member of the genus *Macluravirus* to be fully sequenced was *Chinese yam necrotic mosaic virus* (Kondo and Fujita, 2012). This virus presents the smallest monopartite genome in the family *Potyviridae*. It lacks a P1 leader proteinase and it codes for an HCPro of just 29KDa. Whether this protein has RSS activity or not is still unknown. Macluraviral HCPro appears to be more similar to the bymoviral P2-1 than to other potyvirid HCPro.

The closest relative of HCPro outside the *Potyviridae* family can be found in the picorna-like, fungal-infecting, hypoviruses. Sequence similarity, putative active site and cleavage site composition relate HCPro to p29 and p48 cysteine proteinases of *Cryphonectria* hypoviruses (Choi *et al.*, 1991a; Choi *et al.*, 1991b; Shapira and Nuss, 1991). A study performed by Suzuki *et al.* in 1999 mapped the p29 symptom determinants outside the protease domain, in a region within the N-terminus of the protein. This domain contains four cysteine residues, similar to the conserved residues in the zinc finger domain of HCPro, which are essential for virus viability. Moreover, both p29 and HCPro proteins alter host developmental processes when expressed in the absence of virus infection (Suzuki *et al.*, 2003). Even more important is the fact that p29 has synergistic effects over other fungal viruses (Sun *et al.*, 2006) likely linked, as in the case of HCPro, to the RNA silencing suppression activity that p29 displays in the natural fungal host and in plants (Segers *et al.*, 2006).

Unrelated plant viruses encoding proteins similar to HCPro can be found in the *Closteroviridae* family. They belong to the Sindbis virus-like supergroup and share in common with potyvirids the presence of leader proteinases with C-

terminal papain-like domains, which are also multifunctional factors with apparent crucial domain interplay. Unlike poty- and rymoviral HCPro, these leader proteinases seem to lack RNA silencing suppression activity, but are certainly involved in genome amplification and participate in cell-to-cell movement (Peng *et al.*, 2001). Proteins related to HCPro are also found in animal viruses. Such is the case of alphaviruses, which, as closteroviruses, also belong to the Sindbis virus-like supergroup, and encode leader proteinases sharing remarkable structural homology with HCPro at the level of its cysteine protease domain (Guo *et al.*, 2011).

FUTURE PERSPECTIVES – *Looking forward*

The genome organization of viruses belonging to the family *Potyviridae* is highly conserved in a large core region that starts at the P3 cistron. Coincidentally, mature proteins encoded at this viral segment are all released from polyprotein precursors by proteolytic processing conducted by the NIapro protease (Valli *et al.*, 2015). In contrast, the upstream genomic region is highly variable even among members of the same genus, and encodes proteins that are liberated from the polyprotein precursors by self-cleavage. Thus, it is tempting to speculate that an ancient potyvirid precursor had a simplified genome that only coded for the NIapro-processed module. Although a sound and confident prediction of the *Potyviridae* evolutionary history is out of the scope of this review, we dare to continue speculating that the first step toward contemporary potyvirids was the acquisition of a second genome element in a bymovirus, which includes what would be the first HCPro-related protein: the P2-1 cysteine proteinase. Either as a subsequent step from a bymovirus, or as a parallel event from the proposed potyvirid ancestor, an HCPro-like gene would be incorporated in the viral genome to give rise to the simplest modern monopartite potyvirid: a macluravirus. Then, further evolution would have boosted HCPro size, diversity and functional complexity.

But, what was the primordial function of the proto-HCPro? We do not know, but it is unlikely that such a small protein was able to suppress silencing or help transmission by aphids or other vectors. We do not even know whether or not this function is still conserved by the currently large HCPros from different potyvirid

genera. Research in the barely studied HCPro from macluraviruses and brambyviruses, as well as in P2-1 from bymoviruses, could certainly shed some light on the evolutionary path not only of these multifunctional viral proteins, but also of the entire viral family.

HCPro is a quite well conserved protein in members of the genus *Potyvirus* for which the nucleotide sequences of this factor have been determined so far; it is then surprising the large diversity of this factor within the entire *Potyviridae* family. This could be justified by the assumption that the primordial HCPro was a recently acquired accessory factor, then having some flexibility to evolve and incorporate new functions. In this scenario, HCPro could adopt diverse activities in the different evolutionary lineages that have originated each potyvirid genus. Moreover, several new activities could pyramid in a single protein, as occurred with the HCPro of potyviruses, although the coupling among different protein functions might restrict its ability to evolve (Hasiów-Jaroszewska *et al.*, 2014). On the other hand, HCPro has been shown to be elicitor and target of different plant defence responses; thereby the escape from these responses should also limit its potential to evolve.

The fact that engineered members of the *Potyvirus* genus depleted of HCPro are unable to infect wild type plants, but infect RNA silencing-deficient plants, and that unrelated RNA silencing suppressors are able to functionally replace HCPro, indicates that the main function of the present potyviral HCPro is suppressing the RNA silencing-mediated antiviral defences (Carbonell *et al.*, 2012; Garcia-Ruiz *et al.*, 2010; Maliogka *et al.*, 2012). However, further studies using systems biology approaches will be required to decipher the contribution to the overall silencing suppression of those HCPro activities somehow related to this function (Table 1).

Although silencing suppression-unrelated activities of HCPro are not absolutely essential, they have been shown to be relevant for the viral infection. Further characterization of these activities to understand how they are integrated in the infection process also needs to be the target of future studies. Indeed, the development of appropriate real-time imaging techniques that allow unveiling the HCPro localization dynamics in the infected cell would be especially helpful for this aim.

In spite of being the first HCPro function identified, very little is known about how HCPro plays its role as bridge during aphid transmission. Identifying the HCPro receptor in the aphid stylet and characterizing the dynamic of virion/HCPro/aphid interactions that governs both acquisition and release of viral particles by insects are among the most interesting future challenges of HCPro research.

Finally, whereas the crystal structure of the protease domain of a potyviral HCPro has been solved at 2.0 Å resolution (Guo *et al.*, 2011), no high-resolution structure of the complete HCPro is currently available. Solving the structure of HCPro alone and bound to viral and host co-factors, or even bound to nucleic acids (e.g. siRNAs, miRNAs), would be of great value to understand the multiple functions of this amazing protein.

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FIGURE LEGENDS

Figure 1. "Bridge hypothesis" for aphid transmission of potyviruses. (left) An aphid is feeding from an infected plant. (centre) Longitudinal section of the mandibular stylet (the external flanking maxillae have been omitted to simplify the figure), including the two parallel channels (the food canal that connects to the digestive system, and the salivary canal that allows secretions during feeding) joining at the common duct. (right) An HCPro complex (depicted in a dimeric form) is bound at one end of the viral particle and allows a reversible interaction with potential receptors located over the cuticle lining (internal side of the stylet tip). Note that this figure is just a predictive representation of the viral transmission process based on very limited available experimental data about interactions and the consequent role of HCPro during this process (see text for details). Hence, it cannot be ruled out, for instance, that HCPro/CP interaction might occur all along the viral particle and non-dimeric forms of HCPro play a role in viral transmission.

Figure 2. Potential targets of HCPro in the antiviral RNA silencing pathway. Simplified schematic representation of the RNA silencing-mediated defences in plants that are deployed against RNA viruses. Steps of the cascade at which HCPro from different potyvids may be acting in order to block this defensive response are indicated. DCL: Dicer-like protein; DRB: Double-stranded RNA Binding Protein; HEN1: HUA Enhancer 1; RISC: RNA-Induced Silencing Complex; AGO: Argonaute protein; RDR: RNA-Dependent RNA-polymerase; SGS3: Supressor of Gene Silencing 3.

Figure 3. HCPro structural and functional features. (A) Schematic representation of a representative potyviral HCPro (from *Tobacco etch virus*, TEV) divided into three main regions. Best characterized motifs are shown in squares. Amino acids relevant for a given function, which are conserved at least among the *Potyvirus* genus, are marked with triangles at their corresponding positions. Amino acids relevant for viral movement (marked in light blue) were described before the characterization of HCPro RNA silencing suppression activity, thereby their real role might be miss assigned. Pentapeptide insertions that rendered PPV HCPro poorly functional or non-functional as an RNA silencing suppressor are depicted as

grey circles at the equivalent TEV HCPro positions. A 2D representation of the *Turnip mosaic virus* HCPro structure solved by Guo *et al.* (2011) is encompassing the equivalent C-terminal region of TEV HCPro. Superscript numbers indicate the following references: [1](Carrington *et al.*, 1989a), [2](Oh and Carrington, 1989), [3](Carrington and Herndon, 1992), [4](Atreya *et al.*, 1992), [5](Atreya and Pirone, 1993), [6](Huet *et al.*, 1994), [7](Dolja *et al.*, 1993), [8](Blanc *et al.*, 1998), [9](Kasschau and Carrington, 2001), [10](González-Jara *et al.*, 2005), [11](Shiboleth *et al.*, 2007), [12](Torres-Barceló *et al.*, 2008), [13](Cronin *et al.*, 1995), [14](Valli *et al.*, 2014), [15](Varrelmann *et al.*, 2007). (B) Crystal structure of the cysteine protease domain of *Turnip mosaic virus* HCPro (Guo *et al.*, 2011; PDB code 3RNV). The corresponding L and R domains of papain-like proteases would be represented by the α-helices shown in green and the ß-sheets shown in orange, respectively. Those amino acids highlighted in (A) are also indicated in (B).

Figure 4. Schematic representation of genomic organization in viruses from different genera of the family *Potyviridae*. The long open reading frame is shown as a box divided in mature viral products. PIPO open reading frame is indicated as box below P3. The terminal protein VPg is depicted as a black ellipse. P1a and P1a-like proteins are represented by grey boxes, whereas P1b and P1b-like proteins are represented by black boxes. Features that are not shared by all potyvirids are highlighted in different colours. (A) *Potyvirus* and *Rymovirus* genera. The PISPO open reading frame in sweet potato-infecting potyviruses is indicated as a pale green box below P1. The extra protein HAM between NIb and CP in *Euphorbia ringspot virus* (Knierim *et al.*, 2017) is highlighted in pink. (B) *Tritimovirus* and *Poacevirus*. (C) *Ipomovirus*. The diversity among members of this genus has been reviewed (Dombrovsky *et al.*, 2014). A HAM extra protein (in pink) was also present in a subset of ipomoviruses (D) *Brambyvirus*. The AlkB domain in the P1 from *Blackberry virus Y* is highlighted in pale orange. (E) *Macluravirus*. (F) *Bymovirus*. The RNA2, unique in the *Potyviridae* family, is highlighted in yellow.

Table 1: HCPro-targeted steps of the antiviral RNA silencing pathway.

Targeted Step	Molecular Mechanism	Potyvirus	References
vsiRNA uploading	Sequestration of vsiRNAs	TEV, PPV, PRSV, ZYMV, TuMV	(Garcia-Ruiz *et al.*, 2015; Lakatos *et al.*, 2006; Sahana *et al.*, 2014; Shiboleth *et al.*, 2007; Valli *et al.*, 2011)
vsiRNA methylation	Inhibition of the CH_3-production	PVY, PVA	(Cañizares *et al.*, 2013; Ivanov *et al.*, 2016 ; Soitamo *et al.*, 2011)
	Binding and inactivation of HEN1	ZYMV	(Jamous *et al.*, 2011)
Effector	Down-regulation of AGO1	TEV	(Varallyay and Havelda, 2013)
	Interaction with AGO1	PVA	(Ivanov *et al.*, 2016)
Amplification	Down-regulation of RDR6	SCMV	(Zhang *et al.*, 2008)
Movement of silencing signal	Sequestration of siRNAs?	PVY, TEV	(Delgadillo *et al.*, 2004; Hamilton *et al.*, 2002; Pfeffer *et al.*, 2002)
Induction of endogenous silencing suppressors?	Interaction with rgs-CaM and RAV2	TEV, TuMV	(Anandalakshmi *et al.*, 2000; Endres *et al.*, 2010)

Table 2: Diverse activities of HCPro whose roles in viral infection have not been fully characterized.

Activity	Function	Hypothetical aims	Virus	Reference
Interaction with PAA, PBB, PBE or PAE proteasome subunits	Inhibition of the 20S proteasome	Counteracting a proteasome-based plant defence mechanism	LMV PVY PRSV	(Ballut *et al.*, 2005; Dielen *et al.*, 2011; Jin *et al.*, 2007b; Sahana *et al.*, 2012)
Interaction with NtMinD, NtDXS, CF1β–subunit of chloroplast ATP synthase, Ferrodoxin-5	Reduction of the photosynthesis rate	General weakening of the host	PVY SCMV	(Cheng *et al.*, 2008; Gunasinghe and Berger, 1991; Jin *et al.*, 2007a; Li *et al.*, 2015; Tu *et al.*, 2015a; Tu *et al.*, 2015b)
Interaction with PaCRT	Disturbance of Ca^{+2} binding to PaCRT	Blocking the Ca^{+2}-mediated activation of host defences	PRSV	(Shen *et al.*, 2010)
Interaction with HIP2	Blocking of HIP2 activity	Disturbing some signalling networks of defence responses	PVA PVY TEV	(Haikonen *et al.*, 2013a; Haikonen *et al.*, 2013b)
Formation of cytoplasmic granules	Recruitment of both host and viral factors	Overcoming RNA silencing-based defences. Reaching optimal viral translation.	PVA TuMV	(Hafrén *et al.*, 2015)
Interaction with eIF4E/eIF(iso)4E	Recruitment of translation initiation factors	Reaching optimal viral translation	PVA, PVY, TEV	(Ala-Poikela *et al.*, 2011)
Interaction with VPg and CI	Protein allocation at the tip of virions.	Transmission or movement. Reaching optimal viral translation	ClYVV WSMV PSbMV LMV PVA PPV	(Choi *et al.*, 2000; Guo *et al.*, 2001; Ivanov *et al.*, 2016; Roudet-Tavert *et al.*, 2007; Yambao *et al.*, 2003; Zilian and Maiss, 2011)
Interaction with CP?	Proper formation of viral particles	Coordination of different stages of the viral infection cycle	PPV	(Valli *et al.*, 2014)

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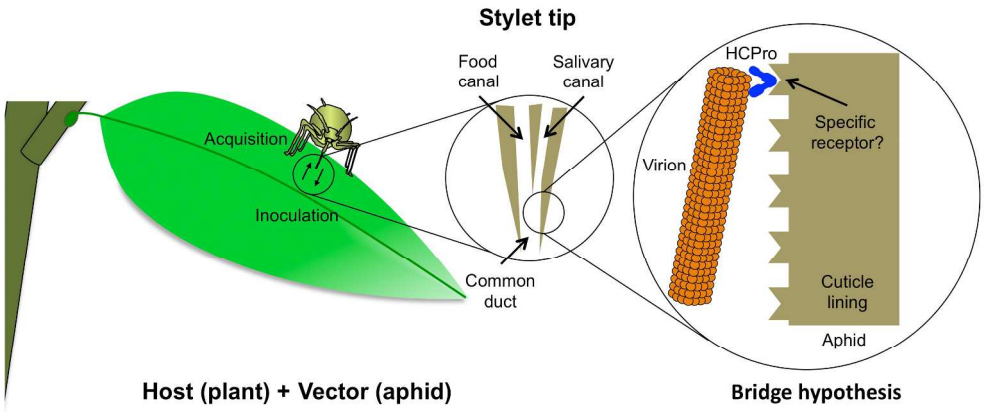

Figure 1. "Bridge hypothesis" for aphid transmission of potyviruses.
Bridge hypothesis
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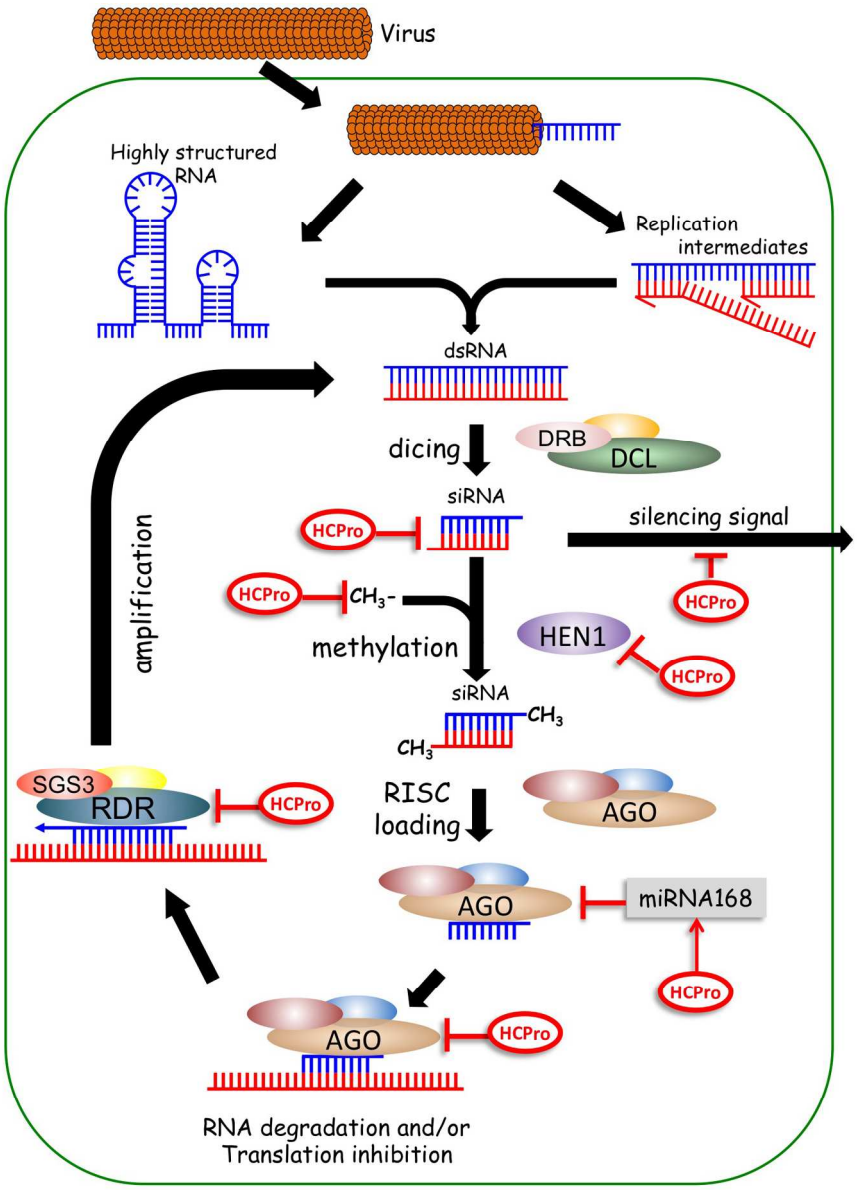

Figure 2. Potential targets of HCPro in the antiviral RNA silencing pathway.
RNA silencing
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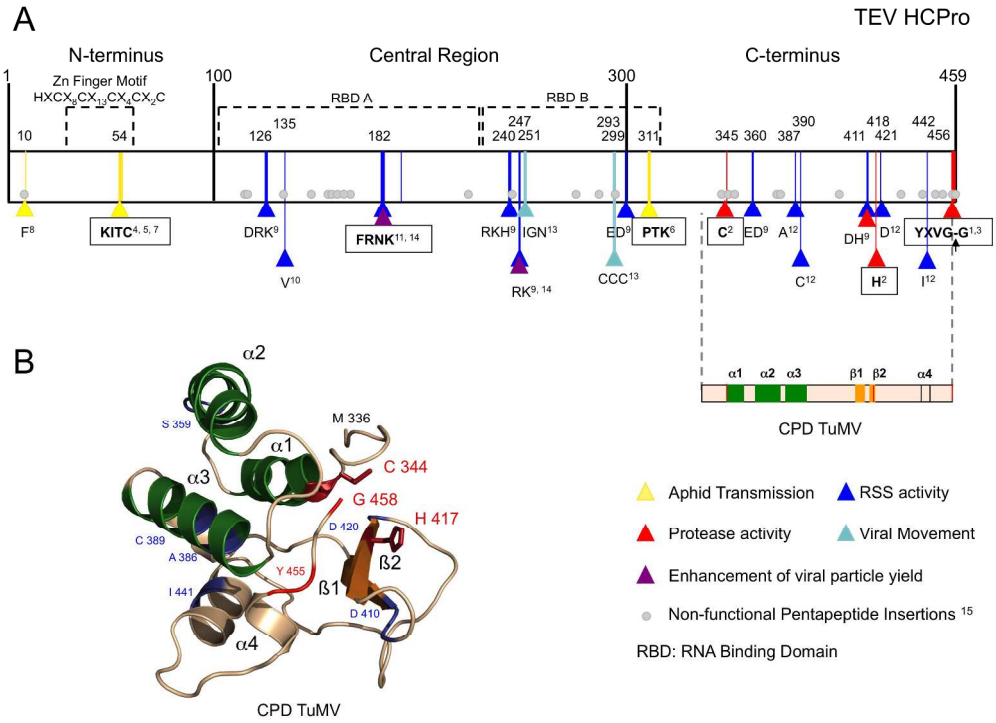

Figure 3. HCPro structural and functional features.
domain
253x190mm (300 x 300 DPI)

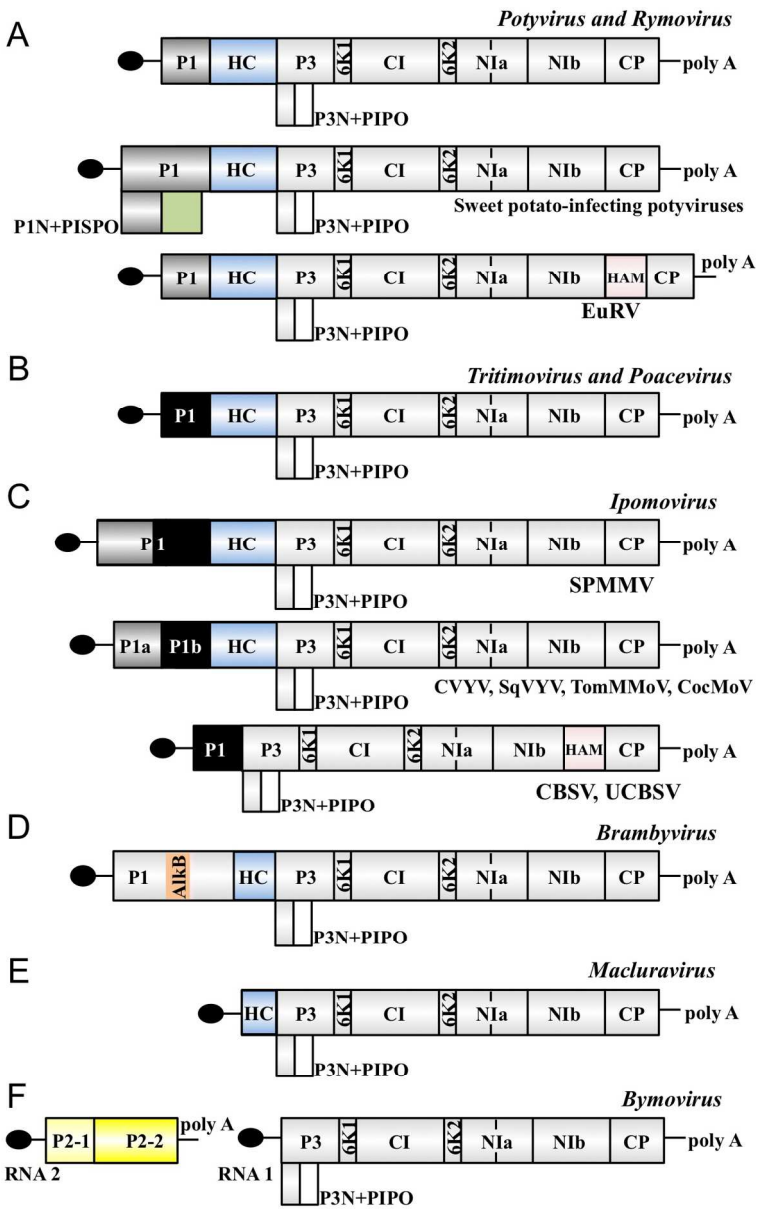

Figure 4. Schematic representation of genomic organization in viruses from different genera of the family Potyviridae.
genome, genomic
120x190mm (300 x 300 DPI)