Molecular and cellular mechanisms underlying potyvirus infection

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Potyviruses represent one of the most economically important and widely distributed groups of plant viruses. Despite considerable progress towards understanding the cellular and molecular basis of their pathogenicity, many questions remain about the mechanisms by which potyviruses suppress host defences and create an optimal intracellular environment for viral translation, replication, assembly and spread. The review focuses on the multifunctional roles of potyviral proteins and their interplay with various host factors in different compartments of the infected cell. We place special emphasis on the recently discovered and currently putative mechanisms by which potyviruses subvert the normal functions of different cellular organelles in order to establish an efficient and productive infection.

Introduction

Potyviruses belong to the family *Potyviridae*, together with seven other genera, and comprise a very important plant virus group agriculturally, economically and biologically. They are widespread in cultivated plants throughout all regions of the world, but as recent metagenomics studies have indicated, they are also found abundantly in the wild (Roossinck, 2012). The International Committee on Taxonomy of Viruses has recognized *Potyviridae* as the second largest plant virus family after *Geminiviridae*. In wild plants, it is the third largest family after the *Partitiviridae* and *Totiviridae* (reviewed by Roossinck, 2012). Potyviruses have a broad geographical distribution, consist of numerous species capable of infecting various host plants and lead to great economic losses. The type member of the genus *Potyvirus*, *Potato virus Y* (PVY), together with *Potato virus A* (PVA; genus *Potyvirus*) and *Potato leaf roll virus* (genus *Polerovirus*) pose the highest threat to potato production worldwide and can reduce crop yields up to 90% (Salazar, 2003). *Plum pox virus* (PPV; genus *Potyvirus*) is also of great economic importance as it causes the most destructive diseases of stone fruit worldwide (García et al., 2014; Sochor et al., 2012). Taking these harmful qualities into consideration, it is clear why PVY and PPV have been selected in a recent survey to be among the top 10 plant viruses with the highest economic and scientific importance (Scholthof et al., 2011). The fact that potyviruses are spread by migrating aphids, and that each potyvirus may be transmitted by many different aphid species and each aphid species may transmit many potyviruses (Gibbs et al., 2008), makes it even more difficult to control and prevent potyvirus infections in agriculture.

Previous comprehensive reviews on the molecular biology of potyviruses date back to more than a decade ago (Rajamäki et al., 2004; Urcuqui-Inchima et al., 2001). In recent years, a better understanding of potyvirus biology has been obtained through the analysis of the host transcriptome, proteome and metabolome during infection (Aguedol-Romero et al., 2008; Alfenas-Zerbini et al., 2009; Babu et al., 2008; Baebler et al., 2009; Prasch & Sonnewald, 2013; Whitham et al., 2003; Yang et al., 2007, 2011), improved knowledge of virus–host protein interactions (Elena & Rodrigo, 2012) and a more comprehensive visualization of the infection process (e.g. Agbeci et al., 2013; Grangeon et al., 2012). Furthermore, constantly improving imaging techniques (reviewed by Huang et al., 2009; Tilsner & Oparka, 2010) make the functional dissection of viral components more thorough and precise. The current challenge is combining these various advances in order to get a better insight into the molecular mechanisms exploited by potyviruses during infection. Here, we discuss our current understanding of the functions of potyviral proteins and their interplay with various host factors in different cellular compartments during the potyviral multiplication cycle.

Regulation of potyviral protein amounts and functions

The genome of potyviruses is a positive-sense ssRNA molecule with its 5′ end covalently linked to a virus-encoded protein genome-linked protein (VPg) and its 3′ end polyadenylated (Fig. 1a). An important property of potyviruses is that 10 out of 11 proteins that they encode are derived from a single polyprotein precursor (Rajamäki et al., 2004). The other protein, P3N-PIPO, is separately synthesized from a small ORF (Chung et al., 2008). Such a genome expression strategy makes it challenging to understand how potyviruses regulate their protein levels at each stage of infection. In comparison, other viruses commonly regulate their protein production
through special translational strategies, such as the use of subgenomic RNAs (Szubka-Solińska et al., 2011) or ribosomal frameshifting (e.g. Brault & Miller, 1992; Mäkinen et al., 1995). In potyviruses, all proteins, except P1, helper component protease (HC-Pro) and P3N-PIPO, are produced theoretically in an equimolar ratio, necessitating the use of multiple strategies to dynamically regulate viral protein concentrations at each stage of the infection cycle.

A good example of potyviral protein regulation concerns VPg and its fusion partner, Nla-Pro protease (Fig. 1b). The first important mechanism regulating VPg concentration is the proper timing of its proteolytic processing. The slow release of VPg from the Nla fusion, consisting of the N-terminal VPg domain and the C-terminal Nla-Pro protease domain, is required for the infectivity of the Tobacco etch virus (TEV; genus Potyvirus) (Schaad et al., 1996). Furthermore, the interaction of the VPg domain with the Nla-Pro domain increases the protease turnover in Pepper vein banding virus (PVBV; genus Potyvirus) (Mathur et al., 2012). The VPg-mediated structural and functional modulation of Nla-Pro may, therefore, regulate viral proteolytic activity at particular stages of the virus life cycle. Another layer of VPg functional regulation is provided by its structural flexibility (Grzela et al., 2008; Hébrard et al., 2009; Rantalainen et al., 2008, 2011). Such flexibility allows conformational adaptation when binding to the anionic phospholipid bilayer (Rantalainen et al., 2009) or its various protein binding partners. In a recent study, PVBV VPg has been shown to possess ATPase activity, which is enhanced in the presence of Nla-Pro in cis (Mathur & Savithri, 2012). This suggests that the interaction with Nla-Pro induces a structural change in VPg, which in turn leads to the increased ATPase activity. VPg phosphorylation (Hafren & Mäkinen, 2008; Ivanov et al., 2001) adds yet another tool to the arsenal of regulatory mechanisms. The addition of phosphate groups to one or more residues in VPg by cellular kinases changes their charge and steric size, and as a result, possibly triggers some as yet unknown mechanisms. However, phosphorylation of PVBV Nla-Pro inhibits its proteolytic activity (Mathur et al., 2012). Finally, VPg may be targeted to various cellular compartments for a particular function, either separately or as part of a dedicated protein complex. Potyviral VPg fused to Nla-Pro has been localized to viral replication complexes (VRCs) (Thivierge et al., 2008) as well as to the nucleus (Restrepo et al., 1990). VPg is also present in virions (Oruitexbarria et al., 2001); more specifically, in one end of the filamentous particle (Puustinen et al., 2002), where it can associate with a tip structure consisting of the cylindrical inclusion protein (CI) (Gabrenia-Verkhovskaya et al., 2008) and HC-Pro (Torrance et al., 2006). As already mentioned above, one of the means to regulate VPg functions is through specific intermolecular interactions. Indeed, VPg has many cellular and viral binding partners including eukaryotic initiation factor 4E (eIF4E), poly(A)-binding protein (PABP) (Léonard et al., 2004), potyviral VPg-interacting protein (PVIP) (Dunoyer et al., 2004), eukaryotic elongation factor 1A (eEF1A) (Thivierge et al., 2008), fibrillarin (Rajamäki & Valkonen, 2009), a host RNA helicase RH8 (Huang et al., 2010), viral RNA (Merits et al., 1998), P1 (Merits et al., 1999), HC-Pro (Roudet-Tavert et al., 2007), P3 (Merits et al., 1999), CI (Tavert-Roudet et al., 2012), Nilb (Hong & Hunt, 1996; Li et al., 1997), coat protein (CP) (Shen et al., 2010a; Zilian & Maiss, 2011) and itself (Oruitexbarria et al., 2001). Similarly to VPg regulation, the amounts, functions and localization of other potyviral replication proteins are controlled by many different mechanisms, as discussed later in this review.
At later stages of infection, the viral genome is encapsidated into virions, allowing the virus to survive in the extracellular environment. The sole structural protein required for this process is the CP. The current understanding of the mechanisms underlying potyviral CP regulation during viral translation/replication and genome encapsidation has been reviewed elsewhere (Ivanov & Mäkinen, 2012), and will not be discussed here. Another viral process that requires CP-mediated genome encapsidation is aphid transmission. At the molecular level, aphid transmission is controlled by the interactions between specific amino acid motifs in CP, HC-Pro and a putative receptor in an aphid stylet (reviewed by Rochon, 2007).

**Potyvirus-induced modifications of host gene expression**

The information required to subvert the intracellular environment to benefit viral infection, such as shutting down host defences and ensuring the local and systemic spread of the virus, is stored not only in the viral but also in the host genome. Therefore, one way for the virus to execute the above tasks is to hijack host gene transcription or alter the stability of certain cellular mRNA. Transcriptome data analysis has shown that different sets of host transcripts are up- and downregulated during potyvirus infection. Early after inoculation of resistant and susceptible potato cultivars with the necrotic strain of PVY, a significant upregulation of signalling and WRKY transcription factor transcripts was observed in the susceptible cultivar, whereas upregulation of genes involved in defence compound synthesis and biotic stress response [e.g. PR (pathogenesis-related) genes] was observed in the resistant cultivar (Baebler et al., 2009). Gene expression analysis of *Arabidopsis thaliana* plants infected with *Turnip mosaic virus* (TuMV; genus *Potyvirus*) revealed upregulation of genes involved in cell rescue, defence, virulence (e.g. heat-shock genes), protein synthesis and turnover during later stages of the infection. The analysis also showed downregulation of the genes involved in chloroplast and cell wall functions in correlation with the increasing virus accumulation (Yang et al., 2007).

In addition to the transcriptome, the host proteome has also been studied in response to plant virus infection (Di Carli et al., 2012). Host proteins can be either directly or indirectly associated with the infection. We define direct involvement as the participation of a particular host protein in the formation of virally induced protein–protein or ribonucleoprotein (RNP) complexes and indirect involvement as the participation of a host protein in a signalling or defence pathway activated by the virus. The current understanding is that potyvirus-induced effects are not as pronounced at the proteome level as they are at the transcriptome level. For example, only 28 proteins were expressed differentially during *Soybean mosaic virus* (SMV; genus *Potyvirus*) infection in soybeans (Yang et al., 2011). Among these were proteins active in protein synthesis and degradation, signal transduction, disease defence, and the regulation of energy metabolism.

Potyvirus infection activates host genes that are also induced by various abiotic stresses, such as drought, salinity, high temperature and wounding (Agudelo-Romero et al., 2008). However, abiotic stresses themselves promote potyvirus infection. For example, TuMV RNA accumulation increases under heat stress (Prasch & Sonnewald, 2013) and PVA gene expression is upregulated under salt stress (Suntio & Mäkinen, 2012). Plants frequently respond to abiotic stresses through Ca$^{2+}$ signalling (e.g. Kader & Lindberg, 2010), which in turn leads to induction of Ca$^{2+}$ signalling genes (e.g. Kudla et al., 1999). Interestingly, treatment of plants with calcium activates PVA gene expression, and promotes local and systemic spread of the virus (Suntio & Mäkinen, 2012). Ca$^{2+}$ signalling genes, such as those for calmodulin and calmodulin-like protein, are among the genes upregulated at the onset of PVY infection in a susceptible potato cultivar (Baebler et al., 2009). Similarly, 4.9% of the genes upregulated during infection with *Pepper yellow mosaic virus* (genus *Potyvirus*) are involved in Ca$^{2+}$-mediated signal transduction (Alfenes-Zernibi et al., 2009). Furthermore, TEV HC-Pro binds to a calmodulin-related protein, rgs-CaM, which has three EF-hand Ca$^{2+}$-binding motifs in its C-terminal domain (Anandlakshmi et al., 2000) and is upregulated during TEV infection. HC-Pro of *Papaya ringspot virus* (PRSV; genus *Potyvirus*) interacts with yet another Ca$^{2+}$-binding protein, calreticulin, which is upregulated at both the mRNA and protein levels in the early stages of PRSV (Shen et al., 2010b) and SMV (Yang et al., 2011) infections. On the basis of the above findings, it can be hypothesized that the upregulation of Ca$^{2+}$ signalling and abiotic stress-related genes serves to modify the intracellular environment in a way that is beneficial to the virus. As stated above, potyviruses are transmitted by aphid vectors. Interestingly, aphids feeding on a plant also activate calcium signalling genes (Botha et al., 2010), which likely promotes the initiation of infection after aphid transmission. Molecules transporting potyvirus-induced signals from the cytoplasm to the nucleus, and the transcription factors responding to these signals, are mostly unknown. Therefore, an emerging and interesting area of research is the analysis of potyviral protein functions in the nucleus.

**Potyviral proteins in the nucleus**

Potyviruses encode several proteins that are translocated into the nucleus. The two most thoroughly studied nuclear proteins are the multifunctional protein NiA and the viral RNA-dependent RNA polymerase NiB. Despite their involvement in viral replication on the surface of the cytoplasmic membranes, these proteins accumulate predominantly in the nucleus of infected cells (Baunoch et al., 1988, 1991; Restrepo et al., 1990; Riedel et al., 1998) and contain independent nuclear localization signals (Carrington et al., 1991; Li & Carrington, 1993; Rajamäki & Valkonen, 2009; Schaad et al., 1996). As their name implies, the NiA and NiB proteins of some potyviruses are able to form amorphous or crystalline nuclear inclusions (NIs) in infected cells (Baunoch et al., 1988; Dougherty & Hiebert, 1980; Schaad et al., 1996).
Knuhtsen et al., 1974; Riedel et al., 1998). In addition to NLa and Nlb, three more potyviral proteins have been detected in the nucleus. Two of these proteins, viral CI helicase and multifunctional HC-Pro, are predominantly cytoplasmic, but a small portion is also found in the nucleus (Riedel et al., 1998). The third and least studied protein, P3, has been detected in the nuclei of cells infected with TEV (Langenberg & Zhang, 1997); however, the P3s of Tobacco vein mottling virus (TVMV; genus Potyvirus) and PRSV have been found only in the cytoplasm (Rodriguez-Cerezo et al., 1997) or in association with the endoplasmic reticulum (ER) (Eiamtanasate et al., 2007).

As potyviruses replicate in the cytoplasm, the rationale behind the nuclear transport of their proteins is not immediately obvious. One scenario where such transport could be useful is when there is a need to sequester excess non-structural proteins from the cytoplasm. As mentioned above, all potyviral proteins, except P1, HC-Pro and P3N, PIPO, are theoretically produced in an equimolar ratio. However, the presence of equimolar amounts of structural and non-structural proteins in the same cellular compartment may be counterproductive for viral infection. For example, virally encoded enzymes are clearly not needed in the same amounts as the CP. Sequestration of such ‘overproduced’ proteins may be achieved by different mechanisms, such as targeted proteasomal degradation or post-translational modifications, although active transport to another cellular compartment appears to be the most obvious and straightforward solution. The nucleus is a highly suitable cellular compartment for this purpose, because it is effectively separated from the cytoplasm by the nuclear envelope and is impermeable to large molecules that are unable to actively traverse the nuclear pores. The notion that the nucleus may serve as a sequestration site for NLa and Nlb is also supported by the high levels of their nuclear accumulation and the formation of nuclear inclusions (Baunoch et al., 1991; Restrepo et al., 1990; Riedel et al., 1998), especially towards the later stages of infection.

In recent years, new data have emerged suggesting that several potyviral non-structural proteins play active roles in the nucleus. The first and rather surprising finding is that the NLa protein, a sequence-specific proteinase, also possesses sequence-non-specific DNase activity (Anindya & Savithri, 2004). This finding raises the interesting possibility that NLa may be targeted to chromatin to degrade the host cell DNA at late stages of infection (Anindya & Savithri, 2004). If such targeting indeed takes place, NLa may also interact with chromatin-associated transcriptional regulators. Support for this scenario comes from the observation that the VPg domain of NLa interacts with a cysteine-rich plant PVIP protein potentially involved in transcriptional control through chromatin remodelling (Dunoyer et al., 2004). Originally identified in pea, the PVIP protein is homologous to the OBERON1 and OBERON2 proteins from A. thaliana (Saiga et al., 2008; Thomas et al., 2009). Both OBERON1 and OBERON2 contain a PHD-finger domain involved in the regulation of the chromatin structure (Taverna et al., 2007) through specific binding to trimethylated histone H3 at lysine 4 (H3K4me3) near actively transcribed genes (Lee et al., 2009; Li et al., 2006; Peña et al., 2006; Shi et al., 2006; Wysocka et al., 2006). The role of OBERON1 as a transcriptional regulator has been confirmed experimentally in a recent study showing that the protein binds to actively transcribed chromatin in vivo (Saiga et al., 2012). Furthermore, being a proteinase, NLa can potentially cleave its chromatin-associated binding partners. However, this would require that they contain endogenous protease recognition motifs, which is not very likely but cannot be excluded. Taken together, the above results suggest that NLa may alter host gene expression in a way beneficial to the virus through the hijacking/inactivation of transcriptional regulators, such as members of the OBERON (PVIP) family. This would explain why a mutation in the VPg domain of NLa that blocks the interaction with PVIP correlates with reduced viral movement (Dunoyer et al., 2004). The finding that NLa interacts with PVIP is especially interesting in light of a recent discovery that another family of plant RNA viruses replicating in the cytoplasm, carlaviruses, encode a dedicated viral transcription factor, which translocates into the nucleus to hijack cellular transcription (Lukhovitskaya et al., 2013).

Another potyviral non-structural protein, HC-Pro, also interacts with a transcriptional regulator. HC-Pro specifically binds to the ethylene-inducible transcription factor RAV2 in vitro and in vivo, and the interaction is thought to function in the suppression of virus-induced gene silencing through the induction of endogenous silencing suppressor genes (Endres et al., 2010). The intracellular location where the two proteins meet is not yet known, but several scenarios are possible. One scenario is that HC-Pro binds to RAV2 directly in the nucleus to hijack the RAV2-mediated transcriptional program. The second is that HC-Pro, which is found predominantly in the cytoplasm (Mlotshwa et al., 2002; Riedel et al., 1998), interacts with the cytoplasmic pool of RAV2 and the resulting protein complex is then actively transported to the nucleus, where it alters RAV2 binding to its DNA target sites, modifying the RAV2 transcriptional programme. In the third scenario, the interaction serves to sequester RAV2 in the cytoplasm, away from its essential functions in the nucleus. Finally, the interaction might trigger a post-translational modification(s) of RAV2 or its cellular binding partner(s). This modification(s) may, in turn, interfere with the RAV2 nuclear transport or its binding to DNA target sites in the nucleus.

Yet another line of evidence in support of the active role of potyviral non-structural proteins in the nucleus comes from a study describing the nucleolar localization of NLa and its interaction with fibrillarin (Rajamäki & Valkonen, 2009). As fibrillarin is involved in processing pre-rRNA, the interaction may interfere with host defences at the level of ribosomal assembly. Alternatively, the nucleolar targeting of NLa and its interaction with fibrillarin may serve to suppress virus-induced gene silencing (Rajamäki & Valkonen, 2009). Two other proteins interacting with NLa in the nucleolus are...
the translation initiation factor eIF(iso)4E and PABP (Beauchemin & Laliberté, 2007; Beauchemin et al., 2007). Interestingly, PABP also interacts with the second predominately nuclear potyviral protein Nib (Dufresne et al., 2008). This is, perhaps, not surprising, given that Nib and Nla form specific complexes in the yeast two-hybrid assay (Darós et al., 1999; Guo et al., 2001; Hong et al., 1995; Li et al., 1997), in vitro (Fellers et al., 1998) and in vivo in the nuclei of infected cells (Ivanov et al., 2014). The significance of the interactions between Nla, Nib and translation factors in the nucleus is not yet known, but they might play a role in the potyvirus-induced host gene shut-off (Beauchemin & Laliberté, 2007; Wang & Maule, 1995).

To conclude, a growing body of evidence suggests that the roles of potyviral non-structural proteins in the nucleus are not limited to the simple sequestration of excess protein from viral replication sites, but rather involve complex molecular interactions aimed at suppressing host defence responses and creating an optimal intracellular environment for viral reproduction.

Modified intracellular environment for viral translation and replication

In order to establish a successful infection, potyviruses must ensure that their RNA is translated efficiently by host ribosomes. Cellular stress signalling activates reprogramming of cellular cap-dependent translation in favour of internal ribosome entry site (IRES)-mediated translation (reviewed by Spriggs et al., 2008). The primary mechanism underlying such reprogramming is the phosphorylation of the α-subunit of eIF2 (eIF2α, reviewed by Holcik & Sonenberg, 2005). In plants, eIF2α phosphorylation is thought to be catalysed by a PKR-like kinase activity induced by viral dsRNA replication intermediates (Crum et al., 1988; Langland et al., 1995). Viral RNA released from virions is not capped, but rather covalently linked to VPg at its 5’ end (Oruetxebarria et al., 2001). Therefore, potyviruses use the cap-independent IRES mechanism to initiate protein synthesis (Basso et al., 1994; Gallie, 2001; Levis & Astier-Manifacier, 1993; Niepel & Gallie, 1999; Zeenko & Gallie, 2005), although other mechanisms based on scanning from the 5’ end of viral RNA are also possible (Gallie, 2001; Niepel & Gallie, 1999). In agreement with the notion that potyviral RNA translation is IRES-dependent, PVA RNA and the capped fluc mRNA have been demonstrated to be translated by two different mechanisms (Eskelin et al., 2011; Suntio & Mäkinen, 2012).

As the infection progresses, increasing amounts of viral proteins must be correctly folded in order to avoid ER stress, which may in turn lead to autophagy or cell death through a signalling cascade called the unfolded protein response (UPR) (reviewed by Howell, 2013). In addition, potyvirus infection induces selective changes in host gene expression, rather than a complete shut-off of protein synthesis. These factors justify the need for molecular chaperones, such as the heat-shock protein 70 (HSP70), to assist with the correct folding of viral and host proteins. Ensuring sufficient chaperone capacity may be one of the reasons why the expression of HSP70 is induced with the onset of viral replication (Aranda et al., 1996). The fact that TuMV infection upregulates genes involved in UPR (Prasch & Sonnewald, 2013) suggests that potyvirus infection may indeed cause a mild ER stress. This is in agreement with an earlier study where infection with Potato virus X (PVX; genus Potexvirus) caused ER stress and was accompanied by an enhanced UPR (Ye et al., 2011). Interestingly, an inhibitor of PKR kinase, P58IPK, interacts with potyviral CI and may prevent eIF2α phosphorylation-mediated cell death during potyvirus infections (Bilgin et al., 2003). Taken together, these results emphasize the importance of the delicate balance between sufficient viral protein production and acceptably low levels of ER stress in infected cells.

Plant positive-sense RNA viruses replicate in association with cytoplasmic membranes, transforming them into virus-induced membranous compartments, often called viral factories (Verchot, 2012). The requirement for a membrane-derived micro-environment for replication arises from the need to protect viral RNA from silencing and other host defence mechanisms, and provide a scaffold to assemble viral non-structural and host proteins into a functional replication complex (den Boon & Ahlquist, 2010). The general consensus is that potyviruses assemble their VRCs on membranous vesicles at ER exit sites (ERES) using hijacked components of the secretory pathway (reviewed by Patarroyo et al., 2012). These vesicles then move along microfilaments to chloroplasts and fuse with chloroplast membranes, as has been shown for TuMV VRCs (Cotton et al., 2009; Wei & Wang, 2008; Wei et al., 2010a). In agreement with this scenario, PVA VRCs follow similar intracellular trajectories (Fig. 2). Furthermore, viral dsRNA has been detected in TuMV-induced vesicles (Cotton et al., 2009), emphasizing their importance for viral replication.

Transient expression of the membrane-associated 6K2 protein induces formation of VRC-like structures even in the absence of a viral infection, suggesting that this protein is essential for virus-induced membrane modifications (Beauchemin et al., 2007; Schaad et al., 1997; Thivierge et al., 2008). Furthermore, the formation of 6K2-induced vesicles triggers chloroplast aggregation (Wei et al., 2013). The plant SNARE protein Syp71 facilitates VRC fusion with the chloroplast membranes (Wei et al., 2013) – a process essential for successful viral infection (Wei et al., 2010a). However, some of the most fundamental and interesting questions remain unanswered. How do potyviruses hijack the early secretory pathway? What are the host components recruited by viral proteins during VRC formation? What are the molecular mechanisms regulating this process? Many important questions also relate to the timing of viral replication. Fig. 2 illustrates how the 6K2-induced vesicles develop in the course of a PVA infection in Nicotiana benthamiana leaves. In the newly infected cells, only scattered individual 6K2-induced vesicles are present (Fig. 2, zone 1). As the infection progresses, the vesicles
start to aggregate with chloroplasts (Fig. 2, zone 2) and at later stages fuse into large perinuclear globular structures (Fig. 2, zone 3). It is not yet clear whether all of the above 6K2-associated membranous structures are active in replication, because the replication occurs only in a specific time window (Raghupathy et al., 2006; Wang & Maule, 1995). Therefore, it cannot be excluded that the replication ceases at the very late stages of infection, despite the presence of the 6K2-induced membranous structures.

Interestingly, 6K2 vesicles derived from the large perinuclear structures are transported along microfilaments towards the cell wall and into adjacent cells through plasmodesmata (PD) (Agbeci et al., 2013; Grangeon et al., 2013). Future studies will determine whether these vesicles (see Fig. 3) are indeed infectious in the adjacent cells. There is some evidence, however, that argues against the vesicle-mediated mechanism of cell-to-cell movement. For example, the vesicle-mediated transport mechanism does not fit easily into the existing picture of potyviral cell-to-cell movement. At present, it is difficult to understand how the three potyviral proteins involved in cell-to-cell movement, i.e. CI, CP and P3N-PIPO (Carrington et al., 1998; Dolja et al., 1994; Wei et al., 2010b), would function in a vesicle-mediated viral RNA transport system. In addition, the transported vesicles may not be derived from the late perinuclear structures, as the intercellular movement of potyviruses involves the formation of pinwheel inclusions positioned centrally over PD apertures (Fig. 3, Roberts et al., 1998), but these inclusions are already disassembled before the 6K2-induced perinuclear structures are formed (Wei et al., 2010b).

**Potyviral replication in VRCs**

The sequence of reactions involved in the amplification of viral RNA consists of the recruitment of viral and host components to the correct membranes, VRC assembly, negative-strand RNA synthesis, positive-strand RNA synthesis and the release of progeny RNA. The formation of the potyviral replication complex is initiated by the 6K2–Vpg-Pro precursor (Schaad et al., 1997). As a membrane-spanning protein, 6K2 tethers Nla to the ER membranes. The interaction between Nla and Nlb, in turn, recruits viral replicase activity to the forming membrane-associated replication complex (Hong & Hunt, 1996; Li et al., 1997). The current consensus is that the secondary structures in the 3′ UTR of viral RNA, together with the 3′ part of the CP-encoding region and PABP, direct the Nib replicase to the 3′ end of the genome to initiate negative-strand RNA synthesis (Haldeman-Cahlil et al., 1998; Wang et al., 2000). A membrane-associated polyprotein-processing intermediate, CI–6K2, may be partially responsible for recruiting components of the replication complex to the membranes (Merits et al., 2002). This is supported by the fact that the CI–6K2 protein cleavage site is processed very slowly in PVA and that this intermediate exists in infected cells even longer than the CI–6K2–Nla intermediate (Merits et al., 2002). The P3 protein of TEV forms punctate inclusions that originate from ERESs and are trafficked to the Golgi apparatus by the early secretory pathway, similar to 6K2 vesicles. The fact that these P3-associated structures colocalize with 6K2 vesicles (Cui et al., 2010), and that P3

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**Fig. 2.** Virus-induced structures at different stages of infection. (a) *N. benthamiana* leaves were agro-infiltrated with PVA carrying a second copy of the 6K2 gene fused to yellow fluorescent protein (YFP). Agro-infiltration was carried out at low OD600 (0.0005) to initiate infection from single cells. The figure shows the changes in virus-induced subcellular structures that occur throughout the different stages of infection. Zone 1 consists of recently infected cells containing mostly cytoplasmic free VRCs. Zone 2 consists of cells infected for ~24–48 h, containing both free and chloroplast-bound VRCs. Virus-induced chloroplast aggregates start to appear in these cells. Zone 3 surrounds the primary infection site and represents the longest infected area of the leaf (>48 h post-infection). All Zone 3 chloroplasts are labelled with YFP, confirming their association with VRCs. Many chloroplasts also aggregate into perinuclear structures. Bar, 100 μm. (b) Magnified images of virus-induced structures in the three infection zones explained above. Upper panels show the overlay of the YFP signal from the viral 6K2 fusion protein (green), chloroplast autofluorescence (red), and cyan fluorescent protein signal from both nuclei and cytoplasm (blue). Lower panels show the virus-derived YFP signal alone. Cell nuclei are marked with ‘N’ and virus-induced perinuclear structures are indicated by arrows. Bars, 15 μm.
interacts with CI, NIa and NIb (Merits et al., 1999), suggests that P3 plays a role in VRC formation. Whilst the essential role of HC-Pro in suppression of RNA silencing is well established, PVA HC-Pro has also been identified in VRCs (Ala-Poikela et al., 2011) and, therefore, might participate in viral replication through a mechanism as yet unknown.

Much of our knowledge about bromo- and tombusvirus replication is derived from yeast assays and cell-free replication assays with yeast extracts (Janda & Ahlquist, 1993; Pogany et al., 2008). Although such powerful experimental tools are not yet available for potyviruses, several enzymatic activities involved in potyviral replication have been dissected by reverse genetics and biochemical analyses. The catalytically active site of the NiB replicase contains the conserved GDD motif found in many RNA-dependent RNA polymerases from diverse plant and animal viruses (Kamer & Argos, 1984). Viral replication likely begins with the NiB-mediated uridylylation of VPg (Anindya et al., 2005; Puustinen & Mäkinen, 2004), which may act as a primer for RNA synthesis in a manner similar to that of poliovirus VPg (Murray & Barton, 2003; Paul et al., 1998). Mutation of the uridylylated tyrosine residue in TVMV VPg is lethal for the virus (Murphy et al., 1996) and replication of another potyvirus, PVA, likely involves uridylylation of two alternative tyrosine residues (Rantalainen et al., 2011).
Potyviral CI is a member of the DExH/D-box family of ATP-dependent RNA helicases. Its function is to unwind complementary RNA strands in the 3'→5' direction (Lain et al., 1990, 1991) and therefore it plays an important role during viral replication (Fernández et al., 1997). The current understanding of the role of CI in replication and movement has been reviewed by Sorel et al. (2014).

Recently, a hypothetical mechanism has been proposed for the regulation of the NIb repulsive case of TuMV based on the post-translational covalent attachment of the small ubiquitin-like modifier (SUMO) polypeptide to NIb (Xiong & Wang, 2013). The Arabidopsis SUMO-conjugating enzyme, SnSCE1, has been shown to interact with TuMV NIb in the yeast two-hybrid assay and in planta. Furthermore, co-localization of transiently expressed TuMV NIb and SnSCE1 has been demonstrated in the cytoplasm and in the nucleus of N. benthamiana cells. Although it remains to be determined whether NIb is indeed SUMOylated in the context of viral infection, the mutation of a potential SUMOylation site in the SETC1-binding domain of TuMV NIb reduced virus accumulation in infected plants (Xiong & Wang, 2013). As many viruses are known to exploit host SUMOylation pathways for their own benefit (Everett et al., 2013), it is possible that a similar SUMOylation-dependent mechanism might be utilized by potyviruses to regulate their replication.

**Molecular events subsequent to potyviral replication**

Several host proteins involved in translation associate with replication complexes of positive-strand RNA viruses (Ahlquist et al., 2003). These include eIF4E, eIF(iso)4E, PABP, eEF1A and RH8 (Ala-Poikela et al., 111; Beauchemin & Laliberte, 2007; Beauchemin et al., 2007; Huang et al., 2010; Thivierge et al., 2008). Whether any of these proteins are involved in viral replication per se remains to be determined. One possibility is that they assist in directing the replicative viral RNA to replication-associated translation (Cotton et al., 2009; Hafren et al., 2010). Two host proteins, HSP70 and acidic ribosomal protein P0, are upregulated during viral infection (Aranda et al., 1996; Vuorinen et al., 2010; Whitham et al., 2003) and co-purify with the PVA-induced membrane-associated RNP complexes (Hafren et al., 2010, 2013). A functioning HSP70/HSP40 system has been suggested to prevent premature particle assembly at the early stages of infection, thus promoting viral RNA replication and translation (Hafren et al., 2010). P0, however, is required to achieve high virus titers in infected cells, but is not essential for viral spread (Hafren et al., 2013). Despite being present in the membrane-associated VRCs, the primary function of P0 is to regulate translation of PVA RNA.

Following its release from VRC, replicated potyviral RNA can be directed to translation, encapsidation or degradation (reviewed by Mäkinen & Hafren, 2014; Fig. 3). However, little is known about what determines the RNA's fate at this point. One of the roles attributed to the multifunctional protein HC-Pro is suppression of RNA silencing. TuMV devoid of HC-Pro is non-infectious and its infectivity is restored only in an RNA-silencing-deficient genetic background (Garcia-Ruiz et al., 2010). Along the same lines, viral RNA and protein accumulation are reduced in HC-Pro mutants defective in silencing suppression (Kasschau & Carrington, 2001). These findings suggest that in the absence of the RNA silencing suppression activity of HC-Pro, a major part of potyviral RNA becomes susceptible to small interfering (si) RNA-mediated degradation. It was discovered recently that TEV 6K2, a marker of potyviral VRCs, co-localizes with the membrane-associated siRNA bodies (Jouannet et al., 2012). This observation led the authors to suggest that the siRNA bodies represent a point of convergence between viral replication and host defence mechanisms.

In plants, mRNA decay and RNA silencing mechanisms overlap both spatially and functionally (reviewed by Christie et al., 2011). Plants with a mutated DCP2 (decapping enzyme 2), which is a key component of the mRNA decay pathway, show an enhanced silencing phenotype. The phenotype can be reversed by infecting the plant with TuMV, presumably through the action of the RNA silencing suppressor HC-Pro (Thran et al., 2012). These results imply a link between RNA silencing and mRNA decay, which in turn suggests that the progress of potyvirus infection may be affected by both pathways. Yet another pathway which may potentially interfere with potyvirus infection is proteasome-dependent RNA degradation. This possibility is supported by the observation that HC-Pro of Lettuce mosaic virus (genus Potyvirus) modulates the RNase activity associated with the 20S proteasome (Dielen et al., 2011). In addition, inhibition of the host 20S proteasome leads to the increased accumulation of PRSV (Sahana et al., 2012). Future research will likely shed more light on the roles of these RNA degradation pathways in the regulation of potyviral RNA metabolism during infection.

**Potyviral translation**

In the infected cell, viral RNA must be able to compete with endogenous mRNAs for the host cell translation machinery and associated translation factors. Unlike cellular mRNAs, potyviral RNA is not capped, but is instead attached covalently to the genome-linked protein VPg. The presence of such an unusual structure at the 5' end of viral RNA may initially suggest that VPg could play a role in the initiation of viral RNA translation. However, as discussed above, 5' UTRs of several potyviruses facilitate IRES-mediated translation initiation (Basso et al., 1994; Levis & Astier-Manifacier, 1993; Niepel & Gallie, 1999). Therefore, VPg appears not to be required for translation initiation, but rather may be essential for genome stabilization. As IRES-mediated translation initiation does not rely on the canonical cap recognition and scanning mechanisms, it usually does not require the participation of all translation initiation factors. This creates a competitive advantage for
the virus because translation of its genomic RNA may proceed even in the absence of some translation initiation factors. The functional dependency of IRES within the 5’ UTR of TEV on translation initiation factors has been studied using in vitro translation assays partially depleted in eIF4E, eIF(iso)4E, eIF4G, eIF(iso)4G, eIF4A and eIF4B (Gallie, 2001). Under such competitive conditions, the TEV 5’ UTR promoted translation much more efficiently than a control leader sequence of equal length. This may be explained by assuming that either the TEV 5’ UTR recruits the limiting eIFs more efficiently or that it does not require them for translation. Supplementation of the depleted translation mix with purified eIF4G was enough to abolish the translational advantage of the TEV 5’ UTR (Gallie, 2001), implying that eIF4G was recruited more efficiently to TEV IRES than to the control leader sequence. More recently, eIF4G has been shown to bind directly to the 5’ proximal pseudoknot of the TEV 5’ UTR with an ∼20- to 30-fold higher affinity than eIF(iso)4G (Ray et al., 2006). Additionally, eIF4A, a DEAD-box-containing RNA helicase, and its activator eIF4B appear to play moderate roles in TEV translation (Gallie, 2001). Furthermore, potyviral VPgs interact with eIF4E or eIF(iso)4E, depending on the virus and host species (Lénard et al., 2000; Wittmann et al., 1997). This interaction is essential for successful potyvirus infection and its disruption leads to the development of virus resistance (reviewed by Robaglia & Caranta, 2006). Although the exact mechanism behind the interaction is still unknown, possible explanations have been discussed in a recent review (Wang & Krishnaswamy, 2012).

The addition of recombinant VPg or NIa to in vitro translation reactions has been shown to inhibit cap-dependent translation of reporter constructs (Eskelin et al., 2011; Grzela et al., 2006; Khan et al., 2008). This inhibition could be caused by the sequestration of eIF4E and eIF(iso)4E through binding to VPg. Kinetic assays showed a slower accumulation of reporter proteins in reactions that were supplemented with PVA VPg (Eskelin et al., 2011). When the potyviral 5’ UTR was placed in front of the reporter mRNAs, expression of VPg in trans led to a slightly enhanced translation both in vitro and in vivo (Eskelin et al., 2011; Khan et al., 2008). This can be explained by the eIF4E-independent translation initiation from the potyviral 5’ UTRs via an IRES-mediated mechanism. A peptide containing the NTP-binding site of PVA VPg was identified in a complex with eIF(iso)4E, indicating that the site is involved in the interaction (Grzela et al., 2006). In agreement with this finding, mutations at the NTP-binding site of PVA VPg reduced the protein’s ability to inhibit translation (Eskelin et al., 2011).

VPg plays an important role in the translation of the full-length potyviral RNA in planta. PVA VPg boosted translation of viral RNA to a much greater extent than that of monocistronic reporter mRNA, even when the monocistronic mRNA was fused to the PVA 5’ UTR (Eskelin et al., 2011). However, the boost in viral RNA translation was still dependent on the PVA 5’ UTR but not the 3’ UTR. Ectopic VPg expression led to the simultaneous enhancement of viral RNA translation and accumulation. Interestingly, members of the eIF4E family appear to play a role in the VPg-mediated enhancement of viral gene expression, as silencing of eIF4E and eIF(iso)4E blocks the VPg-mediated stimulation (Eskelin et al., 2011). This finding is supported by the increased virus accumulation when PVA VPg is co-expressed with eIF(iso)4E (Hafren et al., 2013). PVA VPg has been shown to be a gene silencing suppressor (Rajamäki & Valkonen, 2009), and this newly discovered VPg function can be used to redirect the replicated viral RNA away from degradation and direct it back to translation.

Riboproteomic studies show that the ribosome composition is dynamic and can change in response to external stimuli (Hummel et al., 2012). There is growing evidence that subpopulations of heterogeneous and functionally specialized ribosomes are present in different plant tissues and are likely involved in the selection of mRNAs for translation (Byrne, 2009; Schippers & Mueller-Roeber, 2010; Whittle & Krochko, 2009). In view of these findings, it is interesting that potyvirus infection activates transcription of genes encoding ribosomal proteins (Alfenas-Zerbini et al., 2009; Dardick, 2007; Yang et al., 2007). For example, infection of N. benthamiana with PPV activated transcription of genes encoding proteins of both ribosomal subunits (Dardick, 2007). However, not all ribosomal protein paralogues were similarly upregulated in the infected cells, suggesting a complex pattern of ribosomal protein expression during PPV infection. In the same way, various members of the ribosomal protein family were differentially induced in TuMV-infected Arabidopsis plants (Yang et al., 2007). The strongest effects on gene expression were observed in cells with the highest levels of virus accumulation. Interestingly, the virus-induced activation of ribosomal protein gene expression did not increase the amount of ribosomes in the infected cells (Yang et al., 2009). This suggests that the newly produced ribosomal proteins perform other functions or that the ribosome composition changes in response to viral infection. Whether these potentially modified ribosomes exhibit a preference for the translation of viral RNA or defence-related mRNAs remains to be studied. Yet another important finding is that potyviral NIa accumulates in the cell nucleolus (Rajamäki & Valkonen, 2009), where rRNA synthesis and ribosome biogenesis take place. In the future, it would be interesting to determine whether NIa can interfere with these processes.

A global reduction in translation typically occurs in response to various cellular stresses, and manifests itself as an increased accumulation of ribosomal subunits and monosomes, accompanied by the simultaneous decrease in polysome levels. A comparison of the polysome profiles of TuMV-infected and healthy Arabidopsis plants did not find any significant differences (Moeller et al., 2012), which provides an additional line of evidence that potyvirus infection does not inhibit translation globally. A common observation in potyvirus-infected cells is that the upregulated transcripts are bound to polysomes, indicating that they are translated
efficiently. However, some of the studied genes showed increased transcription but reduced polysome association or vice versa, demonstrating selective translation (Moeller et al., 2012).

Proteomic studies of potyvirus-infected tissues have not reported major virus-induced changes in ribosomal protein accumulation (Clemente-Moreno et al., 2013; Díaz-Vivancos et al., 2008). Only one ribosomal protein, RPS12, showed reduced accumulation in PPV-infected peach leaves (Yang et al., 2011). As ribosomal proteins are small basic proteins, they are not well separated in standard two-dimensional gel systems; therefore, additional differentially regulated ribosomal proteins could perhaps be identified by modifying the gel systems used for their detection.

TuMV infection foci were reduced in inoculated leaves and systemic movement was abolished or severely reduced upon silencing of RPS2, RPS6, RPL7, RPL13 or RPL19 (Yang et al., 2009). The silencing of these genes did not appear to affect the ribosome amounts, suggesting that these ribosomal proteins may function in processes other than translation. Additionally, the ribosomal stall proteins P0, P1, P2 and P3 appear to be important for potyvirus infection, since their silencing reduces PVA accumulation (Hafre´n et al., 2013). The specific role of P0 in the VPg-mediated PVA translation was discussed earlier in this review and the importance of P1–P3 for PVA infection is likely explained by their essential roles in viral translation.

Concluding remarks

Infection with potyviruses involves complex molecular processes which take place in several cellular compartments, and requires the participation of many host genes and proteins. One of the future challenges is to understand the crosstalk between various virus-induced processes in the cytoplasm and host gene expression in the nucleus. Whilst the immediate/early events of potyvirus infection are still largely unknown, the emerging view is that signalling networks, likely including Ca²⁺ signalling, transduce virus-induced signals to the nucleus, triggering changes in host gene expression. This hypothesis raises many intriguing questions. Which signalling cascades and transcription factors are activated in response to viral infection? Do viral proteins play an active role in the transcriptional reprogramming of host gene expression in the nucleus? What are the exact roles of host genes that are activated or repressed? What are the transcriptional mechanisms underlying the virus-induced modification of the intracellular environment? Yet another future goal will be to develop new research tools to aid in the understanding of potyviral pathogenicity mechanisms. Such tools may include methods to isolate various protein–protein and RNP complexes from different compartments of the infected cell. New cell biological and biochemical assays will be required to determine the functions of each complex and its individual components during viral infection. Whilst much is already known about the 6K2-induced cellular structures, there is still more to be learned about the replication and movement functions associated with these structures and their regulation.

Despite many advances, our knowledge of the fate of replicated viral RNA is still limited. For example, we do not really know how viral RNA is released from VRCs. It is also unclear what mechanism(s) determines whether replicated viral RNA will be recruited to polysomes to serve as a template for new rounds of translation, whether it will be carried to the PD for transport into neighbouring cells, encapsidated into progeny virions or even targeted for degradation. Although modern potyvirus research offers many fundamental and challenging questions to virologists, it is of the utmost importance to use the results of this research in plant-breeding programmes to control economically important viral diseases.

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