Potato virus Y HCPro Localization at Distinct, Dynamically Related and Environment-Influenced Structures in the Cell Cytoplasm

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Potyvirus HCPro is a multifunctional protein that, among other functions, interferes with antiviral defenses in plants and mediates viral transmission by aphid vectors. We have visualized *in vivo* the subcellular distribution and dynamics of HCPro from *Potato virus Y* and its homodimers, using green, yellow, and red fluorescent protein tags or their split parts, while assessing their biological activities. Confocal microscopy revealed a pattern of even distribution of fluorescence throughout the cytoplasm, common to all these modified HCPros, when transiently expressed in *Nicotiana benthamiana* epidermal cells in virus-free systems. However, in some cells, distinct additional patterns, specific to some constructs and influenced by environmental conditions, were observed: i) a small number of large, amorphous cytoplasm inclusions that contained α -tubulin; ii) a pattern of numerous small, similarly sized, dot-like inclusions distributing regularly throughout the cytoplasm and associated or anchored to the cortical endoplasmic reticulum and the microtubule (MT) cytoskeleton; and iii) a pattern that smoothly coated the MT. Furthermore, mixed and intermediate forms from the last two patterns were observed, suggesting dynamic transports between them. HCPro did not colocalize with actin filaments or the Golgi apparatus. Despite its association with MT, this network integrity was required neither for HCPro suppression of silencing in agropatch assays nor for its mediation of virus transmission by aphids.

Members of the genus *Potyvirus* are filament-shaped, plus-sense RNA viruses that express a single polypeptide that undergoes proteolytic cleavage to generate the final products, although one small essential gene (*P3N-PIPO*) expresses itself via translational frameshifting (Chung et al. 2008). The proteases involved in this processing are the P1 protein, which is

the first product from the polyprotein that detaches itself at its C terminus from the following HCPro (Verchot and Carrington 1995a; Verchot et al. 1991): HCPro, a papain-like protease that detaches itself at its C terminus from the rest of the polyprotein (Carrington and Herndon 1992); and the small nuclear inclusion body protein (NIa), which cleaves the remaining sites (Carrington et al. 1988; García et al. 1989). *Potyvirus* polyprotein translation is thought to be closely coupled to viral replication which, as in many RNA viruses, takes place in replicative complexes associated with membrane vesicles that originate from the endoplasmic reticulum (ER). In the specific case of potyviruses, these vesicles are transported from the ER via actin filaments toward the chloroplasts for enhanced replication (Wei and Wang 2008; Wei et al. 2013). The transport of potyviral protein products from replication-translation vesicle factories to other parts of the cell may be mediated by routes such as the secretory pathway or the cell microtubules (MT) or microfilaments, although our knowledge of these processes is still fractionary (Sorel et al. 2014).

HCPro is a multifunctional protein that, in addition to its protease activity, is involved in the suppression of the antiviral gene silencing defenses of the host, among other functions (Anandalakshmi et al. 1998; Brigneti et al. 1998; Canto et al. 2002; Johansen and Carrington 2001; Rajamäki et al. 2005; Valli et al. 2006). It is also required in the transmission of potyviruses by their insect aphid vectors; hence its original name of “helper component” (Govier and Kassanis 1974). HCPro has been traditionally divided into three domains: the N-terminal domain associated with aphid transmission (Blanc et al. 1997; Canto et al. 1995b) and its interaction with proteasomal units (Jin et al. 2007a; Sahana et al. 2014), the central domain associated with the suppression of silencing function (Shiboleth et al. 2007), and the C-terminal domain of the protein that harbors its protease activity (Carrington and Herndon 1992) and interaction domains to plant HIP2-like MT-associated proteins (Guo et al. 2003; Haikonen et al. 2013a and b).

Soluble HCPro from several potyviruses extracted from infected plants has been shown by size chromatography to be formed mainly by homodimers and further aggregates (Plisson et al. 2003; Ruíz-Ferrer et al. 2005; Tena et al. 2013; Thornbury et al. 1985), which could be relevant to some of the biological functions of the protein. Self-interactions of HCPros have also been demonstrated by yeast two-hybrid assays (Y2H) (Urcuqui-

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Inchima et al. 1999) or visualized in the plant cell cytoplasm by bimolecular fluorescence complementation (BiFC) (Ala-Poikela et al. 2011; Sahana et al. 2012; Zheng et al. 2011; Zilian and Maiss 2011).

Despite being one of the most studied plant virus proteins, relatively little is known about the precise modes by which HCPro undertakes these two principal functions of silencing suppression and mediation of viral transmission by vectors. As regards silencing suppression, it is known that HCPro interacts in vitro with long RNAs (approximately 200 nucleotides) and small RNAs (Chapman et al. 2004; Lakatos et al. 2006; Maia and Bernardi 1996; Mangrauthia et al. 2009; Mérai et al. 2006; Shibolet et al. 2007; Urcuqui-Inchima et al. 2000) but at protein/RNA molar ratios much higher than the 2:1 characterized for the 2b and P19 suppressors of *Tomato bushy stunt virus* (TBSV) (González et al. 2012; Vargasson et al. 2003) and *Cucumber mosaic virus* (CMV), thus making it unlikely that HCPro interferes with antiviral silencing in the same way as P19 and 2b do; that is, by means of sequestering small interfering RNAs. On the other hand, HCPro interacts in vitro with the RNA methyltransferase HEN-1, inhibiting it (Jamous et al. 2011). Suppressor activity of HCPro also seems influenced by the P1 protein (Canto et al. 2002; Johansen and Carrington 2001; Pruss et al. 1997; Rajamäki et al. 2005; Valli et al. 2006; Verchot and Carrington 1995a and b). That P1 detachment is essential for HCPro suppressor activity in agropatch assays was recently demonstrated (Pasin et al. 2014). However, a direct role for P1 on HCPro suppressor activity either as cofactor or by influencing its conformation during translation to improve its stability has been excluded and it appears, instead, that its effect on HCPro can be explained by cis-acting elements that favor HCPro translation in the upstream presence of P1, without ruling out a possible role of P1 in protecting HCPro from degradation (Tena et al. 2013).

As regards viral transmission by vectors, HCPro is required to be taken by the vector prior to or simultaneously with virions for transmission to occur. HCPro can interact with the homologous viral coat protein in vitro, as well as in plant extracts (Blanc et al. 1997; Roudert-Tavert et al. 2002). Of not clear significance to transmission, HCPro has been found to be present at one end of a tiny minority (less than 3%) of *Potato virus Y* (PVY) and *Potato virus A* (PVA) virions (Torrance et al. 2006).

HCPro interaction with plant factors and cytoplasmic structures involved in processes other than gene silencing is extensive: it binds a host rgs-CaM factor, which directs its degradation through autophagy (Nakahara et al. 2012) to the *Arabidopsis thaliana* transcription factor RAV2 (Endres et al. 2010) to components of the proteasome, which participate in another type of antiviral defense (Ballut et al. 2005; Dielen et al. 2011; Sahana et al. 2012), with translation initiation factors (Ala-Poikela et al. 2011; Freire 2014), with chloroplast factors (Cheng et al. 2008; Jin et al. 2007b), and with MT-binding HIP2-type proteins (Guo et al. 2003; Haikonen et al. 2013).

As regards the subcellular loci where several HCPro functions and interactions take place, early electron microscopy immunolocalization studies situated PVY HCPro in the cytoplasm of fixed infected mesophyll cells forming amorphous inclusions (Baunoch et al. 1990). Studies using fixed cowpea protoplasts found HCPro from *Cowpea aphid-borne mosaic virus* (CABMV) diffused throughout the cytoplasm (Mlotshwa et al. 2002). In live tissues, HCPro from *Turnip mosaic virus* (TuMV) tagged with green fluorescent protein (GFP) was also found to localize to the cytoplasm of epidermal *Nicotiana benthamiana* cells as filaments, around the nucleus, and in what could be the ER; whereas, in protoplasts from transgenic *N. benthamiana* plants expressing GFP targeting the ER (line

16c) (Ruíz et al. 1998), untagged HCPro from TuMV induced morphological changes in the ER structure, suggesting an interaction between HCPro and this structure (Zheng et al. 2011). Furthermore, PVA HCPro interaction with the host HIP2 factor has been visualized by BiFC, coating in vivo MT (Haikonen et al. 2013b). Thus, evidence from the literature with regard to HCPro subcellular targets and its accumulation originates from different potyviruses and hosts and is diverse, pointing at different subcellular structures and features.

In this work, we have used HCPros modified with fluorescent proteins to visualize the protein in the living cell and its dynamics in vivo, while characterizing their biological properties using activity assays. Our initial hypothesis was that this approach could allow us to link observations on subcellular targets of HCPro to its biological functions, by disrupting those targets and assessing their effect on the activity assays. Unlike previous works by others, we carried out a comparative study on the subcellular distribution of PVY HCPro using different, functionally characterized constructs fused to GFP and yellow and red fluorescent protein (YFP and RFP, respectively) tags or their split halves to obtain a more comprehensive picture of HCPro subcellular whereabouts and dynamics. We found that, in a virus-free transient expression system, HCPro distributed evenly throughout the cytoplasm. However, for some constructs and in some cells, we could also identify accumulation in distinct structures: as a few large, irregular inclusions; as numerous evenly distributed dot-shaped inclusions of similar size associated with both cortical ER and MT; and, finally, colocalizing extensively with the MT cytoskeleton. Although, in some individual cells, localization at one particular structure was prevalent, in other cells mixed and intermediate patterns could be found, suggesting that they evolved from one into another and, thus, were dynamically linked. Furthermore, the presence of these structures was influenced by environment stresses. We also tested the need for HCPro functions of its interactions with defined subcellular structures by disrupting them and performing biological activity assays. Potential biological implications of our results are discussed.

RESULTS

Fluorescent tags affect HCPro biological activities to different degrees.

To study the subcellular distribution of PVY HCPro in vivo, we tagged it with fluorescent proteins or their split parts (Fig. 1). C-terminal tagging of HCPro included GFP and monomeric (m)RFP (Fig. 1, constructs P1-HCPro-GFP and P1-HCPro-mRFP, respectively). In these C-terminal fusion constructs, the second glycine at the HCPro protease cleavage site (tyrosine-X-valine-glycine/glycine) had been replaced by tyrosine-arginine-valine-glycine/alanine, a motif shown previously not to be cleaved by *Tobacco etch virus* (TEV) HCPro in rabbit reticulocyte lysates (Carrington and Herndon 1992). However, Western blot analysis of the expressed proteins showed that both had the same size as the native protein (Supplementary Fig. S1, Western blot, third and fourth lanes versus second lane). The most likely explanation for these data is that, after translation of the fusion product, the fluorescent tags became detached from HCPro. Thus, our C-terminal tagging approach proved inefficient to its purpose.

N-terminal taggings of HCPro with GFP, mRFP, split (s)YFP (sYN and sYC), and split monomeric (sm)RFP (sRN and sRC) parts (Fig. 1) were stable, and fusion proteins had the expected size in Western blots (Fig. 2; data not shown). All the constructs had an additional hexahistidine motif in frame, located either at the end of the N terminus of the fusion construct (plus a starting methionine) in the case of GFP- or sYFP-

tagged constructs, or between the fluorescent tag and HCPro in the case of mRFP- or smRFP-tagged constructs (Fig. 1).

Suppression of the silencing of a free *GFP* reporter by these N-fusion constructs in agroinfiltration patch assays showed that tags affected this HCPro activity to varying degrees. Suppression was very weak in both of the GFP-tagged constructs (Fig. 2A, leaves in the upper panel and Western blot panel). By contrast, the sYFP-tagged constructs showed stronger suppressor activities; in particular, construct 6x-sYN-HCPro or when both N- and C-terminal parts were combined. The accumulation of construct 6x-YC-HCPro was also weak in infiltrated tissue but it rose when it was coexpressed with construct 6x-YN-HCPro (Fig. 2A, leaves in the middle panel, and Western blot panels). In contrast to the GFP constructs, the mRFP-tagged construct displayed suppression of silencing activity (Fig. 2B); in particular, that from the combined smRFP-tagged constructs (Fig. 2C). For this high preservation of suppressor activity and for their good fluorescence response under confocal microscopy, the use of BiFC smRFP-tagged HCPro constructs was preferred, when possible, in our *in vivo* subcellular localization studies.

Regarding mediation of virus transmission by aphids, we tested 6x-HCPro and 6x-GFP-HCPro after their large-scale purification from plant tissue infected with PVX-P1-6x-HCPro or with PVX-P1-6x-GFP-HCPro, respectively. Although purified 6x-HCPro successfully mediated transmission of PVY virions in aphid membrane feeding tests, 6x-GFP-HCPro did not (Supplementary Fig. S2). Therefore, the tagging of HCPro with GFP had a severe negative effect on both activities, silencing suppression in agropatch assays and mediation of virus transmission by insects. Tagging with mRFP and smRFP was less detrimental to suppressor activity. These two latter constructs were not tested for aphid transmission activity.

HCPros with fluorescent tags accumulate in distinct yet dynamically related as well as environment-influenced structures in epidermal cells.

We used confocal microscopy of our N-terminal tagged constructs to visualize HCPro in subcellular structures within liv-

ing cells. We had GFP and mRFP-tagged constructs as well as constructs that were tagged with split parts of either YFP (sYFP) or mRFP (smRFP). In the latter two cases, visualized fluorescence would correspond only to the interacting homodimers of HCPro that had complementary tags (sYN + sYC tags; sRN + sRC tags) as a result of BiFC.

Confocal imaging of single epidermal cells showed that, in many cells, fluorescence derived from constructs P1-6x-GFP-HCPro and mRFP-6x-HCPro distributed smoothly throughout the cytoplasm, negatively staining some unidentified vesicle structures (Fig. 3, upper panels). In addition, large irregular inclusions were observed in the case of the GFP-tagged construct but not in the case of the mRFP-tagged one (Fig. 3, upper left panel, arrows)

Confocal imaging at low magnification covering fields of approximately 20 *N. benthamiana* epidermal cells transiently expressing GFP- or sYFP-tagged HCPros showed, in both cases, a similar pattern of distribution, in which most of the fluorescence appeared distributed throughout the cytoplasm; in particular, as the large inclusions aforementioned of irregular or amorphous shape. Interestingly, the transient presence of heterologous suppressors of silencing, provided with the intention of boosting the accumulation of HCPro, such as TBSV P19 or the CMV 2b protein, had the effect of reducing both the number and size of these irregular inclusions throughout the cytoplasm without increasing the overall accumulation of HCPro (Fig. 3, second and third rows of panels from the top, respectively). At the same low magnification level, a few irregular inclusions were also found in smRFP-tagged HCPro, usually one or two inclusions per cell, much less than those found in GFP- or split YFP-tagged constructs. The presence of heterologous suppressors P19 or 2b was also detrimental to their number and size (Fig. 3, fourth row of panels from the top; data not shown). These data show that the formation of large irregular inclusions can be influenced by factors from the environment in which the constructs are expressed.

In some epidermal cells, instead of the diffuse distribution of fluorescence throughout the cytoplasm, other different and distinct patterns were found: fluorescence from smRFP-tagged constructs was found in some cells distributed as dots of similar size, regularly positioned in a mesh pattern. In some of these cells, the dots appeared interconnected by filaments of red fluorescence of a similar diameter. In other cells, only the smooth red filaments were visible (Fig. 4, second row of panels from the top). The presence of fluorescent filaments connecting dots was also demonstrated for mRFP-, sYFP-, and GFP-tagged HCPros (Fig. 4, upper and lower rows of panels, respectively; data not shown). We found cells harboring these dots and filaments plus their intermediate forms mainly but not exclusively when infiltrated tissue was exposed to some stresses; in particular, volatile components from common nail varnish used as sealant at the edges of the infiltrated plant tissue several minutes prior to confocal viewing. It must be highlighted that none of the epidermal cells imaged had direct contact with the varnish, they were fully turgid, and they had their subcellular structures visually unaltered (Fig. 5, integrity of the ER). Cells with filaments could also sometimes be found in tissue treated with saturated CO₂ atmosphere (data not shown). By contrast, 50 aphids left overnight on the infiltrated patch before confocal viewing failed to induce the filament pattern (data not shown). These responses were specific to HCPro constructs, because transiently expressed free mRFP or split mRFP-tagged 2b protein dimers did not alter their diffuse cytoplasmic and nuclear localization under the same stresses (data not shown; Supplementary Fig. S3). We did not identify which component of the nail varnish was responsible for the response. For example, one of its volatile components,

Fig. 1. N-terminal and C-terminal tagging of HCPro for fluorescence studies *in vivo*. Schematic representation of the different constructs tagged with green fluorescent protein (GFP), N-terminal and C-terminal split yellow fluorescent protein parts (sYN and sYC, respectively), monomeric red fluorescent protein (mRFP), or N-terminal and C-terminal split mRFP parts (sRN and sRC, respectively) at either the N or the C terminus of HCPro, and in the upstream presence or absence of P1 protein during translation.

n-butyl acetate, can be a component in insect pheromones. However, we tested the two components (n-butyl acetate and ethyl acetate) separately and neither induced the HCPro filament distribution pattern in our experimental conditions (data not shown).

To identify whether the HCPro-derived fluorescence as dots or filaments or as large inclusions colocalized with defined subcellular structures, we used transgenic *N. benthamiana* plants that constitutively expressed GFP fused to *A. thaliana* α -tubulin (Tua-GFP plants) (Gillespie et al. 2002), GFP targeting the cortical ER (line 16c) (Ruiz et al. 1998), and a Lifeact TagRFP fusion targeting actin. To visualize the Golgi apparatus, we also transiently overexpressed a binary vector expressing mCherry protein with a targeting signal to this structure (Nelson et al. 2007). In the green-fluorescing backgrounds of the 16c and Tua-GFP transgenic plants, we transiently expressed split red HCPro constructs and detected the BiFC-derived fluorescence of the interacting homodimers. As we had previously

seen in nontransgenic plants (Fig. 4), we could find fluorescence from sRN-6x-HCPro + sRC-6x-HCPro BiFC distributing as dots, filaments, or intermediates in some epidermal cells of both stress-treated 16c and Tua-GFP plants (Fig. 5). In 16c plants, the cortical ER fluoresced green in a characteristic polygonal net (Fig. 5, upper green and overlay panels). Although the BiFC-derived red fluorescence from interacting HCPros did not overlap with that from the ER, images showed that the red dots appeared nevertheless anchored somehow or linked to the ER scaffold (Fig. 5, overlay panels in upper row). The red filaments seemed, by contrast, unrelated to the ER structure (Fig. 5, overlay panel in second row from the top). In stressed Tua-GFP plants, many epidermal cells displayed a filamentous green fluorescent MT cytoskeleton (Fig. 5, green and overlay panels in third and fourth rows from the top). In some of the cells, the HCPro BiFC-derived red filaments but not many of the red dots matched exactly the green MT fluorescence (Fig. 5, overlay panels in third and fourth rows from the top; data

Fig. 2. Suppression of silencing in agroinfiltration assays by N-terminally tagged HCPro constructs: a binary vector expressing a *green fluorescent protein* (GFP) reporter was agroinfiltrated together with the empty binary vector pROK2 (right side of leaves) or together with binary vectors expressing *Potato virus Y* (PVY)-derived constructs. **A**, P1-6x-GFP-HCPro, 6x-GFP-HCPro, 6x-sYN-HCPro, 6x-sYC-HCPro or 6x-sYN-HCPro, plus 6x-sYC-HCPro (left side of upper left to bottom right leaves, respectively) and upper Western blot panel in which a polyclonal GFP antibody was used. The increase in GFP-derived fluorescence and on its accumulation was greatest in presence of P1-6x-HCPro and of 6x-sYN-HCPro, either alone or in combination with 6x-sYC-HCPro. By contrast, GFP tagging greatly reduced or abolished HCPro suppressor activity in this type of assay. Note that the serum also detects the GFP and split yellow fluorescent protein (sYFP)-tagged HCPro constructs. **B**, P1-6x-HCPro and mRFP-6x-HCPro (lower right side of leaf and upper patches, respectively) and right side Western blot panel using an anti 6xhistidine tag antibody. **C**, P1-6x-HCPro, sRN-6x-HCPro, sRC-6x-HCPro, and sRN-6x-HCPro plus sRC-6x-HCPro (as labeled by the infiltrated patches). In contrast to the 6x-GFP tag, the monomeric red fluorescent protein (mRFP)-6x tag did not prevent suppressor activity, nor did the tags of the split mRFP parts (corresponding patches in leaves, and Western blot panel to GFP). In all Western blot panels, lane H shows a sample extracted from a healthy plant and lane M = prestained markers for molecular weight, indicated in kilodaltons (kDa).

not shown). In addition, green fluorescence derived from the GFP-tagged α -tubulin transgene product was also found in the large red irregular inclusions (Fig. 5, arrow in overlay panel in bottom row). Thus, large irregular HCPPro inclusions contain tubulin.

In epidermal cells of red actin transgenic plants, green fluorescence derived from the BiFC of transiently expressed 6x-sYN-HCPPro + 6x-sYC-HCPPro constructs did not colocalize with the red fluorescence associated with the plant actin filaments (Fig. 6, upper panels). Likewise, coexpression of 6x-sYN-HCPPro + 6x-sYC-HCPPro with an m-cherry construct designed to target the Golgi stacks detected no sign of colocalization (Fig. 6, lower panels).

Disruption of the MT network does not prevent HCPPro suppression of silencing activity or its mediation of viral transmission by insect vectors.

To test whether the integrity of the MT network would be required for HCPPro biological activities that we could test in activity assays (suppression of silencing in patch assays or mediation of viral transmission by insect vectors), we disrupted the MT cytoskeleton using colchicine, as described by Wright and associates (2007). Three days after agroinjection of smRFP-HCPPro constructs in *Tua*-GFP plants with colchicine in the infiltration buffer, green fluorescence derived from transgenic tubulin was distributed as dots or blobs instead of as filaments in epidermal cells, under stress conditions. By contrast, green fluorescence was distributed as filaments in the patches from the same plants injected with buffer lacking colchicine (Fig. 7A). In both cases, red fluorescence derived from BiFC of smRFP-tagged HCPPro colocalized with the green tubulin, as either dots or filaments (Fig. 7A).

To test for the suppression of silencing activity of HCPPro under undisrupted versus colchicine-disrupted MT cytoskeleton, we carried out a 3-day suppression of silencing patch assay using a free GFP reporter and suppressor P1-6x-HCPPro, as well as CMV 2b and TBSV P19. The test was performed both in nontransgenic and in *Tua*-GFP-transgenic *N. benthamiana* plants. In all cases, suppression of the partial silencing of the transiently expressed GFP reporter was comparable in the presence and in the absence of colchicine (Fig. 7B, upper versus lower patches in left side of leaves; compare GFP fluorescence derived from the reporter under UV light). This happened even if the amount of colchicine injected ($5 \mu\text{M}$) was 10 times that required for efficient disruption of the MT (Fig. 7B, lower panel). Thus, integrity of the MT cytoskeleton is not required for suppressor of silencing activity in patch assays.

To test the potential requirement of intact MT cytoskeleton for HCPPro to mediate aphid transmission, we injected aphid-transmissible, PVY-infected *N. benthamiana* leaves with either colchicine or buffer without this substance and, 3 days after injection, we carried out viral transmission tests with aphids. Transmission efficiencies were similar in both cases, at 100%. Furthermore, we carried out one experiment in which we injected $5 \mu\text{M}$ colchicine, 10 times the amount required to disrupt the cytoskeleton, and the results were similar (Fig. 7C).

DISCUSSION

Members of the genus *Potyvirus* are among the most successful and economically relevant plant viruses to agriculture, despite their translation strategy leading to the expression of almost all their protein factors in equimolar amounts. Because it appears that not all these proteins would be required in similar amounts at any given time during the viral infection cycle, this strategy would not seem efficient, unless turnover of the different viral products and their accumulation in specific

structures for storage, removal, or degradation within the cell at different times or under different environmental conditions was closely integrated and programmed. In this regard, several potyvirus proteins are known to form inclusion structures in

Fig. 3. Visualization of the distribution of fluorescence derived from green fluorescent protein (GFP)-tagged HCPPro (construct P1-6x-GFP-HCPPro) from monomeric red fluorescent protein (mRFP)-tagged HCPPro (construct mRFP-6x-HCPPro), or from the bimolecular fluorescence complementations (BiFC) between the two split yellow fluorescent protein (sYFP)-tagged HCPPro (constructs 6x-sYN-HCPPro and 6x-sYC-HCPPro) or the two split monomeric red fluorescent protein (smRFP)-tagged HCPPro (constructs sRN-6x-HCPPro and sRC-6x-HCPPro) transiently expressed by agroinfiltration in fields of epidermal cells of *Nicotiana benthamiana* leaves. Fluorescence derived from the GFP and mRFP tags is found throughout what appears as the cytoplasm; in particular, in large irregular inclusions (arrows in panels) that were absent in the case of construct mRFP-6x-HCPPro (upper left and right panels, respectively). Addition of heterologous viral suppressors of silencing such as the tombusviral P19 or cucumoviral 2b protein had the effect of making these inclusions smaller, without increasing the steady-state levels of HCPPro, as indicated in the Western blot panel for construct P1-6x-GFP-HCPPro (third lane, P19 added versus second lane, empty vector added) or for the BiFC pairs 6x-sYN-HCPPro + 6x-sYC-HCPPro and sRN-6x-HCPPro + sRC-6x-HCPPro (third and fourth rows of panels from the top, respectively). Size bars are indicated in the bottom right corner.

different subcellular compartments such as, among others, the nuclear inclusion proteins NIa and NIb within the nucleus, the characteristic plasmodesmata (PD)-associated pinwheels formed by the cylindrical inclusion (CI) helicase protein in the cytoplasm (Sorel et al. 2014), or the cytoplasm-scattered amorphous inclusions of HCPro (Baunoch et al. 1990).

Despite extensive literature, our overall view of the turnover of potyviral proteins or the structures they form and their biological significance is rather poor. That is, are these structures functional sites or only places to remove excess protein? Are these structures reversible or do they contain host factors such as proteins or nucleic acids that may interfere with the progress of the infection or, alternatively, that may need recruiting for use at some further step of the viral infection? How do all of these proteins travel there from the translation sites? In this regard, research into the CI helicase has shown how this protein, in addition to its enzymatic role in replication, is involved in the translation of the vesicle-associated viral replication factories from the ER toward the protoplast membranes via actin

filaments (Wei et al. 2010a), while it also translocates via the ER-Golgi secretory pathway toward the PDs, where it anchors through its interaction with viral P3N-PIPO and is involved somehow in the local movement of the virus (Wei et al. 2010b).

In contrast to the CI protein, we have a less integrated view of the subcellular dynamics of HCPro interactions, translocation pathways, accumulation, and their relation to its several functions. HCPro is a factor important to processes such as viral replication or local movement but two roles make it essential to the virus cycle: as a strong suppressor of antiviral gene silencing and as a helper factor in the horizontal transmission of the virus by vectors. Microscopy observational data from previous works with different potyviral HCPros have individually linked it to the cytoplasm, distributing diffusely or forming inclusions (Baunoch et al. 2002; Mlothswa et al. 2002; Sahana et al. 2012, 2014; Zheng et al. 2011; Zillian and Maiss 2011). It has also been suggested that it may affect ER structure (Zheng et al. 2011), and it has been found to bind in Y2H and *in vivo* (BiFC) to a protein (HIP2) associated with the cellular

Fig. 4. Close-up view at high magnification within the cytoplasm of individual epidermal cells in which fluorescence distributes as dots of similar size arranged in a regular, reticulate pattern (leftmost panels), as combinations of fluorescent dots and connecting filaments, or mainly as filaments (central and rightmost panels, respectively). The horizontal arrow above the set of panels indicates the increase in filament- versus dot-associated fluorescence and does not suggest any direction to protein traffic. Size bars for panels are indicated in the bottom right corner.

sRN-6x-HCPro
sRC-6x-HCPro in transgenic plants expressing green fluorescing cortical endoplasmic reticulum

sRN-6x-HCPro
sRC-6x-HCPro in transgenic plants expressing green fluorescing α -tubulin

Fig. 5. Visualization of the subcellular distribution of fluorescence derived from the bimolecular fluorescence complementation (BiFC) between the two split monomeric red fluorescent protein (smRFP)-tagged HCPros (constructs sRN-6x-HCPro and sRC-6x-HCPro) transiently expressed by agroinfiltration in epidermal cells of transgenic *Nicotiana benthamiana* leaves that show their cortical endoplasmic reticulum (ER) fluorescing green (Line 16c) (Ruíz et al. 1998) or their microtubule (MT) cytoskeleton fluorescing green by expressing green fluorescent protein (GFP)-tagged α -tubulin (Tua-GFP plants) (Gillespie et al. 2002). Panels show either monochrome green (from ER or MT in transgenic plants) or red (from BiFC HCPro transiently expressed constructs) fluorescence, or overlays of both colors for colocalization studies. HCPro fluorescence either as dots (upper top left panel and enlarged area) or as filaments (upper middle panels) did not colocalize with that from the ER (middle panels). However, HCPro fluorescent dots appeared anchored to the ER scaffold (upper top panels). By contrast, HCPro distribution as filaments matched perfectly that of the MT cytoskeleton but not when distributing as dots (lower panels). Note that, interestingly, tubulin-derived green fluorescence did colocalize with that from HCPro in large irregular inclusions (arrow in lower panels). Size bars for corresponding panels appear at the bottom right corners.

MT, an interaction that is important to virus infection (Guo et al. 2003; Haikonen et al. 2013a and b). Our present work's contribution to PVY HCPPro subcellular whereabouts and dynamics in vivo uses fluorescently tagged constructs whose biological activities as suppressors have been assessed (Fig. 2). Although several studies have been published involving tagged HCPPro for in vivo localization or protein–protein interaction studies using confocal or epifluorescent microscopy or Y2H assays, surprisingly little has been done to assess the effect of these tags and modifications on the biological activities of the tagged constructs in planta. To our knowledge, ours is the first assessment of suppressor activity of the different tagged HCPPro studied, using agropatch assays.

With our N-terminal-tagged constructs, we could visualize fluorescence derived from both single HCPPro molecules and the interacting homodimers that carried complementary BiFC tags. Our work was carried out in a virus-free system and, thus, it does not replicate the infected cell environment. As a transient expression system, there was no infection front or infected rearward to distinguish, either. Even so, our results were remarkably in agreement with previous published observations, and data from the different constructs we have used were consistent overall. PVY HCPPro showed a diffuse presence throughout the cytoplasm, as well as large irregular inclusions (Fig. 3). These inclusions could be the equivalent of amorphous inclusions observed by immunomicroscopy (Baunoch et al. 1990). We did not observe them for construct mRFP-6x-HCPPro but this might be because the tag prevented their formation, the same way that the GFP tag interfered with the suppressor activity of HCPPro. However, because construct mRFP-6x-HCPPro displayed good suppressor activity in agropatch assays, the irregular inclusions are seemingly not required for

this function. Two features of these large inclusions can be underlined. The first is that, in the presence of heterologous suppressors of gene silencing such as P19 or 2b protein, both their number and size diminish drastically (Fig. 3). We do not know the reason for this, but we could speculate that a host factor, either protein or nucleic acid, required for the formation of these inclusions is prevented from doing so by the heterologous suppressors. In any event, it shows that inclusion formation (their number and size) is dependent on factors external to HCPPro itself and suggests that they may not be as irreversible or permanent as thought. The second observation is that, in *Tua*-GFP transgenic plants, these large inclusions contain GFP-tagged α -tubulin (Fig. 5). This suggests that these inclusions could be linked to the MT cytoskeleton. In this regard, it appears relevant to mention that caulimovirus aphid transmission helper factor P2 (the equivalent to potyviral HCPPro as an aphid helper transmission factor) accumulates in a tubulin- and virion-containing large inclusion that, in response to insect probing, disperses throughout the cell cytoplasm using the MT cytoskeleton, facilitating transmission (Martinière et al. 2013).

We also found two additional characteristic patterns of subcellular localization of PVY HCPPro. One was a not previously described pattern of small dots of similar size, distributed regularly throughout the cytoplasm, which in 16c ER-fluorescent transgenic plants appeared clearly associated or anchored to the ER (Fig. 5). ER-linked structures that bear visual resemblance to these have been reported for other virus proteins such as *Tobacco mosaic virus* movement protein (Gillespie et al. 2002) which, on its way to the proteasome for degradation, also associates with MT or, in the case of potyviruses, the 6K protein (Wei and Wang 2008), which would be related to the genesis of sites of viral replication-translation. In our virus-

Fig. 6. Visualization of the subcellular distribution of fluorescence derived from the bimolecular fluorescence complementation (BiFC) between the two split yellow fluorescent protein (sYFP)-tagged HCPPro constructs (6x-sYN-HCPPro and 6x-sYC-HCPPro) transiently expressed in epidermal cells of transgenic *Nicotiana benthamiana* leaves that constitutively express a Lifeact TagRFP fusion targeting actin in the upper row of panels, or together with a binary vector that expresses mCherry protein with a targeting signal to Golgi stacks (Nelson et al. 2007) in the lower row of panels. Panels show either monochrome green (from BiFC-derived HCPPro fluorescence) or red (from red actin filaments or from Golgi) fluorescence, or overlays for colocalization studies. No colocalization between green and red fluorescence was observed in both cases. Size bars for corresponding panels appear at the bottom right corners.

A sRN-6x-HCPro
sRC-6x-HCPro in Tua-GFP transgenic plants expressing green fluorescent α -tubulin

Fig. 7. Disruption of the microtubule (MT) cytoskeleton and its effect on HCPro suppression of silencing activity and on its mediation of virus transmission by aphids. **A**, Visualization of the subcellular distribution of fluorescence derived from the bimolecular fluorescence complementation (BiFC) between the two split monomeric red fluorescent protein (smRFP)-tagged HCPros (constructs sRN-6x-HCPro and sRC-6x-HCPro) transiently expressed by agroinfiltration in epidermal cells of transgenic *Nicotiana benthamiana* leaves that express green fluorescent protein (GFP)-tagged α -tubulin (Tua-GFP plants) (Gillespie et al. 2002). Agrobacteria was infiltrated in the absence or presence in the agroinfiltration solution of colchicine at 0.5 μ M (upper and lower rows, respectively). Colchicine had the effect of disrupting the MT cytoskeleton into dots (lower green panel). However, red fluorescence from BiFC of HCPro constructs still colocalized with that of the tubulin in those dots. **B**, Suppression of the silencing in agropatch assays of a binary vector expressing a *green fluorescent protein* (GFP) reporter agroinfiltrated together with the empty binary vector pROK2 (right side of leaves) or together with binary vectors expressing tombusviral P19, *Cucumber mosaic virus* 2b protein, *Potato virus Y* (PVY)-derived P1-6x-HCPro construct or the combined sRN-6x-HCPro plus sRC-6x-HCPro constructs (left side of leaves): suppressor effects on the transient levels of fluorescence derived from the reporter did not appear affected adversely by the colchicine. **C**, Transmission by aphids of PVY from systemically infected *N. benthamiana* leaves. Leaves had been infiltrated 3 days prior to the transmission experiment with water (as control) or with colchicine (0.5 μ M in experiment 1 and 5 μ M in experiment 2). Five aphids were used per receptor plant. Transmission was 10 out of 10 plants in all cases.

free system, PVY HCPro localized to these structures without need of interaction with another viral factor. The second pattern showed HCPro-derived fluorescence as filaments which, in *Tua*-GFP plants, overlapped the fluorescent MT (Figs. 5 and 7). Interestingly, the ER-associated dots of HCPro were also connected to the MT cytoskeleton (Fig. 5, third row of images). Our results are in agreement with observations by Haikonen and associates (2013a and b) that showed, through Y2H, that HCPro from both PVY and PVA bound to the MT-associated factor HIP2. Visualization by BiFC of the PVA HCPro/HIP2 interacting pair showed a filamentous distribution (Haikonen et al. 2013b) similar to our colocalization of fluorescence from HCPro or its homodimers with that from transgenic fluorescent tubulin (Figs. 5 and 7A). Therefore, PVY HCPro BiFC homodimers may colocalize with the MT through this same host factor.

In addition to both patterns, we found numerous intermediate forms that showed both HCPro fluorescence in ER-linked dots as well as MT-linked filaments which, in our opinion, could be better explained if there was a dynamic connection between both patterns (Fig. 4). We do not know the direction of the protein transport (from dots or the diffuse cytoplasm toward MT or vice-versa) or whether it is bi- or multidirectional. An important feature of the MT-associated pattern is that it appears mainly but not exclusively after the live tissue has been subjected to stresses. Haikonen and associates (2013b) found the filamentous pattern in epidermal cells overexpressing both split YFP-tagged PVA HCPro-HIP2 BiFC constructs but not in cells expressing fluorescent HCPro, in which, instead, fluorescence spread diffusely throughout the cytoplasm. They suggest that low cellular levels of endogenous HIP2 could be responsible for the absence of a tubular pattern in the latter case. However, in our results, relocation of PVY HCPro toward the MT was extensive and took place within minutes of the added stress in those cells affected (Figs 4, 5, and 7) and, if mediated by HIP2, it would have to necessarily imply that the latter was not a limiting factor. Alternatively, HCPro coating of MT could also take place through another, HIP2-independent route. What physical process makes HCPro relocate to the MT in response to the stress? We could speculate that a host factor is released from a place where it has no contact with HCPro, or that it suffers some structural alteration that allows it to take place.

We finally tested whether the integrity of the MT cytoskeleton was required for HCPro suppressor of silencing activity or for its mediation of viral transmission by aphids. For that, we disrupted the cytoskeleton by providing colchicine as described (Wright et al. 2007). MT disruption in *Tua*-GFP plants did not prevent colocalization of PVY HCPro with green tubulin in dot-like structures (Fig. 7A), indicating that colocalization did not require an MT tubular architecture. In agroinfiltration assays, we could not find a negative effect of MT disruption on the HCPro suppressor activity on the transient expression of a GFP reporter (Fig. 7B) or on the viral transmission by aphids from a PVY-infected plant (Fig. 7C); in both cases, even at doses that were 10 times higher than those needed to completely disrupt the MT. Therefore, despite the HCPro-HIP2 interaction being important to PVA infectivity (Haikonen et al. 2013b), in our experimental conditions, suppression of silencing or mediation of viral transmission by vectors functions did not require the integrity of the MT network. These results were unexpected and leave open the quest for a biological role of the binding of HCPro to MT under stress and its transit through that network for further research.

In conclusion, we have shown that, in addition to a diffuse presence throughout the cytoplasm, PVY HCPro displays several distinct subcellular localization patterns which can be

influenced by the cellular environment: as large irregular, tubulin-containing inclusions; as novel, dot-sized ER- and MT-associated structures; and, finally, coating the MT in response to stresses. We also show that there is a continuum between the two latter forms that support the existence of HCPro transport between both. By contrast, in a system free from other virus components, we found no evidence of HCPro trafficking through the secretory pathway or following actin filaments. Despite its association with MT, the integrity of this tubular cytoskeleton was not necessary for HCPro suppression of silencing activity in agroinfiltration assays or for its mediation of PVY transmission by aphids from infected plants.

MATERIALS AND METHODS

Plasmid constructs.

For transient expression in plants, proteins were cloned into the 35S promoter-polylinker-Nos terminator T-DNA cassette of pROK2-based binary vectors. Cloning of construct P1-HCPro with the *P1HCPro* sequence from PVY has already been described (Canto et al. 2002). Cloning of construct P1-6x-HCPro has also been described, as was the determination that it has suppressor activity in agroinfiltration assays similar to that of the native protein (Tena et al. 2013). To generate constructs P1-HCPro-mRFP and P1-HCPro-GFP, the *P1-HCPro* sequence without a terminator codon was amplified by polymerase chain reaction (PCR) with appropriate oligonucleotides, digested with *NheI*, and cloned into *XbaI*-linearized pROK2, in frame with either the *mRFP* or *GFP* genes that had previously been inserted at the *XbaI*-*SacI* sites of the pROK2 polylinker. For the generation of GFP- and sYFP-tagged constructs, *HCPro* sequences were amplified by PCR using appropriate oligonucleotides and cloned into pROK2 harboring *GFP* or pROK harboring sequences corresponding to sYFP N or C terminal halves already described (González et al. 2010) at the *XbaI*-*BamHI* sites of their respective polylinkers, to generate intermediate constructs GFP-HCPro, sYN-HCPro, and sYC-HCPro. To add an N-terminal tag of methionine plus six histidines (Met-6xHis; ATG-CAT-CAC-CAT-CAT-CAT-CAC) to these three constructs as well as to construct HCPro, the sequences corresponding to the tags, if present, plus the first 5' 330 nucleotides of the *HCPro* sequence were amplified by PCR with four appropriate 5' oligonucleotides that contained the Met-6xHis sequence and a common 3' oligonucleotide (nucleotides 319 to 339). The four PCR fragments were digested with *NheI* and *XhoI* (a unique site present at nucleotide 330 of the *HCPro* sequence) and inserted into the above-mentioned constructs and linearized with *XbaI*-*XhoI* in place of the original sequences, thus generating constructs 6x-HCPro, 6x-GFP-HCPro, 6x-sYN-HCPro, and 6x-sYC-HCPro, respectively. To insert the *Met-6xHis* sequence between the *P1* and *HCPro* sequence or between the *P1* and *GFP-HCPro* sequences, the *P1* sequence was amplified by PCR with an appropriate 5' oligonucleotide and a 3' oligonucleotide complementary to the end of the *P1* sequence plus a serine codon and an *NheI* site (CAGTTT[P1]AGC[serine]GCTAGC[NheI site; proline-serine]). On the other hand, two sequences from constructs 6x-HCPro and 6x-GFP-HCPro were amplified by PCR using a 5' oligonucleotide containing the *Met-6xHis* tag sequence with an added *NheI* site upstream, and an appropriate 3' oligonucleotide. These two PCR fragments were cleaned, digested with *NheI*, and ligated in vitro to the *NheI*-digested P1 PCR fragment. The ligation products were then used to amplify two PCR fusion fragments using the P1 5' oligonucleotide and the 3' oligonucleotide at the *HCPro* sequence (nucleotides 319 to 339). The fusion fragments thus obtained contained a *P1*-(*Ser-Pro-Ser-Met-6xHis-Met*)-*GFP* or *HCPro* sequence at the fusion

sites. The two fusion PCR products were digested with *Bam*HI and *Xho*I and cloned into equally linearized construct P1-HCPro, generating constructs P1-6x-HCPro and P1-6x-GFP-HCPro, respectively. For large-scale purifications of P1-6x-HCPro and of P1-6x-GFP-HCPro from plants, both sequences were amplified by PCR with appropriate oligonucleotides and cloned into a binary construct expressing an infectious *Potato virus X* (PVX) vector (construct pGR 107) (Lu et al. 2003), linearized with *Cla*I and *Sma*I. To obtain HCPro constructs tagged at their N-termini with the mRFP 1 or its N-terminal and C-terminal splits for BiFC, plus 6 histidines, mRFP or its 5' and 3' split part sequences were amplified by PCR with appropriate oligos and cloned into *Xba*I- and *Bam*HI-linearized pROK2 to create intermediate constructs pROK2-smRFP1, pROK2-split mRFPN (encoding the protein 168 N-terminus amino acids), and construct pROK2-split mRFPN (encoding a methionine plus the 58 C-terminus amino acids), respectively, following Zillian and Maiss (2007). On the other hand, the HCPro sequence was amplified by PCR from construct P1-6x-HCPro using a 5' oligo that contained a *Bam*HI site followed by 6 histidines and a 3' oligo complementary to the 3' end of HCPro coding sequence, followed by a stop codon and a *Kpn*I site. This fragment was cloned into constructs pROK2-smRFP1, pROK2-split mRFPN, and pROK2-split mRFPN and linearized with *Bam*HI and *Kpn*I to generate binary constructs mRFP-6x-HCPro, sRFPN-6x-HCPro, and sRFPN-6x-HCPro, respectively. All constructs were fully sequenced prior to experimental use. The 2b sequence from CMV var. Fny was amplified by the PCR with appropriate oligos and cloned into *Bam*HI- and *Sac*I-linearized constructs pROK2-split mRFPN and pROK2-split mRFPN to create constructs sRFPN-2b and sRFPN-2b, respectively, which express the CMV 2b suppressor fused to the split parts of the mRFP.

Plant material.

This work used *N. benthamiana* plants, either wild-type or transgenic, for the constitutive expression of GFP targeting the cortical ER (line 16c) (Ruiz et al. 1998), of GFP fused to *A. thaliana* α -tubulin (Tua-GFP) (Gillespie et al. 2002), or a Lifeact TagRFP fusion targeting actin filaments. Lifeact TagRFP plasmid was made by primer extension PCR, as described by Berepiki and associates (2010). Gateway adapters were added by PCR with primers 5'-GGGGACAAGTTTGTACAAAAA GCAGGCTGCATGGGTGTGCGAGATTTGATCAAG-3' and 5'-GGGGACCACCTTTGTACAAGAAAGCTGGGTCTTACT GTACAGCTCGTCCATGCC-3', and the resulting PCR product was recombined into pDONR207 (LifeTechnologies). After sequence verification, Lifeact TagRFP was recombined into pGRAB.TEVL (SC1046), a derivative of pGreen0229 containing a 35S promoter and a TEV leader sequence upstream of the Gateway cassette. pGRAB.TEVL Lifeact TagRFP plasmids were mobilized into *Agrobacterium* strain AGL1+psoup and used to transform *N. benthamiana* leaf segments as described by Horsch and associates (1985), with Basta-resistant plants being regenerated and screened for expression by fluorescence microscopy.

Expression of proteins in plants from binary vectors, and purification of HCPro and PVY.

For transient expression assays, binary vectors were transferred to the same batch of electrocompetent *Agrobacterium tumefaciens* C58C1 derived from a single colony, to prevent bacterial variability. Cultures were grown to exponential phase in Luria-Bertani medium with antibiotics at 28°C. For infiltration, each bacterial culture harboring a different T-DNA was diluted to a final optical density of 0.2 at 600 nm. Different cultures harboring different T-DNAs were then combined and

infiltration of the mixtures was performed on fully expanded leaves of nonflowering *N. benthamiana* plants using a syringe, as described (Canto et al. 2002). Large-scale purifications of P1-6x-HCPro and of P1-6x-GFP-HCPro were performed from plants infected with constructs PVX-P1-6x-HCPro and PVX-P1-6x-GFP-HCPro, as described (Tena et al. 2013). A purified virion prep from an aphid-transmissible PVY isolate (Scottish ordinary variety PVY-O; Scottish Agricultural Science Agency), from which the P1-HCPro sequence used in this study derived (Canto et al. 2002), was obtained from systemically infected *N. benthamiana* plants as described, omitting the sucrose gradient final step (Moghal and Francki 1976).

Local suppression of silencing in agroinfiltration patch assays.

A free GFP reporter gene expressed from a binary vector under the control of the 35S promoter was expressed transiently in an *N. benthamiana* leaf, either co-infiltrated with the empty binary vector pROK2 or with another vector expressing a protein to be tested for suppression of silencing activity. To monitor the effect of the second protein on the levels of fluorescence derived from the transiently expressed free enhanced GFP, leaves were illuminated at 3 to 6 days postinoculation (dpi) with a Blak Ray long-wave UV lamp (UVP, Upland, CA, U.S.A.) and photographed as previously described (González et al. 2010).

Imaging of fluorescence from tagged proteins in live cells and tissues, and chemical treatments.

Epidermal cells in *N. benthamiana*-infiltrated leaves were monitored live for fluorescence derived from GFP-, mRFP-, smRFP-, mCherry-, or sYFP-tagged proteins transiently expressed at 3 to 5 dpi. Imaging was conducted with Leica SP2 and SP5 (Leica Microsystems GmbH, Heidelberg, Germany) confocal laser-scanning microscopes and software, using fresh leaf tissue and $\times 63$ magnification oil immersion objectives. Excitation and emission settings in nanometers were the following: GFP (488 and 500 to 530); BiFC sYFP (514 and 530 to 550); and mRFP, BiFC smRFP, and mCherry (561 and 575 to 640). To visualize the cortical ER, actin filaments, or MT cytoskeleton transgenic *N. benthamiana* plants constitutively expressing GFP fused to *Arabidopsis thaliana* α -tubulin (Tua-GFP plants) (Gillespie et al. 2002), GFP targeting the cortical ER (16c plants) (Ruiz et al. 1998) and a Lifeact TagRFP fusion targeting actin were also used. To visualize the Golgi apparatus, a binary vector expressing mCherry protein with a targeting signal to this structure was used (Nelson et al. 2007). In colocalization studies, every single frame was imaged consecutively for green and red fluorescence. These green and red simultaneous frames were then Z-axis-stacked to create final projections.

Chemical treatments for MT disruption were performed with colchicine (Sigma-Aldrich C9754) at the dilution of 0.5 μ M, as described by Wright and associates (2007), unless otherwise stated. The disrupting agent was infiltrated simultaneously with the *Agrobacterium* solutions. Exposure of infiltrated tissue to two different supermarket brands of common nail varnish (Vera Valenti, classic; Vera Valenti, San Fernando de Henares, Spain; and Deliplus, gel acabado brillo; You Cosmetics, Gava, Spain) was achieved by directly sealing the edges of the fresh tissue to the microscope slide prior to confocal viewing. Exposure of infiltrated tissue to organic solvents (n-butyl acetate and ethyl acetate) was achieved by keeping freshly cut tissue inside a wet chamber saturated with fumes from either of these two chemicals for 1 h at room temperature, prior to confocal viewing. Exposure of infiltrated tissue to saturated CO₂ atmosphere was achieved as described by Martinière and associates (2013).

Immunoblot detection of proteins.

Total protein content from infiltrated tissue was ground with a pestle in extraction buffer (0.1 M Tris-HCl [pH 8], 10 mM ethylenediaminetetraacetic acid, 0.1 M LiCl, 1% β -mercapto-ethanol, and 1% sodium dodecyl sulfate [SDS]), mixed 1:1 with 2 $\times$ Laemmli buffer, and the sample was incubated for 10 min at 95°C under shaking and clarified in the bench microfuge for 5 min before fractionation in SDS polyacrylamide gel electrophoresis 10% gels. Gel proteins were wet-blotted in Tris-glycine buffer onto Hybond-P polyvinylidene difluoride membranes (Amersham, GE Healthcare, Buckinghamshire, U.K.) For immunological detection of GFP, a rabbit GFP polyclonal antiserum kindly donated by G. Cowan (James Hutton Institute, Dundee, U.K.) was used. For detection of 6x-HCPro, a mouse monoclonal antiserum to 6-histidines was used (Sigma-Aldrich, St. Louis). In the Western blot shown in Figure 5C, a rabbit polyclonal antiserum against PVY HCPro was used (Canto et al. 1995a). Blotted proteins were detected using commercial alkaline phosphatase-conjugated secondary antibodies and SigmaFast BICP/NBT substrate tablets (Sigma-Aldrich).

Aphid transmission tests and exposure of leaf tissue to aphids.

Transmission tests of PVY from either infected *N. benthamiana* fresh leaf tissue or from solutions of purified virions through stretched parafilm membranes, the latter in combination with purified HCPro, was performed using a clone of the aphid *Myzus persicae* and as described (Canto et al. 1995b). To expose infiltrated tissue to aphids, 50 aphids were harvested and placed under confinement on an infiltrated patch of approximately 15 mm in diameter for 16 h, at room temperature in a wet chamber, prior to confocal viewing.

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