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Virus Transmission—Getting Out and In

Stéphane Blanc

UMR Biologie et Génétique des Interactions Plante-Parasite (BGPI),
INRA-CIRAD-ENSAM, TA 41/K, Campus International de Baillarguet,
34398 Montpellier cedex 05, France
blanc@supagro.inra.fr

Abstract Logically, most plant viruses being vector-transmitted, the majority of viral transport mechanisms associated to the transmission step have been approached through the study of virus-vector relationships. However, in the case of non-vector vertical transmission through the seeds, some viruses have evolved specific patterns to colonize either the gametes or the embryo, thereby connecting viral transport within the plant to that in between plants. Moreover, though it may appear counter intuitive and has been largely overlooked, some specific virus accumulation within cells or organs, as well as specific control of multiple infections of single cells, can also directly affect the success and efficiency of vector transmission, again connecting viral transport mechanisms inside and outside the host plants. This work summarizes the data available on viral transport outside the plant in various vectors, and also highlights a few available examples and proposes hypotheses for illustrating the concept that some viral trafficking within plants is specifically intended to prepare ulterior acquisition by the vectors.

1 Introduction

Besides replicating in cells and trafficking from cell-to-cell and long distance, when invasion of the host plant is completed, viruses have found very diverse ways to move on and jump into the outside world, seeking another host plant. This adventure involves various steps and sophisticated modes of transport, not only for travelling safely in the big outdoors, but also, before and after, for preparing to leave and securing efficient installation, respectively. In contrast to intracellular or symplastic intercellular trafficking within plants, viral transport between plants implies one additional major difficulty: the repeated passage through cell walls, both for getting out of an infected plant and back into a healthy one. While some very rare viruses can autonomously and passively exit and enter adjacent plants from wounds via non-specific mechanical transmission, the vast majority have adopted a strategy that uses plant-feeding invertebrates as transport devices, which easily ensures the passage through cell walls and also allows the virus to cover considerable distances between host plants in the environment.

Because of its tremendous impact on epidemiology, virus transmission has been intensely studied for nearly a century (Doolittle and Walker 1928) in

different scientific disciplines (for reviews see Nault 1997; Gray and Banerjee 1999; Van den Heuvel et al. 1999; Blanc 2004). The development of molecular biology marked a big turning point in this scientific field, allowing the identification and characterization of the numerous viral determinants involved in transmission, and a few counterpart "receptors" in the corresponding vectors. In the near future, cell biology and imaging also promise great returns in this field; despite their limited use to date, they have already informed on some mechanisms of viral transport within the vector and even within plants, that are clearly specific to the step of transmission.

The transport of virus particles or viral proteins that is related to plant-to-plant passage includes specific within-plant phenomena allowing the colonization of embryos in vertical seed-transmission, and efficient interaction with specific vectors in horizontal transmission. In the latter, the virus can have a steady interaction with vectors, "sticking" somewhere and waiting for release when an appropriate destination is reached, but can sometimes also traffic through the vector cells, implying mechanisms different from those existing in the plant cells that are described in other chapters of the present volume. Still related to vector-transmission, a largely overlooked phenomenon is being uncovered: viruses can develop interactions with the host plant, involving protein or viral particle transport processes, that are specifically destined to prepare and optimize acquisition by the vector in the infected source plant or facilitate the initiation of de novo infection in the inoculated healthy plant.

This work reviews known molecular mechanisms and cellular processes, occurring in either plants or vectors, that contribute to the successful transport of viruses from one host plant to the next. While some aspects have long been investigated and deserve continued research efforts, others are just being discovered and will be highlighted as they represent promising future prospects.

2
Virus Transport Involved in Non-Vector Transmission

Vertical transmission through seeds is a phenomenon relevant to about 15% of plant virus species (Hull 2001). A tremendous amount of data is available concerning the list of virus-host combinations where seed transmission can occur, as well as on the dramatic variations in the percentage of infected seeds observed either with different virus isolates in a given host, or with a single isolate in different host species or ecotypes (Mink 1993).

With the exception of TMV, and presumably other tobamoviruses, which externally contaminate the seed coat and are later transmitted mechanically to the germinating plants (Broadbent 1965), the most frequent case is infection of the embryo, via two distinct but sometimes co-existing pathways.

Embryo infection can occur indirectly before fertilization, by infection of the gametes, or after fertilization by direct invasion of the seed tissues (Maule and Wang 1996). Both pathways are summarized and discussed below, as both could rely on specific transport mechanisms.

2.1 Indirect Embryo Colonization by Early Infection of Gametes

Several virus species, for instance cryptic viruses (Kassanis et al. 1978), some tobraviruses (Wang et al. 1997) and nepoviruses (Hull 2001), readily infect gametes, and this is believed to be positively correlated to a rather uncommon property in plant viruses, i.e. the capacity to invade meristematic cells (Maule and Wang 1996). It would be interesting to understand what specific mechanisms allow or prevent a viral presence in meristem cells subsequently leading to gamete infection and vertical transmission.

Meristem exclusion of some RNA viruses has been indirectly related to post-transcriptional gene silencing (PTGS) (Foster et al. 2002), and this was recently confirmed for Potato virus X (PVX) in *Nicotiana bentamiana* (Schwach et al. 2005). The authors of this latter study have shown that virus accumulation in meristematic cells is prevented by the action of the RDR6 cellular RNA-dependent RNA-polymerase. In the same report, RDR6 is proposed to relay the long-distance silencing signal reaching the apical growing points, by promoting rapid production of a secondary siRNA at the site of virus entry. From these data, we could reason that the ability of some viruses to infect gametes depends not on specific mechanisms of viral transports into the meristem, but rather on circumvention of PTGS in this tissue. The case of *Barley stripe mosaic virus* (BSMV), which is known to indirectly infect embryos by early colonizing of gametes (Maule and Wang 1996), and where the viral determinant of seed-transmission was shown to be the protein γb (Edwards 1995), a protein later characterized as a PTGS suppressor (Yelina et al. 2002), is consistent with this scenario (for detailed information on PTGS, see the work by T. Hohn et al., in this volume).

This PTGS-related mechanism of meristem exclusion, however, may not apply to all virus species, as inspired by a recent work on the early development of the *Arabidopsis thaliana* embryo (Kim et al. 2005). In this work, the authors demonstrate the rapid establishment of specific boundaries that separate symplastic sub-domains prefiguring shoot apex, cotyledons, hypocotyls and roots. Interestingly, they also observed that the movement protein of TMV (P30) cannot dilate embryonic plasmodesmata and overcome these boundaries between subdomains. One could imagine that a similar putative boundary around the meristematic symplastic domain could later prevent TMV entry. This provides another hypothetical mechanism of meristem exclusion that could apply to TMV, which interestingly is not affected by the RDR6-related PTGS discussed above (Schwach et al. 2005). This putative

meristem boundary could possibly be overcome by some gamete-infecting viruses, implying unknown specific mechanisms of viral transport at this level.

2.2 Direct Infection of the Embryo by Invasion of Seed Tissues

Besides the early infection of gametes, another pathway for embryo colonization occurs after fertilization by sequential virus movement into the seed, from the micropylar region of the maternal testa, to the endosperm, suspensor and finally the embryo. This route is also used by the above-mentioned BSMV, and is the exclusive mode of seed transmission for the best-studied case, *Pea seed borne mosaic virus* [PsbMV, (Wang and Maule 1992)].

One major conceptual problem long discussed in this pathway of direct embryo colonization centres on the fact that the virus can reach the micropylar region of the testa by genuine cell-to-cell movement in a symplastic maternal tissue (reviewed in Hull 2001). The same is true for movement from the suspensor to the embryo, as the suspensor derives from early embryonic cell divisions, and symplastic connections also exist at this level. The problem is passage of the virus from maternal to embryonic cells, between which symplastic connections are severed early during meiosis. This barrier was believed to allow the passage of small nutrient molecules by apoplastic transport at the maternal-filial interface, where transfer cell wall projections were observed in the endosperm (Tegeder et al. 1999, 2000). Thus, there was no possible anatomically based explanation for the passage of virus from testa to endosperm, and from endosperm to suspensor cells, until the question was carefully reinvestigated by electron microscopy specifically targeting the ultrastructure of the micropylar region (Roberts et al. 2003). In this study, the cylindrical inclusions induced by PsBMV infection were used as markers of putative symplastic connections, as the same authors had previously shown that these were positioned in the close vicinity of plasmodesmata (Roberts et al. 1998). Cylindrical inclusion bundles, arranged perpendicular to cell walls separating maternal testa and endosperm, were clearly visible and labelled by a PsBMV antiserum. Although proper plasmodesmata could not be observed, the authors interpreted occasional distortion of the cell wall, near the cylindrical inclusions, as reminiscent of plasmodesmal cavities. This result suggests a possible means of virus transfer between maternal tissues and endosperm that requires further investigation to decide whether these symplastic connections are constitutive or specifically induced by seed-transmitted viruses (Roberts et al. 2003). The last problematic barrier to be elucidated is that between endosperm and suspensor cells. The same authors described regions of the embryo sheath, at the base of the suspensor cells, which are discontinued and punctuated with pore-like structures, putatively allowing the transfer of large molecular weight complexes, including viruses. These “pore-like” connections were previously

unknown, and whether viral transport at this level is passive or requires specific active processes, remains to be investigated.

3
Virus Transport Involved in Vector-Transmission

Unlike animal viruses, where hosts are mobile and often come into contact with each other, plant viruses need to cover the often large distances separating their fixed hosts. Hitch-hiking with the invertebrate parasites of plants provides both rapid transportation and secure housing. While the majority of plant viruses rely simply on controlling the timely retention in, and release from, a specific unique location in the vector, a few others have developed a more intricate relationship that also involves specific transport processes as part of a dynamic cycle within the vector body. The mechanisms of virus-vector relationships are logically most often studied outside the plant, and reviews on the subject are published frequently (Nault 1997; Van den Heuvel et al. 1999; Gray and Banerjee 1999; Harris et al. 2001; Pirone and Perry 2002; Blanc 2004). However, the viral processes that occur within the plant, before and after the vector intervention, to prepare for efficient acquisition and ensure successful inoculation, have been largely ignored, though some specific transport events may play an important role. This section will first summarize the diversity of the strategies encountered in virus-vector interactions leading to plant-to-plant transport of viruses, and then highlight the few data available on within-plant mechanisms preceding the way out and accompanying the way in.

3.1
Transport in Vectors

3.1.1
Transport of Circulative Viruses

The term "circulative" was first introduced by Sylvester (1956) and again by Harris (1977) to describe viruses that undergo part of their life cycles within the body of the vector. The term applies to viruses transmitted by arthropod vectors such as mites and mostly insects. Circulative viruses are acquired by vectors feeding upon infected plants. The viruses then traverse the gut epithelium at the midgut or hindgut level (for examples see Reinbold et al. 2003; de Assis Filho et al. 2005), and are released into the haemolymph. The viruses can then adopt various pathways to join and enter the salivary glands, where they are released in the saliva and finally inoculated into healthy hosts, initiating new infection. The latent period—the time required for the virus to complete this cycle—depends on the virus-vector pair and numerous other

factors, including temperature, and can range from several hours to several days in length.

Obviously, circulative transmission implies that the virus traffics through diverse cellular barriers, where the existence of specific transport mechanisms has long been proven experimentally. The gut epithelium, separating the gut lumen and the haemocoel, was unequivocally demonstrated, several decades ago (Storey 1933), to be the first specific barrier encountered by viruses in their insect vectors. *Maize streak virus* (MSV; *Geminiviridae*) could be efficiently transmitted by a non-vector leafhopper species that fed on infected plants provided that breaks were induced in the gut epithelium by repeated needle punctures. A number of more recent works involving intra-thoracic injection of viruliferous solutions into vectors have confirmed that this barrier can stop many plant viruses. Having successfully passed through the gut, the virus must then make its way into the haemocoel cavity, or through various organs and tissues, in order to reach the salivary glands. Some viruses are actually blocked during this process, as they are sometimes readily detected in the haemolymph but never reach the salivary glands, again indicating the involvement of specific transport mechanisms. Finally, for those virus-vector pairs that are compatible at the two above-mentioned barriers, failure during passage through the salivary glands can at last disable transmission success. The circulative transmission mode is divided into two subcategories depending on whether the virus can replicate in its vector (circulative-propagative transmission) or not (circulative-non-propagative transmission).

Transport of Circulative-Propagative Viruses

This category of plant viruses is the exact homologue of arboviruses in vertebrates. The virus families concerned are *Rhabdoviridae*, *Reoviridae* and *Bunyaviridae*, all having member species associated with animals and plants, plus one genus specifically restricted to plant hosts: *Marafivirus*.

In compatible virus–vector associations, once the cells of the gut epithelium are infected virus particles are released in the haemocoel cavity, where they can infect numerous organs and tissues of the vector, including the salivary glands. The viruses can either diffuse in the haemolymph and concomitantly infect different organs, or follow a precise pattern of spread from organ to organ, as demonstrated for rhabdoviruses, for which the infection is believed to progress in, and spread from, the central nervous system (Hogenhout et al. 2003). In all these cases, the viral transport mechanisms involved are related to those necessary for the infection of an animal host (insect) by a virus, and are discussed in several recent reviews (Mellor 2000; Blanc 2004; Kuno and Chang 2005; Ullman et al. 2005; Redinbaugh and Hogenhout 2005); hence, we believe they are outside the scope of the present volume, particularly the scope of this work.

Transport of Circulative-non-Propagative Viruses

This category of virus-vector interaction is very specific to plant viruses and involves peculiar mechanisms of viral transport, both for passing through gut and salivary gland barriers, and during transfer in the haemocoel cavity. Note that only member species of the family *Luteoviridae* are known with certainty to be transmitted this way. Species of the family *Geminiviridae* are often assigned to the group of circulative non-propagative viruses, but because this assignment is becoming increasingly unclear, I will briefly discuss this case at the end of the section.

The very first step in the luteovirus-vector interaction is specific binding of the virus to the gut epithelium. Although the viral "ligands" are somewhat characterized, very little is known of the putative corresponding receptors (Gray and Gildow 2003). Recently, an elegant study used chimeras between two poleroviruses, transmitted by distinct aphid species, to investigate this question on the virus side (Brault et al. 2005). The authors of this study have convincingly shown that the minor capsid protein (the capsid protein fused to an extension read-through domain, RTD) was certainly participating in receptor recognition. Indeed, the two poleroviruses used, *Beet western yellows virus* (BWYV) and *Cucurbit aphid-borne yellows virus* (CABYV), are retained at specific sites in the digestive tract of their respective vector: the midgut for BWYV and both midgut and hindgut for CABYV. In infectious chimeric clones, exchanging the RTD domain of the two viruses resulted in a change in both the transmitting vector species and the gut tropism, as demonstrated by electron microscopy. The RTD domain, as well as the major coat protein, has been subjected to extensive mutagenesis associated with infectivity and transmission testing. It obviously remains difficult to draw definitive conclusions regarding the precise mode of action of these viral proteins within the vector. The intricate interplay between capsid protein and RTD domain, likely involved at different vector cellular specific barriers, remains largely unresolved, and is very comprehensively reviewed in Gray and Gildow (2003).

Recently, the counterpart receptor in the vector gut epithelium has been sought by applying far-western techniques to one- or two-dimensional protein electrophoresis gels of various aphid extracts (Seddas et al. 2004). Three proteins interacting with the domain RTD of BWYV were identified—Rack-1, GAPDH3 and actin—and proposed to participate in a membrane complex used as a receptor by the virus and/or in an ulterior transcytosis phenomenon (see below). Whether additional aphid proteins are required for the full process and whether the three proteins already identified intervene at the level of the gut barrier, the salivary glands, or both, will require further investigation.

Despite the lack of full understanding of the molecular process, a series of impressive electron microscopy and molecular studies have described in detail the route of luteovirus particles within the vector body and across cellular layers. For all luteoviruses, and at both gut and salivary gland barriers,

the cellular mechanisms of cell penetration, crossing and exit appear globally similar, as confirmed by numerous consistent publications (for a detailed review, see Gray and Gildow 2003). There are two noticeable differences, however, between crossing the barriers of the gut and the salivary glands, (1) the endo-/exocytosis phenomenon described below functions in opposite directions, and (2) an extracellular basal lamina surrounding the accessory salivary glands seems to be a specific obstacle that must be overcome by luteoviruses, via unknown transport mechanisms (Pfeiffer et al. 1997). Once the virus reaches either the apical membrane of the gut epithelium, or the basal membrane of the accessory salivary gland cells, and attaches to the specific receptors, it provokes an invagination of the plasmalemma, forming small coated virus-containing vesicles (Gildow 1993; Pfeiffer et al. 1997). Soon after budding, the coated vesicles deliver the virus particles to a larger uncoated membrane endosomal compartment (Fig. 1)—a step that was easily observed at the gut level but was less evident at the salivary gland level. Interestingly, as in other cases of endo-/exocytosis phenomena, luteoviruses mostly escape the route of degradation of internalized material ending in lysosomes. Instead, the virus particles become concentrated in the endosomes, and de novo elongated uncoated vesicles are repacked and transported to the basal or apical membrane, in gut and accessory salivary gland cells, respectively.

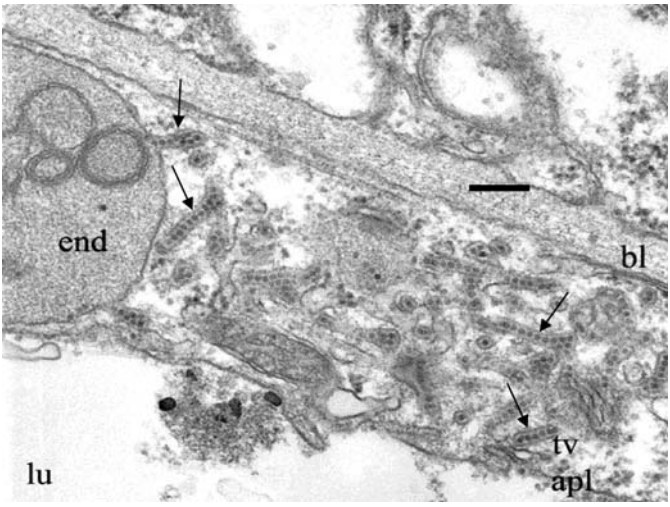

Fig. 1 Transcytosis of CABYV in hindgut cell of the aphid vector *Myzus persicae*. Luteovirus present in the gut lumen (lu) are internalised from the apical plasmalemma (apl) and transported to the basal lamina (bl) in a complex pattern involving different vesicular structures, described in the text. A network of uncoated tubular vesicles is visible (tv indicated by *arrows*), sometimes connected to the endosome (end). *The bar* represents 100 nm. The photograph is gracefully provided by Catherine Reinbold and Véronique Brault (INRA, Colmar, France)

The elongated vesicles, which contain visually spectacular lines of virions (Fig. 1), finally fuse with plasma membranes and release the virus either into the heamocoel cavity or into the lumen of the salivary ducts. As an alternative to this generally accepted model involving clathrin-coated endocytosis, it has recently been proposed that BWYV could be internalized in gut epithelial cells by macropinocytosis, the polarized transport along the cytoskeleton being ensured by aphid protein partners (Seddas et al. 2004). However, this latter speculation awaits experimental support.

Despite the extensive observation of luteovirus particles in their insect vectors by several authors during the past 30 years, none were ever observed suspended in the hemolymph or associated with any organ other than the gut or the accessory salivary glands. Because ultrastructural observations of organs, as well as monitoring of viral titres after luteovirus acquisition by aphids, provided not even the slightest indication of viral replication, it is generally acknowledged that virus particles diffuse passively into the haemocoel cavity, to move from their point of release towards specific receptors likely located on the basal lamina of the accessory salivary glands (Pfeiffer et al. 1997). Not much is known about this hemolymph transfer, and the hypothesis of "passive diffusion" does not motivate intensive studies. Questions are often raised about the possible impact of the insect immune system on luteoviruses at this step of their life cycle (discussed in Gray and Gildow 2003). A pioneering study provided the very relevant information that a major protein of the hemolymph, the symbionin, was required for efficient virus transmission (Van den Heuvel et al. 1994). A homologue of the *Escherichia coli* chaperone GroEL, the symbionin is produced in aphids by endosymbiotic bacteria of the genus *Buchnera*, and massively secreted in the hemolymph. Aphid treated with antibiotics, and hence deprived of symbionin, have a significantly reduced ability to transmit *Potato leaf roll virus* (PLRV). A similar phenomenon was later demonstrated for other luteoviruses, and even unrelated geminiviruses, as briefly discussed below (Van den Heuvel et al. 1999; Akad et al. 2004). Consistently, direct evidence of a physical interaction between symbionin and the RTD domain of luteovirus particles was reported in several species (Filichkin et al. 1997; van den Heuvel et al. 1997), and virus mutants deleted in this RTD domain were less persistent in the hemolymph. The authors concluded that the symbionin likely exhibits protective properties, masking the virus to the immune system and maintaining its integrity during transfer through the hostile hemolymph environment, or alternatively ensuring correct folding facilitating transfer into the salivary glands (also discussed in Van den Heuvel et al. 1999; Akad et al. 2004). These hypotheses are not accepted by all authors (Gray and Gildow 2003), for several reasons: The symbionin interacts non-specifically with many different virus species, with no correlation between affinity and the success of aphid transmission (van den Heuvel et al. 1997); RTD/symbionin binding has never been demonstrated in vivo; the absence of symbionts perturbs the overall physiology

of the aphid, which could result in their being less efficient vectors without necessarily invoking any specific role for symbionin. Whether or not this mechanism is relevant to viral transport, it represents the only data ever reported on the transport of luteo- or geminiviruses in the hemolymph of their insect vectors.

Members of the family *Geminiviridae* have long been considered as circulative non-propagative viruses, transmitted either by leafhoppers or whiteflies, but recent results largely question this assumption. Exhaustive data analogous to those described for luteoviruses are not available, although a similar cycle from the gut, through the hemolymph, to the salivary glands is clearly established (Lett et al. 2002), as is the possible involvement of symbionin-like proteins (Morin et al. 1999, 2000; Akad et al. 2004). In particular, it is remarkable that no characteristic geminate virus particles have ever been observed in the hemolymph or within any organs, not even gut and salivary gland cells. While no evidence for viral replication within the vector could be obtained in the genus *Mastrevirus* (Bosque-Perez 2000), both transovarial vertical transmission (Ghanim et al. 1998) and venereal horizontal transmission (Ghanim and Czosnek 2000) occur in whitefly contaminated with *Tomato yellow leaf curl virus* (TYLCV), a member species of the genus *Begomovirus*.

Furthermore, an interesting study has shown that eggs of whitefly bombarded by TYLCV genomic DNA later hatch into virus-transmitting insects (Goldman and Czosnek 2002). These features being usually associated with viruses that replicate within their vectors, more work is required to definitively understand the transmission strategy of the *Geminiviridae* family. A non-canonical virus-vector interaction may exist there, involving unusual mechanisms of viral transport, but there are no data at present on which to propose any sound alternative hypothesis.

3.1.2 Transport of Non-Circulative Viruses

As stated above, non-circulative viruses do not operate a proper cycle within the body of their vectors. They simply attach to receptor sites located externally on the vectors—the alimentary/salivary canal of the mouth parts or the foregut region in the case of arthropods or nematodes (Hull 2001; Pirone and Perry 2002)—and wait until the vector has moved to another plant, where they contrive to be released to initiate a new infection. When vectors feed on plants, viruses are usually released together with the saliva (Martin et al. 1997) or during egestion (Harris 1977). Thus, the viral transport mechanisms associated with this type of virus-vector interaction are restricted to the action of interacting virus ligands and vector receptors. Comparable phenomena have been described in a wide variety of vector species found in fungi (where somewhat analogous processes operate, as described below), nema-

todes, and arthropods, collectively transmitting nearly half of the plant virus species described so far.

Viral Ligands

Viral protein motifs directly involved in the attachment to vector receptors have been characterized in rare cases. The frequent occurrence of both transmissible and non-transmissible isolates in the same virus species, has greatly facilitated the identification of viral gene regions involved in vector-transmission and reverse genetic approaches have also been successful. However, providing direct proof that the identified motifs are indeed responsible for direct attachment to the vector receptors has proven to be much more complicated and is seldom achieved. The best established cases, described below, indicate that the coat protein is not always the protein that recognizes the receptors, a non-structural additional component most often being involved.

One straightforward experiment to distinguish if the coat protein directly recognizes the receptor involves setting up protocols where the vector can acquire purified virus particles. The two best-studied cases are *Cucumber mosaic virus* (CMV, *Cucumovirus*) transmitted by aphids (Pirone and Perry 2002), and *Cucumber necrosis virus* (CNV, *Tombusvirus*) transmitted by fungi (Rochon et al. 2004). Amino acid changes in a precise motif of the coat protein of CMV were demonstrated to differentially affect the transmission efficiency by different aphid species (Perry et al. 1994, 1998; Liu et al. 2002). It was at first very tempting to hypothesize that the targeted amino acids were likely located in the domain directly binding to specific receptors in the vector stylets. Unfortunately, however, additional work from the same research group revealed that these changes affected the stability of virions, thus possibly indirectly disabling transmission efficiency (Ng et al. 2000, 2005). In CNV, which is transmitted by a root-parasitic fungus, virions are specifically retained at the surface of the zoospore coat, and inoculated into the plant upon cell wall digestion and fungal penetration. An interesting structural phenomenon was revealed during attachment of virions onto the fungi-vector zoospore (reviewed in Rochon et al. 2004). Amino acids playing key roles at this step were identified in the shell, near the three-fold axis contact zone between caspomers of the virus particle (Kakani et al. 2001). The same authors later demonstrated a conformational change of the shell when binding to the zoospore, resulting in swelling of virions (Kakani et al. 2004). One hypothetic effect of swelling was proposed to be the migration of the three subunits (of the three-fold axis) away from each other, exposing the inner domain associated with RNA, and thereby facilitating RNA release during inoculation into the new plant host. Sole participation of the coat protein in vector recognition has been demonstrated in a number of viral genera: aphid-transmitted *Cucumovirus*, *Alfamovirus* and *Carlavirus* (Pirone and Megahed 1966; Weber and Hampton 1980), fungus-transmitted *Tombusvirus*, (Rochon et al. 2004),

nematode-transmitted *Nepovirus*, and a single *Crinivirus* species transmitted by whitefly (Ng et al. 2004).

A very frequent observation is that purified virus particles are not readily transmissible. This was explained in the early 1970s in a series of elegant studies by Govier and Kassanis (Kassanis and Govier 1971a, 1971b; Govier and Kassanis 1974) investigating the aphid transmission of potyviruses. They convincingly discovered the existence of a non-structural protein, encoded by the virus, that was mandatory for vector-transmission. This viral protein was designated the "helper component" (HC) and the phenomenon was later demonstrated to be prominent in non-circulative plant viruses (Pirone and Blanc 1996). One interesting property of HC is the possibility of independent acquisition, in the absence of virus particles, thus demonstrating that HC can directly attach to the receptors in the vector mouth parts. The commonly accepted mode of action is illustrated by the "bridge hypothesis" (Pirone and Blanc 1996): two distinct domains of HC recognize and bind receptors in the vector and protein motifs on the coat protein, respectively, thus creating a molecular bridge between vector and virus. Although HCs have been shown to be also involved in the genera *tritimovirus* (Stenger et al. 2005), *waikavirus* (Hibino and Cabauatan 1987; Hunt et al. 1988), *Tobravirus* (MacFarlane 2003), and presumably *Closterovirus* (Pirone and Blanc 1996; Ng et al. 2004), for transmission by mites, leafhoppers, nematodes, aphids and perhaps whiteflies, respectively, the best characterized are definitely those mediating aphid-transmission of the two genera *Potyvirus* and *Caulimovirus*.

The HC of potyviruses is a multifunctional protein designated HC-Pro, which has recently received much attention due to its capacity to suppress post-transcriptional gene silencing (PTGS, Brigneti et al. 1998). Moreover, HC-Pro also plays a decisive role in viral transport within the plant, both for cell-to-cell and long-distance movement (Cronin et al. 1995; Saenz et al. 2002). Purification of HC-Pro allowed its biochemical and structural characterization (Thornbury et al. 1985; Plisson et al. 2003; Ruiz-Ferrer et al. 2005), and numerous mutagenesis studies have considerably enriched our understanding of the structure-function relationships of this complex molecule (Raccah et al. 2001). The massive amount of data available will be restricted here to those related to vector transmission, the involvement of HC-Pro in within-plant movement and suppression of PTGS being documented in other parts of this volume. Again exploiting naturally existing non-transmissible strains, with subsequent validation by mutagenesis, two key domains involved in the process of aphid-transmission have been identified (reviewed in Raccah et al. 2001). On the one hand, a conserved KITC amino acid motif located near the N-terminus of HC-Pro has been shown to be involved in binding to aphid stylets (Wang et al. 1996), but whether this involvement is direct or indirect remains undetermined (Blanc et al. 1998). On the other hand, the bridge hypothesis was confirmed by two complementary studies demonstrating direct binding between the conserved amino acid motifs DAG

and PTK, located at the N-terminus of the coat protein and in the central region of HC-Pro, respectively (Blanc et al. 1997; Peng et al. 1998).

In the genus *Caulimovirus*, nearly all research efforts have focused on the type-member species *Cauliflower mosaic virus* (CaMV). Lung and Pirone first evidenced the existence of an HC (Lung and Pirone 1973, 1974), which was later identified as the product of viral gene II, P2 (Armour et al. 1983; Howarth et al. 1981; Woolston et al. 1987). The expression of functional P2 in a heterologous system did not support the in vitro concomitant acquisition, and subsequent transmission, of purified virions (Blanc et al. 1993b), indicative of the requirement of another unknown additional component that was presumably lost upon virus purification. This hypothesis was later confirmed and the "missing" component was found to be the viral product of gene III, P3 (Leh et al. 1999). The participation of a third factor, interacting with HC and virion was intriguing, as it had so far not been reported elsewhere and could somehow question the general validity of the bridge hypothesis. A series of biochemical and structural analyses succeeded in unravelling the mode of action of P3, demonstrating perfect agreement with a hypothesis of non-structural proteins forming a molecular bridge between virus and vector. A recent report establishing the three-dimensional structure of the P3-virion complex has shown that P3 passes from a soluble tetrameric form (Leclerc et al. 1998) to a complex network around the virion (Fig. 2), anchored in pores located around capsomers (Plisson et al. 2005). This conformational change in P3 arranges its N-terminus as anti-parallel dimers exhibiting a high affinity for the C-terminus of P2 as demonstrated earlier (Leh et al. 1999; Drucker et al. 2002). This model is consistent with previous results showing binding of a large C-terminal domain of P3 to unknown motifs of the coat protein (Leh

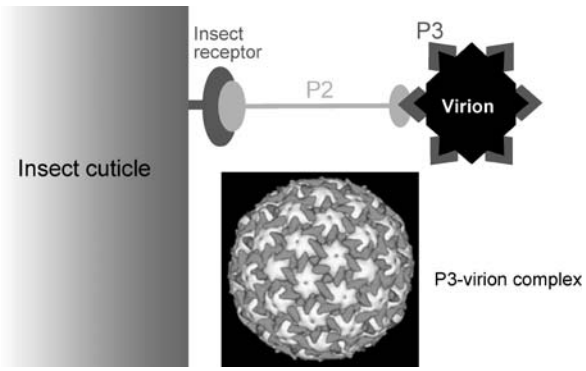

Fig. 2 Transmissible complex of *Cauliflower mosaic virus*. The viral protein P2 attaches both the putative receptor, on the cuticle lining the alimentary canal within the vector's stylets, and P3 intimately associated to the virus particle. *Inset* shows details of P3 (*dark grey*) distribution around the virion shell (*light grey*) The *inset* is adapted from Plisson et al. 2005

et al. 2001). P2 is then the HC of CaMV that recognizes and binds receptors, thereby connecting the P3-virion complexes to the vector (Fig. 2). Replacement of the amino acid at position 6 of P2 was recently reported either to reduce transmission by all aphid species tested, specifically affect only some of them, or abolish all transmission, depending on the substituting residue (Moreno et al. 2005a). The authors argued that this position is part of the domain directly attaching to the receptors in the aphid mouthparts.

Many totally unrelated genera use HC for their transmission, suggesting that this strategy of virus-vector interaction has evolved independently more than once (Froissart et al. 2002). It is then puzzling that molecular mechanisms as complex as those uncovered in *Caulimovirus* and *Potyvirus* are so often adopted by plant viruses. The only explanation proposed so far invokes the need for viruses to move from plant-to-plant in "groups" rather than alone and is explained further below (Pirone and Blanc 1996; Power 2000). The possible sequential acquisition of HC and virions (or P3-virion complexes for CaMV) introduces an interesting phenomenon designated HC-transcomplementation (Fig. 3; Froissart et al. 2002): an HC encoded by a genome X can assist the transmission of a virus particle containing a genome Y. This, together with the fact that vectors usually probe the host plant several times at several locations, or successively probe several different plants, theoretically allows an efficient HC (perfectly adapted to vector receptors) to mediate transmission of virions acquired in various locations

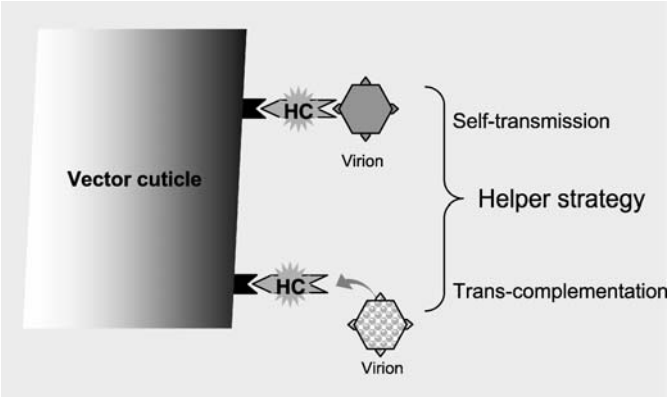

Fig. 3 Schematic representation of HC-transcomplementation in the vector transmission of plant viruses. The HC can be acquired alone, prior to virion, and attach the putative vector receptor. In this case a HC encoded by a genome X (for instance that encapsidated in the gray virion), can subsequently assist the transmission of a genome Y of the same population, encapsidated in the dotted virion. This possible sequential acquisition of HC and virion is symbolised by the *arrow*. It has been demonstrated experimentally that HC and virion can be acquired in different infected cells or even different hosts (see text). This figure is adapted from Froissart et al. 2002

of the same plant, or in different plants. Compared to a single acquisition at one location, the resulting viral sample transported by the vector would be more representative of the variability in the virus population, and hence would maintain a higher fitness in the viral lines moving over time from plant-to-plant. It is evident that this hypothesis applies better when the virus has to constantly adapt to fluctuating vector populations. The HC strategy would then be beneficial at the viral population (or quasi-species) level, the level at which selection has been experimentally shown to operate (Vignuzzi et al. 2006). Considering viral transport from plant-to-plant, this hypothesis is extremely interesting because it opens fields of investigation that have not been explored so far. Once it is admitted that a virus can select mechanisms because they influence the viral pool that is collected by the vector, such mechanisms may be looked for not only in the virus–vector interaction but also in the plant–virus interaction. On the other hand, some reported and unexplained observations in plant–virus relationships may also be interpreted in this viewpoint. More detailed related arguments and the specific example of CaMV are discussed in the following section.

Vector Receptors

Available data on vector receptors used by non-circulative viruses are very scarce. It is surprising that the most abundant literature related to virus transmission by homopteran vectors does not provide any clues, even as to the chemical nature of the attachment sites in the vector anterior alimentary tract. Paradoxically, the only tangible information available was recently obtained on the far less studied fungal transmission. The receptors of CNV, located at the surface of the zoospore of the vector *Olpidium bornovanus*, were demonstrated to be glycoproteins, the oligosaccharide part of the molecules more specifically containing mannose and/or fucose derivatives (Kakani et al. 2003).

The location of attachment sites of non-circulative-viruses in homopteran vectors appears to be divided. While some viruses have been directly observed, by electron microscopy, on the cuticle lining the lumen of the foregut (reviewed in Nault and Ammar 1989; Nault 1997), most species are presumably retained at the very tip of the maxillary stylets (Pirone and Perry 2002). These two possible locations are at the base of a long-standing controversy concerning the process of virus release in new host plants. Viruses retained in the foregut will necessarily flow out upon undocumented regurgitation (egestion) phenomena (Harris 1977; Powell 2005), whereas both egestion and salivation could wash out viruses located at the tip of the stylets. Indeed, the alimentary and salivary canals are differentiated all along the core of the maxillary stylets, except at the very distal extremity, where they fuse into a common duct of only a few micrometres long. The efficient inoculation of viruses of the genera *Cucumovirus* (Martin et al. 1997), *Potyvirus* (Martin

et al. 1997), and perhaps *Caulimovirus* (Moreno et al. 2005b), has been shown to occur readily during the first sub-phase on intracellular activity of aphid stylets, corresponding to salivation (Powell 2005). Hence, while salivation can satisfactorily explain the release of viruses using putative receptors located at the tip of the stylets, the cases of those located in the foregut requires further investigation. Whether differential conditions in sap and saliva promote subsequent attachment and release of viruses, or a specific enzyme activity in the saliva cleaves off the viral proteins or the receptor itself is totally unknown.

3.2 Traffic within the Plant before Acquisition by the Vector

The transport of viruses or viral elements within the plant is documented in the other parts of this volume. The aim of this section is to demonstrate how viral transport within- and between plants can sometimes be intimately related, despite being investigated separately. From all the literature on vector transmission it is always considered that a virus usually "does what it has to" inside the host plant, and that the vector will collect it where and as it is. Here, I would like to invert this point-of-view and stress that viruses could also do things in plants that specifically prepare for and optimize their encounter with vectors. This vision was inspired by the analysis of a series of works published on CaMV, and by a specific more recent investigation in our laboratory (Drucker et al. 2002).

3.2.1 The Case of the Electron-Lucent Inclusion Body of CaMV

Naturally occurring non-transmissible isolates of CaMV either lack gene II (Howarth et al. 1981) or harbour a mutation therein (Gardner et al. 1981). The mutant isolates CM1841 and Campbell both have the same substitution at amino acid position 94 of P2 (here designated $P2_{94}$) that does not alter the functionality of CaMV HC in aphids (Blanc et al. 1993a). Electron microscopy has demonstrated that P2 accumulates in characteristic electron-lucent inclusion bodies (elIB) in infected plant cells, and that CaMV strains carrying $P2_{94}$ lack such inclusions (Espinoza et al. 1991). Altogether these data indicate that the non-transmissibility of isolates CM1841 and Campbell is not related to the lack of $P2_{94}$ activity in the aphid vector, but rather to its incapacity to form proper elIB in plant cells. Drucker and collaborators have re-investigated this question and demonstrated that elIB function as stores of P2, keeping it apart from P3-virion complexes, which are sequestered in another inclusion (the electron-dense inclusion body, edIB). This cellular process prevents the formation of the total transmissible P2-P3-virion complex, which will be completed only when the vector sequentially collects elIB (containing P2) and P3-virion complexes in a series of successive probing in different cells, thus

favouring HC-transcomplementation (Drucker et al. 2002). The elIB is dispensable for virus infectivity in plants (Espinoza et al. 1991) and it is therefore assumed that its only function is the regulation of aphid transmission. All CaMV proteins are thought to be produced inside or at the periphery of the electron dense inclusions (Hohn and Fütterer 1997), suggesting that the components of the electron-lucent bodies, particularly P2, are exported from the former, transported to and accumulated in the latter. Because P2 has been shown to bind plant microtubules (Blanc et al. 1996), we hypothesize that microtubules could be used as trails for this specific transport from edIB to elIB (Alexandre Martinière, Stéphane Blanc and Martin Drucker, unpublished), but this remains to be formally demonstrated. Through this example, although it may appear counter intuitive, it becomes clear that some viral transports within plant cells can only be functionally explained by their ultimate role in vector-transmission. Unfortunately, to the best of our knowledge, no other examples of this phenomenon have been thoroughly documented so far.

3.2.2 Other Examples to be Investigated

In the light of the recognition of the specific role of elIB in the aphid-transmission of CaMV, many other possible adaptations in various plant-virus species relationships should be investigated. Apart from viral replication and processes related to whole plant colonization, particular phenomena, developing at different paces, could participate in the optimization of vector-transmission.

It is widely known that virus titre can vary dramatically, not only within different organs and tissues of the host plant, but also in a timely fashion during the infection cycle. Some of these variations, particularly late in infection, could reflect specific viral in planta adaptations to vector feeding behaviour. For instance, *Maize streak virus* (MSV) accumulates into enormous virion crystals in the nuclei of infected cells, which are likely ingested by the leafhopper vector when searching for the vascular bundles (Bosque-Perez 2000). Whether the mechanisms explaining this massive concentration of virions is a viral adaptation for more efficient vector-transmission, or just a consequence of excessive production during the infection cycle has not been investigated.

Another example of possibly overlooked adaptations is the frequent formation of numerous and sometimes complex viral protein inclusions at late stages of cell infection. Like the elIB of CaMV, some of these inclusions may play a specific role rather than simply being aggregated remnants of the replication wave front that has passed and moved on (Riedel et al. 1998). The HC-Pro of potyviruses has been mentioned to accumulate in many different inclusions late in infected cells (Riedel et al. 1998). Since this protein is

multifunctional, it is likely that a soluble form may act early in the replication, movement or suppression of PTGS, whereas other forms associated with other viral or host factors in various inclusions may assume specific functions, including vector-interaction.

One most certainly relevant trait directly linked to viral transport in plants, and surely impacting the viral pools taken up by vectors, is the rate of co-infection of cells by several variants of the viral population. Indeed, homopteran vectors usually operate by probing of superficial tissue cells, and simply leave and continue their search when they do not sense a suitable host. This superficial short probing has been described countless times as the specific step where non-circulative virus acquisition occurs. The number of viral genome variants present in single cells could thus directly influence the genetic content of the viral sample transmitted by vectors and hence, as proposed and discussed for HC-transcomplementation (Pirone and Blanc 1996; Roossinck 1997; Froissart et al. 2002; Power 2000), the rate of cell multiple infection could also be a trait precisely regulated by specific virus adaptation.

The spatial separation of closely related genetic variants in different cells has been reported for several RNA viruses (Hull and Plaskitt 1970; Dietrich and Maiss 2003; Jridi et al. 2006), but the actual mechanisms explaining this situation have not been elucidated. On the opposite, co-existence of several genomic variants of *Tomato yellow leaf curl geminivirus* (TYLCV) has been reported to concern about 20% of the host plant infected cells (Morilla et al. 2004), and might even be the rule in the case of *Cauliflower mosaic virus* (Baptiste Monsion, Alberto Fereres and Stéphane Blanc, unpublished results). Two categories of hypotheses can be forwarded and illustrate means by which a virus can regulate (prevent or promote) cell entry or replication of secondary infecting variants. The first one (1) relies on the capacity of viruses to both elicit and circumvent plant defences, and the second (2) on the regulation of their own cell-to-cell trafficking.

1. The suppression of post-transcriptional gene silencing, could be relaxed or maintained in late stages of the virus replication cycle, thus respectively preventing or allowing secondary infection. Consistently, post-transcriptional gene silencing has been shown to prevent secondary infection in some cases of "cross-protection" between RNA viruses (Ratcliff et al. 1999; Dietrich and Maiss 2003). Unfortunately, similar data on Gemini- or Caulimoviruses, where secondary infection is likely possible, are so far unavailable. Another interesting hypothesis, related to plant defence process (inspired by the review by Boevink and Oparka 2005), concerns the callose deposition closing the plasmodesmata, and preventing virus movement. The TGB2 protein of PXV has been shown to interact with host proteins involved in callose degradation, thus possibly interfering with the closing of plasmodesmata. It is interesting to note that, in this hypothesis, depending on the maintenance of such TGB2 activity

in infected cells, PVX could either open or close the way for secondary infection.

2. Other possibilities, to regulate single or multiple infections of cells, are related to the very diverse and complex mechanisms of cell-to-cell movement described in other parts of the present volume. These mechanisms are of particular interest, especially considering their regulation in late phases of the replication cycle, once the first genomes on the spot have replicated and moved away. In the best-studied example of TMV, it is clear that the movement protein has a very complex mode of action, and plays different roles during the kinetics of the virus replication cycle (Boevink and Oparka 2005). While gating plasmodesmata early in infection, and thereby allowing the transfer of viral genomes to adjacent cells, the TMV movement protein appears to be rapidly inactivated (Oparka et al. 1997) by phosphorylation events (Waigmann et al. 2000; Trutnyeva et al. 2005) and later degraded (Szecsi et al. 1999), through the 26S proteasome pathway (Reichel and Beachy 2000). It would be interesting to test whether the movement protein, when inactivated and still retained in plasmodesmata, can block the passage of new incoming viral variants, thus controlling secondary infection.

Altogether, though largely speculative, the above discussion suggests that viruses may have developed means for controlling their traffic in the host plant, not only at the leading edge of colonizing infection but also later, in infected tissues promoting or preventing secondary multiple infection. This latter phenomenon is poorly studied, but it directly connects with virus transmission from plant-to-plant, as it determines the pool of genome variants available in single cells and taken up by the vectors.

3.3
Traffic within the Plant Immediately after Inoculation by the Vector

Often, as is the case for aphids, vectors can introduce their mouth-parts within a cell with very limited damage, and inject viruses. Even in these non-destructive inoculation events, viruses must reach the cell compartment where they can initiate the new infection cycle. This problem is more acute for DNA viruses, which are released into the cytoplasm and must translocate to the nucleus before any transcription and/or replication events can take place. Since decapsidation occurs either at the nuclear pores or even within the nucleus (Whittaker and Helenius 1998; Whittaker et al. 2000), the virus particles inoculated by vectors must target the nucleus, without relying on an additional viral non-structural gene product. In the genus *Geminivirus*, a non-structural protein designated Nuclear Shuttle Protein (NSP) is believed to promote within-cell transportation of viral DNA from the nucleus to the cytoplasm, and perhaps vice versa (Sanderfoot and Lazarowitz 1996; Fontes

et al. 2004), during the infection cycle. Nevertheless, the coat proteins of some geminiviruses have been demonstrated to autonomously traffic between nucleus and cytoplasm (Kunik et al. 1998; Unseld et al. 2001), and this property could act early after vector-transmission. A similar unclear situation has been described for *Cauliflower mosaic virus* where nuclear targeting has been described not only for the coat protein (Karsies et al. 2002; Champagne et al. 2004), but also for non-structural viral products (Haas et al. 2005). In all cases, whether the movement functions involved in the normal course of cell-to-cell colonization and those acting in the very early stages following inoculation by vectors are distinct remains to be investigated.

In some particular cases, vector feeding is dramatically damaging or even kills cells, implying an immediate translocation of injected viruses towards adjacent live cells where they can initiate infection. This particular situation is certainly best illustrated and documented for beetle-transmitted viruses, though other vectors have a destructive feeding behaviour. Beetles acquire and retain a large number of virus species, which have very stable virus particles. However, despite the fact that all these viral species can be detected in the beetle regurgitant, deposited upon feeding on host plants, only some are efficiently transmitted (Gergerich and Scott 1991). This observation led to the conclusion that the success of virus transmission by a beetle vector depends on "permissive" plant-virus interaction, immediately after deposition in wounded cells, rather than on specific virus-beetle interaction. This intriguing phenomenon has been investigated by Gergerich and collaborators, in a series of works reviewed in Gergerich (2001). A high amount of RNAse activity has been found in beetle regurgitant, which was demonstrated to block the infection by non-beetle-transmissible viruses. Hence, those viral species that are efficiently transmitted are likely capable of translocation in the vascular system, and/or transfer to unwounded cells, away from the RNAse activity. Unfortunately, the putative specific mechanisms of viral transport have not been investigated in detail.

4 Concluding Remarks

The viral transports involved in plant-to-plant transmission have been extensively studied through the elucidation of the intricate molecular and cellular mechanisms of the virus-vector interaction. If one excludes the circulative propagative transmission, where the virus-vector relationship resembles the infection of an alternative arthropod host, two important questions still stand as major black boxes. The first is the transcytosis of luteoviruses across the gut and salivary barriers, a specific transport process that has been described only in plant circulative viruses, and where the viral determinants are not fully characterized and the host cell interacting partners only hypothetical.

The second important prospect is the identification of the vector receptor(s) used by the majority of plant virus species in non-circulative transmission. Its precise location in vector mouth parts, its chemical nature, and whether different virus species use different or a single ubiquitous molecule are questions perfectly illustrating the cruel lack of data in a scientific field of major interest for plant pathology and epidemiology.

Finally, two major unexplored concepts, directly connecting the viral transport within plants and that in between plants, deserve to be developed and carefully addressed.

1. The viruses can certainly adapt specific strategies for accumulation and storage in certain cell or plant compartments, in the form of defined macromolecular complexes, thereby optimizing the chances and efficiency of acquisition by the vectors. These adaptations can be, for instance, increased concentrations at the right places and timings, specific targeting to inclusions or cell compartments and accumulation in transmissible complexes recruiting viral and host factors. In all cases, this possibility should be kept in mind in order to correctly interpret some viral traffic in the host plant, that is not evidently related to the cell-to-cell or long distance movements during plant colonization. This transport phenomenon devoted to optimal ulterior acquisition by the vector could therefore occur at different time points, later during the infection cycle.
2. While virus movement is most often, if not always, investigated at the leading edge of infection, it would be extremely interesting to address what happens later in the infected tissues, where the viruses could or could not re-enter and replicate in previously infected cells. Recent data demonstrate unambiguously that a potyvirus does not traffic the same way in a healthy or a chronically infected tree (Jridi et al. 2006). This aspect is of major importance because it determines the possibility of mixing of viral variants within a single host. The possibility, or the lack of, encounter of viral genomes in multiply infected cells not only impacts the sampling of the virus population by the vector (as discussed above), but also some of the most important traits in the biology of viruses, such as complementation (Froissart et al. 2004) and recombination (Froissart et al. 2005; Jung et al. 2002; Bocharov et al. 2005). It is likely that different viruses, with totally different life cycles, have adopted strategies either promoting or preventing the multiple infections of single cells. The mechanisms by which a virus, replicating in one cell, would either allow or block the secondary infection by its close relatives are totally unknown, and represent an exciting ground for future research.

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