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Viral Transport in Plants

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Viral Transport in Plants

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Editors

Manfred Heinlein studied biology and obtained his Ph.D. at the University of Cologne in 1992. Following a two year period as a post-doc continuing his studies on maize transposons in Cologne, he went for postdoctoral studies to the Scripps Research Institute (La Jolla, CA, USA), where he initiated his research on plant virus movement. In 1997, he went to Basel (Switzerland) where he became group leader at the Friedrich Miescher Institute for Biomedical Research (FMI) and habilitated (venia docendi) in Molecular Biology at the University (in 2001). In 2003, he obtained a professorship funded by the Swiss National Science Foundation to join the Botanical Institute in Basel as assistant professor. At the end of the same year, he became group leader at the Institut de Biologie Moléculaire des Plantes (IBMP-CNRS) in Strasbourg. His major research interests focus on cell-to-cell communication in plants, particularly on the mechanism and role of RNA transport in systemic gene regulation and RNA virus infection.

Elisabeth Waigmann is currently group leader at the Max F. Perutz Laboratories, a joint venture between the Medical University of Vienna and the University of Vienna, located at the Vienna Biocenter, Austria. She studied Chemistry, with specialization in Biochemistry, at the University of Vienna, and only later on became increasingly interested in cell biology. Her ongoing research focuses on transport processes in and between cells, with plant viruses as a model system.

Preface

Plants, as sessile organisms, are exposed to a multitude of stresses ranging from pathogens to environmental conditions. Not surprisingly, plants have developed sophisticated pathways to respond to and cope with those stresses. Plant viruses and viroids are pathogens and, as their life cycle is largely dependent on the plant host, they provide a valuable doorway to glimpse and analyze the intricate net of host-pathogen interactions. Of course, research on plant viruses provides insight into plant defense reactions. However, they also represent important tools for the analysis of the mechanisms involved in the transport of macromolecules through plasmodesmata, the channels in the plant cell wall through which adjacent cells communicate. In this book we provide a state-of-the-art overview about processes involved in virus transport and start by taking a view on the mechanisms involved in the transmission of viruses from plant to plant. Then we narrow our focus on single infected plants with the question how plant viruses and viroids exploit plasmodesmata and other host cell components to spread cell-to-cell and systemically. Since viruses trigger defense responses of the plant, a chapter is dedicated to the description of the battle between viruses and host plants that rages on the field of post-transcriptional gene silencing. Finally, the book ends by highlighting research performed in the model plant Arabidopsis, which serves as a valuable host for genetic approaches to identify novel factors involved in virus–host interactions.

To optimize entry into and transmission between plants, most plant viruses utilize insect or nematode vectors. Consequently, the majority of viral transport mechanisms associated to the transmission step has been approached through the study of virus–vector relationships. The chapter "Virus Transmission – Getting Out and In" by Stéphane Blanc concisely summarizes our knowledge on viral transport between plants by various vectors, and highlights a few examples in more detail. Blanc illustrates the concept that some viral trafficking within plants is specifically intended to prepare ulterior acquisition by the vectors, thereby providing a direct link to the following chapters dealing with *in planta* movement.

The chapter "Tobacco Mosaic Virus – a Model for Macromolecular Cell-to-Cell Spread" by Elisabeth Waigmann, Mirela Curin and Manfred Heinlein, illuminates the central role of the virus-encoded movement protein (TMV-MP)

in the cell-to-cell movement of TMV. As a pioneer among plant viruses, this virus has served as a favorite research object for more than a hundred years. Still firmly anchored at the forefront of research, TMV breaks the ground for novel insights into the principle mechanisms involved in the cell-to-cell transport of macromolecules, with implications that may go far beyond the field of virology.

While TMV movement involves the transport of the viral genome in a non-encapsidated form, i.e. as a ribonucleoprotein particle, other viruses move between cells in the form of entire virus particles. This type of movement involves the formation of transport tubules within plasmodesmata, which is summarized in the chapter "Tubule-Guided Movement of Plant Viruses" by Christophe Ritzenthaler and Christina Hofmann. The chapter describes the functional relevance of these tubules in the transport of viruses, speculates on models for this movement mechanism and discusses the host components that seem to contribute to this type of transport.

Mechanistically complex and clearly distinct from cell-to-cell transport are plant viral strategies for systemic movement and infection of the whole plant. In their chapter "Spread Throughout the Plant: Systemic Transport of Viruses" the authors Shoko Ueki and Vitaly Citovsky emphasize that systemic movement is indeed more than just the summary of numerous cell-to-cell movement events, since it involves many different types of cells and tissues, requires different cellular factors and proceeds at much higher speed than local movement. Moreover, systemic movement provides insight into the ability of the virus to interact with plant defense responses. For example, several viral and host factors involved in systemic movement act through the suppression of RNA silencing, which targets the viral genome for degradation.

A highly elegant model system for studies on intra- and intercellular transport are viroids, small non-coding and non-encapsidated RNA molecules that are able to replicate and systemically infect plants. Due to their lack of protein components, viroids are particularly dependant on the plant cellular machinery and may therefore represent the best model system to elucidate endogenous intercellular RNA transport processes. Biao Ding and Asuka Itaya summarize in the chapter "Intracellular and Intercellular Transport of Viroids" recent progress in the characterization of viroid structures and host proteins but also critically discuss issues that need to be addressed in future investigations.

As mentioned above, RNA silencing constitutes an important plant defense mechanism against viruses. Thus, no book on viral transport in plants can abstain from including a chapter on RNA silencing. The chapter by Thomas Hohn, Rashid Akbergenov and Mikhail Pooggin entitled "Production and Transport of the Silencing Signal in Transgenic and Virus-Infected Plant Systems" narrates the fascinating story of the race between silencing and virus replication.

Tyrell Carr and Steven A. Whitham dedicate their chapter "An Emerging Model System: Arabidopsis as a Viral Host Plant" to the molecular biologist's

pet plant, *Arabidopsis thaliana*. An amazing array of viruses has already been shown to infect one or more Arabidopsis ecotypes. Thus, although Arabidopsis has only recently entered the scene of plant virus research, it opens the door to genetic and reverse genetic approaches that are not feasible or practical in many agronomically important hosts. Indeed, a number of host genes involved in virus replication and spread have already been identified in Arabidopsis.

Overall, the book is intended for a readership of advanced students, teachers and interested researchers, and is intended to fill the gap that is created by the lack of information in many standard textbooks on the topic of plant viruses. We wish to particularly thank the authors who have contributed to the book with their chapters, especially for their enthusiasm and diligence in providing a state-of-the-art overview on the manifold fascinating aspects of viral transport in plants. We also thank Springer, represented by Christina Eckey and Anette Lindqvist, as well as series editor David Robinson, for undertaking the ambitious effort to create the "Plant Cell Monographs" series, dedicated to various highly interesting aspects of plant biology.

February 2007

Elisabeth Waigmann
Manfred Heinlein

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Virus Transmission—Getting Out and In

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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.

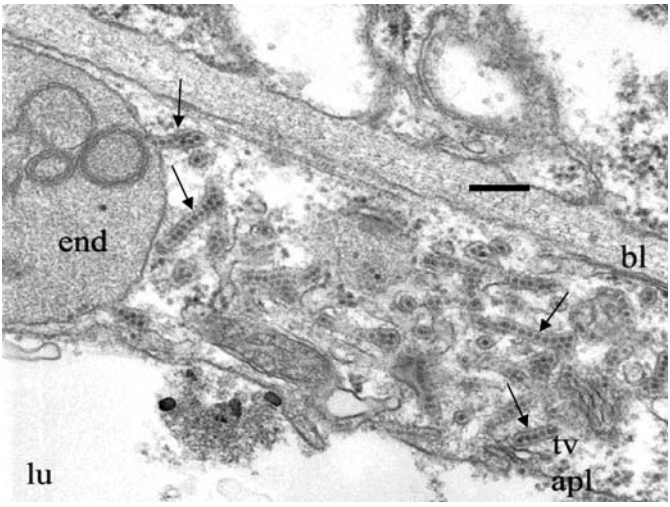

Fig. 1 Transcytosis of CABYV in hindgut cell of the aphid vector *Myzus persicae*. Luteovirus present in the gut lumen (lu) are internalised from the apical plasmalemma (apl) and transported to the basal lamina (bl) in a complex pattern involving different vesicular structures, described in the text. 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

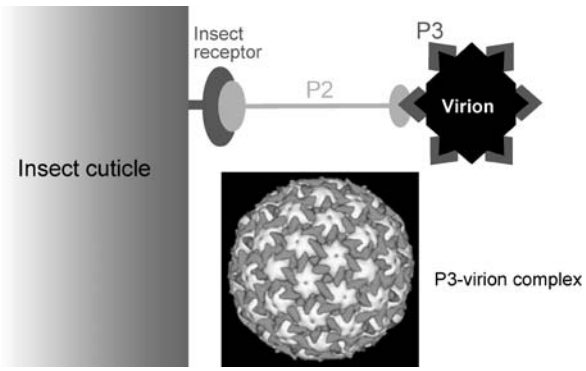

Fig. 2 Transmissible complex of *Cauliflower mosaic virus*. The viral protein P2 attaches both the putative receptor, on the cuticle lining the alimentary canal within the vector's stylets, and P3 intimately associated to the virus particle. *Inset* shows details of P3 (*dark grey*) distribution around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

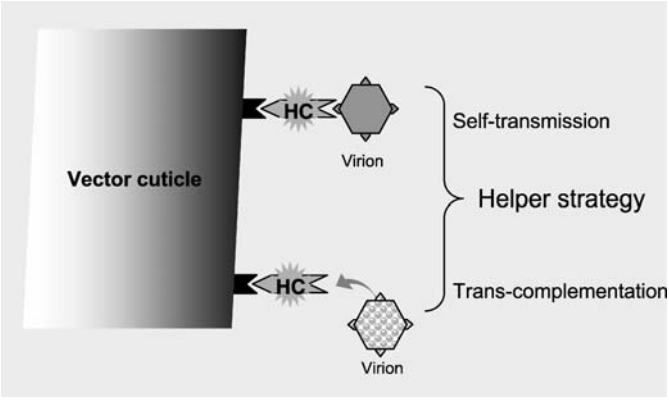

Fig. 3 Schematic representation of HC-transcomplementation in the vector transmission of plant viruses. The HC can be acquired alone, prior to virion, and attach the putative vector receptor. In this case a HC encoded by a genome X (for instance that encapsidated in the gray virion), can subsequently 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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Tobacco Mosaic Virus – a Model for Macromolecular Cell-to-Cell Spread

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Abstract Macromolecular cell-to-cell transport in plants occurs through complex intercellular channels, the plasmodesmata. Plant viruses pirate these natural plant communication channels for their own spread from an infected cell to a neighboring healthy cell. Viral movement proteins are the major agents in promoting this process. *Tobacco mosaic virus* is the most extensively studied plant virus and can therefore be viewed as a model system for cell-to-cell transport. In this chapter we summarize knowledge about mechanistic properties of the movement protein of *Tobacco mosaic virus* and discuss the potential involvement of other viral and cellular components in the intercellular transport process.

1 Introduction

Tobacco mosaic virus (TMV), the first virus ever known, was detected more than a century ago and has served as a model for ground-braking research in virology and molecular biology. TMV was the first virus visualized in the electron microscope and henceforth was intensely studied to gather information that could also be applied to other viruses. As a consequence of these intense studies, the coat protein of the virus was the second protein fully sequenced (after insulin). Moreover, TMV RNA became the first plant viral genome to be sequenced completely (Goelet et al. 1982) and the second plant viral RNA (vRNA) that was completely cloned as cDNA. TMV also served as model to establish engineered resistance in transgenic plants (Powell-Abel et al. 1986). In 1987, the 30 kDa protein of the virus was the first protein to be functionally identified as a movement protein (TMV MP; Deom et al. 1987) and its requirement for the cell-to-cell progression of virus infection through plasmodesmata became established. Although in more recent years it became clear that most, if not all, plant viruses encode such proteins, the MP of TMV has remained at the forefront of research aimed at understanding the mechanism by which a virus moves from cell to cell. Moreover, since TMV spreads its RNA genome through plasmodesmata in non-virion form,

it serves as a model for studying the cellular mechanism by which RNA and protein macromolecules target plasmodesmata. Macromolecular communication through plasmodesmata has essential roles during plant development as well as in the orchestration of plant defense responses, and involves the controlled cell-to-cell and systemic trafficking of a whole range of RNA and protein macromolecules, including non-cell-autonomous transcription factors, RNA-based silencing signals, and messenger RNAs (Haywood et al. 2002; Heinlein 2002; Heinlein and Epel 2004; Huang et al. 2005; Jackson 2005; Kim 2005; Lucas et al. 2001; Tzfira et al. 2000; Wu et al. 2002; Yoo et al. 2004). Thus, research to determine the mechanism by which TMV MP and RNA target plasmodesmata and spread between cells holds the promise for new insights into the mechanisms involved in intercellular communication and plant development as well as in viral disease. Current and further studies also include analysis of the interface between virus and plant defense responses, such as RNA silencing. Given that viruses encode proteins that function as silencing suppressors and that several of these proteins have been previously implicated in movement, it will be important to address the question of whether virus movement is exclusively due to the interaction of the virus with cellular transport processes or whether movement may also result as a manifestation of successful suppression of defense responses by the virus (Carrington 1999). Although, so far, other viral systems have been more successful in contributing to the analysis of silencing pathway components and viral silencing suppressors (see chapter on silencing by Thomas Hohn, in this volume), the combination of established TMV cell biology with the cellular analysis of silencing pathway components will likely contribute important insights into those plant:virus interactions in the future.

In this book chapter we summarize our current understanding of TMV cell-to-cell movement and describe the role of various viral proteins and host factors in this process. Clearly, the movement protein TMV MP is the major actor and consequently the central part of this chapter is devoted to its manifold functions. Nonetheless, the emerging contributions of other viral proteins such as the coat protein and the replicase, as well as the contributions of host factors, whose number is steadily increasing, are also discussed.

2
Plasmodesmata: Structure and Composition

For cell-to-cell spread, *Tobacco mosaic virus* pirates plasmodesmata (Oparka 2005), complex cell-to-cell communication channels in the plant cell wall that provide cytoplasmic continuity between adjacent cells. The ultrastructure of plasmodesmata has been defined by numerous electron microscopy studies (for example Botha 1992; Ding et al. 1992). The plasma membrane delineates the plasmodesmal pore, which is traversed in its axial center by the ap-

pressed membrane of the endoplasmic reticulum (ER) termed desmotubule. Plasma membrane and desmotubule are densely covered with globular particles (Ding et al. 1992) that segment the region between plasma membrane and desmotubule, the cytoplasmic sleeve, into eight to ten channels (Ding et al. 1992). These channels are considered to function as conduits for diffusion of molecules between cells. The cell wall or neck region surrounding the plasmodesmal orifices is speculated to participate in the control of molecular traffic through the channel (Olesen 1979; Overall and Blackman 1996; White et al. 1994).

Unlike the ultrastructure, the molecular composition of plasmodesmata is poorly defined. Several strategies have been employed to elucidate plasmodesmal composition:

- Direct biochemical approaches aimed at extracting plasmodesmal proteins (for example, Epel et al. 1995; Kishi-Kaboshi et al. 2005; Turner et al. 1994)
- Use of antibodies against known proteins suspected to reside at plasmodesmata
- Expression of cDNA libraries to express random proteins fused to GFP and subsequent selection for punctuate localization of fusion proteins to the cell wall, a pattern indicating plasmodesmal localization
- Screening for host factors interacting with viral movement proteins

Here, we will focus on recent findings, as a more complete picture has been presented elsewhere (for example, Aaziz et al. 2001; Waigmann et al. 2004).

The biochemical approach to purifying plasmodesmal proteins from plant extracts led to identification of a 41 kDa protein within mesocotyl cell wall fractions of *Zea mays* (Epel et al. 1996b) and in plasmodesmal protein-enriched fractions from *Arabidopsis* (Sagi et al. 2005). Recently, this protein was shown to represent a member of the class 1 reversibly glycosylated polypeptides (^{C1}RGP) protein family (Sagi et al. 2005). ^{C1}RGPs localize to the Golgi and plasmodesmata, suggesting that these proteins are secretory proteins that are delivered to plasmodesmata via the Golgi apparatus. Within plasmodesmata, ^{C1}RGPs might be attached to the plasma membrane facing the cytoplasmic sleeve and could thus be involved in establishing the size exclusion limit of plasmodesmata (Sagi et al. 2005). Biochemical enrichment of a plasmodesmal protein fraction also led to purification of a casein kinase I (CKI) activity from *N. tabacum* suspension culture cells that was able to phosphorylate TMV MP, in line with previous findings that show TMV MP phosphorylation *in planta* (Lee et al. 2005; see also Sect. 3.4). Since the corresponding *N. tabacum* gene could not be identified conclusively, the 13 CKI-like (CKL) genes from *Arabidopsis* were analyzed for subcellular localization. Indeed, in transient expression assays in tobacco leaves, CKL6 co-localized with TMV MP in cell wall-associated puncta, suggesting its plasmodesmal localization (Lee et al. 2005).

The antibody-based strategy pinpointed cytoskeletal components (Overall et al. 2000; Reichelt et al. 1999; White et al. 1994) and calcium-binding proteins such as centrin and calreticulin as plasmodesmal proteins (Baluska et al. 1999; Blackman et al. 1999) Interestingly, calreticulin, an ER-resident protein reported to accumulate in plasmodesmata of the maize root apex (Baluska et al. 1999) was also shown to interact with TMV MP (Chen et al. 2005; see also Sect. 3.4).

Viral expression of a GFP-fused cDNA library derived from *N. benthamiana* roots revealed 12 GFP fusion proteins (PD01-12) potentially localizing to plasmodesmata (Escobar et al. 2003). Plasmodesmal localization was confirmed for one of these proteins, PD01, by immuno gold labeling with antiserum against GFP. Since some of these proteins might localize to plasmodesmata in response to the viral infection strategy used for their transient expression, only additional experiments can clarify the significance of these results (Escobar et al. 2003).

How far have we come on the road towards complete knowledge of plasmodesmal composition? Considering that plasmodesmata are dynamic entities, flexible in structure and permeability, plasmodesmal complexity may be similar to that of a nuclear pore with its more than 100 structural proteins. If so, the number of known plasmodesmal components represents only the beginning of a list that awaits completion in the future.

3
TMV MP, a Protein of Manifold Qualities

3.1
TMV MP Structure and Single-Stranded Nucleic Acid Binding

TMV MP consists of 268 amino acids and is the type member of a large group of viral MPs with a molecular mass of approximately 30 kDa, termed the "30K superfamily". The members of the 30K superfamily share only low sequence similarity (Koonin et al. 1991, Melcher 1990) but may share a common three-dimensional structure. Unfortunately, not even for the intensely studied TMV MP, has a three-dimensional structure been elucidated by X-ray crystallography, which is primarily due to the fact that recombinant TMV MP is not readily soluble and has a strong tendency to aggregate. Thus, structural models of TMV MP rely on bioinformatic predictions or on a combination of CD spectroscopy and biochemical methods. Bioinformatic prediction based on the family consensus sequences of MPs from 18 families revealed a common core consisting of four α-helices and seven β-elements flanked by variable N- and C-terminal domains (Melcher 2000). The N-terminal region was generally variable in range and structure, whereas the C-terminal region was predicted to be a predominantly random coil. For TMV MP, it has been shown

that deletion or mutation of sequences within the common core region affect the ability of the protein to bind RNA or to bind microtubules. This region of the protein also contains predicted transmembrane domains and functionally tested determinants for the interaction of the protein with microtubules and its targeting to plasmodesmata (see Sect. 3.3 and Fig. 1). Intramolecular complementation of a dysfunctional Pro81Ser amino acid exchange mutation by distant Thr104Ile and Arg167Lys exchange mutations indicates that the core region folds into a compact tertiary structure which allows distant primary sequence and secondary structure elements to interact (Deom and He 1997; Boyko et al. 2002, Fig. 1). The C-terminal random coil structure of the protein is dispensable for cell-to-cell movement (Berna et al. 1991), and may act as a flexible tail that regulates access to those functional domains. In line with this assumption, the carboxyterminus of TMV MP harbors three phosphorylation sites (Citovsky et al. 1993, Fig. 1), which have been shown to play a role in regulation of TMV MP subcellular localization and function in *N. tabacum* (Trutnyeva et al. 2005; Waigmann et al. 2000; see also Sect. 3.4)

Experimental evidence derived from studies using purified recombinant TMV MP (Brill et al. 2000) is in good agreement with the predicted core structure. CD spectroscopy of urea- and SDS-solubilized TMV MP demonstrated

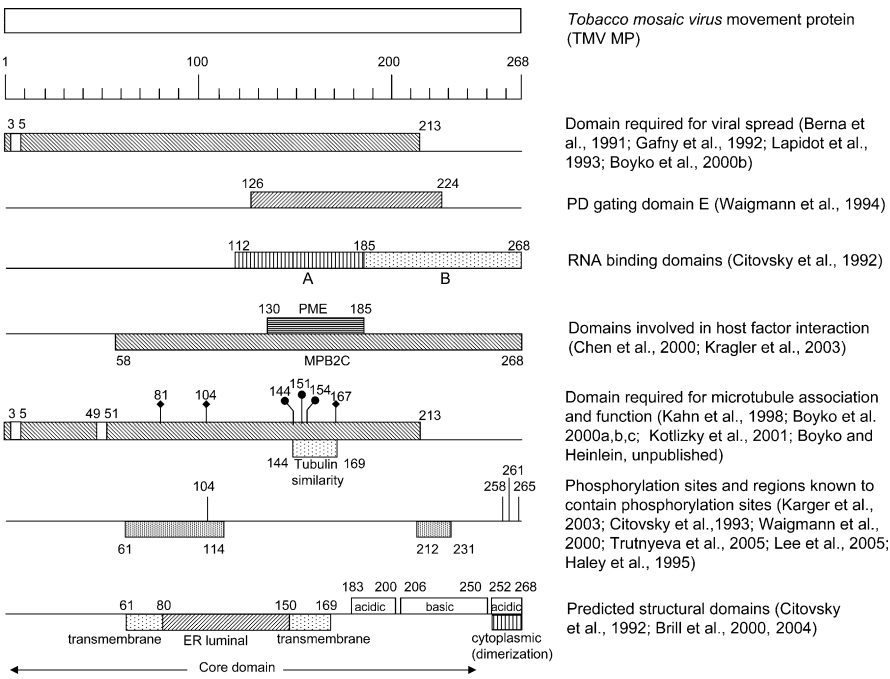

Fig. 1 Overview of TMV MP regions involved in various functions of the protein. For detailed description, see text; • ts mutations in MT association, ♦ required for function

a high α-helical content suggesting an ordered tertiary structure. Trypsin digestion followed by mass spectroscopy revealed two cleavage resistant, highly hydrophobic domains which might constitute two putative membrane spanning-regions. These regions could cause the TMV MP to behave as an integral membrane protein in biochemical fractionation experiments (Reichel and Beachy 1999). The C-terminal part of TMV MP (amino acids 250–268) was highly sensitive to trypsin treatment, indicating that this part of the protein is accessible to the solvent. Analytical ultracentrifugation suggested that the TMV MP forms homodimers. Upon trypsin digestion, dimers were converted into monomers, suggesting that the C-terminal part of TMV MP is involved in dimerization (Brill et al. 2004). In a topological model, the two potential transmembrane domains of TMV MP span the ER membrane, thereby imposing a U-like conformation onto the protein, whereas the short N- and longer C-terminal regions are exposed to the cytosol (N_{cyt}-C_{cyt} topology; Brill et al. 2000); the C-termini link two adjacent molecules into a dimer, potentially via charge–charge interactions (Brill et al. 2004).

MPs of other viral groups that are not part of the 30K superfamily may share structural features with TMV MP. For example, carmoviruses encode two small proteins, p7 and p9, involved in cell-to-cell movement of the virus (Hacker et al. 1992; Li et al. 1998). *Carnation mottle virus* (CarMV) p7 is a soluble protein that binds to RNA, whereas CarMV p9 is an intrinsic membrane protein with two transmembrane helices imposing a U-like conformation onto the protein. The short N- and long C-terminus of p9 are exposed to the cytosol enabling the C-terminal region to interact with CarMV p7, thereby providing membrane localization to the p7-RNA complex (Sauri et al. 2005; Vilar et al. 2002). The topology proposed for the CarMV p7/p9 is remarkably similar to that proposed for TMV MP, suggesting that despite sequence differences, some conserved structural and topological elements exist that may be important for movement protein function.

One of several functional hallmarks of MPs is their ability to bind nucleic acids, a feature first shown for TMV MP. TMV MP binds single-stranded (ss) RNA and DNA in a strong, cooperative, and sequence-non-specific manner (Citovsky et al. 1990, 1992). Mutational analysis of TMV MP revealed two independently active binding domains (Fig. 1) located between amino acid positions 112–185 (binding domain A) and 186–268 (binding domain B) of this 268-residue long MP (Citovsky et al. 1992). Nucleic acid binding domains have been identified in many viral MPs (for review see Waigmann et al. 2004); however, no conserved amino acid motif was revealed. Interestingly, nearly all MPs have only one RNA binding domain, the only exceptions being TMV MP and *apple chlorotic leaf spot trichovirus* (ACLSV) MP, which is also characterized by two adjacent independently active RNA binding domains (Isogai and Yoshikawa 2005).

What may be the role of MP binding to nucleic acids? The ultimate function of MPs is to mediate transport of the viral genome from cell-to-cell via

the size restrictive channels of plasmodesmata, thereby enabling the infection to spread within a plant. Binding of MP to ss nucleic acid is a direct means of physically associating the MP to its cognate viral genome. Based on electron microscopy and atomic force microscopy data, recombinant TMV MP seems to bind ss nucleic acids in a "bead-on-the-string"-like fashion thereby forming extended ribonucleoprotein particles (vRNP; Citovsky et al. 1992; Kiselyova et al. 2001) compatible with the size exclusion limit of dilated plasmodesmata (Waigmann et al. 1994). Thus, complexes between MPs and viral genomes most likely represent intermediates of the movement process, shaped in a transportable form. Interestingly, those complexes are non-translatable and non-replicable in vitro and in isolated plant protoplasts (Karpova et al. 1997), which indicates that they are diverted from translation and replication and dedicated to cell-to-cell movement. Inhibition of translation and replication of TMV RNA is eliminated in plant tissues (Karpova et al. 1997), potentially following TMV MP phosphorylation after passage through plasmodesmata (Karpova et al. 1999) by a cell wall-associated protein kinase (see Sect. 3.4; Citovsky et al. 1993, Lee et al. 2005).

3.2
Subcellular Localization of TMV MP

The cell-to-cell transport of the proposed vRNP is likely to depend on specific mechanisms that target and align the complex to plasmodesmata. Indeed, plasmodesmata in young (sink) leaves are characterized by a large size exclusion limit (SEL) and are able to transport protein macromolecules; yet MPs are required for virus spread (Oparka et al. 1999) suggesting that vRNA movement depends on MP and probably on additional MP-interacting host functions. First attempts to localize TMV MP in infected cells and to identify intercellular targets of the protein employed immuno-electron microscopy (Atkins et al. 1991; Meshi et al. 1992; Moore et al. 1992; Tomenius et al. 1987) and biochemical fractionation using virus-infected tissues and MP-transgenic plants (Deom et al. 1990; Moore et al. 1992; Moser et al. 1988). These studies indicated the presence of TMV MP in cell wall- and plasma membrane-rich fractions as well as in branched plasmodesmata. More recent biochemical analyses suggest that the TMV MP is associated with the microsomal fraction as an integral membrane protein (Reichel and Beachy 1998).

More insight into in vivo associations of TMV MP with host cell structures could be achieved by the analysis of infection sites caused by CP-deficient TMV derivatives expressing the TMV MP as a functional GFP fusion protein (TMV-MP:GFP; Heinlein et al. 1995, 1998; Epel et al. 1996a; Padgett et al. 1996). Infection in leaves of susceptible *Nicotiana* species with TMV-MP:GFP produces radially expanding fluorescent infection sites. The leading edge of these sites reflects the front of the spreading infection, as was shown by experiments involving manual incisions to the leaf lamina. These incisions, if

made just beyond the leading edge of fluorescence, interrupted further spread of infection but allowed further spread if made just behind the leading fluorescent cells (Oparka et al. 1997). These results also indicated that in tissue infected with TMV-MP:GFP visible amounts of MP:GFP or of MP:GFP-vRNA complexes occur only within the limits of infected cells and do not move cell-to-cell far ahead of infection. In contrast, in the absence of infection, when TMV MP was either microinjected (Waigmann and Zambryski 1995; Waigmann et al. 1994) or transiently expressed (Kotlizky et al. 2001), the TMV MP was shown to spread extensively cell-to-cell. This extensive movement underscores the role of TMV MP to modify plasmodesmata and to mediate macromolecular movement between cells. The lack of detection of extensive spread of virus-encoded MP:GFP between cells at the leading front of infection is likely due to the fact that cells at the leading front of infection just undergo the onset of infection during which only a very low amount of TMV MP is present.

The infection sites caused by TMV-MP:GFP appear in the form of fluorescent rings that surround a dark center. The fluorescent rings are continuously growing. Since at early stages of the infection MP:GFP fusion protein has been produced in areas of the ring that are later converted into the dark center, growth as a fluorescent ring indicates a short half life of the MP:GFP. This observation is consistent with fluorimetric measurements using infected protoplasts, which demonstrated that MP:GFP accumulated only transiently during infection, with a peak at about 24 h post-infection (hpi) (Epel et al. 1996a; Padgett et al. 1996). Similar timing of accumulation and subsequent degradation of the protein has also been observed in infected protoplasts using microscopy (Heinlein et al. 1998), immunoblot (Szécsi et al. 1999) or pulse-labeling (Hirashima and Watanabe 2001) analyses. This pattern of a short period of accumulation and subsequent degradation is probably specific for the TMV MP since treatment of virus-infected protoplasts with inhibitors of the 26S proteasome leads to accumulation of ubiquitinylated TMV MP, but not of ubiquitinylated replicase or CP (Reichel and Beachy 2000). Constructs in which the CP was reintroduced (TMV-MP:GFP-CP) were characterized by a drastic reduction in the TMV-MP:GFP expression level and infection sites appeared as faintly fluorescent disks (Heinlein et al. 1998; Szécsi et al. 1999). Since this much lower expression of MP:GFP does not impair the efficiency of vRNA spread, the transient accumulation of high levels of TMV-MP:GFP shown by TMV-MP:GFP and the even higher level of TMV MP accumulation during wild-type TMV infection (Szécsi et al. 1999) is not required for vRNA movement in *Nicotiana* species. Indeed, it was demonstrated earlier that only 2% of the wild-type level of TMV MP expressed during TMV infection is required for movement (Arce-Johnson et al. 1995). The role of TMV MP accumulation is not known. However, it is conceivable that high levels of TMV MP play accessory roles during TMV infection in *Nicotiana* species or in other hosts of TMV.

Since cells at the leading front of a radially expanding infection site represent the earliest stages of infection and since progressively more inner cell layers in the infection site represent progressively later stages of the infection (Heinlein et al. 1998; Oparka et al. 1997), the examination by fluorescence microscopy of cells within infection sites produced by TMV-MP:GFP in *N. benthamiana* leaves reveals a time course of accumulation and localization of TMV MP in infected cells. During early stages of the infection, TMV-MP:GFP accumulates in plasmodesmata and also associates with the ER. Later on, the protein is visualized in association with ER-associated inclusion bodies and microtubules. Finally, detectable MP:GFP fluorescence disappears from all locations except from plasmodesmata (Heinlein et al. 1998). Similar associations were observed in cells infected with the related *Tomato mosaic virus* strain Ob (Heinlein et al. 1995; Padgett et al. 1996). Infection sites of TMV-MP:GFP-CP that produce lower levels of TMV-MP:GFP showed strongly fluorescent plasmodesmata, whereas fluorescence associated with microtubules and bodies was observed with much reduced frequency (Heinlein et al. 1998). Thus, under low expression conditions, MP:GFP still accumulates in plasmodesmata but much less at microtubules and bodies. This implies that the accumulation of TMV-MP:GFP to visible levels in bodies and on microtubules is not required for the spread of infection (Heinlein et al. 1998).

3.3
TMV MP in ER-Derived Inclusion Bodies

The inclusion bodies derived from cortical ER (Heinlein et al. 1998; Mas and Beachy 1999; Reichel and Beachy 1998) likely represent sites of viral replication and protein synthesis, since they contain replicase (Heinlein et al. 1998) and vRNA (Mas and Beachy 1999), and also accumulate CP (Asurmendi et al. 2004). Thus, the inclusion bodies have lately also been referred to as virus-replication complexes (VRCs) (Asurmendi et al. 2004; Kawakami et al. 2004; Liu et al. 2005). Consistent with their membraneous nature, earlier studies have shown that TMV replication complexes, and also *Potato virus X*-replication complexes, co-purify with membrane extracts from infected cells (Doronin and Hemenway 1996; Nilsson-Tillgren et al. 1974; Osman and Buck 1996; Ralph et al. 1971; Watanabe and Okada 1986; Young and Zaitlin 1986; Young et al. 1987). Membranes are also the site of replication of other viruses, such as *Brome mosaic virus* (Restrepo-Hartwig and Ahlquist 1996), *Tobacco etch virus* (Schaad et al. 1997), *Peanut clump virus* (Dunoyer et al. 2002), *Grapevine fanleaf virus* (Ritzenthaler et al. 2002) and poliovirus (Bienz et al. 1994). Association of virus replication with membranes may support the configuration of the replication complex (Osman and Buck 1996, 1997), or may represent a means for compartmentalization, in order to coordinate and regulate efficient virus translation, replication and movement, and also to protect

the virus against the innate defense responses of the host. ER-aggregation leading to the formation of inclusion bodies is likely mediated by TMV MP (Ferralli et al. 2006; Reichel and Beachy 1998) and may imply a role of TMV MP in the enhancement of virus replication. On the other hand, ER aggregation may also be caused by protective defense responses of the plant. Such responses are exemplified by Mx proteins that function as mediators of innate resistance to RNA viruses in animals and humans by trapping and sorting viral components to subcellular locations, where they become unavailable for further virus propagation (Haller and Kochs 2002).

Several lines of evidence suggest that the formation of inclusion bodies from infected ER might be dispensable for replication and movement. For example, bodies do not form in the absence of TMV MP (Mas and Beachy 1999, Reichel and Beachy 1998). Yet, TMV mutants that lack TMV MP replicate normally (Meshi et al. 1987). Moreover, a TMV derivative encoding a mutant but functional TMV-MP:GFP was reported to cause infection in *N. benthamiana* leaves despite the absence of TMV-MP:GFP-containing inclusion bodies (Boyko et al. 2000c). This finding is consistent with the absence of TMV-MP:GFP-containing bodies in most cells infected with TMV-MP:GFP-CP (Heinlein et al. 1998).

3.3.1 Role of ER in TMV Spread

Independent of a potential role of the ER-derived inclusion bodies during infection, the ER network has important functions. In fact, viral RNA of an MP-deficient TMV construct was shown to be localized to ER, suggesting ER association as an intrinsic property of vRNA and/or replicase (Mas and Beachy 1999) and, thus, a critical role of ER in the initiation of cellular infection and virus replication. Recent studies indicate that the viral replicase coding region has a role in cell-to-cell spread of the virus (Hirashima and Watanabe 2001; see Sect. 4.2). One implication of this finding may be that the ER-resident replicase participates in movement by conveying replicated viral genomes to the MP for vRNP complex formation. However, since replicase also acts in the suppression of RNA silencing (Ding et al. 2004; Kubota et al. 2003), its effect on movement may be indirect and be founded on successful counter-defense against defense reactions of the host. Recent studies on initially infected cells of mechanically inoculated leaves suggested that TMV MP-associated inclusion bodies/VRCs may represent the form by which TMV moves in between cells (Kawakami et al. 2004; see Sect. 4.1). However, although the virus may move in association with membranes and replication factors, it remains obscure whether the observed spread of very large TMV-MP:GFP bodies into adjacent cells indeed represented plasmodesmata-mediated movement or rather leakage of cytoplasm, i.e., through gaps in the cell wall caused by mechanical inoculation.

3.3.2
Association of TMV MP with the Cytoskeleton

Although the accumulation of TMV-MP:GFP on microtubules is likely dispensable for infection as is discussed above, several in vivo studies indicated that the association of TMV MP with microtubules is nevertheless strongly correlated with the function of TMV MP in movement (Boyko et al. 2000a,b,c, 2002; Kotlizky et al. 2001). Moreover, using infected protoplasts and a combination of antibody labeling and in situ hybridization procedures, Más and Beachy (1999) showed that vRNA localizes to microtubules in a TMV MP-dependent manner (Mas and Beachy 1999). A subsequent study, again in protoplasts, demonstrated the mislocalization of vRNA in cells expressing a mutant, non-functional TMV MP (TAD5; Kahn et al. 1998; Fig. 1) that binds vRNA but fails to associate with microtubules (Mas and Beachy 2000). Thus, the MP:microtubule complexes observed during later stages of infection may reflect a functional interaction between TMV MP and tubulin during the movement process, even though such complexes are not routinely observed at the front of infection. This may be due to the small amount of TMV MP produced at the infection front, which may not be sufficient to allow visualization of the MP:microtubule interaction. Temperature-sensitive mutations in the TMV MP that simultaneously affect microtubule association and the function of TMV MP in vRNA movement were shown to map to a small domain in TMV MP with structural similarity to the M-loop of α, β, and γ-tubulin (Boyko et al. 2000a; Fig. 1). The tubulin M-loop is essential for the formation and stability of microtubules since it directly contacts the N-loop of tubulin molecules in the adjacent microtubule protofilament (Nogales et al. 1999). The mimicry of the tubulin M-loop may allow direct interaction of TMV MP with free or assembled tubulin of either isoform, including γ-tubulin, and may also pinpoint the TMV MP as a binding target for tubulin cofactors. Interestingly, when TMV MP is expressed in mammalian cells, it not only binds microtubules but also interferes with the recruitment of γ-tubulin to the centrosome (Boyko et al. 2000a; Ferralli et al. 2006). Although plant cells do not concentrate microtubule nucleation events in centrosomes but rather nucleate microtubules at dispersed cortical sites (Murata et al. 2005) and at the nuclear membrane (Schmit 2002; Seltzer et al. 2003), these findings may imply a possible interaction of TMV MP with microtubule organizing complexes or microtubule-organizing sites. In fact, at least in infected protoplasts, the TMV MP localizes to fixed peripheral punctate sites that are aligned to microtubules (Heinlein et al. 1998). Since the pattern of dispersed γ-tubulin sites in plants is also microtubule-aligned (Murata et al. 2005), it will be interesting to see whether or not TMV MP interacts with these γ-tubulin sites or with other markers of microtubule nucleation. An interaction of TMV MP with microtubule-nucleating sites would be reminiscent of the movement process recently described for retroviruses

such as Human-T-lymphotropic virus (HTLV-1), which involves a reorganization of microtubules and the relocation of the microtubule-organizing center to cell–cell contacts leading to the formation of a "virological synapse" (Derse and Heidecker 2003; Igakura et al. 2003). In fact, TMV-MP:GFP fluorescence in TMV-infected cells often concentrates in "paired bodies", i.e., in cell-wall associated localizations within infected cells that are aligned across the cell wall at sites of plasmodesmata (Padgett et al. 1996). It is tempting to speculate that a microtubule reorganizing activity of TMV MP might be involved in forming a virological synapse at the site of plasmodesmata. It should be noted, however, that the region of similarity with the tubulin M-loop overlaps with the region of TMV MP predicted to function as a transmembrane domain (Brill et al. 2000, 2004; Fig. 1). This could indicate that the TMV MP can assume different conformations and that microtubule and membrane association represent alternating events. Since TMV MP potentially forms a dimer (see Sect. 3.1), one could even speculate that one TMV MP subunit interacts with tubulin whereas the other subunit interacts with the ER. This way, TMV MP could form an ER-to-microtubule bridge that could align the ER and microtubules to facilitate microtubule- and TMV MP-mediated transport of vRNA-associated ER membranes (Ferralli et al. 2006). Preliminary observations in leading cells of infection sites suggest an association of TMV-MP:GFP with particles that translocate along cytoskeletal tracks (V. Boyko, A. Sambade, and M. Heinlein, unpublished observations). Whether these particles represent the proposed vRNA-associated ER membranes that are targeted to plasmodesmata and into non-infected cells remains to be shown.

Recent studies involving the treatment of plants with microtubule-disrupting agents led to the conclusion that microtubules are not required for the spread of TMV (Gillespie et al. 2002; Kawakami et al. 2004). However, since these studies did not provide unequivocal evidence that the treatments disrupted all microtubules and all tubulin-based activities, this conclusion is questionable (Seemanpillai et al. 2006). Although plants expressing GFP fused to *Arabidopsis* TUA6 (GFP-*tua*) demonstrate that the GFP-tagged microtubules are absent in drug-treated tissues (Gillespie et al. 2002), microtubules or microtubule fragments made of endogenous tubulin are still present (Seemanpillai et al. 2006). Thus, although an intact microtubule cytoskeleton seems not to be required for vRNA movement by TMV MP, a role of tubulin-based activities in the function of TMV MP cannot be ruled out (Seemanpillai et al. 2006).

The association of TMV MP with microtubules has been observed in protoplasts and mammalian cells transfected with TMV MP-encoding DNA constructs (Boyko et al. 2000a; Ferralli et al. 2006; Heinlein et al. 1998; Kotlizky et al. 2001) and, therefore, can occur independent of virus infection. Whereas in animal cells TMV MP seems to accumulate exclusively at microtubules, the accumulation of TMV MP on microtubules in plants appears

to be regulated and takes place only at certain stages of infection. Thus, it seems likely that specific plant host factors are involved in controlling TMV MP accumulation at microtubules in plants. The binding of TMV MP to microtubules also occurs in vitro (Ashby et al. 2006) and, as described above, in vivo experiments using temperature-sensitive mutants indicated a potential role of tubulin mimicry in this association (Boyko et al. 2000a). The highly fluorescent in vivo complexes that occur during late infection by TMV-MP:GFP appear to be in a non-dynamic state as they resist the treatment with cold, freezing and thawing, as well as with high concentrations of calcium and sodium salts (Boyko et al. 2000a). Similarly, in transfected mammalian cells, the TMV MP:microtubule complex resists treatment with cold as well as with high millimolar concentrations of microtubule-disrupting agents such as colchicine or nocodazole (Ferralli et al. 2006). As mentioned above, the role of these complexes is not known and may be the result of overaccumulation of TMV MP.

In addition to interaction with microtubules, TMV MP was also shown to interact with actin (McLean et al. 1995). This observation has received less attention and hence has not been expanded on. However, the general significance of intact actin filaments for cell-to-cell spread of TMV was addressed experimentally by drug studies (Gillespie et al. 2002; Liu et al. 2005) and actin silencing (Liu et al. 2005), which led to substantially reduced cell-to-cell movement of the virus (see also Sect. 4.2). These studies suggest that actin filaments may be involved in cell-to-cell transport, perhaps by targeting viral replication complexes towards plasmodesmata, as has been proposed in a recent model (Liu et al. 2005).

3.3.3
Is the Accumulation of TMV MP on Microtubules Connected to Degradation?

Since the TMV-MP:microtubule complexes occur before the level of cellular TMV MP diminishes, it has been suggested that the complex may target the TMV MP for degradation (Padgett et al. 1996). During infection the TMV MP occurs in high molecular weight forms, which is consistent with polyubiquitinylation of the protein (Reichel and Beachy 2000). A role of the ubiquitin/26S proteasome (Ub/26S) pathway in the degradation of TMV MP also gained support by the observation that treatments of plants with inhibitors of the 26S proteasome cause changes in the subcellular localization of MP (Gillespie et al. 2002). Recent studies, however, have shown that microtubule-associated TMV MP, in contrast to MP present in whole extract, is not ubiquitinylated. Moreover, treatments of infection sites with microtubule-disrupting agents do not transform the ring-like pattern of TMV-MP:GFP fluorescence of infection sites into a disk-shaped pattern. Thus, although these studies confirm that TMV MP is a substrate for ubiquitinylation and degradation by the 26S proteasome, the microtubules seem not to be involved in this process

(Ashby et al. 2006). However, it was also shown that microtubule-associated TMV MP interferes with motor-driven motility in vitro (Ashby et al. 2006). Therefore, it may be possible that these complexes interfere with the trafficking of signal molecules involved in antiviral plant defense responses, such as the non-cell-autonomous RNA silencing signal (Dunoyer et al. 2005; Heinlein 2005; Himber et al. 2003). In combination with the down-regulation of the SEL of plasmodesmata late in infection (Oparka et al. 1997) this activity may function in protecting the viral RNA in newly infected cells at the leading front of the infection site. The formation of the TMV MP:microtubule complex as well as the down-regulation of plasmodesmal SEL may also function in blocking the backward movement of vRNA into already infected cells, thus assuring that the virus spreads efficiently and unidirectionally into non-infected tissues.

3.3.4
TMV MP Targeting to Plasmodesmata

Another important question to answer is how the MP itself is targeted to plasmodesmata, since this process can be independent of microtubule association (Boyko et al. 2000a; Kahn et al. 1998). The subcellular localization of TMV MP in protoplasts is affected by treatment with the secretory pathway inhibitor Brefeldin A (Heinlein et al. 1998), which suggests a role of the secretory pathway. Only recently, a combination of drug treatments implicated the cortical ER and the actin cytoskeleton in targeting TMV MP to plasmodesmata (Wright et al. 2006). A potential role of secretory vesicles in the cell wall targeting of viral MP in the absence of virus infection has been demonstrated for the MP of tubule-forming *Grapevine fanleaf virus* (GFLV) in BY-2 cells. The protein targets newly formed plasmodesmata during cytokinesis by using a Brefeldin A-sensitive pathway that involves interactions with the t-SNARE syntaxin KNOLLE as well as phragmoplast microtubules (Laporte et al. 2003). Recent studies have demonstrated a role of the secretory actin-ER-driven and endocytic pathways in the plasmodesmal targeting of triple gene block (TGB) movement proteins of Hordei- and Potex-like viruses (Cowan et al. 2002; Haupt et al. 2005; Morozov and Solovyev 2003; Solovyev et al. 2000; Zamyatnin et al. 2004). Although TMV replicates in association with the ER network, a potential role of the secretory pathway in TMV movement has not yet been fully addressed. A role of secretory vesicles in the pathway that targets TMV MP to plasmodesmata may be suggested by results indicating that the TMV MP interacts with PME (Chen et al. 2000; Dorokhov et al. 1999), an enzyme that is targeted to the extracellular cell wall matrix. As suggested for the MP of GFLV (Laporte et al. 2003), the MP of TMV could interact with such secretory cargo to hitch a ride on secretory vesicles in order to reach the cell wall.

3.4 Gating and Cell-to-Cell Transport

Plasmodesmata can dynamically alter their size exclusion limit in response to intrinsic developmental and physiological signals and thus control communication within and between symplastic domains. The seminal discovery that the MP of TMV localizes to plasmodesmata and mediates the cell-to-cell trafficking of TMV RNA provided the first evidence for the existence of proteins able to manipulate plasmodesmata. Direct proof of the TMV MP's ability to "gate" plasmodesmata (i.e., to increase the plasmodesmal SEL) was obtained by a series of microinjection experiments. Purified TMV MP injected into plant cells enabled cell-to-cell trafficking of large fluorescently labeled dextrans that would otherwise have been confined to the microinjected cell (Waigmann et al. 1994). Movement occurred within minutes of microinjection; thus, these experiments allowed a glimpse into the dynamics of interaction between the TMV MP and plasmodesmata. Furthermore, microinjection of fluorescently labeled TMV MP protein provided proof for the TMV MP's ability to move between cells itself (Nguyen et al. 1996). We now know that most, if not all, viruses encode proteins with the ability to interact with plasmodesmal channels. Like the MP of TMV, many other MPs have been shown to alter the conductivity of plasmodesmata and to traffic between cells. Recent studies have shown that MPs share this capacity with endogenous non-cell-autonomous proteins (NCAPs) that are found in the phloem or which function non-cell-autonomously in cell fate determination during plant development (Lucas and Lee 2004). A non-cell-autonomous pathway protein (NCAPP1) was isolated that binds phloem NCAPs and is located to the ER in close proximity to the plasmodesmal orifice. A mutant form of this protein was shown to block the trafficking of NCAPs including MPs. Based on this finding it is speculated that the interaction of MPs and other NCAPs with plasmodesmata involves proteins such as NCAPP1, which assist in the interaction with cellular constituents that lead the way to the channel (Lee et al. 2003; Lucas and Lee 2004). The mechanism by which TMV MP modifies the SEL of plasmodesmata and how the vRNA is transported through the channel is unknown. In vitro association between single stranded nucleic acids and TMV MP result in the formation of elongated and unfolded RNA:protein complexes (see Sect. 3.1), indicating that the movement process may involve the unfolding and linearization of vRNA. There is also evidence that NCAPs must unfold in order to move from cell to cell (Kragler et al. 1998). The fact that MP accumulates in plasmodesmata may suggest that the movement process involves a transport structure formed by MP within the plasmodesmal channel. In fact, TMV forms MP-containing membrane protrusions in infected protoplasts (Heinlein 1998, 2002a), which is very reminiscent of the "tubule-forming" viruses that are known to assemble tubules made of MP within plasmodesmata to allow the cell-to-cell transport of virions (Kasteel

et al. 1997; van Lent et al. 1991; Wellink et al. 1993). However, unlike the MPs of tubule-forming viruses, the MP of TMV has been correlated with the deposition of a MP-associated fibrous substructure in the central cavity of plasmodesmata rather than with the formation of a tubule.

3.5 Host Factors

Plant viruses probably utilize endogenous pathways for their intra- and intercellular transport processes, thereby relying on interaction of viral movement proteins with plant endogenous proteins. TMV MP has been shown to interact with an ever-increasing number of host factors such as actin (McLean et al. 1995) and tubulin (Heinlein et al. 1995; McLean et al. 1995; discussed in Sect. 3.3.3), a cell-wall associated pectin methylesterase, microtubule-associated protein MPB2C, calreticulin, and plasmodesmal associated kinase (PAPK1).

The cell-wall localized enzyme pectin methylesterase (PME; Dorokhov et al. 1999; Chen et al. 2000) was isolated as an interaction partner of TMV MP by a renatured blot overlay assay from tobacco cell wall protein fractions. PME enzymatically affects cell wall porosity, pH, and ion balance (Nairn et al. 1998; Pressey 1984) and may utilize the ER as a transport pathway to the cell wall (Gaffe et al. 1997). By yeast two-hybrid analysis it was shown that TMV MP binds PME with a domain spanning amino acids 130–185 (Fig. 1). TMV encoding a TMV MP mutant lacking this region failed to move cell-to-cell *in planta*, suggesting that interaction of TMV MP with PME is required for viral cell-to-cell movement (Chen et al. 2000). However, deletion of more than 50 amino acids from the core region of the protein may also have caused inactivation by disrupting the overall tertiary structure of the protein. Nevertheless, several mechanisms by which PME may participate in TMV cell-to-cell movement are under discussion (Chen et al. 2000; Waigmann et al. 2004):

1. PME might provide ER localization to TMV MP in trans, thereby mediating its transport along the ER to plasmodesmata
2. PME may act as a cellular receptor for TMV MP, thereby mediating TMV MP localization at the host cell wall
3. TMV MP binding may interfere with PME activity, altering the cell wall ion balance, which could in turn induce changes in plasmodesmal permeability and enable viral cell-to-cell movement

Another TMV MP interacting protein, MPB2C, has been isolated using a membrane-based yeast screening system (Kragler et al. 2003). MPB2C represents a novel plant-specific protein localizing at microtubules in a discrete punctuate pattern that also co-localizes with TMV MP at microtubule-associated sites (Kragler et al. 2003). Transient expression of MPB2C mediated increased accumulation of TMV MP at microtubules coupled to a de-

crease in cell-to-cell transport activity of TMV MP. Also, MPB2C did not interfere with cell-to-cell transport of a movement-enhanced TMV MP mutant, TMV MP^{R3}, reported to show restricted microtubule localization (Gillespie et al. 2002). Collectively, these results suggest that MPB2C is not required for, but plays a negative role in, TMV movement (Kragler et al. 2003). To strengthen this concept, the MPB2C gene was silenced in *Nicotiana* plants (Curin et al. 2006). Indeed, upon MPB2C silencing, cell-to-cell movement of transiently expressed TMV MP and spread of TMV were unimpaired. Strikingly, a nearly complete loss of accumulation of transiently expressed TMV MP on microtubules was observed in silenced plants indicating that MPB2C is involved in accumulating TMV MP at microtubules (Curin et al. 2006). These findings also further support the concept that the accumulation of high levels of TMV MP on microtubules in late stages of infection is dispensable for movement. These findings do not exclude the possibility that microtubules may have a more active role in movement in early infection.

A biochemical approach where TMV MP was used as a specific ligand resulted in identification of calreticulin as a TMV MP interacting host factor (Chen et al. 2005). Calreticulin is a calcium-sequestering ER-resident protein which accumulates in ER-containing plasmodesmata (Chen et al. 2005; see also Sect. 2). In transgenic plants overexpressing calreticulin, TMV spread was substantially reduced. Also, the subcellular distribution of transiently expressed TMV MP was changed, with TMV MP being accumulated at microtubules (Chen et al. 2005). What could be the underlying cause for the changed TMV MP accumulation pattern in calreticulin-overexpressing cells? Plasmodesmata might be overloaded by calreticulin, which prevents TMV MP from reaching its ultimate plasmodesmal destination. Consequently, TMV MP can no longer exit the cell through plasmodesmata, and may therefore accumulate on microtubules, either because they constitute a component of the pathway that targets plasmodesmata or because they represent just another natural cellular interaction structure for the MP. Since the subcellular distribution was addressed in the absence of virus infection, it has not yet been elucidated whether the change in TMV MP subcellular distribution triggered by calreticulin overexpression in the context of transient expression is related to the inhibitory effect of calreticulin overexpression on TMV spread (Chen et al. 2005).

Protein phosphorylation represents a mechanism for regulating protein function. Since phosphorylation of TMV MP has been demonstrated in vivo and in vitro (Citovsky et al. 1993; Haley et al. 1995; Karpova et al. 1999; Waigmann et al. 2000; Watanabe et al. 1992), the quest to reveal the exact function of these phosphorylation events and to isolate the responsible kinase(s) has been ongoing. The best-studied phosphorylation event is phosphorylation of the three Ser/Thr residues at the C-terminus of TMV MP (Citovsky et al. 1993; Trutnyeva et al. 2005; Waigmann et al. 2000; Fig. 1). Since a TMV MP mutant lacking this phosphorylatable region retained movement ability in its

host plants (Berna et al. 1991; Boyko et al. 2000c), C-terminal phosphorylation of TMV MP is obviously not essential for viral spread. Moreover, a TMV phosphorylation mimicking mutant, with all three phosphorylatable residues replaced by negatively charged Asp or Glu, showed reduced viral spread in *N. tabacum* but not in other tested *Nicotiana* species (Trutnyeva et al. 2005; Waigmann et al. 2000). Thus, C-terminal phosphorylation probably represents a mechanism to negatively regulate TMV spread in a host-dependant manner (Waigmann et al. 2000). Interestingly, transient expression studies aimed to clarify the role of each single phosphorylation site for TMV MP movement in *N. tabacum* revealed that phosphorylation on one of the three Ser/Thr residues may even positively affect intercellular movement of the protein, while a negative effect on cell-to-cell transport can only be observed after phosphorylation on two or all three Ser/Thr residues of the protein (Trutnyeva et al. 2005). Potentially, during viral infection, TMV MP phosphorylation might be a sequential event. The virus might use the first phosphorylation event to promote TMV MP transport and thus the transport of its vRNA, while subsequent phosphorylation events inactivating the TMV MP transport function might limit the negative effect of TMV infection for the host plant at later stages (Trutnyeva et al. 2005). A kinase possibly responsible for these phosphorylation events has been recently isolated biochemically using TMV MP as a bait (Lee et al. 2005). This kinase, termed plasmodesmal-associated protein kinase (PAPK), resides at plasmodesmata and specifically phosphorylates TMV MP at its C-terminus in vitro. Further studies utilizing this kinase will likely provide new insights into the role and regulational properties of the C-terminal phosphorylation sites of TMV MP.

4
Role of Coat Protein and Replicase in TMV Cell-to-Cell Transport

Besides the MP, TMV also encodes a coat protein and a replicase that is produced in a long 183 kDa and a short 126 kDa form. While numerous transport studies have focused on TMV MP, the role of the other two viral components in cell-to-cell transport has been more thoroughly investigated only in the last few years, and will be summarized below.

4.1
Coat Protein

Previous observations reported that the presence of the TMV coat protein (CP) is not required for TMV replication or cell-to-cell movement (Siegel et al. 1962; Takamatsu et al. 1987). However, more recent studies suggest a connection between CP and the formation of movement competent complexes involved in spread of TMV. TMV might spread between cells in the form of

VRCs composed of MP, replicase, and genomic viral RNA (Asurmendi et al. 2004; Kawakami et al. 2004). VRCs were observed to rapidly move through the cytoplasm and to associate with plasmodesmata. Since VRC movement was sensitive for actin disrupting agents, the VRCs were proposed to migrate via the actin cytoskeleton towards plasmodesmata, where they move into neighboring cells as viral movement complexes (VMC). CP was speculated to perform a regulatory role in VRC establishment, thereby also influencing the generation of VMCs and hence, cell-to-cell movement (Asurmendi et al. 2004). In addition, studies based on CP^{T42W}, a coat protein mutant that cannot form infectious particles but shows increased subunit interaction, suggest a connection between CP and the amount of MP production (Bendahmane et al. 1997, 2002). Plants expressing CP^{T42W} are more resistant to TMV infections than non-transgenic and wild-type CP-transgenic plants. Resistance is characterized by smaller TMV infection sites and reduced levels of TMV MP. Supporting studies in BY2 cells confirm that CP^{T42W} transgenic BY2 protoplasts infected with TMV accumulate less MP than infected wild-type BY2 protoplasts, whereas CP transgenic BY2 protoplasts produce even more MP. Potentially, CP positively influences the production of MP, perhaps by enhancing the level of subgenomic mRNA encoding the MP (Asurmendi et al. 2004; Bendahmane et al. 2002). On the other hand, when the CP gene was reintroduced into a viral CP-lacking TMV-MP:GFP construct a strong reduction of MP:GFP production was observed in infected cells (Heinlein et al. 1998; Szécsi et al. 1999). Nevertheless, these results suggest that the CP might interface with MP-mediated transport at two levels: by regulating the amount of TMV MP production, and by regulating the establishment of VRCs. Thus, although the CP is dispensable for cell-to-cell movement of the viral RNA, the protein may play a regulatory role in influencing TMV MP expression and activity.

4.2 Replicase

As common for viral RNA replicases, TMV replicase consists of a N-terminal methyl transferase domain, a helicase domain, and an RNA-dependent RNA polymerase domain, the latter of which is only present in the 183 kDa form but not in the shorter 126 kDa form of the protein. The N-terminal methyl transferase domain is separated by a non-conserved region from the helicase domain. The two virus-encoded replicase proteins interact (Goregaoker et al. 2001) and are found in replication complexes isolated from infected plants (Osman and Buck 1996; Watanabe et al. 1999). The 183 kD protein alone is sufficient for replication in protoplasts, although replication efficiency is strongly increased if both replicase proteins are expressed (Ishikawa et al. 1986; Lewandowski and Dawson 2000). A chimeric virus, TMV-hel, consisting of TMV-U1 and the helicase domain of TMV-R, showed defects

in cell-to-cell transport even though genome replication as well as synthesis and accumulation of TMV MP in protoplasts were similar to TMV-U1 (Hirashima and Watanabe 2001). The defect in cell-to-cell movement could not be complemented in transgenic plants expressing TMV MP, but could be complemented when the non-conserved region of the replicase was also encoded by the chimeric virus (Hirashima and Watanabe 2003). Naturally occurring revertants of TMV-hel that regained cell-to-cell movement capacity showed amino acid changes either in the helicase region or in the neighboring non-conserved region of the replicase. Overall, these results indicated an involvement of the replicase in TMV cell-to-cell transport (Hirashima and Watanabe 2001, 2003). Since the movement protein is essential for cell-to-cell transport it is likely that replicase interacts with TMV MP. However, the nature of this interaction remains unknown, as well as the mechanism by which replicase would support cell-to-cell transport.

Another approach to the study of the involvement of replicase in TMV cell-to-cell transport utilizes a defective RNA (dRNA)/helper virus system. In this system, the dRNAs encoded various truncated versions of the replicase whereas the helper virus encoded both the full length 126 kDa and 183 kDa forms of the replicase. For efficient movement of dRNAs, expression *in cis* of the 126 kDa replicase or the N-terminal 258 amino acids of the replicase was required, whereas smaller versions of the replicase were unable to support cell-to-cell movement of the dRNA. This failure could not be complemented *in trans* by the full length replicase proteins produced by the helper virus, suggesting that the nascent dRNA encoded replicase might be required to bind to dRNA *in cis* to facilitate intercellular movement (Knapp et al. 2005).

Yet another line of evidence argues for involvement of the 126-kDa form of the replicase in viral transport. The 126-kDa protein is a component of VRCs, which were reported to align with and traffic along microfilaments (Kawakami et al. 2004; Liu et al. 2005). Based on their observations, Liu et al. (2005) suggested that the 126 kDa replicase not only influences the size of VRCs, potentially via its ability to form oligomers, but also mediates the interaction between VRCs and microfilaments, and movement of VRCs along the microfilament network. Furthermore, disassembly of microfilaments by drugs or an actin-silencing strategy resulted in substantially reduced cell-to-cell movement of the virus. Although the effects of actin disruption can be manifold, these results led to a model that implicated the 126 kDa replicase directly in cell-to-cell movement: the replicase may mediate intracellular transport of VRCs via microfilaments towards plasmodesmata, where the TMV MP may then take over and move the viral RNA through plasmodesmata (Liu et al. 2005).

Overall, we may have to significantly broaden our view on how cell-to-cell movement of TMV RNA is achieved. If VRCs, which physically integrate the viral RNA with the CP, the MP, and the replicase, constitute the actual translocation unit for intercellular transport, either a direct involvement or

a modulating influence of the CP and replicase in cell-to-cell transport is a distinct possibility.

5 Discussion

Within this book chapter, we provide an overview on current knowledge of cell-to-cell movement of TMV genomic RNA. Even though an ever-growing number of host factors and other viral proteins have been found to participate in or modulate the transport process, the key player was and is the movement protein, TMV MP. Over the years, the number of known functions of the TMV MP has been steadily increasing. At present, TMV MP is known to bind to RNA, to localize to the cell wall, to gate plasmodesmata, to bind to microtubules, to interact with the ER (potentially by integrating into the ER membrane), and to interact with several host factors. In many cases, domains or regions within the TMV MP sequence that are required or involved in a particular function have been experimentally defined. Figure 1 presents an overview of the TMV MP regions involved in the various functions. Generally, to render TMV MP fully functional in viral spread, only the carboxyterminal 55 amino acids are dispensable, whereas amino acids 1–213 are required. However, when looking at the molecular requirements for individual TMV MP functions, a more detailed picture emerges. RNA binding is mediated by two independently acting domains, A and B (Citovsky et al. 1992), but only domain A is required in its intact form for viral spread. Gating domain E, which is required to increase plasmodesmal size exclusion limit (Waigmann et al. 1994), overlaps with RNA binding domain A and partially with RNA binding domain B (Fig. 1). This close molecular link between gating and RNA binding might indicate that functional interaction with plasmodesmata is only possible when TMV MP is bound to RNA. It is not known yet which part of the TMV MP confers onto the protein itself the capacity to move between cells. However, a hint may be obtained from the series of microinjection experiments performed to define domain E (Waigmann et al. 1994). In these experiments, TMV MP-mediated movement of fluorescently labeled dextrans between cells was scored. Dextrans were able to spread into numerous cells not directly connected to the microinjected cell, which implied that not only the dextrans, but also the microinjected TMV MP, moved between cells in order to gate plasmodesmata in more distant cells. Therefore, domain E is most likely not only required for gating plasmodesmata but also for mediating cell-to-cell movement of TMV MP itself.

Also overlapping with RNA binding domain A and gating domain E is a region that interacts with host factor PME, a pectin methyl esterase that modulates pH and ion balance and alters cell wall porosity (Chen et al. 2000). Interaction between TMV MP and PME seems to be required for cell-to-cell

spread of TMV. PME might function in transporting the TMV MP towards plasmodesmata, and/or by altering cell wall porosity at the sites of plasmodesmata, thereby inducing changes in plasmodesmal permeability (see Sect. 3.4). In particular, the latter hypothesis is very attractive in the light of the direct overlap between gating domain E and the binding region between TMV MP and PME (Fig. 1), since it could imply that binding of TMV MP to PME is necessary to achieve gating.

Interaction between microtubules and TMV MP has received a lot of attention and was consequently studied in detail. This interaction not only occurs in plant cells but is conserved upon expression of TMV MP in mammalian cells and in vitro, indicating that MP can function as a genuine microtubule-associated protein (MAP) that binds to microtubules through direct interactions (Ashby et al. 2006). The analysis of a series of TMV derivatives encoding progressive aminoterminal and carboxyterminal deletion mutations in TMV MP fused GFP indicated that the interaction of microtubules requires amino acids 1–213 of the TMV MP, i.e.,the same major part of TMV MP that is also required for its function in TMV movement (Boyko et al. 2000c, Fig. 1). The analysis of a series of internal three amino acid deletion mutations indicated that amino acids 49–51 are important for microtubule association and function of the TMV MP (Kahn et al. 1998, Fig. 1). Moreover, deletion of amino acids 3–5 inactivates the protein (Gafny et al. 1992; Lapidot et al. 1993) and causes its constitutive accumulation on microtubules (Kotlizky et al. 2001; Fig. 1). Interestingly, expression of this dysfunctional MP in transgenic *N. benthamiana* plants reduces microtubule association of virus-encoded MP (Kotlizky et al. 2001), a finding that may provide a partial explanation for virus resistance observed in *N. tabacum* plants expressing this dysfunctional protein (Cooper et al. 1995; Lapidot et al. 1993). Amino acid residues Pro 81, Thr 104, and Arg 167 have been genetically shown to functionally interact and to be required for interaction between microtubules and TMV MP (Boyko et al. 2002). Interestingly, amino acid residue Thr 104 was also identified as a phosphorylated residue (Karger et al. 2003; Fig. 1). Phosphorylation at this site is not essential for MP function, since replacement of Thr 104 by the non-phosphorylatable Ala does not affect viral movement. However, substituting Thr 104 with negatively-charged phosphorylation-mimicking amino acid residue Asp strongly inhibits cell-to-cell spread of the mutant virus in *N. tabacum* plants; thus, phosphorylation at Thr 104 may serve as an inactivation mechanism. However, it has not yet been revealed whether phosphorylation at Thr 104 may also influence the MP–microtubular interaction. In vivo assays using TMV derivatives encoding temperature-sensitive MPs (with mutations in amino acid positions 144, 151, and 154 as depicted in Fig. 1) fused to GFP have provided direct correlations between the association of MP:GFP with microtubules and its activity in viral RNA cell-to-cell movement (Boyko et al. 2000a; Boyko and Heinlein, unpublished results). Interestingly, temperature-sensitivity of

all three MP mutants is caused by specific single-amino acid exchange mutations (Ls1: Pro154Ser; Ni2519: Arg144Gly; GV1: Gly151Val) in a domain with structural similarity to the M-loop of α-, β-, and γ-tubulins (Boyko et al. 2000a; Boyko and Heinlein, unpublished results). The M-loop is involved in microtubule assembly and stabilizes the microtubule by forming M-loop/N-loop bridges between adjacent microtubule protofilaments (Nogales et al. 1999). Therefore, it is possible that the TMV MP mimics the M-loop to facilitate direct contacts with tubulin, to interfere with its assembly, or to be by itself recognized as a binding target of tubulin, tubulin cofactors, or microtubule-associated proteins.

Host factor MPB2C is a microtubule-associated protein involved in accumulating TMV MP at microtubules (Kragler et al. 2003; Curin et al. 2006). Interaction between MPB2C and TMV MP is not required for cell-to-cell movement, but exerts a negative effect on TMV MP cell-to-cell transport in a transient expression assay. A TMV MP region stretching from amino acid 58 to the carboxyterminus of the TMV MP is sufficient for binding to MPB2C (Fig. 1; Kragler et al. 2003); however, no minimal interacting region has yet been defined.

To a certain degree, functional studies have been complemented by structural studies even though the success of structural studies has been hampered by the TMV MP's low solubility and tendency to aggregate. Using a combination of various biophysical and biochemical methods, two transmembrane regions have been identified that may mediate TMV MP integration into the ER membrane (Brill et al. 2000). The carboxyterminus of TMV MP probably forms a flexible tail that might be involved in dimerization. Interestingly, the region implicated in interaction with microtubules (amino acids 144–169) overlaps nearly precisely with one of the transmembrane regions spanning from amino acid 150 to amino acid 169 (Fig. 1; see Sect. 3.3.2). Potentially, integration of TMV MP protein into the ER membranes and binding to microtubules are two mutually exclusive events. How the TMV MP might choose between the two localizations is not known yet. However, the influence of host factors such as MPB2C or kinases that could modulate the conformation of the TMV MP is a possibility.

Indeed, the TMV MP is known to be phosphorylated *in planta* at several Ser/Thr sites (Fig. 1). Phosphorylation might provide a regulatory level that allows the TMV MP to engage in its various functions in a coordinated manner even though domains assigned to individual functions show a considerable overlap. With the recent identification of plant kinases that recognize TMV MP as a substrate for phosphorylation (see Sect. 3.4), this important aspect will be revealed in the near future.

The role of microtubules in the TMV transport process has been heavily discussed. So far it remains unclear whether microtubules have indeed a direct causal role in the cell-to-cell movement process, or whether the interaction may occur as a consequence or in parallel to this process. To gain

further insight it would be important to investigate the localization and function of wild-type MP as well as the location of viral RNA in newly infected cells at the front of the spreading infection site. However, such in vivo analysis in newly infected cells is at present hampered by the low level of TMV MP expression as well as by the fact that infection spreads from one cell into the next within only 4 h, which provides a very limited time frame for MP:GFP to accumulate to detectable levels. A new approach to visualize MP:GFP in relation to the movement process has recently been taken by Kawakami et al. (2004) by concentrating their analysis on primary infected cells of mechanically inoculated leaves. Unlike movement between cells at the front of spreading infection sites, movement from primary infected cells into adjacent cells occurs at a rather late stage of virus replication. Thus, movement occurs in the presence of detectable amounts of TMV MP. However, while this system offers the advantage that TMV MP can be visualized, it may be possible that initial virus spread occurs through gaps in the cell wall produced upon mechanical inoculation, rather than through plasmodesmata. Moreover, since only very little TMV MP is required for movement (Arce-Johnson et al. 1995) it may be difficult to dissect the fraction of TMV MP actually involved in the movement process. With these limitations, new insights will have to await the development of novel experimental approaches or more sensitive fluorescence detection techniques.

While microtubules have a potential role during early stages of infection, it seems clear that a fully intact microtubule cytoskeleton is not required for the spread of infection. Several studies have shown that plant leaves treated with microtubule-disrupting agents still allow TMV movement (Ashby et al. 2006; Gillespie et al. 2002; Kawakami et al. 2004). Although the inhibitors do not disrupt all microtubules (Seemanpillai et al. 2006), these studies have shown that movement continues when the microtubular cytoskeleton is greatly affected. In contrast, treatment with drugs that disrupt the actin cytoskeleton led to a reduction in the cell-to-cell spread of TMV infections (Gillespie et al. 2002; Liu et al. 2005) and may point to a major involvement of actin filaments in the movement process, perhaps in addition to a role of microtubules. The ability of TMV to move between cells in which the majority of microtubules are disrupted may reflect the possibility that localized tubulin-dependent processes at plasmodesmata-proximal sites suffice for TMV RNA movement and the spread of infection into adjacent cells. In fact, TMV establishes several ER-associated infection sites in direct vicinity to plasmodesmata (Padgett et al. 1996). Given that:

Both the MP and the viral genome are expressed to high levels during infection (Arce-Johnson et al. 1995; Padgett et al. 1996).

Virus movement requires very few virus particles (Li et al. 2002; Sacristan et al. 2003).

Local events at one of the many plasmodesmata that connect a cell with adjacent cells may suffice for virus movement the inhibition of virus move-

ment may indeed be very difficult to achieve unless a full disruption of the transport mechanism can be established.

Further studies are also required to uncover the role of the accumulation of TMV MP on microtubules late in infection. The finding that microtubule-associated TMV MP is not ubiquitinylated argues against the proposal that the late microtubule complex is involved in proteasome-mediated degradation (Ashby et al. 2006). However, the complex is probably not, at least not directly, involved in TMV movement either, since the complex occurs in cells behind the infection front (Heinlein et al. 1998) and TMV variants that develop the late complex in only few cells can still move cell-to-cell (Gillespie et al. 2002, Heinlein et al. 1998). Also, increased accumulation of TMV MP on microtubules by overexpression of host factor MPB2C resulted in significant reduction of TMV MP cell-to-cell movement (Kragler et al. 2003). Based on results of in vitro experiments it seems possible that the complex interferes with motor-dependent motility along microtubules (Ashby et al. 2006). Blocking the microtubule-dependent pathway may help to ensure that viral movement occurs forward into non-infected cells and not backward into already infected cells. Alternatively, accumulation of TMV MP at microtubules might serve as an effective means to limit the availability of TMV MP for entry into the plasmodesmata trafficking pathway as well as binding to viral RNA. The latter may be particularly important at late stages of infection, where packaging of viral RNA by coat protein into viral particles may be more important than cell-to-cell trafficking, and removal of the competing TMV MP may be desirable (Waigmann et al. 2004). Yet another proposal to be further tested is that microtubule-associated TMV MP is involved in the down-regulation of viral replication and subsequent pathogenesis at late stages of infection. This could be achieved, for example, by the sequestration of host factors to microtubules that are involved in TMV replication and/or translation (Ashby et al. 2006).

Our understanding of the TMV MP-mediated transport process of viral RNA through plasmodesmata has come a long way since its study began more than 20 years ago. Still, the actual mechanism of translocation through the plasmodesmal channel remains obscure. Open questions such as the mechanism of translocation through plasmodesmal channels, or the biological role of microtubular accumulation of TMV MP, will entice researchers to strive to elucidate the remaining mysteries of the multifunctional TMV MP. TMV as a model system will thus remain at the forefront of plant viral research.

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Tubule-Guided Movement of Plant Viruses

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Abstract Plant viruses move from cell to cell through plasmodesmata, which are complex gatable pores in the cell wall. While plasmodesmata normally allow the diffusion of only small molecules, they can be biochemically or structurally modified by virus-encoded movement proteins to enable the passage of either infectious ribonucleoprotein complexes or entire virus particles. In the latter case, the movement protein forms a transport tubule inside the plasmodesmal pore or at the surface of isolated cells. In this review, we describe the functional relevance of the tubules in the transport of viruses, speculative models for this movement mechanism, as well as the host components that seem to contribute to this type of transport.

1 Introduction

Successful propagation of viral infection in host plants comprises distinct and sequential stages: the initial penetration of the virus by mechanical wounding or vector transmission, the replication of the viral genome, and its transport from the initially infected cell into adjacent neighboring cells, a process referred to as local or cell-to-cell movement. Ultimately, propagation to the entire plant results from a chain of events altogether termed systemic movement that consists of viral entry into the vascular tissue, systemic invasion through the phloem stream, and unloading of the virus into non-infected tissues. Within this complex series of events, cell-to-cell movement is certainly one of the most important bottlenecks a virus has to overcome for successful invasion of its host. To do so, viruses exploit plasmodesmata (singular, plasmodesma), microscopic cell wall-embedded channels that provide symplastic continuity throughout most of the plant (for recent reviews Haywood et al. 2002; Heinlein 2002a; Lucas and Lee 2004; Roberts and Oparka 2003; Zambryski and Crawford 2000).

Plasmodesmata can be divided into two major groups (Ehlers and Kollmann 2001; Haywood et al. 2002). The primary plasmodesmata form during cytokinesis, whereas the secondary plasmodesmata develop between cells that are not necessarily clonally related (for recent reviews see Alfonso et al. 2006; Ehlers and Kollmann 2001; Heinlein and Epel 2004; van Bel and van

Kesteren 1999). Although plasmodesmata are subjected to large variations in size, structure, and composition depending on the tissue and the stage of development, they all appear to show a common basic structural architecture consisting of three main elements, the plasma membrane, the cytoplasmic sleeve and the desmotubule, all of which show continuity to the adjoining cells (Ding et al. 1992; Ehlers and Kollmann 2001; Heinlein and Epel 2004; Overall and Blackman 1996, Figs. 1A and 2A; see also Waigmann et al. 2007, in this volume. The plasma membrane inside plasmodesma is continuous with the cellular plasmalemma whereas the cytoplasmic sleeve is enclosed by the plasma membrane and is an extension of the cytosol. The desmotubule is a tightly woven phospholipid bilayer directly connected to the endoplasmic reticulum of each of the adjacent cells, thus forming an endomembrane

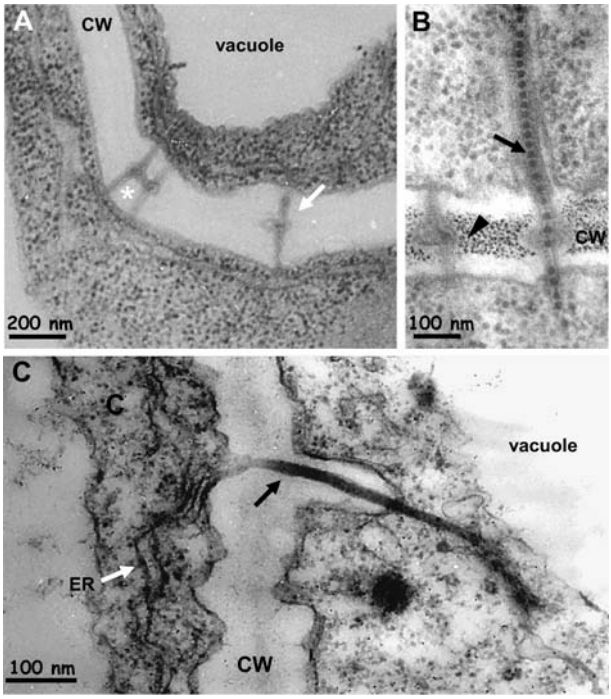

Fig. 1 Transmission electron microscopy images of plasmodesmata in *Chenopodium quinoa* leaf tissue. **A** Transversal section through a leaf showing simple (*white arrow*) and branched plasmodesmata (*) spanning the cell wall. **B** Plasmodesma in GFLV-infected leaf tissue modified by a tubule filled with individually detectable icosahedrical GLFV-virions (*black arrow*). As indicated by the *black arrowhead*, infected tissue also contains non-modified plasmodesmata. **C** Longitudinal section of transgenic tobacco BY-2 cells expressing GFP:MP (GFLV), showing the presence of a tubule (*black arrow*). Thus, tubule formation does not require viral factors other than MP. Since the cells are not infected, the tubule is devoid of any virion particles

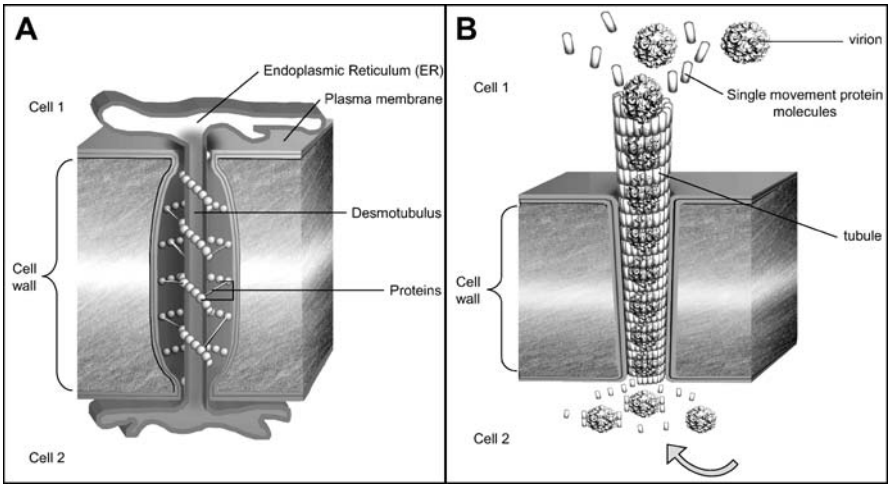

Fig. 2 Schematic representation of a plasmodesma in healthy tissue (**A**) and in tissue infected with a tubule-forming virus (**B**). **A** Non-modified plasmodesma. The plasma membrane and the ER are continuous through the channel. The ER forms a central rod-like structure called the desmotubule. Proteins are embedded in the desmotubule and in the plasma membrane, and also form spoke-like structures connecting both membranes. The cytoplasmic annulus between the ER and the desmotubule likely functions as the major conduit for intercellular communication. **B** MP-modified plasmodesma. The desmotubule is replaced by a tubular structure composed of multiple MP-subunits. The tubulus serves to transport virions into non-infected cells. As indicated by the *grey arrow*, the tubule assembles in the infected cell (Cell 2) and disassembles for the release of the virions in the adjacent, non-infected cell (Cell 1)

continuum. Longitudinal views indicate that plasmodesmata are often narrowed at either end forming a so-called collar, or neck constriction (Olesen and Robards 1990). This constriction is thought to result from the deposition of callose between the plasma membrane and the wall in response to stresses such as plasmolysis or physical wounding (Radford et al. 1998) and may be one point of regulation of molecular flow from cell to cell.

The size exclusion limit of the different types of plasmodesmata can be measured using fluorescent tracer molecules. Initial studies of plasmodesmata gave rise to the idea that only small molecules of less than 1 kDa such as sugar and amino acid can easily pass through plasmodesmata by passive diffusion (Terry and Robards 1987; Tucker 1982). More recent investigations revealed that plants possess a macromolecular trafficking system, facilitating the plasmodesmal movement of endogenous RNA molecules such as silencing RNA molecules as well as proteins such as transcription factors (Heinlein 2002a; Heinlein and Epel 2004; Jorgensen et al. 1998; Lucas et al. 1995; Lucas and Lee 2004; Oparka 2004; Roberts and Oparka 2003). In general, proteins that can move between plant cells have been termed non-

cell autonomous proteins (NCAPs) (Lee et al. 2003). Some proteins, such as green fluorescent protein (GFP) or the transcription factors LEAFY, can pass through plasmodesmata by simple diffusion without requiring a specific interaction with components of the plasmodesmal pore (Oparka et al. 1999; Roberts and Oparka 2003; Wu et al. 2003). However, of the many NCAPs identified to date, most appear to show selective transport through plasmodesmata and also to increase the size exclusion limit of the plasmodesmal pore (Lucas and Lee 2004; Oparka 2004). Similar properties apply also to NCAPs of viral origin termed "movement proteins (MPs)". Originally discovered using temperature-sensitive mutants of *Tobacco mosaic virus* (TMV) (Deom et al. 1987; Meshi et al. 1987), they were given this name because they potentiate the transport of viruses from cell-to-cell (Atabekov and Dorokhov 1984).

The pioneering work that led to the discovery of the 30K MP of TMV gave rise to similar investigations on other plant viruses. It soon became evident that MPs are a general feature of plant viral genomes (Carrington et al. 1996; Lucas and Gilbertson 1994; Maule 1991). Based on their primary structure, viral MPs have been divided into at least four superfamilies, the largest of which being the "30K" superfamily, named after the 30K MP of TMV (Koonin and Dolja 1993; Koonin et al. 1991; Melcher 2000; Mushegian and Koonin 1993). Although structurally related, cell-to-cell movement of viruses belonging to the 30K superfamily can be divided into two main categories that are exemplified by TMV and *Cowpea mosaic virus* (CPMV, genus *Comovirus*). Thus, TMV MP alters the size exclusion limit of plasmodesmata without inducing obvious ultrastructural changes and mediates cell-to-cell transport of a complex of viral RNA and MP by a mechanism that does not involve the viral coat protein (CP) (see Waigmann et al. 2007, in this volume). This mechanism is probably closest to the one that regulates the trafficking of cellular NCAPs that has been extensively reviewed recently (Heinlein and Epel 2004; Lucas 2006; Waigmann et al. 2004). On the other hand, MPs from CPMV and other closely related virus such as *Grapevine fanleaf virus* (GFLV) target plasmodesmata to form tubular structures or nanotubules through which virions are transported from cell to cell (Fig. 1B; for recent review van Lent and Schmitt-Keichinger 2006). A third intermediate category is formed by viruses belonging to the family *Bromoviridae* whose MP assembles into tubules to promote the movement of CP/RNA ribonucleoprotein complexes rather than entire virus particles (Palukaitis and Garcia-Arenal 2003; Sánchez-Navarro and Bol 2001; Sánchez-Navarro et al. 2006). In this chapter, priority will be given to the tubule-guided movement of virions and how this process may relate to other types of mechanisms of cell-to-cell movement.

2
Tubule Formation, an Intrinsic Property of Some Movement Proteins within the 30K Superfamily

First evidence of ultrastructural modifications of plasmodesmata during viral infection was provided by Esau, who detected virus-like particles located within both modified plasmodesmata and mature, functional, sieve elements (Esau 1968). Similar tubule-like structures were subsequently observed with numerous plant viruses belonging to different families and genera with either RNA or DNA genomes. Examples are found predominantly within the *Comoviridae*, *Bromoviridae* and *Caulimoviridae* (Table 1). Based on these collective studies, the concept emerged that some plant viruses move their genomic material between cells in an encapsidated form through specialized structures termed tubules or nanotubules (Fig. 2B).

The tubule-forming capacity is an intrinsic property of some MPs belonging to the 30K superfamily. Evidence was first provided using CPMV. Early electron microscopy studies of plant cells infected with CPMV revealed the presence of long tubular structures that often extended from the entry of plasmodesmata in one cell into the cytoplasm of a neighboring cell and that contained a single row of virus-like particles (van der Scheer and Groenewegen 1971). Genetic data later revealed that both the coat proteins and the RNA2-encoded 48K protein are essential for cell-to-cell movement of CPMV (Wellink and van Kammen 1989). Immunogold labeling with an antibody against the 48K protein showed that this protein is part of the tubules (van Lent et al. 1990), suggesting that it probably functions as the MP of CPMV by building tubules through plasmodesmata for the transport of virus particles. Further studies revealed that plasmodesmata are not required for tubule assembly, since their formation was also observed in protoplasts, isolated plant cells deprived of their cell wall and, consequently, lacking plasmodesmata. Thus, in CPMV-infected cowpea protoplasts, plasma-membrane lined virus-containing tubules protrude up to tens of micrometers from the cell surface into the medium (van Lent et al. 1991). Once it was established that tubules could not only form in plant tissues but also in protoplasts, it became much easier to identify the viral determinants required for tubule formation and virus movement. Thus, CPMV capsid proteins were shown to have no role in the morphogenesis of the tubules, since a mutant virus which failed to produce the capsid proteins was still capable of inducing tubules that appeared identical to those formed upon infection apart from the presence of virions (Kasteel et al. 1993). On the other hand, virus mutants that fail to produce the 48K protein or that produced a truncated 48K protein were no longer able to induce tubules, suggesting that at least the 48K protein is essential for tubule formation (Kasteel et al. 1993). Final demonstration that the 48K MP is the only viral protein needed for tubules formation was provided by using a 35S promoter-driven 48K expression system in protoplasts (Wellink et al. 1993).

Table 1 Examples of plant viruses whose MPs belong to the 30K superfamily and assemble into tubule-like structures. Plant viruses are taxonomically grouped according to virus family and genus. References of publications demonstrating tubule-formation by their MPs are shown

Family	Genus	Virus	Refs.
Comoviridae	*Comovirus*	*Cowpea mosaic virus* (CPMV)	van der Scheer and Groenewegen 1971; (van Lent et al. 1990)
		Bean pod mottle virus (BPMV)	Kim and Fulton 1971
	Nepovirus	*Grapevine fanleaf virus* (GFLV)	Kalasjan et al. 1979; Ritzenthaler et al. 1995; Stussi-Garaud et al. 1994
		Arabis mosaic virus (ArMV)	Stussi-Garaud et al. 1994
		Tomato ringspot virus (TomRSV)	Wieczorek and Sanfaçon 1993
Bromoviridae	*Oleavirus*	*Olive latent virus 2* (OLV2)	Castellano 1987; Grieco et al. 1999
	Alfamovirus	*Alfalfa mosaic virus* (AMV)	Godefroy-Colburn et al. 1991; van der Wel et al. 1998
	Ilarvirus	*Tobacco streak virus* (TSV)	Martelli and Russo 1985
	Cucumovirus	*Tomato aspermy virus* (TAV)	Francki et al. 1985
Caulimoviridae	*Caulimovirus*	*Cauliflower mosaic virus* (CaMV)	Kitajima and Lauritis 1969; Linstead et al. 1988
		Dahlia mosaic virus (DMV)	Kitajima et al. 1969
	Badnavirus	*Commelina yellow mottle virus* (ComYMV)	Cheng et al. 1998
Sequiviridae	*Sequivirus*	*Parsnip yellow fleck virus* (PYFV)	Cheng et al. 1998
Geminiviridae	*Begomovirus*	*Euphorbia mosaic virus* (EuMV)	Kim and Lee 1992
Bunyaviridae	*Tospovirus*	*Tomato spotted wilt virus* (TSWV)	Kormelink et al. 1994

The above-mentioned pioneering work on CPMV performed in the groups of van Kammen and Goldbach paved the way to the discovery of numerous other MPs with tubule forming capacity. In agreement with the predicted function of tubules in virus movement, all the MP of the aforementioned tubule-forming viruses, when tested, assembled into tubules upon expression in protoplasts, including the MPs of GFLV (Ritzenthaler et al. 1995) (Fig. 3A,B), *Olive latent virus 2* (OLV2) (Grieco et al. 1999), *Cauliflower mo-*

saic virus (CaMV) (Perbal et al. 1993), *Tomato spotted wilt virus* (TSWV) (Storms et al. 1995) and *Alfalfa mosaic virus* (AMV) (Kasteel et al. 1997; Zheng et al. 1997). These studies revealed that the MP is the only viral requirement for tubule assembly. Amazingly, a number of these proteins, when tested, maintained their capacity to form tubules when expressed in insect cells (Kasteel et al. 1996; Storms et al. 1995). In all respects, tubules formed in insect and plant cells appear to be similar. Remarkably, the infectivity data of scanning deletion mutants that revealed the existence of a large C-terminal domain necessary for tubule formation (Thomas and Maule 1995a) largely mirrored those obtained with MP mutants expressed in insect cells (Thomas and Maule 1999) emphasizing the importance of tubule formation in aiding virus movement. Thus, Maule and colleagues nicely established that the majority of the CaMV MP (aa 1 to 282) is required for tubule formation, whereas the C-terminus could project into the lumen of the tubule to interact with the virions (Thomas and Maule 1995a, 1999).

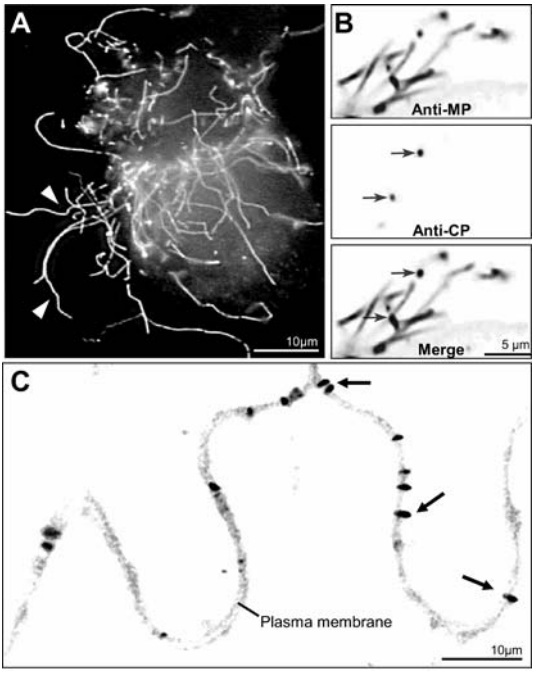

Fig. 3 Localization of GFLV MP and coat protein **A** GFLV-infected BY-2 protoplast stained with anti-MP(GFLV)-antibody. The antibody stained tubules (*white arrowheads*) protruding from the cell surface. **B** Surface of GFLV-infected BY-2 protoplast stained with anti-MP-antibody (*top panel*: anti-MP) and anti-coat protein antibody (*middle panel*: anti-CP). The merged image (*bottom panel*: Merge) reveals detection of the viral coat protein at the tubule extremities. **C** *N. benthamiana* epidermal cells expressing an MP : RFP fusion protein. Red fluorescent tubules (*arrows*) are present in the epidermal cell wall

Rather unexpectedly, a number of other MPs within the 30K family were able to produce tubules at the surface of protoplasts, although tubules have never been found in infected tissues. This includes members of the *Bromoviridae* family such as *Cucumber mosaic virus* (CMV) and *Brome mosaic virus* (BMV) (Canto and Palukaitis 1999; Kasteel et al. 1997) and also the flexuous viruses *Apple chlorotic leafspot trichovirus* (ACLSV) (Satoh et al. 2000), *Grapevine berry inner necrosis virus* (GINV) (Isogai et al. 2003) and *Apple stem grooving capillovirus* (ASGV) (Isogai et al. 2003). The specific case of these viruses that require the CP for movement and show a discrepancy between plants (no tubules) and protoplasts (tubules) will be discussed more specifically below. More intriguing is the tubule-forming capacity of the MP of TMV in infected protoplasts (Heinlein et al. 1998a; Mas and Beachy 1998). Tubules were never found in plasmodesmata of TMV-infected plants. Moreover, TMV is the type member of the viruses that are thought to move as ribonucleoprotein complexes without the functional requirement of the CP (Dawson et al. 1988; Knapp et al. 2001). In transgenic plants, TMV MP is found as fibrillar material in secondary Pd cavities (Ding et al. 1992), while upon expression in multicellular cyanobacteria this MP forms tubular arrangements of fibrils that traverse the septum between cells (Heinlein 2006; Heinlein et al. 1998b). Fibrillar material has also been reported to be present in plasmodesmal pores modified by the MP of TSWV (van Lent and Schmitt-Keichinger 2006), suggesting that TSWV could move in a similar manner as TMV. In agreement with this view, Storms and co-workers established that the MP of TSWV (NSm) had similar effects on plasmodesmal gating as TMV (Storms et al. 1998). A similar mode of action may also be indicated by the finding that the NSm protein can complement movement-defective TMV (Lewandowski and Adkins 2005). While this also demonstrates that virion formation is not required for NSm to traffic TMV RNA (Lewandowski and Adkins 2005), previous experiments suggested that nucleoprotein complexes of TSWV viral RNA and N protein move through tubules, as deduced from the interaction between the N and NSm proteins and their co-localization within tubules (Soellick et al. 2000; Storms et al. 1995). More importantly, this finding questions the requirement of N protein for the movement of TSWV RNAs and, more generally, the relevance of tubules in virus movement. One possible explanation for these observed discrepancies could be that some viruses use two alternative movement strategies to establish systemic infection: movement as a viral RNA-MP complex, as exemplified by TMV (see Waigmann et al. 2007, in this volume), or alternatively, movement as complete virions by a tubule-guided mechanism as exemplified by CaMV and BMV (Jansen et al. 1998; Thomas and Maule 1995b). Such explanation could also account for the fact that the MPs of a number of tubule-forming and virion-transporting viruses like CPMV (Carvalho et al. 2004), CaMV (Citovsky et al. 1991), AMV (Schoumacher et al. 1992a,b) BMV (Jansen et al. 1998) and TSWV (Soellick et al. 2000) are

able to bind nucleic acids in a sequence non-specific manner in vitro or to fix GTP (Carvalho et al. 2004; Li and Palukaitis 1996) like TMV (Citovsky et al. 1990; Li and Palukaitis 1996). These common biochemical properties may reflect structural similarities between members of the 30K superfamily as already suggested by Melcher (2000). However, despite the recent attempts at resolving the three-dimensional structure of TMV MP (Brill et al. 2000, 2004), the first crystal structure of a MP within the 30K superfamily is still being awaited.

3
Tubules in the Transport of *Bromoviridae*

In the family *Bromoviridae*, studies on cell-to-cell movement have mainly been focused on AMV, BMV, CMV and *Prunus necrotic ringspot virus* (PNRSV, genus *Ilarvirus*). For BMV, CP is required for cell-to-cell and long-distance movement (Rao and Grantham 1995, 1996; Schmitz and Rao 1996), although in some hosts, limited cell-to-cell movement occurs if high concentrations of inoculum are applied (Flasinski et al. 1995). Despite the fact that tubules have never been detected in infected tissues, the presence of tubules containing BMV particles extending from transfected protoplasts (Kasteel et al. 1997) together with results from mutational analyses (Okinaka et al. 2001; Schmitz and Rao 1996) support the notion that BMV moves cell-to-cell in the form of virions. For CMV, all five of the proteins encoded by the three genomic RNAs affect the movement of the virus (Palukaitis and Garcia-Arenal 2003). However, the 3a protein encoded by CMV RNA 3 is considered to be the primary movement protein (Canto et al. 1997; Kaplan et al. 1995; Nagano et al. 1997) and the CP has been proven to be required for cell-to-cell movement (Suzuki et al. 1991), even in the epidermis of inoculated leaves (Canto et al. 1997). However, the ability to form virions is not a prerequisite for cell-to-cell movement (Kaplan et al. 1998). In addition, the capacity of the 3a MP of CMV to bind RNA as initially demonstrated by Li and Palukaitis (1996) appears to be essential to promote virus movement (Palukaitis and Garcia-Arenal 2003). Thus, despite the ability of the MP to generate tubules on the surface of protoplasts (Canto and Palukaitis 1999), it is assumed that CMV moves as a ribonucleoprotein complex (Palukaitis and Garcia-Arenal 2003).

The transport mechanism of AMV, which requires RNA3-encoded proteins MP and CP, has been reported to share characteristics with those of both TMV and CPMV (Kasteel et al. 1997; Sánchez-Navarro and Bol 2001). Thus, while CP mutants defective in virion assembly are still able to move cell-to-cell (Sánchez-Navarro and Bol 2001), virus particles are clearly detected within tubules protruding from wild-type AMV-infected protoplasts (Kasteel et al. 1997). Also, the capacity of the MP to promote cell-to-cell and

systemic movement of the virus in plants was shown to be strictly correlated with the ability of the MP to assemble into tubules (Sánchez-Navarro and Bol 2001). More precisely, the 44 C-terminal amino acids of the MP were shown by mutational analyses to be dispensable for tubule assembly and cell-to-cell movement of AMV. Nevertheless, this C-terminal domain is still able to confer specificity to the transport process, as it allowed BMV MP to promote AMV movement upon replacement of the C-terminal domain of the MP of BMV by that of AMV MP (Sánchez-Navarro and Bol 2001). To gain further insight in such transport specificity, additional chimeric RNA 3 mutants with the AMV MP gene replaced by the corresponding MP gene of PNRSV, BMV, CMV, TMV or CPMV were recently tested (Sanchez-Navarro et al. 2006). It appeared from this survey that all RNA 3 hybrids carrying the extended C-terminal 44 aa of AMV MP were functional and that this region is able to interact specifically with AMV virus particles in vitro. Remarkably, the replacement of the CP gene in RNA 3 by a mutant gene encoding a CP defective in virion formation did not affect cell-to-cell transport of the chimeras with a functional MP, thereby clearly demonstrating that virus particles are not required for the cell-to-cell movement mediated by the MP of either AMV, BMV, CPMV or TMV. The most likely explanation for this phenomenon would be that the two mechanisms described in the 30K superfamily could represent two variants of the same viral transport system, where the C-terminus of the MP could be adapted to recognize the cognate CP. It is likely that the same rule applies also to non-*Bromoviridae* such as CPMV, GFLV and CaMV. Strikingly, the C-terminal part of the MP of all these viruses is involved in coat protein recognition and binding. For CPMV it was shown that the MP C-terminus is located on the inside of the tubule (van Lent et al. 1991), thus in close proximity to the virus particles. Incorporation of virions into the tubule was disturbed with a C-terminal deletion mutant of the MP, giving rise to "empty" tubules (Lekkerkerker et al. 1996), i.e., tubules without virus particles. Furthermore, Carvalho et al. (2003) showed specificity of MP binding to CPMV virions, but not to capsids of BMV, TMV or of the related Comoviruses *Cowpea severe mosaic virus* (CPSMV) and *Red clover mottle virus* (RCMV) (Carvalho et al. 2003). Moreover, in blot overlay assays the MP specifically bound to only one, the large, of the two CPMV coat proteins. GFLV movement is likely also to be governed by a specific interaction between tubule and virions, as suggested by the results obtained with chimeric constructs between GFLV and the closely related *Arabis mosaic virus* (ArMV). Virus spread only occurred when the 9 C-terminal residues of the MP were of the same viral origin as the coat protein (Belin et al. 1999). As already mentioned, in the case of CaMV, a C-terminal mutant MP was identified that kept its ability to form tubules, but was unable to support virus movement, suggesting that the ten last C-terminal amino acids of the MP are involved in interactions with the virus particles (Thomas and Maule 1995a).

4
Intracellular Trafficking Pathways and Mechanisms of Tubule Assembly

With the new possibilities offered by the green fluorescent protein (GFP) and its variants to investigate protein trafficking in living cells, attention has turned to the question of how intracellular transport and targeting of MP to plasmodesmata may occur. Viruses that infect plants have developed a variety of strategies to move from cell to cell and are heavily dependent on endogenous host transport systems during movement, as with regard to all other aspects of their life cycles. Pioneering work with TMV MP has demonstrated a close association of the MP with multiple host components. Several models for the movement of TMV have been proposed by which the viral RNA together with the MP could be transported to plasmodesmata in association with the endoplasmic reticulum, microtubules and microfilaments (for recent reviews see Boevink and Oparka 2005; Heinlein 2002b; Heinlein and Epel 2004; Lucas 2006). However, the precise involvement of the cytoskeleton and endomembrane system in the spread of TMV infection remains a subject of intense studies (see Waigmann et al. 2007, in this volume). Even the unlikely hypothesis that cytoplasmic streaming could support the rapid diffusion of infectious TMV ribonucleoprotein complexes to plasmodesmata cannot be ruled out under present circumstances (Boevink and Oparka 2005). Concerning the smaller family of viruses that employ the tubule-guided movement, the analysis of their trafficking mechanisms has focused essentially around CPMV, GFLV, CaMV and AMV.

The use of cytoskeletal inhibitors like Latrunculin B (inhibits the assembly of actin filaments) and Oryzalin (inhibits the assembly of microtubules) as well as inhibitor of secretion like Brefeldin A (BFA), (Nebenführ et al. 2002; Ritzenthaler et al. 2002) have been particularly helpful to unravel the targeting mechanisms of several tubule-forming MPs. Thus, it was shown in protoplasts transiently expressing a MP-GFP fusion of CPMV and CaMV, that neither a functional secretory pathway nor an intact cytoskeleton is required for MP targeting to the plasma membrane (Huang et al. 2000; Pouwels et al. 2002). However, BFA severely inhibited tubule formation, suggesting that vesicle transport is needed for tubule formation or more likely that BFA interfered with the targeting of an essential host protein to the plasma membrane (Huang et al. 2000; Pouwels et al. 2002). For AMV, it was also shown that MP transport to the cell wall and tubule assembly do not rely on an intact cytoskeleton (Huang et al. 2001a), but data on the role of the endomembrane system are not yet available. Support for a stepwise process in CPMV movement was provided by additional mutational analyses of the MP (Carvalho et al. 2004; Pouwels et al. 2003). It is suggested that the MP would first diffuse from the place of synthesis to the plasma membrane as a dimer where it then accumulates in punctuate structures before assembling into tubules, possibly in a similar manner to microtubules as suggested from the

GTP-binding capacity of the MP (Carvalho et al. 2004; Pouwels et al. 2003, 2004). During this assembly process, virions are specifically included within tubules (Fig. 4). It is not known yet whether MP and virions are cotransported along the same pathway or whether they use different routes that converge at the entry of tubules. However, it has been proposed that in the adjacent cell, the tubule destabilizes, thereby releasing the virions for further infection (Pouwels et al. 2003). A similar process may also apply to the intra- and intercellular movement of GFLV as demonstrated using GFP:MP fusion protein in tobacco epidermal or BY-2 cells (Figs. 1C and 3C), although in contrast to CPMV, CaMV, and AMV, a role for microtubules and secretion in the assembly of tubules is established (Laporte et al. 2003). The use of a polarized system (stably transformed tobacco BY-2 cells) for GFLV analyses versus the analysis of MP expression in protoplasts for CaMV, CPMV and AMV, could well account for some of the observed discrepancies. Indeed, for GFLV, treatment with Oryzalin alone or together with Latrunculin B did not abolish tubule formation within cross walls of BY-2 cells, but resulted in their addi-

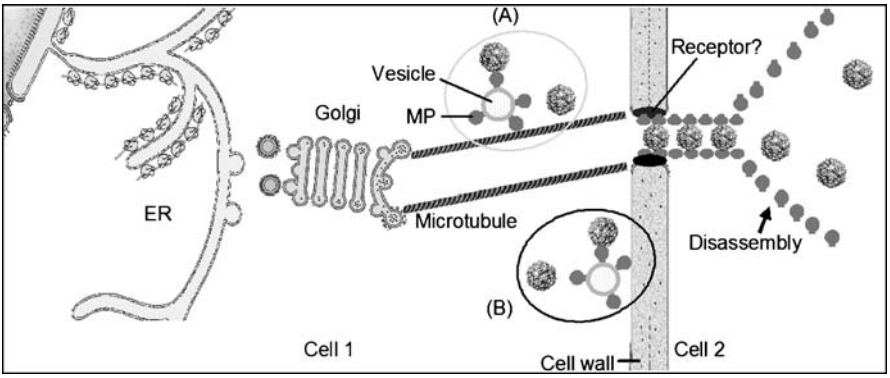

Fig. 4 Schematic representation of the intracellular trafficking and cell-to-cell movement steps during infection with tubule-forming viruses. After penetration and decapsidation of virion particles within the initially infected cell (Cell 1), the viral genome is translated and replication starts. Following virion assembly, virions are transported to plasmodesmata. It remains to be determined whether virions are co-transported with MP. Two basic mechanisms of MP-targeting to plasmodesmata have been described for different viruses. One transport model (*A – grey circle*), which is exemplified by GFLV, suggests that MP is transported on Golgi-derived secretory vesicles along microtubules (Laporte et al. 2003). The other mechanism (*B – black circle*) applies, for example, to CPMV and involves the transport of MP first to the plasma membrane and then to plasmodesmata (Carvalho et al. 2004; Pouwels et al. 2003, 2004). It remains to be determined whether a specific receptor for MP is implied in the mentioned mechanisms and where it is located. Once at the cell periphery at sites probably related to plasmodesmata, MP self-assembles into tubules by which viral particles move from cell to cell. The release of virions in the non-infected cell may be mediated by tubule disassembly (Cell 2). Elements of the figure are not drawn to scale

tional assembly at ectopic sites (Laporte et al. 2003), a phenomenon that may not be visible when working with protoplasts due to changes in the tubulin cytoskeleton upon protoplast preparation (Tylicki et al. 2003). Concerning the involvement of the secretory pathway in GFLV tubule formation, BFA treatment did not abolish tubule formation but severely decreased their numbers (Laporte et al. 2003), as also observed for the tubules formed by CPMV and CaMV MPs (Huang et al. 2000; Pouwels et al. 2002). However, contrarily to CPMV, GFLV MP was strongly redistributed to the cytoplasm upon BFA treatment, suggesting that the MP could traffic along the secretory pathway in a manner similar to membrane-bound protein cargo (Fig. 4). This hypothesis is further supported by the intrinsic membrane properties of the GFLV MP and its ability to physically interact with the cytokinesis-specific syntaxin KNOLLE (Heese et al. 2001; Laporte et al. 2003; Lauber et al. 1997). A way to address this issue more precisely than simply by using BFA, which has multiple cellular targets (Nebenführ et al. 2002), would be to use specific inhibitors of secretion such as dominant negative mutants of the Arf1 and Sar1 GTPases involved in the COPI and COPII vesicular trafficking pathways, respectively (daSilva et al. 2004; Takeuchi et al. 2000, 2002; Xu and Scheres 2005; Yang et al. 2005). In addition, analysis of the interactions between viral MPs and host factors should provide further insight into the movement process. For TMV several MP-interacting host proteins have been identified, such as tubulin (Ashby et al. 2006; Heinlein et al. 1995), actin (McLean et al. 1995), pectin methylesterase (Chen et al. 2000; Dorokhov et al. 1999), KELP, a putative transcriptional co-activator that modulates host gene expression during pathogenesis, (Matsushita et al. 2001), calreticulin (Chen et al. 2005) or the microtubule-associated protein MPB2C (Kragler et al. 2003) (for recent reviews see Boevink and Oparka 2005; Lucas 2006; Oparka 2004; Waigmann et al. 2004) (also see Waigmann et al. 2007, in this volume).

For tubule-forming viruses also, host factors with affinity for the MP have been found. In a yeast two-hybrid screening with the MP of TSWV, Soellink and coworkers found interactions with DnaJ-like chaperones (Soellick et al. 2000). These proteins have functions including protein transport in organelles and the regulation of the chaperone heat-shock protein Hsp70. Remarkably, the latter protein is also involved in the translocation of Closteroviruses (Alzhanova et al. 2001). In addition, TSWV MP was shown to bind proteins with homologies to myosin and kinesin, suggesting an involvement of molecular chaperones in the attachment of TSWV nucleocapsids to the cytoskeleton for subsequent intracellular trafficking (von Bargen et al. 2001). A yeast two-hybrid screen led to the identification of a vesicular-associated membrane protein (VAMP) termed MPI7 that binds CaMV (Huang et al. 2001b). In sequence, MPI7 is related to mammalian Rab acceptor proteins (PRA1), a family of proteins binding Rab GTPases and vSNARE, components implicated in the regulation and proper delivery of transport vesicles (Nebenführ 2002). The protein was localized to punctuate spots at the cell periph-

ery, probably representing plasmodesmata, and in vivo association between the MP and MPI7 was confirmed by fluorescence resonance energy transfer (FRET) (Huang et al. 2001b). Using a GST-pull down approach, CaMV MP was also recently shown to bind the virion-associated protein (VAP) through a C-terminal coiled-coil domain (Stavolone et al. 2005). Immunogold electron microscopy revealed that the VAP and viral movement protein colocalize on CaMV particles within plasmodesmata (Stavolone et al. 2005). Thus, although not proven, the CaMV MP together with VAP and possibly virions might interact to transport vesicles via MPI7 during their delivery to plasmodesmata, in a similar manner to GFLV (Fig. 4). The same transport mechanism could also apply to CPMV, as it was shown that its MP binds GTP and that this binding is required for MP targeting and tubule formation (Carvalho et al. 2004). Although no GTPase activity could be demonstrated for the MP, the GTP-binding activity may become significant if the "grab a Rab" model proposed by Oparka for selective transport of MP to the plasmodesmata is considered (Oparka 2004). Rab GTPases, which play a role in specificity of vesicle transport (Nebenführ 2002; Rutherford and Moore 2002), could carry the MP together with a cargo vesicle to the plasma membrane and at the same time, by GTP hydrolysis, could provide the molecular switch to start MP polymerization. At the plasmodesmata, specific interactions between v-SNARE (soluble N-ethylmaleimide-sensitive factor adaptor protein receptors) and t-SNARE complexes then make the vesicles fuse with the plasma membrane. The vesicles could even transport necessary enzymes for cell wall degradation to enlarge the plasmodesmatal channel or to form secondary channels for virus transport.

Better understanding of the vesicular transport pathways involved in secretion and endocytosis as well as of cytoskeleton-driven transport mechanisms should soon provide new ideas and details about the molecular mechanisms and routes employed by MP and virions for their delivery to the plasma membrane and plasmodesmata. Similarly, resolving the *modus operandi* of plasmodesmatal cell-to-cell transport through the identification of novel plasmodesmal proteins is currently under progress. Altogether, these approaches will converge to provide new hints to the manner by which viruses hijack and modify these pores.

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Spread Throughout the Plant: Systemic Transport of Viruses

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Abstract Viral long distant transport is an essential step for systemic infection. Because the process involves different types of highly differentiated vascular-associated cells, the virus systemic movement is regulated differentially at each tissue interface. In this chapter, we review current knowledge about viral systemic transport process in non-Arabidopsis hosts. We especially focus on viral and host factors participating in viral systemic transport. We also briefly overview the effect of RNA silencing, the host innate immunity, on viral systemic movement.

1 Introduction

After replication and accumulation at the local infection sites, viruses have to travel to uninfected, systemic tissues via the vasculature in order to establish systemic infection. This process, which involves entering into, traveling through, and exiting from the vasculature into uninfected tissue, is collectively termed "systemic movement". Systemic movement is not just an aggregation of numerous cell-to-cell movement processes as occurs in local infection; rather, cell-to-cell and systemic movement are two different modes of viral translocation. During systemic movement, the virus crosses several different types of cells, including mesophyll (MS), bundle sheath (BS), vascular parenchyma (VP), and companion cells (CC), as well as sieve elements (SE) of the vascular system. The involvement of so many different types of tissues and cells in systemic movement stands in stark contrast to local movement, which occurs between only relatively few tissues, such as MS and epidermis, or within uniform population of the cells of the same tissue. Due to the involvement of a number of different cells, systemic movement is expected to be more complex than local movement at the molecular level. Moreover, the rates of the two types of movement are quite different. Generally, local movement is a relatively slow process (e.g., 5–15 μm/h, see Gibbs 1976), presumably restricted by the rate of viral replication. In contrast, long-distance movement through the vascular system is rather rapid (e.g., 50–80 mm/h (see Gibbs 1976), as it occurs with the flow of photoassimilates and, in some

Table 1 Virus–host combinations that develop limited virus systemic movement[a]

Virus	Host	Inoculated leaves				Systemic leaves				Refs.
		ME	BS	CC	SE	SE	CC	BS	ME	
CCMV	Resistant soybean strains	⇒|								Goodrick et al. 1991
CMV	Transgenic plants expressing CMV replicase	⇒	⇒|							Wintermantel et al. 1997
TAV Florida strain	Cucumber	⇒	⇒|							Thompson et al. 1998
CP-less TMV	*N. tabacum*	⇒	⇒|							Ding et al. 1996
RCNMV with mutant MP	*N. edwardsonii* and cowpea	⇒	⇒|							Wang et al. 1998
Move through internal/external phloem										
BGMV	*N. benthamiana*		⇒	⇒	⇒	⇒	⇒	⇒		Morra et al. 2000; Qin et al. 2001
AbMV	Bean		⇒	⇒	⇒	⇒	⇒	⇒		Levy et al. 2003
	Susceptible hosts		⇒	⇒	⇒	⇒	⇒	⇒		Barker 1987; Barker 1989
Move only through external phloem										
PLRV	Resistant hosts		⇐	⇐	⇐	⇐	⇐	⇐		Barker et al. 1986; Derrick et al. 1992, 1997
PepMoV-FL	Resistant pepper strain		⇐	⇐	⇐	⇐	⇐	⇐		Guerini et al. 1999

[a] In susceptible hosts, the indicated viruses, except for PLRV, move from non-vascular ME to different vascular-associated and vascular cell types (BS, VP, CC, SE) of the inoculated leaves and, through SE, to systemic leaves, which they first enter through the vasculature and then unload into ME. In restrictive hosts, viral movement is arrested at different cellular boundaries, which usually are specific for a host-viral strain combination as shown in this Table. Arrows mark the cell types between which the indicated viral strain can move in the corresponding restrictive host, and solid vertical lines indicate the cell boundaries at which the viral movement is arrested

cases, does not require viral replication (Susi et al. 1999; Wintermantel et al. 1997). Moreover, these two types of movement require different sets of viral proteins, suggesting that local and systemic movements utilize different host molecular machineries, especially during entrance to and traffic through the plant intercellular connections, plasmodesmata (PD). The involvement of highly specialized host tissues and multiple viral factors in systemic transport has impeded direct experimental approaches, such as protein microinjection or transient gene expression by microbombardment, to study this transport process at the molecular level. Therefore, the viral systemic movement has been studied by analyzing the accumulation of viral product in systemic leaves: a lower accumulation of viral product in remote tissue may be attributed to either impaired systemic movement of the virus or systemic acquired resistance (SAR). The possibility of SAR can be eliminated by confirming the establishment of secondary infection of the same viral strain in systemic leaves.

This experimental design, however, cannot always define the true reason for the SAR-independent lack of viral product in systemic tissue. This is because plant innate immune response to viral infection often includes RNA silencing, which many viruses counter by encoding RNA silencing suppressors (Baulcombe 2002, 2004; Bisaro 2006; Marathe et al. 2000; Moissiard et al. 2004; Qu et al. 2005; Scholthof 2005; van der Boogaart et al. 1998; Voinnet 2001; Wang et al. 2005). Since viral RNA silencing suppressor is encoded by the moving virus itself, it presumably functions most efficiently at the infection front, where cell-to-cell movement of the virus into uninfected tissue takes place. Therefore, it is difficult to distinguish the reasons for inefficient viral accumulation: is it impaired viral movement, or inhibited viral accumulation based on inefficient viral suppression of RNA silencing at the infected site?

In this chapter, we review current knowledge about the viral and host factors participating in viral systemic movement in non-Arabidopsis host plants, and demonstrate how studies of restriction of systemic movement in specific hosts (Table 1) is used to define cellular boundaries that represent barriers for viral movement. We also briefly describe viral and host factors that were once assumed to be involved in viral translocation during systemic movement, but were then revealed to be involved in suppression of host RNA silencing of the virus.

2 Viral Factors Required for Systemic Movement

Viral systemic movement in a non-Arabidopsis host is often studied using host–virus combinations that show defective systemic accumulation (reviewed in Ueki et al. 2006; Waigmann et al. 2004). Systemic infection of a viral

strain can be restricted in certain hosts, while closely related strains can infect the same host systemically (Ueki et al. 2006; Waigmann et al. 2004). In many cases, the differences in systemic movement can be attributed to sequence variation(s) in a viral factor(s) required for viral systemic movement (Ueki et al. 2006, and references therein; Waigmann et al. 2004). These observations demonstrate that the viral factors are indeed involved in systemic movement, possibly via close interaction with the host machinery (Ueki et al. 2006, and references therein; Waigmann et al. 2004). In addition, compatibilities of these viral factors to the host machinery may define the host susceptibility to the viral strain. Table 1 summarizes several examples of the virus–host combinations that result in limited viral movement.

Among these viral systemic movement factors, some are not actually required for the translocation of viral genome during systemic infection, but for suppression of RNA silencing. For example, the 126-kDa tobamovirus protein and potyvirus HC-Pro have long been designated "systemic movement factors", and it is only recently that these two factors were found to actually be viral suppressors for RNA silencing; as such they do not aid in the movement itself, but in the accumulation of virus.

In this section, we discuss viral factors that are assumed to aid in the translocation process per se during long-distance movement. The involvement of RNA silencing suppressors for systemic movement is discussed in Sect. 5.

2.1 Movement Proteins

The term "movement protein" (MP) is normally used for viral factors that are required for local, cell-to-cell movement. However, in some cases, these factors are also required for the viral systemic transport, possibly exercising an additional function(s) to enable systemic movement. For example, the BR1 and BL1 MPs of bipartite geminiviruses, such as *Bean common mosaic virus* (BCMV), *Tomato golden mosaic virus* (TGMV), and *African cassava mosaic virus* (ACMV), aid in systemic transport (Jeffrey et al. 1996; Schaffer et al. 1995; von Arnim et al. 1993), and triple gene block protein 1 (TGBp1) MPs of hordeiviruses and potexviruses, which function during cell-to-cell movement, are required for systemic infection as well (Kalinina et al. 2001; Lough et al. 2001; Solovyev et al. 1999). Similarly, *Cucumber mosaic virus* (CMV) MP is involved in both local and systemic movement of the virus (De Jong et al. 1995; Kaplan et al. 1997; Li et al. 2001; Sanz et al. 2000; Takeshita et al. 1998). In many of these cases, however, the local and systemic MP activities can be uncoupled. For instance, point mutations in *Red clover necrotic mosaic virus* (RCNMV) MP prevent the virus systemic movement, while they do not affect its cell-to-cell movement (Wang et al. 1998). Moreover, MPs of some luteoviruses, such as *Bean yellow dwarf virus* (BYDV) and *Potato leaf roll virus*

(PLRV), are involved in systemic transport (Chay et al. 1996; Lee et al. 2002), possibly by associating with the specialized deltoid-shaped PD that connect CC with SE in some hosts (Schmitz et al. 1997). In other, less restrictive hosts, MP is not required for the vascular transport of luteoviruses, e.g., PLRV and *Beet western yellow luteovirus* (BWYV) (Lee et al. 2002; Ziegler-Graff et al. 1996). These results suggest that luteoviral MPs may, at least in part, determine the host specificity of the viral systemic movement.

2.2 Coat Proteins

Coat protein (CP) is a viral factor known to be required for systemic movement of the vast majority of viral species, including tobamoviruses (e.g., *Tobacco mosaic virus* (TMV) (Dawson et al. 1988; Holt et al. 1991; Osbourn et al. 1990; Saito et al. 1990; Siegal et al. 1962; Takamatsu et al. 1987)), dianthoviruses (e.g., RCNMV (Vaewhongs et al. 1995; Xiong et al. 1993) and *Carnation ring spot virus* (CRSV) (Sit et al. 2001)), tombusviruses (e.g., *Tomato bushy stunt virus* (TBSV) (Desvoyes et al. 2002; Scholthof et al. 1993), *Cucumber necrosis virus* (CuNV) (McLean et al. 1993), and *Cymbidium ring spot virus* (CymRSV) (Dalmay et al. 1992; Huppert et al. 2002)), geminiviruses e.g., *Maize streak virus* (MSV) (Boulton et al. 1989; Boulton et al. 1993; Liu et al. 2001; Liu et al. 1999), TYLCV (Noris et al. 1998), BYDV (Liu et al. 1998), *Beet mild curly top virus* (Soto et al. 2005), *Bean golden mosaic virus* (BGMV) (Pooma et al. 1996) and TGMV (Brough et al. 1988; Gardiner et al. 1988) alfamoviruses, e.g., *Alfalfa mosaic virus* (AMV) (Spitsin et al. 1999; van der Kuyl et al. 1991), cucumoviruses (e.g., CMV (Takeshita et al. 1998; Taliansky et al. 1995)), bromoviruses (e.g., *Brome mosaic virus* (BMV) (Rao et al. 1996)), luteoviruses (e.g., BWYV (Mutterer et al. 1999; Ziegler-Graff et al. 1996)), potexviruses (e.g., *White clover mosaic virus* (Lough et al. 2001), *Potato virus X* (PVX) (Santa Cruz et al. 1998)), and potyviruses (e.g., *Tobacco etch virus* (TEV) (Dolja et al. 1994, 1995), *Tobacco vein mottling virus* (TVMV) (Lopez-Moya et al. 1998)), and *Pea seed-borne mosaic virus* (PSbMV) (Andersen et al. 1998). Consistent with the role of viral CPs in systemic movement, the occurrence of encapsidated particles of diverse viruses, for example, TMV (Ding et al. 1996; Esau et al. 1967), *Cucumber green mottle mosaic tobamovirus* (CGMMV) (Simon-Buela et al. 1999), BWYV (Esau et al. 1972a,b), BYDV (Gill et al. 1975; Jensen 1969), CMV (Blackman et al. 1998), and PLRV (Schmitz et al. 1997; Shepardson et al. 1980), in the vasculature or vascular fluid obtained from infected plants suggests that these viruses move through the vascular system as assembled virions.

However, encapsidation may not be a prerequisite for the systemic transport of many other viruses, because several viral strains that lack encapsidation activity are still able to move systemically. For example, umbraviruses, which do not produce CP, move systemically, possibly by forming a ribonucle-

oprotein complex between the viral genomic RNA and a viral protein encoded by ORF3 (Taliansky et al. 2003b). Furthermore, even in a number of viruses that produce CP, elimination of this protein (e.g., in RCNMV (Xiong et al. 1993), TBSV (Scholthof et al. 1995), CuNV (McLean et al. 1993), and TGMV (Brough et al. 1988; Gardiner et al. 1988)) or disruption of its encapsidation capacity in CP mutants (e.g., in *Cowpea chlorotic mottle bromovirus* (CCMV) (Schneider et al. 1997) and CymRSV (Dalmay et al. 1992; Huppert et al. 2002)) does not abolish systemic infection of some hosts. On the other hand, the systemic movement ability of some CP mutants of several viruses, such as TMV (Culver et al. 1995; Dawson et al. 1988), CRSV (Sit et al. 2001), RCNMV (Xiong et al. 1993), TGMV (Pooma et al. 1996), and TEV (Dolja et al. 1994; Dolja et al. 1995), is impaired, although they retain their encapsidation activity. Therefore, in many virus–host combinations, the CP function in viral encapsidation can be uncoupled from its function in systemic movement. The latter function of CP may involve interaction with and modification of host factors, in order to facilitate the systemic traffic of viral components.

In addition to their major CP component, some viral capsids contain minor constituents that may also play a role in the systemic movement. For example, the capsid of the luteovirus BWYV consists of two protein species: a major 22-kDa component, p3, and a minor 74-kDa component, the read-through protein (RT) p74; p74 is synthesized by suppressing translational termination of p3, which allows the translation to continue to the adjacent ORF5, thereby producing an additional read-through protein domain (RTD) (Bahner et al. 1990; Brault et al. 1995; Filichkin et al. 1994; Martin et al. 1990; Wang et al. 1995). BWYV mutants that do not produce RTD are still encapsidated, forming virions, but they exhibit reduced systemic infection in *Nicotiana clevelandii*, suggesting that the RTD is required, by as yet unknown mechanism, for efficient systemic transport of the virus (Mutterer et al. 1999).

2.3 VPg of Potyviruses

Another viral factor involved in systemic movement is the potyvirus viral genome-linked protein (VPg), which is covalently attached to the 5′ end of viral genomic RNA and is essential for viral replication activity (reviewed in Revers et al. 1996; Urcuqui-Inchima et al. 2001). VPg has been identified as a factor required for systemic movement and as a host-range determinant in TEV strains for *Nicotiana tabacum* (Schaad et al. 1996, 1997), and in *Potato virus A* (PVA) strains for *Nicanda physaloides* and potato plants (Räjamaki et al. 1999, 2002, 2003). In addition, a point mutation in *Turnip mosaic virus* (TuMV) VPg, substituting phenylalanine at position 12 with methionine, impairs local and systemic movement of TuMV in *Nicotiana benthamiana* and Arabidopsis, demonstrating that this amino acid residue of VPg is crucial for systemic movement of the virus (Dunoyer et al. 2004).

How does VPg facilitate systemic transport? VPg has been shown to interact with the eukaryotic initiation factor-4E (eIF4E) in vitro and in planta (Leonard et al. 2000, 2004; Robaglia et al. 2006; Schaad et al. 2000; Wittmann et al. 1997), and eIF4E has been shown to move from cell to cell (Gao et al. 2004b). Therefore, it is tempting to speculate that the potyvirus VPg interacts with endogenous eIF4E, such that the host factor aids the virus movement (see also Sect. 3.4). Since VPg binds covalently to the 5′ end of viral genome RNA, eIF4E-VPg-viral genome may move cell to cell as a complex after the replication in single cells. In addition to eIF4E aiding VPg to mediate viral movement, VPg may modulate the biochemical activity of eIF4E by increasing the binding affinity of eIF4E to another initiation factor, eIF4G, and reducing it toward mRNA cap (Michon et al. 2006).

Additional, novel potyvirus VPg-interacting proteins (PVIPs), which have a PHD finger-like cysteine-rich domain (Schindler et al. 1993), have been identified from pea, Arabidopsis and *N. benthamiana* (Dunoyer et al. 2004, see also Sect. 3.5). Possible involvement of the VPg–PVIPs interaction during the systemic infection process suggests that PVIPs may represent another class of host factors involved in the potyviral systemic movement process (Dunoyer et al. 2004).

Thus, although VPg is involved in the replication process (reviewed in Revers et al. 1996; Urcuqui-Inchima et al. 2001), it may also function as a crucial factor for the viral systemic movement process. Because no evidence exists to suggest that VPg facilitates viral movement by impairing host resistance, e.g., RNA silencing, VPg may represent a bona fide movement factor required for systemic translocation of the virus. In contrast, HC-Pro was once assumed to be a systemic movement factor, and was later revealed to be a RNA silencing suppressor (Kasschau et al. 2001).

2.4 Umbravirus ORF3 Proteins

Umbraviruses are unusual in that they do not encode a conventional CP and, thus, do not form true viral particles in infected tissues (reviewed in Robinson et al. 1999). Nevertheless, umbraviruses rapidly establish systemic movement in compatible hosts. One of the viral factors, the ORF3 protein of *Groundnut rosette virus* (GRV), supports long-distance transport of both GRV RNA and the genomic RNA of a CP-less mutant of an unrelated virus, TMV (Ryabov et al. 1999). In addition, when the GRV ORF3 protein is expressed from chimeric TMV in place of TMV CP, designated TMV(ORF3), it binds the TMV(ORF3) RNA and facilitates its transport through the host plant vasculature, demonstrating that the GRV ORF3 protein can systemically translocate heterologous RNA molecules, presumably in the form of ribonucleoprotein complexes (Taliansky et al. 2003a). Moreover, chimeric TMV strains expressing the ORF3-encoded proteins from other umbraviruses, such as *Pea ena-*

tion mosaic virus-2 (PEMV-2) and *Tobacco mottle virus* (TMoV), instead of TMV CP, move systemically in *N. benthamiana* and *N. clevelandii*, but not in *N. tabacum* (Ryabov et al. 2001b). Because *N. benthamiana* and *N. clevelandii* are systemic hosts for PEMV-2, TMoV, and TMV, whereas *N. tabacum* is a systemic host only for TMV and not for the two umbraviruses (Ryabov et al. 2001b), the ORF3 protein may also determine the host specificity of the systemic transport process. While the mechanism underlying this ORF3 protein function is still unclear, simple protection of the viral RNA from cellular nucleases may not play a major role in establishing the host range of systemic transport because the ORF3 protein–RNA complexes are stable in cell extracts of both *N. benthamiana*, in which the ORF3 protein supports systemic infection, and *N. tabacum*, in which it does not (Ryabov et al. 2001b).

Electron microscopic studies have shown that in vivo, within infected cells, binding of the ORF3 protein to RNA produces filamentous ribonucleoprotein particles with a helical structure, albeit not as uniform as classical virions (Taliansky et al. 2003a). In vitro, the ORF3 protein forms oligomers and binds RNA, consistent with its RNA-binding activity in vivo (Taliansky et al. 2003a). The ORF3 protein–RNA complexes are detected in all types of cells and are abundant in phloem-associated ones, especially in CC and immature SE (Taliansky et al. 2003a); this accumulation of ORF3 protein within the host plant vasculature is consistent with biological role of this protein as a facilitator of umbraviral systemic transport.

In addition, a recent study has shown that when transiently expressed, umbravirus ORF3 protein is targeted to nuclei, preferably nucleoli (Kim et al. 2004). The relationship of this newly discovered cellular localization of ORF3 to its function as a systemic movement factor has yet to be clarified.

3
Host Factors Involved in Systemic Movement

Besides the effectors encoded by the genome of the invading virus, the process of systemic movement involved host cell components, which often directly interact with the viral factors during movement. To date, several host factors involved in viral systemic movement have been identified from *Arabidopsis thaliana* using reverse genetic analysis. However, due to lack of genomic sequence information, our knowledge about plant factors involved in viral systemic movement in many non-Arabidopsis plant species is quite limited.

3.1
Pectin Methylesterase (PME)

PME has been identified as a cell-wall protein that interacts with tobamovirus MP in the course of cell-to-cell movement (Chen et al. 2000; Dorokhov et al.

1999). The role of PME in viral systemic movement has also been demonstrated using antisense suppression of its gene in tobacco plants, which preferentially occurs within the vascular tissues (Chen et al. 2003). TMV accumulation in uninoculated leaves of these PME-antisense plants is significantly delayed, indicating impaired systemic transport of this virus. Since no differences were detected in the vascular loading and unloading of a fluorescent solute between the PME-antisense plants and wild-type tobacco, PME is not involved in the phloem transport of solutes (Chen et al. 2003). Immunofluorescence confocal microscopy analysis demonstrated that, in the PME-antisense plants, TMV virions enter the host vasculature but fail to exit into uninoculated non-vascular tissues (Chen et al. 2003). Therefore, in the PME-antisense plants, TMV unloading from the vasculature is significantly impaired, whereas its loading into the tissue remains intact. The mechanism by which the MP–PME interaction affects the viral movement remains unknown, but it has been suggested that PME, via biochemical modification of pectins, may loosen the cell wall around PD, allowing the PD to open more easily (Boevink et al. 2005), or it may simply help transport MP to or anchor it at the cell wall, potentially in the vicinity of PD (Boevink et al. 2005; Chen et al. 2000).

3.2 cdiGRP, Callose, and β-1,3-Glucanase

Besides proteins required for the movement process, the host plants produce factors that negatively regulate viral systemic movement; one such factor is a cadmium-induced glycine-rich protein (cdiGRP), discovered in tobacco plants (Ueki et al. 2002). Identification of cdiGRP was based on the observations that systemic movement of tobamoviruses, such as TVCV and TMV, is blocked in tobacco plants pretreated with low concentrations of the heavy metal cadmium, while local virus movement in these plants is not affected (Citovsky et al. 1998; Ghoshroy et al. 1998). This inhibitory effect of cadmium ions is tobamovirus-specific because systemic movement of TEV was not impaired by the same treatment (Ghoshroy et al. 1998); interestingly, however, cadmium treatment also inhibited the systemic spread of RNA silencing in *N. tabacum* and *N. benthamiana* plants (Ueki et al. 2001). Cadmium-induced inhibition of systemic viral spread occurs by a SAR-independent mechanism because it is also observed in *NahG*-expressing transgenic plants (Citovsky et al. 1998) which are unable to accumulate salicylic acid and develop SAR (Gaffney et al. 1993). Immunodetection of tobamoviral CP revealed that the virus accumulates in the vasculature of uninoculated, systemic leaves but not in the surrounding MS cells, indicating that, in cadmium-treated plants, the spreading virus enters, but does not exit, the host plant vascular system (Citovsky et al. 1998).

The cdiGRP cDNA was isolated by a PCR-based subtraction cloning strategy as a tobacco gene whose expression was induced by a low concentration

of cadmium ions whereas high, toxic amounts of cadmium did not induce the *cdiGRP* gene (Ueki et al. 2002). Cadmium-induced expression of cdiGRP is tissue-specific, with the protein found mainly in the cell walls of the plant vascular bundle. Importantly, constitutive expression of cdiGRP in transgenic plants significantly reduces tobamoviral systemic movement in the absence of cadmium, whereas antisense suppression of cdiGRP allows the virus to spread systemically, even in cadmium-treated plants (Ueki et al. 2002). cdiGRP does not restrict viral movement directly. Instead, this protein induces – by an as-yet unknown mechanism – callose accumulation on PD on the cell walls of the phloem; these callose deposits, in turn, most likely reduce viral transport from the phloem into the surrounding non-vascular cells (Ueki et al. 2002). Callose is a 1,3-β-D-glucan (Stone et al. 1992) deposited at the collar region of the PD (Northcote et al. 1989). Degradation of callose is thought to increase PD permeability (Botha et al. 2000; Northcote et al. 1989), whereas its deposition is believed to restrict intercellular transport (Bucher et al. 2001; Delmer et al. 1993; Iglesias et al. 2000) by relaxing or constricting, respectively, of the PD collar sphincter. Thus, callose may represent a polysaccharide plant cell wall component that restricts viral systemic movement through PD, most likely by reducing PD permeability.

A cdiGRP-interacting protein, GrIP, was identified from a *N. tabacum* cDNA library by two-hybrid screening using cdiGRP as bait (Ueki et al. 2005). Like cdiGRP, GrIP is expressed in vascular tissue and accumulates in the cell wall (Ueki et al. 2005). Interestingly, accumulation of cdiGRP protein and callose, with or without the cadmium ion treatment, was enhanced in GrIP-overexpressing transgenic plants relative to wild-type plants, demonstrating that GrIP is involved in the regulation of cdiGRP expression/accumulation (Ueki et al. 2005). Since the levels of the *cdiGRP* mRNA were not affected by GrIP expression, GrIP must regulate the accumulation of the cdiGRP protein at the post-transcriptional level (Ueki et al. 2005). Because GrIP binds to cdiGRP in vitro and in vivo, this GrIP–cdiGRP interaction may stabilize and/or help cell wall targeting of cdiGRP (Ueki et al. 2005). Therefore, GrIP, together with cdiGRP and callose, may control PD transport in *N. tabacum*.

The amount of callose in the cell walls is directly controlled by the balance of two opposing enzymatic activities: callose synthase, which produces callose, and β-1,3-glucanase, which hydrolyzes it (Kauss 1985, 1996). Plant callose synthases are still poorly characterized, whereas β-1,3-glucanases have been better studied. Plant β-1,3-glucanases are grouped into three classes according to their structure (reviewed in Beffa et al. 1996a; Leubner-Mezger et al. 1999). Class I β-1,3-glucanases are basic proteins localized in the vacuole of MS and epidermal cells; class II and III β-1,3-glucanases are acidic isoforms secreted into the cell walls. Class II β-1,3-glucanases include the pathogenesis-related (PR) proteins PR2, PR N, and PR O, and class III consists of a single member, PR-Q′ (reviewed in Beffa et al. 1996a; Leubner-Mezger et al. 1999). By virtue of their ability to regulate the amount of callose, which then restricts intercellular

transport, β-1,3-glucanases can be regarded as cellular factors controlling viral movement. Indeed, TMV infection of tobacco plants elevates β-1,3-glucanase activity, which presumably enables more efficient viral movement (reviewed in Beffa et al. 1996a). Conversely, antisense suppression of β-1,3-glucanase in Nicotiana species results in increased callose deposits in the cell wall (Beffa et al. 1996b), reduced PD permeability (Iglesias et al. 2000), and delayed local and systemic movement of such viruses as TMV, *Tobacco necrosis virus*, and PVX (Beffa et al. 1996b; Iglesias et al. 2000). Similarly, overexpression of the β-1,3-glucanase coding sequence from a TMV-based vector facilitates viral movement, whereas antisense expression of the same sequence delays viral movement in the inoculated leaf (Bucher et al. 2001). Thus, induction of callose accumulation by an abiotic stimulus, e.g., cadmium ions via cdiGRP (Ueki et al. 2002), or by antisense suppression of β-1,3-glucanases (Beffa et al. 1996b; Iglesias et al. 2000), negatively regulates systemic and/or cell-to-cell transport of plant viruses. Potentially, GrIP, cdiGRP, β-1,3-glucanases, and callose represent a multicomponent system that controls PD transport by constricting/relaxing the callose sphincter at the collar regions of PD.

3.3
Tomato Mosaic Virus CP-Interacting Protein-L (IP-L)

A tobacco protein that interacts with tobamoviral CP in vitro, *Tomato mosaic virus* (ToMV) CP-interacting protein-L (IP-L), was identified by screening a tobacco cDNA library using the yeast two-hybrid system (Li et al. 2005). The isolated cDNA was identical to an elicitor-responsive protein from *N. tabacum*, and was also highly homologous to senescence-related proteins from tomato and pepper (Li et al. 2005). The *IP-L* gene expression is markedly increased by inoculation of ToMV and PVX (Li et al. 2005). Importantly, when expression of *IP-L* was suppressed by virus-induced gene silencing (VIGS) utilizing a PVX-based vector in *N. benthamiana* plants, infection of ToMV was significantly delayed, demonstrating that a high level of IP-L is required for efficient systemic infection in the host (Li et al. 2005). Though the molecular mechanism of its action has not been elucidated, IP-L may represent a factor that enhances viral systemic movement, and/or the process of viral replication. Since tobamoviral CP is involved in systemic movement (Dawson et al. 1988; Holt et al. 1991; Osbourn et al. 1990; Saito et al. 1990; Siegal et al. 1962; Takamatsu et al. 1987), the CP-interacting IP-L may be involved in viral systemic movement as well.

3.4
Eukaryotic Initiation Factor-4E (eIF4E)

eIF4E binds specifically to the 5′-CAP structure of mRNA to initiate the translation process in the host cell cytoplasm (Robaglia et al. 2006). The eIF4E

proteins from a compatible host plant interact with potyviral VPg in vitro and in vivo (Leonard et al. 2000, 2004; Robaglia et al. 2006; Schaad et al. 2000; Wittmann et al. 1997). Moreover, recent studies have suggested that incompatibilities of host eIF4E isoforms with potyviral VPg may underlie naturally occurring host resistance to potyvirus by restricting its systemic movement. For example, the *pvr2* locus in pepper, which confers recessive resistance to strains of *Potato virus Y* (PVY), corresponds to the host *eIF4E* gene (Ruffel et al. 2002). Consistent with this idea, PVX-based transient expression of *eIF4E* from a susceptible pepper host (Yolo Wonder strain) restores the systemic movement of PVY in a resistant host (Yolo Y strain) (Ruffel et al. 2002). A similar observation was obtained from an analysis of lettuce resistance to *Lettuce mosaic virus* (LMV) (Nicaise et al. 2003): when *eIF4E* from susceptible, tolerant, and resistant hosts were sequenced, variations in the sequence were found near the predicted CAP-recognition pocket of the protein (Nicaise et al. 2003). Transient expression of eIF4E from the susceptible host, in this case by simultaneous expression of the protein and the virus from a recombinant LMV vector with the *eIF4E* sequence inserted between *LMV-P1* and *LMV HC-Pro* genes, restored systemic infection of the virus in the resistant host, again demonstrating that eIF4E from a susceptible host is sufficient to complement the systemic infection of the virus in a resistant host (Nicaise et al. 2003). Moreover, eIF4E sequence variations have been demonstrated to underlie the resistance mechanism of several pea strains to *Pea seed-borne mosaic virus* (PSbMV) (Gao et al. 2004a,b). These results demonstrate that the compatibility between the host factor and VPg may be crucial to efficient potyviral systemic infection. Because PVY replication occurs in isolated protoplasts from a resistant pepper strain with a *pvr2* genotype, which carries a mutated *eIF4E* gene, the viral resistance based on the incompatibility between the viral VPg and host eIF4E may not be exclusively due to impaired viral replication (Arroyo et al. 1996). Moreover, eIF4E itself can move from cell to cell (Gao et al. 2004b), suggesting that this host protein binds to the viral VPg, which in turn covalently associates with the 5′-CAP structure of the viral genome, and aids VPg in the translocation of the viral genome complex to the neighboring cells, possibly by interacting with and gating PD (see also Sect. 2.3).

Whether the eIF4E–VPg interaction defines the potyviral systemic mobility, rather than cell-to-cell movement, is still uncertain. As described in Sect. 2.3, potyviral VPg may act as a host-range determinant by limiting the viral systemic movement. Based on this idea, the molecular interactions that involve VPg and determine the virus systemic mobility should occur specifically during the systemic translocation, rather than during the cell-to-cell movement process. Potentially, the host eIF4E that participates in the potyviral systemic infection is specifically expressed in vascular-associated tissues, and thus is involved in viral systemic movement via the vasculature. Alternatively, eIF4E may be involved mainly in the cell-to-cell movement, while

another, as yet unknown, host factor(s) facilitates long-distance translocation via its interaction with the viral VPg.

3.5 Potyvirus VPg-Interacting Protein (PVIP)

PVIP is another host protein that may interact with potyviral VPg in the processes of cell-to-cell and viral systemic movement. PVIP was identified by yeast two-hybrid screening of a pea cDNA library with PSbMV VPg as bait (Dunoyer et al. 2004). The PVIP protein has no homology to any proteins with known function, and appears to be plant-specific (Dunoyer et al. 2004). A small family of genes in Arabidopsis (*AtPVIP*s) and a gene from *N. benthamiana* (*PVIPnb*) exhibited a homology to the pea *PVIP* (*PVIPp*) gene at the protein level (Dunoyer et al. 2004). These proteins display differential interactions with VPgs from different strains of potyvirus; VPg from PSbMV, TuMV, and LMV interacts with AtPVIP1, AtPVIP2, PVIPp, and PVIPnb, whereas VPg from TEV, *Cowpea mosaic virus* (CPMV), *Tomato black ring virus* (TBRV), and *Grapevine fan leaf virus* (GFLV) does not (Dunoyer et al. 2004). Within the (Wintermantel et al. 1997) amino acid residues of TuMV VPg, deletion of the 66 N-terminal residues abolishes interaction of the VPg with AtPVIPs, demonstrating the involvement of this VPg domain in the interaction with AtPVIPs (Dunoyer et al. 2004). When the sequence of this VPg domain was compared in different potyviruses, several amino acid variations were found. When, based on this information, the phenylalanine residues at position 12 within TuMV VPg was substituted with methionine to mimic the sequence of TEV VPg, the interaction of TuMV VPg with AtPVIP1, AtPVIP2, PVIPp, and PVIPnb in a two-hybrid system was abolished, indicating that this amino acid residue is crucial for the specificity of the virus–host VPg–PVIP interaction (Dunoyer et al. 2004). Importantly, the mutant TuMV strain with the phenylalanine-to-methionine substitution in VPg displayed a significant delay in its local and systemic infection of *N. benthamiana*, suggesting the involvement of the TuMV VPg–PVIPnb interaction the infection process (Dunoyer et al. 2004). Moreover, RNAi suppression of the *AtPVIP1* and *AtPVIP2* genes dramatically inhibited TuMV systemic infection and disease symptom development, demonstrating the importance of these host factors for the viral infection (Dunoyer et al. 2004).

4 Cellular Route for Viral Systemic Movement

Viral systemic movement comprises six major consecutive steps:

1. Virus translocation from MS to BS cells
2. Penetration into the VP through the BS

3. Entry into the phloem CC/SE complex (or, for some viruses, into xylem-associated cells from the VP, see Sect. 4.4)
4. Rapid transport to systemic uninfected plant organs through the phloem SE (or xylem, in some cases)
5. Unloading from the CC/SE complex into uninfected VP
6. Egress from the VP through BS cells and into the MS cells of systemic plant organs

Some of the boundaries between the different cell types involved in these steps of the systemic transport can block translocation of some viral strains (Table 1), demonstrating that these boundaries can serve as natural barriers for those viruses. Moreover, viruses can enter the host vasculature through both major and minor veins whereas they exit the vasculature only from the major veins (Cheng et al. 2000; Santa Cruz et al. 1998), suggesting that the process of virus unloading may be more restrictive than that of virus loading (see also sections Sect. 4.1 and Sect. 4.5). This notion is supported by the observations that PME and cdiGRP/cadmium treatments restrict tobamoviral systemic movement by blocking the viral egress from, but not entry into, the vasculature (Chen et al. 2003; Citovsky et al. 1998; Ghoshroy et al. 1998; Ueki et al. 2002) (see Sect. 3.1 and Sect. 3.2).

For their movement through the host vasculature, plant viruses are thought to take the same route that the plant utilizes for transport of its photoassimilates (Leisner et al. 1993a,b). Tracking radioisotope-labeled sucrose and low molecular weight fluorescent dye in host plants has demonstrated that photoassimilates are transported from lower, fully expanded leaves (source) to the upper, young leaves (sink) (Leisner et al. 1993b; Oparka et al. 2000; Roberts et al. 1997; Santa Cruz et al. 1999). Similarly, tracking systemically moving viruses, such as *Cauliflower mosaic virus* (CaMV) (Leisner et al. 1993c) or GFP-expressing recombinant tobamoviruses and PVX (Cheng et al. 2000; Santa Cruz et al. 1998), showed that these viruses, and presumably many others, move through the phloem from source leaves to sink tissues.

Viruses can enter two structurally different types of phloem – the internal and the external phloem – for their upward and downward movement, respectively (for details, see Andrianifahanana et al. 1997; Cheng et al. 2000; Guerini et al. 1999). Having entered the phloem SE, viruses move in two opposite directions: upward to the sink leaves and downward to the roots. The upward movement occurs significantly faster than the downward spread (Andrianifahanana et al. 1997; Cheng et al. 2000) (see also Sect. 3.4). As a consequence, sink leaf tissues represent the major and preferential target for viral systemic movement.

4.1
Leaf Veins Utilized for Viral Entry Into and Exit out of the Vascular System

The leaf veinal system is classified into major (classes I-III) and minor veins (classes IV and smaller) (Roberts et al. 1997). GFP-expressing recombinant TMV was used to define the routes for vascular invasion of viruses in the source leaves of *N. benthamiana*: viral loading occurred both in minor (classes IV and V) and in major veins (classes III and larger). Thus, all vein classes function equally as gateways for TMV entry into the vasculature of the source leaves (Cheng et al. 2000). In contrast, virus unloading appears to be more selective. First, the virus unloads from major veins, but not from minor ones. Second, virus unloading patterns change during the course of development, i.e., during the sink-to-source transition. In tobacco leaves, this transition occurs basipetally, from apex to base, so that the apical part of the leaf starts functioning as a source when the basal part is still a sink (Roberts et al. 1997; Turgeon 1989). In such transitioning leaves, GFP-expressing recombinant PVX and TMV are unable to unload into the source portions of the leaf (Cheng et al. 2000; Roberts et al. 1997). The sink-to-source transition events probably alter leaf vasculature at the molecular level (van Bel et al. 2003a,b), and these developmental changes presumably block, by an as yet unknown mechanism, the ability of the virus to exit the minor veins of the leaf vasculature.

Interestingly, in the dicotyledonous *N. benthamiana* plant, the specific patterns of virus unloading and vein involvement mirror those of the fluorescent solute carboxyfluorescein (CF), although viral unloading occurs considerably slower than that of the much smaller CF (Roberts et al. 1997). Parallels in the unloading of viruses and solutes have also been found in monocotyledonous plants, such as barley, in which unloading patterns of CF and GFP-expressing recombinant *Barley stripe mosaic virus* (BSMV) display a striking similarity, with both CF and the virus exiting major longitudinal veins, but not transverse veins (Haupt et al. 2001). Therefore, in both dicots and monocots, the virus appears to hijack the physiological route that the plants have evolved for export of photoassimilates from source to sink tissues.

4.2
Invasion of the Vasculature Across the BS/VP Boundary

During the vascular-invasion process, the first cell type that the virus encounters is the BS. Presumably, viruses enter BS cells by a cell-to-cell movement mechanism whereas viral transport from the BS into the VP occurs by a different pathway. Indeed, TMV MP, which is sufficient to gate PD in nonvascular tissues (Ding et al. 1992; Tomenius et al. 1987; Waigmann et al. 1994), accumulates within the PD between the BS and VP, but does not increase the permeability of PD at this intercellular boundary (Ding et al. 1992). Thus, the

BS/VP boundary in the inoculated leaf may represent the first barrier encountered by viruses during their long-distance movement.

Illustrating the biological relevance of the BS/VP barrier, viral transport can be specifically blocked at this cellular interface in some hosts. For example, CCMV systemic movement is arrested in the BS cells of a resistant cultivar of soybean, and this restriction is responsible for the resistant phenotype (Goodrick et al. 1991). Transgenic tobacco plants that overexpress CMV replicase do not support systemic CMV infection due to the block in viral translocation from BS to the VP (Wintermantel et al. 1997). Similarly, in cucumber cotyledons, a chimeric cucumovirus strain expressing the CP of the Florida strain of *Tomato aspermy virus* (TAV), whose systemic movement is restricted in cucumber plants, accumulates in the BS cells but is not observed in the VP (Thompson et al. 1998). These data suggest that the PD at the BS/VP boundary are equipped with a restrictive mechanism(s) that blocks the systemic movement of incompatible viral strains.

4.3
Entry into the CC/SE Complex Across the VP/CC and/or BS/CC Boundaries

Once in the VP, plant viruses proceed into the CC/SE complex. To this end, they must first enter the CC, crossing the VP/CC boundary. The existence of this boundary is inferred from experiments with a CP-less TMV mutant which is able to cross the BS/VP boundary and accumulate in the VP, but remains excluded from the CC (Ding et al. 1996). Interestingly, the mutant TMV strain with truncated CP, which is deficient in encapsidation activity, still shows systemic movement, suggesting that the CP exerts an unknown function for entrance into the CC from the VP, possibly by interacting with specific host machinery at this intercellular boundary (Ding et al. 1996) (see also Sect. 2.2). Moreover, point mutations in RCNMV MP prevent viral systemic movement, whereas cell-to-cell movement of the mutants remains unaffected; since these systemic-movement-defective mutants accumulate only at low levels in the CC/SE complex in inoculated leaves, their systemic movement most likely is arrested because of impaired virus loading into or accumulation within the CC/SE complex (Wang et al. 1998).

In some cases, such as in minor leaf veins in the Nicotiana species, virus may load into the CC/SE complex directly from BS cells that contact CC and are not separated from them by VP, as in major veins of many plant species (Ding et al. 1995; Santa Cruz et al. 1998). In the case of PVX infection in *N. benthamiana*, viral CP, which is known to be required for the cell-to-cell movement, is found associated with PD at the BS/CC and BS/VP interfaces, but not at the VP/CC interface (Santa Cruz et al. 1998). This may suggest that the virus preferentially enters the CC directly from the BS cells in minor veins in Nicotiana, bypassing the VP (Santa Cruz et al. 1998). Collectively, these observations suggest that the VP/CC boundary (and/or BS/CC bound-

ary, in some cases) in the inoculated leaf represents the second barrier to viral systemic movement.

4.4 Viral Movement Across the CC/SE Boundary and Through the Conduit

In the phloem CC/SE complex, the virus passes from CC into SE, where it presumably utilizes pressure-driven flow of photoassimilates for rapid long-distance movement to systemic sink leaves. Since the enucleated SE are perforated at both longitudinal ends, they provide an unrestricted and uninterrupted path for the long-distance transport of various macromolecules and solutes throughout the plant. Since some viruses do not require replication for their efficient systemic movement (Susi et al. 1999; Wintermantel et al. 1997) and are transported systemically as virions (see Sect. 2.2), these viruses may move through SE in, at least partially, an encapsidated form. Other viruses, such as PVA and *Sweet potato chlorotic stunt virus* (SPCSV), may undergo decapsidation and replication while moving systemically because they are susceptible to RNA silencing during this transport, implying the exposure of the viral genome and replication within the components of the CC/SE complex (Germundsson et al. 2006; Kreuze et al. 2005). Interestingly, in minor leaf veins in *N. benthamiana*, PVX virion, which is required for viral cell-to-cell movement in most tissues, localizes within PD at the BS/VP and BS/CC, but not at the CC/SE boundary (Santa Cruz et al. 1998). Thus, at the CC/SE interface, unlike at others where PVX takes the form of a virion to pass, the virus may use a non-virion transport intermediate (Santa Cruz et al. 1998), suggesting that the CC/SE boundary in the inoculated leaf represents the third potential barrier to systemic movement of, at least, some viruses.

Although the size-exclusion limit of PD at the CC/SE boundary is larger than at other boundaries (Kempers et al. 1993, 1997), these PD may still need to be modified by the viral movement factors; indeed, MPs of several plant viruses, such as PLRV and CMV, localize to the PD at the CC/SE boundary (Blackman et al. 1998; Hofius et al. 2001; Schmitz et al. 1997), presumably modulating these channels for viral passage. Similarly, PVX may utilize a viral component(s) other than the CP/virion to enlarge the PD and enter SE (Santa Cruz et al. 1998). Furthermore, GFP-tagged CMV expressed from a CC-specific promoter of *Commelina yellow mottle virus* (ComYMV) is transported into the SE, indicating its ability to gate the PD that connect these cells; this transport is specific because dimeric GFP, which is also expressed from the ComYMV promoter, remains confined to CC (Itaya et al. 2002). Consistent with the MP role during viral transport from CC into SE, in CMV-infected *N. clevelandii* CMV virions are found in SE but not in CC, suggesting that CMV genomes translocate into the SE as MP–RNA complexes and form virions only within the SE (Blackman et al. 1998).

Although most viruses are known to use the phloem for systemic movement, some, such as *Rice yellow mottle virus* (RYMV) (Opalka et al. 1998) and CGMMV (Moreno et al. 2004), have been reported to move through xylem components. Accumulation of *Soilborne wheat mosaic virus* (SBWMV) has also been demonstrated in the xylem, suggesting the involvement of this tissue in SBWMV systemic movement (Verchot et al. 2001).

As described in Sect. 4, long-distance transport proceeds at different rates and in two directions: upward movement is faster, and downward movement is slower (Andrianifahanana et al. 1997; Cheng et al. 2000). Tracing the movement of TMV, *Pepper mottle potyvirus* (PepMoV), and PLRV demonstrated that these two movement modes occur through structurally different types of phloem – external and internal (Barker et al. 1986; Cheng et al. 2000; Derrick et al. 1992, 1997; Guerini et al. 1999). The external and internal phloem in the transport veins of petioles and stems of such plant families as *Solanaceae, Cucurbitaceae* and others derive from the abaxial (facing away from the axis of the plant and located on the underside of the leaf) and adaxial (facing toward the axis of the plant and located on the upper side of the leaf) phloem, respectively, of the major leaf veins (Cheng et al. 2000; Turgeon 1989). In *N. benthamiana* inoculated with a GFP-expressing recombinant strain of TMV, GFP fluorescence is detected in the external phloem and external phloem-associated cells of the stem internode below the inoculated leaf, and exclusively in the internal phloem and internal phloem-associated cells of the stem internode above the inoculated leaf. These two opposing venues of viral transport are almost independent because only little traffic is detected between the internal and external phloem of the stem (Cheng et al. 2000). Similarly, systemic infection of pepper plants by the Florida isolate of PepMoV (PepMoV-FL) follows a defined pattern of downward movement through the external phloem and upward movement through the internal phloem (Andrianifahanana et al. 1997), whereas the virus-resistant pepper cultivar *Capsicum annuum* cv. Avelar allows downward movement of PepMoV-FL through the external phloem, but restricts upward movement through the internal phloem, resulting in young stem tissues that are virus-free (Guerini et al. 1999). Finally, differential involvement of the internal and external phloem in viral systemic movement was also shown using PLRV-resistant potato plants in which virus is restricted to the internal phloem, whereas both internal and external phloem display PLRV accumulation in the susceptible potato plants (Barker et al. 1986; Derrick et al. 1992, 1997). Thus, plant viruses may move to the roots, downward from the inoculated leaf, through the abaxial phloem of leaves and external phloem of petioles and stems, but utilize the adaxial leaf phloem and internal petiole and stem phloem for their upward movement to the sink leaves.

4.5 Virus Unloading from the Phloem into Systemic Organs

For most viruses, unloading from the phloem into the surrounding non-vascular tissues of systemic, uninoculated organs and propagation in these tissues is the last step in establishing efficient systemic infection. One exception to this rule are "phloem-limited" viruses that are confined to the vascular components and do not appear in systemic MS tissues, such as luteoviruses (Mayo et al. 1996; Smith et al. 1999), some, but not all (Michelson et al. 1997; Morra et al. 2000; Rogers et al. 2001), bipartite (Morra et al. 2000; Qin et al. 2001) and monopartite geminiviruses (Rojas et al. 2001), bipartite closteroviruses (Wisler et al. 1998), and others. These viruses may be limited to the phloem because of their blocked unloading into systemic non-vascular tissues, limited replication in these tissues after unloading and/or because some of them do not encode a bona fide MP (Briddon 2003). These phloem limitations can be removed by coinoculation of a second virus, which provides viral functions that the phloem-limited virus lacks and that are required for infection of non-vascular tissues; studies of the mechanisms underlying this in-*trans* complementation can provide useful insights into the molecular causes of phloem limitation.

For example, luteoviruses, following direct injection into phloem cells by aphids, spread within the phloem but do not leave the host vasculature, although they can replicate in protoplasts derived from non-vascular tissues (reviewed in Mayo et al. 1996; Smith et al. 1999). Coinfection of *N. clevelandii* or *N. benthamiana* with a mixture of PLRV luteovirus and an unrelated PVY potyvirus results in a higher titer of PLRV and its more frequent occurrence within MS cells (Barker 1987, 1989), suggesting that potyviral factors facilitate phloem unloading of PLRV. The potyviral determinants that alleviate luteoviral phloem limitation have not been identified; however, they probably do not include HC-Pro, the potyviral RNA silencing suppressor, because transgenic *N. benthamiana* plants expressing PVA HC-Pro do not promote the occurrence of luteoviruses in MS cells (Savenkov et al. 2001).

The phloem limitation of PLRV may derive from a combination of the host RNA silencing against this virus and other, as yet uncharacterized, processes. This notion is based on the observations that phloem unloading of PLRV into MS tissues is induced following coinoculation by a cucumovirus CMV(ORF4) strain, which is a chimeric CMV expressing the ORF4-encoded GRV MP instead of CMV MP, but not by a mutated CMV(ORF4) with blocked expression of the viral RNA silencing suppressor 2b (Ryabov et al. 2001a). On the other hand, PLRV spread beyond the phloem was promoted, via an unknown mechanism, by coinoculation with PEMV-2, but not with CMV, TMV, PVY, PVX, some of which encode viral RNA silencing suppressors (Ryabov et al. 2001a). Moreover, a recombinant PVX that expresses GRV MP did not rescue PLRV movement, suggesting that the RNA silencing suppressor of PVX

and MP of GRV are unable to allow PLRV unloading from the vasculature (Ryabov et al. 2001a). Thus, PLRV may be restricted to the phloem by a coalescence of two factors: lack of ability to unload from the phloem per se, and failure to accumulate in the MS due to the host defense reactions.

In the case of bipartite geminiviruses, BGMV remains largely confined to the vascular tissues of *N. benthamiana* whereas several other bipartite geminiviruses, such as *Cabbage leaf curl virus* (CabLCV), TGMV, unload into the surrounding MS (Morra et al. 2000; Qin et al. 2001). When BGMV is coinoculated with TGMV, it gains the ability to infect MS cells, suggesting that inoculation with TGMV alleviates BGMV phloem limitation (Morra et al. 2000). The TGMV factors that allow systemic BGMV infection include a *cis*-acting, non-coding TGMV BRi element upstream of the BR1 (formerly BR1) ORF and at least one of the two *trans*-acting factors, the AL2 protein and the BR1/BL1 proteins (formerly BR1/BL1) encoded by DNA-B of the virus (Morra et al. 2000). A later study suggested that AL2, in association with host factors, acts through the BRi region to enhance the TGMV BR1 gene expression (Qin et al. 2001). Since the BGMV genome also encodes BR1 and BL1, which represent geminiviral MPs, phloem limitation of BGMV may in fact not be caused by the lack of movement function per se. Instead, it may be based on tissue incompatibility of viral gene expression and the resulting low levels of BGMV BR1/BL1 production, insufficient for allowing movement into MS cells. Similarly, DNA-B of a non-phloem limited *Bean dwarf mosaic virus* (BDMV) overcame the phloem limitation of *Abutilon mosaic virus* (AbMV) in bean (*Phaseolus vulgaris*) whereas, in the reciprocal combination, DNA-B of AbMV failed to confine DNA-A of BDMV within the phloem (Levy et al. 2003). Thus, AbMV DNA-B, which encodes the BR1/BL1 geminivirus movement factors, is not the sole determinant of phloem limitation of the AbMV while BDMV DNA-A likely encodes additional determinants important for BDMV movement beyond the phloem (Levy et al. 2003).

Another example of complementation of viral systemic transport by coinoculation with an unrelated virus is restoration of movement of the potyvirus isolate PepMoV-FL through the internal phloem of *C. annuum* cv. Avelar plants by cucumovirus strain CMV-KM (Guerini et al. 1999). In this host, PepMoV-FL does not move within the internal phloem at all, as opposed to just being restricted in phloem unloading into MS (Guerini et al. 1999). CMV-KM truly promotes PepMoV-FL's phloem movement because it does not enhance PepMoV-FL accumulation in plant protoplasts, indicating that the presence of CMV-KM does not simply block the host cell defense reactions against PepMoV-FL (Guerini et al. 1999). Also, systemic spread of a long-distance-movement-deficient M strain of CMV (M-CMV) in zucchini squash (*Cucurbita pepo*) was rescued by coinoculation with either *Zucchini yellow mosaic potyvirus* strain A (ZYMV-A) or its attenuated variant ZYMV-AG (Choi et al. 2002). In this case, however, it is unclear whether the rescuing potyvirus provided a bona fide movement function or the RNA silencing-

suppressing activity of its HC-Pro protein (reviewed in Revers et al. 1996; Urcuqui-Inchima et al. 2001).

To date, two host factors have been identified that may affect viral unloading: PME and cdiGRP (Chen et al. 2003; Ueki et al. 2002). As described in detail in sections Sect. 3.1 and Sect. 3.2, PME is required for the systemic transport of tobamoviruses whereas cdiGRP negatively regulates this process. Both proteins appear to affect viral unloading into the non-vascular tissues, such that reduced levels of PME expression or elevated levels of cdiGRP expression in the tobacco vasculature "trap" the virus within the phloem of the systemic leaves (Chen et al. 2003; Ueki et al. 2002). These observations suggest that viral systemic movement may be a directional process employing different molecular pathways for entry into and exit out of the host plant phloem. Moreover, the differences in vascular loading and unloading of plant viruses are also evident from the afore described (see Sect. 4.1) observations of functional equivalence of different vein classes for virus entry and their lack of equivalence for its exit (Cheng et al. 2000). Thus, macromolecular transport into the plant vasculature may occur by a relatively loosely regulated process, whereas transport out of the vasculature may be more selective and/or tightly regulated (see also Sect. 4).

Interestingly, some plant organs, such as the apical shoot meristem, appear to restrict the movement of viruses, such as TMV, and remain permanently virus-free (Cheng et al. 2000). Recent studies have demonstrated that this phenomenon may not be due to blocked viral entrance into the area, but, instead, may be based on suppression of viral replication in the restrictive tissues by the host RNA silencing defense response (Xie et al. 2001) (see also Sect. 5).

5
The Effect of RNA Silencing, the Host Innate Immunity, on Viral Systemic Movement

Typically, the ability of a virus to move systemically is assessed by measuring the levels of viral proteins and/or genomes in systemic tissues. The absence of viral products in uninoculated, systemic leaves, with a normal level of local accumulation in the inoculated leaf, is presumed to be based on SAR and/or blocked systemic movement. Traditionally, when the possible involvement of SAR and hypersensitive reactions can be ruled out, the absence of virus in systemic leaves is postulated to be due to a block in viral systemic movement. However, recent progress in understanding molecular mechanisms of the plant innate immune response by RNA silencing and its inhibition by RNA silencing suppressors encoded by many plant viruses (reviewed in Baulcombe 2001; Baulcombe 2002, 2004; Bisaro 2006; Dunoyer et al. 2005; Scholthof 2005; Soosaar et al. 2005) has revealed that some of the seemingly "blocked viral

systemic movement" is more likely to represent a blocked viral accumulation due to RNA silencing rather than inhibition of the viral transport per se, and that the normal viral spread often requires suppression of RNA silencing by the virus.

For example, the CMV 2b (Soards et al. 2002) and TBSV p19 proteins (Scholthof et al. 1995) modulate viral spread by counteracting RNA silencing (Soards et al. 2002; Voinnet et al. 1999). In fact, many viral factors, once thought of as determinants of local and/or systemic movement, have been revealed to function as RNA silencing suppressors. Specifically, PVX p25, one of the TGB proteins required for cell-to-cell movement (Angell et al. 1996; Beck et al. 1991), also acts as an RNA silencing suppressor, the function that is required for efficient local spread of PVX (Bayne et al. 2005). Also, the TMV 126-kDa protein was considered to be a host range determinant that restricts viral systemic movement. For example, in *N. tabacum* cv. Xanthi nn, TMV Holm's masked strain (TMV-M) accumulates only at low levels in vascular tissues of the inoculated and uninoculated systemic leaves, whereas the TMV-U1 strain – which differs from TMV-M mainly in the sequence of its 126-kDa protein (Shintaku et al. 1996) – accumulates to high levels in both types of leaves of the same host (Ding et al. 1995; Nelson et al. 1993). Based on these data, the attenuated symptoms were attributed to a combination of low replication efficiency and suppression of viral systemic movement, and the 126-kDa protein was implicated in these effects (Chen et al. 1996; Ding et al. 1995; Nelson et al. 1993). Recently, however, the 126-kDa protein has been shown to suppress RNA silencing in *N. tabacum* and *N. benthamiana*, indicating that the lack of TMV-M movement is most likely due to this viral strain's weaker ability to suppress host RNA silencing (Ding et al. 2004).

Another example of functional reassessment of viral protein activity from a systemic movement factor to RNA silencing suppressor is the potyviral HC-Pro protein. HC-Pro was originally found to be involved in polyprotein processing (Carrington et al. 1989), long-distance movement (Cronin et al. 1995; Kasschau et al. 1997; Klein et al. 1994), and efficient replication at the single-cell level (Kasschau et al. 1997). A later study demonstrated correlation of the systemic mobility and replication efficiency of TEV HC-Pro mutants with their capacity for suppression of RNA silencing, suggesting that HC-Pro functions as an RNA silencing suppressor, and that this function is responsible for the HC-Pro effects on viral movement (Kasschau et al. 2001).

Furthermore, RNA silencing may also be responsible for exclusion of apical meristems from viral infection The RNA silencing is mediated by the host RNA-dependent RNA polymerases (RDRs), such as RDR1, which is involved in host defense against TMV and PVX (Xie et al. 2001), and RDR6, which has been implicated in host resistance against cucumoviruses (Mourrain et al. 2000). When GFP-expressing PVX was inoculated on a *N. benthamiana* line with RNAi-silenced RDR6, the virus invaded apical meristems, which remained largely virus-free in the wild-type plants (Schwach et al. 2005). These

results demonstrated that the meristem exclusion, once assumed to be due to a "transport barrier" for viral invasion (Foster et al. 2002), is actually based, at least in part, on the host RNA silencing activity. Indeed, growing, meristematic regions of plants are thought to represent strong photosynthetic sinks and, by implication, preferred transport destinations of RNA silencing signals (Schwach et al. 2005).

6 Concluding Remarks

Viral systemic movement in non-Arabidopsis hosts is often studied using, as experimental systems, virus–host combinations that show limited systemic viral infection (see Table 1 for examples of such combinations). In most cases, this type of host resistance is very specific, i.e., the host is resistant to a few specific isolates of a virus, but not to other closely related strains. One possible explanation for this specificity is that the host plant cannot tolerate alterations in its intercellular transport machinery that are dramatic enough to impede the movement of a wide spectrum of viruses. In other words, viruses may have evolved to "pirate" the fundamental intercellular transport pathways that are essential for the physiology of the host plant itself for their own spread, making it impossible for the host to completely block these venues of viral spread.

The process of viral systemic movement has long since attracted the attention of many plant biologists and virologists; yet, its detailed molecular mechanisms and pathways remain obscure. Based on the information reviewed in this chapter, two main gaps in our understanding of viral systemic movement are immediately clear: except for very few cases, host factors that participate in the movement per se have not been identified, and the molecular events that allow viruses to cross PD within and between different tissues and cell types are unknown. The main reason for this lack of knowledge may be the complexity of the system. The involvement of different types of unique vascular-associated cells and more than one viral factor for movement complicates experimental approaches. Moreover, the involvement of host defense, such as RNA silencing that is not directly related to transport through PD, in the process of viral systemic accumulation is liable to cause misinterpretation of the experimental results. Segregation of viral systemic "movement" from the overall systemic infection process – which is the sum total of replication, movement, host defense, and viral counter-defense reactions – is vital for elucidating the systemic translocation process.

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Intracellular and Intercellular Transport of Viroids

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Abstract Viroids are small, noncoding and nonencapsidated RNAs that infect plants. To establish systemic infection, viroid genomes or their derivatives must interact directly with cellular factors. There is increasing evidence that subcellular localization and systemic trafficking of viroid RNAs are regulated, likely via interactions between viroid RNA *cis* elements and specific cellular proteins. Here we summarize recent progress on the characterization of viroid structures and host proteins that may play important roles in trafficking. We also discuss critical issues that need to be addressed in future investigations.

1 Introduction

Viroids are small (250–400 nt), single-stranded and circular RNAs that infect plants. Approximately 40 species of viroids are known to date and they are classified into two families: The Pospiviroidae family that replicates in the nucleus and the Avsunviroidae family that replicates in the chloroplasts (Tabler and Tsagris 2004; Góra-Sochacka 2004; Flores et al. 2000, 2005). The systemic infection process of viroids encompasses several distinguishable steps: import into the nucleus (the Pospiviroidae) and chloroplasts (the Avsunviroidae), replication within these organelles, export out of these organelles, cell-to-cell movement, entry into the vascular tissue, long distance transport within the vascular tissue, and finally exit from the vascular tissue to infect neighboring cells (Ding et al. 2005). Unlike viruses, viroid RNAs have no protein-coding capacity and are not encapsidated within protein or membrane shells. Therefore, these RNAs most likely utilize pre-existing cellular machineries for systemic trafficking to establish infection. In the simplest scenario, virioid RNA can be envisaged to have evolved distinct *cis* elements that interact with specific cellular factors to accomplish trafficking within a cell, from cell to cell, and from organ to organ to establish systemic infection.

Viroid trafficking is still a rather fresh field with many fundamental questions unanswered and new research tools to be developed. Yet the small size of the RNAs, their experimental tractability and reliance on host factors

for trafficking make viroids attractive tools for uncovering the basic plant mechanisms that regulate RNA trafficking within and between cells (Ding et al. 2005). Here we highlight recent progress on the characterization of viroid structures and host proteins that may play important roles in trafficking. We also discuss critical issues that need to be addressed in future investigations.

2
Organellar Import and Export of Viroid RNAs

Despite their pivotal importance for the initiation of viroid replication, how organellar import of viroid RNAs occurs in a cell is poorly understood. Two approaches have been developed to investigate nuclear import of Potato spindle tuber viroid (PSTVd). In a cellular approach, Woo et al. (1999) characterized nuclear import of fluorescently labeled PSTVd in vitro transcripts in permeabilized tobacco BY2 protoplasts. They showed that import of these transcripts could be inhibited specifically in the presence of nonlabeled transcripts of PSTVd (Woo et al. 1999). In a molecular approach, Zhao et al. (2001) showed that PSTVd could act in *cis* to direct nuclear import of a fusion RNA in Nicotiana benthamiana leaves. All of these results imply the existence of a *cis* element in PSTVd that serves as a nuclear import signal. This signal, as well as the cellular factors that recognize this signal, remain unknown. Nuclear export of any viroid RNAs has not been explored.

Mechanisms for the chloroplastic import or export of viroids in the Avsunviroidae are not understood. It may be speculated that these viroids utilize a pre-existing system for entering and exiting the chloroplasts. Elucidating the underlying mechanisms may have broad ramifications in the basic biology of chloroplasts and in chloroplast–cytoplasm communications.

3
Strand Polarities of Viroid RNAs Control Subcellular Localization

PSTVd replicates via an asymmetric rolling circle in which RNAs of the (+)- and (–)-strand polarities are produced (Branch and Robertson 1984; Fig. 1). Based on fluorescence in situ hybridization studies, Qi and Ding (2003) showed that the (–)-strand PSTVd RNAs were localized in the nucleoplasm whereas the (+)-strand PSTVd RNAs were present in the nucleolus as well as the nucleoplasm. These results suggest that the (+)-PSTVd RNAs synthesized in the nucleoplasm are selectively transported into the nucleolus. It is likely that these RNAs contain a *cis* element, which remains to be identified, that directs their nucleolar import. The nucleoplasmic localization of the (–)-strand PSTVd RNAs may be attributed to the lack of a nucleolar

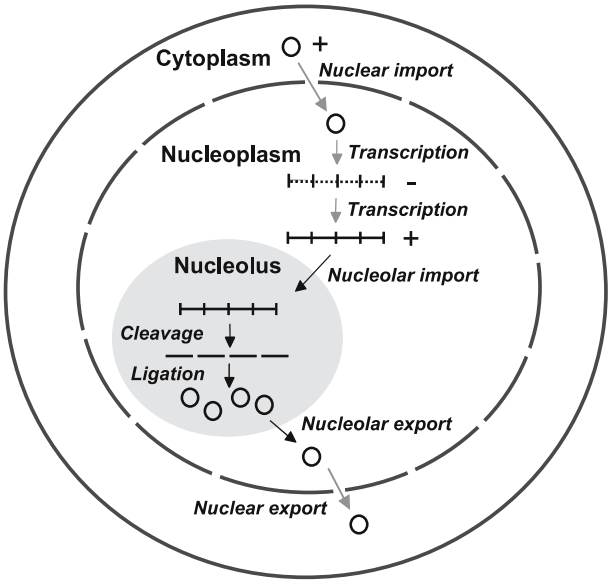

Fig. 1 Model for the replication of PSTVd. The incoming (+)-circular genomic RNA is imported from the cytoplasm into the nucleus, where it is transcribed into concatemeric linear (–)-strand RNAs, which then serve as the replication intermediate to synthesize concatemeric, linear (+)-strand RNAs. These (+)-strand RNAs are subsequently imported into the nucleolus for cleavage and ligation to form the circular molecules. Some circular molecules are exported out of the nucleolus and further out of the nucleus to enter the cytoplasm. Some of these molecules traffic into neighboring cells (not shown) to embark on systemic infection. (Modified from Qi and Ding 2003)

import signal, presence of a nucleoplasmic localization signal, or both. Elucidating the viroid RNA *cis* elements as well as the cellular factors that are responsible for the distinct subnuclear localization patterns will be essential to understand the infection of viroids in the Pospiviroidae family. It will also generate fundamental knowledge about subnuclear trafficking/localization of cellular RNAs.

4 Polarized Subnucleolar Localization of PSTVd RNAs

Detailed examination of PSTVd localization within the nucleolus in comparison with that of some small nucleolar RNAs (snoRNAs) revealed striking patterns of polarized localization (Qi and Ding 2003). Previous studies showed that U3 snoRNA was localized evenly in subnucleolar compartments (Beven et al. 1996). Strikingly, in over 10% of the infected cells, the (+)-PSTVd RNA and U3 were localized in separate domains within the nucleolus. In

each case when the (+)-PSTVd was localized in one part of the nucleolus, the U3 snoRNA was localized in the remaining part. Thus, the (+)-PSTVd RNA may compete with U3 snoRNA for certain nucleolar factors, with the consequence that the U3 is displaced from a part of its normal nucleolar domain. Another small nucleolar RNA, U14, also exhibited similar redistribution within the nucleolus in the presence of (+)-PSTVd in some cells. The significance of this polarized localization of PSTVd RNAs in relation to snoRNAs remains to be understood.

5
Viroid RNA *Cis* Elements Mediate Cell-to-Cell Trafficking

When fluorescently labeled PSTVd RNA transcripts were injected into mesophyll cells, they moved rapidly into neighboring cells. In contrast, the transcripts injected into symplasmically isolated guard cells were retained in such cells. These findings provided strong evidence that PSTVd traffics through plasmodesmata (Ding et al. 1997). Cell-to-cell trafficking of PSTVd RNA between mesophyll cells does not appear to occur by diffusion, but is directed by *cis* elements residing in the RNA. The evidence came from the observation that PSTVd can mediate trafficking of a heterologous fusion RNA (Ding et al. 1997).

Recent studies provided genetic evidence for the existence of *cis* elements in regulating unidirectional trafficking of PSTVd between specific cell types. Two PSTVd strains, $PSTVd^{NT}$ and $PSTVd^{NB}$, differ by five nucleotides. Using in situ hybridization, Qi et al. (2004) showed that in young systemically infected tobacco leaves, $PSTVd^{NB}$ was detected in all types of cells. In contrast, $PSTVd^{NT}$ is found only in the phloem and bundle sheath cells. This difference in the distribution patterns is attributed to the inability of $PSTVd^{NT}$ to traffic out of the bundle sheath cells. Interestingly, when a leaf matures to certain stage, $PSTVd^{NT}$ can move into mesophyll cells (Qi et al. 2004). Mutational studies showed that four of the five $PSTVd^{NB}$-specific nucleotides are all required and sufficient to potentiate trafficking of $PSTVd^{NB}$ from bundle sheath into the mesophyll. Because $PSTVd^{NT}$ introduced into epidermal cells by rub-inoculation or biolistic bombardment could establish systemic infection, the four $PSTVd^{NB}$-specific nucleotides are not required for trafficking from epidermal cells into the phloem for systemic spread. Thus, trafficking between the bundle sheath and mesophyll in opposite directions is differently regulated, likely involving different RNA motifs (Qi et al. 2004; Fig. 2). *Cis* elements that remain to be identified and plant development also appear to regulate trafficking of cellular RNAs (Haywood et al. 2005).

Altogether, these studies lead to a model in which cell-to-cell trafficking of an RNA is mediated by specific *cis* elements. Furthermore, results from PSTVd analyses indicate that trafficking of an RNA across different cellu-

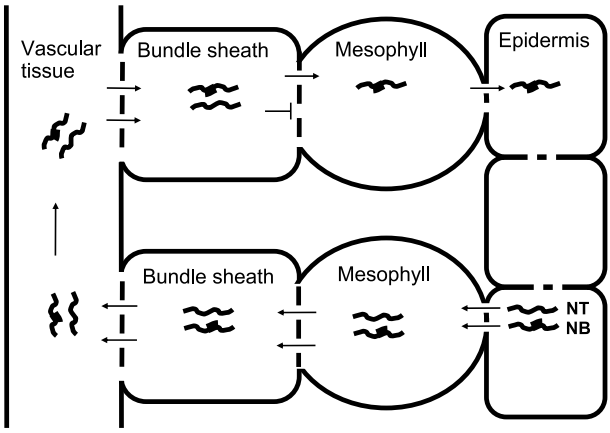

Fig. 2 *Cis* element-mediated unidirectional trafficking of PSTVd across bundle sheath–mesophyll boundary. When inoculated into an epidermal cell in a young tobacco leaf, both $PSTVd^{NT}$ and $PSTVd^{NB}$ replicate and traffic through mesophyll, bundle sheath, and finally into the vascular tissue for long distance transport. In a systemically infected young leaf, both PSTVd stains exit the vascular tissue to enter bundle sheath. While $PSTVd^{NB}$ traffics further into all cell types to establish infection, $PSTVd^{NT}$ remains in the bundle sheath due to inhibited trafficking into the mesophyll. The *rectangle mark on the wavy bar* indicative of $PSTVd^{NB}$ represents the *cis* element that mediates trafficking from the bundle sheath to the mesophyll. *Arrows* show the directions of trafficking. (Based on Qi et al. 2004)

lar boundaries and in opposite directions are uniquely regulated (Ding et al. 2005).

6 Long-Distance Trafficking of Viroid RNAs

Palukaitis (1987) reported the first study on the long distance trafficking of viroid RNAs. His analysis of the systemic infection patterns of PSTVd in tomato in comparison with the known source-to-sink transport patterns of photoassimilates indicated that PSTVd is transported within the phloem. Zhu et al. (2001) used in situ hybridization to localize PSTVd to the phloem, providing cellular evidence for the phloem pathway for long distance transport. In the shoot apices, PSTVd is selectively transported into sepals but not into the other floral organs (Zhu et al. 2001, 2002). Because all floral organs are strong sinks for photoassimilates, these observations imply that a phloem-based mechanism recognizes PSTVd and transports it into the sepals. It is also possible that another phloem-based mechanism actively prevents PSTVd from entering such floral organs as petals, stamen, and ovary. Importantly, phloem-based mechanisms also appear to transport cellular RNAs (Haywood

et al. 2005) and proteins (Aoki et al. 2005) to selective sink organs. Thus, further studies on the long distance trafficking of viroid RNAs should provide useful insights into the basic mechanisms of phloem-mediated transport of a wide range of macromolecules.

7
Cellular Factors that May Facilitate Viroid RNA Trafficking

Because *cis* elements apparently play a critical role in mediating trafficking of viroid RNAs, such *cis* elements may well interact with cellular factors. Such factors remain unknown. However, recent work has revealed a number of cellular proteins as promising candidate factors for viroid trafficking. The first is the phloem lectin PP2 from cucumber (CsPP2), which binds Hop stunt viroid (HSVd) in vitro (Gómez and Pallás 2001; Owens et al. 2001). However, the binding is not specific to HSVd, because the protein also binds a viral RNA (Owens et al. 2001). Recent co-immunoprecipitation experiments demonstrated in vivo interactions between CsPP2 and HSVd in infected cucumber plants (Gómez and Pallás 2004), providing evidence for the functional significance of the interactions. In support of this notion, CsPP2 (Golecki et al. 1999; Gómez and Pallás 2004) and HSVd (Gómez and Pallás 2004) can both traffic from rootstocks into scions in heterografts and the CsPP2 has an RNA-binding motif (Gómez and Pallás 2004).

The tomato protein VIRP1 (Martinez de Alba et al. 2000) interacts with the right-terminal regions of PSTVd and HSVd (Maniataki et al. 2003; Gozmanova et al. 2003). Specifically, the interaction involves two asymmetric internal loop motifs of PSTVd (Gozmanova et al. 2003). This interaction may be important for infection because mutations in these motifs that disrupt VIRP1 binding inhibit infection (Gozmanova et al. 2003). Other mutations in the right-terminal domain of PSTVd that abolish infection of tomato by mechanical inoculation but not by agroinoculation (Hammond 1994) also compromise interactions with VIRP1, suggesting that the interaction between VIRP1 and the right-terminal domain of PSTVd is likely important for trafficking (Maniataki et al. 2003).

It is also of great interest that candidate proteins that may play a role in the trafficking of viroids of the Avsunviroidae family have emerged. Gómez et al. (2005) recently found two proteins that bind Avocado sunblotch viroid (ASBVd). One is the previously characterized CmmLec17 (Dinant et al. 2003) and the other a 14 kDa protein. The CmmLec17 moves long distances from rootstocks to scions in heterografts, raising the possibility that this protein may be involved in ASBVd trafficking.

In summary, some strong candidate cellular proteins for viroid trafficking have been uncovered. Conclusive evidence for their role in viroid trafficking will await further genetic and molecular studies.

8
Future Prospects

Recent development in methodology has allowed rapid progress in the characterization of viroid RNA *cis* elements and cellular proteins that may play crucial roles in the subcellular and systemic trafficking of viroids from both the Pospiviroidae and Avsunviroidae families. A clear future goal in this area is to further determine the nature of these *cis* elements and the cellular proteins that interact with these elements. Importantly, because distinct *cis* elements might be involved in trafficking between specific cells and in a particular direction, it follows that cell-specific factors may be involved. Elucidating these *cis* elements and the cognate cellular factors should help establish a solid conceptual framework for investigating the regulated trafficking of viroid, viral and cellular RNAs.

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Production and Transport of the Silencing Signal in Transgenic and Virus-Infected Plant Systems

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Abstract In plants, RNA interference constitutes an important defense mechanism against viruses, transposons, and transgenes. Viruses, on the other hand, use suppressors to counteract silencing. In contrast to mammalian systems, silencing in plants spreads systemically through the whole organism. Since also viruses spread and consequently produce suppressors systemically, a race between silencing and virus replication occurs. Apparently, successful viruses win the race, but only until they reach the tissue around the meristem. There, the silencing mechanism is most capable by efficiently amplifying the silencing signal, bona fide 21-nt siRNA, leading to the well-known phenomenon of meristem exclusion.

1 Introduction

Double-stranded (ds)DNA and single-stranded (ss)RNA can be converted into each other by transcription and reverse transcription and are usually not recognized as foreign in living cells. In contrast, double-stranded (ds)RNA is recognized in eukaryotic cells as foreign and leads to specific reactions, such as the interferon response in mammals (He 2006) and gene silencing in most eukaryotes including plants (Zamore and Haley 2005; Carrington 2005). Gene silencing can be directed against viruses, transposons, and transgenes (Voinnet 2005a; Baulcombe 2005; Wang and Metzlaff 2005), but is also used for regulation of gene expression to control stress response, development, flowering, and other processes (Jones-Rhoades et al. 2006). Central to the silencing process are dicers or "dicer-like" (DCL) enzymes that cleave double-stranded regions in RNA, i.e. dsRNAs or back-folded ssRNA hairpins, into small double-stranded fragments, called small interfering (si)RNAs and micro (mi)RNAs, respectively, or jointly, sRNAs. Cloning of plant sRNAs revealed size classes ranging from 19 to 25 nucleotides (Llave et al. 2002; Gustafson et al. 2005), but in Northern blot analysis, plant sRNAs are predominantly seen as 21, 22, and 24nt bands for siRNAs (Xie et al. 2005a;

Gasciolli et al. 2005; Akbergenov et al. 2006) and as bands of 21 to 24nt for miRNAs. Double-stranded sRNAs have 2-base-long 3′ overhangs, are phosphorylated at the 5′-end and methylated at the 2′-OH group of the 3′-ribose (Yu et al. 2005; Ebhardt et al. 2005; Yang et al. 2006; Akbergenov et al. 2006). sRNAs are originally formed as duplexes consisting of the effective guide- and the non-functional passenger-strand. Studies in *Drosophila* showed that the guide strand is usually the one whose 5′-terminus has lower base-pairing stability (Rand et al. 2005) and for *Drosophila* it was shown that its selection is guided by the protein R2D2, which binds the siRNA end with the strongest ds character (Tomari et al. 2004).

For post-transcriptional gene silencing (PTGS), siRNAs are incorporated into RNA-induced silencing complexes (RISC) containing an "argonaute" (AGO) family protein (in plants usually AGO1; Baumberger and Baulcombe 2005). siRNA-guided RISC cleaves cognate RNAs endolytically, whereby the passenger strand of the siRNA duplex is the first victim (Rand et al. 2005). Similarly, target RNAs are recognized and regulated by miRNAs also incorporated in AGO-containing RISC complexes. In most cases, plant miRNA-RISC causes cleavage as with siRNA-RISC, in a few plant cases and in most mammalian cases miRNA-RISC inhibits translation of target mRNA (Pillai et al. 2005). Plant transacting siRNAs (tasiRNAs) act like miRNAs, but are produced from perfect dsRNA precursors (see below).

"Repeat associated" siRNAs (rasiRNA) derived from repetitive genes, are presumably produced in the nucleus and form with a distinct AGO protein (in plants, AGO4; Zilbermann et al. 2004) an RNA-induced transcriptional silencing (RITS) complex, which causes RNA-dependent DNA methylation (RdDM, Cao and Jacobsen 2002; Cao et al. 2003), histone modification (Lindroth et al. 2004; Probst et al. 2004), and chromatin remodeling (Amedeo et al. 2000; Kanno et al. 2004) on cognate chromatin and, consequently, transcriptional gene silencing (TGS).

dsRNA precursors of siRNAs, if not introduced deliberately or expressed from inverted repeats, can originate from ill-defined aberrant RNA, e.g. cap- or polyA-deficient mRNA by the action of endogenous RNA-dependent RNA polymerases (RDR: Wassenegger and Krczal 2006). For PTGS, mainly RDR6 is involved. RDR6 (together with DCL4) acts also in signal amplification during systemic silencing (Schwach et al. 2005) and in the production of tasiRNAs (Dunoyer et al. 2004; Xie et al. 2005a; Gasciolli et al. 2005).

2 Multiple Silencing Pathways in Plants

In plants, most of the silencing-related genes occur in multiple copies. Thus, four DCL-, six RDR-, and ten AGO genes have been found in the *Arabidopsis* genome, as well as two genes for the large subunit of POL IV (Table 1).

Table 1 Proteins involved in silencing

Gene	Synonyms	Proteins	Functions	Refs.
RNAi pathways and post-transcriptional gene silencing				
DCL1		Dicer	miRNA, 21nt siRNA	Xie et al. 2005a
DCL2		Dicer	reserve enzyme, 22nt siRNA	Akbergenov et al. 2006
DCL3		Dicer	nuclear activity, 24nt siRNA, rasiRNA	Xie et al. 2004
DCL4		Dicer	relay amplification, 21nt siRNA, tasiRNA	Dunoyer et al. 2004; Xie et al. 2005b; Gasciolli et al. 2005
HYL-1			dsRNA binding, supports DCL1	Han et al. 2004; Kurihara et al. 2006; Lu & Fedoroff 2000
DRB4			dsRNA binding, supports DCL4	Adenot et al. 2006
HEN1			methylates 2′-OH of sRNAs	Chen et al. 2004; Yang et al. 2006
RDR1		RdRp	unknown	
RDR2		RdRp	nuclear activity, 24nt siRNA, rasiRNA	Xie et al. 2004
RDR3a		RdRp	unknown	
RDR3b		RdRp	unknown	
RDR3c		RdRp	unknown	
RDR6	*SDE1, SGS3*	RdRp	relay amplification, 21nt siRNA; tasiRNA	Schwach et al. 2005
AGO1		Slicer	miRNA usage	Baumberger and Baulcombe 2005
AGO4		Slicer	Component of RITS complex	Zilberman et al. 2004
AGO7	*ZIPPY*	Slicer	tasiRNA production	Hunter et al. 2003; Peragine et al. 2004
HST	*HASTY*		tasiRNA production	Hunter et al. 2003; Peragine et al. 2004
Transcriptional gene silencing				
POL IVa	*NRPD1a* + *NRPD2a*	Polymerase IVa	TGS, copies aberrant RNA?	Vaughn and Martienssen 2005; Onodera et al. 2005; Herr et al. 2005
POL IVb	*NRPD1b* + *NRPD2a*	Polymerase IVb	TGS, interacts with AGO4	Kanno et al. 2005
NRPD2a	*DRD2*		second largest SU of POL IVb and POL IVa	Huettel at al. 2006
NRPD1b	*DRD3*		largest SU of POL IVb	Huettel at al. 2006
NRPD1a	*SDE4*		largest SU of POL IVa	Huettel at al. 2006
DRM1			Non functional?	Chan et al. 2006

Table 1 (continued)

Gene	Synonyms	Proteins	Functions	Refs.
DRM2			De novo RNA dependent DNA methylation	Cao et al. 2003; Chan et al. 2006
CMT3		Chromomethyl-transferase	CNG methylation, de novo and maintainance	Cao et al. 2003; Chan et al. 2006
DRD1	*SWI/SNF*-like		Chromatin remodelling; acts with *DDM1/2* and *CMT3*	Kanno et al. 2004; Chan et al. 2006
MET1		Methyl transferase	maintainance of CG methylation	Cao and Jacobsen 2002; Cao et al. 2003
DDM1	*SOM, Somniferous*		"Deficient in DNA methylation", chromatin remodelling	Brzeski and Jerzmanowski 2003; Mittelsten-Scheid et al. 2002
MOM1	*Morpheus molecule*		Maintainance of intermediate heterochromatin	Amedeo et al. 2000; Habu et al.2006
KYP	*Kryptonite*		H3K9 methylase	Lindroth et al. 2004
HDA6			Histone deacetylace	Probst et al. 2004

Furthermore, several of the plant dsRNA binding proteins (DRBs), most prominently HYL1 and DRB4, are thought to be involved in modulation of Dicer activity (Hiraguri et al. 2005). The corresponding gene products seem to be involved in specialized silencing pathways (Vaucheret 2005; Brodersen and Voinnet 2006), although certain redundancy is possible (Henderson et al. 2006), and multiple silencing pathways are involved in the silencing of viruses (see below). Each of the DCL-enzymes, produce predominantly a certain size class of siRNAs or miRNAs (Xie et al. 2004, 2005a; Gasciolli et al. 2005; Akbergenov et al. 2006; Blevins et al. 2006). The predominant size of miRNAs created by DCL1 is 21 nt, but if bulges occur in pre-miRNA hairpins, they can be larger (up to 24 nts, Kurihara and Watanabe 2004). DCL2, DCL3, and DCL4 produce siRNAs of 22, 24, and 21 nts, respectively (Fig. 1). The two size classes of siRNAs originally distinguished (Hamilton et al. 1999, 2002) can now be resolved into three major size classes produced by four pathways (Fig. 2).

- **DCL4** seems to be the enzyme mainly involved in post-transcriptional silencing (RNA interference; Dunoyer et al. 2005) and defense against both, DNA- and RNA viruses (Akbergenov et al. 2006, Deleris et al. 2006, Bouche et al. 2006, Blevins et al. 2006). Together with RDR6 it functions in amplification of the systemic silencing signal throughout the plant (see below, Fig. 6). Furthermore, DCL4 is involved in endogenous tasiRNA production (Vazquez et al. 2004; Gasciolli et al. 2005; Peragine et al. 2006: Xie et al. 2005a; Yoshikawa et al. 2005). tasiRNAs function like miRNAs in targeting

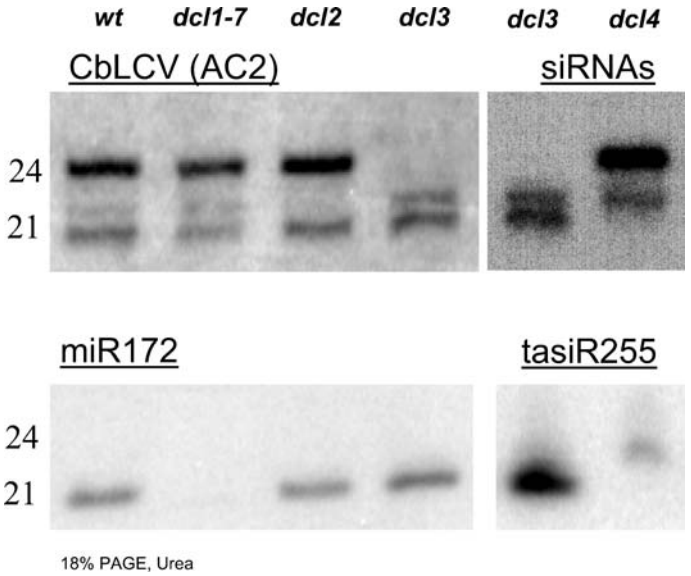

Fig. 1 siRNA species derived from a DNA virus produced in *Arabidopsis dcl* mutants. Small RNAs were isolated from *Cabbage leaf curl virus* (CbLCV)-infected *A. thaliana* plants, separated on 18% PAGE/urea and probed with a small viral DNA fragment. Wild-type plants and mutant plants affected in *DCL* genes were used. In wild-type plants three bands indicating siRNAs of 24, 22, and 21nt were found. Note that in each of the *dcl2*, *dcl3*, and *dcl4* mutants one band is missing (22, 24, and 21nt, respectively) and that other bands compensate for the loss by increased intensity. For controls, gels were stripped and probed with antisense DNA to miRNA R172 and tasiRNA 255, which are known to depend on DCL1 and DCL4, respectively. Similar gel electrophoresis analyses of sRNAs are shown in Akbergenov et al. (2006) and Blevins et al. (2006)

specific transcripts in trans. DCL4, as well as DCL2 and some DCL1-derived siRNAs are complexed with AGO1 to form an "RNA-induced silencing complex" (RISC). Interestingly, TAS3-derived tasiRNA production depends also on another AGO gene named AGO7/Zippy (Adenot et al. 2006).

- **DCL3** acts in the nucleus. Its products, 24nt "repeat associated" rasiRNAs, are derived from repeated genes and loaded onto a transcriptional gene silencing (RITS) complex, which includes a distinct AGO protein (AGO4; Zilberman et al. 2004; Qi et al. 2006), presumably POL IVb (Li et al. 2006; Pontes et al. 2006; Huettel et al. 2006) and the chromatin remodeling factor DRD1 ("defective in RNA dependent DNA methylation"; Huettel et al. 2006). RITS acts on cognate chromatin, causing RNA-dependent DNA methylation (RdDM, Cao et al. 2003; Ekwal 2004; Wassenegger 2005), histone modification (Lindroth et al. 2004; Probst et al. 2004) and, consequently, transcriptional gene silencing (TGS) (Fig. 4). Recent studies revealed that an amplification cycle involving POL IVa produces most of

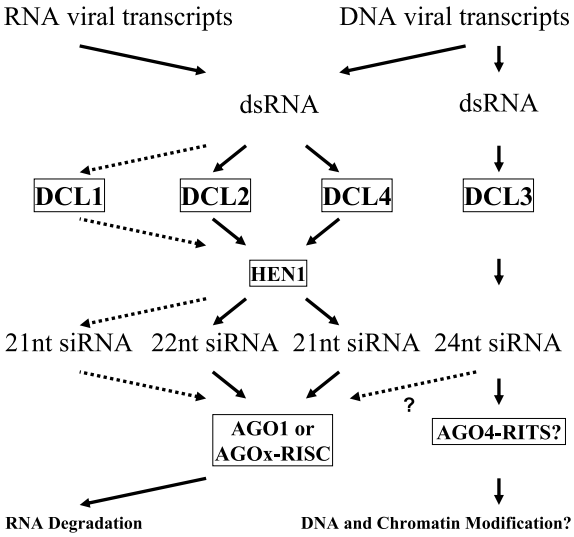

Fig. 2 General silencing pathways involved in antiviral defense. Viral siRNAs are mainly produced by DCL4 and DCL2 in response to RNA viruses and, additionally, by DCL3 in response to DNA viruses. To a lesser extent and with sequence preferences also DCL1, which normally produces miRNAs, is involved. Details of this model are discussed in the text and in Akbergenov et al. (2006), Deleris et al. (2006), and Blevins et al. (2006)

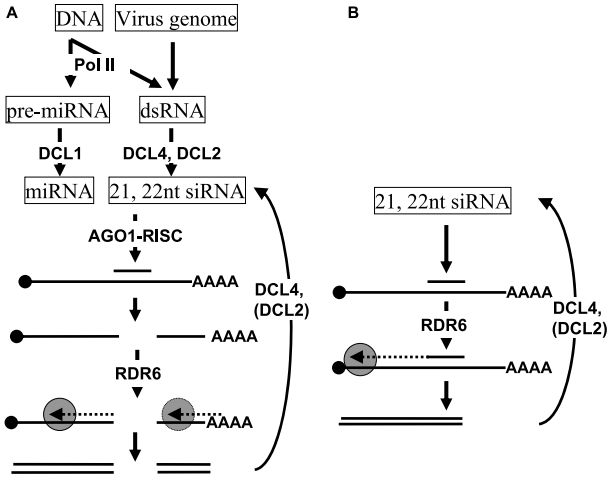

Fig. 3 Models for PTGS amplification cycles. **A** Present model: siRNA as part of a RISC complex leads to target RNA cleavage and the resulting poly(A)-less 5′-fragment and the uncapped 3′-fragment can be recognized as "aberrant" RNA, a target for RDR6 to produce dsRNA, which in turn serves as a source for DCL4- (and perhaps also DCL2-) mediated siRNA production. In contrast to the original model (**B**), the present model can explain bidirectional transitivity. **B** Original model: siRNA serves as a primer for RDR6-mediated dsRNA production leading to the formation of secondary siRNAs

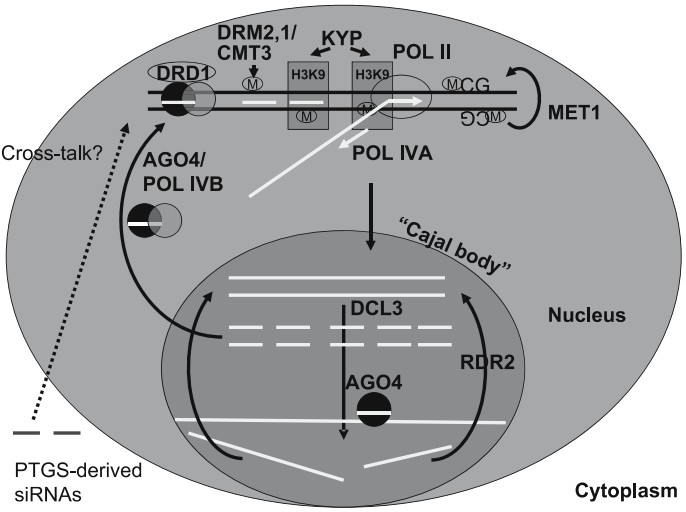

Fig. 4 Amplification cycles in TGS. The model considers two amplification loops: One is located in the intra-nucleolar Cajal bodies and depends on DCL3, RDR2, and AGO4 (or AGO7)-RISC. The second amplification loop located in the nucleus starts with aberrant transcripts from heterochromatic DNA, which are thought to be converted to dsRNA by the action of Pol IVa and fed into the intra-nucleolar Cajal bodies for 24nt siRNA production and further amplification. From these bodies, 24nt siRNA is transported to cognate chromatin as part of "RNA-induced transcriptional silencing (RITS) complexes" involving POL IVB and AGO4. With the help of chromatin remodeling factor DRD1 ("defective in RNA-dependent DNA methylation 1") de-novo methylation is performed by domains-rearranged-methylase (DRM) 1 and 2 on all cytosines and chromomethylase (CMT)3 on the ones in CNG context. DNA methylation is followed by histone H3K9 methylation directed by Kryptonite (KYP) (Schwach et al. 2005; Huettel et al. 2006; Li et al. 2006; Pontes et al. 2006). In subsequent generations CG-methylation is preserved by DNA methylase MET1. A crosstalk between the PTGS and TGS pathways might lead to the transition of post-transcriptionally to transcriptionally silenced states, as observed by Kloeti et al. (2002) and Fojtová et al. (2003, 2006)

the plant genome-derived dicer substrate (Chan et al. 2004; Vaucheret 2005; Pontier et al. 2005; Herr et al. 2005; Onodera et al. 2005; Kanno et al. 2005). Further siRNA amplification occurs in the intra-nucleolar Cajal bodies involving RDR2, a RISC-complex including AGO4 and DCL3. Note that in this model AGO4 is used dually, as part of a RITS- and as part of the RISC-complex (Qi et al. 2006; Fig. 4).

- **DCL1** is involved in two steps of miRNA processing, i.e. in Drosha-like cleavage of the original pri-miRNA to produce one or more distinct pre-miRNAs (Xie et al. 2004; Jones-Rhoades et al. 2006) and in the excision of a miRNA/miRNA* duplex from the pre-miRNA stem-loop. In both steps, the dsRNA-binding protein HYL1 (for "hyponastic leaves"; Lu and Fedoroff 2000) helps in recognition and/or correct processing of the miRNA

precursors (Kurihara et al. 2006). Both processes seem to occur in the nucleus, while miRNAs act in the cytoplasm. For their export, an exportin ("HASTY") is required (Park et al. 2005). In contrast, in mammals pri- and pre-miRNA processing occur separately in the nucleus and the cytoplasm, and for pri-miRNA processing a separate RNaseIII (Drosha) is required. DCL1 and HYL1 are probably also involved in loading plant miRNAs onto AGO1 (Baumberger and Baulcombe 2005; Qi et al. 2005) to form RISC complexes. miRNAs target transcripts and tasiRNA precursors and either cleave them (for tasiRNA: Allen et al. 2005) or interfere with initiation of their translation.

- **DCL2** seems to be a reserve enzyme, which is used when other DCLs are mutated or suppressed. In *dcl3* mutants, for instance, the loss of 21nt endogenous tasiRNA is compensated by DCL2-dependent production of 22nt tasiRNAs (Gasciolli et al. 2005; Xie et al. 2005a). Likewise, the loss of geminivirus derived 24nt siRNAs in *dcl3* and of 21nt siRNA in *dcl4* is compensated by an increased amount of DCL2-derived 22nt viral siRNAs (Blevins et al. 2006; Akergenov et al. 2006). *Turnip crinkle virus* specifically suppresses DCL4 function (Deleris et al. 2006) and as a consequence, siRNA production becomes in early stages of infection dependent on DCL2. In contrast to these compensating activities, DCL2 has also antagonistic activities, i.e. it inhibits weakened DCL1 alleles (Bouche et al. 2006).
- **DCL1, DCL2, and nat-siRNA**. Despite the large intergenic spaces in the eukaryotic genome, many convergent gene pairs occur, whose transcripts create sense-antisense overlaps. Naturally, the overlapping transcripts can form dsRNA and be processed into siRNAs, in this case called natural antisense transcript-derived siRNAs (nat-siRNAs) that can target either one of the convergent genes and thus lead to efficient means of gene regulation. Such a pair was analyzed by Borsani et al. (2005) in *Arabidopsis* and found to be involved in regulation of proline synthesis and, in consequence, salt tolerance. The authors observed several 21 siRNAs and a single 24nt siRNA. The latter depended genetically on RDR6, SGS3, POL IVa, DCL1, and curiously, DCL2, which normally produces 22nt siRNAs. The authors propose that the 24nt siRNA is used in a RISC complex to cleave the target mRNA, thus setting the phase for RDR6- and DCL1-dependent 21nt siRNA formation. It is not clear whether the DCL2-dependent formation of 24nt siRNAs is indirect, whether a cofactor influences the product size of DCL2 action, or whether DCL2 is a priori flexible for product length.
- **HEN1** is involved in all four pathways by methylating the 3′-termini of miRNAs and siRNAs at the 2′-OH group (Yang et al. 2006; Li et al. 2005; Yu et al. 2005; Akbergenov et al. 2006; Ebhardt et al. 2005), and thereby contributing to their stability and availability.

3 Systemic Spreading of Silencing

While silencing in mammalians seems to be restricted to single cells, silencing in nematodes and plants can spread to the entire organism or most of it (reviewed by Mlotshwa et al. 2002; Voinnet 2005b). Systemic spreading of the silencing state was first shown by Voinnet and Baulcombe (1997) for a GFP transgene in *Nicotiana benthamiana* and by Palauqui et al. (1997) for nitrate reductase and nitrite reductase endogenes in *Nicotiana tabacum*. Spreading of silencing can easily be observed after local induction of a reporter transgene or endogene. Two excellent systems to observe short- and long-range spreading of silencing are the GFP transgenic *N. benthamiana* line 16c (Voinnet and Baulcombe), in which silencing can be induced by agroinfiltration of a GFP expressing plasmid (Fig. 5A), and the *Arabidopsis* "chlorata" system

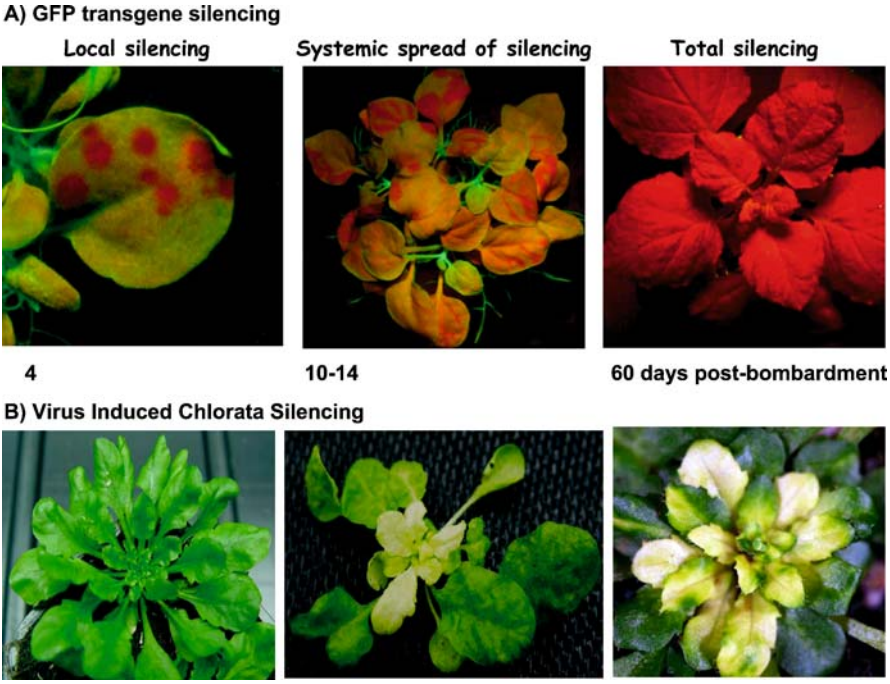

Fig. 5 Visualization of silencing. **A** Silencing of a GFP transgene (green fluorescence) with local overproduction of GFP from a plasmid. Fluorescence patterns indicating original local silencing centers (areas showing lack of green GFP fluorescence and visible red chlorophyll fluorescence), the *onset* of systemic silencing, and full systemic silencing are shown. **B** Virus-induced silencing (VIGS) of the *Chlorata* gene in growing tissue of *Arabidopsis thaliana* plants infected with a *Cabbage leaf curl virus* derivative expressing *Chlorata* sequences. Tissues undergoing *Chlorata* silencing do not produce chlorophyll and, therefore, appear white. *Left*: uninfected control plant

(also named "sulfur"; Papenbrock et al. 2000), in which silencing of the Chlorata I subunit of Mg-chelatase can be induced by a dsRNA transgene or by geminivirus vectors (Peele et al.2001; Dunoyer et al. 2005; Fig. 5B). In the former case, loss of green fluorescence and, in the latter, loss of leaf coloration are the hallmarks of silencing.

Like for viruses or viral nucleic acids (Heinlein and Epel 2004), the silencing signal is thought to move from cell to cell through plasmodesmata (Ruiz-Medrano et al. 2004) and for greater distances through the vascular system (Voinnet and Baulcombe 1997), including graft junctions (Palauqui et al. 1997). Silencing does not spread into mature guard cells, which have lost their plasmodesmal connections to neighboring cells (Wille and Lucas 1984), providing circumstantial evidence that plasmodesmata are in fact involved (Voinnet et al. 1998). The signal must be a nucleic acid or its derivative because it mediates a nucleotide sequence-specific effect. Originally it was proposed that a 24-26nt siRNA is the silencing signal (Hamilton et al. 2002) but the signal might also include other single- or double-stranded siRNAs, and different RNA species could be involved in short range and vascular transport (review: Mlotshwa et al. 2002). Up to now, no systemic transport of miRNA-directed silencing has been reported.

According to a current model (Fig. 6), systemic spreading of silencing occurs in distinct steps: In the first step, "short-range cell-to-cell movement", which accounts for initial, local spread of silencing (Fig. 5A), siRNAs move from an originally triggered center independently of the presence of homologous transcripts and of RDR6-mediated amplification through a layer of 10 to 15 consecutive cells (Himber et al. 2003). Theoretically, all types of siRNAs could be involved in this phase and their main function would be their incorporation into RNA-induced-silencing-complexes (RISC) and targeting of cognate RNA sequences for degradation. Consequently, radial areas of silencing can be observed, for instance, as localized absence of green fluorescence in leaves in the case of GFP-transgene silencing (Fig. 5A).

The second step, "long-range cell-to-cell movement", depends on relay amplification, requiring cognate transcripts and the concerted action of the RNA-dependent RNA polymerase RDR6, the putative RNA helicase SDE3, and the dicer DCL4. In this phase, siRNAs are thought to recognize the cognate transcripts and to act as primers for RNA-dependent transcription, which produces dsRNA (Mourrain et al. 2000; Vaistij et al. 2002; Himber et al. 2003).

If RNA-dependent transcription continues into sequences not present in the original inducer, also these sequences are converted in dsRNA and thus give rise to siRNAs and silencing, a process termed "transitivity". Interestingly, transitivity occurs not only in 3′-to-5′, but also in the 5′-to-3′ direction. There are two hypotheses how siRNAs could prime dsRNA formation. According to one model, which does not explain 5′-to-3′ transitivity, the siRNA acts as a primer for RDR6 (Fig. 3B). According to another model (Fig. 3A;

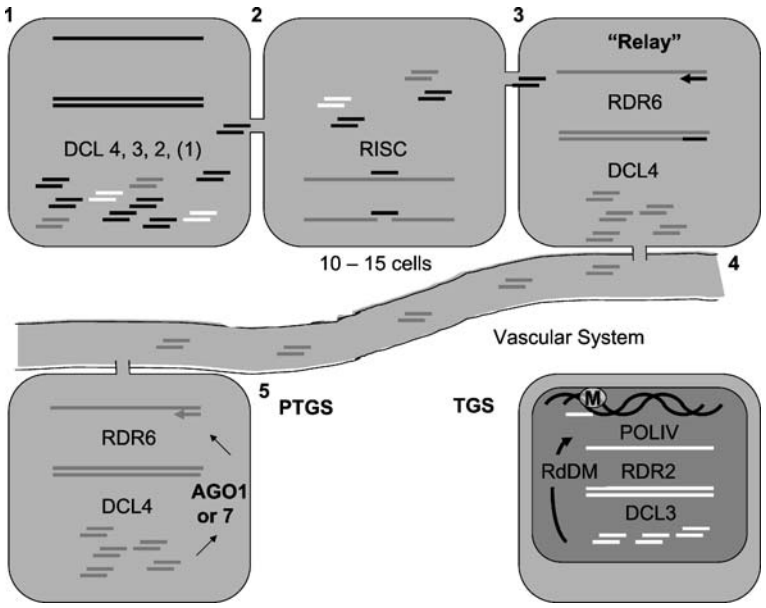

Fig. 6 Systemic silencing model. Silencing of a transgene (or virus gene) is initiated randomly by producing dsRNA by RDR action. Specific dicers cleave dsRNA to siRNAs of specific length (symbolized in *white, grey, and black lines*). siRNAs associate with an RNA-induced silencing complex (RISC) to cleave cognate mRNAs either in the original cell (step 1) or after transport into neighboring cells (step 2). Occasionally, an amplification cycle is induced, whereby dsRNA production is induced by the action of RNA-dependent RNA polymerase (RDR6) (step 3). The resulting secondary dsRNA is mainly cleaved by DCL4. Probably, DCL4-produced 21nt siRNA is the predominant signal transported through the phloem and loaded into adjacent tissue, although the nature of this signal is disputed (step 4). Likely, silencing activities are especially strong in newly grown tissue where 21nt siRNAs are amplified in a self-perpetuating cycle (step 5). Nuclear activities of Pol IV, RDR2, and DCL3 in 24nt siRNA production, RNA-directed DNA methylation (RdDM), and transcriptional silencing are shown in the lower right cell. The additional involvement of AGO, DRB, and HEN proteins is not shown

Petersen and Albrechtsen 2005), explaining both 3′-to-5′ and 5′-to-3′ transitivity, the siRNA as part of a RISC complex leads to target RNA cleavage and the resulting poly(A)-less 5′-fragment and the uncapped 3′-fragment could be recognized as "aberrant" RNA and used as a template for primer-independent transcription by RDR6. In any case, the resulting dsRNA(s) is cleaved by DCL4 to yield secondary siRNAs that should be predominantly 21nt in length. These siRNAs spread to layers of adjacent cells until another relay station is established. Thus, silencing of the whole leaf can be seen as a consecutive series of re-iterated relay amplifications.

Thirdly, the silencing signal feeds into the phloem, where it can be transported over long distances (Fig. 6, step 4), thus allowing silencing to spread

systemically and to cause silencing throughout the whole plant (Fig. 5). Phloem-mediated long-distance movement of the silencing signal follows the source-to-sink route used also for the transport of carbohydrates and most viruses. Analytical quantities of phloem sap can be harvested from cucurbits (Balachandran et al. 1997) and these saps could be analyzed for their contents in siRNAs. In fact a variety of small RNAs ranging from 18 to 25nt in length could be detected and cloned. Several of these corresponded to RNAs derived from cucurbit endogenes or a viral coat protein transgene, whereas others were identified as putative orthologs of *A. thaliana* miRNAs (Yoo et al. 2004). Furthermore, phloem sap derived from *Cucumber yellow closterovirus*-infected pumpkin contains large amounts of 20–21nt virus-derived siRNAs in both sense and antisense orientation.

Viral RNA is transported through plasmodesmata and through the phloem. In some cases viruses move as virions and in other cases as nucleic acid:movement protein complexes. It is likely that also siRNAs move as complexes with transport proteins. In fact, in pumpkin phloem extracts a candidate for such a movement protein was found and termed phloem-derived small RNA binding protein (PSRP)1. PSRP1 is 21 kD in size and specifically expressed in the phloem (Yoo et al. 2004). Future experiments should address whether this protein is a transporter for both siRNAs and miRNAs. A system specifically allowing study of phloem transport of the silencing system is the silencing by a transgene expressing the chlorata hairpin construct under the control of the phloem specific sucrose transporter (SUC) promoter (SUC-SUL). SUC-SUL silencing results in a white stripe of $\sim$ 10 to 15 cells along the vascular veins and this phenotype can also be transmitted to grafted scions (Himber et al. 2003; Dunoyer et al. 2005; Review: Voinnet 2005).

Transport through the phloem likely occurs without amplification, but also without much signaling loss, since scions deficient in RDR6 allow a similar 10 to 15 cell layer spreading of GFP silencing as wild-type scions do (Schwach et al. 2005), but further spreading in recipient leaves depends on RDR6, i.e. signal amplification.

While silencing of the *N. benthamiana* GFP-transgenic line 16c depends on triggering by a GFP-expressing plasmid, other GFP-transgenic lines are silenced spontaneously. In some cases this leads only to small silenced spots lacking sufficient amounts of silencing signal for the spreading of silencing. In other cases enough signal is produced and silencing spreads through all parts of the plant (Kalantidis et al. 2006).

4
Silencing in Virus–Plant Interactions: Plant Defense

It is now generally accepted that silencing and silencing suppression are major weapons governing plant-virus interactions. Obviously, successful virus

infections occur in a minority of cases, since most viruses have a narrow host range and plants can defend themselves successfully against a majority of viral invaders. Silencing as an antiviral strategy has also been suggested for nematodes (Wilkins et al. 2005), while in mammals other antiviral strategies dominate. Probably, systemic spreading of silencing as it occurs in plants and nematodes, but not in higher animals, is required to turn RNA interference into an effective antiviral weapon.

Although common mechanisms of innate immunity, including resistance gene-mediated hypersensitive response (HR) and systemic acquired resistance (SAR), play a great role in plant defense, the sequence-specific silencing of viral invaders might be the most efficient mode of antiviral defense in plants. Original observations that can now at least partially be explained by silencing include (i) recovery, in which a severely infected plant recovers from the virus and produces healthy new growth, and (ii) cross-protection, whereby a plant pre-inoculated with a mild virus strain becomes resistant to subsequent inoculation by a related severe strain (Valle et al. 1988).

On the basis of cross-protection, Sanford and Johnston (1985) introduced the concept of "pathogen-derived resistance", i.e. deriving resistance from the parasite's own genome. Competitive inhibition of a virus function by overproduction of a defective or even a normal version of its gene products is an effective means to debilitate the virus. To set an example for this principle, tobacco plants transgenic for the *Tobacco mosaic virus* (TMV) coat protein gene were analyzed. Some of the plants were very efficiently protected from TMV infection, presumably because the excess coat protein inhibited the uncoating of infecting virus particles (Bendahmane et al. 1997, leading to earlier references). "Coat protein-mediated resistance" and other virus protein-mediated resistances were applied to gain protection from many other viruses (Fitchen and Beachy 1993). The results were interpreted in terms of protein–protein interaction in the case of the *Tobacco etch potyvirus* (TEV). Lindbo and Dougherty (1992) provided a control where the transgene-derived RNA was of full length but untranslatable due to a frame-shift close after the start codon. Surprisingly also this construct provided specific resistance to and only to the cognate virus ("RNA-mediated resistance", reviewed by Lindbo and Dougherty 2005). Thus, cross-protection through silencing in *trans* can be achieved both by a complete and by a partial virus genome, as long as cognate targets are available.

While in transgene-mediated cross protection a host-encoded RNA silences the intruding virus, in a related process, "virus-induced gene silencing" (VIGS), a payload incorporated into a viral genome silences the cognate transgene or endogene (Kumagai et al. 1995; English et al. 1996). VIGS is a well-established tool to study gene function and also to study the mechanisms of silencing. Thus, studies involving VIGS led to the detection of siRNAs as a key component of silencing (Hamilton and Baulcombe 1999). VIGS also functions to repress the causing virus itself, leading to recovery

from virus infections showing that silencing is a natural defense mechanism. Thus, viruses are both inducers and targets of the silencing process (Covey et al. 1997).

While silencing of viruses was usually studied at the post-transcriptional level, early reports show that viroids, which replicate in the nucleus, can silence a cognate integrated DNA version by DNA methylation, a hallmark for transcriptional gene silencing (TGS; Wassenegger et al. 1994; Pelissier et al. 1999). Transcriptional gene silencing is also triggered by the minichromosomes of gemini- and caulimoviruses, which accumulate in the plant nucleus (discussed by Covey and Al-Kaff 2000). For example, *Cauliflower mosaic virus* (CaMV) infection can lead to transcriptional silencing of a herbicide resistance gene driven by the CaMV 35S in oilseed rape (Al-Kaff et al. 2000). Furthermore, accidentally integrated forms of caulimoviruses, such as *Petunia vein clearing virus* in *Petunia hybrida* are transcriptionally silenced (Richert-Poeggeler et al. 2003; Noreen et al. 2007).

5 Silencing in Virus–Plant Interactions: Viral Counter-attack

Viruses counteract silencing by evasion, e.g. by minimizing production and exposure of dsRNA, as well as by suppression, i.e. by interfering with the silencing pathway (Moissiard and Voinnet 2004; Voinnet 2005a). Original observations that can now be explained in terms of silencing suppression concern synergistic infections, e.g. the classical case of *Potato virus X* (PVX) and *Potato virus Y* (PVY) double infections, which cause much more severe symptoms than the individual infections. Pairs of viruses belonging to unrelated families can obviously profit mutually from their synergistic infections. In elegant experiments, it was found that a specific potyvirus protein, the protease helper component (HcPro) of *Tobacco etch virus* (TEV), could be the cause of the severity of PVX/PVY double infections (Shi et al. 1997). HcPro was shown not only to aggravate virus infections, but also to inhibit transgene silencing and VIGS (Anandalakshmi et al. 1998; Kaschau and Carrington 1998; Brigneti et al. 1998).

Synergism can also arise from related viruses, as exemplified by the severe co-infections of Cassava by *East African cassava mosaic virus* and *African cassava mosaic virus*, where the AC2 suppressor of the one and the AC4 suppressor of the other virus allow for a hyper-virulence of the pair (Vanitharani et al. 2004).

Following this lead, many more viral silencing suppressors were detected. In fact, it might be that a silencing suppressor belongs to the outfit of every successful plant virus (Voinnet et al. 1999; Roth et al. 2004; Dunoyer et al. 2004; Deleris et al. 2006) and even of some animal viruses (Li et al. 2002; Keene et al. 2004; Delgadillo et al. 2004; Bucher et al. 2004). Silencing suppres-

sors are usually multifunctional proteins, serving also as main components of virus replication and movement, such as coat protein, replicase, movement protein, etc (Table 2). They have nothing obvious in common, with the exception that most of them have ssRNA and/or dsRNA binding activity (Merai et al. 2006; Silhavy and Burgyan 2004; Lakatos et al. 2006) and that many of them, either in viral infections or as a transgene, cause plant abnormalities and developmental defects (Chen et al. 2004; Voinnet 2005). The reason for the latter could be that they not only interfere with the siRNA- but often also with the miRNA-pathway. Silencing suppression can either lead to a decrease (Mallory et al. 2002) or an increase in siRNAs (Chellapan et al. 2005; Silhavy and Burgyan 2004), indicating that in the first case siRNA production and in the second case siRNA usage is inhibited.

Frequently siRNAs and miRNAs are affected similarly, but curiously, in the presence of transgeneously or transiently expressed HcPro, siRNA concentrations are reduced and miRNA concentrations are increased (Mallory et al. 2002; Chen et al. 2004). We offer here the following explanation: both miRNAs and siRNAs are sequestered by HcPro and taken out of action by binding and modification through polyuridylation and prevention of 3′ methylation. However, miRNAs will be continuously delivered from pri- and pre-miRNAs, and since their use is inhibited, they accumulate further. On the other hand, in the presence of HcPro, siRNAs are not used in the amplification cycle that produces more dsRNA and siRNAs. Consequently, not only the use but also the delivery of siRNAs is reduced. Similarly, in viral infections the continuous accumulation of viral siRNAs despite the presence of a suppressor can be explained by the continuing production of viral dsRNAs as a side product of viral replication (see section below).

By binding to ssRNA, suppressors could inhibit dsRNA formation; by binding to large dsRNA, they could inhibit its degradation by dicers (Qu et al. 2003); and by binding to siRNA duplexes, they could inhibit formation of RISC (Lakatos et al. 2006). The latter case was exemplified by a detailed characterization of the tombusvirus p19 complex with ds-siRNA (Silhavy et al. 2002; Lakatos et al. 2004). This complex is very specific in respect to the size of the siRNAs, preferring 21nt siRNA duplexes. Similar complexes involving Potyvirus Hc-Pro or *Beat yellows virus* p21 are also specific for the presence of the 3′ 2nt overhangs (Lakatos et al. 2006). X-ray analysis provided a very detailed picture of the p19-siRNA complex and showed that p19 binds as a homodimer (Vargason et al. 2003; Ye et al. 2003). X-ray analysis of a RNA-p21 complex revealed that p21 binds as an octamer (Ye and Patel 2005).

Silencing suppressors could also act by interactions with host proteins and by manipulation of the host transcriptome. CMV-encoded 2b interacts with AGO1 and thereby blocks its function (Zhang et al. 2007). HcPro recruits calmodulin-related protein (rgsCaM) from host plants and this protein, if over-expressed, mimics suppression by HcPro (Anandalakshmi et al. 2000).

Table 2 Viral silencing suppressors

Virus name	Virus family	Genome	Suppressor protein	Function in silencing	Other known function	Refs.
TCV	Carmo	RNA (+)	p38		Coat protein	Qu et al. 2003, Thomas et al. 2003 Deleris et al. 2006
BYV,CTV	Clostero	RNA (+)	P20/21	siRNA binding	replication enhancer	Lu et al. 2004, Ye &Patel 2005, Lakatos et al. 2006, Reed et al. 2003
			P23		nucleic acid binding pr.	Lu et al. 2004, Ye & Patel 2005, Lakatos et al. 2006, Reed et al. 2003
			CP		Coat protein	Lu et al. 2004, Ye & Patel 2005 Lakatos et al. 2006, Reed et al. 2003
CoMV	Como	RNA (+)	S		Small coat protein	Canizares et al. 2004, Liu et al. 2004
CMV,TAV	Cucumo	RNA (+)	2b	blocks AGO1	Movement protein	Kasschau and Carrington 1998 Zhang et al. 2007
BYMV	Hordei	RNA (+)	gb		Movement pr., Repl. Enh.	Yelina et al. 2002
BWYV	Luteo	RNA (+)	P0			Voinnet et al. 1999
PCV	Peclu	RNA (+)	P15		Movement Protein	Dunoyer et al. 2002
BWY	Polero	RNA (+)	P0			Pfeffer et al. 2002 Zhang et al. 2007
PVX	Potex	RNA (+)	P25		Movement Protein	Dunoyer et al. 2000
TuMV,PVY,TEV	Poty	RNA (+)	HcPro	siRNA binding/ modification	helper component/ protease	Anandalakshmi et al. 2000, Lakatos et al. 2006, Ebhardt et al. 2005
RYMV	Sobemo	RNA (+)	P1		Movement protein	Voinnet et al. 1999
TMV, ORMV	Tobamo	RNA (+)	P30		replication	Kubota et al. 2003, Akbergenov et al. 2006
TBSV,CRV	Tombus	RNA (+)	P19	siRNA binding		Lakatos et al. 2006, Baulcombe and Molnar 2004

Table 2 (continued)

Virus name	Virus family	Genome	Suppressor protein	Function in silencing	Other known function	Refs.
TYMV	Tymo	RNA (+)	P69		Movement protein	Chen et al. 2004
RHB	Tenui	RNA (–)	NS3			Bucher et al. 2003
TSW	Tospo	RNA (–)	NSs		Transactivator	Takeda et al. 2002, Bucher et al. 2003
RDV	Phyto	dsRNA	Pns10	upstream of dsRNA?		Cao et al. 2005
CaMV	Caulimo	Reversi	TAV?	siRNA production	Transactivator/Viroplasmin	Blevins et al. 2006
ACMV, EACMV	Gemini	DNA	AC2	activates host suppressor	Transactivator	Trinks et al. 2005, Vanitharani et al. 2004
			AC4		Replication enhancer	Vanitharani et al. 2004
b satelite	Gemini sat.	DNA	bC1		DNA binding protein	Cui et al. 2005

To suppress RNAi, *Red clover necrotic mosaic virus* (RCNMV) (RCNMV) needs both viral RNA and replicase, and a model was presented according to which the viral replicase sequesters DCL enzymes as part of the replication complex, using it as a helper helicase and depleting it for its silencing function (Takeda et al. 2002).

Strong *Arabidopsis* transcriptome modification is induced by the action of the geminivirus transactivator protein AC2, and one of the strongly overproduced proteins, Werner-like protein 1 (WEL-1) was shown to be a host-encoded silencing suppressor, if expressed transiently in a *N. benthamiana* silencing indicator line (Trinks et al. 2005). The *Arabidopsis* RNAse L inhibitor (RLI) was identified as another host-encoded silencing suppressor (Sarmiento et al. 2006).

Some suppressors, such as HcPro (Ebhard et al. 2005) and an unknown factor of *Oilseed rape mosaic virus* (ORMV; Akbergenov et al. 2006) inhibit not only the usage but also the Hen1-mediated methylation of sRNAs. Whether this is due to inhibition of the enzyme or due to masking of the substrate in the sRNA-suppressor complex is not yet known.

The universal need for silencing suppressors in plant viruses raises the question if also viroids, which consist of non-coding circular RNA, can suppress silencing and how they would do it (Wang et al. 2004). Viroids have alternative RNA-folds involving a high proportion of base pairing. One or more of these structures could very well act as an RNA-based suppressor providing a decoy for dicers or other enzymes involved in the silencing pathway. In fact, also virus-derived RNAs could act as suppressors, as was suggested by Voinnet (2005). Specific small RNAs are used by some animal viruses, e.g. adenoviruses, as a decoy to interfere with another antiviral strategy, interferon-mediated resistance, by complexing protein kinase K (Katze 2002). In fact, strong evidence was presented that adenovirus VA1 RNA not only inhibits PKR, but also interferes with siRNA and miRNA biogenesis (Lu and Cullen 2004).

6
Systemic Spread of Viral Silencing: a Race Between Silencing and Silencing Suppression

The severity and host range of virus infections can be interpreted at least in part in terms of a race between systemic silencing and silencing suppression. This race is often influenced by environmental factors. Most prominently, high temperature seems to assist silencing whereas low temperature seems to assist silencing suppression (Szittya et al. 2003; Chellapan et al. 2005; Qu et al. 2005).

The race between silencing and viral silencing suppression occurs in five stages: initiation, short distance cell-to-cell movement, long distance cell-

to-cell movement, long distance movement through the vascular tissue, and meristem invasion (Dunoyer and Voinnet 2005).

We assume that not only virus replication but also silencing is initiated in the originally infected cell. Presumably, sufficient silencing suppressor is produced at this stage to counteract silencing. However, the process creates a mobile signal spreading into the adjacent cells. As stated above, if not amplified, this spreading covers about 15 cell layers and seems not to be sufficient to prevent the systemic movement of viral infection in susceptible plants.

The obvious way to evaluate the necessity of suppressors for systemic virus movement would be to create viruses mutant in the suppressor. However, this approach is usually difficult due to the multifunctional nature of the suppressor proteins and their usual need for other essential viral functions. An exception is the case of *Turnip crinkle virus* (TCV), where the coat protein (p38) acts as the suppressor and can be removed. The resulting virus infects wild-type *Arabidopsis* plants only if applied in massive amounts (Deleris et al. 2006). In contrast, strong plant susceptibility is restored in *Arabidopsis dcl4* mutants (Deleris et al. 2006). In another case, Bayne et al. (2005) screened a collection of random mutants of the *Potato virus X* (PVX) suppressor and movement protein p25, all of which compromised viral cell-to-cell movement. However, a class of these mutants could be complemented by a different suppressor protein, showing that they had retained movement function and that silencing suppression is required even for short distance spreading out of the infection center.

If surplus amounts of silencing signal are produced, e.g. by transiently over-expressing hairpin constructs cognate to the viral genome in infected plants, silencing can win over suppression, leading to recovery from virus infection (Pooggin et al. 2003, Akbergenov et al. 2006; Fig. 7).

Spreading of transgene- or antisense-induced silencing beyond the 15-cell zone requires amplification of the silencing signal through the action of RDR6 and DCL4, as discussed above. During virus silencing such amplification might also occur, but is not required, since the spreading virus could initiate new silencing events in every cell it arrives in (Akbergenov et al. 2006; Blevins et al. 2006). Sufficient dsRNA arises as a by-product of viral replication. RNA viruses obligatorily produce sense and antisense copies of their genomes that could form dsRNA feeding into the silencing pathway, despite viral mechanisms minimizing the availability of free RNA. Plant DNA viruses do not need any antisense transcripts for their replication. Nevertheless both gemini- and pararetrovirus infections give rise to sense and antisense transcripts derived from viral coding and non-coding regions. In geminiviruses, bidirectional Pol II promoters normally generate converging left- and rightward transcripts. Prior to cleavage and polyadenylation, the primary viral transcripts potentially extend beyond their overlapping terminators, which would create elongated antisense RNA from both directions extending even into the promoter region itself (Shivaprasad et al.

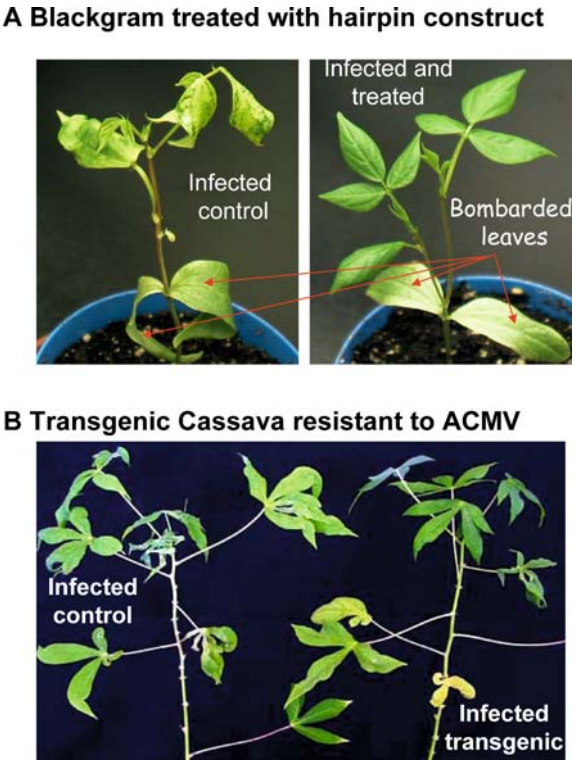

Fig. 7 Synthetic virus resistance. **A** Immunization of blackgram plants against *Mungbean yellow mosaic virus* (MYMV) infection. One week after infection with MYMV, blackgram plants were inoculated with an empty vector (*left*) and with a plasmid expressing a hairpin construct with sequences homologous to the MYMV promoter region (*right*). The control plant developed strong symptoms, while the plant expressing the hairpin construct recovered from infection (from Pooggin et al. 2003). **B** Recovery of transgenic Cassava plants from *African cassava mosaic virus* (ACMV) infection. Cassava plants were transformed with a hairpin construct containing sequences homologous to the ACMV promoter region. Single transformants were selected and tested. Transgenic plants were originally infected and showed disease symptoms, but later recovered from the disease (Akbergenov et al. 2006). A non-transgenic (*left*) and a transgenic plant (*right*) infected with ACMV are shown (Figure kindly provided by Vanderschuren H, Zhang P and Gruissem W, ETH, Zürich)

2005). Pararetroviruses produce coding transcripts only in the sense direction, however, our recent evidence suggests that also antisense transcription occurs (Blevins et al. 2006), presumably because the strong enhancer elements can act bidirectionally. Despite the availability of viral dsRNA and its use to reinitiate silencing in the invaded cell, many viruses may produce enough suppressor also at this stage to allow their replication and movement.

The long-distance movement of viruses and silencing signals through the vascular tissue probably involves neither virus replication nor signal amplification, although after reloading into upper leaves both virus replication and signal amplification resumes as for long-distance cell-to-cell movement.

Up to this point systemic viral infections are very robust and in fact almost insensitive to mutations in individual RDRs and DCLs, the former because viral dsRNA can be formed as a side product of virus replication and antisense transcription, as mentioned above, and the latter because of dicer redundancy. DNA virus infections in *Arabidopsis* plants generate three size classes (21, 22, and 24nt) of virus-derived siRNAs, which are produced by size-specific action of DCL4, DCL2, and DCL3, respectively, as can be traced by using a set of *dcl* single, double, and triple mutants (Akbergenov et al. 2006; Blevins et al. 2006). Apparently, DNA viruses are targeted by all four plant dicers, including the nuclear DCL3 producing 24nt siRNAs. A 21nt siRNA can be produced both by DCL4 and DCL1 and, therefore, the activity of DCL1 becomes most obvious in *dcl4* mutants. Interestingly, DCL1 seems to be choosy in its dsRNA targets, preferring, for example, CaMV caulimo- over *Cabbage leaf curl geminivirus* (CaLCuV)-derived dsRNA, and the structured CaMV 35S RNA leader region over the CaMV coding RNA. Interestingly, the elimination of 21- or 24nt size-classes of geminiviral siRNAs in dcl4 and dcl3 mutants, respectively, is compensated by increased accumulation of the DCL2-dependent 22nt class. In RNA virus infections, the 24nt siRNA band is usually not seen, probably because its generator DCL3 functions mainly in the nucleus and because the DCL4-dependent 21nt viral siRNA is a predominant species. However, in certain cases, when DCL4 activity is impaired by viral suppression, DCL2 (and possibly other dicers) can take over an antiviral defense.

7
Invasion of Newly Growing Tissue and Meristems by Viruses and Silencing Signal

Viruses usually cannot invade meristems and this exclusion is in fact used by the breeder to obtain virus-free plants through meristem culture. Meristem exclusion, and perhaps also poor penetration of newly growing tissue surrounding the meristem, might be caused by inhibition of virus replication or virus RNA movement into this tissue, or by an enhanced capacity for gene silencing, or by a combination of both. Recent reports indicate that viral RNA can invade perimeristematic tissue but that a very proficient silencing mechanism involving efficient and fast amplification of a silencing signal makes the virus the looser. This mechanism involves RDR6 and DCL4. If RDR6 is knocked down in *Nicotiana benthamiana*, PVX, PVY and *Cucumber mosaic virus* (CMV) in the presence of its Y-satellite, but not *Tobacco*

rattle virus (TRV), TMV, *Turnip circle virus* (TCV) and CMV in the absence of its satellite cause hypervirulence and invasion of perimeristemic tissue. This can be seen by the infection with viral vectors carrying an active GFP reporter gene (Schwach et al. 2005). Even a broader palette of viruses, including TMV, CMV, and TCV were found in similar experiments to invade the growing tissue at elevated temperatures (Qu et al. 2005). Likewise, geminivirus-induced gene silencing (gemini-VIGS) of newly growing *Arabidopsis* tissue depends on RDR6 (Muangsan et al. 2004; Peele et al. 2001) and DCL4 (Blevins et al. 2006), as shown for geminivirus vectors carrying sequences cognate to genes active in proliferating tissue, i.e. proliferating cell nuclear antigen (PCNA) and the CHLI subunit of Mg-chelatase (Fig. 5B). The dependence on DCL4 and RDR6 for the ingression of virus-mediated silencing into the growing tissue relates to a similar requirement already for long-distance cell-to-cell movement in the case of hairpin- or excess-RNA induced silencing (Dunoyer et al. 2005). It remains open, whether in either of these cases, DCL4 is required to create the signal in the donor cell (Voinnet 2005b) or to initiate dsRNA synthesis in the acceptor cell.

Apparently the silencing signal—most likely 21nt viral siRNA produced by DCL4 (Blevins et al. 2006)—moves ahead of the viral infection front in growing tissue. As soon as viral RNA invades this tissue, it is immediately recognized by the signal and converted into dsRNA by RDR6. Likewise, the failure of some of the viruses to invade the growing tissue in the RDR6 mutant plants might be due to the compensatory action of other RDR enzymes and those might be less efficient at elevated temperatures. The leakiness of this effect might be due to the presence of other RDR activities. For instance, RDR1 has been shown to contribute to the defense of tobacco plants against TMV infection (Xie et al. 2001) and the lack of a functional RDR1 ortholog in *N. benthamiana* has been attributed to the hypersensibility of *N. benthamiana* to several virus infections (Yang et al. 2004).

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An Emerging Model System: *Arabidopsis* as a Viral Host Plant

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Abstract The model plant *Arabidopsis thaliana* has been established as a host for representatives of many of the major groups of plant viruses. These viruses use a variety of strategies to replicate and traffic their genomes within compatible host plants. The development of *Arabidopsis* as a host for these diverse viruses provides opportunities to apply genetic and reverse genetic approaches to the complex interactions that are not feasible or practical in many of their agronomically important hosts. In this chapter, we summarize the amazing array of viruses that have been shown to infect one or more *Arabidopsis* ecotypes, describe natural variation in the ability of these ecotypes to support systemic infections, and discuss host genes that have been identified through a variety of approaches that are involved in movement or limit virus spread in *Arabidopsis*.

1 Introduction

Arabidopsis thaliana has served as an important model for numerous plant processes, including interactions with plant pathogens (Meyerowitz and Somerville 1994). As described in this chapter, *Arabidopsis* is becoming increasingly useful as a model host for the study of compatible and incompatible host–virus interactions. Surprisingly, *Arabidopsis* can serve as a host for an amazing array of viruses representing all the genome types, dsDNA, ssDNA, (+) RNA, (–) RNA, and ambisense RNA (Table 1). Although the numerous viruses that infect *Arabidopsis* are not limited to those that are adapted to cruciferous plants, it seems that such viruses are the most efficient at infecting *Arabidopsis* in an experimental setting.

The motivation for studying viral infection processes and resistance mechanisms in *Arabidopsis* is that one can exploit the facile genetics, efficient transformation, genome sequence, and genomic resources for understanding the genetic, molecular, and ultimately the biochemical foundations of host–virus interactions. Much work in the area of disease resistance has shown that there is a great deal of conservation in the factors that mediate defense between *Arabidopsis* and crop hosts, and we expect that this will also be the case for host–virus interactions. Ultimately, it will be most useful to apply the fundamental knowledge gained through the use of the model weed

Table 1 Viruses that infect *Arabidopsis*

Virus	Strain(s)	Refs.
Genus: *Begomovirus* (ssDNA)		
Cabbage leaf curl virus (CLCV; CaLCuV)	N/A [a]	Hill et al. 1998
Genus: *Bromovirus* ((+) ssRNA)		
Brome mosaic virus (BMV)	M1	Dzianott and Bujarski 2004
Cow pea chlorotic mottle virus (CCMV)	M1	Fujisaki et al. 2004
Spring beauty latent virus (SBLV)	ATCC PV-369	Fujisaki et al. 2004
Genus: *Carmovirus* ((+) ssRNA)		
Turnip crinkle virus (TCV)	M	Simon et al. 1992
Genus: *Caulimovirus* (dsDNA)		
Cauliflower mosaic virus (CaMV)	CM4-184, CM1841, W260	Leisner and Howell 1992
Genus: *Curtovirus* (ssDNA)		
Beet curly top virus (BCTV)	CFH, Logan	Lee et al. 1994
Genus: *Cucumovirus* ((+) ssRNA)		
Cucumber mosaic virus (CMV)	O, Y	Takahashi et al. 1994
Genus: *Nepovirus* ((+) ssRNA)		
Tobacco ringspot virus (TRSV)	Grape, Bud blight, and uncharacterized field isolate	Lee et al. 1996
Genus: *Polerovirus* ((+) ssRNA)		
Beet western yellows virus (BWYV)	N/A [a]	Pazhouhandeh et al. 2006
Cucurbit aphid-borne yellows virus (CABYV)	N/A [a]	Pazhouhandeh et al. 2006
Genus: *Potexvirus* ((+) ssRNA)		
Potato virus X (PVX)	N/A [a]	Cooper et al. 2003
Genus: *Potyvirus* ((+) ssRNA)		
Lettuce mosaic virus (LMV)	AF199, E, 0	Revers et al. 2003
Plum pox virus (PPV)	NAT, R, PS, Soc, EA	Decroocq et al. 2006
Potato virus Y (PVY)	3	Whitham et al. 2000
Tobacco etch virus (TEV)	HAT, Madison, ST1	Mahajan et al. 1998; Whitham et al. 2000
Tobacco vein mottling virus (TVMV)	N/A [a]	Whitham et al. 2000
Turnip mosaic virus (TuMV)	UK-1	Martin Martin et al. 1999
Genus: *Tobamovirus* ((+) ssRNA)		
Oilseed rape mosaic virus (ORMV)		Martin Martin et al. 1997
Tobacco mosaic virus (TMV)	U1, Cg, L, cr	Arce-Johnson et al. 2003; Dardick et al. 2000; Dorokhov Yu et al. 1994; Ishikawa et al. 1991
Turnip vein clearing virus (TVCV)	N/A [a]	Lartey et al. 1997

Table 1 (continued)

Virus	Strain(s)	Refs.
Genus: *Tobravirus* ((+) ssRNA)		
Tobacco rattle virus (TRV)	PPK20	MacFarlane and Popovich 2000
Genus: *Tospovirus* ((–) & (+/–) RNA)		
Tomato spotted wilt virus (TSWV)	N/A[a]	German et al. 1995
Genus: *Tymovirus* ((+) ssRNA)		
Turnip yellow mosaic virus (TYMV)	N/A[a]	de Assis Filho and Sherwood 2000

[a] N/A Information about the specific strain was not available

to develop novel means of controlling plant-virus infection. A recent example that supports this rationale comes from the study of the *TOM1* and *TOM3* genes, which were first identified as genes required for *Tobacco mosaic virus* (TMV) replication in *Arabidopsis* (Yamanaka et al. 2002). When the tobacco orthologs of *TOM1* and *TOM3* were simultaneously silenced by RNA interference (RNAi) in transgenic tobacco, the resulting tobacco plants had broad-spectrum resistance to TMV strains (Asano et al. 2005). It may be possible to develop resistance by this strategy in many crops, because homologs of *TOM1* and *TOM3* are found in many plant species. For a recent review on virus resistance that includes *Arabidopsis* resistance genes and their mechanisms, the reader is referred to (Kang et al. 2005).

While there have been numerous recent advances in our understanding of the host factors involved in plant virus replication through the use of both yeast and *Arabidopsis* as model hosts (Nagy and Pogany 2006; Noueiry and Ahlquist 2003; Whitham and Wang 2004), the characterization of host factors involved in virus movement has lagged behind. There is a great potential for contributions of *Arabidopsis* to this aspect of virology through the use of genetic and reverse genetic approaches. In addition, investigating the trafficking of host proteins involved in plant development, such as Leafy, may also help to shed light on the host factors that participate in virus movement (Zambryski 2004).

In this chapter, we focus on the role of *Arabidopsis* as a model for studies of both cell-to-cell and systemic virus movement. As each virus–*Arabidopsis* interaction is presented, we identify some major themes corresponding to different avenues of research that take advantage of natural variation among the ecotypes and/or exploit the available genomic and reverse genetic resources. There is a great deal of ecotype diversity within the *A. thaliana* species. Thus, the behavior of different viruses or even strains of a single virus in combination with collections of ecotypes has led to the identification of many novel phenotypes related to replication, movement, or resistance. It is often possible

to genetically characterize, map, and clone the corresponding genes. Mutagenesis of *Arabidopsis* has also been a successful way to generate new alleles of genes or identify additional genes that affect movement phenotypes. More recently, identification of host proteins from *Arabidopsis* or of homologs from other plant species that interact with viral movement proteins has led to reverse genetic approaches for studying the functions of specific host genes in viral movement.

2
Natural Ecotypic Variation in Susceptibility to Viruses

The porte d'entrée into developing *Arabidopsis* as a model host for any given virus has been to screen several ecotypes for systemic infection and to determine whether there are differential responses amongst them. Screening genetically distinct *Arabidopsis* ecotypes for their virus infection phenotypes has led to the discovery of novel genes involved in pathogenesis. The aim of this section is to provide a review of the interactions between diverse viruses and *Arabidopsis* ecotypes. A list of viruses that infect *Arabidopsis* along with information about strain(s), genus, and genome type are provided in Table 1. Characterized and unknown host factors that regulate susceptibility, tolerance, and resistance that were identified from ecotype-specific interactions are discussed.

2.1
Turnip Crinkle Virus (TCV)

Among the first reported observations of phenotypic variation among *Arabidopsis* ecotypes to virus challenge was that the ecotype Di-0 (Dijon) was resistant to TCV strain M, whereas the Col-0 ecotype and others were susceptible (Simon et al. 1992). The majority of Di-0 plants were symptomless in response to TCV whereas a population of inoculated plants developed necrotic lesions resembling a hypersensitive response (HR). Using an in situ hybridization approach it was found that symptomless Di-0 plants restricted TCV accumulation to the midribs of inoculated leaves. This phenotype was also observed in leaves opposite to the infected leaves, indicating that TCV underwent short-distance movement but was systemically restricted in Di-0 plants. The effect of light exposure was also investigated revealing that nearly all plants grown under 16–24 h of light were resistant to TCV infection. In contrast, an 8-h day length that promoted slower plant growth rendered Di-0 plants less resistant to TCV.

Interestingly, among Di-0 plants resistance was not homogenous since individuals were susceptible to TCV (Dempsey et al. 1993). Homogenous susceptible and resistant lines were subsequently identified and designated as Di-3

and Di-17, respectively. Di-3 plants accumulated wild-type levels of TCV while Di-17 plants had resistance that increased according to plant age. For example, 20–56% of 16 day-old plants were resistant whereas 90–100% of 20 day-old plants were resistant to TCV. In Di-17, TCV accumulation was restricted to the HR lesions or the margins of lesions. The inheritance of TCV-induced HR in Di-17 was attributed to a single dominant locus designated *HRT* (*HR to TCV infection*) and was mapped to chromosome 5 (Dempsey et al. 1997).

TCV resistance was shown to be mediated by *HRT* which was found to encode a coil-coiled (CC), nucleotide-binding site (NBS), and leucine-rich repeat (LRR) class resistance (R) protein (Cooley et al. 2000). Although *HRT* controls HR in Di-17 plants, it is not exclusively responsible for resistance to TCV since many progeny from a Di-17 and Col-0 cross were susceptible to TCV regardless of HR activation (Kachroo et al. 2000). Further genetic analysis provided evidence that a recessive locus designated *rrt* (*regulate resistance to TCV*) was necessary in addition to *HRT*. Thus, the combination of *HRT* and *rrt* is needed for complete inhibition of TCV replication and movement. Beyond the scope of this chapter are the viral and host proteins that trigger HR and resistance to TCV, including the TCV CP and an *Arabidopsis* TCV-CP-interacting protein (TIP), and the various host signaling components required for resistance (Chandra-Shekara et al. 2004, 2006; Choi et al. 2004; Kachroo et al. 2000; Ren et al. 2000, 2005).

2.2 Cucumber Mosaic Virus (CMV)

The advantage of screening various *Arabidopsis* ecotypes is the potential for finding differences among ecotypes in response to virus strains or isolates. An example of this approach was demonstrated by measuring the susceptibility of 12 different ecotypes in response to the CMV strains O and Y (Takahashi et al. 1994). Both isolates of CMV were infectious in 11 of the 12 ecotypes tested including Col-0, but CMV-Y infection was not detected in the ecotype C24 unlike CMV-O. C24 plants developed HR to CMV-Y within 21–27 h after inoculation. Genetic analysis of the resistance trait in C24 demonstrated that it was conferred by a single dominant gene that was designated *RCY1* (*resistance to Cucumber mosaic virus strain Y 1*). Interestingly, progeny from reciprocal crosses between C24 × Col-0 and C24 × La (Landsberg ecotype) yielded differences in resistance to CMV-Y. In particular, C24 × Col-0 progeny developed HR to CMV-Y, thereby blocking virus movement to non-inoculated upper leaves. In contrast, some progeny from C24 × La cross developed HR to CMV-Y and accumulated low levels of coat protein (CP) in systemic leaves. From genetic analysis the authors concluded that a recessive modifier gene derived from the La ecotype parent inhibited *RCY1* function.

Similar to TCV-induced resistance in Di-17 plants, CMV-Y-induced resistance in C24 plants is elicited by the CP, and the corresponding RCY1 R protein

is a member of the CC-NBS-LRR class (Takahashi et al. 1994, 2001, 2002). Interestingly, *RCY1* (CMV-Y) and *HRT* (TCV) are allelic, but they mediate different virus-specific resistance responses (Takahashi et al. 2002).

2.3 Cauliflower Mosaic Virus (CaMV)

In response to CaMV isolates CM4-184, CM1841, and W260, *Arabidopsis* ecotypes were found that differed in symptom development or that responded in a similar manner to virus inoculation (Leisner and Howell 1992). In the Rsch-4 (Rschew-4) ecotype, differences were observed for the ability of CaMV isolates to cause symptoms. The CM4-184 and CM1841 isolates produced mild symptoms compared to the W260 isolate. The symptoms were marked by increased chlorosis and vein-clearing. The W260 isolate did cause symptoms in the Tsu-0 ecotype, whereas neither CM4-184 nor CM1841 produced any visible systemic symptoms. More interesting were the ecotypes En-2 (Enkhelm-2), Sv-0 (Sveboelle-0) and Wil-2 (Wilna-2) that appeared symptomless to all CaMV isolates tested. Among the three ecotypes, growth and development was slower for En-2 and Sv-0 than for Col-0. Leaf skeleton hybridization studies of CM4-184 infection in the En-2 ecotype showed that the CM4-184 isolate largely accumulated in the inoculated leaf, but at low levels in systemic cauline leaves compared to Col-0. This observation suggested that CaMV replication was not impaired in En-2 plants but that overall systemic movement was compromised.

Furthermore, the potential effects of plant development in limiting systemic CaMV movement were ruled out after it was demonstrated that systemic movement of CaMV was blocked in En-2 plants inoculated at various leaf stages (Leisner et al. 1993). En-2 was also confirmed to be resistant to CM1841 (Callaway et al. 1996). With regard to resistance, it appeared that a single semi-dominant locus on chromosome 1 designated *CAR1* (*Cauliflower mosaic virus resistance 1*) was responsible for this response (Callaway et al. 1996). It was also reported by Callaway et al. (1996), that the ecotype Bla-14 (Blanes-14) was resistant to infectious CM1841 sap but, unlike En-2, Bla-14 was weakly resistant to agroinfiltrated CM1841. Through genetic analyses, it was found that Bla-14 resistance to CM1841 was also conferred by a locus on chromosome 1, suggesting that the loci from En-2 and Bla-14 were allelic for resistance to CaMV isolates. Interestingly, it was not clear why Bla-14 was not resistant to CaMV-CM1841 agroinfiltration.

2.4 Tobacco Etch Virus (TEV)

The potyviruses are the largest group of viruses that infect herbaceous and woody plants. To establish that TEV was systemically infectious in *Arabidop-*

sis, 10 ecotypes were screened, and it was found that TEV accumulated and moved locally in the inoculated leaves of all ecotypes (Mahajan et al. 1998). Ecotypes that supported systemic TEV infection included C24, La-*er* (Landsberg erecta), Di-G (Dijon-G), and Cvi-0 (Cape Verdi Islands-0), but they displayed no detectable symptoms. The differential ability of the ecotypes to support systemic TEV infection suggested that resistant ecotypes were either deficient in a host factor that would normally facilitate TEV systemic transport, expressed a host factor that interfered with TEV systemic transport, or activated resistance to TEV infection. Genetic analysis of the resistance trait demonstrated that it was conferred by a single dominant locus designated *RTM1* (*restricted TEV movement 1*) that mapped to chromosome 1. *RTM1*-mediated resistance to TEV was shown to be active in the Col (Col-0 and Col-3) and Ws-2 (Wassilewskija-2) ecotypes but not in the C24 and La-*er* ecotypes (Chisholm et al. 2000). Both C24 and La-*er* carry recessive *rtm1* alleles that confer susceptibility to TEV.

Genetic analysis of *RTM1* has been performed with both naturally occurring alleles present in different ecotypes and through the use of Col-0 mutants generated by ethyl methanesulfonate (EMS) that have loss of resistance phenotypes (Chisholm et al. 2000; Mahajan et al. 1998; Whitham et al. 1999). These studies have led to the characterization and cloning of *RTM1*. In C24, the *rtm1* allele contained four codon substitutions and a 26-nt deletion in the 119-nt intron, whereas in La-*er*, the *rtm1* allele contained a codon substitution that resulted in premature termination. *RTM1* encodes a jacalin-like lectin protein similar to those found in *Artocarpus integrifolia* and *Maclura pomifera*. Search results also found that the RTM1 protein was similar to several myrosinase-binding proteins (MBPs) from *Brassica napus* and *Arabidopsis* that co-purify with myrosinase, an enzyme that catalyzes hydrolysis of glucosinolates (Geshi and Brandt 1998). Studies have suggested that lectins, MBPs, and myrosinases are involved in defense mechanisms against pathogens like fungi and insects (Geshi and Brandt 1998; Peumans and Van Damme 1995). The direct function of the RTM1 protein in restricting TEV systemic movement is not known.

A second locus designated *RTM2* was discovered among EMS-mutagenized Col-0 plants that supported TEV systemic infection (Whitham et al. 1999). Genetic analysis of four *Arabidopsis* ecotypes revealed that in addition to Col, the ecotypes C24, La-*er*, and Ws-2 also contained the *RTM2* allele. Although *RTM2* was present in C24 and La-*er* plants, *RTM2* alone was not sufficient in restricting TEV systemic access, signifying that both *RTM1* and *RTM2* are necessary for resistance to systemic movement (Mahajan et al. 1998; Whitham et al. 1999). To test the specificity of *RTM1*/*RTM2* restriction of TEV systemic movement in Col-3 and Ws-2, three strains of TEV (HAT, Madison, and ST1), two strains of *Potato virus Y* (PVY) and one strain of *Tobacco vein mottling virus* (TVMV) were used (Whitham et al. 2000). All TEV isolates tested were unable to move systemically in Col-3 and Ws-2 plants. Conversely, PVY

and TVMV systemically infected Col-3 and Ws-2 plants and all potyviruses tested systemically infected C24 and La-*er* plants. To further investigate TEV and the *RTM1*/*RTM2* system, Col-0, *rtm1* and *rtm2* mutants were co-infected with TEV and CMV-Y or TVMV. Neither virus enabled TEV to overcome the Col-0 *RTM1*/*RTM2* system, but in *rtm1* and *rtm2* mutants, CMV was shown to stimulate TEV systemic infection suggesting a possible synergistic interaction.

RTM2 was mapped to a position on chromosome 5, cloned, and found to encode an unusual small heat shock-like protein (smHSP). The predicted RTM2 protein comprises an α-crystallin domain (characteristic of small molecular weight HSPs), a potential transmembrane domain, and an extensive C-terminal extension not found in other smHSPs (Whitham et al. 2000). *RTM2* is not induced by heat shock and does not contribute to thermotolerance in heat shock assays, indicating that it has other functions within the plant. As with the RTM1 protein, the direct activity of RTM2 protein is not known. A third locus designated *RTM3* was also isolated among Col-0 mutants that supported TEV systemic movement; however, this gene has not yet been cloned (S. Whitham, M. Yamamoto, and J.C. Carrington, unpublished). From these studies, systemic resistance to TEV infection is dependent on at least three RTM proteins that directly or indirectly interact with TEV-encoded proteins that facilitate long-distance movement. The RTM1 and RTM2 proteins were localized to phloem-associated cells and sieve elements where they might directly interact with TEV proteins to restrict virus movement (Chisholm et al. 2001).

2.5
Turnip Mosaic Virus (TuMV)

Among vegetable viruses, TuMV is ranked as one of the most destructive pathogens (Tomlinson 1987). In response to the TuMV strain UK-1, all susceptible ecotypes tested, including Col-0, developed similar symptoms such as stunting and aborted flowers (Martin Martin et al. 1999). The ecotypes Bay-0 (Bayreuth-0), Di-0, Er-0 (Erlangen-0) and Or-0 (Oranienstein-0) were determined to be resistant. Resistance among the Di-0, Er-0 and Or-0 populations tested was heterogeneous, because two-thirds of the plants accumulated TuMV systemically, whereas TuMV was found only in the inoculated leaves of the remaining one-third of plants. The Bay-0 population tested was homogenous for resistance to TuMV infection. Using protoplasts isolated from inoculated Bay-0 leaves, it was shown that individual cells supported virus replication. The ability of TuMV to replicate but not move suggested that cell-to-cell movement was compromised. Genetic analysis of the inheritance of resistance in Bay-0 or the other heterogeneous ecotypes has not yet been published.

2.6 Lettuce Mosaic Virus (LMV)

Variation among 35 *Arabidopsis* ecotypes in response to LMV isolates, LMV-AF199, LMV-E, and LMV-0 has also been studied (Revers et al. 2003). The ecotypes Cvi-0 and Cvi-1 were resistant to LMV-AF199, possibly a result of their inability to support either virus replication or cell-to-cell movement. In contrast, the ecotypes Wt-1 (Wietze-1), Ll-0 (Llagostera-0), and the Col ecotype (Col-0, Col-3, and Col-5) inhibited systemic movement of LMV-AF199. Overall, the majority of ecotypes screened supported LMV-AF199 infection compared to isolates LMV-0 and LMV-E. Mechanisms allowing LMV isolates to overcome resistance were investigated using genetic analysis and the ecotype Nd-1 (Niederzenz-1). Nd-1 plants were initially found to block systemic accumulation of wild-type LMV-0. When the VPg of LMV-0 was replaced with the VPg from either LMV-E or LMV-AF199, recombinant LMV-0 moved systemically. This proved that a resistance-breaking determinant was localized in the viral VPg of LMV isolates, generally explaining why some ecotypes are resistant to one or two isolates but not all three.

The variety of restricted movement phenotypes observed for LMV in the *Arabidopsis* ecotypes were genetically characterized. Local resistance to LMV-0 in Col-5 plants was conferred by a single dominant gene designated *LLM1* (*local resistance to LMV*) located on chromosome 1. Local resistance to LMV-AF199 in the ecotype Cvi was likely due to a single recessive locus. The restriction of systemic movement of LMV-AF199 in Col-5 plants was expected to involve two non-linked dominant genes. Interestingly, it is possible that RTM1/2/3 can also function to restrict LMV-AF199 movement in Col and Ws (Decroocq et al. 2006). Both ecotypes blocked LMV systemic infection whereas *rtm1*, *rtm2*, and *rtm3* mutants suppressed this phenotype. Consequently, the *RTM* system is effective against a range of potyviruses.

2.7 Plum Pox Virus (PPV)

PPV is a major pathogen of peach, apricot, and plum orchards (Nemeth 1994). To further identify host factors involved in plant–potyvirus interactions, several *Arabidopsis* ecotypes were challenged with one of five PPV strains (NAT, R, PS, Soc, and EA) (Decroocq et al. 2006). All ecotypes screened were susceptible to at least one PPV strain and various degrees of symptoms were observed. PPV-R induced severe symptoms in La-*er* but not in Col-0, Col-3, and Col-5. The genetic basis of severe PPV-R symptoms in La-*er* was suggested to be a multigenic trait. In resistant ecotypes, the local or systemic movement of PPV was restricted. Interestingly, nearly all of the ecotypes tested were resistant to PPV-SoC because it was not detected in inoculated leaves. To determine if resistance was controlled by an *R* gene or by

some other mechanism, *rar1* and *sgt1b* single and double mutants were inoculated with PPV-SoC. The *RAR1* and/or *SGT1* gene products have been shown to be essential in *R*-gene function (Shirasu and Schulze-Lefert 2003). These mutants supported local PPV-SoC infection, but resistance to systemic infection was not affected suggesting that two levels of resistance might be active. In response to other isolates, Col and Cvi-1 plants restricted systemic infection by PPV-EA and PPV-PS, respectively. Restriction of PPV-EA movement in Col-0 was conferred by a dominant locus whereas resistance to PPV-PS was conferred by a recessive allele designated *rpv1* (*restricted plum pox virus 1*). As shown with TEV and LMV-AF199, RTM1/2/3 possibly restricts PPV-EA systemic movement (Decroocq et al. 2006). Hence, a variety of host genes can control resistance to PPV in *Arabidopsis*.

2.8 Beet Curly Top Virus (BCTV)

Col-0 plants infected with the CFH or Logan isolates of BCTV (a geminivirus) become stunted and have deformed inflorescence structures (Lee et al. 1994). Between the two, BCTV-CFH infection appeared to result in more severe symptoms in Col-0. Among 46 *Arabidopsis* ecotypes screened, it was found that all were infected by BCTV-CFH and symptoms developed more rapidly in these plants in comparison to those infected with BCTV-Logan. The ecotypes Ms-0 (Moscow-0) and Pr-0 (Praunheim-0) were resistant to BCTV-Logan. Resistance was confirmed by quantitative analysis of viral DNA in aerial tissues and cultured inflorescence stem pieces of Ms-0 and Pr-0 plants. In accordance with the lack of symptom expression, BCTV-Logan was not detected in aerial portions of Ms-0 and Pr-0 inoculated plants. Data from transient replication in inflorescence stem pieces of Ms-0 and Pr-0 plants revealed that BCTV-Logan accumulated to similar levels in Ms-0 and Pr-0 as in Col-0 stem pieces. These data indicated that replication of BCTV-Logan was not inhibited but rather its systemic movement was blocked in Ms-0 and Pr-0 plants. Resistance to BCTV-Logan in Ms-0 and Pr-0 is conferred by distinct recessive loci, but it is not known if these genes are linked.

The authors also reported that the ecotype Sei-0 (Seis am Schlern-0) accumulated 20-fold more BCTV-Logan and two- to sixfold more BCTV-CFH than Col-0. Further analysis of the Sei-0 ecotype was not provided in the study. In an independent study, the ecotype Cen-0 (Caen-0) was shown to be tolerant to BCTV-CFH, based on the lack of symptoms, although viral DNA was detected in systemic tissues (Park et al. 2002). The response of the Cen-0 ecotype to BCTV-CFH was not tested in the study conducted by Lee et al. (1994). From genetic analysis, the authors concluded tolerance to BCTV-CFH in Cen-0 was attributed to a recessive locus. It was speculated that symptom development was suppressed potentially due to the inability of key host and viral factors to interact. From these ecotype screens for differential responses to BCTV

and subsequent genetic analyses, it is feasible that several genes that regulate resistance, tolerance, or susceptibility can be isolated.

2.9
Oilseed Rape Mosaic Virus (ORMV)

Tobamoviruses are among the most well-studied viruses infecting plants, and viruses within this group have been shown to infect the Col-0 ecotype with different degrees of success. ORMV was shown to be highly virulent on Col-0, and it systemically infected nearly all ecotypes tested (Martin Martin et al. 1997). Out of the 116 ecotypes screened, An-1 (Antwerpen) and Wc-1 (Westercelle-1) were found to restrict ORMV systemic movement in a dose-dependent manner. This was demonstrated by testing the response of An-1 and Wc-1 plants to three different concentrations of ORMV inoculum ($2\,mg\,mL^{-1}$, $0.2\,mg\,mL^{-1}$ and $0.02\,mg\,mL^{-1}$). At the highest concentration, symptoms appeared on An-1 and Wc-1 plants. In contrast, the ten- and 100-fold dilutions of ORMV did not result in symptoms or systemic virus movement. It should be noted the dose-dependent study was conducted in a growth chamber. This is noteworthy, because An-1 and Wc-1 were two of 15 ecotypes that did not develop symptoms when grown in the greenhouse and were classified as resistant candidates. When all 15 ecotypes were grown and inoculated in a growth chamber, only An-1 and Wc-1 failed to develop symptoms or accumulate ORMV systemically in response to diluted ORMV inoculum. The reported findings are not necessarily unexpected since environmental conditions do influence virus-induced symptom expression, and virus load is generally thought to be a factor involved in susceptibility. In terms of genetic analysis, the authors did not examine the inheritance of this trait.

2.10
Tobacco Mosaic Virus (TMV)

TMV strain U1 poorly infects the Col-0 ecotype compared to other strains or tobamoviruses, such as ORMV, that are adapted to crucifers. To isolate potential host factors involved in regulating *Arabidopsis* susceptibility to TMV-U1, 14 *Arabidopsis* ecotypes were screened (Dardick et al. 2000). TMV-U1-infected ecotypes were grouped into three distinct classes: rapid, intermediate, and slow accumulators. Out of the ecotypes examined, only Sha (Shahdara) facilitated rapid systemic movement along with distinct symptoms compared to the others. The majority of the remaining ecotypes, including Col-1, supported intermediate levels of TMV-U1 in systemic tissues when compared to Sha, and the remainder accumulated little or no virus. TMV-U1 accumulation did not consistently differ in protoplasts derived from Sha, Col-1, and Tsu-1, representing the rapid, intermediate, and delayed sys-

temic movement classes, respectively. These results suggested that more rapid spread of TMV-U1 in Sha plants was not necessarily due to increased replication but more likely caused by more rapid cell-to-cell transport.

Genetic analysis of progeny derived from crosses between Sha and Tsu-1 identified a single dominant locus controlling the rapid movement phenotype. The authors further suggested that symptom development might be linked to a recessive gene based on the outcome of TMV-U1 challenged F1 and F2 populations. TMV-U1 accumulated rapidly in systemic tissues of F1 plants, but these plants did not develop symptoms as in the Sha parent. In addition, the majority of infected F2 plants did not resemble the infected phenotype of Sha plants. The explanation for this observation was that a recessive gene regulated symptom development in infected Sha plants. It should be noted that in the *Arabidopsis* NASC ecotype collection Shahdara is also spelled Shakdara and other spellings may include Shokhdara and other variations (Loudet et al. 2002). It has been reported that the ecotypes Btg-2 and Uk-4 (Umkirch-4) also rapidly accumulate TMV-U1 compared to other ecotypes tested (Arce-Johnson et al. 2003).

TMV-Cg infection was examined among 14 ecotypes (Arce-Johnson et al. 2003), and it was shown to systemically infect all ecotypes tested without causing major disease symptoms. The highest levels of viral CP were found in Bla-2, C24, No-0 (Nössen-0), and Po-1 (Poppelsdorf-0). Further analyses of these ecotypes were not discussed. However, the authors focused on factors that potentially influence TMV-U1 infection in *Arabidopsis*. Because TMV-Cg, but not TMV-U1, was highly infectious in systemic tissues among all ecotypes, the authors reasoned that infection differences could be attributed to the viral movement protein (MP). To examine this possibility, the authors constructed a hybrid TMV-U1 virus in which the U1 MP was replaced with the Cg MP. Unfortunately, replacing the MP in TMV-U1 did not result in systemic spread. Further studies are warranted to determine host or viral factors that control TMV-U1 infection in *Arabidopsis*, including the ecotypes that allow rapid systemic accumulation.

2.11
Tobacco Ringspot Virus (TRSV)

TRSV infection was tested in 97 *Arabidopsis* ecotypes and the majority of these appeared to be tolerant, including Col-0 (Lee et al. 1996). Tolerance was marked by the ability of infected plants to grow, develop, and produce seed similarly to non-infected control plants. In contrast, TRSV caused severe symptoms among some ecotypes including Estland and H55, which died within 10 days of inoculation. Interestingly, the tolerant and sensitive ecotypes accumulated similar levels of TRSV. TRSV isolates used in the study were the grape strain, the bud blight strain, and an isolate collected from an infected soybean field. Through genetic analysis of reciprocal crosses between

Col-0 and the Estland and H55 ecotypes, it was determined that a single dominant locus designated *TTR1* (*tolerance to Tobacco ringspot 1*) located on chromosome 5 was responsible. From this study, useful information was gained that will assist in the uncovering of host factors regulating tolerance and symptom expression.

2.12 Spring Beauty Latent Virus (SBLV)

Interestingly enough, plants that are either tolerant or sensitive to virus infection can accumulate similar levels of a given virus. Isolation of genes that control symptom development is of interest since it furthers our understanding of how viruses and plants coexist. SBLV was shown to systemically infect all 63 *Arabidopsis* ecotypes screened (Fujisaki et al. 2004). Symptom expression among the ecotypes was variable with the majority of ecotypes being classified as displaying either no or mild symptoms. The S96, Fr-2 (Frankfurt-2), Ei-2 (Eifel-2), and Abd-0 (Aberdeen-0) ecotypes expressed severe symptoms in response to SBLV. Quantification of SBLV accumulation demonstrated that susceptibility was not linked to sensitivity among the ecotypes. To determine the genetics of SBLV-induced symptoms, reciprocal crosses were made among S96, Col-0, and Ei-2. From the S96 $\times$ Col-0 cross, the authors suggested that SBLV-induced symptoms were controlled by a single semi-dominant locus that was designated *SSB1* (*symptom development by SBLV infection 1*) and was mapped to chromosome 4. An allele of *SSB1* was also involved in symptom development in Ei-2, demonstrating that independent ecotypes can carry an allele of this locus conferring sensitivity to SBLV.

2.13 Brome Mosaic Virus (BMV)

To determine if *Arabidopsis* could support BMV infection, 20 ecotypes were screened (Dzianott and Bujarski 2004). The overall purpose of this study was to identify susceptible ecotypes for analysis of BMV RNA replication and recombination. Surprisingly, more than half of the ecotypes supported replication including Col-0, but it was not stated whether BMV-infected plants developed symptoms. Among the susceptible ecotypes, Col-0 was the best host for BMV although its replication was not high when compared to barley, the natural systemic host of BMV. To enhance BMV replication in the Col-0 background, the authors inoculated Col-0 plants expressing P1/HC-Pro, a potent viral suppressor of RNA interference (RNAi). Enhanced accumulation of BMV suggested that RNAi suppressed BMV accumulation in wild-type Col-0. The authors also concluded that stage of plant development and environmental conditions were important considerations when evaluating BMV infection.

2.14
Tomato Spotted Wilt Virus (TSWV)

Viruses that have wide host ranges are ideal pathogens to test for their ability to infect *Arabidopsis*. TSWV has been found to infect more than 400 species of plants (monocots and dicots) representing 50 families (German et al. 1995). A study of the interactions between *Arabidopsis* Col-0 and TSWV concluded that TSWV was infectious, based on positive dot-blot and ELISA data and the appearance of chlorotic spots, a common symptom of TSWV-infected plants. The earliest detection of chlorotic spots occurred 3 weeks post-inoculation (wpi) followed by severe chlorosis and stunting at 4 wpi (German et al. 1995). In the study, only Col-0 was tested against TSWV, however, the authors did mention an interest in testing additional *Arabidopsis* ecotypes for TSWV infection and in screening for mutants that suppress TSWV-induced symptom expression. As described above for several other viruses, screening additional ecotypes might reveal differences in susceptibility and symptom expression that can potentially be used to identify novel host factors.

3
Identification of Host Mutants with Altered Virus Movement Phenotypes

The recovery of mutants that alter susceptibility to viral infections is useful to further our understanding of viral and host factors that regulate infection. Discussed in this section are forward genetics studies that utilized mutant *Arabidopsis* populations to identify novel loci that affect virus movement.

3.1
vsm1 (*virus systemic movement 1*)

Turnip vein clearing virus (TVCV) is highly infectious in *Arabidopsis* and produces robust disease symptoms (Lartey et al. 1997). From a screen of EMS-mutagenized Col plants, a virus systemic movement mutant (*vsm1*) was recovered that only supported local TVCV infection (Lartey et al. 1998). To determine whether systemic resistance in the *vsm1* mutant was due to interference in infectious TVCV virion formation, TVCV particles were isolated from inoculated leaves of wild-type and *vsm1* plants and were RNase treated. Following treatment, TVCV particles were inoculated onto leaves of *Nicotiana tabacum* cv. Xanthi-nc, a local lesion host of TVCV. Findings demonstrated that TVCV virions isolated either from wild-type or *vsm1* plants induced HR and that lesions were overall equal in size. In addition, extracts prepared from stem and systemic tissues of wild-type and *vsm1* plants (rosette-leaf inoculated) confirmed that the *vsm1* mutants restricted TVCV systemic movement since no HR was detected. At this time, the cloning of *VSM1* has not been reported.

3.2
vid1 (*virus inducible dwarf 1*)

Screening of additional EMS-mutagenized Col plants resulted in the discovery a conditional mutant that displayed severe dwarfism in response to TVCV infection (Sheng et al. 1998). Accordingly, this mutant was designated *vid1*, and it was shown that TVCV infection stimulated production of numerous secondary inflorescence stems, whereas in the absence of TVCV infection the conditional mutants were similar in size and structure to wild-type. With regard to stem structures, TVCV infection was shown to reduce the length of stem cortical cells and compress the internodes. Additional studies revealed that TVCV-induced dwarfism and stimulation of inflorescence stem production in mutants were not dependent on growth stage since the phenotype was observed in mutant plants infected before or after bolting. Interestingly, there were no major differences in TVCV accumulation and movement between wild-type and mutant plants. To examine the effects of hormone treatment on TVCV infection in *vid1* plants, auxin or gibberellic acid were applied. Because auxin treatment restored the wild-type infection phenotype, it was concluded that an auxin pathway was compromised in *vid1* plants. From genetic analysis, it was suggested that a recessive mutation in a single gene conditioned this TVCV-induced phenotype. Although virus accumulation was not altered in the mutant the overall phenotype (severe stunting) was similar to phenotypes expressed among ecotypes that are hypersusceptible to certain viruses.

3.3
asc1 (*acceleration of symptom by CaMV*)

Various approaches have been used to identify host factors that regulate virus movement in plants. A novel genetic screen was used to identify *Arabidopsis* mutants that suppressed mutations in the MP (gene I) of the CM1841 isolate that blocked or delayed infection (Callaway et al. 2000; Thomas and Maule 1995a,b). The infectivity and reversion frequencies of this viral mutant in Col-0 plants (non-mutagenized or mutagenized prior to infection) revealed that three single codon substitution mutants were infectious and two multiple codon substitution mutants were not infectious among the mutants tested (Callaway et al. 2000). Interestingly, progeny derived from a virus mutant designated ER2A were recovered that restored virulence without reversion but by second-site mutations. These secondary mutations included sites in the vicinity of the original ER2A mutation and throughout the C-terminus of gene I.

In response to recombinant CaMV inoculation (N6, N7, or N13), Col-0 plants became infected, indicating that these sites were responsible in restoring virulence in the ER2A background mutant. Compared to wild-type CaMV, recombinant CaMV containing the primary and secondary mutations delayed the induction of symptoms on Col-0 plants. As a result, a search for

host suppressors that accelerated symptom development was initiated. Out of 15 symptom-accelerated M_2 suppressor mutants recovered, only two mutant lines (5–2 and 15B) were consistent for this phenotype in the M_3 generation. Symptoms produced on these mutant lines in response to CaMV N7 included severe stunting. Quantitative trait loci (QTL) analysis suggested that a unique, recessive locus designated *asc1* on chromosome 1 of suppressor line 5–2 was responsible for accelerated symptom expression and hypersusceptiblity to CaMV N7.

3.4
Screening for Mutants that Alter Subcellular Targeting of CMV MP

In another clever twist on screening for host mutants that affect viral movement, Sasaki et al. (2006) made transgenic *Arabidopsis* plants that expressed the CMV MP fused to GFP (MP::GFP) under control of an epidermis-specific promoter. GFP fluorescence in the epidermal cells was observed in punctuate foci at the cell wall, presumably in association with plasmodesmata. The authors mutagenized these transgenic plants and used microscopy to screen 11 056 M2 seedlings for altered distribution of MP::GFP fluorescence with the goal of identifying host factors that affected the ability of CMV MP to localize to the plant cell wall. Of the mutants with altered subcellular targeting of MP::GFP, three had mutations in the MP itself. Interestingly, the independent mutations clustered within a region of the MP rich in cysteine and histidine, suggesting a role for zinc (Zn^{2+}) binding. The MP was shown to bind zinc and that the mutants had weaker interactions with zinc than wild-type MP, which correlated with the ability to localize to the plasmodesmata. Mutation of these amino acids in the MP of an infectious CMV clone resulted in recombinant viruses that caused reduced or no symptoms. Although a novel host factor was not identified in this study, the Zn^{2+} binding activity of CMV MP was correlated with its physiological activities in mediating virus movement and disease development.

4
Application of Reverse Genetic Approaches to Discover the Roles of Candidate Genes in Virus Movement

Studies probing the interactions of viral and host proteins offer the opportunity to identify specific interactions that are essential in promoting viral infection. The identification of interacting proteins by yeast two hybrid (Y2H) or biochemical approaches yields candidate genes that may potentially have direct functions in the activity of viral movement proteins. Whether the candidate genes originate from *Arabidopsis* or another plant species, reverse genetic studies in *Arabidopsis* can typically be employed to study their func-

tions. The only caveat is that a candidate gene identified from another plant species must have a homolog in the *Arabidopsis* genome. Discussed in this section are studies that utilize reverse genetic approaches in *Arabidopsis* to study the function of candidate genes in virus movement.

4.1 AtNSI (*Arabidopsis thaliana* Nuclear Shuttle Protein Interactor)

Geminiviruses are DNA viruses that replicate in the nuclei of plant cells and as such have distinct, yet highly coordinated, intracellular and intercellular mechanisms for transporting their genomes. The nuclear shuttle protein (NSP) transports viral DNA between the nucleus and cytoplasm while the MP transports NSP–genome complexes from cell to cell (Noueiry et al. 1994; Pascal et al. 1994; Sanderfoot et al. 1996). Using Y2H analysis coupled with protein pull-down assays, the NSP of *Cabbage leaf curl virus* (CaLCuV or CLCV, geminivirus) was found to interact with an *Arabidopsis* acetyltransferase protein referred to as AtNSI (*Arabidopsis thaliana* nuclear shuttle protein interactor) (McGarry et al. 2003). It was also observed that AtNS1 and NSP of *Squash leaf curl virus* (SqLCV) interacted, suggesting that AtNSI might have a general function in geminivirus infections. Functionally, acetyltransferases are involved in regulating chromatin remodeling (histones) and protein–protein interactions through acetylation. Likewise, AtNSI was shown to acetylate calf thymus histones in vitro.

To identify protein candidates acetylated by AtNS1 in vitro, nuclear-targeted CaLCuV proteins including NSP were tested. Interestingly, out of the five nuclear-targeted viral proteins, only the CP was acetylated, however a stable interaction between AtNSI and CP was not detected. Based on this finding, it was proposed that NSP bound to the viral genome recruits AtNSI to a ternary complex with the genome-bound CP in order to disrupt CP binding (Carvalho and Lazarowitz 2004; Carvalho et al. 2006; McGarry et al. 2003). According to the model, disruption of CP binding allows NSP to transport the viral genome from the nucleus. Partial support for this model was evident in CaLCuV-infected transgenic *Arabidopsis* lines over-expressing AtNSI (McGarry et al. 2003). Compared to wild-type, AtNSI over-expression lines accumulated increased levels of CaLCuV. With regard to AtNSI expression and localization, it is predominantly expressed in the leaves (developmentally regulated) and accumulates in the nucleus (AtNSI expression studies in *Nicotiana benthamiana*) (Carvalho et al. 2006; McGarry et al. 2003).

Using mutational and truncation analyses, the interactions between NSP and AtNSI in promoting CaLCuV infection were further investigated. Analysis of NSP revealed that a 33-aa region (aa 150–187) was needed for AtNSI binding (Carvalho and Lazarowitz 2004). In particular, CaLCuV mutants NSP^{E150G}, NSP^{I164T}, or NSP^{D187G} were shown to mildly infect *Arabidopsis* compared to wild-type virus. This suggested that NSP mutants were unable to

stably recruit AtNSI to disrupt CP binding according to the proposed model to facilitate viral genome nuclear entry. Additional data supported this concept since NSP mutants retained DNA binding affinity, accumulated in the nucleus, and interacted with CaLCuV MP.

When AtNSI was subject to mutational analysis, an 88-aa region overlapping the acetyltransferase domain (aa 107-194) was identified for NSP interaction (Carvalho et al. 2006). Since AtNSI mutants, AtNSI^{I107T}, AtNSI^{K136E}, and AtNSI^{D194G} were unable to stably interact with NSP, defects in AtNSI mutants pertaining to folding and stability were tested against AtNSI plant interacting proteins (At6–8 and AtENA) (Carvalho et al. 2006). From this study, it was found that all three AtNSI mutants interacted with At6–8 and AtENA, indicating another host factor was involved. Further examination demonstrated that these mutant proteins were enzymatically inactive, because CaLCuV CP was not acetylated in their presence. Together with Y2H data that displayed self-interaction of AtNSI (wild-type) it was concluded that oligomerization was necessary to promote acetylation since mutant proteins lacked this interaction and activity. Interestingly, after detecting a decrease in CP and calf thymus histone acetylation in the presence of NSP, it was discovered that CaLCuV NSP inhibited AtNSI activity in vitro. Inhibition of AtNSI activity was likely a result of NSP interference in AtNSI assembly. Therefore, it was proposed that NSP binds the oligomerization sites of AtNSI preventing the formation of AtNSI complexes that target plant proteins. Under this revised model, NSP recruits AtNSI monomers that acetylate CP, thereby weakening the interactions between CP and the viral genome, allowing NSP to transport the viral genome from the nucleus. Thus, NSP and AtNSI interactions and the acetylation of CP are necessary for successful CaLCuV infection in *Arabidopsis*.

4.2 Host Proteins Interacting with Potyvirus Proteins

Using the Y2H system, proteins from pea (*Pisum sativum* L. cv. Scout) were found to interact with the VPg of *Pea seed borne mosaic potyvirus* (PSbMV) (Dunoyer et al. 2004). One strongly interacting protein identified as PVIPp (*Potyvirus* VPg-interacting protein from pea) was similar to two cysteine-rich *Arabidopsis* proteins (At5g48160; PVIP1 and At3g07780; PVIP2). To examine whether VPgs of several *Arabidopsis*-infecting potyviruses could interact with these two proteins, Y2H analysis was performed. The TuMV VPg was shown to interact with both cysteine-rich proteins. Deletion analysis of TuMV VPg was performed to determine regions necessary for VPg–PVIP interactions. These interactions required amino acids 1–16 and 42–66 in the N-terminal region of TuMV VPg. *Arabidopsis* RNAi lines with reduced expression of PVIP1 or PVIP2 showed restricted movement of TuMV (wild-type) when compared to wild-type plants. TuMV levels were reduced in the inoculated leaves of the mutants indicating that cell-to-cell movement was restricted. Similar re-

sults were also observed in *N. benthamiana* after inoculation with TuMV containing a mutation in VPg that blocked its interaction with PVIP-related proteins. However, when the TuMV VPg mutant was delivered by agroinfiltration, a reduction in TuMV levels was not observed locally but systemically. Collectively, the *Arabidopsis* and supporting *N. benthamiana* data suggested that VPg interactions with specific cysteine-rich proteins are necessary for the movement of TuMV and possibly other potyviruses.

The Y2H approach has also been applied to identify host proteins interacting with the CI (cylindrical inclusion) protein of PPV (Jimenez et al. 2006). The CI, which is involved in replication and movement, of PPV interacted with a *N. benthamiana* protein named PSI-K, a component of photosystem I. Additional tests showed that the *Arabidopsis* PSI-K protein could also interact with a potyvirus CI protein suggesting that this may be a general feature of plant–potyvirus interactions. Subsequently, the functions of NbPSI-K and AtPSI-K were tested by RNA silencing and knockout mutation, respectively. The results in both species demonstrated that loss of PSI-K resulted in increased accumulation of PPV and more rapid spread of the virus, suggesting an inhibitory function for PSI-K in potyvirus infection.

4.3 Plasmodesmal Associated Protein Kinases (PAPK) and TMV MP

To identify proteins that potentially regulated cell-to-cell trafficking of macromolecules, such as viral ribonucleoprotein complexes, a biochemical approach was utilized to identify tobacco PAPKs that phosphorylated the TMV MP (Lee et al. 2005). The purified tobacco PAPK was determined to be a member of the casein kinase I family, but the corresponding tobacco cDNA was not identified. This problem was overcome by identifying the orthologous *Arabidopsis* gene, which was one of at least 14 members of the casein kinase I family in *Arabidopsis*. Subsequently, the CLK6 protein was shown to possess all the properties expected of the tobacco casein kinase including localization to the cell wall, co-localization with the TMV MP, and the ability to phosphorylate TMV MP. The conserved function of these casein kinases in *Arabidopsis* and tobacco provides the opportunity to utilize a variety of molecular and genetic approaches to assess the role of the casein kinase in virus movement and macromolecular trafficking of non-cell autonomous proteins.

5 Conclusions and Future Perspectives

Many questions remain with respect to the biochemical underpinnings of plant-virus movement. These will be obtained through a variety of ap-

proaches that will continue to include *Arabidopsis* as an important model, because of the potential to combine genetics, molecular biology, and cell biology to understand host–virus interactions. The natural variation in movement phenotypes has not been fully exploited since many of the genetic loci that have been identified that affect movement have not yet been cloned. Presumably, identification of the proteins encoded by these genes will lead to new insights into the mechanisms of virus movement.

Important insight into host–virus interactions can also come from studying resistance traits that do not conform to the typical form mediated by dominant resistance genes (e.g., eIF4e or eIF(iso)4E). Those genes that render *Arabidopsis* resistant to local or systemic movement but are not associated with "classical" defense responses are the most interesting targets in this respect. The naturally existing alleles of such genes can be complemented by additional mutant searches for altered infection phenotypes. Many such mutant screens have already been attempted. However, it is clear that the numbers of plants screened in these virus assays does not approach the scale of mutant screens often used in the study of plant development or response to abiotic stimuli. This is because the screens are typically much more time-consuming and cumbersome, requiring plants to be grown in soil and reach an appropriate stage of development, and that inoculation is extremely efficient. Thus, the throughput of virus mutant screens is typically relatively low. It has been postulated that many of the host genes required for virus movement will be necessary for plant growth and development because of their roles the local and systemic trafficking of proteins and RNA. Nevertheless, opportunities may be present if one is persistent in doing a large-scale mutant screen. For example, only one recessive *rtm2* or *rtm3* allele was identified out of about 81 150 M2 plants, but these would not have been identified on the scale of most mutant screens performed for host–virus interactions, which involve at most approximately 25 000 plants. High throughput screening methods such as the use of selectable viruses may help to overcome this important limitation (Whitham et al. 1999). The use of T-DNA knockouts may not be effective in cases where loss of gene function is lethal, but perhaps EMS-induced point mutations can generate alleles of genes that will yield a virus infection phenotype, but not severely alter plant growth and development.

We currently have very little understanding of how resistance mechanisms function to limit virus movement. The identification and cloning of recessive and dominant genes that function to inhibit virus movement will provide new insight into these phenomena. Recessive genes that restrict virus movement may indicate the lack of a host factor, or an allele of that factor that is not able to interact appropriately with the virus to support movement. Dominant genes of this category may inhibit required interactions or be involved with as-yet uncharacterized resistance pathways. Cloning and further characterization of these genes will provide novel insight into the host requirements for

movement. This knowledge may also be used to design novel control strategies to combat virus infection in crops.

With respect to resistance mechanisms mediated by dominant *R* genes, it is unknown what aspect of the HR plant defense response is actually restricting virus infection, but there must be some associated factors that inhibit viral replication and/or movement. Even in susceptible interactions, many viruses activate what appear to be basal plant defenses (Huang et al. 2005; Love et al. 2005; Whitham et al. 2003). CaMV activates at least three basal defense pathways, but is still able to systemically infect susceptible *Arabidopsis* ecotypes (Love et al. 2005). This raises a question of whether viruses might encode other suppressors of basal plant defenses in addition to the silencing suppressors to combat plant innate immunity mechanisms in order to replicate and move successfully.

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