Chapter 13
Protein-Protein Interactions in Plant Virus Movement and Pathogenicity

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13.1 Introduction

Viruses generally consist of a rather small number of molecular components including a few proteins, sometimes a membranous envelope, and an RNA or DNA genome encompassing a very limited set of coding sequences. For every step in the viral life cycles such as replication of the viral genomes, transcription/translation of viral gene products, intra- and intercellular movement and virus assembly and transmission, viruses make use of the biosynthetic and regulatory capacities of the host cells.

The rigid nature of plant cell walls and the lack of a cardiovascular system prevent plant viruses, in contrast to animal viruses, from spreading within the infected organism by cell lysis or budding and subsequent passive transport in a liquid circulation system. Similarly, the lack of a circulating antibody system has brought about RNA silencing mechanisms as the major antiviral defence strategy applied by plant cells. Due to these two main differences in viral infections between plant and animal systems, plant viruses have evolved specialized proteins and protein functions. On the one hand, so-called movement proteins utilize plant host structures and mechanisms to facilitate intra-cellular, inter-cellular and long-distance transport; on the other, silencing suppressors serve to counteract and escape the host plant antiviral defence machineries.

Virus movement and the suppression of silencing cannot be separated unambiguously. Historically, a number of different plant viral proteins have been termed "movement proteins" based on the observation that defects in the respective genes affect cell-to-cell movement and limit systemic spread. In some cases it is now becoming clear that the genuine function of some of these movement proteins might rather be suppression of silencing. While it is obvious that the suppression of silencing is the prerequisite for the establishment of an infection of a cell during the

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movement process, there is evidence that many viral proteins are multifunctional, and that the functions in movement and suppression of silencing may be separable (Bayne et al. 2005). Advances in research in both fields in recent years not only have greatly improved our understanding of the molecular basis of plant viral infection cycles, but also have revealed insight into as yet poorly understood cellular processes. Long-distance transport of RNA and/or proteins, and micro RNA-based control mechanisms are emerging as ancient fundamental regulatory processes underlying plant development and the processing of environmental signals (Lough and Lucas 2006; Murchison and Hannon 2004).

The appropriation of cellular functions and regulatory machineries often is accomplished by physical interaction between viral and host proteins facilitating a redirection of the host protein's function to serve a function in the viral life cycle. The investigation of host proteins interacting with viral proteins has proven to be a very promising approach to dissect the molecular basis of viral infections and to understand how viruses integrate in the complex structural and regulatory networks controlling plant growth and development.

13.2 Cell-to-Cell Movement of Plant Viruses

Plant viral movement proteins facilitate cell-to-cell and long-distance transport of viral structures and thereby allow the virus to establish a systemic infection in the host plant. In general, movement of viruses in plants does not involve extra-cellular stages, but rather occurs via plasmodesmata, the specialized, plant-specific intercellular structures connecting the cytoplasms of adjacent cells. Historically, plasmodesmata were regarded as simple channels allowing passive trafficking of low-molecular weight growth regulators and nutrients. An emerging picture now is that plasmodesmata are highly complex structures that regulate the selective trafficking of macromolecules (Lucas and Lee 2004).

Recent progress, in the analysis of cell-to-cell and long-distance transport processes in plants – stimulated by the investigation of virus movement in plants – revealed a complex communication network based on the transport of signalling molecules. The trafficking of proteins, RNA or ribonucleotide-protein complexes might be a fundamental means of plants to control development and to communicate and respond to environmental signals (Lough and Lucas 2006; Lucas and Lee 2004). A growing body of evidence supports the notion that proteins that move through plasmodesmata, so-called non-cell-autonomous proteins (NCAPs), can contribute to patterning and the establishment of cell fate in plant tissues (Gallagher and Benfey 2005; Lough and Lucas 2006; Lucas and Lee 2004). In fact, the immunological relatedness of CmPP16, an NCAP isolated from *Cucurbita maxima* phloem sap, and the movement protein of *Red clover necrotic mosaic virus* was interpreted as an indication of a potential common evolutionary origin of viral movement proteins and NCAPs (Xoconostle-Cazares et al. 1999). The observation that viral movement proteins compete with endogenous NCAPs for the

plasmodesmata trafficking machinery supports the notion that plant viruses have evolved movement proteins to "hitch-hike" this NCAP trafficking pathway (Lucas 2006).

The mechanisms and molecular machineries involved in the inter-cellular and long-distance movement of NCAPs, viruses or other macromolecules are only beginning to be understood, but research in this field has been greatly stimulated by the investigation of the molecular interactions of viral movement proteins, especially by the identification of plant host proteins interacting with viral movement proteins (Boevink and Oparka 2005; see Table 13.1).

Table 13.1 Plant proteins interacting with viral movement proteins

Virus	Movement protein	Biological function(s)	Interacting host proteins	References for interaction
CLCV	NSP	Virus movement, nuclear shuttling	AtNSI1 acetyltransferase	Carvalho and Lazarowitz (2004); McGarry et al. (2003)
TGMV TCrLYV	NSP	Virus movement, nuclear shuttling	LeNIK, GmNIK, NIK1, NIK2, NIK3 LRR receptor-like kinases	Fontes et al. (2004); Mariano et al. (2004)
TMV	MP, p30	Virus movement	KELP, MBF1, transcriptional co-activators; NtRIO kinase PME MPB2C Calreticulin PAPK kinase	Chen MH et al. (2000, 2005); Dorokhov et al. (1999); Kragler et al. (2003); Lee et al. (2005); Matsushita et al. (2001, 2002); Yoshioka et al. (2004)
TuMV	VPg	RNA replication and translation, virus movement, virulence factor	PVIP PHD finge cysteine-rich protein	Dunoyer et al. (2004)
ToMV	CP	Coat protein, virus movement	IP-L	Li et al. (2005)
TCV	p8	Virus movement	Atp8	Lin and Heaton (2001)
PPV	CI	Replication, virus movement	Photosystem I PSI-K protein	Jimenez et al. (2006)
TSWV	NSm	Virus movement	AtA39, NtDNAJ_ M541 At4/1	Soellick et al. (2000); von Bargen et al. (2001)
PMTV	TGB2	Virus movement	TIP1, TIP2, TIP3; RME-8 J-domain protein	Fridborg et al. (2003); Haupt et al. (2005)
TBSV	P22	Virus movement	HD-ZIP	Desvoyes et al. (2002)
GFLV	MP	Virus movement	KNOLLE syntaxin	Laporte et al. (2003)
CPMV	60 K	Virus movement	VAP27-1/2 SNARE	Carette et al. (2002)
CaMV	MP	Virus movement	MP17 rab-acceptor	Huang et al. (2001)

Intercellular movement of macromolecules involves as the first step the intracellular translocation of the proteins, RNAs or nucleo-protein complexes to the cell periphery and the sites of the plasmodesmata. In contrast to other translocation or localization events such as transport into chloroplasts or mitochondria, or targeting to the endoplasmic reticulum (ER) and the secretory pathway, no recognizable conserved sequence patterns or structural elements have been identified so far, targeting proteins to plasmodesmata. There is now increasing evidence that different pathways can direct proteins to the plasmodesmata, involving cytoskeletal elements and/or the endomembrane system. Both ways have recently been shown to be employed by viruses as well.

The second step is the translocation of the macromolecules through the plasmodesmata. Still, only very few structural components of plasmodesmata have been identified up to date. Therefore, the mechanism and the selectivity of plasmodesmatal transport are largely unknown. However, some of the identified host proteins interacting with viral movement proteins allow developing hypotheses on the molecular mechanisms involved in this step of viral movement.

Long-distance transport of viral structures usually occurs via the plant vasculature. This is probably the least understood step in plant viral movement and clear molecular data about the mechanisms involved are not available at present.

13.3 The Role of the Cytoskeleton

In order to move from the site of replication to the plasmodesmata at the cell periphery, viruses make use of the transporting capacities of the plant cytoskeleton. More than ten years ago plant viral movement proteins had already been demonstrated to co-localize with and bind to both, microtubules and actin filaments (Heinlein et al. 1995; McLean et al. 1995). However, the exact roles of these two cytoskeletal elements in the intracellular translocation and the intercellular transport of viral structures are just emerging. The most extensively investigated plant viral movement protein is the P30 protein from TMV. Fusions of P30 with the green fluorescent protein (GFP) have been analyzed with respect to intracellular localization, colocalization with cytoskeletal elements, and dynamics during infection and movement processes (Boyko et al. 2000; Epel et al. 1996; Heinlein et al. 1995; Mas and Beachy 2000; Padgett et al. 1996; Reichel et al. 1999). A conserved amino acid sequence motif has been identified within the sequences of tobamoviral movement proteins resembling a region in tubulin that was proposed earlier to mediate lateral contacts between microtubules (Boyko et al. 2000). This finding indicates that tobamoviral movement proteins bind to microtubules by mimicking tubulin interaction and assembly surfaces, thereby presumably competitively displacing γ-tubulin, and probably make use of microtubule polymerization to drive the transport process (Boyko et al. 2000; Wick 2000).

The TMV movement protein has furthermore been shown to interact with MPB2C, a previously uncharacterized microtubule associated plant protein (Kragler et al. 2003).

In accordance with the idea of microtubule-based intra- or intercellular transport, At4/1, a protein with homologies to myosin/kinesin motor proteins, has been found to interact with NSm, the movement protein of *Tomato Spotted Wilt Virus* (TSWV), a virus with a negative/ambisense ssRNA genome (von Bargen et al. 2001).

However, the role of microtubules in viral movement is still not quite clear, and it is very likely that different viruses utilize different cellular structures and mechanisms for intra- and inter-cellular movement. In the case of the TMV movement protein it has been shown that MPB2C is not required for movement but is necessary for microtubule association (Curin et al. 2007). In fact, MBP2C seems to be a negative effector of intracellular movement, and there is evidence that microtubules are dispensable for inter-cellular movement (Kragler et al. 2003). These and other recent findings indicate that microtubules and microtubule-associated factors might be involved in degradation of movement proteins, and that a transport function necessary for translocation of movement proteins and viral structures to the cell periphery and the plasmodesmata might rather be provided by the microfilaments of the actin cytoskeleton in connection with the endomembrane system (Boevink and Oparka 2005; Liu et al. 2005).

So far, no direct physical interaction between viral movement proteins and actin filaments has been demonstrated. However, co-localization and application of actin-destabilizing drugs indicate an association of for example the TMV movement protein with actin filaments (McLean et al. 1995). Similarly, an association of the movement proteins TGB2 and TGB3 of Potato mop-top virus (PMTV) have been shown to co-align with the actin cytoskeleton and, upon application of the actin-depolymerizing drug latrunculin, subcellular localization of these two proteins was changed and movement was abolished (Haupt et al. 2005).

Beet yellows virus requires five viral proteins for movement, including a homolog of a class of eukaryotic heat shock proteins of approximately 70 kDa, Hsp70 h (Peremyslov et al. 1999). Recently this protein has been found to form motile granules that are associated with actin microfilaments, and translocation to plasmodesmata was dependent on an intact actin cytoskeleton (Prokhnevsky et al. 2005). The involvement of Hsp70 proteins in viral movement is interesting because this class of proteins has been shown to be involved not only in protein folding but also in protein translocation processes (Young et al. 2003). The function of Hsp70 proteins is dependent on co-chaperones such as proteins of the DnaJ family, which are required for stable binding of Hsp70 to their substrates. Intriguingly, several plant viral movement proteins have been shown to interact with proteins containing a J-domain conserved in DnaJ proteins (Haupt et al. 2005; Soellick et al. 2000; von Bargen et al. 2001). These findings indicate a potentially general role of Hsp70 in viral movement either in connection with transport along the cytoskeleton or in the process of partial unfolding of viral structures that may be required for passage through plasmodesmata.

In addition to the direct recruitment of the host cell's transport machineries there are indications that hint at an indirect way of trafficking by "hitch-hiking" a protein that itself is transported into the neighbouring cell via the plasmodesmata. Recently, an interaction between the TBSV movement protein P22 and a plant homeodomain leucin zipper (HD-ZIP) protein has been identified (Desvoyes et al. 2002). One of

the first non cell-autonomous proteins identified in plants is the homeodomain protein KNOTTED1, that has been shown to move between cells via plasmodesmata (Lucas et al. 1995). Therefore, the proposition in the case of the TBSV-P22 protein is that by way of interacting with the HD-ZIP protein it may be transported to and possibly passaged through the plasmodesmata (Desvoyes et al. 2002). So far, the mechanism of how homeodomain proteins move to and traffic through plasmodesmata is not known. However, recently a microtubule-associated protein has been identified interacting with and regulating the intra-cellular localization of homeodomain proteins of the three amino acid loop extension (TALE) class (Hackbusch et al. 2005). Whether this intriguing novel protein family might be a candidate for a component of intra- or intercellular transport machineries remains to be investigated.

13.4 Involvement of the Endomembrane System in Virus Movement

Plamodesmata are plasma membrane-lined inter-cellular channels that establish continuity of the cytoplasms of adjacent plant cells. Although the molecular structure and composition of plasmodesmata is still obscure, it is well established that parts of the endomembrane system, the so-called desmotubules, extend through these channels from one cell to the other.

An emerging picture is that both inter-cellular trafficking of endogenous signalling molecules like NCAPs, and virus movement might proceed with involvement of the endomembrane transport system (Boevink and Oparka 2005). Specific targeting of some cellular proteins to the plasmodesmata via a Golgi-dependent pathway has been revealed recently (Sagi et al. 2005).

There is a growing body of evidence that many steps in plant viral life cycles may be connected with cellular endomembrane systems. Virus replication often is associated with the ER, and a number of viral proteins facilitating movement similarly locate to the ER.

The TMV P30 movement protein and the TMV 126/183-kDa protein, both required for TMV movement, have been shown to locate to the ER (Heinlein et al. 1998). Similarly, an association of the movement protein (p6) of beet yellow closterovirus with the ER has been observed (Huang and Zhang 1999; Peremyslov et al. 2004).

Several host proteins involved in the secretion pathway or membrane trafficking events have been identified recently as interaction partners of plant viral movement proteins, supporting the view that the dynamic endomembrane system plays a central role in viral movement processes.

Movement of Grapevine fanleaf virus (GFLV) virions proceeds through tubules, formed by the virus-encoded movement protein, that penetrate modified plasmodesmata. Tubule formation is dependent on a functional secretory pathway, and the GFLV movement protein physically interacts with KNOLLE, a syntaxin

involved in cytokinesis (Laporte et al. 2003). Syntaxins are membrane proteins belonging to the SNARE family that regulates vesicle targeting and fusion (Chen YA and Scheller 2001). Interestingly, the Cowpea mosaic virus 60 K movement protein interacts with VAP33/SNARE (Carette et al. 2002), indicating that Syntaxin/SNARE-mediated vesicle fusion, targeting or trafficking is a mechanism for intracellular translocation that different viruses take advantage of.

In addition to interacting with the syntaxin KNOLLE, the movement protein of GFLV has been shown to colocalize with calreticulin-containing foci (Laporte et al. 2003). This again might be a common theme in movement processes of different plant viruses, because recently a direct interaction between the TMV movement protein p30 and calreticulin has been described (Chen MH et al. 2005). Calreticulin is a ubiquitous calcium-binding chaperone involved in integrin-mediated cell adhesion in animals (Coppolino et al. 1997). In plants, calreticulin has been shown to localize to the ER and to plasmodesmata. The interaction with p30, co-localization in vivo and the observation that over-expression of calreticulin affects p30 localization and TMV movement have led to the conclusion that calreticulin might be functionally involved in viral movement (Chen MH et al. 2005). Indirect support for the idea of an involvement of calreticulin in plant viral movement comes from the finding that one of the two movement proteins of turnip crinkle virus (TCV) interacts with a protein designated Atp8, containing so-called RGD motifs (Lin and Heaton 2001). These motifs are known to function as cell-attachment sequences that are recognized by integrins which in turn may be bound by calreticulin (D'Souza et al. 1991). Direct or indirect binding of movement proteins to calreticulin might serve for anchoring viral movement complexes to peripheral attachment sites which frequently are associated with plasmodesmata (Boevink and Oparka 2005).

MPI7, an *Arabidopsis thaliana* protein interacting with the cauliflower mosaic virus (CaMV) movement protein (MP) provides more, albeit indirect, evidence for a possible involvement of membrane or vesicle trafficking in viral movement processes. Direct interaction between MP and MPI7 in yeast and in planta has been demonstrated by yeast two-hybrid analyses and fluorescence resonance energy transfer (FRET) (Huang et al. 2001). Biological significance and a potential important role of this interaction in viral infectivity were inferred from the observation that two amino acid exchanges in the MP that were shown previously to abolish infectivity likewise disrupted the interaction. Furthermore, an infectious second-site mutant that differed from the non-infective mutant by only a single amino acid restored the interaction in the yeast two-hybrid system (Huang et al. 2001). MPI7 has homologies to a class of mammalian Rab acceptor proteins. Rab proteins, in turn, are small ras-like GTP binding proteins now known to function in both constitutive and regulated exocytosis, as well as in endocytosis and transcytosis. Proteins of the Rab class tether incoming vesicles to the correct target organelle contributing to the specificity of membrane trafficking and the proper flow of cargo within the cell (Zerial and McBride 2001).

Potyviruses have three overlapping genes encoding the so-called "triple-gene block" (TGB) proteins that function together to promote virus cell-to-cell

movement. TGBp2 of poa semilatent virus, as well as TGBp2 and TGB3p3 of potato virus X (PVX) and potato mop top virus (PMTV) have recently been shown to locate to the ER (Haupt et al. 2005; Solovyev et al. 2000). PMTV TGBp3, and similarly a number of different viral movement proteins contain a conserved amino acid motif that was shown to be essential for correct targeting of the proteins (Haupt et al. 2005; Laporte et al. 2003). In animals, similar amino acid motifs have been shown to be recognized by clathrin-coated vesicle adaptors at the Golgi and the plasma membrane. Moreover, the movement proteins of Potato mop-top virus physically interact with a conserved RMA-8 family of J-domain proteins essential for endocytic trafficking (Haupt et al. 2005).

Thus, these recent data strongly support the idea that intracellular movement of plant viruses proceeds via hitch-hiking the host's membrane and vesicle trafficking pathways.

13.5 Modification of and Passage Through Plasmodesmata

A characteristic generally associated with plant viral movement proteins is their ability to increase the size exclusion limits (SEL) of plasmodesmata. Plasmodesmata connecting mesophyll cells usually have an SEL of approximately 60 kDa, i.e. they are permeable to molecules of up to 60 kDa. Plasmodesmal SEL may change depending on cell type and developmental stage, and in mature tissue, for example, SEL may be as low as 1 kDa or less (Imlau et al. 1999). Although numerous viral movement proteins have been shown to possess the ability to increase this SEL, the exact mechanism of how this is accomplished is largely unknown. Here again, the identification of host factors interacting with plant viral movement proteins might be helpful in understanding the molecular basis of plasmodesmal dynamics. The deposition of callose to close plasmodesmata during wound responses and defence reactions is supposed to have a function in antiviral defence by blocking systemic viral spread (Beffa and Meins 1996). It is therefore an intriguing finding that the PVX TGB2 protein interacts with three ankyrin repeat-containing proteins, TIP1, TIP2 and TIP3, that in turn interact with beta-1,3-glucanase (Fridborg et al. 2003). Beta-1,3-glucanase is a callose-degrading enzyme that thereby may regulate plasmodesmal SEL, suggesting that a potential strategy of PVX to gate plasmodesmata is to accelerate callose degradation.

The TMV movement protein has been shown to interact with a pectin methylesterase (PME) (Chen MH et al. 2000; Dorokhov et al. 1999). While the functional implication favoured by the authors was that the main function of this interaction might be to recruit TMV movement protein to the cell periphery, more recently, it has been speculated that on the contrary, the movement protein might recruit the activity of PME to loosen the cell wall surrounding plasmodesmata to increase the SEL (Boevink and Oparka 2005).

Long-discussed regulatory steps in the passage through plasmodesmata include protein phosphorylation by plasmodesmata-associated kinases and an influence of

local Ca^{2+} concentrations (Citovsky et al. 1993; Roberts and Oparka 2003). The recent finding of an interaction between the TMV movement protein and calreticulin might provide a link to calcium-dependent regulatory processes (see above). Furthermore, PAPK, a plasmodemata localized member of the casein kinase I family has been shown recently to specifically recognize and phosphorylate the TMV movement protein and a number of endogenous NCAPs, a modification that has been shown previously to be important for TMV movement protein function (Citovsky et al. 1993; Lee et al. 2005).

13.6 Movement and Pathogenicity

The identification of a particular plant virus protein as a movement protein is based often on observations of loss-of-function, using infectious cDNA clones of viruses, whereby mutation of the gene encoding the protein leads to an inability of the virus to move out of the initial infected cell (i.e. debilitated in local or cell-to-cell movement) or from the inoculated leaf into other leaves (i.e. debilitated in systemic or long-distance movement). Gain-of-function approaches include co-infection studies where various viruses were shown to be able to enhance the movement of other viruses, with the complementation leading to local movement of the dependent virus in an otherwise non-host plant (Malyshenko et al. 1989). In another approach, movement-viable viruses have been constructed in which the movement gene of one virus has been directly replaced with that of a different virus (Dejong and Ahlquist 1992; Ryabov et al. 1999). It is also possible to complement the movement of otherwise defective (mutated) viruses by inoculating them to transgenic plants that themselves express the virus movement protein (Kaplan et al. 1995). Approaches such as these have also been used widely to assign a pathogenicity function to particular virus proteins, where pathogenicity may signify an increase in virus replication/accumulation as well as stimulation of symptom production, both in terms of intensity and of distribution throughout the plant (Brigneti et al. 1998; Liu et al. 2002; Yelina et al. 2002). Further investigation of the mechanism of action of different plant virus pathogenicity proteins has revealed an association between the ability of the virus to overcome host defence responses, specifically RNA silencing (also known in plants as post-transcriptional gene silencing) and the involvement of virus proteins in the process of virus movement (see Table 13.2).

13.7 Antiviral Defence by RNA Silencing

The term RNA silencing refers to an enzymatic process occurring in plant (and other organisms) cells where RNA molecules are targeted in a sequence-specific manner for cleavage and further degradation (Brodersen and Voinnet 2006). The initiator for RNA silencing is double-stranded (ds) RNA, which in the case of

Table 13.2 Plant proteins interacting with viral silencing suppressors

Virus	Suppressor	Biological function(s)	Interacting host proteins	References for interaction
TEV PVY	HC-Pro	Systemic movement, transmission by aphids, genome amplification	Rgs-CaM HIP1, HIP1	Anandalakshmi et al. (2000); Guo D et al. (2003)
CMV TAV	2b	Systemic and cell-to-cell movement, Pathogenicity	LytB, karyopherin α, TLP1	Ham et al. (1999); Kim et al. (2005); Wang et al. (2004b)
TBSV	p19	Pathogenicity, cell-to-cell and systemic movement	ALY (Hin19)	Park et al. (2004; Uhrig et al. (2004)
PVX	p25	Cell-to-cell movement, egress from veins in systemic leaves, RNA helicase	TIP1, TIP2, TIP3	Fridborg et al. (2003)
BWYV CABYV PLRV	P0	Symptom production, virus accumulation	SKP1, SKP2	Pazhouhandeh et al. (2006)
TCV	CP	Capsid formation, virus movement	TIP	Ren et al. (2000)
ToMV TMV	126 kDa	RNA-dependent RNA polymerase, virus movement	PAP1/IAA26, AAA AtPase, 33 K subunit of photosystem II, P58^{IPK}	Abbink et al. (2002; Bilgin et al. (2003); Padmanabhan et al. (2005)
ACMV TGMV TYLCV BCTV	AC2, AL2, C2, L2	Pathogenicity, activation of virus gene expression	SNF1 kinase, ADK	Hao et al. (2003); Wang et al. (2003)

viruses may be provided as an intermediate of replication or by base pairing of regions of the single-stranded genomic or messenger RNA (Molnar et al. 2005). The initiator dsRNA molecules are cleaved into 20–26 nt ds small interfering (si)RNAs by RNaseIII-domain-containing Dicer proteins, or Dicer-like (DCL) proteins in plants. *Arabidopsis thaliana* encodes four DCL proteins, which have distinct but overlapping roles in the processing of dsRNA from various sources. The ds siRNAs are unwound and one strand is incorporated in the RNA-induced silencing complex (RISC) which contains, among others, Argonaut (AGO) proteins. The single-stranded siRNA in RISC base pairs with its complementary target RNA, which is then cleaved by the AGO component. The RNA silencing system is efficiently triggered by virus RNA, and can effectively damp down if not completely prevent virus infection. This action is associated with the phenomenon of recovery in which plants fight off an initially strong infection, e.g. with nepoviruses

or tobraviruses, and reach a state where they contain extremely low levels of virus and are protected from further infection by the same or a very similar virus (Ratcliff et al. 1997). The introduction of techniques such as the transient expression of virus and reporter proteins in plants using infiltration with *Agrobacterium tumefaciens* cultures, the production of transgenic plants expressing virus proteins, and the creation of hybrid viruses expressing genes from different sources have led to the understanding that the pathogenicity proteins of many viruses interfere in some way or other with RNA silencing, and are now commonly referred to as silencing suppressor proteins (Palukaitis and MacFarlane 2006; Voinnet 2005a).

13.8 Plant Proteins Interacting with Viral Silencing Suppressors

Many of the virus encoded silencing suppressor proteins had previously been shown to be involved in virus movement (see Table 13.2). Further studies revealed that some proteins interfere with local RNA silencing, whereas others prevent silencing in systemic infected leaves. In addition, grafting experiments have shown that some of the proteins interfere with the initiation of silencing whereas others interrupt the movement of a silencing signal that is necessary for propagation of the silenced state (Roth et al. 2004; Voinnet 2005b). Information on the precise mechanism of action of most silencing suppressors is not available. However, recent studies have shown that dsRNA-binding, including siRNA-binding may be common to many different suppressors (Lakatos et al. 2006; Merai et al. 2006). In fact the crystal structure of two suppressors, one complexed with siRNA, have been obtained (Vargason et al. 2003; Ye and Patel 2005).

Mainly by applying the yeast two-hybrid system, a number of host proteins have been identified that bind to various silencing suppressor proteins. However, while the interactors of viral movement proteins have led to some conclusive hypotheses on the mechanisms of viral movement (see above), the functions of most of the proteins interacting with viral silencing suppressors are unknown. Therefore, there is still no general picture of how these cellular targets of silencing suppressors actually integrate in the complex network the RNA silencing process.

One of the most studied suppressor proteins is the helper component-proteinase (HC-Pro) that is encoded by potyviruses such as TEV and PVY. This protein is multifunctional, being required for virus transmission by aphids, viral polyprotein processing and systemic movement (Maia et al. 1996). HC-Pro also is a strong silencing suppressor that binds to ds siRNAs (Lakatos et al. 2006), interferes with 3 methylation of another class of small RNAs, miRNAs (Yu et al. 2006), and inhibits the ribonuclease activity of the 20 S proteasome (Ballut et al. 2005). How many of these functions are directly related to suppression of silencing is not known. However, the introduction into HC-Pro of mutations that affected long-distance movement and genome amplification also inhibited silencing suppression activity, whereas mutations that inactivated the proteolytic activity of HC-Pro had no effect on silencing suppression (Kasschau and Carrington 2001). The TEV

HC-Pro was found to interact with a calmodulin related protein called rgsCaM (regulator of gene silencing-calmodulin-like protein) whose expression was upregulated by HC-Pro (Anandalakshmi et al. 2000). Over-expression of this protein in plants also led to suppression of silencing, suggesting that it might be an endogenous suppressor and that the calcium-signaling pathway might play a role in silencing. Two other proteins, HIP1, a RING-finger protein, and HIP2, with no identifiable functional motifs, bind to HC-Pro in yeast, although the significance of these interactions is not known (Guo D et al. 2003).

Mutations in the gene encoding the P19 protein, present in tombusviruses such as TBSV and CymRSV, affect cell-to-cell and systemic movement of the virus in a host-specific manner, as well as symptom production (Scholthof et al. 1995a,b; Turina et al. 2003). The p19 protein is a very strong silencing suppressor that binds siRNAs in vitro and in vivo and was suggested to function solely by sequestration of these molecules without the involvement of any host proteins (Lakatos et al. 2004). Nevertheless, yeast two-hybrid experiments revealed that P19 interacts with members of the ALY family of RNA-binding proteins, which in animals are involved in export of RNAs from the nucleus (Park et al. 2004; Uhrig et al. 2004). In plants, expression of P19 leads to re-localization of two of the four ALY proteins from the nucleus to the cytoplasm. By contrast, the two ALY proteins that remain in the nucleus themselves sequester the P19 protein in the nucleus (Canto et al. 2006). This relocalisation of P19 inhibits its activity as a silencing suppressor. Whether the suppression activity of P19 is directly responsible for its influence on virus movement is not clear, although mutations in P19 that affected silencing suppression activity also affected virus movement and interaction of P19 with ALY (Chu et al. 2000; Uhrig et al. 2004).

Mutation of the gene encoding the 2b protein of CMV does not affect virus replication in protoplasts (Soards et al. 2002) but does affect the degree of movement of the virus in tobacco and cucumber (Ding et al. 1996). The 2b protein functions as a silencing suppressor, which differs in its activity to HC-Pro and TBSV P19 as it interferes with the long range spread of the silencing signal away from the point of initiation (Guo HS and Ding 2002). In doing so the 2b protein enters the cell nucleus, where it also reduces methylation of DNA sequences. Mutation of sequences necessary for nuclear localisation of the 2b protein affects its ability to suppress silencing and to promote a pathogenic synergistic interaction with ZYMV (Wang et al. 2004b). Yeast two-hybrid studies have identified a prokaryotic LytB homologue from tobacco that interacted with the CMV 2b protein in yeast (Ham et al. 1999), a karyopherin α protein from *Arabidopsis* that is likely involved in nuclear import of the 2b protein (Wang et al. 2004a), and a tobacco thaumatin-like protein (TLP1) whose expression is upregulated by CMV infection (Kim et al. 2005). In this latter example TLP1 also interacted in yeast with the CMV movement and capsid proteins.

The CP (P38) of TCV suppresses local silencing and prevents the accumulation of siRNAs (Qu et al. 2003). A 25 amino acid region at the N-terminus of the protein, that is sequestered inside assembled virus capsids, was shown to be important for suppression activity as well as for interaction with the TIP, a transcription factor

from *Arabidopsis thaliana* (Ren et al. 2000). Furthermore, the CP:TIP interaction is required for a hypersensitive resistance response in Arabidopsis. However, single amino acid mutations in the N-terminal regions can separate the TIP-binding and suppression activities of the CP, suggesting that TIP may not be involved in the silencing pathway (Choi et al. 2004). Mutations were introduced into P38 that retained suppressor function but abolished encapsidation. Movement of TCV did not require encapsidation but did require P38-mediated silencing suppression (Deleris et al. 2006).

Poleroviruses, which include PLRV, BWYV and CABYV, are aphid transmitted viruses that accumulate only within the phloem system of plants. This tissue limitation is likely to be due in part to their lack of a particular movement function, as co-infection with an umbravirus, PEMV-2, or with PVX expressing the movement protein (ORF4) of PEMV-2, enabled PLRV to move out of the phloem into the mesophyll tissue (Ryabov et al. 2001). The P0 protein of poleroviruses is a silencing suppressor, and mutation of the gene encoding this protein greatly reduces or abolishes accumulation of viral RNA (Pfeffer et al. 2002; Sadowy et al. 2001). The polerovirus P0 protein interacts via an F-Box-like motif with AtSKP1 and AtSKP2 (ubiquitin E3 ligases), and mutation of the F-box motif in P0 prevented interaction with SKP1/SKP2 and inhibited the silencing suppression activity of P0 (Pazhouhandeh et al. 2006). Knock-down of SKP1 in *Nicotiana benthamiana* by virus-induced gene silencing (VIGS) made these plants resistant to PLRV infection. These results suggest that P0 might function as an F-box protein potentially directing ubiquitination and degradation by the 26 S proteasome of an essential component of the host posttranscriptional gene silencing machinery.

The 126 K protein of TMV, and its homologue in other tobamoviruses, is a component of the viral replicase. It contains motifs associated with methyltransferase and RNA helicase proteins, and has a role in cell-to-cell movement of the virus (Hirashima and Watanabe 2001). The 126 K protein is also a suppressor of RNA silencing (Kubota et al. 2003). Three different yeast two-hybrid studies have isolated different host proteins that interact with the helicase domain of this protein. In the first study, interaction was found with the tobacco AAA ATPase and with the 33 K subunit protein of the oxygen-evolving photosystem II complex (Abbink et al. 2002). Silencing by VIGS in *Nicotiana benthamiana* of the ATPase gene decreased TMV accumulation twofold and also reduced accumulation of PVX and AMV. Silencing of the 33 K subunit gene led to a tenfold increase in TMV accumulation as well as an enhancement of PVX and AMV accumulation. In the second study, the TMV helicase domain interacted with the *Arabidopsis* AUX/IAA protein PAP1/IAA26 which is a putative regulator of plant auxin genes involved in plant development (Padmanabhan et al. 2005). Silencing of PAP1 induced symptoms similar to those seen during virus infection, and infection of plants with TMV prevented the normal accumulation of PAP1 in the nucleus which led to a disruption in the expression pattern of auxin-stimulated genes. The third study used a yeast three-hybrid approach to identify proteins that complexed with the TMV helicase domain and the tobacco N gene, a TMV-resistance gene (Bilgin et al. 2003). This identified $P58^{IPK}$, which inhibits cell death mediated by a double-stranded RNA-activated

protein kinase (PKR), that in animals is part of the interferon response to virus infection. Plants in which the P58^{IPK} gene was silenced or mutated were hypersusceptible to TMV and tobacco etch virus resulting in plant death upon infection with these viruses, suggesting that the virus does not inhibit P58^{IPK} activity but in some way modulates it to prevent the induction of cell death.

Geminiviruses, which are comprised of single-stranded DNA rather than RNA also encode a silencing suppressor protein (Voinnet et al. 1999). The suppressor protein of ACMV is the AC2 protein, which is a transcriptional activator protein involved in CP expression. The homologous protein from TGMV is called the AL2 protein, and the homologue from TYLCV is called the C2 protein (Dong et al. 2003). Transgenic plants expressing AL2 or the positional homologue L2 from BCTV are more susceptible to these viruses and to TMV, an unrelated RNA virus (Sunter et al. 2001). AL2 and L2 interact in plants with SNF1 kinase which controls the activity of a range of metabolic pathway transcriptional activators and repressors in response to nutritional and environmental stress (Hao et al. 2003). Overexpression of SNF1 causes enhanced resistance to geminivirus infection, and the AL2 and L2 proteins bind SNF1 to inhibit its kinase activity in vitro and in vivo (in yeast). In a further yeast two-hybrid screen TGMV AL2 and BCT L2 proteins were also shown to interact with adenosine 5 phosphotransferase (ADK) (Wang et al. 2003). The viral proteins inactivate ADK in vitro and in vivo, as also occurs in transgenic plant expressing these proteins or in plants infected with geminiviruses. SNF1 is activated by 5 AMP; therefore, these observations indicate that global regulation of metabolism by SNF1 might be part of antiviral defences, and the inactivation of ADK and SNF1 by the geminivirus proteins might represent a dual strategy to counter this defense.

13.9 Conclusion

The molecular investigation of plant viruses has stimulated and promoted the recent advance in understanding of two fundamental cellular processes underlying the coordination of developmental programs and the response to both environmental cues and pathogen challenge: RNA silencing, as a part of the general microRNA pathways regulating gene expression, and the long-distance communication networks in plants based on the intercellular trafficking of signalling macromolecules. Protein interaction studies using viral movement proteins and silencing suppressors have now identified a large number of plant proteins providing a valuable source of information about the molecular basis of these two processes. Interactors of silencing suppressors indicate functional links between RNA silencing and calcium signalling, nuclear shuttling, global regulation of metabolism, auxin action and protein degradation. While these protein interaction data still not connect to give a complete picture of the RNA silencing process, a more conclusive idea of the mechanisms of intercellular movement through plasmodesmata is emerging, involving the participation of the cytoskeleton and the endomembrane system, as well as the action of chaperones and cell wall-modifying enzymes.

Abbreviations: ACMV, African cassava mosaic virus; AMV, Alfalfa mosaic virus; BCTV, Beet curly top virus; BSMV, Barley stripe mosaic virus; BWYV, Beet western yellows virus; BYSV, Beet yellow stunt virus; BYV, Beet yellows virus; CABYV, Cucurbit aphid-borne yellows virus; CTV, Citrus tristeza virus; CMV, Cucumber mosaic virus; CPMV, Cowpea mosaic virus; CymRSV, Cymbidium ringspot virus; PCV, Peanut clump virus; PEMV-2, Pea enation mosaic virus 2; PLRV, Potato leafroll virus; PoLV, Pothos latent virus; PSLV, Poa semilatent virus; PVX, Potato virus X; PVY, Potato virus Y; RDV, Rice dwarf virus, RHBV, Rice hoja blanca virus; RYMV, Rice yellow mottle virus; SBWMV, Soilborne wheat mosaic virus, TAV, Tomato aspermy virus; TBSV, Tomato bushy stunt virus; TCV, Turnip crinkle virus; TEV, Tobacco etch virus; TGMV, Tomato golden mosaic virus; TMV, Tobacco mosaic virus; ToMV, Tomato mosaic virus; TRV, Tobacco rattle virus; TSWV, Tomato spotted wilt virus; TYLCV, Tomato yellow leaf curl virus; TYMV, Turnip yellow mosaic virus; ZYMV, Zucchini yellow mosaic virus

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