Tatjana Kleinow *Editor*

Plant-Virus Interactions

Molecular Biology, Intra- and Intercellular Transport

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1 Interaction of Movement Proteins with Host Factors, Mechanism of Viral Host Cell Manipulation and Influence of MPs on Plant Growth and Development

Katrin Link and Uwe Sonnewald

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Abstract

Even more than 100 years after Eduard Tangl first described plasmodesmata (PD) as "open communications" between protoplasts of endosperm cells (Carr 1976), the components of plasmodesmata as well as plasmodesmata-located proteins still remain enigmatic. Considering the fact, that this system is also co-opted by various plant viruses to enable the viral spread throughout the plant, the interaction of viral movement proteins (MPs) with plant host factors inevitably has to target the transport to or through plasmodesmata. This chapter attempts to give an overview concerning experimentally demonstrated interactions of movement proteins with host factors and possible mechanisms of viral host cell manipulations.

Abbreviations

AbMV	Abutilon mosaic virus
ACMV	African cassava mosaic virus
AltMV	Alternanthera mosaic virus
BaMV	Bamboo mosaic virus
BBSV	Beet black scorch virus

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BDMV	Bean dwarf mosaic virus
BIFC	Bimolecular fluorescence complementation
BMCTV	Beet mild curly top virus
BMV	Brome mosaic virus
BRET	Bioluminescence resonance energy transfer
BYV	Beet yellows virus
CaLCuV	Cabbage leaf curl virus
CaMV	Cauliflower mosaic virus
CMV	Cucumber mosaic virus
CTMV-W	Crucifer tobamovirus
CWMV	Chinese wheat mosaic virus
ER	Endoplasmic reticulum
FRAP	Fluorescence recovery after photobleaching
GarVX	Garlic virus X
GFLV	Grapevine fanleaf virus
gRNA	Genomic RNA
GRV	Groundnut rosette virus
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LMV	Lettuce mosaic virus
MALDI-TofMS	Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry
MNSV	Melon necrotic spot virus
MOWSE	Molecular weight search
ORMV	Oilseed rape mosaic virus
PepMV	Pepino mosaic virus
PM	Plasma membrane
PMTV	Potato mop-top virus
PPV	Plum pox virus
PsbMV	Pea seed-borne mosaic virus
PSLV	Poa semilatent virus
PVA	Potato virus A
PVX	Potato virus X
PVY	Potato virus Y
RNP	Ribonucleoprotein
RSV	Rice stripe virus
SP	Signal peptide
SqLCV	Squash leaf curl virus
TBSV	Tomato bushy stunt virus
TCrLYV	Tomato crinkle leaf yellows virus
TCV	Turnip crinkle virus
TEV	Tobacco etch virus
TGMV	Tomato golden mosaic virus
TLCV	Tomato leaf curl virus
TMV	Tobacco mosaic virus
ToMV	Tomato mosaic virus

TRV	Tobacco rattle virus
TSWV	Tomato spotted wilt virus
TuMV	Turnip mosaic virus
TVCV	Turnip vein clearing virus
TVMV	Tobacco vein-mottling virus
TYLCSV	Tomato yellow leaf curl Sardinia virus
TYLCV	Tomato yellow leaf curl virus
VIGS	Virus-induced gene silencing
VRNA	Viral RNA
WT	Wild type
Y2H	Yeast two-hybrid
ZYMV	Zucchini yellow mosaic virus

1.1 Interaction of Movement Proteins with Host Factors and Mechanisms of Viral Host Cell Manipulation

Even more than 100 years after Eduard Tangl first described plasmodesmata (PD) as "open communications" between protoplasts of endosperm cells (Carr 1976), the components of plasmodesmata as well as plasmodesmata-located proteins still remain enigmatic. Considering the fact, that this system is also co-opted by various plant viruses to enable the viral spread throughout the plant, the interaction of viral movement proteins (MPs) with plant host factors inevitably has to target the transport to or through plasmodesmata. This chapter attempts to give an overview concerning experimentally demonstrated interactions of movement proteins with host factors and possible mechanisms of viral host cell manipulations.

Table 1.1 summarizes most of the interactions known between viral proteins involved in movement processes and host proteins including the methods used for identification/confirmation and the putative function/influence of the respective interaction. Here we put special emphasis on PDLP1 (*P*lasmo*d*esmata-*L*ocated *P*rotein 1), which represents one of the few examples of a plasmodesmata-located protein that exerts direct receptor-like functions for movement proteins of tubule-forming viruses (Amari et al. 2010). After identification of the protein in cell wall proteomics of Arabidopsis suspension cell cultures (Bayer et al. 2006), the plasmodesmal localization was validated via C-terminal GFP fusion and confocal laser scanning microscopy (CLSM) analysis as well as colocalization with callose and tobacco mosaic virus (TMV)-MP30 (Thomas et al. 2008). The protein belongs to a plant-specific family of eight members, all predicted type 1 membrane proteins with an N-terminal signal peptide followed by a large region including two DUF26 domains, a single transmembrane domain (TMD) and a short cytoplasmic tail. Interestingly, the 21aa long TMD alone is sufficient to mediate targeting to PD when fused to GFP. PD targeting of PDLP1 depends on the secretory pathway as shown by pharmacological and genetic studies using Brefeldin A and coexpression with Sar1[H74L], a GTPase-defective mutant of Sar1p (Thomas et al. 2008),

Table 1.1 Interactions between viral proteins involved in movement processes and host proteins

Host factor	Viral protein	Identified/confirmed by	Putative function/influence	Reference
PD and cell periphery				
PDLP	GFLV-MP2B	Survey of subcellular targeting of cell wall-associated membrane proteins derived from highly purified Arabidopsis cell walls		Thomas et al. (2008)
		FRET-FLIM	Receptor-like properties for tubule forming MPs	Amari et al. (2010)
		Knockout mutants		
PME	TMV-MP30	Blot overlay binding assay with cell wall fraction of *N. tabacum* and $(His)_6$-tagged TMV-MP		Dorokhov et al. (1999)
		Affinity purification		
	TMV-MP30	Blot overlay binding assay with cell wall fraction of *N. tabacum* and TMV-MP	PD delivery?	Chen et al. (2000)
	TVCV MP	Affinity purification	PD modification?	
	CaMV-MP	Y2H		
	CWMV 37K	BIFC		Andika et al. (2013)
		Y2H		
AtP8	TCV MPp8	Y2H of *Arabidopsis thaliana* cDNA library	Unknown	Lin and Heaton (2001)
		Blot overlay binding assay	2 Arg-Gly-Asp cell-attachment sequences → recognition by integrins?	

TIP	PVX TGB12K	Y2H of *N. tabacum* cDNA library	Regulation of PD SEL by interaction with ß-1,3-glucanase	Fridborg et al. (2003)
		Blot overlay binding assay		
	CMV 1a	Yeast Sos recruitment system (SRS)	Formation of CMV replication complex by anchoring complex to tonoplast	Kim et al. (2006)
		Coimmunoprecipitation		
Calreticulin	TMV-MP30	Affinity purification with cell wall fraction of *N. tabacum*	Entry to ER secretory pathway via SP?	Chen et al. (2005)
	TVCV MP	FRET	Influence on PD permeability?	
		Blot overlay binding assay		
		Y2H		
PAPK1	TMV-MP30	Isolation of a plasmodesmal-enriched cell wall protein (PECP) fraction from tobacco suspension-cultured cells	Regulation of MP function via phosphorylation	Lee et al. (2005)
	BDMV BC1	Phosphorylation assays		
SIUPTG1	TLCV V1	Y2H of *Solanum lycopersicum* cDNA library	Cell wall (hemicellulose) biosynthesis	Selth et al. (2006)
	ACMV AV2	Coimmunoprecipitation	Transport to PM, PD or Golgi?	
	TYLCSV V1		Glycosylation of V1 or factors involved in viral movement	
NbSTKL	BaMV	cDNA-AFLP experiment	Phosphorylation of TGBp1?	Cheng et al. (2013)
		Tobacco rattle virus (TRV)-mediated VIGS		

(continued)

Table 1.1 (continued)

Host factor	Viral protein	Identified/confirmed by	Putative function/influence	Reference
Cytoskeleton				
Actin as PD component	CMV-MP	Inhibitor treatment + FITC-dextran movement	Increase of PD SEL by F-actin severing	Su et al. (2010)
	TMV-MP30	Blot overlay binding assay		
		Cosedimentation experiments		
		Immunofluorescence labelling		
Actin	TMV-MP30	Colocalization in tobacco protoplasts	Transport of vRNA to PD	McLean et al. (1995)
		Inhibitor studies		
		Cosedimentation experiments		
		Pulldown experiments		
		FRAP + inhibitor treatment	Trafficking of MP to PD	Wright et al. (2007)
	BYV Hsp70h	Inhibitor treatment	PD localization of Hsp70h	Prokhnevsky et al. (2005)
Actin	CaMV P6	Colocalization studies with different subcellular markers + inhibitor treatment	Delivery of P6-containing inclusions + virions to PD	Harries et al. (2009a)
	TMV, PVX, TBSV	Inhibitor treatment	Actin-dependent viral spread	Harries et al. (2009b)
	MNSV MPp7B	Colocalization studies with different subcellular markers + inhibitor treatment	Trafficking to PD involves ER, Golgi apparatus and actin cytoskeleton	Genoves et al. (2010)
	TSWV N			Feng et al. (2013)
Class VIII myosins	BYV Hsp70h	Inhibitor treatment	PD localization of Hsp70h	Prokhnevsky et al. (2005)
		Dominant-negative inhibition of different myosins		Avisar et al. (2008)
Myosin VIII-1	RSV NSvc4	Inhibitor treatment	Trafficking to PD	Yuan et al. (2011)
		Dominant-negative inhibition of different myosins		

Myosin XI-2	TMV	TRV-mediated VIGS	Intercellular movement of TMV	Harries et al. (2009b)
Class XI myosins	GFLV-MP2B	Inhibitor treatment	Delivery of PDLP receptors to PD	Amari et al. (2011)
XI-K and XI-2		Dominant-negative inhibition of different myosins		
	TuMV MP6K_2	Chemical and protein inhibitors	Intercellular movement of TuMV	Agbeci et al. (2013)
		TRV-mediated VIGS		
At-4/1	TSWV-NS_M	Y2H of *A. thaliana* cDNA library using C-terminally truncated NS_M polypeptide as a bait	Unknown	von Bargen et al. (2001)
		Blot overlay binding assay	Similarities to myosin-, kinesin- and ankyrin-like proteins	Paape et al. (2006)
EB1a	TMV-MP30	Colocalization studies	Formation of MP:microtubule complexes during late stages of infection	Brandner et al. (2008)
		Pulldown assay		
		Blot overlay binding assay		
		FRET-FLIM	Manipulation of microtubule polymerization by targeting of EB1	
Tubulin, MT	TMV-MP30	Colocalization studies	vRNA movement	Heinlein et al. (1995)
γ-Tubulin			Targeting of MP for degradation?	Heinlein et al. (1998)

(continued)

Table 1.1 (continued)

Host factor	Viral protein	Identified/confirmed by	Putative function/influence	Reference
Tubulin, MT	TMV-MP30	Colocalization in tobacco protoplasts	vRNA movement	McLean et al. (1995)
γ-Tubulin		Depolymerization of microtubules		
		Pulldown experiments		
		Amino-acid substitutions in MP	vRNA movement	Boyko et al. (2000a, b, c, 2002, 2007)
		Temperature shift treatment		
		Colocalization		
		TMV derivatives expressing N- and C-terminal deletion mutants of MP fused to GFP		
		Immunofluorescence assay in African green monkey kidney (COS-7) cells	Stabilization of microtubules by MP association	Ferralli et al. (2006)
		Inhibitor treatment	Centrosomal release and noncentrosomal nucleation of MT	
		Coimmunoprecipitation		
		Colocalization studies	vRNA movement	Ashby et al. (2006)
		Microtubule-depolymerizing drugs	Stabilization of microtubules by MP	
		FRAP		
		Cosedimentation experiments		
		Multiple marker three-dimensional confocal and time-lapse video microscopies	Microtubules as scaffold for anchorage and assembly of ER-associated MP	Sambade et al. (2008)
		FRAP + inhibitor treatment		
		Microtubule polymerization inhibitors	vRNA movement	Seemanpillai et al. (2006)
		Tobacco mutant plants generated by T-DNA activation tagging and tolerant to microtubule assembly inhibitor	vRNA movement	Ouko et al. (2010)
		Colocalization	Involvement of nucleation-driven processes in virus movement	

MPB2C	TMV-MP30	Yeast Sos recruitment system (SRS) of *N. tabacum* cDNA library	Negative regulator of MP30 function/movement	Kragler et al. (2003)
		Blot overlay binding assay	Accumulation of TMV-MP at microtubules	Curin et al. (2007)
		MPB2C silenced *N. benthamiana* plants	Negative regulator of ORMV movement	Ruggenthaler et al. (2009)
	ORMV	GFP-AtMPB2C overexpression in *A. thaliana*	Stabilization of microtubules	
Vesicle trafficking/intracellular translocation				
KNOLLE	GFLV-MP2B	Coimmunoprecipitation	Cytokinesis-specific syntaxin, targeting of GFP:MP to the cell plate	Laporte et al. (2003)
MP17	CaMV-MP	Y2H of *Arabidopsis thaliana* cDNA library with N-terminal third of the CaMV-MP	Vesicle trafficking	Huang et al. (2001)
		FRET		
NbNACa1	BMV MP3a	Screening of *Nicotiana benthamiana* cDNA library by far-western blotting	Regulation of BMV MP localization to PD	Kaido et al. (2007)
		Coimmunoprecipitation		
		NbNACa1 silenced *N. benthamiana* plants		
SYTA	CaLCuV MP	Yeast Sos recruitment system (SRS) of *Arabidopsis thaliana* cDNA library	Regulation of endocytosis and endosome recycling at the plasma membrane	Lewis and Lazarowitz (2010)
	TMV-MP	Pulldown assays		
	SqLCV MP	SYTA knockdown line		
		Dominant-negative mutants		

(continued)

Table 1.1 (continued)

Host factor	Viral protein	Identified/confirmed by	Putative function/influence	Reference
GDI2	TMV 126 kDa	Y2H of *Arabidopsis thaliana* cDNA library with helicase domain	Rab GDP dissociation inhibitor protein	Kramer et al. (2011)
		Coimmunoprecipitation	Alteration of vesicular trafficking?	
		TRV-mediated VIGS		
µA-adaptin	CaMV-MP	Pulldown assay	PD targeting	Carluccio et al. (2014)
Chaperones				
NtMPIP1	TMV-MP30	Y2H of *N. tabacum* cDNA library	Mediating interaction between TMV-MP and NTH201	Shimizu et al. (2009)
		Blot overlay binding assay		
		TRV-mediated VIGS		
NbMPIP1	ToMV-MP	Y2H of a tomato cDNA library	MP protein stability	Du et al. (2013)
	TMV-MP	Pulldown assays		
		Firefly luciferase complementation imaging assay		
		Coimmunoprecipitation		
		TRV-mediated VIGS		
NtCPIP (DNAJ-like)	PVY CP	Y2H of *N. tabacum* cDNA library	Recruitment of heat shock protein 70 chaperones for viral assembly and/or cellular spread?	Hofius et al. (2007)
		Pulldown assay		
		BIFC		
		Dominant-negative mutants		
DNAJ-like	TSWV-NS_M	Y2H of *N. tabacum* and *Arabidopsis thaliana* cDNA library as well as *L. esculentum* cDNA library	Recruitment of Hsp70s?	Soellick et al. (2000)
		Pulldown assay		von Bargen et al. (2001)
	RSV MPpc4	Y2H of rice cDNA library		Lu et al. (2009)
		Coimmunoprecipitation		

cpHSC70-1	AbMV MP	Y2H of *Arabidopsis thaliana* cDNA library	Chaperone	Krenz et al. (2010, 2012)
		$MP_{1–116}$		
		BIFC		
Hsc70.3	PepMV CP	Y2H of *Solanum lycopersicum* cDNA library	Viral assembly and/or cellular spread?	Mathioudakis et al. (2012)
		BIFC		
Hsp70	TYLCV CP	Coimmunoprecipitation	Chaperone	Gorovits et al. (2013)
Hsp20	RSV MPpc4	Y2H against rice cDNA library	Preventing aggregation of unfolded Pc4 for transport and renaturation after passage through PD	Lu et al. (2009)
		Coimmunoprecipitation		
CCT8	ORMV	Mutant screen for KNOTTED1 transport deficiency Arabidopsis knockout plants ctt8-1	Component of type II chaperonin complex	Fichtenbauer et al. (2012)
			Refolding after transport through PD to ensure functionality?	
RME-8	PMTV TGB2	Blot overlay assay of *N. tabacum* cDNA library	Contains DnaJ domain	Haupt et al. (2005)
			Endocytic recycling?	
CDC48	TMV-MP30	Pulldown assay	Promotes MP degradation	Niehl et al. (2012)
	ORMV MP	FRET-FLIM	Control of virus movement by removal of MP from ER transport pathway	
		Coimmunoprecipitation		
Nucleus				
NIG	CaLCuV NSP	Y2H of *Arabidopsis thaliana* cDNA library	Modulating NSP nucleocytoplasmic shuttling + facilitating MP–NSP interaction in the cytoplasm	Florentino et al. (2006)
	TGMV NSP	Pulldown assay		Carvalho et al. (2006)
	TCrLYV NSP	Coimmunoprecipitation assays		
		NIG overexpression		

(continued)

Table 1.1 (continued)

Host factor	Viral protein	Identified/confirmed by	Putative function/influence	Reference
MBF1	ToMV MP	Far-western screening of *N. tabacum* cDNA library	Transcriptional coactivator	Matsushita et al. (2002)
	CTMV-MP			
KELP	ToMV MP	Far-western screening of *N. tabacum* and *Brassica campestris* cDNA libraries	Transcriptional coactivator	Matsushita et al. (2001)
	CTMV-MP	Blot overlay binding assay	Modulation of host gene expression?	
	CMV-MP			
	ToMV MP	KELP overexpression	Inhibitory effect on MP localization and function	Sasaki et al. (2009)
HFi22	TBSV p22	Y2H of *Nicotiana tabacum* cDNA library	Homeodomain leucine-zipper protein	Desvoyes et al. (2002)
		Blot overlay binding assay	Transport through PD?	
			Preventing activation of expression of one or more broad-acting defence genes?	
Fibrillarin	GRV ORF3	TRV-mediated VIGS	Formation of viral ribonucleoprotein particles (RNPs)	Kim et al. (2007)
		Blot overlay binding assay		Canetta et al. (2008)
	PSLV TGBp1	Blot overlay binding assay	Formation of viral RNPs	Semashko et al. (2012)
	BBSV P7a	BIFC		Wang et al. (2012)
Importin α	BBSV P7a	Blot overlay binding assay	P7a targeting to nucleus	Wang et al. (2012)
		BIFC		
MP modification				
RIO kinase	ToMV MP	Far-western screening of cDNA clones from a phage expression library of *Nicotiana tabacum*	MP phosphorylation	Yoshioka et al. (2004)
	CMV-MP			

LeNIK/GmNIK	TGMV NSP	Y2H of *L. esculentum* and *Glycine max* cDNA library	Leucine rich-repeat receptor-like kinase	Mariano et al. (2004)
	TCrLYV NSP	Blot overlay binding assay		
	CaLCuV	Coimmunoprecipitation	NSP phosphorylation?	
NIK1-3 (*A.th.*)		Y2H	Suppression of NIK-mediated antiviral responses	Fontes et al. (2004)
		Pulldown assays		
		NIK knockout lines		
NsAK	CaLCuV NSP	Y2H of *Arabidopsis thaliana* cDNA library	Proline-rich extensin-like receptor protein kinase (PERK)	Florentino et al. (2006)
	TGMV NSP	Pulldown assays		
	TCrYLC NSP	NsAK knockout lines	Regulation of NSP function	
CK2	ToMV MP	In vitro phosphorylation assays + different stimulatory and inhibitory factors	MP phosphorylation	Matsushita et al. (2000)
	PVX TGBp1			Matsushita et al. (2003)
				Modena et al. (2008)
	PVA CP	In-gel kinase assays and LC-MS/MS	Regulation of RNA binding activity of CP	Ivanov et al. (2003)
	TMV MP30			
Chloroplast				
CHUP1	CaMV p6	Y2H screen of an *A. thaliana* cDNA library	Chloroplast movement to PM on microfilaments	Angel et al. (2013)
		Coimmunoprecipitation		
		TRV-mediated VIGS	Supporting CaMV infection	
AtpC	TMV replicase	Isolation of viral replicase-enriched fractions from TMV-infected *Nicotiana tabacum* plants, SDS-PAGE, MALDI-TofMS, nano-LC-MS/MS, MOWSE	ATP-synthase γ-subunit	Bhat et al. (2013)
		TRV-mediated VIGS	Viral spread	

(continued)

Table 1.1 (continued)

Host factor	Viral protein	Identified/confirmed by	Putative function/influence	Reference
Other				
NbPCIP1	PVX CP	Y2H of *Nicotiana benthamiana* cDNA library	Viral replication	Park et al. (2009)
	ToMV CP	Pulldown assay		
		BIFC		
		Overexpression		
		TRV-mediated VIGS		
Histone H3	BDMV NSP and MP	Gel overlay assays with protein extracts of BDMV-infected *Nicotiana benthamiana* plants and transgenic MP-expressing tomato plants	Component of a geminiviral movement-competent vDNA complex?	Zhou et al. (2011)
	TYLCV CP	Blot overlay binding assay		
	BMCTV CP	Pulldown assay		
	BDMV CP	In vivo coimmunoprecipitation		
IP-L	ToMV CP	Y2H of *Nicotiana tabacum* cDNA library	Viral long-distance movement	Li et al. (2005)
		Blot overlay binding assay		
		TRV-mediated VIGS		
PVIPp	PSbMV VPg	Y2H of *Pisum sativum* cDNA library	Viral movement	Dunoyer et al. (2004)
	TuMV VPg	RNA interference	Transcriptional regulation through chromosome remodelling?	
	LMV VPg			
AtNSI	CaLCuV NSP	Y2H of *Arabidopsis thaliana* cDNA library	Acetyltransferase, acetylation of viral CP	McGarry et al. (2003)
	SqLCV NSP	Pulldown assay		
		Coimmunoprecipitation from Sf21 insect cells	Regulation of nuclear export of the viral genome	Carvalho and Lazarowitz (2004)

PSI-K	PPV CI	Y2H of *Nicotiana benthamiana* cDNA library	Subunit of photosystem I	Jimenez et al. (2006)
	TVMV CI	RNA interference	Inhibition of CI function?	
2bip	CMV 2b	Y2H of tobacco cDNA library	Unknown	Ham et al. (1999)
eIF4E	TuMV VPg	Yeast LexA interaction trap of an *A. thaliana* cDNA library	Regulation in the initiation of translation	Wittmann et al. (1997)
				Leonard et al. (2000)
		ELISA-based interaction assay	Viral movement	Leonard et al. (2004)
		Copurification by metal chelation chromatography		Gao et al. (2004)
	TEV NIa	Yeast LexA interaction trap of a TMV-infected *Lycopersicum esculentum* cDNA library		Schaad et al. (2000)
				Robaglia and Caranta (2006)
PsbO1	AltMV TGB3	Y2H of *Arabidopsis thaliana* cDNA library	Photosystem II oxygen-evolving complex protein	Jang et al. (2013)
		BiFC	GTPase activity	
NtTLP1	CMV 1a	Y2H of *Nicotiana tabacum* (CMV-infected) cDNA library	Thaumatin-like protein, PR protein	Kim et al. (2005)
	CMV CP	Pulldown assay	Regulation of protein activity?	
	CMV-MP	Coimmunoprecipitation using cotransformed Arabidopsis protoplasts	Antiviral factor by inhibition of interactions?	
Tcoi1	CMV 1a	Y2H of *Nicotiana tabacum* cDNA library	Methyltransferase	Kim et al. (2008)
		BRET analysis	Regulation of CMV1a function and viral replication/spread via methylation?	
		Coimmunoprecipitation using cotransformed Arabidopsis protoplasts		
		Antisense-Tcoi1 transgenic plants		
P17	GarVX p11	Y2H of garlic cDNA library	Unknown	Lu et al. (2011)
		BIFC		

(continued)

Table 1.1 (continued)

Host factor	Viral protein	Identified/confirmed by	Putative function/influence	Reference
Remorin	PVX TGBp1	Split-ubiquitin assay	Negative regulator of virus movement	Raffaele et al. (2009)
		Pulldown assay		Perraki et al. (2012)
		Transgenic tomato lines containing sense or antisense REM cDNA sequences		
CAT1	PepMV TGBp1	Y2H of *Lycopersicum esculentum* cDNA library	Catalase	Mathioudakis et al. (2013)
		Pulldown assay	Increase of H_2O_2 scavenging	
		BIFC	Negative regulator of plant defence mechanisms	
		TRV-mediated VIGS		
PCaP1	TuMV P3N-PIPO	Y2H of *Arabidopsis thaliana* cDNA library	Hydrophilic cation-binding protein	Vijayapalani et al. (2012)
		Coimmunoprecipitation	Membrane-binding function for movement complex?	
		BIFC		
		PCaP1 knockout mutants (pcap1)		
NTH201	TMV	TRV-mediated VIGS	Class II KNOTTED1-like protein	Yoshii et al. (2008)
		Overexpression	Facilitation of MP accumulation and VRC formation	

Modified after Niehl and Heinlein (2011)

respectively. To test whether PDLP1 would be involved in the control of PD size exclusion limits, monomeric GFP was bombarded into single cells of Arabidopsis lines with altered PDLP1 expression level. This revealed that in PDLP1-overexpressing lines the observed dwarf-like phenotype correlated with an impaired diffusion of free GFP into the surrounding cells, whereas crosses between knockout lines of distinct members of the PDLP family led to increased distribution of the fluorophore (Thomas et al. 2008). Amari et al. (2010) could establish a connection between PDLP1 and the intracellular movement of the tubule-forming grapevine fanleaf virus (GFLV) (Laporte et al. 2003). After expression of GFLV movement protein (GFLV-MP2B) fused to GFP in a pdlp1–pdlp2–pdlp3 triple mutant, the number of cells exhibiting tubular structures was reduced by 46 % compared to wild-type (WT) plants. This phenomenon was associated with an impaired viral spread of RFP-tagged GFLV and also cauliflower mosaic virus (CaMV), another representative of tubule-forming viruses (Perbal et al. 1993; Stavolone et al. 2005). In contrast, the systemic movement of the Arabidopsis-infecting oilseed rape mosaic virus (ORMV), a close relative of TMV whose cell-to-cell movement is tubule independent (Mansilla et al. 2006; Ruggenthaler et al. 2009), was not altered (Amari et al. 2010).

By the use of the Förster resonance energy transfer–fluorescence-lifetime imaging microscopy (FRET-FLIM) technique, the group could show that PDLP1 – and also the other PDLPs – and GFLV-MP2B directly interact at the base of the tubule. Significant FRET was also verified for the movement protein P1 of CaMV (CaMV-P1), but not for TMV-MP30 that colocalizes with PDLP1 at PD but uses a different strategy for its intercellular movement. Furthermore, inhibition of ER export – and thus prevention of PDLP1 trafficking to PD – resulted in a diminution of tubule formation and instead distribution of GFP:2B (GFLV-MP2B with an N-terminal GFP fusion) fluorescence in the nucleus and the cytosol, whereas PD localization of TMV-MP30 was unimpeded (Amari et al. 2010). These results refute the theory that GFLV-MP2B might be a secretory cargo itself or that it harbours an independent PD targeting signal. The interaction of PDLPs with membrane-embedded GFLV-MP and CaMV-MP might be mediated by the transmembrane domain of PDLPs directly at PD or on their way to PD via the endoplasmic reticulum (ER) (Laporte et al. 2003; Pouwels et al. 2003) as they only possess a short cytosolic C-terminal domain (Thomas et al. 2008).

With the identification of the PDLP protein family as one important plasmodesmal component and the beginning understanding of their cellular function, the first step towards a functional understanding of PD constituents has been made. However, PDLPs are only one example of probably a larger number of proteins building functional and structurally diverse PDs which remain to be elucidated. The reported interactions between viral movement proteins and PDs will help to identify additional PD proteins.

1.2 Influence of MPs on Plant Growth and Development

Plant viruses have evolved various strategies for their intra- and intercellular movement. Amongst others, they encode specialized movement proteins (MPs) which facilitate transport of the viral RNA to and through plasmodesmata (PD). The localization of movement proteins at plasmodesmata and their potential interaction with host proteins involved in macromolecular trafficking or plasmodesmal constituents made these proteins important tools for studying the still unsolved plasmodesmal components and mechanisms of cell-to-cell trafficking of macromolecules. However, most of the research was performed with transient expression systems, and there are only a few examples for the generation of stable transgenic plants expressing viral movement proteins. In the following chapter, we want to summarize results obtained for the influence of movement proteins on plant growth and development.

The most intensively studied protein involved in viral movement, the movement protein MP30 of the tobamovirus TMV, was the first to be stably expressed in plants. Constitutive expression of MP30 under control of the 35S promoter significantly altered growth and development of transgenic tobacco plants.

Based on dye distribution experiments, using fluorescein isothiocyanate (FITC)-labelled dextrans (F-dextrans), size exclusion limits (SEL) of PD were found to be increased in these transgenic plants. PDs of transgenic plants allowed movement of F-dextrans with an average molecular mass of 9400 Da. In contrast, the size exclusion limit for the control plants resided between 700 and 800 Da (Wolf et al. 1989; Ding et al. 1992). Deom et al. demonstrated that TMV-MP30 was expressed in both young and mature leaves, but was only able to increase the SEL of PD in mesophyll cells of mature tobacco leaves (Deom et al. 1990). Immunogold labelling for MP30 showed that the protein exclusively binds to secondary plasmodesmata between various nonvascular cells and also between bundle-sheath and phloem parenchyma cells, where it was unable to affect SEL of PD. Plasmodesmata within the vascular tissue were not labelled (Ding et al. 1992).

Counterintuitive to the increased SEL, sugar measurements and pulse-chase experiments following $^{14}CO_2$-labelling revealed that the presence of the movement protein in transgenic tobacco plants led to an inhibition of sucrose export and an accumulation of carbohydrates in source leaves during the day. During the dark, breakdown of accumulated carbohydrates was accelerated as compared to control plants (Lucas et al. 1993; Balachandran et al. 1995; Olesinski et al. 1995). Under ambient conditions, photosynthetic performance of transgenic tobacco plants was not altered compared to control tobacco plants, and significant differences could only be obtained under elevated atmospheric CO_2 concentrations (Lucas et al. 1993). Additionally, total plant biomass was not drastically altered in transgenic plants, but lower root-to-shoot ratios for the transgenic plants conveyed a reduced carbon allocation to the roots (Lucas et al. 1993; Balachandran et al. 1995, 1997).

The same effect in terms of an increased SEL and decreased shoot-to-root ratio could be obtained for tobacco plants expressing the movement protein of cucumber moasic virus, CMV-MP; however, plants had reduced levels of soluble sugars and starch in their source leaves (Vaquero et al. 1994; Shalitin et al. 2002).

By means of reciprocal grafts made between control and TMV-MP-expressing plants and by expression of TMV-MP under constitutive and tissue-specific promoters, Balachandran et al. (1995) established that the mesophyll tissue is the site of action for MP30. In the same study, transgenic tobacco plants expressing deletion mutants of TMV-MP were analysed for their ability to increase SEL and to alter carbon allocation. It could be shown that the influence of the movement protein on biomass partitioning was independent of its capability to increase the SEL.

Contrasting results were obtained, when TMV-MP was expressed in transgenic potato plants under the control of the light-regulated ST-LS1 promoter. In this case, sucrose and starch accumulated to significantly lower levels during the day, and the sugar efflux from petioles was higher compared to control plants. According to the authors, this could be explained either by the use of the different promoters or the fact that TMV-MP may exert its influence on PD and carbon allocation by different modes due to a difference in plasmodesmal protein composition in tobacco and potato plants (Lucas et al. 1996; Olesinski et al. 1996).

Almon et al. could show that TMV-MP – when expressed under the phloem-specific rolC promoter – also increases the carbohydrate levels in source leaves and alters assimilate allocation between the various plant organs in transgenic potato plants (Almon et al. 1997). The authors concluded that plasmodesmata might be involved in a supra-cellular communication network between the companion and mesophyll cells that tightly regulates the carbon allocation (Balachandran et al. 1995; Lucas et al. 1996). High levels of the movement protein at plasmodesmata, especially within companion cells or phloem parenchyma cells, could perturb the trafficking of endogenous signalling molecules and thus alter carbon partitioning (Almon et al. 1997).

To investigate whether the observed alterations of TMV-MP regarding alteration of PD SEL as well as carbohydrate allocation and biomass partitioning are general features for viral movement proteins, Herbers et al. switched the system to the movement protein of potato leafroll virus (PLRV-MP17) – a phloem-restricted luteovirus (Herbers et al. 1997). Immunogold-labelling experiments using transgenic potato plants expressing MP17 fused to six histidine residues suggested an affinity of MP17 to PD of phloem tissue (Tacke et al. 1993; Schmitz et al. 1997). Furthermore, former studies with transgenic potato plants expressing MP17 with an *N*-terminal extension of 20 aa provided evidence that the presence of MP17 mediates a broad-spectrum resistance against several unrelated plant viruses (Tacke et al. 1996).

The same construct was used for stable expression of MP17 in transgenic tobacco plants under the control of the 35S CaMV promoter. In contrast to the non-phenotypic TMV-MP tobacco plants, MP17-expressing plants showed severe growth retardation, the development of bleached intercostal regions and sometimes also necrosis on lower leaves. Similarly to TMV-MP expression of MP17 resulted in increased levels of carbohydrates in phenotypically affected source leaves 12 h after the beginning of the light period, with glucose (increased by a factor of 9–18) accumulating to higher levels as compared to fructose and sucrose (factor 3–5) or starch (factor 3–5). Determination of steady-state levels of phosphorylated

intermediates revealed a slight increase in UDP-Glc, a decrease in ATP and unchanged levels of hexose phosphates, Glc-6-P, Frc-6-P and Glc-1-P, in these leaves (Herbers et al. 1997).

It is known that an accumulation of carbohydrates may influence photosynthetic rates by direct or indirect feedback inhibition mechanisms (Goldschmidt and Huber 1992). Indeed, the photosynthetic capacity was significantly decreased under both ambient and saturating light intensities. In parallel, carboxylation efficiency of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) and expression of photosynthetic genes decreased.

Comparing physiological consequences of TMV-MP, CMV-MP and MP17 expression seems to pinpoint to different mode of actions of the different movement proteins. This could be explained by different PD preferences of the proteins. PLRV normally is phloem restricted, while TMV and CMV can replicate outside phloem tissue in mesophyll and other cell types. This opens the possibility that MP17 binds to other host factors as TMV-MP and CMV-MP and thereby may influence cellular processes in a different manner. Alternatively, different expression levels of the different movement proteins could explain the observed phenotypic differences. At least in case of MP17 a clear dose dependence of the phenotype and hence sugar accumulation could be shown (Herbers et al. 1997). Therefore, the observed inhibition of photosynthesis could be due to an indirect effect of high sugar accumulation. In several studies sugar accumulation was accompanied by enhanced expression of PR genes and decreased expression of photosynthetic genes (Herbers et al. 1995, 1996a). By means of western and northern blot experiments, elevated expression of defence-related genes, e.g. for a ß-glucanase belonging to class II of PR proteins and PRQ-specific transcripts encoding an acidic chitinase (class III), could be verified. Furthermore, MP17 transgenic tobacco (Herbers et al. 1997) and potato (Tacke et al. 1996) plants were characterized by a reduced viral susceptibility. In potato it could be shown that this is not due to an RNA-based mechanism, but depends on expression of the movement protein. In tobacco, virus resistance correlated strongly with sugar accumulation. Hence, the observed broad-spectrum virus resistance may have been caused by induction of high-sugar-mediated defence mechanisms, rather than directly by the MP17 protein. This leaves the question of how MP17 expression alters carbohydrate export from photosynthetic active leaves. In tobacco and potato, phloem loading occurs apoplasmically. In the leaf, sucrose moves from mesophyll cell to mesophyll cell via plasmodesmata until it reaches the phloem parenchyma. In the phloem parenchyma, sucrose is most likely unloaded by sucrose efflux carriers (Chen et al. 2012) into the apoplasm. Apoplasmic sucrose is subsequently transported into companion cells by sucrose/H^+ symport. Companion cells are highly symplasmically connected to sieve elements in which sucrose is transported to distant sink tissues. Uptake of sucrose into companion cells was shown to be energy dependent (Sonnewald 1992; Lerchl et al. 1995). To test whether MP17 expression would affect sugar metabolism in vascular tissue, soluble sugars and phosphorylated intermediates were measured in vascular tissue enriched samples. This analysis revealed a significant decrease in the levels of soluble sugars, hexose phosphates, UDP-Glc, ATP and ADP. To further investigate whether MP17 would

affect PD function in a cell type-specific manner, leaves of 2-week-old tobacco plants expressing MP17 were harvested and PD structure was investigated by electron microscopy (Herbers et al. 1997). While PD structure in vascular tissue was significantly altered, PD structures between mesophyll cells were found to be indistinguishable from the untransformed control. This led the authors to conclude that MP17 might impair the trafficking between cells of the vascular tissue rather than the symplastic transport between mesophyll cells. In this context, it was speculated that trafficking of the SUT1 mRNA between companion cells and sieve elements (Kühn et al. 1997) might be hampered by perturbation of PD structure. Although perturbation of PD structure in phloem cells is still likely to be the cause for the observed sucrose export block of MP17 expressing plants, trafficking of SUT1 mRNA between companion cells and sieve elements has recently been challenged. By immunolocalization, Schmitt et al. (2008) clearly demonstrated that SUT1 exclusively accumulates in companion cells of tobacco, potato and tomato plants, making a cell-to-cell movement of mRNA unnecessary.

To further analyse a possible causal relationship between MP17 protein amount and the perturbation of the carbohydrate metabolism, Hofius et al. (2001) studied non-phenotypic tobacco plants expressing a MP17:GFP fusion protein under the control of the 35S promoter to existing phenotypic MP17 transgenic lines (Herbers et al. 1997). PD localization of MP17:GFP was analysed by CLSM analysis and immunogold labelling. This study revealed MP17 targeting to PD connecting various cell types in the mesophyll and vascular tissue with the latter being not targeted by TMV-MP. Pressure injection of Lucifer yellow (457 Da) and FITC-dextrans (9.7 and 12 kDa) revealed that plasmodesmal SEL was increased up to 12 kDa in mesophyll cells from transgenic tobacco plants compared to control plants. Unfortunately, the technique did not allow to measure SEL of vascular cells.

The absence of a phenotype was accompanied by a decline of carbohydrate levels, which – in the case of glucose and starch – correlated with MP17 levels. Interestingly, the spread of PVY was increased in the non-phenotypic lines. It has been shown that changes in the expression of defence genes can be mediated by carbohydrates (Herbers et al. 1995, 1996a, b, 2000; Herbers and Sonnewald 1998). In accordance with this, induction of several tested PR-protein transcripts exclusively occurred in phenotypic transgenic lines (Herbers et al. 1997).

In summary, low expression of MP17 led to decreased levels of carbohydrates and enhanced symplasmic sucrose transport towards vascular tissue, whereas high expression of MP17 resulted in carbohydrate accumulation, an export block for sucrose and growth retardation. This effect on carbohydrate levels was independent from the increased SEL of PD in the mesophyll. It is possible that MP17 might influence the function of PD in the vascular bundle – reflected by its affinity to phloem cells – in an expression level-dependent manner. Indeed, in phenotypic MP17-expressing tobacco plants, phloem PD were altered but mesophyll PD were not. However, TMV-MP had the same effects on carbohydrate levels, although it was not able to bind to phloem PD (Ding et al. 1992), indicating more indirect metabolic effects of the movement protein (Almon et al. 1997).

Since all previous studies concerning the stable expression of viral movement proteins in plants were performed in solanaceous species, Kronberg et al. established the expression of MP17 in the model plant *Arabidopsis thaliana* (Kronberg et al. 2007). The transgenic plant with the highest expression level of MP17, Col-16, was characterized by a severe growth retardation and the development of chlorosis (Fig. 1.1a). CLSM analysis and immunogold labelling confirmed the same localization pattern as observed for the transgenic tobacco and potato plants. A representative confocal image showing PD localization of MP17 in source leaves of line Col-16 is given in Fig. 1.1b. Table 1.2 summarizes dose-dependent effects of MP17 expression on growth and development of transgenic *Arabidopsis thaliana* plants.

As expected, sucrose export from leaves of the strongest MP17 expressing line (Col-16) was reduced during vegetative growth of Arabidopsis plants. Rather unexpected, however, was the observation that leaf sucrose export rates of the same plants were significantly enhanced (by 15 %) as compared to the untransformed control during the generative phase. This reversion of the carbohydrate export block during the reproductive growth phase was also reflected by a 2.8-fold increased harvest index (= ratio of seed mass and total plant mass) of Col-16 compared to untransformed Col-0. As the seed mass and the number of siliques did not differ from control plants, the enhanced reproductive output did result from an increased number of seeds per silique presumably as a consequence of reduced ovule abortion due to a higher availability of nutrients (Sun et al. 2004, 2005). The release of the observed export block could be connected to the developmental stage of the leaf. Progressing senescence was accompanied by a retrograde starch accumulation and elevated levels of soluble sugars as well as unimpeded movement of carboxyfluorescein (CF) out of the minor vein network in senescent leaves compared to mature source leaves (Fig. 1.2). This could be attributed to a reduction of MP17:GFP protein levels indicating a senescence-associated alleviation of the plasmodesmal localization of MP17:GFP (Fig. 1.3) as a consequence of specific structural changes of PD during this developmental stage. The non-targeted MP17:GFP might then be a target for degradation by the 26S proteasome as it can be observed in sink leaves (Vogel et al. 2007).

As all experiments described so far, used apoplasmic loaders as expression system for the various viral movement proteins, it remained unclear whether the effect of MP on carbon metabolism was the consequence of a direct or an indirect interaction with the sucrose/H^+ cotransport system. To clarify this question, Shalitin et al. (2002) transiently introduced TMV-MP and CMV-MP into melon plants, i.e. a symplasmic loader, by a novel virus-vector system which makes use of a mutated form of zucchini yellow mosaic virus (ZYMV-AG) that causes almost no symptoms on cucurbits (Gal-On and Raccah 2000). In this case, expression of both proteins caused a significant elevation of sucrose in phloem sap (Shalitin et al. 2002). This result was in agreement with a former study, in which radiolabelling experiments revealed elevated levels of ^{14}C sucrose in the phloem sap of CMV-infected plants (Shalitin and Wolf 2000). The authors concluded that the underlying cause might be a switch in the mode of phloem loading from symplasmic to apoplasmic in those plants. In symplasmic loaders sucrose diffuses symplasmically through PD along the entire route from

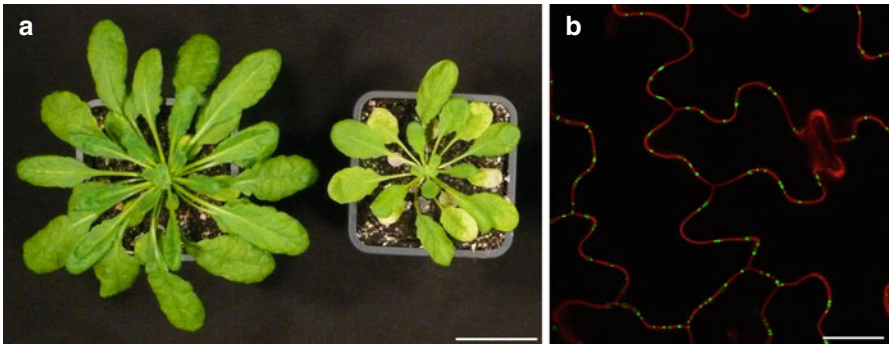

Fig. 1.1 Phenotype of the Arabidopsis line with the highest MP17:GFP expression level. (**a**) Visible growth phenotype of Col-16 grown for 8 weeks under short-day conditions. Compared to wild type Col-0 (*left*), Col-16 (*right*) displays a strong growth retardation and development of chlorosis. Bar indicates 3.35 cm. (**b**) MP17:GFP localization to PD in source leaves of Col-16. Epidermal cells of a source leaf from line Col-16 were scanned for MP17:GFP-derived fluorescence at PD by confocal laser scanning microscopy. MP17:GFP fluorescence is shown in *green*, cell wall stained with propidium iodide in *red*. Bar indicates 20 µm

Table 1.2 Dose-dependent responses of Arabidopsis plants expressing various levels of MP17

	Low expression	Modest expression	High expression
Biomass production	Increased 16–41 %	Slightly reduced	Strongly decreased 13 % of WT
Carbohydrate level	Slight reduction of both sucrose and starch contents	Only minor increase in carbohydrate levels	Strong accumulation in source leaves, export block
Flowering initiation	Accelerated	Accelerated	Delayed flowering by 6 and 7 days relative to the controls
Seed yield	Largely unaltered	Largely unaltered	Increase in seed yield by in average 29.9 %
Capacity of source leaves for Suc export	Slightly higher 20 %	Not tested	Reduced 29–48 % of WT

the mesophyll to the CC–SE complex. In so-called intermediary cells (IC), a specific type of CC in minor veins of cucurbit plants, sucrose is converted to raffinose and stachyose. These sugar molecules exceed the SEL of the connecting PD and are not able to diffuse back into the mesophyll. As a result, the concentration of raffinose and stachyose rises in the phloem (Turgeon and Gowan 1990; Knop et al. 2001; Turgeon and Medville 2004). Nevertheless, the occurrence of ordinary CCs in the larger minor veins provides evidence that apoplasmic loading is still possible in symplasmic loaders and both loading mechanisms may function concurrently in the same plant (Schmitz et al. 1987; Turgeon et al. 1993; Van Bel 1993; Grusak et al. 1996). The presence of the movement protein and its effect on PD SEL might be a trigger for such a switch from symplasmic to apoplasmic loading as a possible defence mechanism of the plant (Shalitin and Wolf 2000).

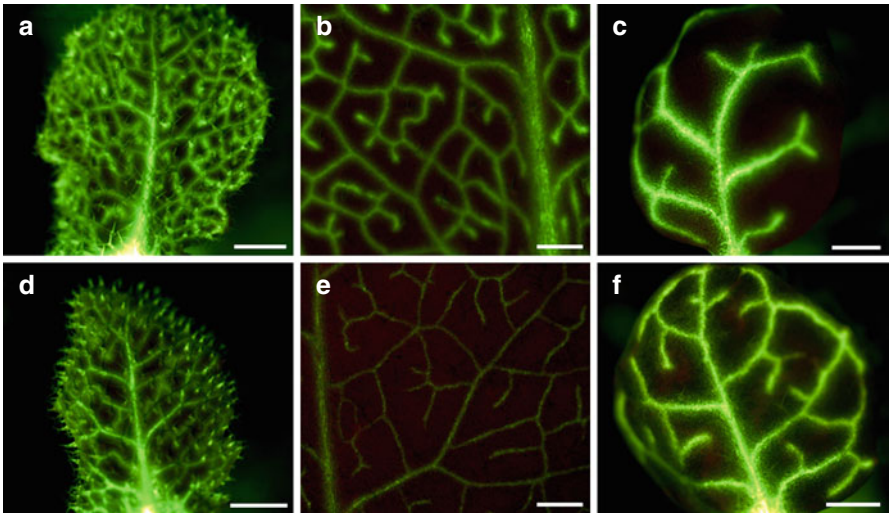

Fig. 1.2 Confocal laser scanning microscopy imaging of carboxyfluorescein unloading from phloem of wild type and Col-16 in different developmental stages**.** Movement of CF from vascular bundles into the surrounding mesophyll tissue was analysed in sink (**a**, **d**), source (**b**, **e**), and senescent (**c**, **f**) leaves 5 min after inserting cut leaves with their petioles in the tracer solution (Kronberg et al. 2007). (**a–c**) Unimpeded unloading of CF could be detected in all developmental stages in wild type Col-0; (**d–f**) CF unloading in Col-16 could be observed in sink (**d**) and senescent (**f**) but not in source (**e**) leaves. Bar indicates 1 mm

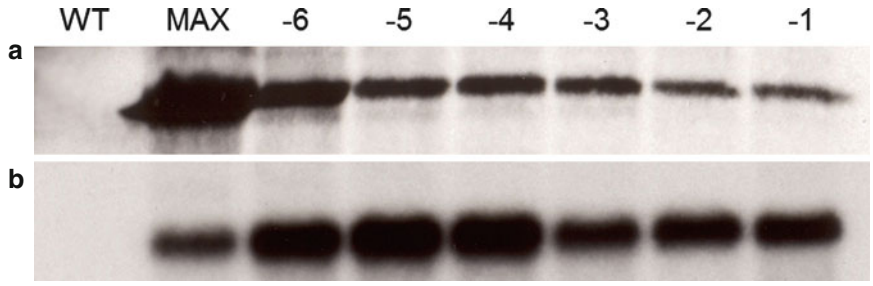

Fig. 1.3 Analysis of MP17:GFP protein and transcript levels in different developmental leaf stages of Col-16. (**a**) Western blot analysis of MP17:GFP protein accumulation. Leaf samples of different developmental stages (*MAX* source leaf; *−6* to *−1* mature leaf to progressing senescent) were homogenized and identical amounts of protein were separated by SDS-PAGE. MP17:GFP was detected using a polyclonal anti-MP17 antibody. (**b**) Northern blot analysis of MP17:GFP transcript accumulation. Each lane contains 25 µg of total RNA isolated from the indicated leaf stages of wild-type and Col-16 plants. Northern blot was hybridized with MP17 cDNA

Nevertheless, the contradiction between the MP-mediated increase in plasmodesmal SEL and the observed export block remained unresolved, and the previous assumed theories were unsatisfying. Rinne et al. (2005) put another complexity on the matter by shifting the focus to callose, a 1,3-ß-D-glucan, and its regulation as a possible explanation for the striking phenotype of *Nicotiana tabacum* plants constitutively expressing the movement protein of the tospovirus tomato spotted wilt virus

(TSWV), TSWV-NS_M (Rinne et al. 2005). The deposition of callose at PD has been shown to be a critical step of basal defence during virus infection and was able to impede viral spread (Allison and Shalla 1974; Shimomura and Dijkstra 1975; Atabekov and Dorokhov 1984; Stone and Clarke 1992; Gechev et al. 2004). This opened the possibility that callose deposition at plasmodesmata might be a first attempt of the plant to counteract the dilation of PD caused by the presence of the movement protein which in turn can be overcome by the virus through activation of glucanases. Two examples strengthened this hypothesis: first, TMV-MP was only capable to increase PD SEL at the infection site, but loses this capability behind the infection front (Oparka et al. 1997), and second, glucanase-deficient plants were more resistant to TMV (Beffa and Meins 1996).

In contrast to TMV and CMV, TSWV belongs like cowpea mosaic virus (CPMV), CaMV and GFLV to the tubule-forming viruses (Vanlent et al. 1991; Perbal et al. 1993; Ritzenthaler et al. 1995; Storms et al. 1995). The expression of TSWV-MP in transgenic tobacco plants induced severe growth retardation including a poorly developed root system, absent internode elongation, impaired leaf expansion and infection-like symptoms. An accumulation of high amounts of starch in source leaves was paralleled by a reduced quantum yield of photosystem II (ΦPSII) and later also by chlorotic lesions in common with a block of LYCH (457 D) cell-to-cell movement and an impaired viral spread of free GFP expressing pPVX::GFP (Baulcombe et al. 1995; Rinne et al. 2005). By electron microscopy and immunolocalization techniques, NS_M could be localized at PD of both source and sink leaf mesophyll, but only source PD showed additional proteinaceous sphincter-like structures (Olesen and Robards 1990) and the presence of callose deposits inside the sphincters visualized by aniline blue staining. As glucanases were not downregulated in the NS_M plants during the light period compared to wild-type plants, the callose deposits seem to originate from the upregulation of callose synthases. However, the circadian rhythm-dependent upregulation of glucanases during the night in control plants was abolished in NS_M transgenic plants (Rinne et al. 2005). Consequently, a temperature shift of TSWV-MP-expressing plants to 32 °C, a treatment which causes an upregulation of basic class I GLU-glucanases (Dinar et al. 1983), gave rise to a complete rescue of the phenotype.

In summary, these results confute the previous opinion that transgenic expression of movement proteins hampers the plant's capability for regulating the transport through PD. Instead the presence of NS_M elicited a defence response in the transgenic plants, i.e. the obstruction of the PD channel by the deposition of callose. Interestingly, although NS_M is able to target PD in sink leaves, both sphincters and callose deposits were not present at these PD, which suggests that the prerequisites for the plant response to continuous expression of MP might be developmentally regulated.

Potato virus X (PVX), a member of the genus Potexvirus, follows another strategy for its intercellular movement. Apart from the viral replicase and the coat protein, the viral genome encodes a so-called triple gene block – consisting of TGB1, TGB2 and TGB3 – required for viral movement (Huisman et al. 1988). Besides the viral gRNA and the coat protein, TGB1 forms a part of the ribonucleoprotein complex which moves cell to cell, whereas TGB2 and TGB3 seem to act as docking proteins for the targeting of PD.

Constitutive expression of PVX TBGp1 – but not TGB2 and TGB3 – in *N. tabacum* plants exerts an influence on proliferating tissues, e.g. the root meristems, which resulted in a stunted growth and decreased leaf number correlating to the expression level (Kobayashi et al. 2004). In contrast to TGB2 and TGB3 plants, a decline in the photosynthetic rate was detectable in TGBp1 plants, which might be attributed to changes in the morphology of chloroplasts, namely, diminished starch deposits and the presence of plastoglobuli-like structures, and the downregulation of photosynthetic genes. Unlike TMV-MP, constitutive TGB1 expression caused a reduced carbohydrate accumulation and had no effect on the overall biomass partitioning. However, the expression of PR genes was elevated in TGBp1 plants, which could not be observed during normal PVX infection of tobacco plants (Kobayashi et al. 2001).

In a recent study, Conti et al. (2012) investigated transcriptional changes induced by TMV-MP expression using microarray analysis (Conti et al. 2012). The expression of TMV-MP in transgenic tobacco lines resulted in an accumulation of hydrogen peroxide (H_2O_2) and superoxide ions (O_2^-). In this context, it has been suggested that reactive oxygen species (ROS) play an important role in plant response to biotic and abiotic stresses (Mittler 2002; Apel and Hirt 2004). Nevertheless, an increase in the expression of detoxifying genes, e.g. the detoxifying cytosolic ascorbate peroxidase (cAPX1), could not be observed, implying that the movement protein is able to interfere with the ROS scavenging system of the plant. This strongly resembled a phenomenon called "gene shut-off", which is characterized by a virus-induced downregulation of host gene expression (Aranda and Maule 1998; Havelda and Maule 2000; Maule et al. 2002; Bazzini et al. 2011; Pallas and Garcia 2011). Another key player in plant disease and pathogen resistance is salicylic acid (SA), inducing especially defence against biotrophic pathogens (Durrant and Dong 2004; Vlot et al. 2009). Indeed, in TMV-MP-expressing tobacco plants, the SA level was elevated as well as the levels of SA-responsive genes, e.g. PR-1, PR-2 and PR-5, whereas the level of jasmonic acid (JA), predominantly acting against necrotrophic pathogens, was not altered. Thus, it seems likely that TMV-MP is able to elicit a defence response mediated by SA.

To test the impact of the movement protein on the infectivity of different pathogens varying in their lifestyle and in defence pathway induction, several infections have been performed. After infection with TMV RNA, the MP-expressing transgenic plants were more susceptible at the early infection stages. This might be attributed to the induced expression of PR-2, a β-1,3-glucanase, which could promote viral spread by abolishing callose deposits at plasmodesmata (Beffa et al. 1996; Epel 2009; Baebler et al. 2011). However, in the later stages the transgenic plants showed enhanced resistance due to recovery processes. This might either result from increased transport of the silencing signal through PD (Vogler et al. 2008) or from the induction of SA and ROS-mediated defence mechanisms. In contrast, TMV-MP-expressing plants were more susceptible to the hemibiotrophic bacterium *Pseudomonas syringae* pv. *tabaci* and the necrotrophic fungus *Sclerotinia sclerotiorum* in evidence of higher bacterial growth rates and greater lesions. The reason might be that the transgenic plants accumulate high levels of ROS and H_2O_2 that are both a prerequisite for the switch to necrotrophic phases and successful infection (Able 2003).

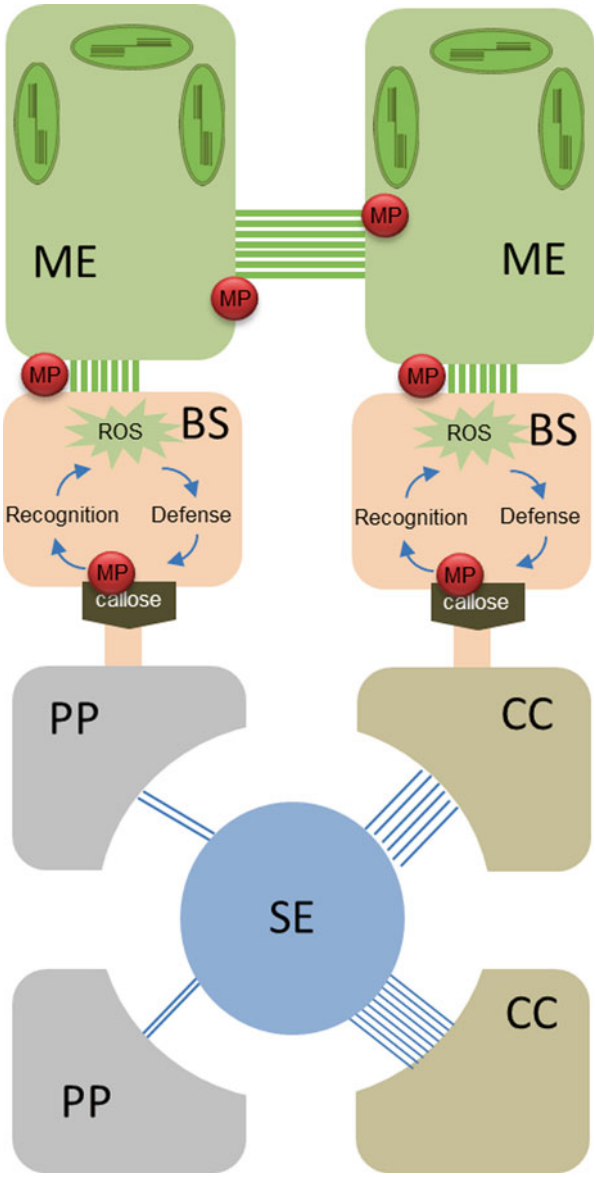

Fig. 1.4 Model of MP induced assimilate accumulation in MP-expressing source leaves. Expression of MP leads to increased SEL of mesophyll cells (*ME*). Upon recognition of MP by the plant immune system, ROS formation is induced in bundle-sheath cells (*BS*) which leads to callose plugging of PDs connecting BS and phloem parenchyma (*PP*) or companion cells (*CC*). Consequently less assimilates are allocated to the sieve elements (*SE*) and hence sugars accumulate in source leaves

Taken all data together, it seems a general feature of movement proteins to increase SEL of targeted plasmodesmata. However, increased SEL seems not to correlate with enhanced or blocked sugar export. Hence, based on yet unknown mechanisms, movement proteins induce additional cellular responses, which are dose and protein dependent. One likely explanation is the recognition of the viral proteins by the plant immune system, which may lead to defence responses (Fig. 1.4). In case of TMV, these responses have been associated with elevated oxidative stress. In another study Hofius et al. (2004) could show that oxidative stress leads to callose deposition most

prominently in vascular tissue and a reduced sucrose export rate. Hence, it seems possible that upon recognition of viral proteins, plugging of plasmodesmata within vascular tissue occurs which is responsible for reduced sucrose export rates. The sensitivity of recognition may depend on the specific viral protein, which can explain the responses to the different movement proteins. Since so far size exclusion limits of mesophyll but not of vascular cells have been determined, it would be important to know how PDs are regulated within the vasculature.

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Viral Transport and Interaction with the Host Cytoskeleton

2

Manfred Heinlein

Contents

Abstract

Viruses depend on host cell functions for replication and for spreading their genomes between cells and throughout the organism. The spread of viruses in plants relies on the ability of virus-encoded movement proteins (MPs) and interacting host components to facilitate the transport of the infectious viral genomes through plasmodesmata (PD), the gatable cytoplasmic cell wall channels through which adjacent cells are connected. Studies in the last 20 years have provided insights into the role of membranes and the dynamic cytoskeleton in the cell-to-cell movement of viruses. Using tobacco mosaic virus (TMV) as an example, this article highlights the specific roles of the actin and microtubule cytoskeletons in supporting the endoplasmic reticulum (ER)-associated assembly and trafficking of viral protein:RNA complexes and their targeting to PD.

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Already 40 years ago, Dales and Chardonnet (1973) proposed that the intracellular transport of animal viruses is mediated by interaction with microtubules (Dales and Chardonnet 1973). Since then, this hypothesis has been confirmed by numerous studies, and it is now clear that animal viruses subvert the microtubule transport system to facilitate their replication and spread (Dohner et al. 2005; Greber and Way 2006; Radtke et al. 2006; Brandenburg and Zhuang 2007; Ward 2011; Dodding and Way 2011). The observation of virion particles near microtubules in plants suggested a role of microtubules in the assembly and cell-to-cell transmission of viruses in plants already in the early 1970s (Kim and Fulton 1971, 1975; Mayhew and Carrol 1974). However, stronger insights into a potential role of microtubules during virus infection in plants had to await the introduction of green fluorescent protein (GFP) as a nonintrusive cellular marker 20 years later. In the mid-1990s, the fusion of GFP to the MPs of the tobamoviruses tobacco mosaic virus (TMV) and tomato mosaic virus Ob (obuda pepper virus) revealed that these proteins associate with microtubules in infected leaf epidermal cells as well as in tobacco BY-2 protoplasts (Heinlein et al. 1995, 1998; Padgett et al. 1996). The association of MP with microtubules in these systems was confirmed by antibody staining (Heinlein et al. 1995, 1998; Boyko et al. 2000a, b) and shown to occur also independent of infection, thus upon transient expression of MP:GFP, or of free MP (detected by immunostaining), in epidermal cells, BY-2 protoplasts, and mammalian cells (Boyko et al. 2000a; Heinlein et al. 1998; McLean et al. 1995; Ferralli et al. 2006). The MP was also shown to bind and stabilize microtubules in vitro, thus indicating that the binding of MP to the polymer occurs through direct interactions (Boyko et al. 2000a; Ashby et al. 2006). The initial observation that MP associates with microtubules (Heinlein et al. 1995) was achieved just a few years after the MP of TMV had been demonstrated to be essential for the cell-to-cell movement of TMV (Deom et al. 1987), to transport the virus independent of coat protein (Takamatsu et al. 1987; Dawson et al. 1988), to bind single-stranded nucleic acids in a sequence-independent manner (Citovsky et al. 1990, 1992), to localize to PD (Tomenius et al. 1987; Ding et al. 1992a; Atkins et al. 1991), and to modify the size exclusion limit of the channel (Wolf et al. 1989, 1991). Based on these findings and consistent with numerous reports at the time that microtubules transport macromolecular complexes, organelles, viruses, and RNA molecules in animal systems (Vale 1987; St Johnston 1995; Langford 1995), it was proposed that the MP forms a viral ribonucleoprotein (vRNP) complex with viral RNA and that microtubules are involved in targeting the vRNP to PD (Heinlein et al. 1998; Carrington et al. 1996; Heinlein 2002). Functional analysis of stable and conditional mutations in MP showed that the ability of MP to associate with microtubules is correlated with MP function in viral cell-to-cell trafficking, thus supporting this model (Boyko et al. 2000a, b, c, 2002, 2007). However, the association of MP with microtubules was usually observed in cells located behind rather than at the spreading infection front of the virus (Heinlein et al. 1998; Padgett et al. 1996). Thus, the way by which microtubules contribute to viral movement remained rather obscure. The proposed direct role of microtubules in serving as a track for vRNP trafficking could not be supported by application of microtubule polymerization inhibitors since such treatments failed to degrade all microtubules (Seemanpillai et al. 2006) and did not inhibit the spread of infection (Kawakami et al. 2004; Ashby

et al. 2006; Seemanpillai et al. 2006; Gillespie et al. 2002), as might be expected given that the spread of infection depends on the successful movement of only very few viral genomes (Tromas et al. 2014; Zwart et al. 2013). Enhanced virus movement seen under specific conditions correlated with either enhanced or reduced microtubule alignment of MP:GFP in independent experiments (Boyko et al. 2000b; Gillespie et al. 2002), thus arguing against a critical role of microtubule-aligned MP. The high amount of MP associated with the highly fluorescent MP:GFP-associated filaments observed late in infection was indeed not expected to play a critical role since only 2 % of the amount of MP produced by TMV infection were shown to be sufficient to support the cell-to-cell movement of the virus (Arce-Johnson et al. 1995). Consistently, TMV derivatives that produced rather low amounts of MP:GFP in the infected cells showed efficient cell-to-cell movement without accumulating the protein along the filaments (Heinlein et al. 1998; Szécsi et al. 1999). Based on the observation that the accumulation of MP along microtubules correlated with the disappearance of MP during subsequent stages, it was suggested that the filaments may participate in a pathway that guides the protein to degradation (Padgett et al. 1996; Gillespie et al. 2002). However, this latter hypothesis did not hold since the degradation of MP was later shown to be fully independent of an intact microtubule cytoskeleton (Ashby et al. 2006).

Taken together, these initial findings indicated that virus movement involves interactions of MP with microtubules but that the MP fraction that accumulated along microtubules and was easily visualized with the help of GFP is not involved in this process. Importantly, it became clear that an understanding of the viral movement process depended on the analysis of cells at the spreading infection front expressing very low levels of MP and that the processes involving high amounts of accumulated MP in cells behind the infection front represented late infection stages and had to be separated.

The low amount of MP associated with function in virus movement in cells at the infection front of the virus was initially difficult to address by microscopy. However, the analysis was then greatly facilitated when microscopical time-lapse imaging techniques and resolution were improved, and fluorescent protein-tagged cellular marker proteins to label the ER (Ruiz et al. 1998), microtubules (Ueda et al. 1999), and actin filaments (Sheahan et al. 2004; Hofmann et al. 2009) became available. As will be described in the subsequent paragraphs, it now appears that microtubules of the cortical array act as a dynamic scaffold for providing localized anchorage, structural support, and functional coordination for TMV replication and movement along the ER-actin network.

2.1 TMV Replication and MP Production and Turnover in Association with Microtubule-Associated, ER-Derived Virus Factories

The TMV RNA genome encodes two replicase subunits (126 and 183 k) and a coat protein (CP, 17.5 k) in addition to the MP (30 k). The 183 k protein is produced by translational read-through of the amber stop codon that terminates translation of the

shorter 126 k protein. Both proteins contain an N-terminal methyltransferase domain and a helicase domain, but only the 183 k protein carries an RNA-dependent RNA polymerase (RdRp) domain in its C-terminal extension. Although the 183 k protein is sufficient for viral replication, the 126 k protein binds to the 183 k protein (Goregaoker et al. 2001; Watanabe et al. 1999) and contributes to replication efficiency (Lewandowski and Dawson 2000). During infection, the 126 k protein accumulates to tenfold higher levels than the 183 k protein (Lewandowski and Dawson 2000) and, like also the small subunit replicases of other related tobamoviruses, acts as the viral suppressor of RNA silencing (VSR), presumably by sequestration of small RNAs (Kubota et al. 2003; Vogler et al. 2007; Csorba et al. 2007; Hu et al. 2011). The CP is dispensable for cell-to-cell movement but plays a role in facilitating efficient long-distance spread of the virus via the vascular phloem (Siegel et al. 1962; Hilf and Dawson 1993; Holt and Beachy 1991), either by stabilizing the vRNP (Taliansky et al. 2003; Spitsin et al. 1999) or by forming virions (Esau and Cronshaw 1967; Simon-Buelo and García-Arenal 1999). The MP binds to the surface of endoplasmic reticulum (ER) membranes (Heinlein et al. 1998; Peiro et al. 2013) as well as to microtubules (Heinlein et al. 1995; Boyko et al. 2000a; Ashby et al. 2006) and may facilitate virus movement and replication through the recruitment of ER membrane- and cytoskeleton-associated host functions.

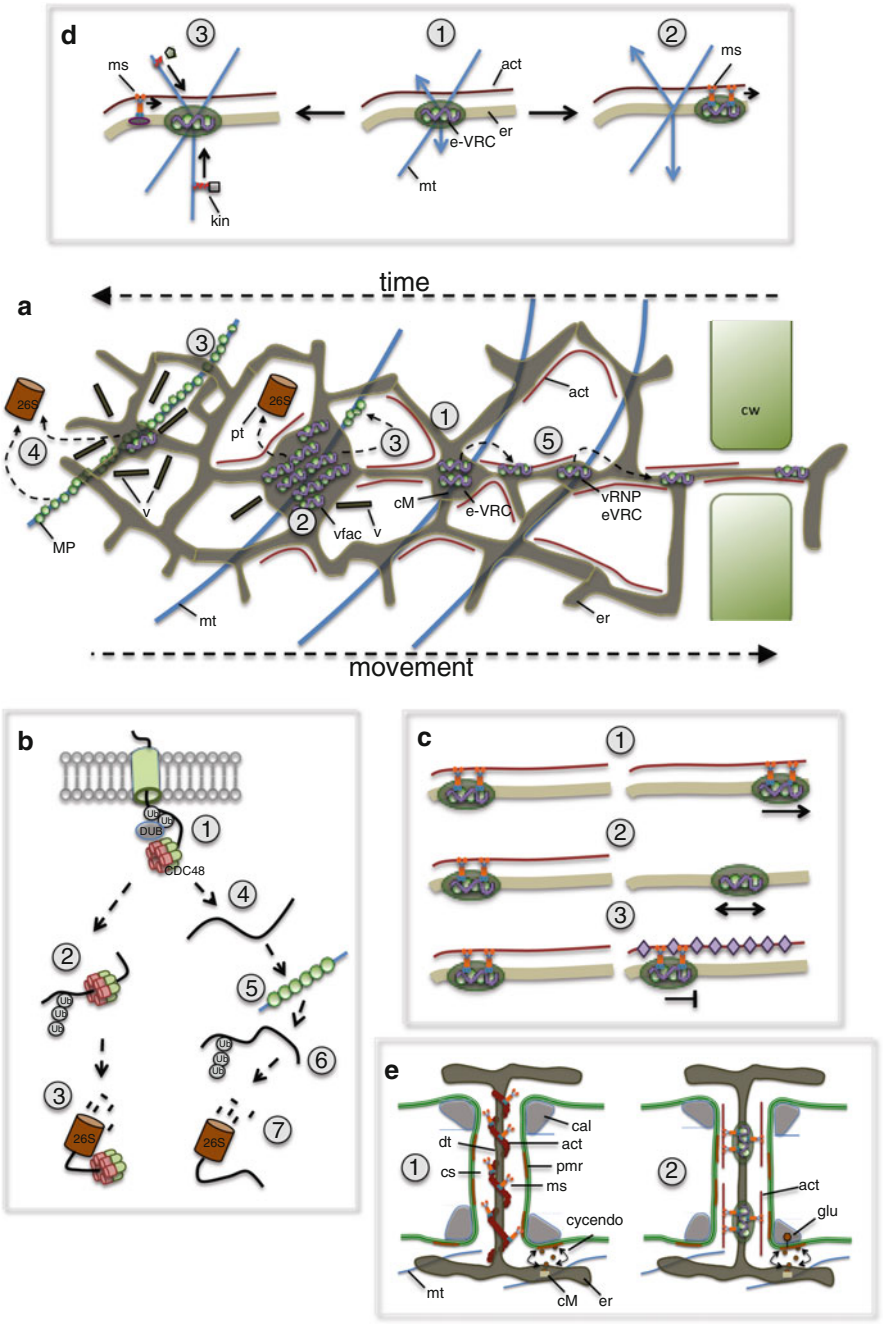
Upon entry of the vRNP into a cell, the viral RNA associates with the ER via its 5′ CAP (Christensen et al. 2009). Subsequent translation of the viral RNA leads to the production of the replicase proteins that cotranslationally bind to the 5′ untranslated region of the viral RNA to initiate replication (Kawamura-Nagaya et al. 2014) and to form viral-replication complexes (VRCs) (Fig. 2.1a; 1) in which the viral proteins are translated from genomic and subgenomic RNAs. Over time, the VRCs congregate into large, ER-derived, microtubule-associated inclusion bodies or "virus factories" (Fig. 2.1a; 2) that accumulate MP, replicase and viral RNA, and produce CP and virions on their surface (Heinlein et al. 1998; Szécsi et al. 1999; Más and Beachy 1999; Asurmendi et al. 2004). The condensation of VRCs into factories has been correlated with the accumulation of high amounts of MP at the ER (Reichel and Beachy 1998) and may involve the ability of the protein to oligomerize (Boutant et al. 2010) and to condense MP-associated ER membranes along microtubules (Ferralli et al. 2006). The MP is only transiently produced (Watanabe et al. 1984). Thus, following initial accumulation in the viral factories, the MP subsequently disappears from these ER inclusions and occurs in the cytosol along microtubules (Fig. 2.1a; 3) before it is degraded (Heinlein et al. 1998) by the 26S proteasome (Reichel and Beachy 2000) (Fig. 2.1a; 4).

The degradation of the MP is followed by recovery of the preinfection morphology of the ER (Reichel and Beachy 1998) and involves active removal of MP from the ER by the activity of the AAA ATPase CDC48 (Niehl et al. 2012) (Fig. 2.1b), a protein also known as p97/VCP (valosin-containing protein) in mammals and yeast (*Saccharomyces cerevisiae*). This protein utilizes its ATPase activity to promote the assembly and disassembly of protein complexes and is pivotal to the processes involved in ER-associated degradation (ERAD) (Baek et al. 2013). The CDC48 ATPase plays an essential role in the dislodgement of misfolded proteins from the

membrane by providing the power for the energy-demanding substrate retrotranslocation into the cytosol to reach the proteasome (Wolf and Stolz 2012; Ye et al. 2001; Liu and Ye 2012). In plants, expression of CDC48B, one of three CDC48 isoforms in *Arabidopsis*, is triggered by accumulation of unfolded proteins and ER stress. Thus, upon virus infection and accumulation of MP at the ER, MP is recognized as a substrate for CDC48B, which binds MP and relocates the protein from the membrane to the cytosol, leading to the transient accumulation of the protein along microtubules (Fig. 2.1a; 3) and its degradation in the cytosol (Niehl et al. 2012) (Fig. 2.1a; 4). Although extraction from the ER may occur as a default mechanism to remove overaccumulating proteins from the ER, this process may also represent a plant defense response against TMV by removing MP from the ER pathway for movement (Niehl et al. 2012, 2013a). In agreement with evidence indicating that ERAD substrates are deubiquitinated for dislocation from the ER (Tsai and Weissman 2011; Ernst et al. 2011) and that CDC48 occurs in association with ubiquitin ligase and deubiquitination activities (Crosas et al. 2006; Rumpf and Jentsch 2006; Liu and Ye 2012), microtubule-aligned MP was found to be free of ubiquitin (Ashby et al. 2006) (Fig. 2.1b; 2). Ubiquitinated MP found in crude cell extracts (Reichel and Beachy 2000) may accumulate in the ER prior to retrotranslocation or in the cytosol upon re-ubiquitination for subsequent degradation.

Given that virus movement requires only low amounts of MP and that virus movement occurs before MP accumulates along microtubules or in factories, the aggregation of the ER and, consequently, the condensation of VRCs into virus factories as well as the CDC48-facilitated degradation of MP are late events that are dispensable for the spread of infection and caused by MP overaccumulation. Thus, why does the MP overaccumulate at all? Accumulation of MP in late VRCs/viral factories is dispensable for TMV cell-to-cell movement as has been demonstrated by deletion mutagenesis of virus-encoded MP (Boyko et al. 2000c). As is described below, the ability of MP to bind microtubules and to recruit ER membranes and even the interaction of MP with CDC48 seem to reflect MP functions involved in TMV movement during early infection stages. Thus, the accumulation of MP in inclusions/factories and even their formation and also the alignment of MP along microtubules and the CDC48-mediated degradation of MP may represent mere consequences of MP overproduction. Nevertheless, overaccumulation of MP may facilitate the aggregation of the ER and the compartmentalization of viral replication and silencing suppression into ER inclusions and factories, which may play an important role in the protection of efficient viral replication and virion formation against host defense responses. Moreover, overproduction of MP may be inhibitory for virus movement and thus play an important role in switching the virus from the movement mode to the replication mode to produce virion progeny. The inhibitory effect of accumulated MP has been seen at various levels. Accumulation of MP in virus factories was correlated with slow TMV movement at low temperature (Boyko et al. 2000a) as well as with inhibited movement of temperature-sensitive TMV at nonpermissive temperature (Boyko et al. 2000a, 2007). Accumulation along microtubules by MP was shown to interfere with kinesin-mediated motility (Ashby et al. 2006), the transport of mobile MP particles (Boyko et al. 2007), and virus

Fig. 2.1 Role of the cortical cytoskeleton in the coordination of replication and movement of TMV in association with the ER. (**a**) Proposed model for the time course of TMV infection in the infected cell. Infection is initiated by attachment of vRNP (complex of viral RNA with MP and potentially also other host and viral proteins) at a cortical microtubule-ER junction (c-MER) (*1*). Attachment is probably mediated through binding of the MP to the microtubule. Translation and replication leads to the formation of an early VRC (eVRC). The eVRC, or a subcomplex of the eVRC (e.g., vRNP potentially associated with viral replicase and host factors), detaches from the c-MER to move along the ER-actin network in a stop-and-go manner from one c-MER to the next (*5*) to finally reach a c-MER at PD (not shown, see **e**) and enter the PD channel for infection of a new cell. The remaining eVRC and other eVCRs that remained anchored at the c-MER develop into a viral factory, which continues to replicate the viral genome (*2*) and produces virion particles. The formation of the factory involves the recruitment of ER membrane with the help of MP (not shown). At this time, infection is already established in the newly infected cell (not shown), and the current cell is now behind the cell at the new leading front of infection. Overproduction of MP in the factory causes ER stress and triggers retrotranslocation of the protein from the ER into the cytosol (*3*) where it either binds along the microtubule or gets degraded by the 26S proteasome. With time, production of MP ceases and all MP accumulates along the microtubule and is finally degraded (*4*). *act* actin, *cM* c-MER, *er* endoplasmic reticulum, *MP* movement protein, *mt* microtubule, *v* virion, *vfac* viral factory, *pt* proteasome, *cw* cell wall. (**b**) Retrotranslocation of MP from the ER to the cytosol. Since MP lacks ubiquitin after retrotranslocation, a DUB-assisted retrotranslocation model is presented here. Removal of the bulky ubiquitin chains from MP by deubiquitination enzyme (DUB) allows MP to enter the narrow pore of the barrel-like CDC48 hexamer (*1*). Following passage through the CDC48 pore, the MP is immediately re-ubiquitinated (*2*) and degraded by the 26S proteasome (*3*). Alternatively, the MP may be liberated from CDC48 (*4*), refolded, and able to accumulate along microtubules (*5*), before it is re-ubiquitinated (*6*) and degraded (*7*). (**c**) vRNP/eVRC movement along the ER-actin network. The ER is associated with actin (ER-actin network) that facilitates macromolecular transport, e.g., transport of the vRNP/eVRC along the membrane with the help of myosin motors (*1*). Myosins may transport the vRNP/eVRC along the membrane by binding it as cargo (as shown) or by moving the complex indirectly with the transport of another cargo (bulk transport, not shown). Upon removal of actin and/or myosin, the efficiency of directional movement of the vRNP/eVRC along the membrane is slowed down (*2*). Overexpression of an actin-binding protein dominantly interferes myosin-mediated transport along the membrane and strongly inhibits the movement of the vRNP/eVRC along the membrane (*3*). (**d**) The potential role of tubulin polymerization during TMV infection. Tubulin polymerization at c-MERs (*1*), or another APM-sensitive event at c-MERs, causes detachment of the eVRCs for movement along the ER (*2*) and allows the formation of MTs and their contribution to an aggresomal mechanism for motor-mediated recruitment of host factors and membranes required for maturation of the eVRC into viral factories (*3*). An aggresomal mechanism may also be involved already in the maturation of eVRCs before detachment (not shown). *act* actin, *er* endoplasmic reticulum, *kin* kinesin with cargo, *ms* myosin with cargo, *mt* microtubule. (**e**) The potential role of actin in vRNP/eVRC transports through the PD channel. The desmotubule is connected to the cortical ER-actin network and associated microtubules. In a noninfected cell (*1*), communication through PD is controlled by callose deposition in the PD neck regions. Actin filaments are associated with the desmotubule and may control desmotubule diameter and thus the width of the cytoplasmic sleeve. Endosomal vesicles that cycle between PD-associated c-MERs and the plasma membrane may contribute to the protein loading and functional programming of plasma membrane rafts and microdomains at PD. TMV trafficking through PD in the infected cell (*2*) is facilitated by a MP-mediated increased in the pD size exclusion limit, presumably involving degradation of callose by β-1,3-glucanase, and may occur along the desmotubule, either by diffusion along the membrane following removal of actin by severing (not shown) or by rearrangement of the actin filaments such that the transport of the vRNP/eVRC can occur with support of the filaments and associated myosin activity (shown). Interaction of MP with synaptotagmin suggests that MP may enter PD, not only with the vRNP/VRC but also independently, by trafficking via vesicles from c-MERs to plasma membrane rafts (not shown). *act* actin, *cal* callose, *cM* c-MER, *cs* cytoplasmic sleeve, *cycendo* cycling endosomes, *dt* desmotubule, *er* endoplasmic reticulum, *glu* glucanase, *ms* myosin, *mt* microtubule, *pmr* plasma membrane raft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e-VRC
vRNP
eVRC
mt
er
b
DUB
CDC48
26S
c
e
dt
cs
cal
act
pmr
ms
cycendo
mt
cM
er
glu

movement (Chen et al. 2005; Curin et al. 2007; Niehl et al. 2012). Moreover, a TMV variant showing reduced accumulation of MP along microtubules exhibited increased cell-to-cell movement properties (Gillespie et al. 2002). Consistent with these findings, the MP of the TMV-related oilseed rape mosaic virus (ORMV) was recently shown to provide more efficient cell-to-cell movement to TMV than its own MP although it accumulates to low levels and neither accumulates in virus factories nor along microtubules (Niehl et al. 2014). Interestingly, in cells at the infection front, both MPs localize to microtubule-associated structures functionally correlated with virus movement, as is described in the following section.

2.2 TMV Movement Is Associated with Mobile MP Particles/VRCs That Are Transported along the ER in a Microtubule-Guided Manner

MP-associated structures that occur in advance of the condensation of VRCs into factories are very small and contain only low amounts of MP. Nevertheless, the importance of these structures became apparent already in early studies when protoplasts infected with viral constructs expressing high and low levels of MP were compared. Whereas infection with highly MP-expressing virus led to the accumulation of MP in factories and along microtubules in a large fraction of the protoplasts, both of these patterns occurred only in a small fraction of the protoplasts infected with weakly MP-expressing virus. In contrast, most, if not all, protoplasts accumulated the MP in association with "small peripheral puncta" irrespective of whether being infected with weakly or strongly MP-expressing virus (Heinlein et al. 1998). The peripheral MP puncta occurred in a regular pattern along immunolabeled or $DIOC_6$-stained cortical ER membranes and along immunolabeled microtubules (Heinlein et al. 1998), thus suggesting that the puncta were localized at ER sites that intersect with microtubules. High-resolution time-lapse video and confocal fluorescence microscopy allowed the detection of similar microtubule-aligned MP puncta, now also referred to as "MP particles", in cells at the virus front of radially spreading infection sites in leaves (Boyko et al. 2007; Sambade et al. 2008). In vivo time-lapse imaging of such cells revealed that some of the MP particles remained fixed in their position, whereas other MP particles moved in a stop-and-go manner along GFP-tagged microtubules (Fig. 2.1a; 5). Moreover, using a conditionally mutant MP, the MP particles were linked to MP function in virus movement. The appearance of the MP particles correlated with the recovery of virus movement, and, thus, with the functional recovery of the conditionally mutant MP, at permissive conditions (Boyko et al. 2007). The formation of the MP particles was independent of infection and was detected also upon transient expression of functional wild-type MP (able to functionally complement a MP-deficient virus for movement) or of a conditional MP mutant, if expressed under permissive conditions (Sambade et al. 2008). Similar to the MP particles formed by MP during infection, also the MP particles formed by transiently expressed MP were either anchored or mobile, the latter undergoing directional stop-and-go movements. Co-expression with cellular markers and observation by

time-lapse microscopy revealed that the MP particles were associated with fluid ER membranes as well as with microtubules, whereby the sites of stable or transient anchorage between movements coincided with junctions between the ER and microtubules (Fig. 2.1a; 5). The ER-associated movements of the MP particles occurred either parallel to microtubules or from one microtubule to the next (Sambade et al. 2008), and recent movies taken in the presence of PDLP1 (Thomas et al. 2008) as a PD marker may indicate that at least some of the MP particles targeted PD (unpublished data). Moreover, by combining the visualization of MP with MS2 RNA-labeling technology, it was shown that the MP particles contained MP mRNA and that the MP mRNA colocalized with MP also in PD (Sambade et al. 2008). These observations provided important supporting evidence for MP particles being the mobile form by which MP targets nucleic acids and potentially viral RNA to PD. Consistent with a role of the actin cytoskeleton in supporting the dynamic movements of the ER and the transport of organelles and other macromolecular complexes along the membrane (Sparkes et al. 2009a, b; Boevink et al. 1998; Hofmann et al. 2009; Nebenführ et al. 1999; Akkerman et al. 2011), the ER-associated movements of the MP particles were slowed down in the presence of the actin polymerization inhibitor Latrunculin B (Sambade et al. 2008) (Fig. 2.1c). This finding is also consistent with a functional involvement of the actin cytoskeleton in supporting the accumulation of MP in PD, as shown by FRAP (Wright et al. 2007). Interestingly, the MP particle movements were highly sensitive to the microtubule polymerization inhibitor amiprophos-methyl (APM). Although MP particle movements appeared to resume after a period of time, this observation suggested a role of microtubule polymerization in the detachment of the ER-associated MP particles from microtubule sites (Sambade et al. 2008) (Fig. 2.1d). Prolonged treatment with microtubule polymerization inhibitor oryzalin led to the accumulation of MP at these sites (swelling of the MP puncta) in protoplasts (Heinlein et al. 1998), which may suggest that the MP particles represent early VRCs that accumulate MP over time. A role of microtubule dynamics in the movements of the MP particles and that the MP particles in infected cells represent VRCs are consistent with the observation that tobacco mutants that are affected in their microtubule polymerization and treadmilling dynamics have a reduced ability to support the spread of TMV (Ouko et al. 2010). The VRC nature of the MP particles in infected cells is also supported by the functional correlation between virus movement and their presence in cells at the virus infection front (Boyko et al. 2007; Sambade et al. 2008) and also by studies indicating that the 126 k/183 k replicase is involved in TMV movement (Hirashima and Watanabe 2001, 2003; Guenoune-Gelbart et al. 2008).

Microtubule polymerization to release the MP particles/VRCs from their attachment sites for further movement along the ER could be induced by MP since MP was shown to interfere with microtubule nucleation activity in mammalian cells (Boyko et al. 2000a; Ferralli et al. 2006) and to (super)stabilize microtubules (Boyko et al. 2000a; Ferralli et al. 2006; Ashby et al. 2006), as well as to interact with the microtubule assembly factors EB1:GFP (Brandner et al. 2008) and γ-tubulin (Sambade et al. 2008) in plants. The MP carries a domain with similarity to α-, β-, and γ-tubulins and amino acid mutations shown to affect microtubule association,

MP particle formation, and MP function in a temperature-sensitive manner (Boyko et al. 2000a, 2007), as well as other mutations affecting microtubule association and function in virus movement (Boyko et al. 2002) localize to this domain (Heinlein 2008). This domain may be essential for interaction with microtubules or with factors that regulate microtubule dynamics and may therefore be involved in locally stimulating microtubule polymerization and the release of VRCs for movement.

Future research may reveal the extent by which the transport of the MP particles/early VRCs may reflect a viral subversion of an existing RNA transport system in plants and whether the mobile MP particles/VRCs share features with RNA transport particles (or "granules") described in other systems. RNA transport in animal systems usually involves active transport supported by the cytoskeleton (St. Johnston 2005), and it will be interesting to determine whether conserved mechanisms play a role. A first hint for conserved features may be provided by the example of translational repression of the transported RNA, which seems to apply to both TMV RNA during intercellular transport in plants (Karpova et al. 1997, 1999) and to transported RNA molecules in other systems (St. Johnston 2005; Kiebler and Bassell 2006; Torvund-Jensen et al. 2014).

2.3 The Role of Actin

The MP particle/VRC movements along the ER are consistent with the dynamic nature of the membrane network (Sparkes et al. 2009a; Griffing 2010) and its ability to allow the trafficking of associated small molecules and protein complexes by lateral diffusion (Guenoune-Gelbart et al. 2008; Runions et al. 2006; Grabski et al. 1993; Martens et al. 2006; Baron-Epel et al. 1988). The ER network is continuous between cells through the desmotubule within PD (Ding et al. 1992b), thus allowing viruses, their MPs, and potentially other non-cell autonomous proteins and RNPs to reach PD and move cell to cell by transport along the membrane. The ER is tightly associated with actin filaments (Sparkes et al. 2009a; Boevink et al. 1998; Ueda et al. 2010), and a role of actin and associated myosin motors in the trafficking of various viral proteins or in the spread of infection by various viruses has been reported (Haupt et al. 2005; Harries et al. 2009a, b; Kawakami et al. 2004; Avisar et al. 2008a; Prokhnevsky et al. 2005; Hofmann et al. 2009; Wright et al. 2007; Amari et al. 2014). Consistent with a role of the ER-associated actomyosin system, TMV movement and motor-dependent Golgi movements were inhibited by overexpression of an actin-binding protein. This inhibition was actin dependent, indicating that the overexpressed actin-binding protein caused the inhibition of TMV movement by a dominant effect, i.e., the obstruction of ER-embedded, motor-mediated cargo trafficking along the filament (Hofmann et al. 2009) (Fig. 2.1c). In contrast, the cell-to-cell spread of TMV infection continued in the absence of intact actin filaments (Hofmann et al. 2009) (Fig. 2.1c) as also did the functionally correlated trafficking of MP particles/early VRCs and the targeting and accumulation of MP at PD (Wright et al. 2007; Sambade et al. 2008), although with significantly reduced efficiency. These findings are consistent with the notion that the membrane alone

provides fluidity for the transport of macromolecular complexes, while the transport efficiency and directionality of transport are increased if supported by an intact actomyosin system (Griffing 2010; Sparkes et al. 2009a). However, the movement of several viruses tested, including TMV, was reduced upon long-term inhibition of actin filaments or myosins (Harries et al. 2009b) indicating the importance of the ER-associated actomyosin network in maintaining the efficiency or directionality of ER-mediated MP/viral RNP diffusion along the membrane.

Given the tight association of the actin-myosin network with the ER, it may not be surprising that the ER-associated late VRCs/viral factories are associated with actin filaments (Liu et al. 2005; Hofmann et al. 2009) and can show actin-supported motile behavior (Kawakami et al. 2004; Liu et al. 2005). The finding that also ectopically expressed 126 k protein localized near actin filaments (Liu et al. 2005) may suggest that this protein interacts with actin or myosin to facilitate VRC transport along the ER. However, as the vicinity of expressed 126 k protein to actin filaments may also be due to association of the protein with the ER, further studies using ER and actin markers may be needed to further support this hypothesis.

Since the ER extends between cells through the desmotubule, it appears likely that the ER-associated actin cytoskeleton plays a role also in regulating or facilitating macromolecular transport through PD (Fig. 2.1e). An association of actin and myosin with PD is supported by immunological evidence (Blackman and Overall 1998; White et al. 1994; Golomb et al. 2008; Reichelt et al. 1999), but it remains questionable how the molecular size dimensions of actin and myosin fit into the narrow channel (Burch-Smith and Zambryski 2012). Nevertheless, actin-depolymerizing agents were found to increase PD aperture (Ding et al. 1996; White et al. 1994), whereas treatment with BDM (2,3-butanedione monoxime), an inhibitor of actin-myosin interaction, led to constriction of PD (Radford and White 1998). Studies in *Tradescantia* (spiderwort) suggested that the SEL of PD is increased when myosin detaches from actin, as induced by BDM, and is decreased when myosin attaches to actin, as induced by NEM (N-ethylmaleimide) (Radford and White 2011). A role of actin filaments in controlling the SEL of PD and also the cell-to-cell movement of viruses was suggested also by the ability of the MPs of TMV and of cucumber mosaic virus to sever actin filaments in vitro and by the observation that the MP-induced increase in the PD SEL depends on this activity and can be inhibited by co-treatment of the plants with the actin filament-stabilizing agent phalloidin (Su et al. 2010). It remains unclear, however, whether the implied MP-triggered reorganization of the actin cytoskeleton leading to the SEL increase has to occur inside PD or could also occur rather elsewhere in the cell.

2.4 Coordination of Microtubule- and Actin-Dependent Events at the ER Membrane

The alignment of the cortical ER network with actin (the ER-actin network) supports the motility and organization of the network as well as lateral trafficking of Golgi stacks and the movement of membrane-associated protein complexes with the

help of myosin motor proteins (Nebenführ et al. 1999; Boevink et al. 1998; Sparkes et al. 2009a; Akkerman et al. 2011; Avisar et al. 2009; Ueda et al. 2010). The microtubules of the nearby cortical array were shown to intersect with the ER-actin network (Sampathkumar et al. 2011) and contribute to its overall structure (Foissner et al. 2009; Langhans et al. 2009). This interconnectivity between the ER-actin network and the microtubule cytoskeleton establishes potential routes for signal transduction and for the targeting and retrieval of molecules to and from specific peripheral sites. The ER at the junctions with microtubules shows increased stability (Peña and Heinlein 2013; Hamada et al. 2012). These junctions may indeed be identical with the previously described "immobile, fixed sites" (Knebel et al. 1990), "persistent ER puncta," or "anchoring points" (Sparkes et al. 2009a, b, c) around which the ER remodels. Such microtubule-associated and stable ER junctions were more recently described as "cortical landmarks" at which various organelles interrupt their actin-driven movements (Hamada et al. 2012). These landmarks appeared to require microtubules for their formation but not for their maintenance, suggesting that these sites contain additional components. As organelles continued to pause at the sites in the absence of microtubules, organelle pausing may not necessarily depend on interaction with the microtubule, but may involve binding to a third component of the landmark complex (Hamada et al. 2012). It has been proposed that the pausing of organelle movement at microtubule-associated pausing sites may increase the probability for organelles to meet and thus to exchange components or that the landmarks may represent sites at which organelles deliver their contents to the surrounding area, for example, to specific domains in the plasma membrane (Peña and Heinlein 2013; Hamada et al. 2012). These ER-associated junctions of the actin and microtubule cytoskeleton may thus provide important cortical hubs at which various motor and membrane-mediated trafficking pathways come together and exchange their cargoes for further distribution or complex assembly. The observation of MP particles/early VRCs at such ER junctions with microtubules may suggest that the MP of TMV and potentially also the MPs of other viruses exploit such "cortical microtubule-associated ER sites (c-MERs)" for protein and membrane recruitment (Peña and Heinlein 2013). Organelles that pause their actin-driven movement along the ER membrane at microtubules are often associated with microtubule-associated proteins or kinesin motor proteins (Cai and Cresti 2012; Wei et al. 2009; Ni et al. 2005; Lu et al. 2005; Liu et al. 1994; Lee et al. 2001) that may facilitate organelle binding to the microtubule. In analogy, MP particles/early VRCs undergoing stop-and-go movements between c-MERs may pause their actin-driven movements along the ER at microtubule sites with the help of the microtubule-binding activity of MP. TMV may exploit c-MERs in two different ways during early and late infection stages (Peña and Heinlein 2013): Initially, the virus may use c-MERs as sites for initial anchorage of viral RNA, local translation, and assembly of early VRCs, some of which subsequently detach from these sites and move directionally in a stop-and-go manner between c-MERs toward PD. Later, thus after some of the early VRCs moved into new cells, the virus may use c-MERs to further recruit host factors and membrane and thus to transform the remaining VRCs into late VRCs/virus factories (Fig. 2.1d).

2.5 Are VRCs and Viral Factories Formed by an Aggresomal Pathway?

It has been suggested that viral protein inclusions are reminiscent of aggresomes and serve as platforms for the generation of viral replication sites (Jackson et al. 2005; Laliberté and Sanfacon 2010; Wileman 2006). Aggresomes are perinuclear inclusion bodies in mammalian cells that contain terminally misfolded proteins, which are eventually cleared by autophagy (Chin et al. 2010; Hao et al. 2013). The similarity of the TMV factories to aggresomes in animal and human cells is supported by the shared dependence on microtubules and microtubule-associated proteins. The formation of the aggresome in mammalian cells involves the CDC48-interacting, tubulin-associated histone deacetylase 6 (HDAC6) and microtubules as well as microtubule minus-end-directed dynein motors that gather and transport aggregated proteins toward the centrosome, which harbors the microtubule organizing center (MTOC) and where autophagosomes and lysosomes are concentrated (Chin et al. 2010; Kawaguchi et al. 2003; Ouyang et al. 2012; Iwata et al. 2005; Lee et al. 2010). Unlike animal cells, plant cells do not have centrosomes and a dedicated single MTOC. In contrast, MTOC activity in plants is dispersed throughout the cortical microtubule array, and microtubules nucleate at existing microtubules (Nakamura et al. 2010). Since the anchoring sites for the development of TMV late VRCs/viral factories appear to be localized at c-MERs, the c-MERs might represent sites of microtubule nucleation and aggresome formation in analogy to the MTOC-associated development and anchoring of the aggresome in mammalian cells (Fig. 2.1d). Although further studies are needed to confirm this hypothesis, the observation that the release of MP complexes/early VRCs from c-MERs can be inhibited by a microtubule polymerization inhibitor (Sambade et al. 2008) may provide first evidence for the presence of microtubule-nucleating activity at c-MERs. Such activity of c-MERs is also suggested by the presence of microtubule branchpoints and crossovers associated with viral factories of different developmental stages, which may be indicative of previous microtubule nucleation activity (Niehl et al. 2013b). Similar to microtubules that emerge from the centrosome and support aggresome formation with their associated motors in mammalian cells, also the microtubules emerging from c-MERs may support the growth of aggresomes with the help of associated motor proteins and thus assist in the formation of viral factories in plant cells (Fig. 2.1d).

The proposed microtubule polymerization-induced release of early VRCs from c-MERs stands in contrast to the proposed microtubule polymerization-dependent aggresomal development of viral factories, which depends on the ability of early VRCs to remain at their positions and not being released for movement, irrespective of microtubule polymerization. To reconcile these two opposing events at c-MERs, the model may be complemented by the proposal that the release of early VRCs from c-MERs involves microtubule growth and subsequent release by severing, whereas the formation of viral factories may involve microtubule growth and the suppression of microtubule severing and thus the maintenance of VRC anchorage at c-MERs. Although this proposal is rather speculative, it is also consistent with

microtubule-associated proteins that can restrict katanin-mediated microtubule severing, such as SPIRAL2 (Wightman et al. 2013). However, such activity may also reside in the MP or in other VRC components such as the 126/183 replicase, which may be revealed by future studies.

As already described, CDC48 may target viral factories for removal and degradation of MP late in infection. However, CDC48 may also play a role in the initial development of viral factories during early infection stages. Indeed, the formation and subsequent degradation of the MP-induced viral factories may be part of the same pathway. Mammalian CDC48 mediates the delivery of proteins either to the aggresome or the proteasome, depending on the proteostatic state of the cell (Ju and Weihl 2010). This could suggest that the development of the TMV factory is functionally linked to MP degradation and the saturation of the MP-degrading proteasome. Thus, at early stages of infection, when only little MP is present at c-MERs, only little MP is misfolded and extracted for degradation by the proteasome. However, at later stages, when more misfolded MP accumulates at c-MERs, the proteasomal degradation pathways may get saturated, which may inhibit CDC48-mediated extraction and, in turn, may cause aggresomal growth of the VRC into a viral factory at the c-MER. Once MP expression within the viral factories ceases, the MP amount left at the aggresomal factory consequently decreases, and extracted MP may accumulate along microtubules in equilibrium with available ubiquitination and proteasome activity until all MP is degraded. Although this model predicts MP clearance by the proteasome, it is also possible that the majority of the MP is cleared together with late viral factories by autophagy. Recent studies indicated the existence of a proteasomal deubiquitination activity that releases ubiquitin chains to activate HDAC6 involved in autophagy-dependent aggresome processing (Hao et al. 2013). Since microtubule-associated MP lacks ubiquitin, MP may act as a donor for ubiquitin chains to activate the processing of viral factories by autophagy.

2.6 Role of the Cytoskeleton in the Context of Other Viruses

Consistent with the interaction of plant viruses with various cellular membranes and membrane-associated transport pathways for replication and spread and given that membrane transport in plants is generally facilitated by the actomyosin system, there are numerous studies indicating the role of the actin cytoskeleton in intracellular and intercellular trafficking of the virus or of viral components (Serra-Soriano et al. 2014; Agbeci et al. 2013; Ribeiro et al. 2013; Tilsner et al. 2012; Genoves et al. 2009, 2010; Yuan et al. 2011; Su et al. 2010; Cui et al. 2010; Harries et al. 2009a, b, c; Cotton et al. 2009; Hofmann et al. 2009; Vogel et al. 2007; Wright et al. 2007; Prokhnevsky et al. 2005; Liu et al. 2005; Haupt et al. 2005; Kawakami et al. 2004; Laporte et al. 2003; Carette et al. 2002). Consistently, several studies also demonstrated a role of myosin motors, which in plants belong to two specific classes, class VIII and class XI (Peremyslov et al. 2011). Avisar et al. showed that inactivation of class VIII myosins impaired the PD localization of the Hsp70 (heat

shock protein, 70 kDa) homolog of beet yellows closterovirus, a virion component required for the cell-to-cell movement (Avisar et al. 2008a). Later also, rice stripe tenuivirus (Yuan et al. 2011) and rice black-streaked dwarf fijivirus (Sun et al. 2013) were described to require myosin VIII for the PD targeting of their MPs. Class XI myosins (XI-2 and XI-K) were implicated in the PD targeting of PDLP1, which is a target of the MP of GFLV, a tubule-forming nepovirus (Amari et al. 2010, 2011). The tomato spotted wilt tospovirus, also a tubule-forming virus, has been reported to use myosin XI-K for transporting its nucleocapsid within the cell, and, consistently, inhibition of this myosin delayed the systemic infection (Feng et al. 2013). Using RNA interference, it was shown that TMV movement depends on the activity of myosin XI-2 and that the silencing of other tested myosins (myosins XI-F, VIII-1, VIII-2) had no effect (Harries et al. 2009b). However, since myosins have redundant roles in trafficking (Prokhnevsky et al. 2008; Vidali et al. 2010; Peremyslov et al. 2010; Avisar et al. 2009; Ojangu et al. 2012), the interference with expression of individual myosins may not lead to a phenotype. Using dominant-negative inhibition by expression of myosin tail domains, which interferes with regulated cargo binding of functionally related myosin paralogs (Peremyslov et al. 2008; Avisar et al. 2008b, 2009, 2012), may lead to the identification of the roles of other specific myosins in virus trafficking that remain undetected so far. Our recent work indeed indicates the requirement for different class VIII and class XI myosins during specific subcellular events involved in TMV spread (Amari et al. 2014). Silencing expression as well as dominant inhibition was used to demonstrate the role of myosins XI-2 and Xi-K in the intercellular spread of turnip mosaic potyvirus (TuMV; potyvirus) (Agbeci et al. 2013).

Microtubules have been addressed in great depth in relation to TMV and other tobamoviruses such as ToMV-Ob (Padgett et al. 1996) and ORMV (Niehl et al. 2014), whereas less is known with respect to their role in the context of other viruses. However, the list of viruses or viral proteins reported to undergo direct or indirect interactions with microtubules is growing and already includes potato-mop-top virus (PMTV) (Shemyakina et al. 2011; Wright et al. 2010), potato virus X (PVX) (Serazev et al. 2003; Cho et al. 2012), potato virus A (PVA) (Haikonen et al. 2013), GFLV (Laporte et al. 2003), CaMV (Martinière et al. 2009, 2013; Harries et al. 2009a; Bak et al. 2013), and the ophioviruses Citrus psorosis virus (CPsV) and mirafiori lettuce big-vein virus (MilBVV) (Robles Luna et al. 2013). Movement of PMTV and PVX depends on three MPs encoded by the triple gene block (TGB). Several models by which TGB proteins facilitate virus movement have been suggested (Verchot-Lubicz et al. 2010). However, it is generally believed that TGB1 binds viral RNA to form a vRNP and that the transmembrane proteins TGB2 and TGB3 facilitate the transport of the vRNP to PD. The TGB2 and TGB3 proteins of PMTV associate with the ER and also with recycling endocytotic vesicles (Haupt et al. 2005), whereas the PMTV TGB1 protein appears to interact with microtubules and forms cortical particles along them (Shemyakina et al. 2011; Wright et al. 2010). Functional studies indicated that the microtubule association of TGB1 correlates with its accumulation at PD. Consistently, PD targeting and the formation of the microtubule-associated TGB1 particles were sensitive to treatments with

microtubule polymerization inhibitor and led to the accumulation of TGB1 along the ER network. Based on these findings, it seems likely that the microtubule-associated TGB1 particles form upon binding of TGB1-RNP complexes to TGB2 and TGB3 at the ER and that the ER-actin network transports the microtubule-associated particles to PD. This model shows resemblance to the model proposed for TMV, namely, the formation of movement-compatible complexes at c-MERs (ER-actin: microtubule junctions) and the subsequent movement of the complexes along the ER-actin network toward PD.

Unlike PMTV, PVX requires its coat protein (CP) in addition to the TGB complex for movement. In vitro experiments demonstrated that the CP binds to microtubules and competes with MAP2 for microtubule binding (Serazev et al. 2003). In agreement with these in vitro data, PVX movement in *N. benthamiana* was inhibited by overexpression of the microtubule-binding protein MPB2C (Cho et al. 2012), which was shown previously to interfere with TMV movement under similar ectopic overexpression conditions (Kragler et al. 2003). PVX movement was also inhibited by overexpression of remorin, a protein localized to plasma membrane rafts (Raffaele et al. 2009). This finding may suggest that the pathway by which PVX and potentially other viruses move from c-MERs to PD involves an intermediate step at the plasma membrane, potentially involving endosomal recycling between the ER at the c-MERs and plasma membrane rafts (Peña and Heinlein 2013).

PVA is a potyvirus recently reported to interact with a microtubule-associated protein through its helper component-proteinase (HC-Pro) (Haikonen et al. 2013). The HC-pro-interacting protein (HIP2) is a potato homolog of the protein SPIRAL2 (SPR2) of *Arabidopsis*, which promotes polymerization-driven microtubule growth and stabilizes microtubule crossovers by controlling microtubule severing (Yao et al. 2008; Wightman et al. 2013). It seems conceivable that HC-Pro recruits HIP2 to support the stabilization of a microtubule scaffold that may be involved in the formation of movement-competent complexes and viral factories, as proposed for TMV (Niehl et al. 2013b).

CaMV is a pararetrovirus that interacts with the microtubule cytoskeleton to facilitate viral transmission by aphids. The P2 protein of this virus is a microtubule-binding protein that is transported via microtubules from virus factories to a transmission body (TB) (Martiniere et al. 2009). Within seconds after aphid feeding, the P2 protein present in the TB and virions present in virus factories are distributed throughout the microtubule array (Martinière et al. 2013). The process is reversible and allows P2 and virions to return to the TB and viral factories upon departure of the aphid (Bak et al. 2013). Thus, microtubules can participate in dynamic processes allowing to rapidly distribute virions and viral proteins in the cell. In addition to playing this important role in TB formation, microtubules may also provide a scaffold for the assembly of the CaMV virus factory as suggested by the observation of a strong, nonmobile association with microtubules of the factory protein P6 (Harries et al. 2009a). This hints to the possibility that similar to TMV, also CaMV has evolved mechanisms to exploit c-MERs for anchorage and assembly of viral complexes.

2.7 Conclusions and Future Perspectives

While it is clear that that the actin network facilitates the trafficking of membranes and other cellular components with the help of myosin motors, there is now emerging evidence that microtubules function in the transient or stable local tethering of organelles (e.g., peroxisomes, mitochondria, P-bodies) and membranes (ER, Golgi, endosomes, plasma membrane) and thus in the functional organization of the plant cell cortex (Brandizzi and Wasteneys 2013; Peña and Heinlein 2013). Localized tethering appears to occur at specific sites in the cortex at which the microtubules of the cortical array intersect with the cortical ER-actin network. As a consequence of this connectivity, these "cortical microtubule-associated ER sites (c-MERs)" likely represent important hubs of myosin and kinesin-mediated trafficking and may function as localized platforms for membrane and organellar cargo exchange, macromolecular assembly, and organized trafficking in both lateral (along the ER between c-MERs) and vertical (e.g., endosomal cycling between c-MERs and adjacent plasma membrane domains) directions. The subcellular events during TMV infection are consistent with the concept that viruses target these sites for anchorage and for the recruitment of membranes and host factors required for replication and guided transport along the ER membrane to PD (Peña and Heinlein 2013). Although viruses may use lateral VRC movement along the ER membrane for their passage through PD, they may also exploit vertical trafficking between c-MERs and the plasma membrane to target PD, as is suggested by reported interactions of MPs of different viruses with cycling endosomes or plasma membrane proteins (Carluccio et al. 2014; Lewis and Lazarowitz 2010; Raffaele et al. 2009; Vijayapalani et al. 2012; Haupt et al. 2005). An endosomal pathway linked to microtubules also plays a role in the cell-to-cell transport of SHORT-ROOT and of other cell-nonautonomous transcription factors in *Arabidopsis* (Koizumi et al. 2011; Wu and Gallagher 2013). It will be interesting to see whether endosomal pathways are linked to microtubules at c-MERs and whether the distribution of c-MERs along the cortical array and their role in specific cargo exchange may contribute to the functional programming and localization of the diverse rafts and protein microdomains present in the plasma membrane (Jarsch et al. 2014), particularly at PD (Faulkner 2013). Future research may also demonstrate whether c-MERs represent general sites for viral access to cellular, cytoskeleton-supported, transport pathways.

Another important aim for future research should be to identify the composition of the MP particles/early VRCs and to determine whether these show resemblance to RNA transport particles in other systems. However, the composition may be expected to be different since RNA transport and localization in various systems usually depend on cis-acting localization elements ("ZIP codes") present in the 3′ UTR of the mRNAs (St. Johnston 2005), whereas the MP of TMV and other MPs bind nucleic acids in a non-sequence-specific manner (e.g., Citovsky et al. 1990, 1991; Osman et al. 1992; Shoumacher et al. 1992; Jansen et al. 1998; Herranz and Pallas 2004) and can facilitate the movement of unrelated viruses (e.g., Morozov et al. 1997). Another important point to address is the role of motor proteins. Several studies have addressed the role of myosins in virus movement. The particular roles

of each implied myosin and the respective cargo transported during the distinct steps of virus infection (e.g., replication or transport) have been investigated for TMV (Amari et al. 2014) but remain to be investigated for other viruses. Moreover, given that aggresomal pathways in animal systems imply the role of minus-end-directed microtubule motors, a role of an aggresomal pathway in the formation of viral factories in plants could be supported by the analysis of a potential association of the factories with minus-end-directed kinesins. Plants encode a large and distinct set of kinesin motors, and some of them appear to play a role in regulating microtubules and cargo transport along microtubules within the cortical array (Zhu and Dixit 2011, 2012; Oda and Fukuda 2013; Cai and Cresti 2012). Finally, the mechanism by which MP interacts with microtubules should be further explored. Although it has been proposed that MP mimics a tubulin domain involved in the interaction of tubulin protofilaments (Boyko et al. 2000a; Heinlein 2002), we still lack detailed structural information about MP and the MP-microtubule complex that could help to verify this hypothesis. Moreover, since the MP was shown to interfere with microtubule nucleation activity in mammalian cells (Ferralli et al. 2006), it remains to be demonstrated whether MP exerts such activity also in plant cells and, if so, by which mechanism. In any case, TMV and other viruses are important pathogens, and more detailed insights into the molecular interactions are needed for the development of new strategies against viral diseases in crops. Research on virus replication and movement will certainly continue to reveal novel aspects of cellular organization and function as well as molecules and interactions involved in intercellular communication and development.

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Virus-Induced Modification of Subnuclear Domain Functions

3

Andrew J. Love, Jane Shaw, and Michael E. Taliansky

Contents

Abstract

The nucleus and subnuclear structures such as Cajal bodies and the nucleolus function as pleiotropic control centres which regulate cellular activities. In recent years it has been found that proteins encoded by diverse genera of plant viruses can localize and interact with components of these structures during the infection process. In some cases such interactions are required for successful replication and systemic spread of the viruses, whereas in other cases these associations are detrimental to virus infection. This chapter aims to discuss the types of interaction at the mechanistic level, to provide the reader with a broad understanding of the role of subnuclear domains during plant virus infections.

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3.1 Introduction

The cell nucleus is a highly dynamic membrane-bound organelle which orchestrates a diverse array of crucial cellular activities such as ribosome subunit biogenesis, DNA replication and RNA synthesis and processing (Trinkle-Mulcahy and Lamond 2007). Given that the nucleus and its compartments play a critical role in the regulation of host plant cell survival and homeostasis, it is not unexpected that components of invading viruses interact with these structures. For example, it is known that RNA reverse transcribing caulimoviruses (Haas et al. 2005), begomoviruses with DNA genomes (Sharma and Ikegami 2009) and negative stranded RNA nucleorhabdoviruses (Tsai et al. 2005) directly replicate in the plant cell nucleus and subvert nuclear functions to promote this end and ensure viral survival. Interestingly, many studies in recent years have found that viruses which do not replicate in the nucleus (such as single-stranded positive-sense RNA viruses; +ssRNA) and so would not be expected to interact with the nuclear structures actually encode components which may target the nuclear compartments in order to promote viral replication, viral cell-to-cell movement and systemic movement and suppress a suite of antiviral defence mechanisms. Understanding at the mechanistic level how viruses subvert nuclear compartments to enhance virulence is important for the development of new virus control strategies.

3.2 Structure and Function of the Nucleus and Nuclear Substructures

The nucleus is an organelle whose complex architecture not only facilitates chromosome replication, division and organization but also integrates and controls a diverse plethora of cellular functions required for survival. It contains regions of condensed heterochromatin, interchromatin and euchromatin, which are arranged appropriately to regulate chromosome segregation, gene expression and DNA replication (Trinkle-Mulcahy and Lamond 2008; Schneider and Grosscheld 2007). Moreover, the nucleus is punctuated with a variety of subnuclear structures whose numbers and morphologies change due to developmental cues or environmental conditions, reflecting that nuclear function and architecture are highly responsive and dynamic (Jolly and Morimoto 1999; Belmont 2003). A variety of these subnuclear structures such as the nucleolus, Cajal bodies (CBs), paraspeckles and splicing speckles have now been identified and their components characterized at the RNA and protein level, such that various functionalities have now been ascribed to them (Boisvert et al. 2007; Rippe 2007; Lamond and Spector 2003; Matera et al. 2009; Cioce and Lamond 2005). The typical morphologies and organization of these subnuclear structures are indicated in a diagrammatic representation produced using confocal microscopy on nuclei labelled with fluorescent markers specific for CBs and nucleoli (Fig. 3.1).

The most prominent of these subnuclear structures is the nucleolus, which is traditionally known to be the site for rRNA gene transcription and processing and

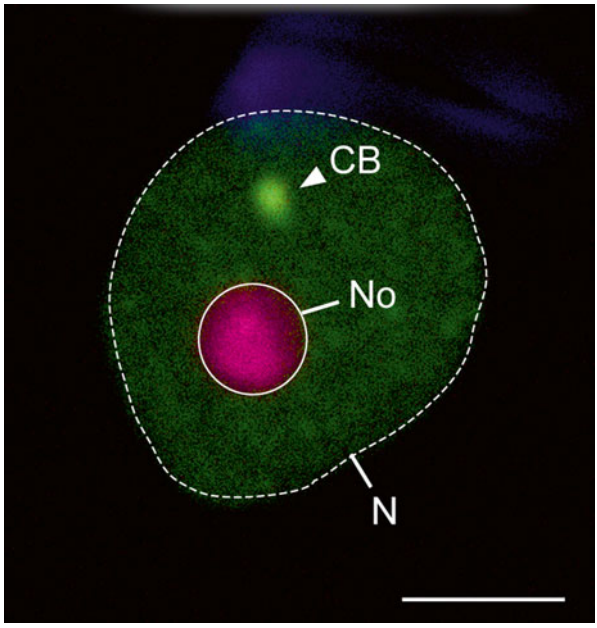

Fig. 3.1 Confocal image of the plant nucleus (*N*) and its subnuclear compartments. Cajal bodies (*CBs*) and the nucleolus (*No*) are labelled with fluorescent markers specific for these compartments; U2B"-GFP for CBs and fibrillarin-mRFP for the nucleolus. Scale bar, 5 μm

the location for incorporation of the rRNAs into ribosomal proteins which form the large and small subunits of the ribosome (Granneman and Baserga 2004; Boisvert et al. 2007; Fatica and Tollervey 2002). Nucleoli are formed at specific points within the nucleus around tandemly repeated segments of chromosomes coding for rDNA, structures known as nucleolar organiser regions (Olson 2004a). Plant nucleoli are often nearly spherical in shape and are composed of three distinct regions which vary in composition and appearance, namely, the fibrillar centre (FC), the dense fibrillar component (DFC) and the granular component (GC) (Shaw and Brown 2004). The FC is composed of a meshwork of fine fibrillar strands and appears as an area of lower contrast in TEM images. The subunits of RNA polymerase I are enriched in this region (Boisvert et al. 2007). Surrounding this is an area of greater electron density, the DFC, which can occupy up to 70 % of the nucleolar volume (Shaw and Brown 2004). As the name suggests, these areas are densely packed with fine fibrils, and it is within this region that pre-rRNAs are modified and spliced by small nucleolar RNPs (snoRNPs; Boisvert et al. 2007). The GC component is found at the outer edge of each nucleolus, and it envelops the DFC region. It contains many small granules, possibly pre-ribosome particles, and it is in this region that the final processing and ribosome maturation take place and it may act as a storage depot for ribosomes before their export to the cytoplasm. The whole process of ribosome maturation is a tightly orchestrated event which involves the formation of a series of intermediate pre-ribosomal complexes and the participation of up to 200 accessory proteins (Granneman and Baserga 2004) and various RNA complexes which both shuttle into and out of the nucleolus (Andersen et al. 2005; Lam et al. 2007). In addition to these functions, the plant nucleolus has been linked to the

recognition of aberrantly spliced mRNAs and is involved in nonsense-mediated decay (Kim et al. 2009) and production of heterochromatic siRNAs involved in transcriptional gene silencing (Li et al. 2006; Pontes et al. 2006). In non-plant systems, the nucleolus has been implicated as a regulator of cell cycle control (Boisvert et al. 2007) and as a stress sensor and response transducer (Rubbi and Milner 2003; Olson 2004b; Boisvert et al. 2007; Boulon et al. 2010), roles which have not yet been ascribed to plant nucleoli.

It would be anticipated that the most copious nucleolar proteins play a pivotal role in these processes, and as such, several of these abundant proteins, like fibrillarin, B23 and nucleolin, have been studied.

Fibrillarin is predominantly found within the fibrillar regions of the nucleolus which are involved in the transcription and processing of rRNA (Warner 1990; Eichler and Craig 1994), but it also localizes to CBs (Raška et al. 1991). It is highly conserved in sequence, structure and function in eukaryotes (Aris and Blobel 1991; Barneche et al. 2000; Reichow et al. 2007; Pih et al. 2000; Wang et al. 2000) and is a key component of box C/D snoRNPs and is essential for rRNA processing (Venema and Tollervey 1999; Carmo-Fonseca 2002; Reichow et al. 2007). The *N*-terminal region contains a glycine- and arginine-rich domain (GAR) (Barneche et al. 2000) which facilitates protein-protein interactions (Jones et al. 2001; Nicol et al. 2000). In addition, fibrillarin has a centrally situated RNA-binding domain (Aris and Blobel 1991; Rakitina et al. 2011) which together with the C-terminal helical structure confers methyltransferase activity (Wang et al. 2000). This methyltransferase activity can direct the 2′-O-ribose methylation of rRNA, one of the processes involved in rRNA maturation (Barneche et al. 2000; Cioce and Lamond 2005; Matera and Shpargel 2006). Interestingly the C-terminal end can also target fibrillarin to other subnuclear structures, such as CBs. Although the role of fibrillarin in CBs is not well elucidated, it likely participates in 2′-O-ribose methylation of small nuclear RNAs (snRNAs).

Another major component of the nucleolus is nucleolin, which is typically found in a variety of cellular compartments but is typically very abundant in the nucleolus. The *N*-terminal domain of nucleolin contains basic and acidic tracts which promote nuclear localization and facilitates rDNA transcription by interacting with rDNA repeats and histone H1. The C-terminal region contains a GAR domain which enables the interaction with ribosomal proteins, and the central portion has RNA-binding capacity (Tuteja and Tuteja 1998). Given the variety of functional domains, nucleolin is involved in a plethora of activities, such as ribosome biogenesis, rRNA transcription, processing and modification and also the shuttling of ribosomal components between the cytosol and the nucleus (Mongelard and Bouvet 2007; Tuteja and Tuteja 1998). The nucleolus also contains B23, which is thought to contribute to ribosome assembly, nucleocytoplasmic protein shuttling (Li et al. 1996) and regulation of rDNA transcription (Okuwaki et al. 2001).

Many nucleolar processes require movement of proteins from the cytosol into the nucleus and from various nuclear compartments into the nucleolus. Proteins of less than 40 kDa can passively diffuse into the nucleus from the cytosol (Hiscox 2007), and those proteins with RNA-binding motifs may nonspecifically locate into the

nucleolus as this nuclear structure contains copious amounts of rRNA (Taliansky et al. 2010). Proteins larger than 40 kDa may enter the nucleus from the cytoplasm via an energetic import which requires monopartite or bipartite tracts of basic amino acids which function as nuclear export signals (Macara 2001; Hiscox 2007). How these proteins then subsequently move from the nucleoplasm to the nucleolus is poorly understood, since there is not a distinct barrier or membrane between these structures. It is possible that some of these proteins display a nucleolar localization signal (NoLS), a poorly characterized motif rich in lysine and arginine residues which promotes localization (Hiscox 2002, 2007). The NoLS is thought to contribute in part to nucleolar localization, but other factors such as what the protein can interact with can influence nucleolar localization; for example, the protein may localize on its own or require association with transcripts transcribed in the nucleolus and/or interaction with other proteins destined for the nucleolus (Hiscox 2002, 2007).

CBs are another important subnuclear structure. They are highly conserved cell components which are found within the nuclei of a diverse range of organisms (Andrade et al. 1991; Tucker et al. 2001; Bevan et al. 1995; Collier et al. 2006; Gall et al. 1995; Yannoni and White 1997; Liu et al. 2009) and are frequently observed in association with the nucleolus. This conservation suggests that CBs may have an essential role within the cell, but elucidating the function of these organelles has proved difficult. CBs are dynamic structures whose number is controlled by environmental and developmental cues (Raška et al. 1991; Andrade et al. 1993; Boudonck et al. 1999; Cioce and Lamond 2005). CBs are most frequently detected in cells with high transcriptional demands, such as neuronal and cancer cells or in cell lines infected by viruses (Morris 2008; Matera et al. 2009), although it is also likely that an increased incidence of CBs may also be detected in plant cells with a heavy transcriptional burden. Their major function is the maturation of certain nuclear RNAs and the modification, assembly and transport of different classes of RNP particles such as snoRNPs, snRNPs, U7 snRNP and telomerase. CBs also play a role in histone mRNA 3′-end processing (Bassett 2012).

The major scaffolding protein required for CB composition, formation and activity is coilin, a multifunctional protein found in CBs but also in the nucleoplasm (Carmo-Fonseca et al. 1993; Lam et al. 2002). Recent work on plant coilin has indicated that it has two sets of basic amino acids in the *N*-terminal domain and one set in the central domain which facilitates interaction with RNA (Makarov et al. 2013). After binding RNA, structural changes occur in the *N*-terminal region of the coilin monomer that promotes multimerization into RNA-coilin aggregates (Makarov et al. 2013). These multimers constitute the scaffolding of the CB, which acts as a framework for sequential modification, assembly and transport of RNP particles. In addition to participating in these functions, work in mammalian cell systems has indicated that coilin may also be involved in additional activities such as regulation of PolI in response to DNA damage (Velma et al. 2010; Gilder and Do 2011). However, it remains to be determined whether or not coilin is involved in these processes in plants.

These nuclear structures and components are quite well characterized in terms of their architecture and function; however, various reports have indicated that plant

virus components may hijack these processes and subvert the nuclear machinery in unusual ways to facilitate the viral invasion process. A considerable number of reports have been published which describe how components from diverse plant virus groups can localize to nuclear structures. In many of these studies, the nuclear localization of the virus component was found to be essential for effective virus replication and dissemination throughout the plant, with a smaller number of publications elucidating the mechanisms of how this may be achieved. This chapter shall discuss these findings in order to describe the role of plant nuclear components in different virus infections from the perspective of cell-to-cell spread, long-distance movement, replication, suppression of silencing and other host defence responses.

3.3 Overview of Plant Virus Infection

Viruses may enter plants either through feeding by insect, soil microbe or nematode vectors or via mechanical damage. Following viral entry, replication occurs prior to the local and long-distance movement of the virus through the plant, which typically precedes development of systemic disease symptoms. Movement of viruses through the plant requires the virions or nucleic acid-protein complexes to initially move from cell-to-cell via plasmodesmata (PD), which are intercellular conduits which span cell walls and links the cytoplasm of contiguous cells. This process is dependent on both host and virus-encoded components (Lucas 2006). Following local cell-to-cell spread, the virus eventually reaches the phloem, a vascular system which transports macromolecules and photoassimilates throughout the plant (Lucas 2006; Oparka 2004). Following entry into the phloem, the virus is disseminated systemically through the plant via the movement of these solutes. Such successful replication and spread of viruses through the plant is also contingent on their evasion or suppression of host defences such as RNA silencing and hypersensitive cell death responses.

3.4 Virus Component-Nuclear Interactions Controlling Systemic Movement and Cell-to-Cell Spread

With regard to nucleus/plant virus component interactions and pathogenesis, one of the most studied areas has been on how such associations control long-distance virus movement, with a significant body of work coming from the studies on ground nut rosette virus (GRV). GRV is a +ssRNA umbravirus which unlike other plant viruses does not encode a coat protein (CP). It encodes an RNA replicase (Taliansky and Robinson 2003), also an ORF4 movement protein (MP) which mediates cell-to-cell movement through PDs (Ryabov et al. 1998) and an ORF3 which binds viral RNA and thus protects it from degradation while permitting long-distance movement through the phloem, leading to systemic infection. The 26–29 kDa ORF3 interacts with viral RNA to form filamentous RNPs which accumulate in the cytosol of plant cells as inclusions (Taliansky et al. 2003). Surprisingly ORF3 protein has

also been detected in nuclei, mainly in nucleoli (Ryabov et al. 1998, 2004). The nucleolar localization and subsequent movement of ORF3 back to the cytoplasm was found to be essential for successful umbravirus infection. Moreover, mutagenesis studies were performed in which mutated leucine- and arginine-rich regions of ORF3 led to compromised nuclear export, prevented the formation of cytoplasmic viral RNPs and blocked long-distance movement (Kim et al. 2007a), thus linking nucleolar localization with viral protein function.

Later studies showed that the ORF3 localization to the nucleolus occurs via interaction with reorganized multiple CB-like structures (CBLs) which fuse with the nucleolus (Kim et al. 2007b). The mechanisms by which ORF3 targets to CBs and promotes the formation of CBLs remain unelucidated; however it is likely that ORF3 hijacks existing host CB transport and distribution pathways. With regard to the ORF3-mediated reorganization of CBs into CBLs and their fusion with the nucleolus, it is a possibility that ORF3 may achieve this by influencing pathways or interactions that are known to control CB form and function. For example, ORF3 may lead to redistribution of CB components (such as coilin, U2B" and fibrillarin; Collier et al. 2006) or their interaction with themselves or additional nucleolar components (Ogg and Lamond 2002; Cioce and Lamond 2005) or via modulating the phosphorylation state of coilin (Sleeman et al. 1998; Lyon et al. 1997). With time, ORF3 moves out of the nucleus into the cytoplasm, and in doing so it binds fibrillarin directly which leads to the redistribution of fibrillarin to the cytosol, where it is not normally found (Kim et al. 2007a, b). This led to the idea that fibrillarin may participate in the formation of cytoplasmic filamentous ORF3 RNPs and also the long-distance systemic movement of the virus. Subsequent fibrillarin knockdown experiments showed that while fibrillarin is required for both these processes, it is not required for the replication or local cell-to-cell spread of the virus (Kim et al. 2007b). In vitro work demonstrated that ORF3 combined with fibrillarin and viral RNA can assemble into architectures reminiscent of the filamentous viral RNPs *formed in planta*, and moreover the in vitro structures had a similar level of infectivity (Kim et al. 2007a, b). The assembly of these structures requires interaction of the ORF3 leucine-rich region of ORF3 with the GAR domain of fibrillarin (Kim et al. 2007b; Jones et al. 2001). Later atomic force microscopy (AFM) studies revealed that the ORF3-fibrillarin complexes forms single layer rings of 18–22 nm which interact with viral RNA, leading to encapsidation and arrangement into a helical morphology (Canetta et al. 2008). These viral RNP structures, when formed in phloem companion cells, are able to enter sieve elements and move throughout the plant to cause systemic infection. Thus, umbraviruses hijack host rRNA and RNP processing machinery to perform unexpected functions in unusual cellular locations to facilitate long-distance virus spread.

A variety of components encoded by other viruses have been shown to localize to the nucleolus and/or interact with key nucleolar components, aspects of which have been found to be crucial for long-distance viral movement. Poleroviruses, +ssRNA viruses which are restricted to cells in the vascular system, encode a major CP and also a minor CP which is formed by occasional translational read through of

the CP gene (Bahner et al. 1990). Although both CPs have an identical NoLS and can be localized to the nucleolus when expressed individually, the minor CP loses the capacity to localize to the nucleolus during potato leafroll virus (PLRV) infection (Haupt et al. 2005). This indicates that PLRV components (RNA or protein) or virus-induced changes in the host may act to restrict movement of the minor coat protein into the nucleolus. It remains to be elucidated what the role of the nucleolar localization of the coat protein plays in the infection process. However, similar to the results obtained with GRV, knockdown of fibrillarin in plants did not affect local accumulation of PLRV on the inoculated leaves but did compromise long-distance movement of the virus and the subsequent development of systemic symptoms, indicating that fibrillarin is involved in long-distance movement of PLRV (Kim et al. 2007b).

Viruses such as pomoviruses (potato moptop virus; PMTV), hordeiviruses (poa semilatent virus; PSLV) and potexviruses encode three proteins required for cell-to-cell and long-distance movement arranged into a triple gene block (TGB) (Morozov and Solovyev 2003). The TGB1 protein of PMTV, which is involved in long-distance movement, typically localizes to the nucleolus. It was found that removal of the 84 *N*-terminal amino acids of the PMTV TGB1 protein compromised its targeting to the nucleolus and abolished long-distance movement of the viral RNAs (Wright et al. 2010; Torrance et al. 2011). The deletion did not affect the local cell-to-cell spread of the virus. With regard to the TGB1 of other viruses, such as PSLV, work by Semashko et al (2012) found that TGB1 could interact with fibrillarin in vitro and in vivo. Taken together this indicates that the nucleolus may also play an important role in the long-distance movement of viruses which encode a TGB.

Another example is the CP of satellite panicum mosaic virus (SPMV; +ssRNA genome), which can associate with the nucleolus, bind RNA and is involved in the systemic movement of the virus (Omarov et al. 2005; Desvoyes and Scholthof 2000; Qi et al. 2008), functionalities which may be considered similar to those of GRV ORF3. Taliansky et al (2010) indicated that similar to GRV ORF3, systemic movement of SPMV is likely dependent on nucleolar localization of its CP.

Work on one of the cell-to-cell movement proteins of beet black scorch virus (BBSV), namely, P7a, revealed that an *N*-terminal R-rich motif determined the nuclear and nucleolar localization of the P7a (Wang et al. 2012). Furthermore, far western analysis demonstrated that P7a could bind fibrillarin, which the authors suggested may be required for targeting to the CBs and nucleolus (Wang et al. 2012). Mutation studies on the R-rich region showed that the nuclear and nucleolar localization played an important role in virulence, for example, the mutant which fails to accumulate in the nucleus had fewer lesions in *Chenopodium amaranticolor* and also reduced replication in protoplasts compared with the wild type, with similar findings observed with mutants which accumulate in the nucleus but not the nucleolus (Wang et al. 2012). The mechanistic aspects of how the nucleolus can influence virulence in BBSV infections remains to be uncovered but could partly involve modulation of the P7a facilitated cell-to-cell movement.

3.5 Viral Subversion of Host Nuclear Machinery and Suppression of Defence Mechanisms

Potyviruses have a +ssRNA genome, which are translated into a large 3000–3350 amino acid polyprotein, that is processed into ~10 proteins via the activity of three virus-encoded proteinases (Rajamäki et al. 2004). Although potyviruses replicate in membranous cytoplasmic structures (Cotton et al. 2009; Schaad et al. 1997), two of the encoded replication associated proteins, namely, nuclear inclusion protein a (NIa) and NIb, have been reported to accumulate in the nucleus (Hajimorad et al. 1996; Restrepo et al. 1990), with significant localization of both in the nucleolus at later stages of infection (Beauchemin et al. 2007; Rajamäki and Valkonen 2009; Baunoch et al. 1991; Restrepo et al. 1990).

With regard to NIa, its C-terminal end constitutes the major viral proteinase (Dougherty et al. 1989), and the *N*-terminal half functions as the VPg which is covalently linked to the viral genomic RNA (Siaw et al. 1985; Oruetxebarria et al. 2001). The two functional regions of NIa are separated by a suboptimal cleavage site, the slow proteolytic cleavage of which is required for successful virus replication. Full-length NIa is typically located in the nucleus (Carrington et al. 1993); however a polyprotein form of NIa with an *N*-terminally attached 6K protein is predominantly localized to the sites of viral replication, namely, the cytoplasmic membrane vesicles; it is thought that the attached 6K precludes movement into the nucleus (Restrepo-Hartwig and Carrington 1994; Cotton et al. 2009; Beauchemin et al. 2007). The bipartite NLS and NoLSs of potyviral NIa are located in the *N*-proximal part of VPg (Rajamäki and Valkonen 2009: Schaad et al. 1996), with both required for nucleolar localization, and either one required for nuclear targeting. The most *N*-terminal NLS is necessary for localization to the CBs (Rajamäki and Valkonen 2009). Various reports on different potyviruses found that mutations which prevent nuclear and nucleolar localization of NIa can perturb replication of these viruses, indicating that such localizations are required for successful virus infection (Schaad et al. 1996; Rajamäki and Valkonen 2009).

Potyviral NIa (or its VPg region) was found to interact with an isoform of the eukaryotic translation initiation factor (eIF(iso)4E), an interaction which is required for virus infectivity (Robaglia and Caranta 2006; Schaad et al. 2000; Léonard et al. 2000). Moreover this interaction and also that of NIa and poly(A)-binding protein 2 (PABP2) was found to occur in the nucleus and nucleolus (Beauchemin and Laliberté 2007; Beauchemin et al. 2007). In contrast, although eIF(iso)4E and PABP2 interactors were found with 6K-NIa, they were localized in cytoplasmic membrane vesicles, implying that these proteins are required in different cellular locations during the infection process. It has been suggested that NIa may directly mediate redistribution of a portion of the PABP2 from its usual cytoplasmic location to the nucleolus and may also direct eIF(iso)4E nucleolar localization (Beauchemin and Laliberté 2007). Interestingly, NIa can interact simultaneously with PABP2 and eIF(iso)4E, which may indicate that they form part of the same complex. eIF4E participates in nuclear export of mRNA, nuclear translation and nonsense-mediated decay (Strudwick and Borden 2002), whereas PABP2 regulates nuclear export of

mature mRNAs and their stability and the initiation of protein synthesis (Mangus et al. 2003). It is therefore quite plausible that potyviruses hijack and exploit these functionalities to favour viral replication and translation over that of the host plant processes (Thompson and Sarnow 2000).

Subsequent work illustrated a potential role of the potyviral VPg and its nuclear localization in suppression of host defence responses. An interesting observation is that potyviral VPgs have been detected ahead of the infection front in potato plants (Rajamäki and Valkonen 2003) and the idea developed that VPg may perturb host defence responses in pre-invasion tissues and render them more susceptible to infection. This suggestion was rendered quite valid since overexpression of VPg in plants disrupted RNA silencing, whereas overexpression of mutant VPgs which are perturbed in nuclear and nucleolar localization failed to interfere with RNA silencing (Rajamäki and Valkonen 2003), indicating that VPg and its appropriate nucleolar localization can indeed suppress the onset of antiviral gene silencing defence mechanisms. Interestingly, studies have shown that fibrillarin can interact with the VPg of potato virus A (PVA) and that depletion of fibrillarin reduces the accumulation of PVA in *N. benthamiana* (Rajamäki and Valkonen 2003). This may imply that the association of VPg with fibrillarin could be required for the suppression of various defence responses such as RNA silencing.

RNA silencing regulates the degradation of different RNA species and allows the plant to tightly control the gene regulation in a wide variety of developmental and environmental processes. RNA silencing involves the formation of dsRNA, which are then targeted for cleavage by DCLs into short siRNA fragments, which catalyze the sequence-specific degradation of RNAs. This process is a potent defence against invading viruses. As discussed previously, the nucleolar localization of potyvirus VPg is a requirement for interference of this antivirus mechanism, which is perhaps not surprising considering that a plethora of host components involved in RNA silencing and biogenesis and processing of small RNAs are located in the nucleolus, CBs and nucleus (Pontes and Pikaard 2008). However, other operational aspects of RNA silencing occur in the cytosol (Ruiz-Ferrer and Voinnet 2009), and viruses have evolved a series of proteins which can disrupt the silencing machinery in this cellular location. For example, the main silencing suppression activity of potyviruses are encoded by HC-Pro, which acts in the cytosol by sequestering small RNAs which mediate the silencing signal (see Costa et al. 2013, for a general review of the activities of silencing suppressors). Similarly, the P19 silencing suppressor protein of the tomato bushy stunt virus (TBSV) tombusvirus acts in the cytosol by sequestering small RNAs; however, host plants can impinge P19 activity by inducing its nuclear and nucleolar relocalization (Canto et al. 2006). It is thought that plant ALY proteins which play a role in mRNA maturation and movement is involved in the P19 relocalization (Canto et al. 2006). Other virus RNA silencing suppressors such as 2b encoded by cucumber mosaic virus (CMV) has the capacity to sequester small RNAs but has also been shown to localize to the nucleus and nucleolus via its NLS and NoLS signals and interact with AGO1 and AGO4, which are core components of the RNA-induced silencing complex that cleaves (slices) targeted RNA (Zhang et al. 2006; Lucy et al. 2000; González et al. 2010). Later work showed that the

nucleolar 2b interaction with AGO1 curtailed its slicer activity, which may partially contribute to suppression of RNA silencing (Feng et al. 2013).

P6 is a multifunctional nucleocytoplasmic protein encoded by the pararetrovirus CaMV (cauliflower mosaic virus) which contains several NLSs and a NES which allows it to dynamically shuttle between the cytosol and nucleus, activities which are essential for infectivity (Haas et al. 2005, 2008; Kobayashi and Hohn 2004). While P6 is also found in the nucleolus, a NoLS has not yet been identified; however its association with components of the 60S ribosomal subunit may facilitate its import into this location, considering that the nucleolus is the assembly site of ribosomal subunits (Leh et al. 2000; Bureau et al. 2004; Park et al. 2001; Haas et al. 2008). P6 acts as a translational transactivator, and it is possible that the interactions with ribosomal components may facilitate the translation of the polycistronic CaMV mRNA. P6 is also a major symptom determinant (Cecchini et al. 1997) and has been shown to act as a plant defence suppressor which can impinge on cell death and salicylic acid (SA)-mediated responses (Love et al. 2012; Laird et al. 2013), impacts which may be a consequence of P6's capacity to perturb RNA silencing mechanisms via interference of dsRNA-binding protein (DRB4) activity (Love et al. 2007; Haas et al. 2008; Laird et al. 2013). Work has demonstrated that the inhibition of these host defence mechanisms require P6 to be localized to the nucleus (Haas et al. 2008; Laird et al. 2013), with the suggestion that nucleolar localization is not necessary for these activities.

Recent work has indicated that coilin may be involved in differentially modulating the response of plants to a range of different viruses (Shaw et al. 2014). Using *N. benthamiana* plants with knocked down coilin levels, it was found that coilin plays a role in reducing the number of infection foci on leaves inoculated with barley stripe mosaic virus (BSMV; hordeivirus), which could delay the subsequent systemic spread of BSMV. In addition it was found that coilin participates in the recovery of emergent tissues from the symptoms produced by infection with tobacco rattle virus (TRV; tobravirus) and tomato black ring virus (TBRV; nepovirus), thus implicating coilin in antiviral silencing pathways. In contrast, with potato virus Y (PVY; potyvirus) and turnip vein clearing virus (TVCV; tobamovirus), coilin promotes symptom development and greatly enhances PVY virus accumulation. The authors suggest that the functions of coilin may be recruited or subverted by different groups of plant viruses to facilitate invasion or in contrast are involved in plant defence mechanisms which suppress host susceptibility to viruses (Shaw et al. 2014).

3.6 Additional Virus-Nuclear Subcompartment Interactions with as Yet Unknown Biological Function

With regard to other plant viruses, localization of encoded components to the nucleus and nucleolus has been reported; however their biological relevance remains poorly understood. For example, the CPs of geminiviruses such as tomato leaf curl Java virus (ToLCJAV) and tomato yellow leaf curl virus (TYLCV) are

nucleocytoplasmic shuttling proteins which are hypothesized to localize the viral genomic DNA to the nucleus (Rojas et al. 2001; Sharma and Ikegami 2009); these proteins also contain NoLSs and are targeted to the nucleolus for unknown activities. It has previously been observed that geminivirus infection leads to shrinkage of the nucleus and nucleolus, separation of granular and fibrillar regions of the nucleolus and development of fibrillar rings, phenotypes reminiscent of cells arrested in early mitosis (Bass et al. 2000). It was suggested that the fibrillar rings contained viral DNA and protein (Kim et al. 1986, 1978), and moreover since virus particles were first detected near the nucleoli or fibrillar bodies, it seemed likely that these could be locations of virus replication and assembly. Later work by Kleinow et al (2009) which used an abutilon mosaic virus (AbMV) lacking a coat protein demonstrated that the CP is not required for these gross changes in nuclear ultrastructure, and as such these changes in structure are not due to virion assembly. These authors suggested that the fibrillar rings likely represent nuclei containing replicating viral DNA complexed with other viral components such as the nuclear shuttle protein (NSP), which also transfers viral DNA between the nucleus and the cytoplasm. For various geminiviruses, it has been shown that the NSP and CP have some level of functional redundancy with regard to viral genome shuttling (Zhou et al. 2007). Other geminivirus components such as Reps (replication initiator proteins) are required for replication and have been observed in nuclei of plant cells but not in nucleolar compartments, as in the case of AbMV (Krenz et al. 2011), which is consistent with the additional role of Rep in host DNA re-replication.

The ourmia melon virus (OuMV; Ourmiavirus) genome comprises 3 plus stranded RNA molecules, RNA1, RNA2 and RNA3 which encode an RNA-dependent RNA polymerase (RdRP), MP and CP, respectively (Rastgou et al. 2009). The CP is not required for cell-to-cell spread and long-distance movement but is necessary for the efficient formation of infection foci at exit sites in leaves distal to the inoculated leaf (Crivelli et al. 2011). Work by Rossi et al. (2014) demonstrated that the that the CP localized to the nucleus and accumulated in the nucleolus, which is an unusual location for viral structural proteins, which likely indicates hitherto unknown functions in addition to that of encapsidation.

The TEV P3 protein localizes to the ER and forms Golgi-associated punctate structures which can traffic along actin filaments and colocalize with replication vesicles, indicating that P3 may play a role in virus replication and movement (Cui et al. 2010). Moreover, P3 has been implicated in virus-host interactions (Eggenberger et al. 2008; Jenner et al. 2003; Johansen et al. 2001) and has also been found to target to the nucleolus; however its biological function in this compartment is unknown.

Work by Herranz et al. 2012 has indicated that the alfalfa mosaic virus (AMV) CP contains a NoLS which also forms part of the RNA-binding domain that is required for viral replication and translation and also a C-terminal domain which may function as a nuclear export signal. These authors suggest that the CP nucleolar localization may modulate or recruit host nucleolar factors to promote AMV infection. Moreover they proposed a model in which the NoLS masking of the

RNA-binding properties could play an important regulatory role in modulating the cytosolic/nuclear balance of CP accumulation and its activities of nucleolar shuttling, RNA binding and virion formation.

NIb, a potyviral encoded RNA-dependent RNA polymerase, is involved in viral replication on cellular membranes in the cytosol (Schaad et al. 1997; Hong and Hunt 1996) and has also been found in the nucleus and nucleolus (Restrepo et al. 1990). Mutation of the NLSs of NIb precluded movement into the nucleus and nucleolus and prevented infection by TEV (Li et al. 1997; Li and Carrington 1995), thus indicating that nuclear and nucleolar targeting of NIb is required for successful viral infection.

NLSs have also been reported for three maize fine streak virus (MFSV) nucleorhabdovirus proteins; however two of these proteins, namely, the nucleocapsid protein and phosphoprotein, are required to interact in order to be targeted to the nucleolus (Tsai et al. 2005), a process which remains mechanistically and functionally unelucidated.

3.7 Summary

Nuclear compartments are a common destination for proteins encoded by a variety of different plant viruses. Many reports have arisen in recent years which demonstrate that such localizations are essential for successful systemic virus infection, although in general the mechanisms behind this are poorly elucidated. However a few studies have uncovered a diverse series of processes by which some virus components can hijack host nuclear proteins to permit pathogenesis. Association of plant viral proteins may in some cases lead to an alteration in the architecture of nuclear structures, or movement of host nuclear components into the cytosol, or alternatively translocation of cytoplasmic factors into the nucleus, or various combinations of these effects. In other cases the viral protein may or may not induce redistribution of host nuclear components but nevertheless associates with them. The relocation/interaction with nuclear proteins allows viruses to harness their functionalities to enable replication, movement and suppression of host plant defence responses. However, several interesting studies have found that plants may on occasion "counterattack" against the virus by redistributing viral components to the nucleus where they are no longer functional, much to the detriment of viral invasion. Taken as a whole, this area of research has uncovered many unexpected and novel functions of nuclear compartments, which has broadened our understanding of their dynamism and the number of cellular roles that they play. In future, we expect yet further unmasking of novel nuclear operational activities influenced by virus infection, knowledge which will hopefully be exploited for the design and implementation of new virus control strategies.

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Regulation of Plasmodesmal Transport and Modification of Plasmodesmata During Development and Following Infection by Viruses and Viral Proteins

4

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Abstract

Plant cells are encased in cellulose precluding direct contact. To enable intercellular communication, plants evolved cell wall-spanning channels called plasmodesmata. Plasmodesmata are essential to facilitate transport of small molecules such as photosynthate, as well as critical signaling macromolecules such as transcription factors and RNAs. Plasmodesmata are indispensible for all stages of plant development, from embryogenesis, through vegetative and reproductive development. Plasmodesmata are not passive channels, but instead they are highly dynamic and change their apertures in response to intracellular signals such as reactive oxygen species, hormones, and chloroplast and mitochondrial homeostasis. To date the best-known mechanism for controlling the degree of plasmodesmata transport is the reversible deposition of callose polysaccharides in the cell wall immediately surrounding plasmodesmata channels. Plant viruses have evolved to counteract innate plasmodesmata regulatory mechanisms and are well-known pirates of plasmodesmata during infectious spread.

4.1 Introduction

Perhaps one of the most critical innovations in the evolution of multicellularity in the early ancestors of plants was the development of channels that facilitate the continuity of cytoplasm between adjacent cells. These channels would provide a route for resource allocation between cells as the early plant developed in three dimensions, allowing cell specialization and differentiation. These cytoplasmic routes for transport were necessary in order to circumvent the limitations that arise from the presence of cell walls.

The existence of structures that directly connect the cytoplasm between adjacent plant cells was first proposed by Albert Pfeiffer in 1879 and observed later that same year by Edward Tangl. These channels were first called plasmodesmata (singular, plasmodesma) by Strasburger in 1901 (Carr 1976). The name plasmodesma is derived from the Latin *plasma*, for fluid, and the Greek *desma,* for bond. The direct observation of plasmodesmata was a revolutionary breakthrough in botany as it challenged the then current view that plant cells acted autonomously (Carr 1976).

Plasmodesmata have been the subject of intermittent scientific investigation since their discovery in 1879. It is now clear that plasmodesmata are not simple cytoplasmic channels for nutrient distribution, but rather form the basis of a complex network for intercellular transport of nutrients and for intercellular communication via the movement of various signal molecules such as RNAs and proteins. Such intercellular trafficking and signaling is essential to all aspects of plant growth and development. Given the cell-to-cell connectivity provided by plasmodesmata, it is not surprising that viruses have evolved to take advantage of these channels for their infection and spread in plant hosts. Indeed, viruses and viral proteins have proved invaluable for investigating the functions of plasmodesmata.

In this chapter, we will discuss factors that regulate plasmodesmata structure and function and how these are influenced by virus infection. We begin with a description of the structure and components of plasmodesmata, describe the functions of plasmodesmata, and then discuss the regulatory mechanisms that control plasmodesmal permeability and formation. Finally, we describe how viruses modify plasmodesmata and their regulatory systems for their local intercellular and eventual systemic spread. Given the scope of this book, it is impossible to cover all literature related to plasmodesmata and we direct the reader to other sources where appropriate.

4.2 Plasmodesmata Structure

A typical plasmodesma has a diameter of 30–50 nm, below the limits of resolution of light microscopy. Transmission electron microscopy (TEM) has therefore been indispensable in elucidating the structure of plasmodesmata. A variety of fixatives and staining protocols have been used to resolve the structure of plasmodesmata. A typical, simple channel is represented in Fig. 4.1. The outer limits of the channel are the plasmalemma of the cells connected by the plasmodesmata. The center of the plasmodesmata is occupied by a structure derived from the endoplasmic reticulum (ER), the so-called desmotubule. Under various conditions, and in various cell types, the desmotubule may be in an open or closed state (reviewed in Overall and Blackman (1996)). The space between the desmotubule and plasmalemma is referred to as the cytoplasmic sleeve. Detailed TEM imaging has also revealed the presence of punctate structures connecting the desmotubule and plasmalemma (Ding et al. (1992) and references therein). A curious feature of the desmotubule is its high protein content (Tilney et al. 1991). It has been proposed that these proteins are required to maintain the extreme curvature of the lipid bilayers that constitute the desmotubules (Tilsner et al. 2011).

Several routes for transport through plasmodesmata have been described. The bulk of plasmodesmal transport occurs via the cytoplasmic sleeve (Fig. 4.1). The movement of small molecules (sugars, amino acids, dextrans) and soluble exogenous proteins like GFP is by passive diffusion. The upper limit on the size of molecules transported by a given plasmodesma is called the size exclusion limit (SEL). Plasmodesmal SEL is dynamic and regulated by developmental and environmental signals (reviewed in Burch-Smith et al. (2011b)). Fluorescently labeled probes and proteins that initially localize to the ER lumen can move between cells via the lumen of the desmotubule in plasmodesmata (Barton et al. 2011; Guenoune-Gelbart et al. 2008; Martens et al. 2006). Lipids and ER membrane proteins may also transit the plasmodesmata via the membranes of the desmotubules (Guenoune-Gelbart et al. 2008; Grabski et al. 1993). However, the plasmalemma of plasmodesmata does not allow intercellular exchange of proteins or lipids and indeed serve as permeability barriers for the movement of lipids (White and Barton 2011; Grabski et al. 1993).

A fascinating property of plasmodesmata is that their structure and function vary temporally and spatially during plant development (reviewed in Burch-Smith et al.

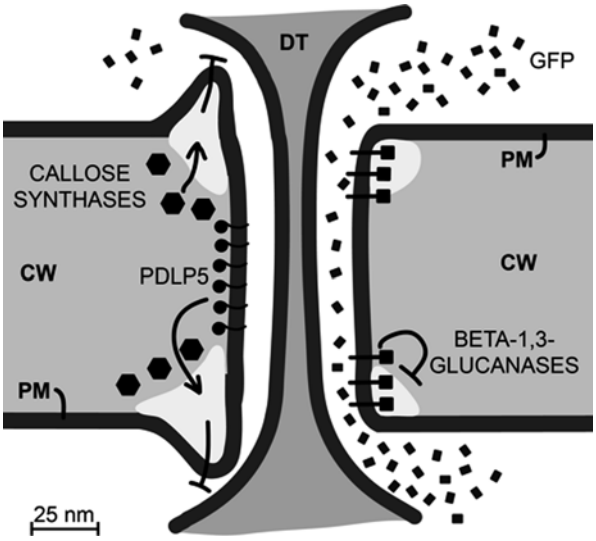

Fig. 4.1 Cartoon model of a plasmodesma. Plasmodesmata are plasma membrane (*PM*)-lined channels that cross the cell wall (*CW*), containing cytosol and a central, tightly compressed strand of ER called the desmotubule (*DT*). The cytosol (*white areas*) is continuous between adjacent cells, allowing small and large molecules to move from one cell to the next. Here, GFP (*small rectangles*) is moving via diffusion through the plasmodesmata. Callose deposition (*light areas*) in the CW bordering the openings of plasmodesmata negatively regulates plasmodesmal transport and is mediated by callose synthases (*hexagons, left*). Degradation of callose bordering plasmodesmata positively regulates their transport and is mediated by β-1,3-glucanases (*dark squares* with PM-spanning tails, *right*). Some plasmodesmal proteins affect plasmodesmal transport through unclear mechanisms, such as PDLP5 (*circles* with membrane-spanning tails, *left*), which here is shown inhibiting GFP transport, possibly by promoting local callose synthesis (This figure was modified from Fig. 4.1 in Brunkard et al. 2013)

(2011b)). Typically, plasmodesmata in immature tissues are single, linear channels, about 30 nm wide and 100 nm long (drawn to scale in Fig. 4.1); these channels may undergo a fission event via Y- or X-shaped intermediates to form twin plasmodesmata (Fig. 4.2a, b) (Faulkner et al. 2008). It is not yet clear whether twin plasmodesmata formation is driven by cell wall deposition (Fig. 4.2a, b) or by insertion of a second ER strand into the desmotubule followed by subsequent deposition of cell wall material between the two ER strands (Faulkner et al. 2008) (Fig. 4.2c).

In mature tissues, plasmodesmata are more elaborately branched channels. The ultrastructures of the individual channels of complex branched plasmodesmata are largely unchanged relative to those of simple channels, consisting of the plasmalemma, ER, and cytoplasm of the cells they subtend. Leaves undergoing the sink-to-source transition provide a striking example of the simple to branched progression of plasmodesmal structural changes. In sink leaves, more than 90 % of plasmodesmata are simple compared to fewer than 30 % in source leaves (Oparka et al. 1999). How simple plasmodesmata become branched is an interesting question that has been addressed primarily by TEM. These studies suggest that a second desmotubule

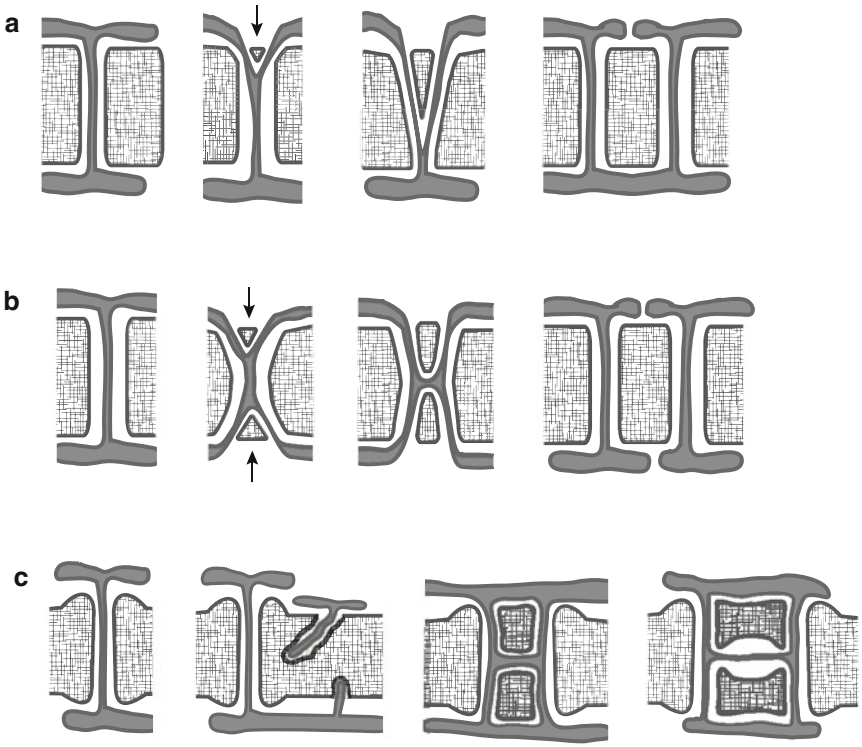

Fig. 4.2 Formation of twin and branched plasmodesmata. (**a**) and (**b**) show cell wall synthesis-mediated initiation of new plasmodesmata via Y-shaped and X-shaped intermediates. *Arrows* indicate sites where new cell wall material is deposited either on one side (**a**) or two sides (**b**) of the plasmodesmata. The images from left to right show continued cell wall deposition that leads to bifurcation of the ER and ultimately to twin plasmodesmata. (**c**) shows ER-mediated initiation of plasmodesmata formation proceeding from left to right. First, ER strands enter the area of the cell wall and then fuse with the ER of the desmotubule in the original plasmodesmata forming an H-shaped "branched" structure. Further expansion of the cell wall leads to larger plasmodesmata that sometimes contain a central cavity (indicated by lack of shading)

is inserted adjacent to a preexisting one by an unknown mechanism, then the membranes at the middle of the desmotubules fuse, and deposition of new cell wall material leads to formation of elaborately branched channels that may contain large central cavities (Ehlers and Grobe Westerloh 2013; Faulkner et al. 2008; Ehlers and Kollmann 2001) (Fig. 4.2c). This change from simple to complex branched plasmodesmata coincides with changes in the transport properties of plasmodesmata: simple plasmodesmata transport larger molecules than complex branched plasmodesmata (Oparka et al. 1999). This is supported by studies performed in a variety of systems and tissues that consistently reveal that simple plasmodesmata generally have larger SELs than more complex plasmodesmata (reviewed in Burch-Smith et al. (2011b)). Indeed, the SEL of a given plasmodesma appears to be dynamic and responds to

environmental and physiological signals. How plasmodesmata alter their channel size or SEL to regulate intercellular trafficking is a pressing question that is the focus of current enquiries.

Besides their morphology, plasmodesmata may also be distinguished on the basis of their origins (reviewed in Ehlers and Grobe Westerloh (2013), Burch-Smith et al. (2011b), Ehlers and Kollmann (2001)). Plasmodesmata may form during cytokinesis, when strands of ER are trapped in the newly forming cell wall, and are called primary plasmodesmata. Plasmodesmata are also found in cell walls that are not clonally related by cell division of a precursor cell. Such plasmodesmata are formed de novo across existing cell walls and are called secondary plasmodesmata. The mechanism of de novo plasmodesmata formation is not known, but several models in Fig. 4.2a–c show how new plasmodesmata may arise adjacent to an existing one. However, how plasmodesmata form completely de novo across cell walls that lack preexisting plasmodesmata is a mystery; it is postulated that cell wall loosening leads to localized weakening of the cell wall and turgor pressure from inside the cell may lead to the insertion of ER between adjacent cells (reviewed in Burch-Smith and Zambryski (2012)). It is essential to note that primary and secondary are indistinguishable on the basis of their structure and morphology. During the modification of simple plasmodesmata to complex branched, the branched plasmodesmata formed are secondary since they are generated in the absence of cell division (Faulkner et al. 2008). Primary and secondary plasmodesmata can only be distinguished if it is known whether they are formed at cytokinesis or in the absence of cytokinesis.

4.3 Plasmodesmata Function

4.3.1 Transport of Products of Photosynthesis

Perhaps one of the most significant transport functions of plasmodesmata is the distribution of nutrients throughout a plant. Sucrose is the principal form of assimilated carbon that is circulated throughout the plant. A major route for sucrose transport from the mesophyll to the vasculature is cell-to-cell movement via plasmodesmata (termed symplastic trafficking) (Ayre 2011). Alternatively, to exit the mesophyll, sucrose may cross the plasma membrane to move into the cell wall space between cells (apoplasm) for subsequent uptake by adjacent cells of the vasculature. In sink tissues, sucrose exits the phloem symplastically or via the apoplasm. Apoplastic movement of sucrose is an energy-dependent process that requires sucrose transporters and cotransport of protons. The identification and characterization of sucrose transporters is an exciting and vibrant area of current research (Ayre 2011; Chen et al. 2012).

The distribution of plasmodesmata between tissues that transport photosynthates is often informative about the strategy used for loading sugars into the phloem, whether it is apoplastic or symplastic. Gamalei conducted the seminal analysis comparing vein structure and plasmodesmal distribution to phloem transport

strategy (Gamalei 1989). He found that woody species contain "open" veins with numerous plasmodesmata connecting the mesophyll and phloem (type 1) and are symplastic loaders. In contrast, herbaceous species tend to have "closed" veins with virtually no plasmodesmal contacts between the mesophyll and the phloem (type 2) and are apoplastic loaders (Gamalei 1989). Recent analyses have largely confirmed Gamalei's findings regarding symplastic loading by woody species (Rennie and Turgeon 2009), but the relationship between plasmodesmal density and phloem loading strategy has proven more tenuous. For example, the type 1 species *Clethra barbinervis* and *Liquidambar styraciflua* are apoplasmic loaders (Turgeon and Medville 2004) despite the presence of numerous plasmodesmata connecting mesophyll cells and between cells of the minor veins. Thus, some plant species appear to have larger numbers of plasmodesmata than would be strictly necessary for photosynthate transport (Turgeon and Medville 2004). It is proposed that some plants may switch between apoplastic and symplastic loading in response to sugar levels in the mesophyll (Rennie and Turgeon 2009). Thus, plasmodesmata may be poised to respond to the photosynthetic status of the tissues they subtend by altering their transport capacities for optimal carbon partitioning.

The most widely accepted model for phloem transport is Münch's pressure flow model (Munch 1930). Briefly, it predicts that the bulk flow of sap through the sieve tubes of phloem is driven by differences in hydrostatic pressure between the source and sink sieve elements. Recent findings, while generally supporting this model, have also called certain aspects into question. To try to reconcile some of these discrepancies, Fisher articulated his high-pressure manifold model of phloem transport (Fisher 2000) that highlights the role of sink plasmodesmata in regulating the rates of phloem unloading (Patrick 2013). Simply put, the rate at which sugars are unloaded in sinks depends on the hydraulic conductance of the plasmodesmata of the symplastic unloading pathways linking sieve elements with phloem parenchyma cells. In most instances, this is a limiting step in the bulk flow of resources from source to sink and hence in carbon partitioning between sinks. The high-pressure manifold model therefore positions plasmodesmata as crucial contributors to crop yield potential, although this is yet to be tested experimentally.

Plasmodesmata are also important for sucrose transport in the nitrogen-fixing root nodules of *Datisca glomerata,* which forms actinorhizal symbioses with actinomycetes of the *Frankia* genus (Schubert et al. 2011), and for nematode parasitism of plant hosts. Cyst nematodes cause catastrophic damage to a variety of crop species and *Heterodera schachtii* is a species that successfully infects the laboratory model dicot Arabidopsis thaliana and the commercial crop tobacco (Hofmann et al. 2010). Infective second-stage juveniles (J2) of *H. schachtii* invade the roots of susceptible plants and induce specialized feeding structures termed syncytia. Syncytium development starts from a single cell and is enlarged by the subsequent fusion of protoplasts formed by the local dissolution of cell walls initiated at plasmodesmata (Sobczak 2008). The role of plasmodesmata in nutrient transport and syncytium development has been controversial, but recent findings suggest that plasmodesmata-mediated intercellular trafficking is essential for syncytium expansion (Hofmann et al. 2010).

4.3.2 Transport of Signaling Molecules

4.3.2.1 Transport of Hormones

The role of plasmodesmata in cell-to-cell movement of phytohormones has been the subject of much speculation and the focus of early studies that has garnered renewed interest. The early work of Arisz (1969) suggested that the auxin, indole acetic acid, could move between cells via plasmodesmata in *Vallisneria*. Another early report suggested that gibberellic acid (GA) moved intercellularly via plasmodesmata (Kwiatkowska 1991); radioactively labeled GA_3 moved from the underlying thallus to cells of the differentiating antheridial cells of *Chara vulgaris* L. As the cells of the antheridia became symplastically isolated over the course of development, symplastic transport subsided.

A recent study reveals that plasmodesmal trafficking of auxin is required for auxin gradient formation and signaling in tropic responses (Han et al. 2014a). One of the major mechanisms for regulating plasmodesmal flux is via the deposition and removal of callose at plasmodesmata (discussed below). Mutants lacking the product of a specific enzyme, GLUCAN SYNTHASE 8 (GSL8) failing to accumulate callose at plasmodesmata, have increased plasmodesmal permeability, and the hypocotyls of these mutants no longer display the phototropic or gravitropic responses known to be mediated by auxin (Han et al. 2014a). Such *gsl8* mutant seedlings allow increased intercellular trafficking of auxin. Modern models for auxin transport largely focus on the role of polar auxin transport (PAT), a transcellular mode of transport that combines apoplastic movement with secretion and endocytosis-based or channel- and carrier-based pathways to move between cells across plasma membranes (Robert and Friml 2009). PAT is widely studied and has been the basis of computational models that attempt to explain how auxin distribution results in various developmental and growth patterns (Band et al. 2014; Beleyur et al. 2013). The finding of an additional symplastic component to auxin flux will prompt a reassessment of how auxin gradients are established and maintained. It will also be interesting to determine whether symplastic auxin transport is limited to the hypocotyl or is found in other regions of the plant including the root and shoot apical meristems. Finally, a role for auxin in control of symplastic transport may explain some of its effects on development by regulating the movement of other key signaling molecules including other hormones and transcription factors.

4.3.2.2 Transport of Transcription Factors

The discovery that transcription factors move intercellularly via plasmodesmata was a major advance in the field of plant developmental biology. Given that plant cells do not move, positional information is key to determining cell fate (van den Berg et al. 1995, 1997). The non-cell-autonomous activity of transcription factors is one way to convey positional information to neighboring cells to reinforce other developmental cues directing a differentiation process (Han et al. 2014b). The KNOTTED1 (KN1) transcription factor was the first non-cell-autonomous transcription factor described. KN1 localized in both tunica and

corpus layers of maize spikelet meristems, but its mRNA was absent from the tunica (Jackson et al. 1994). Symplastic movement of KN1 was confirmed by direct microinjection of fluorescently labeled KN1 into tobacco mesophyll cells (Lucas et al. 1995). KN1 also increased the SEL of plasmodesmata and facilitated trafficking of its own mRNA. Subsequently numerous other developmentally important transcription factors have been found to act non-cell-autonomously (Table 4.1).

Table 4.1 Plant development processes involving plasmodesmata-mediated intercellular trafficking and the molecules trafficked

Process	Description	Molecules trafficked	References
Shoot and leaf development	KNOX family TFs	KNOTTED 1 (KN1), SHOOTMERISTEMLESS (STM), Knotted 1-like homeobox protein 1 (KNAT1)/BREVIPEDICELLUS (BP)	Lucas et al. (1995), Kim et al. (2003)
	Homeodomain-containing TF	WUSCHEL (WUS)	Yadav et al. (2011)
	Transcriptional coactivator	ANGUSTIFOLIA3 (AN3)/GRF-INTERACTING FACTOR 1 (GIF1)	Kawade et al. (2010, 2013)
	Trans-acting siRNA (TAS)	TAS3	Chitwood et al. (2009)
Root development and patterning	GRAS family TF	SHORT-ROOT (SHR)	Helariutta et al. (2000), Nakajima et al. (2001)
	AT-hook TF	AT-HOOK MOTIF NUCLEAR-LOCALIZED PROTEIN 4 (AHL4)	Zhou et al. (2013)
	Basic helix-loop-helix (bHLH) TFs	UPBEAT1 (UPB1)	Tsukagoshi et al. (2010)
		TARGET OF MONOPTEROS 7 (TMO7)	Schlereth et al. (2010)
	MicroRNA (miRNA)	miR165/166	Carlsbecker et al. (2010)
Epidermal patterning	MYB-like TF	CAPRICE (CPC)	Kurata et al. (2005)
	WD-40 repeat protein	TRANSPARENT TESTA GLABRA 1 (TTG1)	Bouyer et al. (2008)
Floral induction and organ development	MADS domain family, B-type TFs	GLOBOSA (GLO)/DEFICIENS (DEF)	Perbal et al. (1996)
		AGAMOUS (AG)	Urbanus et al. (2010)
	Unique TF	LEAFY (LFY)	Sessions et al. (2000)
	Raf kinase inhibitor-like protein	FLOWERING LOCUS T (FT)	Corbesier et al. (2007, Jaeger and Wigge (2007)

4.3.2.3 Transport of Small RNA Molecules

Plant cells produce a variety of small RNA molecules that are critical for development, and the most prominent are the microRNAs (miRNAs). miRNAs act by regulating the expression of transcription factors by binding to conserved sites in their target mRNAs and recruiting specific RNA-induced silencing complexes (RISCs), leading to mRNA degradation (reviewed in Meng et al. (2011)). While many miRNAs act in the cytoplasm of the cells in which they are produced, the cell-to-cell movement of several miRNAs to regulate the activity of essential developmental transcription factors is well documented (see Root Development below).

In plants, another class of developmentally important small RNAs is the *trans-acting* silencing RNAs (ta-siRNAs). Ta-siRNAs are unique in that they require both miRNA and components of the siRNA pathway for their biogenesis (Vaucheret 2006). Ta-siRNAs regulate their target genes by acting in trans to cleave mRNAs with partial complementarity (Peragine et al. 2004). The importance of non-cell-autonomous activity of ta-siRNAs in plant development is illustrated by the action of tasiR-ARF in leaf development (Chitwood et al. 2009). Here, TasiR-ARFs are produced and show highest activity in the adaxial regions of the leaf margins while their levels gradually decrease toward the abaxial side. The specific pattern of tasiR-ARF abundance in the developing leaf predicts the expression pattern of one of its target mRNAs, *AUXIN RESPONSE FACTOR 3* (*ARF3*)*/ETTIN (ETT)*, that acts to promote abaxial identity (Pekker et al. 2005).

Another class of small RNAs that moves between cells in plants is the small interfering RNAs (siRNAs) (Dunoyer et al. 2010). The siRNA pathway targets both endogenous and exogenous molecules for silencing (Chapman and Carrington 2007). A recent study reinforces the significance of plasmodesmata in mediating systemic siRNA movement. Using a seedling grafting system consisting of a GFP reporter scion and a silencing inducing rootstock, Liang and coworkers showed that long-distance silencing in Arabidopsis occurs via reiterated cell-to-cell movement (Liang et al. 2012). In this system, movement of the silencing signal followed the sink-to-source transition in leaves and was restricted to various extents by different symplastic domains. Interestingly, inhibitors of auxin signaling disrupted this cell-to-cell movement of siRNA silencing signaling, while drugs that disrupt the cytoskeleton or vesicle transport had negligible effects on the system (Liang et al. 2012). Virus-derived RNA molecules are a major target of siRNA-mediated silencing, and the role of silencing as a plant antiviral defense strategy has been extensively investigated (reviewed in Szittya and Burgyan (2013)). The significance of siRNAs as antiviral mechanism is underscored by the existence of several viral strategies that disrupt siRNA production or function (Szittya and Burgyan 2013).

4.3.3 Intercellular Communication and Development

Plasmodesmata play a major role in plant development that extends beyond the trafficking of developmentally important signaling molecules. One consequence of the

dynamic control of plasmodesmal SEL or flux is that groups of cytoplasmically coupled cells can be isolated from their neighbors when it is necessary for that group of cells to execute a specific and/or unique developmental program. Such a group of isolated cells is called a symplastic field, and the creation and removal of this symplastic isolation is a recurring feature of many tissues (reviewed in Burch-Smith et al. (2011b)). Here we discuss a few examples, but it should be noted that the use of symplastic fields for development is a widespread phenomenon.

4.3.3.1 Cotton Fiber Elongation

One notable example of the requirement of symplastic isolation for plant development is during cotton fiber elongation. Plasmodesmata connecting the nascent fibers to the underlying epidermis show reversible gating: from 6 days after anthesis (DAA) to 10 DAA symplastic transport was inhibited but resumed by 16 DAA (Ruan et al. 2001). Callose deposition (see below) is critical to facilitate fiber plasmodesmata closure, and simple plasmodesmata were modified to more complex structures. Concomitantly, fiber cell elongation was driven by increased turgor pressure resulting from induced expression of sucrose and K^+ transporters (Ruan et al. 2001). Once plasmodesmal trafficking resumed, fiber elongation ceased, and the length of fibers depends on the duration of symplastic isolation (Ruan et al. 2004).

4.3.3.2 Seeds and Embryos

Studies of plasmodesmata-mediated intercellular trafficking in seeds and embryos reveal the importance of symplastic fields to the execution of developmental programs. The two layers of cells that make up the outer integument of Arabidopsis seeds are connected by plasmodesmata that allow intercellular transport of GFP within the cells of this tissue (Stadler et al. 2005), but there is no movement from these outer layers to the inner integument. Similarly GFP expressed in the inner integuments is able to move between cells of this tissue but not spread to other tissues. The failure of even the low-molecular-weight fluorescent molecule HPTS to move between the inner and outer integuments further emphasizes the absence of trafficking between these two symplastic fields. Again highlighting the importance of plasmodesmata in nutrient trafficking, globular stage embryos are symplastically connected to the suspensor. Over the course of embryo development, there are marked changes in symplastic connectivity (Stadler et al. 2005; Kim et al. 2005a, b). In globular and heart-stage embryos, GFP expressed in the epidermis diffuses freely into all cells of the embryo (Stadler et al. 2005). However, during later of embryogenesis, the torpedo stages, transport is significantly reduced (Stadler et al. 2005). Detailed experiments have mapped out the symplastic fields of embryos, and these are correlated with the early differentiation of certain cells and tissues (Stadler et al. 2005) and major axes of the seedling (Kim et al. 2005b).

4.3.3.3 Shoot Apical Meristem

Detailed time course experiments indicate that plasmodesmal trafficking is temporally regulated at the shoot apical meristem (SAM) during development (Gisel et al. 1999, 2002). There is restricted movement of fluorescent tracers from the vascular

system into young vegetative apices (plants with less than 12 visible leaves), but movement increases dramatically into apices in older plants with more than 12 visible leaves. Remarkably, trafficking into the apex ceases for several days during the vegetative to floral transition in Arabidopsis and then resumes once floral morphogenesis is underway. As the SAM undergoes profound changes in architecture and gene expression to produce floral meristems versus leaves, it may be advantageous to sequester the SAM during the establishment of reproductive development.

A family of eight PLASMODESMATA-LOCATED PROTEIN (PDLP) proteins was identified through proteomic analysis of highly purified Arabidopsis cell walls (Bayer et al. 2006; Thomas et al. 2008). PDLP1, encoded by *At5g43980,* is a type I membrane protein that localizes to plasmodesmata, as do all other PDLP family members (Thomas et al. 2008). PDLP1 also possesses two N-terminal domains of unknown function 26 (DUF26) domains and a short C-terminal domain. Bimolecular fluorescence complementation (BiFC) reveals that the DUF26 domains of PDLP1 are extracellular, with the bulk of the protein located in the apoplast; only a very short C-terminal domain is cytoplasmic. Overexpression of PDLP1 from a constitutive 35S promoter caused a significant decrease in intercellular trafficking of GFP, and conversely loss of two members of the PDLP family leads to increased plasmodesmal permeability. These findings indicate that PDLP proteins likely act at plasmodesmata to regulate plasmodesmal flux. Given the topology of the protein, however, it is not yet known how the major apoplastic domain exerts its regulatory role on plasmodesmata function. Note that PDLP expression patterns correlate with symplastic domains in the Arabidopsis SAM (Bayer et al. 2008), further supporting the role of plasmodesmata in the establishing of symplastic domains to allow development.

Plasmodesmata and intercellular trafficking in the SAM are also regulated in response to environmental cues like seasonal day-length changes that influence development. The frequency of plasmodesmata in the *Sinapis alba* SAM undergoing the floral transition increases dramatically (Ormenese et al. 2000). Interestingly, the vast majority of these newly formed plasmodesmata are secondary, and plasmodesmata formation may be the result of a transient increase in a floral stimulus (Ormenese et al. 2000), most likely cytokinin (Ormenese et al. 2006).

4.4 Regulation of Plasmodesmata

The modern view of plasmodesmata is that of dynamic structures that actively regulate the flux of the molecules they traffic. Plasmodesmata function, structure, and formation all respond to intracellular signals and to environmental signaling (reviewed in Burch-Smith and Zambryski (2012)). Such responses are necessary to ensure effective communication between distant parts of the plant and coordination of responses. Pathogens, especially viruses, are also major triggers for changes in plasmodesmata. Below we discuss factors that have been identified as critical to regulating plasmodesmata function, structure, and formation. What is rapidly becoming clear is that these signals do not act in isolation but are instead integrated into a complex signaling network to produce a single outcome at plasmodesmata.

4.4.1 Callose

One of the best-known mechanisms for regulating plasmodesmal transport is the controlled deposition and/or removal of callose, a β-1,3 glucan polysaccharide comprised of β-1,3-linked glucose residues, at the necks of plasmodesmata. Callose accumulation effectively constricts plasmodesmal necks, inhibiting intercellular traffic. Callose deposition at plasmodesmata is critical during development as well as wounding and pathogen attack (reviewed in Zavaliev et al. (2011) and see also below). During normal development callose is often deposited at plasmodesmata when it is necessary to establish symplastic fields. This is exemplified by the isolation of the SAM of birch during dormancy cycling (Rinne et al. 2001, 2011). Controlled removal of callose is then required for the resumption of intercellular communication. Removal of callose is mediated by β-1,3-glucanases, and some of these have been localized to the plasma membrane at plasmodesmata (Rinne et al. 2011; Levy et al. 2007). In the case of the dormant birch meristem, chilling-induced release from dormancy coincides with the removal of callose sphincters by glycophosphatidylinositol (GPI)-anchored, gibberellic acid-inducible β-1,3-glucanases (Rinne et al. 2011), and symplastic connectivity is restored.

4.4.1.1 Callose Synthesis

The Arabidopsis genome contains 12 genes that code for CALLOSE SYNTHASE (CalS), also called *GLUCAN SYNTHASE-LIKE* (*GSL*) genes (Verma and Hong 2001). However, only a subset of these is responsible for the accumulation of callose at plasmodesmata during normal development. GSL8/CALS10/CHORUS is required for callose deposition in the cell wall surrounding plasmodesmata. The *chorus* mutant has reduced accumulation of callose at plasmodesmata and increased intercellular trafficking (Guseman et al. 2010). This increase in plasmodesmal permeability ultimately results in defective stomatal patterning, presumably due to altered trafficking of signals that serve as positional cues for stomata development. Consistent with these findings, silencing *GSL8* by RNAi leads to reduced callose at plasmodesmata and increased plasmodesmal permeability (Han et al. 2014a). Intriguingly, hypocotyls of seedlings with reduced *GSL8* expression show impaired phototropic and gravitropic responses, again pointing to a role for plasmodesmata in auxin transport (see above and *Phytohormones* below).

A remarkable example of the precision in callose-mediated plasmodesmata restriction is the regulation of the intercellular movement of the SHORT-ROOT (SHR) transcription factor and microRNA165 between the stele and endodermis during root development (Vaten et al. 2011). *GSL12*/*CALS3* is expressed in the stele and root apical meristem (RAM) during normal plant development, and CALS3 localizes to plasmodesmata. Gain-of-function mutations or ectopic expression of *CALS3* leads to accumulation of callose at plasmodesmata in the root and a concomitant decrease in plasmodesmal permeability (Vaten et al. 2011). Notably, the movement of miR165/6 from the ground tissue into the stele and of SHR transcription factor from the stele into the endodermis was strongly decreased in the dominant mutant *cals3-1d* that contains elevated stele-specific plasmodesmal callose.

This defective intercellular transport was associated with a loss of protoxylem cells and other phenotypes consistent with mutations in the *PHABULOSA* (*PHB*) locus that render it insensitive to miR165/166 (Carlsbecker et al. 2010). Interestingly, expression of *CALS3* in the phloem partially suppresses the loss of the phloem-specific CALS7 (Vaten et al. 2011). *cals7* mutants have reduced apical growth and greatly reduced callose in phloem sieve elements (Xie et al. 2011; Barratt et al. 2011). These data together suggest that the CALS enzymes have similar functions and, further, that the spatial regulation of these enzymes is a critical aspect to regulating plasmodesmal function during plant development.

Other proteins that may act in callose deposition are implicated in plasmodesmal function. Proteomic identification of plasmodesmal-enriched proteins from cell walls of Arabidopsis cell suspension cultures revealed several membrane-associated proteins (Bayer et al. 2006). One of the proteins identified is a GPI-anchored protein with in vitro callose-binding activity named PD CALLOSE-BINDING PROTEIN 1 (PDCB1) (Simpson et al. 2009). *PDCB1* belongs to a small family of three genes, *PDCB1–PDCB3,* and the products of all three genes localize to plasmodesmata. Single or double insertional mutants of *pdcb2* and *pdcb3* showed no phenotypic defects, suggesting functional redundancy with PDCB1. In contrast, overexpression of *PDCB1* resulted in increased callose accumulation at plasmodesmata and reduced plasmodesmata-mediated intercellular trafficking. How PDCB proteins may influence callose deposition and accumulation is unclear, since (like PDLP1 mentioned above) they localize to the apoplast and do not themselves contain any catalytic domains (Simpson et al. 2009).

4.4.1.2 Callose Degradation

The removal of callose from plasmodesmata is carried out by β-1,3-glucanases, a group of hydrolytic enzymes encoded by 50 genes in the Arabidopsis genome (Doxey et al. 2007). β-1,3-glucanases are well known for their roles in pathogen defense, but they are also vital for normal plant development where they are involved in processes such as cell division, pollen tube development and growth, overwintering, seed development and germination, and regulation of plasmodesmata (reviewed in Balasubramanian et al. (2012)). The 50 Arabidopsis β-1,3-glucanases can be divided into five classes according to their protein architecture (Doxey et al. 2007). All five classes contain N-terminal secretion signals and a core glycosyl hydrolase family 17 domain (GH-17), and based on the conservation of essential catalytic residues, all fifty proteins likely retain β-1,3-glucan hydrolysis activity (Doxey et al. 2007). Several of these β-1,3-glucanases are predicted to be GPI anchored (Borner et al. 2003) (Elortza et al. 2003) placing the proteins at the extracellular face of the plasma membrane. Gene expression analyses indicate that a large subgroup of 13 β-1,3-glucanases are nonspecifically expressed in tissues with high rates of cell division including the shoot apex and root and are likely involved in cell division and cell wall remodeling. Nine of these 13 proteins are predicted to be GPI anchored, and this has been experimentally confirmed for four of them (Borner et al. 2003). In fact it is likely that the ancestral plant β-1,3-glucanase functioned in cell division or cell wall remodeling (Doxey et al. 2007). Expression analyses also identified five

β-1,3-glucanases as pathogenesis-related (PR) genes based on their responses to fungal infection (Doxey et al. 2007).

Several recent studies have addressed the role of specific β-1,3-glucanases in removing callose from plasmodesmata both during plant development and in response to pathogen infection. One such protein, *A. thaliana* BETA-1,3-GLUCANASE_PUTATIVE PD-ASSOCIATED PROTEIN (AtBG-ppap), was identified through proteomic analysis of a cell wall-enriched fraction from Arabidopsis (Levy et al. 2007). AtBG_ppap possesses an N-terminal secretion signal peptide and a predicted GPI anchor that would specify localization to the plasma membrane and/or endoplasmic reticulum, and it accumulates at plasmodesmata. Expression of *ATBG_PPAP* is highest in flowers and siliques, suggesting that it may belong to a class of plasmodesmata-localized β-1,3-glucanases specifically involved in floral development. However, while *atbg_ppap* knockout mutants accumulated callose at plasmodesmata and reduced intercellular trafficking, no other phenotypic defects were reported (Levy et al. 2007). Other proteomic analyses have identified additional plasmodesmata-localized β-1,3-glucanases (Bayer et al. 2006; Fernandez-Calvino et al. 2011). One of these, PLASMODESMAL-LOCALIZED B-1,3-GLUCANASE 1 (PdBG1), is expressed in xylem pole pericycle (XPP) cells that give rise to lateral roots (Benitez-Alfonso et al. 2013). PdBG1 localizes to plasmodesmata, and plants overexpressing this protein are compromised in their ability to degrade wound-induced callose. In addition to PdBG1, two closely related β-1,3-glucanases, PdBG2 and PdBG3, also localize to plasmodesmata, and the PdBG2 expression pattern suggests it may function in root development. Indeed, *pdbg1* and *pdbg2* double mutants accumulate callose in their roots and have reduced plasmodesmal permeability. Importantly, these mutants have defective lateral root initiation, and lateral root primordia form adjacent to each other with increased frequency compared to wild-type plants. Consistent with these findings, plants overexpressing PDCB1 show defects in lateral root formation. Thus, the action of β-1,3-glucanases at plasmodesmata is central to lateral root formation (Benitez-Alfonso et al. 2013).

Other 1,3-glucanases that function in plant development include the GA-inducible β-1,3-glucanases that degrade callose at plasmodesmata to allow the resumption of intercellular trafficking required for emergence from dormancy in several plants, as discussed previously (Rinne et al. 2011). In cotton, the removal of callose at plasmodesmata in the cotton fiber coincides with a resumption of symplastic connectivity, and the degradation of callose is likely mediated by a fiber-specific β-1,3-glucanase, GhGluc1 (Ruan et al. 2004).

4.4.1.3 Callose and Pathogens

The role of callose in viral infections is discussed in a later section. First, we discuss intense investigations of the roles callose synthases and β-1,3-glucanases in response to other pathogens (reviewed in Zavaliev et al. (2011)). As mentioned, callose is a major regulator of PD function in the syncytia formed by cyst nematodes on the roots of their hosts (Hofmann et al. 2010). There is emerging evidence that callose and plasmodesmata are also crucial components of defense responses to bacterial pathogens. Plants constitutively overexpressing the plasmodesmal proteins PDLP5,

in addition to accumulating callose at plasmodesmata and overproducing the phytohormone salicylic acid (SA), also have enhanced basal resistance to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* ES4326 (Pma) (Lee et al. 2011). Conversely, *pdlp5-1* mutant plants are more susceptible to this pathogen. *PDLP5* expression is induced by Pma infection, and this correlates with increased callose at plasmodesmata and reduced plasmodesmal permeability. PDLP5 may also have a role in infection by TMV (Lee et al. 2011). Plasmodesmata have also been implicated in defense against fungal pathogens. The plasmodesmal protein, LYSIN MOTIF DOMAIN-CONTAINING GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED PROTEIN 2 (LYM2) (Fernandez-Calvino et al. 2011), is an Arabidopsis receptor for chitin (Petutschnig et al. 2010), a pathogen-associated molecular pattern recognized by fungal pathogens. Direct application of chitin or the flagellin-derived peptide flg22 leads to decreased intercellular movement of GFP via plasmodesmata (Faulkner et al. 2013). Reduction in plasmodesmal flux in response to chitin treatment is relieved in *lym2* mutants, indicating that the plasmodesmal response is LYM2 dependent. *lym2* mutants are also more susceptible to infection by the fungal pathogen *Botrytis cinerea* than are wild-type plants, implying that altering cell-to-cell trafficking is a critical component of the defense response to fungal pathogens (Benitez-Alfonso et al. 2013). However, the role of callose in this response has not yet been examined. Considered collectively, these results suggest that intercellular trafficking of signaling molecules is crucial for plant defense against pathogens. Identification of these mobile signals will yield valuable insight into how such responses can be fine-tuned for crop improvement.

4.4.2 Actin-Myosin

The plant cytoskeletal components actin and myosin have both been implicated in regulating plasmodesmal trafficking. Actin was identified in plasmodesmata by immunolocalization with various anti-actin antibodies and techniques (White et al. 1994; Blackman and Overall 1998; Baluska et al. 2001, 2004). Similarly, myosin and the actin-binding proteins tropomyosin and centrin have been localized to plasmodesmata by immunohistochemistry and more recently by fluorescent tagging (see Deeks et al. (2012), White and Barton (2011)). In addition, several actin and myosin isoforms have been identified as part of the plasmodesmal proteome (Fernandez-Calvino et al. 2011). The function of cytoskeletal proteins in regulating plasmodesmata has mainly been examined through the use of various chemical inhibitors of actin polymerization or myosin activity (reviewed in White and Barton (2011)). As such, the results of these studies should be interpreted cautiously due to possible nonspecific effects on other cellular processes involving the cytoskeleton. However, the preponderance of evidence suggests that cytoskeleton regulates plasmodesmal flux by directing and loading cargo into the channels (White and Barton 2011). One long-standing question about the localization of actin and myosin to plasmodesmata is how they could be physically accommodated within the channels given their dimensions (Tilsner et al. 2011).

4.4.3 Intracellular Signaling and ONPS

Given that intercellular communication and transport are essential to life, mutants with compromised plasmodesmata were predicted to result in severe developmental defects, manifesting first during embryogenesis. With this in mind, the Zambryski lab conducted a genetic screen for Arabidopsis mutants with altered plasmodesmata-mediated intercellular transport (reviewed in Burch-Smith and Zambryski (2012)). The *increased size exclusion limit (ise)* mutants were identified by monitoring the transport of 10-kDA fluorescent-dextrans in embryos of embryonic lethal mutants, and *ISE1* and *ISE2* were mapped and cloned (Stonebloom et al. 2009; Kobayashi et al. 2007). In addition to increased plasmodesmal trafficking, *ise1* and *ise2* embryos also contain increased numbers of plasmodesmata (Burch-Smith and Zambryski 2010).

Intriguingly, ISE2 is a nuclear-encoded plastid-localized DEVH-type RNA helicase (Burch-Smith et al. 2011a). Further analyses in *Nicotiana benthamiana* where *ISE2* was silenced with tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) revealed that in addition to increased intercellular trafficking and biogenesis of plasmodesmata, loss of ISE2 leads to defective chloroplasts and, ultimately, leaves become yellow (Burch-Smith et al. 2011a; Burch-Smith and Zambryski 2010). Of further significance chloroplasts in *ISE2*-silenced *N. benthamiana* leaves have a lower redox state compared to those in non-silenced TRV-infected control leaves (Stonebloom et al. 2012). Similar changes to chloroplasts and leaves were observed upon loss of ISE1, a nuclear-encoded mitochondrial DEAD-box RNA helicase (Stonebloom et al. 2009, 2012; Burch-Smith et al. 2011a; Burch-Smith and Zambryski 2010).

Comparative gene expression analyses of *ise1* and *ise2* embryos revealed that the largest class of nuclear genes affected in *both* mutants encodes chloroplast-specific products (Burch-Smith et al. 2011a). Significantly, numerous affected genes encode products critical for three photosynthesis-related pathways: the light reactions of photosynthesis (43 genes), chlorophyll biosynthesis (15 genes), and the Calvin cycle for carbon fixation (12 genes). Dramatically exemplifying the shared phenotype between *ise1* and *ise2*, 70 % of the 43 genes for the light reactions are down-regulated in *both* mutants. Thus, *ise1* and *ise2* may have the same phenotype with regard to plasmodesmata-mediated transport because both mutants have substantially disrupted chloroplast function. These data lead to three important ideas: (1) Chloroplast-mitochondrial cross talk is important, (2) chloroplasts can regulate the formation and function of plasmodesmata, and (3) chloroplast-to-nucleus retrograde signaling is disrupted in the *ise1* and *ise2* plasmodesmal mutants. We postulate the existence of a pathway of organelle-nucleus-plasmodesmata signaling (ONPS) where plastid signals regulate plasmodesmata via nuclear gene expression or by direct signaling to plasmodesmata (Fig. 4.3) (reviewed in Burch-Smith and Zambryski (2012)).

Further evidence for a link between chloroplasts and plasmodesmata comes from other mutants with defective plasmodesmal function. The maize *sucrose export defective1 (sxd1)* mutant fails to export photosynthate from sites of photosynthesis

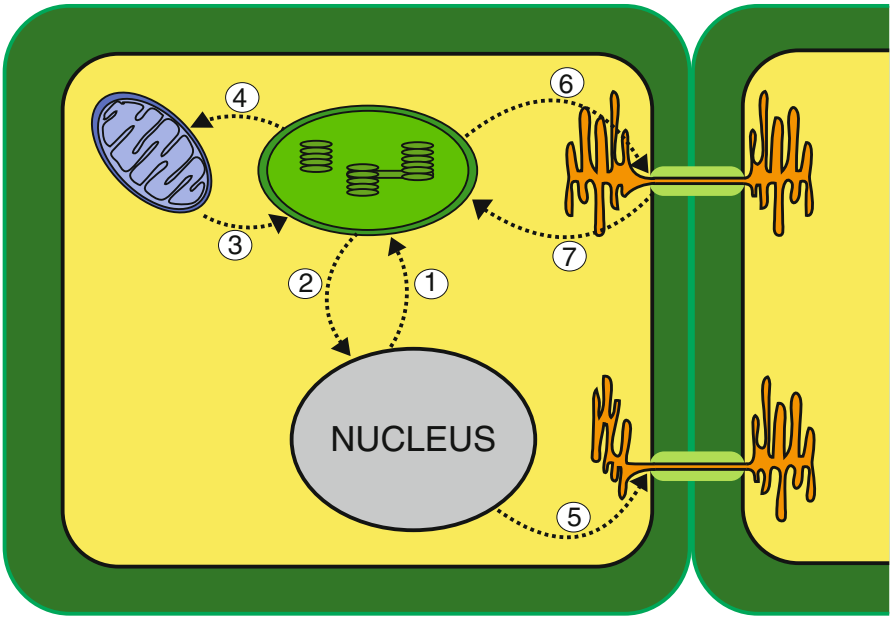

Fig. 4.3 Organelle-nucleus-plasmodesmata signaling. Plasmodesmata function and formation are exceptionally sensitive to intracellular signaling pathways. *Arrows 1–4* indicate previously known signaling pathways, chloroplast and nuclear signaling (*arrows 1 and 2*) and signaling between the chloroplast and mitochondrion (*arrows 3 and 4*). Recent results indicate potential additional signaling processes, such as nucleus-plasmodesmata signaling (*arrow 5*) and chloroplast-plasmodesmata signaling (*arrows 6 and 7*)

(Russin et al. 1996), and there is reduced intercellular trafficking due to accumulation of callose at plasmodesmata at the interface of the bundle sheath (BS) and vascular parenchyma (VP) (Botha et al. 2000). *SXD1* is the maize ortholog of Arabidopsis *VTE1*, encoding a chloroplast tocopherol cyclase required for the production of the antioxidant vitamin E (Porfirova et al. 2002; Provencher et al. 2001). This suggests that redox signals produced by chloroplasts may regulate plasmodesmata. It remains to be determined if there are any changes to plasmodesmal structure/function in *vte1* mutants.

Like the *ise2* and *sxd1* mutants, the *gfp-arrested trafficking (gat) 1* mutant provides support for a relationship between chloroplasts and plasmodesmata. In the *gat* mutants, GFP synthesized in the companion cells of the phloem fails to move through plasmodesmata into the surrounding tissues as in wild-type tissues (Benitez-Alfonso et al. 2009). *gat1, 2, 4,* and *5* mutants are all seedling lethal, with development ceasing about 10 days after germination. *GAT1* encodes a plastid-localized thioredoxin, and *gat1* roots accumulate higher levels of reactive oxygen species (ROS) than wild-type roots. In addition, *gat1* roots accumulate callose at plasmodesmata. Overexpression of *GAT1* results in the opposite phenotype of increased intercellular transport. Thus, like SXD1/VTE1, GAT1 likely functions in redox

homeostasis involving the chloroplasts, and perturbation of plastid redox state results in altered plasmodesmata (see Sect. 4.4.4).

Further evidence for plasmodesmata responding to chloroplast-generated signals derives from analyses of the development of complex plasmodesmata from simple plasmodesmata. Fitzgibbon and coworkers found that accelerating the sink-to-source transition leads to accelerated formation of complex plasmodesmata (Fitzgibbon et al. 2013). One interpretation of these results is that plasmodesmata are responding to leaf carbon balance, such as the supply of photosynthate (sucrose) from source leaves.

Studies of plasmodesmal proteins further reinforce the relationship between chloroplasts and plasmodesmata. It has been long known that transgenic expression of vial MPs leads to reduced photosynthetic carbon assimilation (Lucas et al. 1996). Further, transgenic expression of the tobacco mosaic virus (TMV) movement protein (MP) that targets to PD and promotes local viral cell-to-cell movement leads to increased H_2O_2 accumulation, overaccumulation of SA, and transcriptional induction of SA response and chloroplast ROS-scavenging genes (Conti et al. 2012). Overexpression of the plasmodesmal protein PDLP5 decreases plasmodesmal trafficking (Lee et al. 2011) and leads to chlorosis and overaccumulation of salicylic acid (SA), a phytohormone synthesized in plastids (Wildermuth et al. 2001). Together, these findings imply that chloroplasts are sensitive to alterations in plasmodesmal function. Thus, it appears that not only do chloroplasts regulate plasmodesmata but that chloroplasts in turn are responsive to events at the plasmodesmata (Fig. 4.3).

4.4.4 Reactive Oxygen Species

Reactive oxygen species (ROS) in plant cells include superoxide anion, singlet oxygen, and hydrogen peroxide (reviewed in Foyer and Noctor (2003)). These are generated during the light reactions of photosynthesis in the chloroplasts and by the activity of numerous enzymes. The amount of ROS is controlled by the antioxidative system that acts to prevent unwanted oxidation of cellular components. This antioxidant-buffering capacity utilizing enzymes that regulate the soluble ascorbate and glutathione pools in addition to numerous membrane-associated molecules including tocopherols, carotenoids, and lipoic acids. Redox signaling therefore impacts all aspects of plant biology, particularly development and stress responses. Downstream of the initial ROS-antioxidant interaction, heterotrimeric G-proteins (Joo et al. 2005) and kinases (Gupta and Luan 2003; Kovtun et al. 2000; Rentel et al. 2004) likely mediate the outcomes of redox signaling, including the regulation of nuclear gene expression. Differential ROS production in various cell compartments results in the production of specific redox signals (Foyer and Noctor 2003).

ROS, and by extension redox signaling, have also been strongly implicated in plasmodesmal development and function (reviewed in Benitez-Alfonso et al. (2011)). The first evidence for this connection stems from wheat seedling roots

subjected to anaerobic conditions that leads to increased plasmodesmal SEL allowing the transport of fluorescent tracers of up to 10 kDA, while untreated roots have an SEL less than 1 kDa (Cleland et al. 1994).

In many plant tissues, chloroplasts account for a significant portion of ROS generation, as much as 30 % (reviewed in Foyer and Noctor (2003)). Paradoxically the two plasmodesmata mutants, *gat1* and *ise1*, produce increased amounts of ROS compared with their wild-type counterparts and yet have opposite effects on plasmodesmal transport, decreasing and increasing traffic, respectively (Benitez-Alfonso et al. 2009; Stonebloom et al. 2009). To clarify the role of ROS in regulating intercellular trafficking, careful studies utilizing redox-sensitive GFP reporters coupled to the cellular antioxidant pool of glutathione measured the redox state of various organelles in the mutant backgrounds compared to their wild-type counterparts. These studies unequivocally demonstrated that increased mitochondrial ROS production leads to increased plasmodesmata-mediated intercellular trafficking, whereas increased chloroplast ROS inhibits it (Stonebloom et al. 2012). Further support for the specificity of ROS production having distinct effects on plasmodesmal transport comes from analysis of plants lacking ISE1 or ISE2 (Stonebloom et al. 2012). *ISE1*-silenced *N. benthamiana* plants increase the oxidation state of mitochondria while concomitantly reducing chloroplasts. Likewise, chloroplasts in *ISE2*-silenced leaves are in a reduced or lower redox state. Thus, oxidized mitochondria or reduced chloroplast environments increase plasmodesmal transport. Therefore, the site of ROS production has specific effects on plasmodesmal permeability possibly due to differential activation of downstream signals. In addition, levels of ROS also differentially regulate plasmodesmal function. Low concentrations of hydrogen peroxide (0.6 mM) increase plasmodesmal permeability, whereas tenfold higher concentrations decrease it (Rutschow et al. 2011). Thus, redox signals produced at varying levels by different organelles may be one mechanism used to regulate intercellular trafficking by plasmodesmata.

In support of the role of ROS in regulating plasmodesmata, examination of the plasmodesmal proteome identified several proteins that function in ROS metabolism as putative plasmodesmal components. At least six class III peroxidases were identified (Fernandez-Calvino et al. 2011). Potentially ROS generated by cell wall peroxidases loosens the cell wall to facilitate plasmodesmal development (Liszkay et al. 2003). Reinforcing this idea, class II peroxidases and their substrate hydrogen peroxide (H_2O_2) were detected near plasmodesmata in cambial cell walls that were actively remodeling their plasmodesmal network (Ehlers and van Bel 2010). Thus, plasmodesmata-localized class III peroxidases may regulate the modification of plasmodesmata through their effects on the cell wall. The same proteomic analysis identified multiple thioredoxins as plasmodesmal proteins (Fernandez-Calvino et al. 2011). Another membrane-associated thioredoxin, Trxh9, moves cell to cell (Meng et al. 2010). When expressed in the root endodermis from the

SCARECROW promoter, Trxh9-GFP localizes throughout the entire root. This raises the tantalizing possibility that redox molecules may act as intercellular signals.

4.4.5 Phytohormones

There is increasing evidence that plant hormones are also important regulators of plasmodesmal function. Short-day-induced dormancy in the birch and poplar SAMs is released by the action of gibberellic acid (GA) (Rinne et al. 2001). Indeed, GA_4 induces the expression of the β-1,3-glucanases that degrade callose at the plasmodesmata (Rinne et al. 2011). Further, the role of plasmodesmata in establishing auxin gradients has recently been reported (Han et al. 2014a) (see Sect. 4.3.2.1). These studies also support the existence of an auxin-GSL8-positive feedback circuit that acts to control symplastic connectivity through callose deposition (Han et al. 2014a).

The phytohormone salicylic acid (SA) is typically associated with plant defense responses. However, SA is also in various plant developmental processes including seed germination and flowering and in physiological processes including growth, photosynthesis, and respiration (reviewed in Rivas-San Vicente and Plasencia (2011)). As alluded to earlier, SA can regulate plasmodesmal function. The direct application of SA to Arabidopsis leaves significantly reduces plasmodesmal permeability, as assayed by GFP intercellular movement (Wang et al. 2013). Reduced plasmodesmal flux is accompanied by increased accumulation of callose at PD. Above we discussed the roles of SA and callose in defense responses to various pathogens.

4.5 Modification of Plasmodesmata by Viruses and Viral Proteins

Viruses have evolved to usurp plasmodesmata for their cell-to-cell spread during infection. Intracellular transport of viruses and how they are delivered to plasmodesmata has been discussed in other chapters of this volume. To traffic between cells, viruses encode movement proteins (MPs). MPs target to and specifically interact with plasmodesmata (for reviews see Niehl and Heinlein (2011); Epel (2009); Lucas (2006); Waigmann et al. (2004); Beachy and Heinlein (2000)) to facilitate the intercellular movement of either viral ribonucleotide-protein complexes or intact virions. Both of these cargoes are beyond the SEL of typical plasmodesmata. Here we highlight changes to plasmodesmata that are mediated by viruses and virus-derived proteins to facilitate their transit of plasmodesmata. The emerging picture is that viruses, especially MPs, recruit host proteins to modify plasmodesmata. Table 4.2 summarizes host proteins that modify plasmodesmata during viral

Table 4.2 Host proteins that modify plasmodesmata for viral intercellular trafficking

Protein	Features	Function in viral infection	References
TAG4.4/SAG2.3	Class I β-1,3-glucanase	Removes callose at plasmodesmata	Iglesias and Meins (2000), Beffa et al. (1996)
AtBG_ppap	β-1,3-glucanase, also ER localized	Removes callose at plasmodesmata	Zavaliev et al. (2013)
Class I reversibly glycosylated polypeptides (RGPs)	Plant-specific proteins that self-glycosylate, also Golgi localized	Overexpression leads to callose accumulation at plasmodesmata, loss leads to increased plasmodesmal permeability	Zavaliev et al. (2010), Burch-Smith et al. (2012)
Ankyrin repeat-containing proteins (ANK/TIP1-3)	Multifunctional proteins with functions in chloroplast biogenesis and disease resistance	Bind to PVX and TMV MPs, removes callose at plasmodesmata	Ueki et al. (2010)
Synaptotagmin A (SYTA)	Synaptotagmin family member, calcium and lipid-binding proteins	Binds to CaLCuV and TMV MPs, regulates endocytosis	Lewis and Lazarowitz (2010)
PD CALLOSE-BINDING PROTEIN 1 (PCDB1)	GPI-anchored PM protein, callose-binding ×8 domain faces apoplast	No longer localizes to plasmodesmata during infection, loss of callose accumulation	(Krenz et al. 2012)
PDLP1–PDLP8	Family of eight receptor-like proteins with N-terminal 2xDUF domains in apoplast, small cytoplasmic domain	Interact with GFLV MPs to assist tubule formation	Thomas et al. (2008), Amari et al. (2010)
Remorin	Family of plasma membrane-associated hydrophilic proteins of unknown function that cluster in microdomains	Binds to PVX TGB1, may also directly modify plasmodesmal structure	Raffaele et al. (2009)
Rubisco small subunit (RbcS)	Secretory plant enzymes using H_2O_2 to oxidize secondary metabolites		Zhao et al. (2013)
ATP synthase γ-subunit (AtpC)	Part of the ATP synthesis machinery of chloroplasts	Part of TMV replicase complex, acts to limit viral movement	Bhat et al. (2013)
Rubisco activase (RCA)	Activates Rubisco through formation of carbamate at Rubisco's active site	Part of TMV replicase complex, acts to limit viral movement	Bhat et al. (2013)

trafficking. In addition to altering plasmodesmata, viral proteins themselves can be modified by host proteins for cell-to-cell movement. We direct readers to reviews of this important aspect of viral-plasmodesmata interaction that is outside the scope of this chapter (Lee and Lucas 2001; Niehl and Heinlein 2011).

4.5.1 Callose

The role of callose in virus-plant interactions has long been studied. Early analyses focused on the role of β-1,3-glucanases in viral intercellular movement, and loss of these enzymes results in decreased local cell-to-cell viral spread (Iglesias and Meins 2000; Beffa et al. 1996). Not surprisingly, plants with reduced β-1,3-glucanase activity accumulated callose at plasmodesmata and had reduced plasmodesmal SEL (Iglesias and Meins 2000). Consistent with these findings, overexpression of a β-1,3-glucanase-enhanced viral infection (Bucher et al. 2001). A recent study reinforces the role of plasmodesmata-specific callose in regulating viral intercellular spread. In Arabidopsis, infection by the turnip vein-clearing virus (tobamovirus) strongly induces expression of the pathogenesis-related (PR) β-1,3-glucanases, *AtBG2* and *AtBG3* (Zavaliev et al. 2013). In TMV-infected plants, AtBG2 localized to virus-induced ER bodies that associate with plasmodesmata at the leading edge of infection. While AtBG2 was secreted to the apoplast in response to SA-induced stress, it was not secreted in response to TMV infection. Overexpression of AtBG2 had no effect on TMV intercellular spread, similar to *atbg2* knockout mutants. However, the loss of the plasmodesmata-specific AtBG_ppap led to callose accumulation at plasmodesmata and reduced intercellular spread of TVCV-GFP (Zavaliev et al. 2013).

Ankyrin repeat-containing proteins (ANKs) constitute another class of host proteins that is likely recruited by viruses to gate plasmodesmata and allow intercellular spread. ANKs, also known as TIP1-3, were initially identified through their interactions with the PVX MP (Fridborg et al. 2003). Decreased *ANK* expression by RNAi in tobacco specifically reduced intercellular trafficking of TMV-MP and did not affect cell-to-cell movement of other proteins (Ueki et al. 2010). In support, TMV local spread was delayed in *ANK*-silenced plants, while overexpression of ANK resulted in increased TMV local spread and cell-to-cell spread of TMV-MP. Significantly, although ANK is a cytoplasmic protein, it interacts with TMV-MP at plasmodesmata (Ueki et al. 2010). This ANK-MP interaction is required for removal of callose at plasmodesmata during infection by TMV. Together, these findings suggest that ANK may be a host factor that is uniquely involved in the gating of plasmodesmata by viruses during infection.

4.5.2 Host Secretory System

It has long been known that viruses use host membranes for their replication (see previous chapters in this volume). However, there is increasing evidence that host secretory systems can have crucial roles in viral cell-to-cell movement via

plasmodesmata. While the details are not yet known, it is becoming clear that plant viruses co-opt these endomembranes and transport routes for their own benefit.

4.5.2.1 Endosomes in Viral Protein Transport to Plasmodesmata

The involvement of a synaptotagmin protein, SYTA, in the cell-to-cell spread of two unrelated viruses, cabbage leaf curl virus (CaLCuV) and TMV, shows that the host endocytic machinery may be used for viral intercellular trafficking (Lewis and Lazarowitz 2010). Synaptotagmins are a large family of Ca^{2+} and lipid-binding proteins that function in membrane trafficking. Arabidopsis *SYT1* is essential for plasma membrane repair (Schapire et al. 2008). SYTA binds directly to the MPs of CaLCuV and the related squash leaf curl virus (SqLCV) as well as to TMV-MP, and loss of *SYTA* through knockdown leads to reduced intercellular trafficking of CaLCuV and TMV-MPs (Lewis and Lazarowitz 2010). Further, CaLCuV infection is slowed in the Arabidopsis SYTA knockdown line. SYTA localizes to endosomes and regulates endosome recycling, suggesting that the endocytic pathway is crucial for targeting viral proteins to plasmodesmata. However, it is not clear if the effects on MP movement were caused by their mislocalization, since the authors did not address this question in their study.

4.5.2.2 ER Remodeling

Potato virus X (PVX) encodes three movement proteins in overlapping ORFs called the triple gene block (TGB). Besides the MP, the PVX coat protein (CP) is also required for cell-to-cell movement (Verchot-Lubicz et al. 2010). PVX replication occurs at the ER. TBG2 and TBG3 are integral membrane proteins that localize to ER-derived granules, while TGB1 is a cytoplasmic protein whose localization to plasmodesmata depends on TGB2 and TGB3 (Verchot-Lubicz et al. 2010). A recent study designed to probe the interactions between PVX TGB proteins to mediate movement via plasmodesmata reveals that remodeling the ER membranes at the plasmodesmal orifice is a critical step in the process (Tilsner et al. 2013). This study found that for PVX, both TGB2 and TGB3 target to plasmodesmata and together recruit TGB1 to plasmodesmata (Tilsner et al. 2013). Using state-of-the-art microscopy techniques including 3D-structured illumination microscopy and electron microscopy, the remodeling of the ER near orifices of plasmodesmata at early stages in infection was observed. These plasmodesmata-associated ER structures contain the viral replicase and unencapsidated viral RNA (vRNA) and are therefore likely sites of viral replication. Further, TBG1 directs the CP into the plasmodesmata, but it does so only in the presence of a movement complex, suggesting that vRNA is required for insertion. Together, these results suggest that the processes of viral replication and cell-to-cell movement are linked at the entrances of plasmodesmata. From there, it is likely that interactions between TGB2/TGB3 and the desmotubule allow the transit of the plasmodesmata (Tilsner et al. 2013). Intriguingly, the authors propose that other viruses that move unencapsidated vRNA may use this "coreplicational insertion" strategy to maximize the efficiency of replication and spread.

4.5.2.3 Vesicles and Plasmodesmata

Another recent study highlights the role of the host endomembrane system in intercellular viral transport. Turnip mosaic virus (TuMV) is a potyvirus and as such has no dedicated MP. TuMV replication occurs in mobile vesicles that move along microfilaments (Cotton et al. 2009). These 6 K_2 vesicles, as they are called after the TuMV protein, are derived from earlier perinuclear globular structures that contain ER, Golgi, COPII coatomers, and chloroplasts, in addition to vRNA, and both viral and host proteins (Grangeon et al. 2012). Treatment with latrunculin B to disrupt microfilaments also inhibits intracellular movement of 6 K_2 vesicles and the intercellular movement of TuMV (Cotton et al. 2009). Thus, 6 K_2 vesicles are critical for vRNA movement. Indeed, these 6 K_2 vesicles themselves transit plasmodesmata (Grangeon et al. 2013). Using fluorescently labeled proteins and photoactivation coupled to confocal microscopy, the authors observed movement of the 6 K_2 protein as well as 6 K_2 vesicles containing the viral replicase between *N. benthamiana* cells. Given that the 6 K_2 vesicles localize to the plasma membrane and plasmodesmata, it is assumed that the intercellular movement is via plasmodesmata. Thus, TuMV VRCs likely move cell-to-cell via plasmodesmata, as proposed previously for TMV (Kawakami et al. 2004). However, it is not clear how the SEL of plasmodesmata is altered to accommodate these VRCs or what other changes to PD ultrastructure are necessary to allow passage of the 6 K_2 vesicles.

4.5.3 Plasmodesmal Proteins

4.5.3.1 PDLPs

In addition to their likely roles in development (see Sect. 4.3.3.3), PDLP proteins have also been implicated in viral infection and spread (Amari et al. 2010). Grapevine fan leaf virus (GFLV) belongs to the class of viruses possessing tubule-forming MPs. Coexpression of fluorescently tagged GFLV MP and PDLP1 in *N. benthamiana* demonstrated PDLP1 localization to the cell wall at the base of tubules formed by the MP, the result of a specific and direct interaction (Amari et al. 2010). The same interaction was observed between GFLV MP and all PDLP proteins, suggesting they may all function in tubule formation, a hypothesis that was subsequently confirmed experimentally. Triple mutant *pdlp1/2/3* plants have reduced formation of MP tubules at plasmodesmata and also reduced local and systemic spread of GFLV. Thus, PDLPs act at plasmodesmata to allow the specific assembly of tubules by viruses that use the tubule-forming strategy for intercellular movement.

4.5.3.2 Remorin

Remorin (REM) is another plasmodesmal protein that functions in viral infection. REM was the first protein identified from detergent-insoluble membranes from plants and as such was the first membrane raft protein marker from plants (Simon-Plas et al. 2011). REM is localized to the cytosolic leaflet of the tobacco plasma membrane where it clusters in microdomains (Raffaele et al. 2009). Fluorescently

labeled REM localizes to the PM in a discontinuous pattern representing both these microdomains and plasmodesmata. Consistent with these findings, an Arabidopsis REM family member was identified as part of the plasmodesmal proteome (Fernandez-Calvino et al. 2011). Tomato plants in which REM expression was suppressed by antisense technology accumulate higher levels of PVX compared to wild-type and control plants, while plants overexpressing REM show reduced viral accumulation. These changes in virus accumulation are apparently the result of changes to intercellular spread, as measured by monitoring the size of viral lesions in transgenic plants. Further, REM binds directly to the triple gene block protein 1 (TGBp1) that is a component of the PVX MP (Raffaele et al. 2009). REM has also been found among host rice proteins involved in the life cycle of rice yellow mottle virus (Brizard et al. 2006). Thus, remorin may act at plasmodesmata to regulate both MPs and the structure of the plasmodesmata themselves during viral infection. The role of REM proteins in plant-microbe interactions is under active investigation, and it is becoming apparent that REM functions beyond viral movement (Jarsch and Ott 2011). Indeed, the role of lipids in plasmodesmal function is the focus of renewed interest (Tilsner et al. 2011; Faulkner 2013), and we anticipate important findings regarding the role of lipids in regulating plasmodesmal structure and function.

4.5.3.3 Reversibly Glycosylated Proteins

Proteomic analysis of plasmodesmata of maize mesocotyl cell walls (Kotlizky et al. 1992) identified a 41 kD protein later termed SE-WAP41 (for SALT-EXTRACTABLE WALL-ASSOCIATED PROTEIN 41) (Epel et al. 1996; Sagi et al. 2005). SE-WAP41 was later determined to be a class 1 reversibly glycosylated protein (^{C1}RGP) (Sagi et al. 2005). The Arabidopsis genome encodes four ^{C1}RGPs, and AtRGPS2 is most similar to SE-WAP41. RGPs are Golgi-localized enzymes that have been implicated in cell wall and starch synthesis (Sandhu et al. 2009). However, SE-WAP41 and Arabidopsis RGPs localize to the plasmodesmata when transiently expressed in tobacco leaves, suggesting that the Golgi, in a subset of tissues, specifically delivers them to plasmodesmata. Constitutive expression of AtRGP2 in tobacco transgenics leads to stunting and chlorosis of source leaves (Zavaliev et al. 2010). The chlorosis is apparently the result of starch accumulation and reduced chlorophyll levels. Importantly, these plants accumulated callose at plasmodesmata when compared to controls, and TMV cell-to-cell spread was reduced (Zavaliev et al. 2010). TRV-VIGS of *RGP*s in *N. benthamiana* reveals that reduced expression of these genes leads to increased intercellular diffusion of GFP and increased systemic spread of TMV-GFP, probably due to increased plasmodesmal permeability (Burch-Smith et al. 2012). Together these results support the role of RGPs in regulating plasmodesmal flux during viral infection, likely by modulating levels of callose at plasmodesmata.

4.5.4 Chloroplasts

Two recent studies raise the possibility that chloroplast proteins may also act to modify plasmodesmata to regulate viral intercellular movement. One long-standing

question in plant virology is the identity of host proteins that are constituents of viral replication complexes. In experiments aimed at addressing this question, two nucleus-encoded chloroplast proteins, ATP synthase-γ subunit (AtpC) and Rubisco activase (RCA), were identified as host proteins that are part of the TMV 126/183 kD replicase complex (Bhat et al. 2013). Curiously, infection by TMV and the related tobamovirus TVCV results in significantly decreased mRNA levels of both *AtpC* and *Rca*. Silencing *AtpC* or *Rca* by TRV-VIGS in *N. benthamiana* leads to increased systemic spread of the U1 strain of TMV, revealing enhanced pathogenicity in the silenced plants. Silencing *AtpC* also leads to increased spread of TVCV. Thus, both chloroplast proteins are required to limit tobamovirus spread, but the mechanisms by which they act remain unknown.

Using a different tobamovirus, ToMV, Zhao and coworkers found a role for the small subunit of Rubisco (RbcS) in regulating virus movement, host susceptibility to infection, and *R* gene-mediated resistance (Zhao et al. 2013). The MP of ToMV is the avirulence determinant for the *Tm-22 R* gene. A yeast two-hybrid screen identified RbcS as interacting with ToMV MP, and this interaction was subsequently confirmed by coimmunoprecipitation and pulldown assays. Interestingly, BiFC assays revealed that interaction between the MP and RbcS occurs at plasmodesmata, and there is likely no interaction in the chloroplasts (Zhao et al. 2013). Silencing *RbcS* by TRV-VIGS in *N. benthamiana* leads to increased local susceptibility to both ToMV and TMV but reduces long-distance movement of ToMV. Thus, ToMV MP recruits RbcS to plasmodesmata, and RbcS acts to limit local spread of ToMV but enhance its systemic spread. However, again it is unknown how a chloroplast protein might modify plasmodesmata. In both these studies, physiological changes related to chloroplast function were observed in addition to effects on viral infection and spread. Thus, chloroplast proteins may not themselves directly affect plasmodesmata but rather the changes in plasmodesmata are a consequence of ONPS (see above and Fig. 4.3) due to perturbed chloroplasts and the consequent effects on cellular physiology.

Another intriguing possibility that chloroplasts and chloroplast proteins may modify plasmodesmata during virus infections derves from observations of stromules during infection by Abutilon mosaic geminivirus (AbMV) (Krenz et al. 2012). Stromules (for stroma-filled tubules; Kohler and Hanson 2000) are highly dynamic protrusions of the plastid membrane envelope that likely function in intracellular molecular exchange, signal transduction, and transport (Hanson and Sattarzadeh 2011). In uninfected plants, oligomers of the chloroplast chaperone cpHSC70 are predominantly found in foci in chloroplasts and to a lesser degree in small filaments extending to the cell periphery (Krenz et al. 2010). Upon infection, a network of cpHSC70-labeled stromules proliferates (Krenz et al. 2010) that extends toward the nucleus and the cell periphery, most likely to plasmodesmata (Krenz et al. 2012). Interestingly, in plants infected with AbMV, PDCB1 no longer resides in foci at plasmodesmata but is instead dispersed in the membrane (Krenz et al. 2012). The authors hypothesize that the mobilization of PDCB1 is due to the loss of callose at plasmodesmata, facilitating intercellular trafficking during infection. It will be interesting to see if other viruses also use stromules for infection and intercellular spread. This may be a more widespread strategy for recruiting soluble

chloroplast proteins to sites for replication and then for modifying plasmodesmata for their intercellular movement.

4.6 Conclusions

Clearly plasmodesmata play important roles during plant development, and plant viruses have been extensively utilized to probe plasmodesmata structure and function. Until recently most studies have been focused "at" plasmodesmata. But we need to shift our focus to include monitoring of whole-cell homeostasis in having a significant impact on plasmodesmata, best exemplified by recent evidence that plasmodesmata and chloroplast physiology are intimately linked. Until now very few labs study plasmodesmata, but this situation will undoubtedly change dramatically given the roles of plasmodesmata in so many critical plant processes.

We still know very little about how plasmodesmata form either during or post cell division. We are largely dependent on *still* images afforded by TEM. Higher-resolution imaging strategies such as 3D electron tomography and 3D-structured illumination microscopy will help to illuminate plasmodesmata structure and de novo formation. Imaging technology will likely improve to where we can start to watch plasmodesmata in action via live cell imaging. Indeed, visualization of plasmodesmata dynamics during different developmental stages and during cellular stress is an exciting prospect.

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Plant Defence and Viral Interference

5

Heiko Ziebell

Contents

Abstract

Plants face attack by a number of pathogens, pests and abiotic stresses. Due to their sessile nature, they cannot flee but have to fight their attackers. They have developed numerous mechanisms to fight off pathogens such as viruses. Some of these are genetically determined by resistance genes, coding for factors that might be required for the replication of a virus (recessive resistance genes) or that trigger an active defence via the hypersensitive response to restrict the invading attacker (such as dominant resistance genes). Additionally, adaptive responses such as induced resistance or RNA silencing are further obstacles that might prevent successful infection of a host. In return, viruses have developed several countermeasure strategies in order to infect plants successfully. Some of these strategies are presented in this review.

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5.1 Introduction

Due to their sessile nature, plants are bound to their location and cannot actively evade attacks by pests and pathogens. However, despite the diverse nature of the attackers, plants have developed numerous mechanisms to fight the attacks by, for example, viruses. The defence responses can be pre-existing ('basal') and thus limit the ability of viruses to replicate or spread in a host plant (Carr et al. 2010). Pathogen attack, stresses and chemicals can also induce defence responses that are not present in non-attacked plants ('induced resistance', IR). These responses can often also inhibit or prevent infection of plants by viruses. However, in recent years, it has become apparent that there is no clear cut between basal and IR; many of the signalling pathways are overlapping and also influenced by RNA silencing (Pooggin, this volume, Chap. 6). In addition, viral proteins are often multifunctional and interfere with different host signalling pathways or defence mechanisms. This chapter gives an overview of plant defence mechanisms against viruses. It also addresses how viruses circumvent the defences that plants have put up in place to prevent infection.

5.2 Definitions of Different Resistance Types

Hull (2014) defines in detail the different host reactions towards virus infection. He refers to plants in which a virus cannot replicate (neither in protoplasts nor in plant cells) as immune (non-host resistance). In infectible plants ('hosts'), viruses can infect and replicate in protoplasts. Plants that react upon infection with extreme resistance (ER; see Table 5.1 for list of abbreviations) prevent either multiplication of the virus or restrict replication to the vicinity of the initially infected cell. Resistant plants allow infection of a small zone surrounding the initially infected cell, and necrotic lesions may develop. Susceptible plants allow systemic movement and replication of the virus (Hull 2014; Palukaitis and Carr 2008; Bruening 2006).

5.2.1 Genetic Resistance

Genetic resistance targeting viruses or their vectors is seen as the most effective way to prevent virus infections (Hull 2014; Carr et al. 2010; Maule et al. 2007; Kang et al. 2005). Resistance genes can be used for natural breeding or can be transferred into crop plants by genetic engineering (Hull 2014; Thompson and Tepfer 2010; Gottula and Fuchs 2009; Reddy et al. 2009). Numerous single dominant resistance genes, single recessive genes or multiple genes are currently known to confer resistance to plant virus infection (Maule et al. 2007; Kang et al. 2005). Interestingly, the underlying mechanism is often closely linked to the mode of inheritance; dominant resistance genes are typically involved in active defence by activation of a hypersensitive response (HR), whereas recessive resistance genes correspond to loss or mutations of host factors required to complete the virus' life cycle (Robaglia and Caranta 2006).

Table 5.1 List of abbreviations

Abbreviation	Definition
ADK	Adenosine kinase
AGO1	Argonaute protein 1
AGO4	Argonaute protein 4
ARC	Domain present in apoptotic protease-activating factor-1, R-proteins and *Caenorhabditis elegans* death-4 protein
ARL8	GTP-binding protein in Nicotiana tabacum
ATAF2	Transcription factor in plants
Avr	Avirulence gene
BCTV	Beet severe curly top virus
BED	BED finger domain named after BEAF/DREF proteins of *Drosophila melanogaster*
C4/AC4	C4 protein of begomoviruses
CaMV	Cauliflower mosaic virus
CC	Coiled coil
CMV	Cucumber mosaic virus
COI1	Coronatine-insensitive protein 1
CP	Coat protein
CP/AV1	Coat protein of begomoviruses
DCL2	Dicer-like endoribonuclease 2
DCL3	Dicer-like endoribonuclease 3
DCL4	Dicer-like endoribonuclease 4
DELLA	Repressors of gibberellic acid-mediated responses
eIF4	Eukaryotic initiation factor 4
EIL1	Ethylene insensitive-3-like 1 protein
EIN3	Ethylene insensitive 3; transcription factor
ER	Extreme resistance
ERF	Transcription factor in plants
ETI	Effector-triggered immunity
GFG	Gene-for-gene
GFP	Green fluorescent protein
GRIK	Geminivirus Rep-interacting kinase
HR	Hypersensitive response
IAA	Indole acetic acid IR Induced resistance
JA	Jasmonic acid
JAZ	Repressor protein
LAR	Localised acquired resistance
LIMYB	L10-interacting MYB domain-containing protein
LMV	Lettuce mosaic virus
LRR-RLK	Leucine-rich repeat receptor-like kinase
Meprin	Mammalian tissue-specific metalloendopeptidase
Me-SA	Methyl salicylate

(continued)

Table 5.1 (continued)

Abbreviation	Definition
miRNA	MicroRNA
MP	MP Movement protein
MP/AV2	Movement protein of begomoviruses *on virion-sense strand*
MP/BC1	Movement protein of begomoviruses *on complementary-sense strand*
MYB	DNA-binding domain
MYC2	Transcription factor in plants
NAC	Domain found in NAC protein family
NBS-LRR	Nucleotide-binding site-leucine-rich repeat
N gene	Resistance gene of *Nicotiana glutinosa*
NIK	NSP-interacting kinase
NPR1	Transcriptional cofactor of SA-dependent genes
NPR3	NPR1-homolog
NPR4	NPR1-homolog
NSP/BV1	Nuclear shuttle protein of begomoviruses
PAMP	Pathogen-associated molecular pattern
PAP1	Transcription factor in plants
PPV	Plum pox virus
PR proteins	Pathogenesis-related proteins
PTI	Pathogen-associated molecular patterns-triggered immunity
PVX	Potato virus X
QM	Eukaryotic transcription factor
R	(Dominant) resistance gene
RDR1	RNA-dependent RNA polymerase 1
RDR4	RNA-dependent RNA polymerase 4
RDR6	RNA-dependent RNA polymerase 6
RdRP	RNA-dependent RNA polymerase
REn/AC3	Replication enhancer protein of begomoviruses
Rep/AC1	Replication-associated protein of begomoviruses
RNA	Ribonucleic acid
RPL10	Ribosomal protein 10
RTM1	Resistance gene in *Arabidopsis thaliana*
RTM2	Resistance gene in *Arabidopsis thaliana*
RTM3	Resistance gene in *Arabidopsis thaliana*
Rx-gene	Resistance gene of *Solanum tuberosum*
SA	Salicylic acid
SAM	*S*-adenosyl methionine
SAR	Systemic acquired resistance
SD	Solanaceous domain
siRNA	Small interfering RNA
SNF1	Sucrose non-fermenting 1
TEV	Tobacco etch virus
TGA	Transcription factor in *Arabidopsis thaliana*
TIM	Triosephosphate isomerase

(continued)

Table 5.1 (continued)

Abbreviation	Definition
TIR	Toll/interleukin-1 receptor
Tm-1/Tm-2	Resistance gene of Solanum lycopersicum
Tm-2²	Resistance gene of Solanum lycopersicum
TMV	Tobacco mosaic virus
TNV	Tobacco necrosis virus
TOM1	Transmembrane protein in Arabidopsis thaliana
ToMV	Tomato mosaic virus
ToRSV	Tomato ringspot virus
TPL	Negative co-regulator of jasmonate responses
TRAF	Protein interacting human tumour necrosis factor receptor
TrAP/AC2	Transcriptional activator protein of begomoviruses
TRSV	Tobacco ringspot virus
TYLCCV	Tomato yellow leaf curl China virus
VPg	Viral protein genome-linked
VSR	Viral suppressor of RNA silencing
WRKY	Transcription factor family

5.2.1.1 Recessive Resistance

Plant viruses depend on host factors for replication and production of viral gene products. Hence, the absence of suitable host factors or a mutation of them may lead to resistance to these viruses. This type of resistance is often conferred by recessive resistance alleles (Truniger and Aranda 2009). Most of these recessive genes encode for variants of the eukaryotic initiation factor (eIF) 4, a protein that binds to the m^7G cap structure present at the 5′ end of most eukaryotic messenger RNAs during initiation of translation (Ruffel et al. 2002, 2004, 2006; reviews by Truniger and Aranda 2009; Robaglia and Caranta 2006; Moury et al. 2014). Potyviruses possess a viral protein genome-linked (VPg) at the 5′ end of the genomic RNA that replaces the cap structure of mRNAs (Moury et al. 2014). Binding of the VPg to eIF4 is therefore required for viral replication; absence or alterations of eIF4E or eIF4G can lead to resistance to viruses (Moury et al. 2014; Astier et al. 2007). This form of recessive resistance accounts for almost half of all known resistance genes (Kang et al. 2005; Truniger and Aranda 2009), demonstrating the importance of this recessive trait for plant virus resistance compared to other plant-pathogen systems where resistance traits are predominantly available as monogenic dominant traits (Fraser 1990; Truniger and Aranda 2009). For in-depth information including examples for recessive resistance genes against plant viruses, the reader is referred to the reviews by Truniger and Aranda (2009), Diaz-Pendon et al. (2004), Robaglia and Caranta (2006), Nicaise (2014), Moury et al. (2014), Le Gall et al. (2011), Sanfaçon (2015), to name but a few.

5.2.1.2 Dominant Resistance

About 50 % of known resistance genes are dominant and monogenic (Hull 2014). Control of virus infection is achieved by either triggering a hypersensitive response or by extreme resistance in which the virus is limited to only the infected cell or a few cells surrounding the initially infected one (Palukaitis and Carr 2008;

Maule et al. 2007; Hull 2014; Astier et al. 2007). The majority of dominant resistance genes belong to the class of nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins (Moffett 2009; Nicaise 2014). Interestingly, NBS-LRR proteins are very similar in structure yet can recognise very different and diverse pathogens (Moffett 2009). Many of these genes conferring resistance to viruses, fungi, bacteria, oomycetes, insects and nematodes have been cloned and described in-depth (Sacco and Moffett 2009). The NBS and LRR domains are common to all NBS-LRR proteins, yet they differ, for example, in the conserved ARC domain located between the NBS and LRR domain as well as in the N-terminal domains. The N-terminus can harbour a Toll and interleukin-1 receptor (TIR) homology domain, a coiled coil (CC) domain which is often coupled with additional domains such as a solanaceous domain (SD) or a BED DNA-binding domain (Moffett 2009; Rairdan et al. 2008). However, in some poplar proteins, the CC domain is replaced by the BED domain, and many NBS-LRR proteins of both TIR and CC classes lack any domain N-terminal to the NB domain (Moffett 2009; Kohler et al. 2008). The LRR domain appears to be the primary determinant of recognition specificity (Rairdan and Moffett 2006; Shen et al. 2003; Qu et al. 2006; Collier and Moffett 2009). For more details, the reader is referred to the reviews by Moffett (2009), Sacco and Moffett (2009), Hurley et al. (2014) and Bonardi et al. (2012). Many dominant resistance genes have been described in great detail, such as the *N* gene of *Nicotiana glutinosa* (Whitham et al. 1994), the *Rx* gene of potato (Bendahmane et al. 1999) or the *Tm-2* and *TM2*^{2}genes of tomato (Lanfermeijer et al. 2003, 2004, 2005). Details on these resistance genes can be found in several other reviews (Moffett 2009; Marathe et al. 2002).

5.2.1.3 Models of Interaction

Different working models have been proposed to explain the putative interactions between a pathogen and resistance genes. One of the first models proposed was the gene-for-gene (GFG) model by (Flor 1971), now commonly referred to effector-triggered immunity (ETI). In this model, complementary pairs of dominant genes are required in both the pathogen and the host. The loss or alteration in the host resistance (*R*) or in the pathogen's avirulence (*Avr*) gene would lead to susceptibility and disease of the host (Hull 2014). The interaction of the gene products leads to signal cascades on both the local (restricting the pathogen to its initial infection site, e.g. through HR reactions) and systemic levels (priming other parts of the host for defences, e.g. through systemic HR reactions). Fraile and Garcia-Arenal (2010) discussed the matching allele model. In this model, a match between the host and parasite genes would be required so that recognition of the pathogen would lead to susceptibility and not resistance as in the GFG model. This model would fit some plant-pathogen interactions that could not sufficiently be explained by the GFG model, such as some instances of recessive resistance (Fraile and Garcia-Arenal 2010).

Initially, it was suggested that NBS-LRR and Avr proteins would undergo receptor-ligand interactions. Early attempts to prove this idea failed. Therefore, two other models were used to explain recognition by NBS-LRR: the guard hypothesis

and the decoy model. In the guard model, it is thought that NBS-LRR proteins 'guard' host proteins, the 'guardee' proteins (Jones and Dangl 2006; Moffett 2009). If a pathogen interacts with guardee proteins, alterations to these proteins are perceived by the plants rather than direct detection of the pathogen itself. This perception would start the defence signalling cascade. van der Hoorn and Kamoun (2008) proposed the decoy model. They argued that guardee proteins would be evolutionary unstable in populations polymorphic for *R*-genes. Depending on presence or absence of the guarding proteins, the guardee proteins would either be improved for Avr interaction and detection or evade pathogen effectors to reduce pathogen virulence (van der Hoorn and Kamoun 2008; Moffett 2009). Those conditions would favour decoys interacting with pathogen Avr proteins to facilitate R protein recognition but would not be true virulence targets (Zipfel and Rathjen 2008; van der Hoorn and Kamoun 2008). This means that decoy proteins can be neutral in terms of pathogen virulence, but if they interact with the same effector molecules as the actual virulence targets, this will allow them to act as molecular sensor of pathogen virulence activity as Moffett (2009) describes.

A number of proteins have been identified that interact with Avr and NBS-LRR proteins to facilitate recognition (reviewed by Moffett 2009). However, as no interaction of these cofactors with the LRR domain but instead interaction with the N-terminal domains could be observed, Moffett and colleagues therefore suggested to extend the models by a 'bait-and-switch' model (Collier and Moffett 2009; Moffett 2009). They proposed that not only the LRR domain would play an important role in pathogen recognition but additionally the N-terminal domain (CC, SD and TIR domains). The N-terminal domains could act as molecular baits for the primary interaction with Avr proteins. They proposed that these baits could interact with either guardee or decoy proteins (Collier and Moffett 2009; Moffett 2009).

5.2.1.4 Non-NBS-LRR Resistances Genes

Not all dominant resistance genes encode proteins that belong to the LRR-NBS class and activate defence signalling leading to ER, HR, localised acquired resistance (LAR) and systemic acquired resistance (SAR). For example, the *Tm*-1 gene of tomato encodes a protein that inhibits replication of tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV) in tomato plants and in protoplasts (Fraser and Loughlin 1980; Fraser et al. 1980; Watanabe et al. 1987; Motoyoshi and Oshima 1977; Ishibashi et al. 2007). The TM-1 protein binds to viral replication proteins of ToMV probably after formation of a pre-membrane-targeting complex but not to replication proteins of a resistance breaking strain (Ishibashi and Ishikawa 2013; Ishibashi et al. 2007). The protein is not related to any functionally characterised proteins, but a TIM barrel structure was predicted that is present in a range of enzymes (Ishibashi et al. 2007). However, truncated proteins at the C-terminus still display inhibition activities (Ishibashi et al. 2014; Kato et al. 2013a). Recent studies showed a positive selection of a small region of the TM-1 protein responsible for inhibition of replication (Ishibashi et al. 2012). Ishibashi and Ishikawa (2014) proposed that formation of ToMV replication complexes occurs in the following order: (1) production of replication proteins by translation of viral RNA,

(2) formation of the pre-membrane-targeting complex through binding of the replication proteins to the viral RNA, (3) binding of the complex to membranes, (4) binding of replication proteins to membranes and to transmembrane proteins (TOM1 and ARL8) and (5) initiation of RNA synthesis (Ishibashi and Ishikawa 2014). TM-1 is thought to bind the replication proteins after step 2, does not affect step 3 but can inhibit step 4 (Ishibashi and Ishikawa 2014). Although some structural analyses of TM-1 have been carried out (Ishibashi et al. 2014; Kato et al. 2013b), the exact mechanism and order of binding events remains elusive (Ishibashi and Ishikawa 2014).

Other examples of dominant, non-NBS-LRR resistance genes are the *RTM1*, *RTM2* and *RTM3* genes of certain *Arabidopsis thaliana* ecotypes; where these resistance genes were present, the systemic spread of tobacco etch virus (TEV) was inhibited (Mahajan et al. 1998). It was later found that RTM-mediated resistance was also effective against other *Potyviruses* such as lettuce mosaic virus (LMV) (Revers et al. 2003) and plum pox virus (PPV) (Decroocq et al. 2006). *RTM1* encodes a lectin-like protein (Chisholm et al. 2000), *RTM2* a multidomain protein containing a region similar to plant small heat shock proteins (Whitham et al. 2000) and *RTM3* a protein with meprin and TRAF homology domains as well as a CC domain at the C-terminal end (Cosson et al. 2010). Restriction of movement mediated by RTM proteins is specific to TEV and does not involve HR or SAR (Mahajan et al. 1998; Whitham et al. 2000). Translational fusion of RTM1 and RTM2 with green fluorescent protein (GFP) showed expression in sieve elements of *A. thaliana* plants (Chisholm et al. 2001). Despite an in-depth analysis of natural variation of *RTM* genes, the putative role of each of the RTM protein domains in terms of resistance is still poorly understood (Cosson et al. 2012). Self-interaction of RTM3 and an interaction between RTM1 and RTM3 were confirmed (Cosson et al. 2010), but the interaction of RTM3 and the potyviral coat protein (CP) could not be shown (Cosson et al. 2010). This is interesting as the CP was the determinant to overcome RTM-mediated resistance (Decroocq et al. 2009). In *A. thaliana*, loss of resistance was attributed to non-functionality of one or more alleles associated with amino acid changes in the RTM protein but not changes in *RTM* gene expression (Cosson et al. 2012).

5.2.2 Induced Resistance

Pathogen attack, abiotic stresses or the application of chemicals can stimulate plant resistance responses in non-invaded tissue. The general term for this is known as 'induced resistance' (Gilliland et al. 2006; Hull 2014; Loebenstein 2009; Carr et al. 2010). These reactions might be localised to the site of entrance or affect different parts of the plants that have not been infected or treated. Interestingly, the reactions might also restrict not only the inducing agent itself but they might also be effective against other microbes and viruses (Hammerschmidt 2009). It has now been more than 50 years that Ross initially described the phenomenon of LAR and SAR (Ross 1961a, b).

5.2.2.1 Localised Acquired Resistance and Hypersensitive Response

Ross initially demonstrated that infection of resistant tobacco cultivars containing the *N* gene with TMV led to HR in the inoculated area, clearly visible as local lesions (Ross 1961a). When those leaves were challenged again with TMV, new lesions would only develop in areas outside a zone surrounding the initial lesions; on these few occasions, he could observe newly forming lesions; they were smaller in size (Ross 1961a). Interestingly, the zones resistant to secondary infection were also resistant to other viruses such as tobacco necrosis virus (TNV), tobacco ringspot virus (TRSV) or tomato ringspot virus (ToRSV) (Ross 1961a). Ross (1961a) termed this observation 'localised acquired resistance'. Interestingly, the observed resistance to secondary inoculation was not restricted to the inoculated leaves but also occurred in the upper, non-inoculated leaves; the phenomenon was called SAR (Ross 1961b). Loebenstein (2009) and Loebenstein and Akad (2006) give excellent reviews on local lesions and signal transduction pathways involved in these responses.

5.2.2.2 Systemic Acquired Resistance and Salicylic Acid-Dependent Signalling

Ross made another important observation. As mentioned before, he observed IR in non-inoculated parts of the plants upon challenge inoculation (Ross 1961b). Again, SAR was not restricted to TMV but also worked with other viruses. As has been demonstrated later, induction of SAR often requires a specific host-pathogen interaction, but the induced responses can be highly efficient against a range of other microbes and viruses (see Loebenstein 2009, Carr et al. 2010, Hammerschmidt 2009). One example is the interaction between TMV and the *N* gene in tobacco: TMV infection inhibits the invasion of the plant by other viruses, bacteria, fungi and oomycetes (Carr et al. 2010; Palukaitis and Carr 2008; Lewsey et al. 2009). But how can infection with one pathogen lead to resistance of the plant to other pathogens?

A central role in SAR is occupied by salicylic acid (SA; 2-hydroxybenzoic acid). SA is an important plant hormone involved in regulating physiological functions such as seed germination, photosynthesis and flowering as well as thermogenesis, responses to abiotic stress (heat, cold, drought, salinity) and in particular disease resistance; in-depth reviews are provided by Vlot et al. (2009), Banday and Nandi (2015), Rivas-San Vicente and Plasencia (2011), Boatwright and Pajerowska-Mukhtar (2013), Dempsey et al. (2011), Fu and Dong (2013), Shah and Zeier (2013), Shah et al. (2014) and Spoel and Dong (2012).

The role of SA in SAR has been the focus of research for many years. Although it was known for a long time that SA is involved in plant resistance (White et al. 1983; White 1979), its exact role in antiviral defence is still not fully understood, despite a lot progress in recent years (Boatwright and Pajerowska-Mukhtar 2013; Yan and Dong 2014; Carr et al. 2010). SA has a central role in the signal transduction pathway leading to SAR (Carr et al. 2010; Hammerschmidt 2009; Alvarez 2000). In particular, gene expression and subsequent synthesis of 'pathogenesis-related proteins' (PR proteins) as well as chitinases and glucanases are induced by SA (Ward et al. 1991). A mobile signal is

required from the inoculated leaves that induces resistance in non-inoculated parts of the plants. For a long time, SA was the candidate component, but recent studies suggest methyl salicylate (Me-SA) is the mobile signal (Park et al. 2007; Vlot et al. 2009, 2008a, b; Manosalva et al. 2010; Park et al. 2009; Zhu et al. 2014; Vernooij et al. 1994). Interestingly, Me-SA can also act as airborne signal to prime neighbouring plants against pathogen attack (Koo et al. 2007; Shulaev et al. 1997). Despite intensive studies, the exact nature of SA/methyl-SA signalling remains unclear.

It has been shown that NPR1, a transcriptional cofactor of SA-dependent genes, is involved in regulating SA-induced immune responses (reviews by Fu and Dong 2013 and Spoel and Dong 2012). NPR1 and the NPR1-homologs NPR3 and NPR4 are supposed to be SA receptors with different functions (Caarls et al. 2015; Fu et al. 2012; Wu et al. 2012; Pajerowska-Mukhtar et al. 2013). NPR1 also interacts with TGA transcription factors leading to the co-activation of SA-induced gene transcription. In particular, SA markers such as *PR* as well as *WRKY* transcription factor genes are activated (Wang et al. 2006; Eulgem and Somssich 2007). Other proteins with SA-binding properties were also described although their exact functions remain unclear (see the reviews by Kumar 2014; Yan and Dong 2014). A number of novel SA-binding proteins were identified recently that further suggests that a large number of different proteins are influenced by the presence of SA (Manohar et al. 2015). Additionally, the SA-binding properties of NPR1 were confirmed in that study (Manohar et al. 2015).

5.2.2.3 Jasmonic Acid-Dependent Signalling

Besides SA-mediated defence, jasmonic acid (JA) is another important substance involved in plant defence responses. Whereas SA-mediated defence responses are mainly induced by biotrophic pathogens and some phloem-feeding insects, chewing herbivores and necrotrophic pathogens induce JA-mediated defence responses (Stout et al. 2006; Thaler et al. 2012). Nevertheless, both pathways are interconnected and show a high degree of crosstalk (Caarls et al. 2015; Thaler et al. 2002a, b, 2012).

Major players in the JA-signalling network are JAZ repressor proteins and the F-box protein COI1 (Ballare 2011). In the absence of JA, JAZ repressor proteins associate with the corepressor TPL via adaptor proteins and repress transcription factors such as MYC2, EIN3 and EIL1 (see review by Song et al. 2014). A complex of COI1 and a bioactive form of JA target JAZ repressors for degradation and successively release transcriptional activators. This leads to the activation of JA-responsive genes. Two major signalling pathways are known: the MYC branch, co-regulated by JA and abscisic acid, and the ERF branch, co-regulated by JA and ethylene (see Caarls et al. (2015) for a detailed overview). However, it appears that SA regulates the JA-responsive pathway negatively at the level of gene transcription (van der Does et al. 2013; Spoel et al. 2003); see Caarls et al. (2015) for an in-depth review of SA- and JA-regulated responses in the model plant *A thaliana* as well as other reviews on this topic (Gimenez-Ibanez and Solano 2013; Thaler et al. 2012; Koornneef and Pieterse 2008).

5.2.2.4 RNA Silencing

Another mechanism in plants acting against viral attack is RNA silencing. RNA silencing (or RNAi) is thought to be an ancient antiviral defence mechanism that can be activated by the presence of double-stranded RNA intermediates of viral replication, hairpin structures or aberrant RNAs (Poogin, Chap. 6). The mechanism of RNA silencing and the roles of small RNAs in virus-host interactions are discussed by Pooggin in Chap. 6. There is growing evidence that ETI and pathogen-associated molecular patterns-triggered immunity (PTI) responses are regulated by the small RNA machinery as well as RNA silencing contributing to plant defence against non-viral pathogens (reviewed by Zvereva and Pooggin (2012); Poogin Chap. 6). Several lines of evidence suggest that RNA silencing and other defensive pathways in plants are overlapping and/or interconnected.

RNA-Dependent RNA Polymerases

RNA-dependent RNA polymerases (RdRps) are crucial for the amplification of small interfering RNAs (siRNAs) such as trans-acting siRNAs, secondary-phased siRNAs and heterochromatic siRNAs during RNA silencing and the *de novo* synthesis of double-stranded RNAs (Pooggin, Chap. 6). However, before the roles of RdRps in RNA silencing were discovered, it was noted that virus infection can induce *RdRp* gene expression (Carr et al. 2010). Until now, six RdRps are known in *A. thaliana*, and orthologs have been found in *Nicotiana benthamiana*, *N. glutinosa*, *N. tabacum* and *Medicago truncatula* (Carr et al. 2010; Yang et al. 2004; Yu et al. 2003a; Dalmay et al. 2000; Liu et al. 2009). SA application to plants can also induce *RdRp* expression and activity (Xie et al. 2001), whereas RDR1 in *N. attenuata* plays a role in regulation of JA-responsive genes and its biosynthesis (Pandey and Baldwin 2007). RDR1 of *N. tabacum* is also linked to defence-related genes that are induced by virus infection (Rakhshandehroo et al. 2009).

Viral Suppressors of RNA Silencing and SA Signalling

Several viral suppressors of RNA silencing also interfere with SA-mediated response. In particular, the 2b protein of cucumber mosaic virus (CMV) and the P6 protein of cauliflower mosaic virus (CaMV) are the best-characterised silencing suppressors that interfere with SA signalling (see below). However, also the P1/HCPro silencing suppressor from potyviruses can interfere with SA signalling (Alamillo et al. 2006).

micro RNA Regulation of Resistance Genes

It appears that many *NB-LRR* genes are regulated by microRNAs (miRNAs) as in the case of *M. truncatula* (Zhai et al. 2011). This was also shown in tobacco for the *N*-immune receptor conferring resistance to TMV and confirmed for other NB-LRR genes in solanaceous species (Li et al. 2012; Shivaprasad et al. 2012). In *A. thaliana*, the action of RDR6 and miRNA miR472 controls post-transcriptional expression of *NB-LRR* genes (Boccara et al. 2014). Bhattacharjee et al. (2009) showed that co-expression of a plant NB-LRR protein with its elicitor induced an antiviral response inhibiting translation of viral proteins. The inhibited translation of viral transcripts

was AGO4-dependent. A review on the role of miRNAs and siRNAs in plant immunity was recently published by Katiyar-Agarwal and Jin (2010).

JA and Silencing

The JA-signalling pathways are also influenced by RNA silencing directly or indirectly via interaction with viral silencing suppressors (Pandey and Baldwin 2007; Pandey et al. 2008; Westwood et al. 2014; Lewsey et al. 2010a; Ziebell et al. 2011; Yang et al. 2008); for more details, see the following sections.

5.3 Viral Interference with Defence Signalling

The suppression of RNA silencing by viral proteins has been shown for many plant viruses and was the topic of many reviews. Very little, however, has been published on the suppression of other plant defence responses by viruses. A (nonexclusive) summary of viral proteins interfering with plant defence mechanisms is shown in Table 5.2. Some examples are discussed in more detail in the following paragraph.

5.3.1 Cauliflower Mosaic Virus

Cauliflower mosaic virus (CaMV) is the type member of the *Caulimoviridae* family belonging to the group of pararetroviruses. It has a double-stranded, circular DNA genome, replicating by reverse transcription (Geering and Hull 2012). Its host range is limited to crucifers, such as brassicas and *A. thaliana* (Haas et al. 2002). CaMV infection induces gene expression of SA-, ethylene/JA- as well as reactive oxygen species-responsive genes indicating the involvement of defence-related signalling pathways upon CaMV infection (Love et al. 2005; Callaway et al. 1996). Consistently, *A. thaliana* mutants with constitutively enhanced SA-responsive pathways show enhanced resistance to CaMV (Love et al. 2007b). However, mutants with a defect in SA accumulation did not show enhanced susceptibility to CaMV nor did exogenous application of SA show an effect on CaMV accumulation indicating that SA is not required for antiviral defence (Love et al. 2005, 2007b).

It has been shown that gene VI of CaMV encodes the multifunctional P6 protein (Haas et al. 2002). P6 is involved in nuclear-cytoplasmic shuttling, virus replication; it acts as translational activator, and through association with actin filaments, it is involved in cell-to-cell movement of the virus; additionally, it is a pathogenicity and avirulence determinant as well as a component of the viroplasm (Schoelz and Shepherd 1988; Broglio 1995; Daubert and Routh 1990; Wintermantel et al. 1993; Yu et al. 2003b; Haas et al. 2002, 2005; Ryabova et al. 2002; Harries et al. 2009; Hapiak et al. 2008; Hohn and Futterer 1997; Covey et al. 2000; Cawly et al. 2005; Palanichelvam and Schoelz 2002; Zvereva and Pooggin 2012; Palanichelvam et al. 2000; Angel et al. 2013; Rodriguez et al. 2014a). The avirulence domain has been located at the N-terminal end of P6 (Palanichelvam and Schoelz 2002; Agama et al. 2002; Kobayashi and Hohn 2004). As with many other plant viruses, it has been

Table 5.2 Viral proteins interfering with plant defence responses

Virus species	Genus	Viral interaction partner	Gene function	Plant interaction partner	Reference
Cucumber mosaic virus	*Cucumovirus*	2b	Pathogenicity factor, silencing suppressor	Interference with salicylic acid-mediated responses, jasmonic acid-mediated responses and abscisic acid-mediated responses; indirect mediation of aphid transmission	Ji and Ding (2001), Lewsey et al. (2010a), Zhou et al. (2014), Westwood et al. (2013a, b, 2014), Lewsey et al. (2010b), Ziebell et al. (2011)
Tobacco etch virus	*Potyvirus*	HC-Pro	Silencing suppressor, aphid transmission	Interaction with RAV2 transcription factor; altering salicylic acid-mediated responses	Endres et al. (2010), Alamillo et al. (2006)
Potato virus Y	*Potyvirus*	HC-Pro	Silencing suppressor, aphid transmission	Changes in gene expression: in particular defence-related and hormone-responsive genes	Kovač et al. (2009), Soitamo et al. (2011)
Plum pox virus	*Potyvirus*	HC-Pro	Silencing suppressor, aphid transmission	Altering salicylic acid-mediated responses	Alamillo et al. (2006)
Tobacco mosaic virus	*Tobamovirus*	CP	Coat protein	Suppression of salicylic acid-mediated responses, interacting with DELLA proteins	Rodriguez et al. (2014b)
Tobacco mosaic virus	*Tobamovirus*	Replicase	Replication	Interference with auxin-mediated gene expression; interaction with transcription factor ATAF2 of *Arabidopsis thaliana*	Padmanabhan et al. (2005a, b, 2006, 2008), Wang et al. (2009)

(continued)

Table 5.2 (continued)

Virus species	Genus	Viral interaction partner	Gene function	Plant interaction partner	Reference
Cauliflower mosaic virus	*Caulimovirus*	P6	Silencing suppressor, pathogenicity factor	Inhibition of salicylic acid-dependent signalling; increased expression of jasmonic acid-responsive genes; elicitor of HR; interaction with CHUP1	Love et al. (2012), Palanichelvam et al. (2000), Laird et al. (2013), Angel et al. (2013)
Tomato chlorosis virus	*Crinivirus*	CP	CP, Silencing suppressor	Interacting with *S*-adenosylhomocystein hydrolase thus interfering with RNA silencing	Cañizares et al. (2013)
Rice dwarf virus	*Phytoreovirus*	P2	Component of CP, essential for vector infection and transmission	Reduction of *ent*-kaurene oxidase expression	Zhu et al. (2005)
Tomato golden mosaic virus	*Begomovirus*	Rep	Replication	Interaction with GRIK	Kong and Hanley-Bowdoin (2002), Shen and Hanley-Bowdoin (2006)
Tomato golden mosaic virus	*Begomovirus*	TrAP	Silencing suppressor	Suppression of host defence responses; interaction with ADK; interaction with rgsCaM; inactivation of SNF1 kinase	Sunter et al. (2001), Chung et al. (2014), Wang et al. (2003); Wang et al. (2005), Hao et al. (2003), Yang et al. (2007)
Tomato golden mosaic virus	*Begomovirus*	AC4	Silencing suppression	Interaction with shaggy-related protein kinase and brassinosteroid signalling	Piroux et al. (2007)
Tomato golden mosaic virus	*Begomovirus*	NSP	Nuclear shuttling	Interaction with NIKs; interaction with PERK	Mariano et al. (2004), Florentino et al. (2006)

Virus species	Genus	Viral interaction partner	Gene function	Plant interaction partner	Reference
Cabbage leaf curl virus	*Begomovirus*	Rep	Replication	Interaction with GRIK	Kong and Hanley-Bowdoin (2002), Shen and Hanley-Bowdoin (2006)
Cabbage leaf curl virus	*Begomovirus*	TrAP	Silencing suppressor	Interaction with SnRK1; silencing suppression via ADK inhibition	Shen et al. (2014), Jackel et al. (2015), Buchmann et al. (2009)
Cabbage leaf curl virus	*Begomovirus*	NSP	Nuclear shuttling	Interaction with NIK, interference with NIK-mediated relocalisation of rpL10A, interaction with PERK	Carvalho et al. (2008), Fontes et al. (2004), Florentino et al. (2006), Santos et al. (2009)
Tomato crinkle leaf yellows virus	*Begomovirus*	NSP	Nuclear shuttling	Interaction with NIK; interaction with PERK	Mariano et al. (2004), Florentino et al. (2006)
Tomato leaf curl virus	*Begomovirus*	REn	Replication enhancer	Interaction with NAC transcription factor	Selth et al. (2005)
Tomato yellow spot virus	*Begomovirus*	NSP	Nuclear shuttling	Interaction with NIK	Carvalho et al. (2008), Sakamoto et al. (2012)
Tomato yellow leaf curl Sardinia virus	*Begomovirus*	Rep	Replication	Repression of methylation	Rodríguez-Negrete et al. (2013)
Tomato yellow leaf curl Sardinia virus	*Begomovirus*	TrAP	Silencing suppressor	Inhibition of jasmonic acid-signalling; interference with ubiquitination pathways	Lozano-Duran and Bejarano (2011), Lozano-Duran et al. (2011)

(continued)

Table 5.2 (continued)

Virus species	Genus	Viral interaction partner	Gene function	Plant interaction partner	Reference
β-satellite of Tomato yellow leaf curl China virus	*Begomovirus*	β-C1	Pathogenicity factor, silencing suppression	Suppression of jasmonic acid-responses; methylation interference; interaction with SnRK1; interaction with RFP1 ubiquitin ligase	Yang et al. (2008, 2011), Shen et al. (2011), Zhang et al. (2012), Zhou (2013), Shen et al. (2012)
β-satellite of Cotton leaf curl Multan virus	*Begomovirus*	β-C1	Pathogenicity factor, silencing suppressor	Interaction with multiple host proteins (metabolic and defensive pathways), interaction with ubiquitin-conjugating enzyme	Tiwari et al. (2013), Eini et al. (2009)
African cassava mosaic virus	*Begomovirus*	TrAP	Silencing suppressor	Changes in gene expression; in particular defence-related and hormone-responsive genes	Soitamo et al. (2012)
Beet curly top virus	*Curtovirus*	C2	Pathogenicity factor	Reactivation of cell cycles, inactivation of ADK; enhanced susceptibility; inactivation of SNF1 kinase	Caracuel et al. (2012), Wang et al. (2003), (2005), Lozano-Duran et al. (2012), Sunter et al. (2001), Hao et al. (2003), Buchmann et al. (2009)
Beet severe curly top virus	*Curtovirus*	C2	Pathogenicity factor	Attenuation of SAMDC1 degradation, suppression of DNA methylation-mediated gene silencing	Zhang et al. (2011)
Beet curly top virus	*Curtovirus*	C4	Symptom determinant; cell cycle control	Interaction with shaggy-related protein kinase and brassinosteroid signalling	Piroux et al. (2007)
Wheat dwarf virus	*Mastrevirus*	Rep	Replication	Interaction with GRAB proteins	Xie et al. (1999)

demonstrated that pathogenicity determinants are often linked to silencing suppression activity (Burgyán and Havelda 2011; Csorba et al. 2009). Also in this case, it was also shown that P6 acts as silencing suppressor (Love et al. 2007a; Haas et al. 2008; Shivaprasad et al. 2008). Several lines of evidence suggested that P6 is not only involved in silencing suppression but also in suppression of innate immunity (reviewed by Zvereva and Pooggin 2012).

Early investigations using transgenic approaches showed that expression of P6 in transgenic plants influenced flowering and showed symptom-like phenotypes (Cecchini et al. 1997; Zijlstra et al. 1996; Baughman et al. 1988; Goldberg et al. 1991). Additionally, ethylene- and auxin-dependent signalling responses were modified (Geri et al. 1999, 2004) resembling the responses induced by infection with CaMV (Love et al. 2005). Recently, Joel Milner's group provided evidence that P6 is directly involved in the suppression of SA-dependent gene regulation and enhancement of JA-responsive genes (Love et al. 2012). Based on the observation that infection with CaMV leads to rapid increase of *PR-1* transcripts that decreased with increasing virus titre (Love et al. 2008; Roberts et al. 2007), they postulated that a virus-encoded protein suppressed SA-dependent signalling in infected cells (Love et al. 2012). Indeed, transgenic expression of P6 in *A. thaliana* as well as transient expression of P6 in *N. benthamiana* decreased abundance of SA-dependent transcripts but increased JA-dependent transcripts (Love et al. 2012). Furthermore, P6-expressing plants were more susceptible to virulent and avirulent strains of *Pseudomonas syringae* but less susceptible to *Botrytis cinerea*, a necrotrophic pathogen (Love et al. 2012). There is also a close link to viral long-distance movement as in mutants that accumulated high levels of SA; CaMV movement was inhibited (Love et al. 2007b).

Several lines of evidence show that the N-terminal D-I domain of P6 is responsible for the silencing and SA-suppression activities. For example, *A. thaliana* ecotype Tsu-0 showed resistance to one CaMV strain (CM1841) but not a different one (W260). Swapping of the N-terminal D-I domain of P6 (Li and Leisner 2002), Hapiak et al. (2008) demonstrated that strain CM1841 could overcome resistance and spread systemically when it contained the D-I domain of W260. In addition, pathogenicity in terms of symptom expression did not correlate with the ability to suppress RNA silencing: whereas transgenic expression of P6 of strain D4 had a stronger silencing suppression activity than P6 of CM1841, it only induced mild symptoms in *A. thaliana* Col-0 in contrast to strains CM1841 and W260 YU2003.

In further studies, they were able to map the silencing and SA-dependent defence suppression activities to the N-terminal domain D-I of P6 (Laird et al. 2013). They demonstrated that the distal end of the subdomain 1b was responsible for silencing suppression activity and cell death triggered by SA-signalling but not pathogen-associated molecular pattern (PAMP)-driven PR1a expression indicating that the activities may overlap albeit not being identical (Laird et al. 2013). However, subdomain 1a was required for all activities. They speculated that RNA silencing regulates at least one SA-signalling response (cell death) that can be targeted by CaMV P6, possibly via the viral suppressor of RNA silencing (VSR) activity (Laird et al. 2013).

In summary, P6 is an excellent example for the multifunctionality of viral proteins and shows the complexity in which different defence mechanisms of plants are

interwoven. Not only is P6 able to suppress innate immunity pathways but also adaptive defence pathways such as RNA silencing.

5.3.2 Cucumber Mosaic Virus

Cucumber mosaic virus (CMV) belongs to the *Bromoviridae* family and is the type species of the genus *Cucumovirus*. CMV has an extremely wide host range, infecting more than 85 distinct plant families with around 1,000 species (Palukaitis et al. 1992; Palukaitis and García-Arenal 2003). The genome is tripartite; each of the single-stranded, (+)-sense RNA molecules is encapsidated separately (Palukaitis and García-Arenal 2003). CMV encodes for five proteins: 1a and 2a being components of the replication complex; the 3a protein is a movement protein (MP), and the CP 3b is expressed from the subgenomic RNA4. The latest protein to be discovered is the 2b protein expressed from the subgenomic RNA4a (Peden and Symons 1973; Ding et al. 1994). The 2b protein is a multifunctional protein. It is involved in symptom induction in several host species (Ding et al. 1995; Lewsey et al. 2007; Soards et al. 2002) and influences systemic and local movement of CMV (Soards et al. 2002; Ding et al. 1995; Naylor et al. 1998). In addition, the 2b protein is a suppressor of gene silencing, binds to AGO 1 and AGO 4 but impedes silencing through binding of small RNA species (Zhang et al. 2006; González et al. 2010; Goto et al. 2007). The 2b protein is also known to interfere with miRNAs (Lewsey et al. 2007; Du et al. 2014). Additionally, it inhibits SA-mediated resistance as well as interferes with abscisic acid-mediated signalling (Ji and Ding 2001; Westwood et al. 2013b). The 2b protein is also indirectly involved in plant-vector interactions via manipulation of SA and JA pathways (Ziebell et al. 2011; Westwood et al. 2014).

Lewsey and Carr (2009) investigated more closely the overlap of SA-induced resistance and RNA silencing. They used RNA silencing-deficient *A. thaliana* mutants and found that SA-induced resistance to both CMV and TMV still functioned in mutants lacking DCL2, DCL3 and DCL4, the predominant endonucleases directing cleavage of RNA of these viruses. This indicated that, at least partly, SA-mediated resistance was not dependent on these endonucleases. Therefore, the suppression of SA-mediated defence via the 2b protein must have a different functionality than the RNA silencing suppression properties of 2b. Using DNA microarrays and *2b*-transgenic *A. thaliana* plants, the Carr laboratory showed that the 2b protein interfered with JA-dependent signalling but not JA biosynthesis (Lewsey et al. 2010b). The 2b protein inhibited transcriptome changes that were normally induced by application of MeJA, whereas in the absence of treatment, the abundance of only few transcripts changed. Consistently, infection of plants with wild-type CMV but not with a CMV deletion mutant that was lacking the 2b protein inhibited JA-inducible gene expression (Lewsey et al. 2010b). Interestingly, the expression of most SA-regulated genes was not inhibited by the 2b protein, but in the *2b*-transgenic plants, a number of genes that were normally not affected by SA became SA responsive (Lewsey et al. 2010b). They speculated that these paradoxical finding (facilitation of SA biosynthesis and inhibition of JA-mediated signalling) would promote

vector transmission of the virus by creating favourable conditions of the host (for more details, see Westwood et al. 2013a, 2014; Ziebell et al. 2011). Functional analyses of the 2b protein suggested the ability to prime SA accumulation is unrelated to the silencing suppression activity (González et al. 2010, 2012; Zhou et al. 2014).

5.3.3 Tobacco Mosaic Virus

Tobacco mosaic virus (TMV) is the type member of the genus *Tobamovirus* in the family *Virgaviridae* (Adams et al. 2012). TMV has an undivided genome of approximately 6.3–6.6 kb in size encoding for one structural protein (CP); two nonstructural proteins are responsible for replication (126-kDa and 183-kDa proteins, respectively), and another nonstructural protein facilitates cell-to-cell and long-distance movement (Adams et al. 2012; Liu and Nelson 2013). In tobacco containing the *N* gene (Whitham et al. 1994), the 126-kDa replicase protein is the elicitor of SA-mediated SAR (Abbink et al. 1998; Les Erickson et al. 1999) leading to increase of SA (Malamy et al. 1990). Induced cell death during HR is not responsible for the restriction of TMV in *N*-containing tobacco: using TMV expressing GFP, Murphy et al. (2001) showed that although most cells containing TMV-GFP had died off, there were still viable particles in the vicinity of the lesions. However, the movement of those particles was restricted. In further studies, they found out that SA reduces RNA and CP accumulation of TMV and potato virus X (PVX) (Chivasa et al. 1997; Naylor et al. 1998).

Different interactions between the TMV replicase complex and host plants have been described. For example, the replicase proteins 126/183-kDa interact with auxin response regulator indole acetic acid (IAA)26/PAP1 as well as IAA27 and IAA128. It was suggested that this interaction would alter the localisation and subsequent function of these proteins thus inducing TMV symptoms (Padmanabhan et al. 2005a, 2006, 2008). Several studies showed that the replicase proteins also interfered with RNA silencing. Ding et al. (2004) demonstrated that the 126-kDa protein was sufficient for suppression of RNA silencing. Several domains showed silencing suppression activity independent from their enzymatic activities (Wang et al. 2012). The 122-kDa replicase subunit of a crucifer-infecting TMV strain (cr-TMV) was later found to bind double-stranded siRNAs as well as miRNAs thus interfering with the silencing machinery (Csorba et al. 2007; Várallyay and Havelda 2013).

The 126–183-kDa replicase complex also interferes with the *A. thaliana* NAC domain transcription factor ATAF2 (Wang et al. 2009). ATAF2 belongs to a family of transcription factors involved in developmental processes and defence (Olsen et al. 2005). Wang et al. (2009) showed that interaction of the TMV replicase complex with ATAF2 resulted in the degradation of the transcription factor thus leading to disruption of downstream host defences. An interesting observation was made by Vogler et al. (2008). They found that TMV MP enhanced the spread of siRNAs within host plants thus self-regulating virus propagation through prevention of viral over accumulation.

To complicate the story even further, also the TMV CP interferes with host defence responses. In a recent study, Rodriguez et al. (2014b) found out an

interaction of the CP with DELLA proteins. DELLA proteins are negatively regulating growth and are involved in hormonal crosstalk and interference of SA- and JA-dependent defence signalling (Sun and Gubler 2004; Grant and Jones 2009; Navarro et al. 2008). During TMV-Cg infection, the CP stabilised DELLA protein accumulation in *A. thaliana*. As a consequence, the expression profile of SA-responsive genes was altered and negatively regulated (Rodriguez et al. 2014b).

5.3.4 Begomoviruses

The genus *Begomovirus* (family *Geminiviridae*) comprises of single-stranded circular DNA viruses that are whitefly transmitted (Brown et al. 2009; Fauquet et al. 2003). They can be mono- or bipartite; the two genetic components of the bipartite *Begomoviruses* are called DNA A and DNA B. The genetic component of monopartite *Begomoviruses* resembles DNA A of bipartite ones (Brown et al. 2009). The DNA A virion-sense strand encodes the CP (syn. AV1) and the MP (syn. AV2/V2); the complementary-sense strand encodes the replication-associated protein (Rep, syn. AC1), transcriptional activator protein (TrAP, syn. AC2), replication enhancer protein (REn, syn AV3) and the C4 protein (Brown et al. 2009). DNA B encodes the nuclear shuttle protein (NSP, syn. BV1) on the virion-sense strand and another MP on the complementary-sense strand (MP, syn. BC1) (Brown et al. 2009). *Begomoviruses* are often associated with satellites (α- and β-satellites) that have important roles in symptom induction and pathogenicity as well as suppression of host defences by interfering with RNA silencing pathways but can also influence JA-dependent signalling (Zhou 2013; Cui et al. 2004, 2005; Yang et al. 2008). *Begomoviruses* are becoming a more and more serious threat to agriculture due to a wider spread of its vector and rapid evolution through recombination events (Moriones and Navas-Castillo 2008; Prasanna et al. 2010; Leke et al. 2015; Lefeuvre et al. 2011; Seal et al. 2006; Patil and Fauquet 2009).

Many begomoviral proteins and proteins from related members of the *Geminiviridae* interfere on different levels with a diverse range of host defence and metabolic pathways. Hanley-Bowdoin et al. (2013) have recently reviewed the current knowledge of virus-host interactions between members of the *Geminiviridae* and plant hosts. A brief summary of the current knowledge is given in the following paragraphs and in Table 5.2; for more in-depth information, the reader is referred to the review of Hanley-Bowdoin et al. (2013).

Begomoviruses are no exception to the complex interaction with plant signalling pathways. For example, protein kinases and hormones play important role in plant growth and development as well as in pathogen recognition and defensive signalling. The begomoviral TrAP has been described as main silencing suppressor protein but TrAP can also interfere with JA-mediated defensive signalling (Soitamo et al. 2012). It has been shown that TrAP, a transcription factor required for expression of viral late genes (Sunter and Bisaro 1992, 2003), interacts and inhibits SNF1-related kinase activity. Transgenic plants show therefore enhanced susceptibility phenotypes to geminiviruses and also unrelated viruses (Sunter et al. 2001; Hao et al. 2003). SNF1-related kinases are key regulators of plant development and metabolism and mediate responses to nutritional and environmental stress (Hao

et al. 2003; Baena-Gonzalez et al. 2007; Lu et al. 2007). SNF1-related kinases also negatively regulate several biosynthetic pathways such as fatty acid synthesis, steroid and isoprenoid synthesis, nitrogen assimilation and sucrose synthesis (Hao et al. 2003). The βC1 protein of the tomato yellow leaf curl China virus (TYLCCV) β-satellite also binds to SNF1-related kinases leading to phosphorylation of βC1 and subsequently attenuated infection (Shen et al. 2011).

SNF1-related kinases can also be activated by geminivirus Rep-interacting kinases (GRIK) (Shen et al. 2009). GRIKs have been found in young plant tissues as well as in mature tissues of geminivirus-infected cells (Shen and Hanley-Bowdoin 2006) and interact with Rep (Kong and Hanley-Bowdoin 2002). Shen et al. (2014) found that GRIK-activated SNF1-related kinases phosphorylated TrAP proteins from different geminiviruses leading to a delay in virus accumulation and symptom development confirming its antiviral role in plants.

Adenosine kinase (ADK) is a housekeeping enzyme that is involved in the adenine and adenosine salvage pathway (Wang et al. 2003). The geminiviral TrAP also interacts and inhibits the activity of this kinase (Wang et al. 2003). Interestingly, further studies demonstrated the overlap of RNA silencing with innate defence responses as inhibition of ADK activity by TrAP also counters RNA silencing (Wang et al. 2005). Bisaro's group gave further evidence that TrAP suppression of host defence responses included overlapping mechanisms: they suggested that chromatin methylation-mediated transcriptional gene silencing was used by plants as a defence against DNA viruses counteracted by begomoviral TrAPs through inhibition of global methylation and transcriptional gene silencing (Raja et al. 2008; Buchmann et al. 2009).

For beet severe curly top virus (BCTV), another begomovirus, it was also shown that TrAP can inhibit proteasome-mediated degradation of SAM decarboxylase that stabilises a competitive inhibitor of *S*-adenosyl methionine (SAM) (Zhang et al. 2011). Production of SAM in return is dependent on ADK activity that is inhibited by TrAP (Wang et al. 2005; Wang et al. 2003). SAM production can also be inhibited by interference with begomoviral satellites such as the β-satellite of TYLCCV (Yang et al. 2011). Interestingly, it appears that ADK-dependent suppression of RNA silencing by TrAP appears to be dependent on developmental stages of the host plant and/or responses to stress (Jackel et al. 2015).

The interactions of begomoviral NSP and plant NSP-interacting kinases (NIKs) have been the focus of E. Fontes' group. NSP targets NIK receptors that belong to the leucine-rich repeat receptor-like kinases (LRR-RLK) subfamily LRR-RLK II (Mariano et al. 2004; Fontes et al. 2004); see Fontes (2014) and Santos et al. (2010) for brief summaries. Sakamoto et al. (2012) produced a phylogenic analysis of tomato RLKs and showed that there is a functional and structural conservation between the *A. thaliana* and tomato RLK families. NIK1, NIK2 and NIK3 of *A. thaliana* were targeted by NSP (Fontes et al. 2004), but also tomato orthologs were targeted by NSP (Sakamoto et al. 2012). There are several indicators that NIKs are involved in defence responses: in *A. thaliana*, loss of NIK-function led to a phenotype with enhanced susceptibility to begomovirus infection (Fontes et al. 2004; Santos et al. 2009), whereas overexpression of AtNIK1 in tomato led to attenuated symptoms and a delay of infection (Carvalho et al. 2008). Additionally, binding of

NSP to NIK inhibits its kinase activity thus suppressing NIK-mediated antiviral responses (Fontes et al. 2004; Santos et al. 2009). This kinase activity is important for phosphorylation of the downstream effector, the ribosomal protein L10 (RPL10), a QM-like protein (Rocha et al. 2008). Phosphorylation of RPL10 is required for translocation from the cytosol to the nucleus where it might interfere with viral infection (Carvalho et al. 2008). Using a NIK1 phosphomimetic gain-of-function mutant in a *nik1* knockout background, Zorzatto et al. (2015) observed global translation suppression and translocation of RPL10 to the nucleus. RPL10 interacted in the nucleus with L10-interacting MYB domain-containing protein (LIMYB), a novel MYB-like protein (Zorzatto et al. 2015). Overexpression of LIMYB led to repression of ribosomal proteins and protein synthesis inhibition, decreased viral messenger RNA association with polysome fractions and enhanced tolerance to begomovirus infection. Loss of LIMYB function led to enhanced susceptibility to begomovirus infection (Zorzatto et al. 2015). Interestingly, constitutive activation of NIK1 and LIMYB overexpression did not interfere with SA-mediated signalling or RNA silencing. The authors suggested that host translation suppression could be a novel antiviral defence branch that would be suppressed by NSP interaction (Zorzatto et al. 2015). Interestingly, Fontes's group was also able to transfer the gain-of-function mutant from *A. thaliana* to tomatoes as one of the most affected crop plants by begomovirus infection (Brustolini et al. 2015). The constitutive activation of NIK-mediated antiviral signalling successfully repressed begomovirus infection in tomato plants. As the antiviral response was effective against highly divergent begomoviruses, this strategy might open up new directions to achieve broad virus resistance in crop plants (Brustolini et al. 2015).

Many more components of plant signalling pathways are influenced by begomovirus infection such as shaggy-related kinases, ubiquitylation and ubiquitylation-like pathways as well as silencing pathways. Due to space restraints, the reader is referred to the summary in Table 5.2 as well as the review by Hanley-Bowdoin et al. (2013) and the references within.

5.4 Concluding Remarks

In recent years, we have seen many advances in the field of plant virology. The major milestone was the discovery of RNA silencing and its role in antiviral defence. But as it transpires, the defence mechanisms that plants have developed cannot be seen separately. They appear all to be connected via a complex web of interactions, positive and negative crosstalks and signalling cascades. It is not surprising that viruses also have developed numerous counteracting strategies in order to infect plants successfully. Most, if not all, viral proteins are multifunctional and interfere with plant defence in many ways. There are now numerous examples demonstrating that plant viruses can suppress independent defence pathways that are not necessarily linked, showing that viruses have invented many different toolboxes to circumvent defences. This arms race will continue and give us many more opportunities for surprising discoveries!

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Role of Small RNAs in Virus-Host Interaction

6

Mikhail M. Pooggin

Contents

Abstract

Short-interfering RNAs (siRNAs) and microRNAs (miRNAs) play an important role in regulation of host gene expression and defense against invasive nucleic acids such as transposons, transgenes, and viruses. In plants, siRNA-directed silencing is a major defense mechanism that restricts replication and spread of RNA and DNA viruses as well as viroids and viral satellites. During viral infection, host Dicer-like (DCL) enzymes catalyze production of 21-, 22-, and 24-nucleotide viral siRNA duplexes from longer double-stranded RNA (dsRNA) precursors generated by viral and/or host RNA polymerases. These duplexes are

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sorted by several distinct Argonaute (AGO) proteins to form RNA-induced silencing complexes (RISCs) that can potentially target cognate viral RNA for posttranscriptional gene silencing (PTGS) and, in the case of DNA viruses, also viral DNA for cytosine methylation and transcriptional gene silencing (TGS). To establish successful infection, plant viruses have evolved various mechanisms of silencing evasion as well as silencing suppression through effector proteins that interfere with the biogenesis and/or action of viral siRNAs. Furthermore, viruses appear to manipulate host siRNAs and miRNAs which may contribute to antiviral defense indirectly, through regulation of the host genes mediating silencing and innate immunity.

6.1 Introduction

Plant viruses and viroids are invasive nucleic acids that can replicate in different cellular compartments including nucleus, chloroplasts, and cytoplasm and move for a short distance from cell to cell and for a long distance via vascular system, thereby causing systemic infection of the whole plant (Hull 2002). Viral replication and spread through the plant tissues can potentially be restricted by two major host defense mechanisms, (1) RNA silencing (also known as RNA interference), triggered by recognition of viral double-stranded RNA (dsRNA) molecules, and (2) innate immunity, triggered by recognition of viral proteins or their activities (reviewed by Zvereva and Pooggin 2012). These mechanisms are thought to be responsible for exclusion of many viruses from certain tissues (e.g., shoot apical meristem; Schwach et al. 2005) and for restriction of some viruses to phloem tissues where they can still replicate, move systemically, and be picked up by insect vectors (Hull 2002). RNA silencing has also been implicated in plant recovery from viral infections (Covey et al. 1997; Ratcliff et al. 1999).

The plant RNA silencing machinery can recognize and process perfect dsRNA or hairpin-like RNA secondary structures into 21–24 nucleotide (nt) small RNA (sRNA) duplexes. The processing reaction can be catalyzed by four distinct Dicer-like (DCL) enzymes. DCLs have specialized but partially redundant functions in the biogenesis of different classes of endogenous plant sRNAs, including miRNAs and short interfering RNAs (siRNAs) (Vaucheret 2006). miRNAs and trans-acting siRNAs (tasiRNAs) are encoded by *MIR* and *TAS* genes, respectively, and act *in trans* to repress genes involved in developmental and physiological processes as well as biotic and abiotic stress responses (Bartel 2004; Chapman and Carrington 2007; Sunkar et al. 2007; Ruiz-Ferrer and Voinnet 2009; Seo et al. 2013). The repression is at a posttranscriptional level via sequence-specific cleavage of a target mRNA or inhibition of its translation. Likewise, viral siRNAs accumulating in all virus-infected plants examined so far (first time reported by Hamilton and Baulcombe 1999) have the potential to repress cognate viral RNAs. Furthermore, viral and viroid siRNAs can occasionally target complementary plant mRNAs (Wang et al. 2004, which contributes to symptom development in certain cases (Shimura et al.

2011; Smith et al. 2011). Some of the plant genes targeted by endogenous plant miRNAs or tasiRNAs are additionally repressed by secondary-phased siRNAs (phasiRNAs) which are generated by a combined action of RNA-dependent RNA polymerase 6 (RDR6) producing dsRNA on the miRNA-cleaved mRNA templates and DCL4 processing the resulting dsRNA (Howell et al. 2007). Secondary siRNAs play an important role in amplification and spread of RNA silencing. Once initiated in a single cell, RNA silencing can spread locally from cell to cell and systemically via phloem tissues. The mobile signals that spread silencing of transgenes locally and systemically have an siRNA component (Hamilton et al. 2002; Molnar et al. 2010; Dunoyer et al. 2010), although longer dsRNA molecules have also been implicated (Brosnan et al. 2007). By analogy, mobility of viral siRNAs or their precursors may contribute to antiviral defense: movement of viral siRNAs ahead of the infection front would "immunize" cells and tissues from the incoming virus. Indeed, viral siRNAs were found in phloem exudates, suggesting their systemic movement (Yoo et al. 2004), but a role of mobile viral siRNAs in antiviral silencing remains to be investigated.

In addition to posttranscriptional gene silencing (PTGS) directed by miRNAs and siRNAs, the RNA silencing machinery can repress genes transcriptionally by targeting certain regions of chromosomes for DNA cytosine methylation and histone modification. This leads to formation of heterochromatin which is inaccessible for polymerase II (Pol II) and results in transcriptional gene silencing (TGS) (Law and Jacobsen 2010; Pikaard et al. 2012). Heterochromatic siRNA (hcsiRNA)-directed DNA methylation (RdDM) plays an important role in de novo establishment and maintenance of the repressive chromatin marks and may therefore contribute to transcriptional silencing of plant DNA viruses (reviewed by Pooggin 2013).

To establish successful infection, viruses and viroids must evade or actively suppress both RNA silencing and innate immunity (Zvereva and Pooggin 2012). For silencing suppression, viruses have evolved suppressor proteins that target different components of the RNA silencing machinery and thereby block the biogenesis and/or action of viral siRNAs. In many cases, viral silencing suppressors have initially been identified as pathogenicity determinants and virulence/avirulence factors (Voinnet et al. 1999). By analogy with nonviral pathogens, the virulence/avirulence factors are thought to be viral effectors that suppress basal immune responses in susceptible hosts. In resistance hosts, these effectors are recognized directly or indirectly by immune receptors, which triggers hypersensitive responses and programmed cell death, restricting viral infection to the inoculated cell (reviewed by Zvereva and Pooggin 2012).

Growing evidence indicates that RNA silencing regulates innate immunity responses through the action of miRNAs and/or siRNAs induced by pathogen infections (Zvereva and Pooggin 2012; Pumplin and Voinnet 2013). Viral-silencing suppressors may therefore interfere with the biogenesis or action of such immunity-related plant sRNAs and thereby block immune responses. Alternatively, the plant defense system may exploit the ability of viruses to suppress silencing by placing certain innate immunity genes under the control of PTGS and/or TGS (Pumplin and Voinnet 2013).

Taken together, sRNAs play a major role in virus-host interactions. This chapter will focus mainly on the mechanisms of biogenesis and action of viral siRNAs and the viral strategies of evasion and suppression of antiviral silencing.

6.2 The Biogenesis of Viral siRNAs

6.2.1 Precursors of Endogenous Plant siRNAs

Plant tasiRNAs, phasiRNAs, and hcsiRNAs are processed from perfect dsRNA precursors generated by RDR activity (Fig. 6.1). In contrast, plant miRNAs are processed from Pol II transcripts of *MIR* genes. These single-stranded transcripts form short hairpins or, in some cases, longer hairpin structures which are processed by

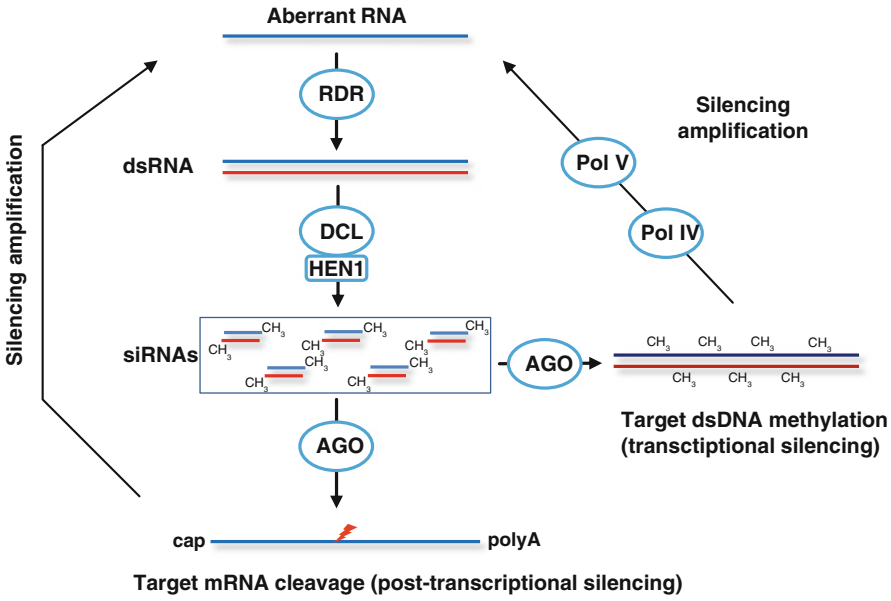

Fig. 6.1 Mechanisms of siRNA-directed gene silencing in plants. Double-stranded RNA (*dsRNA*) is a key trigger of both posttranscriptional and transcriptional gene silencing (PTGS and TGS). DICER-LIKE (*DCL*) enzymes catalyze processing of long dsRNA into short interfering RNA (*siRNA*) duplexes. HUA ENHANCER 1 (*HEN1*) methylates each strand of the duplexes at 2′-hydroxyl of the 3′-terminal nucleotide (*CH3*). One of the duplex strand gets associated with one of the ARGONAUTE (*AGO*) family protein and the resulting RNA-induced silencing complex targets cognate genes for PTGS through mRNA cleavage and/or TGS through DNA cytosine methylation (*CH3*). Positive feedback loops reinforce both PTGS and TGS. In PTGS, the mRNA cleavage products are converted by RNA-dependent RNA polymerase (*RDR*) activity into dsRNA precursors of siRNAs. In TGS, the methylated DNA and the target (to-be-methylated) DNA are transcribed by Pol IV and Pol V, respectively. The Pol IV transcripts are converted by RDR activity into dsRNA precursors of siRNAs, while the Pol V transcripts serve as scaffolds that interact with siRNA-AGO complexes and recruit the DNA methyltransferase

DCL1 and DCL4, respectively (Bartel 2004; Chapman and Carrington 2007; Rajagopalan et al. 2006).

The *Arabidopsis thaliana* genome contains six *RDR* genes, from which only *RDR1*, *RDR2*, and *RDR6* are known to be involved in the biogenesis of endogenous siRNAs (Wassenegger and Krczal 2006; Cao et al. 2014), and the biochemical activities of RDR2 and RDR6 were characterized in vitro (Haag et al. 2012; Curaba and Chen 2008). The biogenesis of tasiRNAs and phasiRNAs is initiated by miRNA-directed cleavage of Pol II transcripts of the non-protein coding *TAS* genes and some miRNA-targeted protein-coding genes, respectively. One of the cleavage products is converted by RDR6 to dsRNA with one or two-nucleotide 3′ overhangs as shown for *TAS* dsRNAs (Rajeswaran and Pooggin 2012a; Rajeswaran et al. 2012). The resulting dsRNA is processed from one or both ends by DCL4 into 21-nt duplexes (and occasionally in 22-nt duplexes) with two-nucleotide 3′ overhangs. Some of these duplexes are sorted by AGO1 or AGO2 and create tasiRNA-RISCs that target genes *in trans* (Axtell et al. 2006; Howell et al. 2007; Rajeswaran and Pooggin 2012a; Rajeswaran et al. 2012). Interestingly, *cis*-acting siRNAs derived from *TAS* genes have also been identified, which are involved in negative and/or positive feedback regulation of tasiRNA biogenesis (Axtell et al. 2006; Rajeswaran and Pooggin 2012a; Rajeswaran et al. 2012).

Presumed dsRNA precursors of hcsiRNAs have not been mapped precisely so far. Available genetic and biochemical evidence suggests that methylated DNA at the RdDM loci is transcribed by plant-specific polymerase Pol IV and the resulting transcripts are converted by RDR2 into dsRNAs (Fig. 6.1). RDR2-dependent dsRNAs are processed predominantly by DCL3 into 24-nt siRNA duplexes which are then sorted by AGO4, AGO6, and AGO9 to form hcsiRNA-RISCs targeting various loci including transposons and other repeats for cytosine methylation and TGS. On the target loci, plant-specific polymerase Pol V generates a scaffold transcript which interacts with hcsiRNA-RISC, a prerequisite for recruitment of de novo methyltransferase activity (Pikaard et al. 2012; Pooggin 2013).

6.2.2 Precursors of RNA Virus-Derived siRNAs

So far precursors of viral siRNAs have not been characterized genetically or structurally as comprehensively as those of tasiRNAs (Rajeswaran and Pooggin 2012a; Rajeswaran et al. 2012). Based on deep-sequencing analysis of viral siRNA populations, two types of precursors were inferred: (1) perfect dsRNAs processed into siRNAs of both sense and antisense polarities that accumulate at high and comparable levels and (2) secondary structures of viral single-stranded RNAs processed into siRNAs of one polarity, which may create a strand bias depending on the relative abundance of sense and antisense viral transcripts. It should be noted, however, that the strand bias in total siRNA populations can be explained not only by siRNA processing from RNA secondary structures but also by differential stability of siRNAs (e.g., preferential stabilization of siRNAs of one polarity by host AGO proteins). Furthermore, technical biases in certain deep-sequencing protocols can lead

to overrepresentation of siRNAs of one polarity in cDNA libraries (Smith et al. 2010). Growing evidence showing roughly equal accumulation of sense and antisense viral siRNAs in RNA(+) virus-infected plants indicates that the majority of viral siRNAs are generated from perfect dsRNA precursors (Donaire et al. 2009; Wang et al. 2010, 2011; Garcia-Ruiz et al. 2010; Seguin et al. 2014). However, strong biases to a positive strand (Molnar et al. 2005; Szittya et al. 2010; Hu et al. 2011) and local biases to either sense or antisense strand (Donaire et al. 2009) have also been reported. Nonetheless, the hotspots of viral siRNA production generally do not map to the regions of strong secondary structures that would be compatible with DCL processing (Szittya et al. 2010), thus supporting the notion that viral siRNAs are processed from perfect dsRNA precursors. Since genetic evidence indicates that DCL4 is a primary dicer mediating the biogenesis of viral siRNAs in RNA virus-infected plants (Deleris et al. 2006; Blevins et al. 2006; Bouche et al. 2006; Fusaro et al. 2006) and because DCL4 prefers long and perfect dsRNA substrates (Nagano et al. 2013), viral siRNAs are likely processed from perfect dsRNA intermediates of RNA virus replication (Fig. 6.2) and/or from viral dsRNAs produced by host RDR activity.

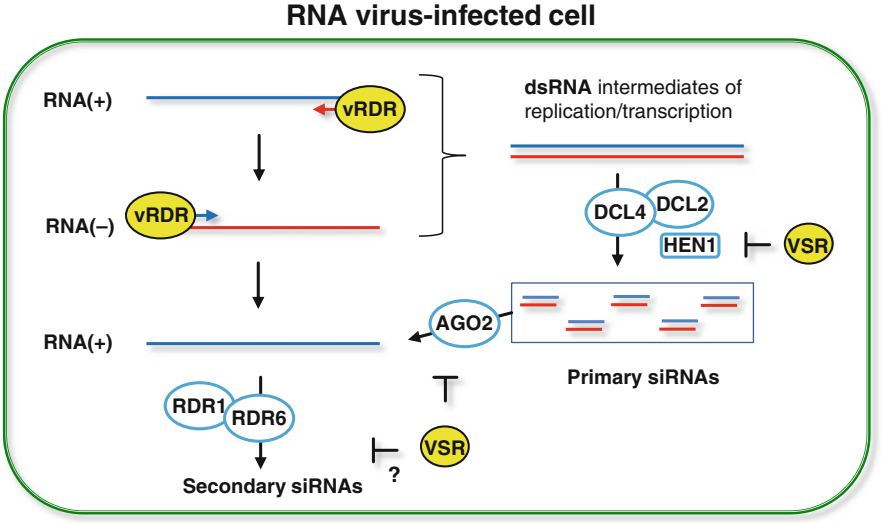

Fig. 6.2 Model for the biogenesis and action of RNA virus-derived siRNAs. During RNA virus replication, viral RNA-dependent RNA polymerase (*vRDR*) generates negative -RNA [RNA(−)] and positive-strand RNA [RNA(+)] on the RNA(+) and the RNA(−) templates, respectively. Both processes produce dsRNA intermediates that are recognized by DCL enzymes. DCL4 is a primary dicer that catalyzes processing of viral dsRNA intermediates into 21-nt siRNA duplexes. DCL2 is a secondary dicer that can take over DCL4 function and generate 22-nt siRNA duplexes. The resulting primary viral siRNAs are associated with AGO2 or other AGO proteins to target cognate viral RNA for cleavage. RDR6 converts the cleavage products into dsRNA precursors of secondary viral siRNAs which reinforce RNA silencing. Viral suppressors of RNA silencing (*VSR*) can interfere with (1) the HEN1-mediated methylation of siRNA duplexes, which would prevent their association with AGOs, (2) the activity of viral siRNA-AGO silencing complexes, or (3) the RDR6-dependent amplification of viral siRNAs

The primary role of DCL4 in production of 21-nt viral siRNAs and in defense against RNA viruses suggested an involvement of RDR6 in generation of viral siRNA precursors. However, RNA virus-infected *Arabidopsis rdr6* mutant plants, in which RDR6-dependent tasiRNA biogenesis is abolished, still accumulate high levels of viral siRNAs, comparable to those in wild-type plants, as was shown for the tobravirus tobacco rattle virus (TRV) (Deleris et al. 2006; Donaire et al. 2008), the carmovirus turnip crinkle virus (TCV) (Deleris et al. 2006), and the cucumovirus cucumber mosaic virus (CMV) (Deleris et al. 2006) and the tobamovirus oilseed rape mosaic virus (ORMV) (Blevins et al. 2006). Likewise, RNA virus-infected *Arabidopsis* single mutants for *RDR2*, *RDR1*, or other *RDR* genes (*3a*, *3b*, *3c*; Wassenegger and Krczal 2006) also accumulated wild-type levels of viral siRNAs (Deleris et al. 2006; Blevins et al. 2006; unpublished data of M.M.Pooggin group).

Interestingly, redundant contribution of *RDR1*, *RDR2*, and *RDR6* to production of wild-type TRV and turnip mosaic potyvirus (TuMV; potyvirus)-derived siRNAs was reported: only in *rdr1/2/6* triple mutant plants (but not in single or double mutants of these three *RDRs*) accumulated reduced levels of viral siRNA accumulation (Donaire et al. 2008; Garcia-Ruiz et al. 2010). Nonetheless, substantial amounts of viral siRNAs of both polarities accumulating in *rdr1/2/6* plants indicate that their precursors are likely RDR-independent perfect dsRNA. Taken together, RDR-independent perfect dsRNAs appear to be a major type of viral siRNA precursors (Fig. 6.2). Differential stability of viral siRNAs likely accounts for the local and, in certain cases, genome-wide strand bias.

The involvement of RDR6 and RDR1 in the biogenesis of secondary viral siRNAs has been demonstrated by using suppressor-deficient RNA viruses, 2b-deficient CMV, and HCPro-deficient TuMV. These mutant viruses could not establish systemic infection on wild-type plants but could do so on *rdr1* and/or *rdr6* plants (Wang et al. 2010, 2011; Garcia-Ruiz et al. 2010). It has been noted that strong silencing suppression by wild-type viruses could mask the contribution of RDRs in viral siRNA biogenesis, which would account for unaltered accumulation of viral siRNAs from wild-type CMV or TuMV in *rdr1* or *rdr6* plants. However, neither HCPro nor 2b are known to block RDR activity. An alternative explanation for RDR involvement in viral siRNA production from suppressor-deficient RNA viruses is that deletions and point mutations abolishing expression or activity of the suppressor protein could potentially lead to destabilization of viral genomic or subgenomic RNAs, which would result in accumulation of aberrant viral transcripts, the templates for RDR activity. In line with this hypothesis, non-translatable transgenes carrying a viral protein sequence, in which the ATG start codon was mutated or part of the coding sequence deleted, are more prone to PTGS than translatable transgenes carrying the wild-type viral protein sequence (Lindbo and Dougherty 2005). Wild-type viral RNAs may not be good substrates for RDR-dependent amplification of viral siRNAs. Likewise, most of the miRNA-targeted plant mRNAs do not spawn secondary siRNAs.

It is worth noting that aberrant viral transcripts sensed by RDRs may overaccumulate owing to potential defects in replication and transcription of suppressor-deficient viruses, especially if the viral suppressor protein serves an auxiliary function in replication or transcription.

6.2.3 Precursors of DNA Virus-Derived siRNAs

In contrast to RNA viruses, DNA viruses do not replicate via dsRNA intermediates and do not code for a viral RNA-dependent RNA polymerase, which implies an involvement of host RDRs in viral siRNA production. However, genetic evidence did not reveal any role for RDR1, RDR2, or RDR6 in the biogenesis of viral siRNAs from cabbage leaf curl virus (CaLCuV) and cauliflower mosaic virus (CaMV), which represent two major families of plant DNA viruses, *Geminiviridae* and *Caulimoviridae*. Indeed, viral siRNAs of three major size classes (21-, 22-, and 24-nt) in CaLCuV- and CaMV-infected *Arabidopsis rdr2* or *rdr6* mutant plants accumulate at wild-type levels (Blevins et al. 2006). Redundant contribution of *RDR1*, *RDR2*, and *RDR6* in viral siRNA production was also ruled out by testing *rdr1/2/6* triple mutant plants (Blevins et al. 2011; Aregger et al. 2012). Likewise, Pol IV and Pol V activities required for the biogenesis and function of endogenous hcsiRNAs are dispensible for production of CaLCuV or CaMV siRNAs (Blevins et al. 2006, 2011; Aregger et al. 2012).

In *Geminiviridae*, Pol II-mediated bidirectional transcription of circular viral DNA generates rightward and leftward transcripts with short overlapping sequences followed by poly(A) tails (Fig. 6.3; reviewed in Pooggin 2013). This short overlap cannot account for RDR-independent production of viral siRNAs throughout the genome including a "non-transcribed" bidirectional promoter region (Akbergenov et al. 2006; Blevins et al. 2006; Aregger et al. 2012; Seguin et al. 2014). In a plausible scenario, readthrough transcription far beyond the poly(A) signal in both directions on the circular viral DNA generates sense and antisense transcripts covering the entire geminivirus genome (Fig. 6.3). Such transcripts or their degradation products can form perfect dsRNA precursors processed by DCLs into siRNAs. Pregenomic RNA (pgRNA) of plant pararetroviruses is generated by Pol II transcription far beyond a poly(A) signal (reviewed by Hohn and Rothnie 2013; Fig. 6.4). In most pararetroviruses, the poly(A) signal is located at a short distance downstream of the pgRNA transcription start site, which allows for efficient readthrough transcription at its first encounter and termination of transcription at the second encounter. Readthrough transcription is therefore expected in geminiviruses, especially for relatively short transcripts covering AC2/AC3 and AV2/AV1 genes (Fig. 6.3), and such readthrough transcripts were detected using a circularization-reverse transcription PCR method (Shivaprasad et al. 2005).

In pararetroviruses, Pol II transcription of viral DNA is monodirectional. All viral proteins are encoded on one strand and translated from pgRNA and its spliced variants. In some genera, Pol II drives transcription of a subgenomic RNA, mRNA for the transactivator/viroplasmin protein (TAV; also known as P6), in the same orientation (Hull 2007; Hohn and Rothnie 2013; Fig. 6.4). Therefore, geminivirus-like, bidirectional readthrough transcription cannot be a mechanism for production of perfect dsRNA precursors of RDR-independent viral siRNAs (Blevins et al. 2006, 2011). However, the CaMV pgRNA promoter may drive erroneous transcription in the opposite orientation (Blevins et al. 2006).

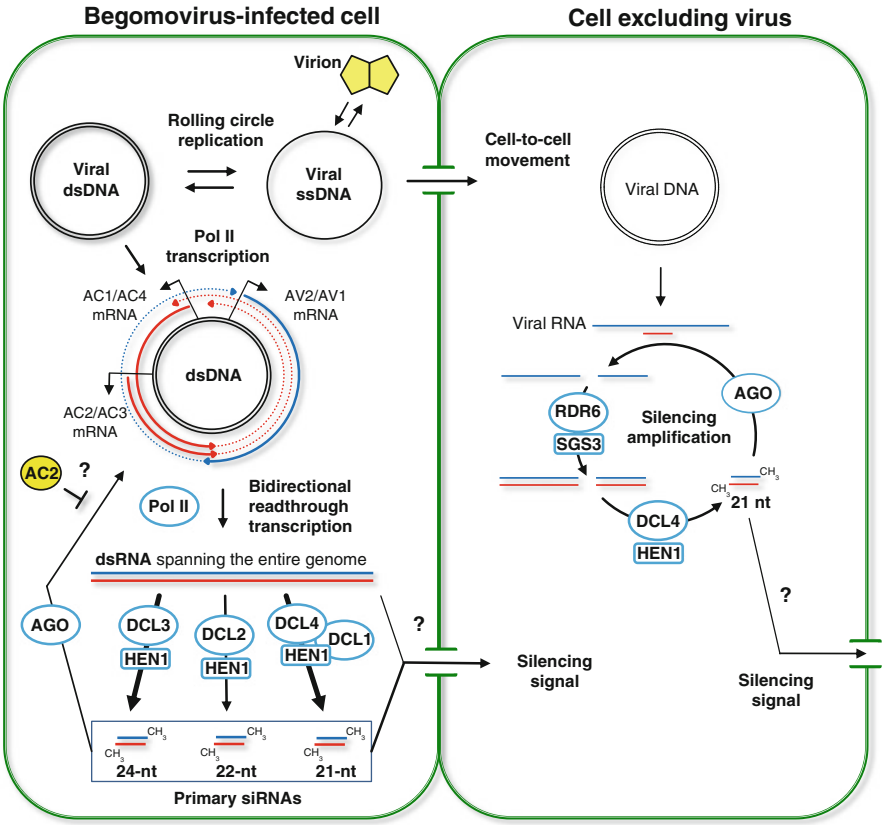

Fig. 6.3 Model for the biogenesis and action of geminiviral siRNAs. Two adjoining plant cells are shown schematically. The initially infected cell (*left*) contains high viral titers. Viral single-stranded DNA released from the virion is converted to double-stranded DNA (*dsDNA*) that serves as a template for rolling circle replication and Pol II-mediated transcription of viral mRNAs (in the case of begomoviruses, the leftward AC1/AC4 and AC2/AC3 mRNAs and the rightward AV2/AV1 mRNA, depicted with red and blue lines with arrowheads, respectively). Double-stranded RNA (*dsRNA*) arises from the viral genome by Pol II-mediated bidirectional readthrough transcription beyond the poly(A) signals (*dotted lines*), i.e., independently of known silencing-related host RNA polymerases. Every DCL digests the dsRNA into distinct size classes of viral siRNAs, with DCL3, DCL4 and DCL2 being favored (in that order). Both viral DNA and viral siRNAs (or long dsRNA) move into the neighboring cell. However, the viral titer remains low, because the RDR6/SGS3/DCL4 pathway amplifies incoming siRNA signal and digests viral transcripts. Viral siRNAs are stabilized by HEN1-mediated methylation. The viral silencing suppressor AC2 directly or indirectly interferes with viral siRNA-AGO complexes targeting viral RNA and DNA for PTGS and TGS, respectively (The model was adopted and extended from Blevins et al. (2006) and Rejeswaran and Pooggin (2012b))

An additional evidence for pervasive transcription of both strands of viral dsDNA in pararetroviruses and geminiviruses comes from bioinformatics analysis of viral siRNA populations showing that non-redundant siRNA species cover the entire circular viral genome in both orientations without gaps (Seguin et al. 2014; Rajeswaran

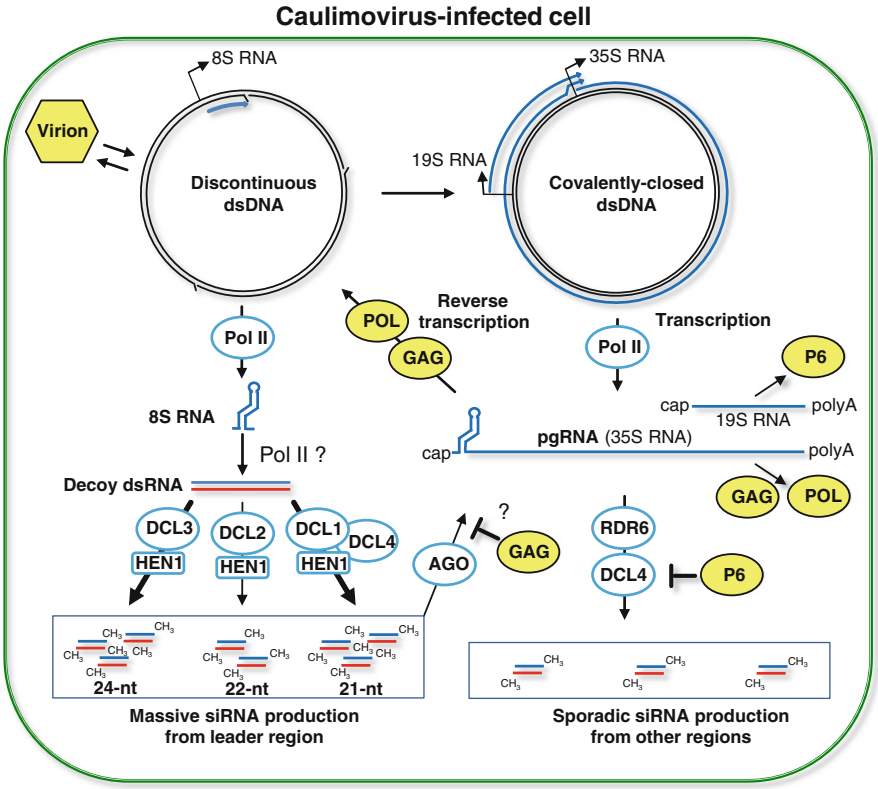

Fig. 6.4 Model for the biogenesis and action of pararetroviral siRNAs. Viral DNA is released from the virion into the nucleoplasm. Gaps in this discontinuous dsDNA left after reverse transcription are repaired by the host repair enzymes to create covalently closed dsDNA. Both repaired and unrepaired forms of viral dsDNA are transcribed by host Pol II. The repaired dsDNA gives rise to pregenomic RNA (pgRNA; also called 35S RNA), which serves as a polycistronic mRNA for coat protein (*GAG*) and reverse transcriptase (*POL*) and a template for reverse transcription. In some genera of pararetroviruses, Pol II transcription from a separate promoter generates subgenomic 19S RNA, mRNA for the transactivator/viroplasmin/silencing suppressor protein (*P6*). Abrupt termination of Pol II transcription at the minus-strand DNA gap (Met-tRNA gap) of the unrepaired dsDNA results in production of 8S RNA (leader RNA). This RNA forms a viroid-like secondary structure which can be converted by Pol II to dsRNA. The resulting dsRNA serves as a decoy to engage all the four DCLs in massive production of 21-, 22-, and 24-nt viral siRNAs, which are then methylated by HEN1 and get associates with AGO protein(s). Stable secondary structure of the pgRNA leader sequence interferes with complementary interaction of viral siRNA-AGO complexes with pgRNA. The viral GAG protein, which initiates packaging of pgRNA in previrions and assists its reverse transcription within previrions, also protects the pgRNA from being targeted by viral siRNAs. The viral protein P6 interferes with production of viral secondary siRNAs by the RDR6/DCL4 pathways (The model was adopted and extended from Blevins et al. (2011) and Rajeswaran and Pooggin (2012b))

et al. 2014a, b). As argued above, this pervasive transcription is likely mediated by Pol II, which generates dsRNA precursors of primary viral siRNAs.

6.2.4 Involvement of Multiple DCLs in Processing of Viral dsRNA Precursors

Three major size classes of viral siRNAs, 21-nt, 22-nt, and 24-nt, accumulate in plants infected with DNA geminiviruses (Akbergenov et al. 2006; Blevins et al. 2006; Rodríguez-Negrete et al. 2009; Kreuze et al. 2009; Yang et al. 2011; Aregger et al. 2012) and pararetroviruses (Blevins et al. 2006, 2011; Noreen et al. 2007; Staginnus et al. 2007; Rajeswaran et al. 2014a, b) as well as nuclear viroids (Di Serio et al. 2010; Seguin et al. 2014). Genome-integrated endogenous pararetroviruses also give rise to 21-, 22-, and 24-nt siRNAs but those are less abundant than viral siRNAs derived from episomal virus causing infection (Noreen et al. 2007). Based mostly on genetic evidence in the model plant *Arabidopsis*, whose genome codes for four DCLs, all the DCLs generate siRNAs from DNA viruses: DCL4 and DCL1 make 21-nt siRNAs; DCL2, 22-nt siRNAs; and DCL3, 24-nt siRNAs (Akbergenov et al. 2006; Blevins et al. 2006, 2011; Aregger et al. 2012). Thus, both the nuclear and the cytoplasmic silencing pathways are induced by DNA virus infection and the four DCLs catalyze processing of viral dsRNA precursors into siRNAs (Figs. 6.3 and 6.4) with potential functions in transcriptional and posttranscriptional silencing of viral gene expression. The contribution of DCL1 in the biogenesis of 21-nt viral siRNAs is more substantial for the pararetrovirus CaMV than the geminivirus CaLCuV (Blevins et al. 2006, 2011; Aregger et al. 2012), likely because of CaMV-mediated suppression of DCL4 activity (see below).

RNA viruses replicating in the cytoplasm spawn mostly 21-nt and 22-nt viral siRNAs (Xie et al. 2004; Molnar et al. 2005; Deleris et al. 2006; Blevins et al. 2006; Bouche et al. 2006; Fusaro et al. 2006; Qu et al. 2008 ; Donaire et al. 2008, 2009; Garcia-Ruiz et al. 2010; Wang et al. 2010; Szittya et al. 2010). DCL4 is a primary antiviral DCL that generates 21-nt viral siRNAs (Deleris et al. 2006; Bouche et al. 2006; Blevins et al. 2006; Fusaro et al. 2006; Garcia-Ruiz et al. 2010). In certain cases such as CMV and TCV, when DCL4 activity is diminished by a viral silencing suppressor, DCL2 takes over processing of viral dsRNA and generates 22-nt siRNAs (Xie et al. 2004; Deleris et al. 2006). If the silencing suppressor gene is deleted or mutated, the primary function of DCL4 in detection and processing of viral dsRNA is restored, which drastically restricts replication and systemic spread of the suppressor-deficient virus (Deleris et al. 2006; Qu et al. 2008; Wang et al. 2010, 2011).

Relative contributions of distinct DCL activities to viral siRNA biogenesis can vary in different host plants. Infection of *Nicotiana benthamiana* and cassava plants with the geminivirus african cassava mosaic virus resulted in different profiles of viral siRNAs, with 24-nt and 21-nt siRNAs dominating in cassava and 22-nt siRNAs dominating in *N. benthamiana* (Akbergenov et al. 2006). Relative accumulation of viral siRNA size classes can also vary in different regions along the viral genome. Thus, 24-nt siRNAs were predominant in a bidirectional promoter region

of the geminivirus genome, whereas 21-nt and 22-nt siRNAs dominated in the transcribed regions, suggesting that the promoter region is targeted for TGS in the nucleus, whereas viral transcripts are targeted for PTGS in the cytoplasm (Akbergenov et al. 2006; Rodríguez-Negrete et al. 2009). However, DCL3-dependent 24-nt siRNAs can also dominate in the transcribed regions of DNA viruses as shown for CaLCuV and CaMV in *Arabidopsis* (Blevins et al. 2006, 2011; Aregger et al. 2012). Interestingly, DCL3 contributes to the biogenesis of RNA virus-derived siRNAs in wild-type plants and more pronouncedly in *dcl2/4* double mutant plants (Deleris et al. 2006; Blevins et al. 2006; Bouche et al. 2006; Fusaro et al. 2006). This implies that DCL3 might move to the cytoplasm upon virus infection or, alternatively, dsRNA precursors might be transported to the nucleus for DCL3 processing. Likewise, nuclear localization of DCL4 and occurrence of PTGS in plant nuclei were reported (Hoffer et al. 2011), suggesting that the biogenesis of DCL4-dependent viral siRNAs might also occur in the nucleus.

A biochemical study using crude extracts from *Arabidopsis* has revealed that DCL3 prefers rather short dsRNA substrates and cannot efficiently process dsRNAs longer than 100 bp. Conversely, DCL4 prefers long dsRNAs and cannot efficiently process dsRNAs shorter than 100 bp (Nagano et al. 2013). This difference may depend on specific recognition of dsRNA precursors by distinct dsRNA-binding (DRB) family proteins (five *DRBs* in *Arabidopsis*). Thus, DRB4 is required for efficient DCL4-mediated processing of tasiRNAs from RDR6-dependent long dsRNA precursors (Vaucheret 2006). DRB3 was reported as a binding partner of DCL3, but it is not required for the biogenesis of DCL3-dependent viral siRNAs in geminivirus-infected plants (Raja et al. 2014).

DNA virus-infected plants display similar profiles of siRNA hotspots along the virus genome for each of the major size classes, suggesting that DCLs might compete for the same dsRNA substrates (Donaire et al. 2009; Blevins et al. 2011; Aregger et al. 2012; Rajeswaran et al. 2014a, b). Given distinct substrate specificities of DCLs, both long and short dsRNA precursors might generated from the siRNA hotspot regions. Relative abundance of distinct dsRNA precursors may correlate with relative abundance of siRNA size classes.

6.2.5 Methylation of Viral siRNAs by HEN1

Endogenous miRNAs and siRNAs are produced by DCLs as short duplexes with two-nucleotide 3′-overhangs. In *Arabidopsis*, both strands of these duplexes are then methylated by the methyltransferase HEN1 at 2′-hydroxyl of the 3′-terminal nucleotide. HEN1-mediated methylation protects mature miRNAs and siRNAs from degradation and oligouridylation (Li et al. 2005; Yang et al. 2006) and is possibly required for AGO loading and formation of active RISC (Csorba et al. 2007). Likewise, viral siRNAs accumulating in the geminivirus CaLCuV- and the pararetrovirus CaMV-infected *Arabidopsis* plants are methylated by HEN1 (Akbergenov et al. 2006; Blevins et al. 2006). The geminivirus ACMV-derived siRNAs are modified at the 3′-nucleotide in cassava and *N. benthamiana* by HEN1-like activities (Akbergenov

et al. 2006). In contrast, *Arabidopsis* and *N. benthamiana* plants infected with the RNA tobamovirus ORMV accumulate non-methylated viral siRNAs (Akbergenov et al. 2006; Blevins et al. 2006), likely because HEN1 activity is inhibited by a viral silencing suppressor that binds sRNA duplexes (Csorba et al. 2007; Kurihara et al. 2007). In addition to tobamoviruses, RNA potyviruses and tombusviruses also inhibit HEN1-like activity in *N. benthamiana* through the action of viral suppressor proteins (Lózsa et al. 2008). Notably, non-methylated siRNAs derived from RNA viruses accumulate without any apparent degradation or elongation by oligouridylation activity (Blevins et al. 2006; Lózsa et al. 2008), suggesting that viral siRNA binding to a suppressor protein may also be a stabilizing factor. Suppressor proteins of DNA viruses including geminiviral AC2 and AC4 and caulimoviral P6 are unable to bind sRNA duplexes (Chellappan et al. 2005; Wang et al. 2005; Shivaprasad et al. 2008), consistent with the methylated state of DNA virus-derived siRNAs.

6.2.6 Amplification of Antiviral Silencing: Primary and Secondary siRNAs

As argued above, RDR-independent primary siRNAs appear to play a major role in antiviral defense in infected cells. However, RDR-dependent secondary siRNAs may play an important role in restricting viral replication and spread to certain types of cells and tissues (Fig. 6.3). Viruses and viroids are generally excluded from shoot apical meristem, and RDR6 is required for meristem exclusion of an RNA potexvirus (Schwach et al. 2005) and a nuclear viroid (Di Serio et al. 2010) in *N. benthamiana*. Furthermore, suppressor-deficient RNA viruses are restricted to inoculated leaves but they can spread systemically and establish robust infection in *rdr6*, *sgs3*, or *dcl4* mutant plants (Deleris et al. 2006; Wang et al. 2011). Since DCL4, RDR6, and SGS3 (an RNA binding protein) are required for the biogenesis of tasiRNAs and secondary siRNAs from some miRNA-targeted mRNAs, the RDR6/SGS3/DCL4 pathway may generate secondary viral siRNAs and thereby prevent virus replication in certain plant cells and tissues. This pathway is also required for cell-to-cell spread of transgene-induced silencing (Himber et al. 2003; Dunoyer et al. 2010). Given the similarities in genetic requirements for the biogenesis of transgene-derived siRNAs and viral siRNAs (Fusaro et al. 2006), primary viral siRNAs produced from RDR-independent dsRNA precursors would target complementary viral transcripts for cleavage and the resulting cleavage products would enter the RDR6/SGS3/DCL4 pathway generating secondary siRNAs. Indirect evidence for involvement of the RDR6/SGS3/DCL4 pathway in the biogenesis of secondary viral siRNAs in DNA virus-infected plants was obtained in virus-induced gene silencing studies (Muangsan et al. 2004; Blevins et al. 2006; Fig. 6.3), but these viral secondary siRNAs accumulate at very low levels (Aregger et al. 2012). Recently, two alleles of an atypical *RDR* gene, an ortholog of the *Arabidopsis RDR 3a*, *3b*, and *3c* genes, were shown to confer resistance to the geminivirus tomato yellow leaf curl virus in tomato plants (Verlaan et al. 2013) and to contribute to the biogenesis of viral siRNAs (Butterbach et al. 2014).

Some viruses code for suppressor proteins that can overcome the RDR6-dependent mechanism of virus exclusion from the meristem. Thus, a suppressor activity of the TRV protein p16 allows the virus to transiently invade the shoot apical meristem early in viral infection (Martín-Hernández and Baulcombe 2008).

6.3 The Action of Viral siRNAs in Silencing

6.3.1 Virus-Induced Gene Silencing (VIGS)

VIGS phenomena provide perhaps the most convincing evidence for repressive action of virus-derived siRNAs. VIGS associated with both DNA and RNA viruses have been described (Lu et al. 2003; Robertson 2004), in which silencing was triggered either by wild-type viruses that have sequence homology to a transgene or an endogenous plant gene or recombinant viruses that harbor a sequence from an endogenous plant gene or a transgene. VIGS can be classified as posttranscriptional (PTGS-VIGS), when the gene transcribed region is homologous to the virus, and transcriptional (TGS-VIGS), when the gene promoter region is homologous to the virus. Thus, infections with wild-type geminivirus and pararetrovirus could silence transgenes driven by the geminivirus promoter (Seemanpillai et al. 2003) and the pararetrovirus promoter and/or terminator (Al-Kaff et al. 1998, 2000), respectively. Likewise, efficient VIGS could be achieved by using recombinant DNA viruses carrying the coding sequences of endogenous genes and transgenes (Peele et al. 2001; Turnage et al. 2002; Muangsan et al. 2004; Purkayastha et al. 2010) and the promoter or enhancer sequences of a transgene (Aregger et al. 2012).

Targeting host genes by wild-type virus-derived siRNAs can potentially be either a deliberate viral strategy to promote infection or an off-target effect of viral siRNAs that have a primary function in silencing cognate viral nucleic acids. The off-target effects of siRNAs derived from viruses, viral satellites, or viroids are likely to be more general and can potentially contribute to development of disease symptoms (Wang et al. 2004; Navarro et al. 2012). Thus, CMV Y-satellite-derived siRNAs target mRNA of the *Chlorata I* (*ChlI*) gene involved in chlorophyll biosynthesis for cleavage and degradation, which results in appearance of yellowing symptoms in CMV-infected tomato plants, owing to reduced accumulation of chlorophyll (Shimura et al. 2011; Smith et al. 2011). VIGS of the *ChlI* gene is not essential for replication and systemic spread of CMV or Y-satellite, but it may provide an advantage for the virus–satellite complex by promoting its insect transmission from plant to plant (Shimura et al. 2011). The deliberate targeting of host genes by viral siRNAs was proposed for CaMV, although this targeting appears to be independent of DCL (and hence viral siRNA) activities (Blevins et al. 2011).

Genetic studies of VIGS in *Arabidopsis* established that viral siRNAs can form active RISCs targeting cognate mRNA in the cytoplasm and cognate DNA in the nucleus. Thus, DCL4- and DCL2-dependent siRNAs derived from RNA virus vectors caused efficient knockdown of homologous plant mRNAs (Deleris et al. 2006). Analysis of *dcl* mutants infected with the geminivirus CaLCuV vector carrying the

ChlI gene fragment revealed that each of the four DCLs generates siRNAs capable of *ChlI* mRNA knockdown (Blevins et al. 2006). Notably, the *ChlI* mRNA knockdown did not require RDR6 or RDR2 activities, which correlated with unaltered production of 21, 22, and 24 nt siRNAs from the *ChlI* sequence inserted in CaLCuV. Nonetheless, total silencing of the *ChlI* gene in newly growing tissues of infected plants required RDR6 as well as SGS3, DCL4, and HEN1 activities (Muangsan et al. 2004; Blevins et al. 2006). Thus, the RDR6/SGS3/DCL4 pathway appears to be involved in amplification of the *ChlI* insert-derived siRNAs and, perhaps, in their systemic spread in new growth including shoot apical meristem where total silencing of the *ChlI* gene expression is thought to be established (Blevins et al. 2006). By analogy, systemic spread of viral siRNAs may immunize the meristematic cells against incoming virus (Fig. 6.3). Interestingly, only very low amounts of secondary siRNAs derived from the *Chl* gene sequences outside of the CaLCuV::*ChlI* VIGS target region could be detected (Aregger et al. 2012). In contrast, abundant RDR6-dependent secondary siRNAs were produced from a *GFP* transgene targeted by CaLCuV::*GFP* virus-derived primary siRNAs (Aregger et al. 2012). This indicates that cleavage products of transgenic *GFP* mRNA are good substrates for RDR6 which generates dsRNA precursors of secondary siRNAs. On the other hand, *ChlI* mRNA and geminiviral mRNAs appear to have evolved to be resistant to RDR6-dependent siRNA amplification (Aregger et al. 2012).

Infections with the wild-type pararetrovirus CaMV can cause both TGS and PTGS of transgenes carrying the CaMV 35S promoter or terminator sequences, respectively (Al-Kaff et al. 1998, 2000). Interestingly, despite effective TGS of the 35S promoter-driven transgenes, Pol II transcription of CaMV minichromosomes in the nucleus was not affected (Al-Kaff et al. 1998, 2000), suggesting that the virus evades TGS. The evasion of TGS correlates with very low levels of viral siRNAs derived from the 35S promoter/enhancer region of CaMV genome (Blevins et al. 2011). It remains to be investigated whether these low levels of siRNAs are sufficient for TGS, or TGS-VIGS is siRNA-independent. Using a geminivirus CaLCuV derivative carrying the CaMV 35S promoter or enhancer sequences, efficient TGS-VIGS of the 35S-GFP transgene was associated with high levels of virus-derived primary siRNAs targeting the promoter/enhancer sequence and barely detectable levels of secondary siRNAs (Aregger et al. 2012). It remains to be investigated if primary viral siRNAs can direct cytosine methylation in this system.

6.3.2 Association of Viral siRNAs with AGO Proteins

In plant and animals, RNA silencing (also known as RNA interference) is directed by a small RNA (miRNA, siRNA, or piRNA) associated with an AGO/PIWI family effector protein (Cenik and Zamore 2011). The plant genomes encode multiple AGOs with diversified functions in endogenous silencing pathways. Ten *AGO* genes of *Arabidopsis thaliana* fall into three clades, *AGO1*/*AGO5*/*AGO10*, *AGO2*/*AGO3*/*AGO7*, and *AGO4*/*AGO6*/*AGO8*/*AGO9*, and all these AGOs possess a catalytic motif required for slicer activity, i.e., sRNA-directed cleavage of

complementary target RNA (Tolia and Joshua-Tor 2007; Mallory and Vaucheret 2010; Carbonell et al. 2012). The *AGO1/5/10* and *AGO2/3/7* clades have been implicated in the cytoplasmic miRNA and tasiRNA pathways, whereas the *AGO4/6/8/9* clade in the nuclear RdDM and TGS pathways. AGOs sort endogenous sRNAs based on size, 5′-terminal nucleotide, and, in some cases, other sequence features. 21/22-nt RNAs with 5′-terminal uridine (5′U), which is characteristic of most miRNAs, are predominantly bound to AGO1, 21/22-nt RNAs with 5′-terminal adenosine (5′A) to AGO2; 21/22-nt RNAs with 5′- terminal cytosine (5′C) to AGO5; and 24-nt 5′A-RNAs to AGO4, AGO6, and AGO9, and 24-nt RNAs with 5′G, 5′U, and 5′C constitute smaller fractions of AGO4-bound sRNAs (Montgomery et al. 2008; Mi et al. 2008; Takeda et al. 2008; Havecker et al. 2010). AGO7 is specifically associated with miR390 (21-nt 5′A-RNA) to mediate the biogenesis of *TAS3* family tasiRNAs (Montgomery et al. 2008; Rajeswaran and Pooggin 2012a).

It is expected that viral siRNAs can be associated with multiple AGOs, because siRNA populations derived from DNA and RNA viruses, viral satellites, and viroids cover the entire viral/viroid genomes in both orientations and comprise 5′U-, 5′A-, 5′G-, and 5′C-siRNAs of the three size classes (21-nt, 22-nt, and 24-nt) (Donaire et al. 2009; Kreuze et al. 2009; Yang et al. 2011; Blevins et al. 2011; Aregger et al. 2012; Seguin et al. 2014; Rajeswaran et al. 2014a, b). Indeed, siRNAs derived from RNA viruses associate with AGO1, AGO2 and AGO5 (Zhang et al. 2006; Takeda et al. 2008; Azevedo et al. 2010; Harvey et al. 2011; Wang et al. 2011). Genetic evidence implicates AGO1, AGO2, and AGO7 in defense against RNA viruses. Thus, *ago1* and *ago2* mutants are hypersusceptible to wild-type CMV infection (Morel et al. 2002; Harvey et al. 2011). Suppressor-deficient CMV could establish systemic infection only on *ago1* and *ago2* plants but not on any other *ago* mutant plants; testing double and triple *ago* mutants also did not reveal contribution of other AGOs in this case (Wang et al. 2011). For suppressor-deficient TCV, the systemic infection was observed not only on *ago1* but also on *ago7* mutant plants (Qu et al. 2008).

Growing evidence indicates that AGO2 is a primary effector protein that binds viral siRNAs and exerts cleavage of viral RNAs (Harvey et al. 2011; Scholthof et al. 2011; Wang et al. 2011). Indeed, AGO2 strongly binds viral siRNAs of both sense and antisense polarities, while AGO1 shows rather loose association with viral siRNAs of only sense polarity (Wang et al. 2011). Furthermore, the catalytic residues of AGO2 are required for restriction of both local and systemic infection by suppressor-deficient TuMV (Carbonell et al. 2012). AGO2 can replace AGO1 in miRNA-directed cleavage of target RNA but not in triggering RDR6-dependent production of secondary siRNA from the cleavage products (Carbonell et al. 2012). Consistent with the latter finding, the antiviral activity of AGO2 in restricting wild-type virus infections (Harvey et al. 2011) is not associated with production of secondary siRNAs. Since accumulation of RDR6-dependent secondary siRNAs from suppressor-deficient CMV is increased in *ago1*, *ago2*, and *ago1*/*ago2* mutant plants (Wang et al. 2011), presumed cleavage of viral RNA by viral siRNA-AGO2 (or AGO1) RISCs is not required for the recruitment of RDR6 activity. This implies that suppressor-deficient viruses produce aberrant transcripts for RDR6 in a viral

siRNA-independent manner. These findings also show that the dicing step itself is not sufficient to restrict RNA virus replication and systemic spread (Wang et al. 2011).

AGO1 can exert its antiviral activity either directly or indirectly, i.e., through association with viral or endogenous sRNAs, respectively. Thus, miR403-AGO1 RISC targets AGO2 mRNA for cleavage and degradation, which reduces AGO2 protein accumulation (Harvey et al. 2011). Suppressor proteins of some RNA viruses interfere with AGO1 activity (see below), which would result in increased accumulation of AGO2, as shown in plants infected with wild-type CMV and TCV (Harvey et al. 2011). However, increased accumulation of AGO2 protein in *ago1* plants (Harvey et al. 2011) does not appear to compensate for the diminished activity of AGO1, since titers of suppressor-deficient CMV in *ago1* were even higher than those in *ago2* plants (Wang et al. 2011).

Little is known on involvement of AGOs in defense against DNA viruses. None of the ten single *ago* mutant lines exhibited increased susceptibility to the pararetrovirus CaMV or the geminivirus CaLCuV infection (R. Rajeswaran and M.M.P., unpublished data). CaMV-derived 21-nt siRNAs can bind AGO1 in *Arabidopsis* (Blevins et al. 2011). Surprisingly, despite massive production of CaMV 24-nt siRNAs (from the pgRNA leader region), only negligible amounts thereof bind AGO4 (Blevins et al. 2011). Consistent with this finding, *ago4* mutant plants did not exhibit increased susceptibility to CaMV and did not accumulate higher levels of viral DNA than wild-type plants (Blevins et al. 2006). Since the CaMV promoter region (upstream of the leader) spawns very little quantities of viral siRNAs (Blevins et al. 2011), those may not be sufficient to direct viral DNA methylation and TGS. Furthermore, 21-nt viral siRNAs bound to AGO1 may not be able to target viral RNA, because the CaMV pgRNA leader sequence from which the majority of 21-24 nt viral siRNAs are derived is highly structured and may therefore be inaccessible to the AGO1-RISC or a presumable AGO2-RISC (Blevins et al. 2011). Likewise, siRNAs derived from dsRNA replicative intermediates of viroids may not be able to target their highly structured circular genomic RNA for cleavage and degradation (Itaya et al. 2007).

For DNA geminiviruses and nanoviruses, AGO-bound siRNAs were not reported so far. CaLCuV infection and DNA accumulation did not differ between wild-type and *ago4* mutant plants (Blevins et al. 2006). In another study, *ago4* mutant plants infected with CaLCuV and beet curly top virus (BCTV) exhibited increased symptom severity in the inflorescence tissues compared to wild-type plants (Raja et al. 2008). Furthermore, unlike wild-type *Arabidopsis*, *ago4* mutant plants infected with suppressor-deficient BCTV (lacking C2) could not recover from virus infection. The recovery phenotype well correlated with hypermethylation of residual viral DNA in the intergenic region (Raja et al. 2008). However, several lines of evidence indicate that viral DNA methylation is AGO4- and viral siRNA-independent and that circular viral dsDNA that serves as a template for Pol II transcription evades de novo methylation owing to rolling circle replication; the only viral DNA form that gets methylated substantially is heterogeneous linear dsDNA, a product of recombination-dependent replication (Paprotka et al. 2011; reviewed

by Pooggin 2013). Consistent with evasion of siRNA-directed DNA methylation and TGS, episomal circular dsDNA of banana streak virus (pararetrovirus) was found to be unmethylated in persistently infected *Musa acuminata* banana plants (Rajeswaran et al. 2014b).

Interestingly, transgenes expressing inverted repeats of a geminivirus intergenic region facilitate plant recovery from infection with the cognate geminivirus (Vanderschuren et al. 2007; Hagen et al. 2008). Likewise, transient expression of dsRNA cognate to the intergenic region of mungbean yellow mosaic virus could cure plant from the viral infection (Pooggin et al. 2003). This suggests that high quantities of artificial siRNAs or, alternatively, long dsRNA cognate to the geminivirus promoter have the potential to interfere with viral replication.

6.3.3 Evidence for Targeting of Viral RNA by Viral siRNA-AGO Complexes

Several studies have investigated if viral siRNAs can direct cleavage of cognate viral RNA. Thus, viral RISC complexes were isolated from virus-infected plants and tested in vitro (Omarov et al. 2007; Ciomperlik et al. 2011). Antiviral RISCs were also reconstituted using exogenous siRNAs and recombinant AGOs in tobacco extracts that support replication of viral RNA (Schuck et al. 2013). The latter study has established that RISCs reconstituted from AGO1, AGO2, AGO3, and AGO5 (but not other AGOs) can target viral RNA for cleavage and thereby inhibit viral replication. Interestingly, the minus strand serving a template for viral plus stand replication (Fig. 6.2) was protected from RISC activity, whereas the plus strand that accumulates at much higher levels was accessible for RISC-mediated cleavage. Furthermore, only unstructured regions of viral plus strands were accessible for RISC-mediated cleavages (Schuck et al. 2013). These findings illustrate silencing evasion strategies evolved by RNA viruses.

Comparison of viral RNA degradome and viral siRNA hotspot profile in the context of viral infection generally reveals little correlation between most frequent cleavage events and highly abundant viral siRNA species (Pantaleo et al. 2007; Miozzi et al. 2013). This implies that cleavages of viral RNA are directed by a few viral siRNA species of low abundance, while the majority of viral siRNAs are inactive. Thus, the action of viral siRNA-AGO complexes can be either suppressed or evaded.

6.4 Viral Strategies of Silencing Suppression and Evasion

Both core and axillary proteins required for viral replication, encapsidation, and movement have been implicated in suppression of RNA silencing. Given distinct nature of these proteins, molecular mechanisms of silencing suppression must be different. Furthermore, single viral proteins were reported to suppress silencing by two and more different mechanisms. The following mechanisms of PTGS

suppression have been proposed for suppressor proteins of RNA viruses (reviewed in Burgyan and Havelda 2011; Incarbone and Dunoyer 2013; Pumplin and Voinnet 2013):

(a) TCV CP (p38) and aureusvirus p14 bind long dsRNA, which would inhibit DCL4 catalyzed processing of viral siRNA precursors.
(b) TCV CP, CMV 2b, tombusvirus p19, potyvirus HCPro, and tobamovirus p122/p126/p130 bind and sequester siRNA duplexes, which would interfere with HEN1 methylation, RISC assembly, and cell-to-cell movement of viral siRNAs.
(c) Closterovirus RNase3 degrades siRNA duplexes.
(d) Polerovirus P0 and potexvirus p25 target AGO1 and possibly other AGO proteins for proteosome degradation.
(e) TCV CP, CMV 2b, and ipomovirus P1 bind AGO1 and thereby inactivate preassembled AGO1-siRNA RISC.
(f) Tombusvirus p19 and perhaps other suppressors induce overaccumulation of miR168 that represses translation of AGO1 mRNA.

It should be noted that many of these mechanisms were investigated in artificial experimental systems and/or with isolated viral proteins. It will be necessary to validate which of the above mechanisms operate in the context of wild-type virus infection in susceptible hosts.

Interestingly, DNA viruses have evolved the mechanisms of silencing suppression and evasion that differ from those evolved by RNA viruses (reviewed by Raja et al. 2010; Pooggin 2013). This reflects the differences in replication cycles of DNA and RNA viruses: DNA viruses can evade the cytoplasmic PTGS pathways by transcribing their DNA in the nucleus. Moreover, the replication mechanisms evolved by DNA viruses (rolling circle replication of gemini- and nanoviruses and reverse transcription of pararetroviruses) help them evade the nuclear RdDM and TGS (reviewed by Pooggin 2013). Nonetheless, DNA viruses possess suppressor proteins that either directly or indirectly interfere with both PTGS and TGS. Thus, geminiviral nuclear protein AC2 can suppress PTGS (Dong et al. 2003; Trinks et al. 2005; Wang et al. 2005), likely by activating transcription of plant genes that negatively regulate RNA silencing (Trinks et al. 2005). Begomoviral AC4 binds single-stranded sRNAs but not siRNA or miRNA duplexes (Chellappan et al. 2005). However, it is unclear how AC4 may exert its PTGS suppression activity (Vanitharani et al. 2004), since single-stranded sRNAs are tightly associated with AGO proteins and are not known to exist in a free form. Begomoviral protein V2 interacts with SGS3 (Glick et al. 2008), which may prevent RDR6/SGS3/DCL4-dependent siRNA amplification and spread of antiviral silencing. V2 and SGS3 both interact with dsRNA carrying a long 5′-overhang and V2 outcompetes SGS3 in binding this substrate (Fukunaga and Doudna 2009).

In addition to suppressing PTGS, geminiviral AC2 homologs were also reported to reverse TGS and interfere with endogenous DNA methylation pathways. Surprisingly, the host components targeted by the AC2 homologs from different

geminiviruses include the cytoplasmic enzymes adenosine kinase (Wang et al. 2005), S-adenosyl-methionine (SAM) decarboxylase 1 (Zhang et al. 2011), and S-adenosyl homocysteine hydrolase (Yang et al. 2011), all involved in the methyl cycle generating SAM, a donor of methyl groups (Raja et al. 2010). Since these host proteins are not absolutely essential for RdDM, their inactivation may interfere with other pathways involving protein or nucleic acid methylation, which indirectly regulate TGS and PTGS. Emerging evidence indicates that direct suppression of RdDM and TGS might promote plant defenses through activation of defense genes repressed by TGS (reviewed in Pumplin and Voinnet 2013). Plants may respond to viral infection by hypomethylating the genome through the action of DNA demethylases (reviewed by Pooggin 2013), which would activate defense genes repressed by TGS. Indeed, global DNA hypomethylation and reversal of TGS were recorded in plants expressing the geminiviral AC2 and the replication initiator protein (Buchmann et al. 2009; Rodríguez-Negrete et al. 2013). Interestingly, viroid infection also leads to reversal of plant DNA methylation (Martinez et al. 2014), indicating that DNA demethylation is a more general plant response to pathogen infection.

In pararetroviruses, only the CaMV P6 protein has so far been established as a silencing suppressor. Mechanistically, P6 interferes with processing of RDR6-dependent dsRNA by DCL4 (Love et al. 2007; Haas et al. 2008; Shivaprasad et al. 2008). P6 does not bind long dsRNA (P.V. Shivaprasad, J. Burgyan, and M.M.P.; unpublished data) and, in line with this finding, does not interfere with silencing induced by RDR6-independent dsRNA expressed from an inverted-repeat transgene (Shivaprasad et al. 2008). Instead, P6 interacts with dsRNA-binding protein DRB4, a partner of DCL4 (Haas et al. 2008). Notably and in contrast to many RNA viruses, CaMV infection and P6 protein expression do not affect the accumulation or activity of AGO1, AGO2, or AGO7 (Blevins et al. 2011; Rajeswaran et al. 2012; Rajeswaran and Pooggin 2012a).

Some pararetrovirus genera do not possess a P6 homolog (Hull 2007), suggesting that other viral proteins may have antisilencing activity. Alternatively, these viruses may employ a different strategy to evade silencing. PgRNA of most plant pararetroviruses has a long leader sequence preceding the first large viral ORF; this sequence folds into a stable secondary structure bypassed by scanning ribosome during the initiation step of pgRNA translation (Pooggin et al. 1999, 2008; Ryabova et al. 2002). In CaMV, massive production of 21-, 22-, and 24-nt viral siRNAs of both sense and antisense polarities, comparable to the entire complement of the host siRNAs and miRNAs, is confined to the 600 bp leader region, while other regions of the 8 kbp CaMV genome spawn only small amounts of viral siRNAs. However, *dcl1/2/3/4* quadruple mutant plants, in which DCL-mediated production of viral siRNAs is nearly abolished, do not exhibit increased susceptibility to CaMV infection. These findings and other lines of evidence suggest that massive production of the leader-derived siRNAs serves as a decoy diverting the silencing machinery from the promoter and protein-coding regions of the CaMV genome (Blevins et al. 2011; Fig. 6.4). The decoy model implies that a large and stable stem-loop structure of the pgRNA leader in pararetroviruses has evolved to be (1) a good substrate for Pol II that generates dsRNA engaging all DCLs in massive production of siRNAs and (2)

a poor target for AGO-RISCs charged by viral siRNAs of antisense polarity. Acquisition of the P6 gene is a later event in the evolution of plant pararetroviruses, which enables a more efficient mechanism of polycistronic translation of pgRNA (reinitiation vs. leaky scanning; Ryabova et al. 2002) and ensures that limited, siRNA-directed cleavage of pgRNA or other mechanisms generating viral aberrant RNAs do not trigger siRNA amplification by the RDR6/SGS3/DCL4 pathway. Furthermore, P6 interferes with innate immunity-based plant defenses (Love et al. 2012; Zvereva and Pooggin 2012). Evidence for a similar decoy strategy in the plant pararetrovirus genera lacking a *P6* gene has been obtained (Rajeswaran et al. 2014a, b). Interestingly, an RNA decoy strategy was also evolved by human adenovirus that expresses short, highly structured viral RNAs to suppress the interferon-mediated antiviral defense and sequester Dicer (Andersson et al. 2005).

Recent evidence suggests that pararetroviruses may evade silencing with a help of CP/GAG protein that specifically binds pgRNA to initiate packaging and reverse transcription. Thus, highly abundant 21-nt siRNAs derived from a retrotransposon *Evade* activated in certain epigenetic mutants of *Arabidopsis* are generated by the RDR6/DCL4-dependent mechanism and loaded onto AGO1 and AGO2. However, these siRNAs are not effective in silencing the retrotransposon transcript, because PTGS is evaded via GAG protein-mediated packaging of the transcript into virus-like particles (Marí-Ordóñez et al. 2013). By analogy, pararetroviral CP/GAG might also protect the viral pgRNA from viral siRNA-directed cleavage and/or translational repression by forming a previrion in which reverse transcription takes place. This analogy can also be extended to RNA viruses whose single-stranded or double-stranded RNA genomes are encapsidated into virions which can effectively protect viral RNA from RISC or DCL activities, respectively.

6.5 A Concert Action of RNA Silencing and Innate Immunity in Antiviral Defense

Innate immunity-based responses play a major role in defense against nonviral pathogens. Transmembrane receptor proteins at the frontline of this defense mediate pattern-triggered immunity (PTI) upon recognition of the conserved, microbe-associated molecular patterns (MAMPs), and/or the host dander-associated molecular patterns (DAMPs). As a counter-defense, microbes inject in the plant cell a number of effector proteins that block PTI-based responses. In resistant hosts some of these effectors are recognized by intracellular receptor proteins of the nucleotide binding-leucine rich repeat (NB-LRR) family, which trigger hypersensitive response (HR) and programmed cell death (PCD), collectively called effector-triggered immunity (ETI) (Boller and Felix 2009; Bonardi et al. 2012). Growing evidence indicates that both PTI and ETI responses are regulated by RNA silencing pathways. Conversely, PTI and ETI contribute to antiviral defense (reviewed in Zvereva and Pooggin 2012). Silencing suppressor proteins encoded by some RNA viruses are recognized the NB-LRR immune receptors, which triggers HR and PCD and prevents virus escape from the inoculated cells. Several lines of evidence suggest that

the action of virulence factors (suppressor/effector proteins) in suppression of both RNA silencing and PTI, manifested as successful viral infections in susceptible hosts, is monitored by the immune system. This monitoring leads to ETI-like responses in susceptible hosts and HR and PCD in resistant hosts. The ETI in resistant hosts can eventually be overcome by resistant-breaking strains that acquire new effectors to suppress ETI. According to an extended zig-zag-zig model (Jones and Dangl 2006; Zvereva and Pooggin 2012), these defense, counter-defense, and counter-counter-defense activities manifest an ongoing arms race between the viral pathogens and their host plants at the evolutionary scale. Interestingly, a large number of the *NB-LRR* resistance genes, which mediate not only ETI but also PTI responses, are under the repressive control of miRNAs and RDR6-dependent phasiRNAs and this repression mechanism is conserved across plant kingdom, including *Medicago*, *Solanum*, *Nicotiana*, and *Arabidopsis* (Zhai et al. 2011; Shivaprasad et al. 2012; Li et al. 2012; Boccara et al. 2014). In *Solanum lycopersicum*, miR486 targets several *NB-LRR*s and the action of this miRNA is blocked by viral and bacterial infections. This suggests that the plant defense system exploits the suppressor activity of viral and bacterial effectors for pathogen-inducible expression of NB-LRR proteins (Shivaprasad et al. 2012). In *Arabidopsis thaliana*, AGO1-miR472 and RDR6-dependent downregulation of a subset of *NB-LRR* genes contributes to repression of PTI, which accounts for an increased basal resistance to bacterial pathogens in *rdr6* and *mir172* mutant lines (Boccara et al. 2014). Viral suppressors of silencing that interfere with AGO1- and/or RDR6-dependent secondary siRNA pathways are expected to enhance both ETI- and PTI-based responses triggered by NB-LRRs. The latter might compensate for the suppression of RDR6-dependent production of secondary viral siRNAs, which would explain the abovementioned observations that *rdr6* mutant plants do not exhibit increased susceptibility to many wild-type viruses.

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