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CHAPTER 5

Involvement of the Plant Nucleolus in Virus and Viroid Infections: Parallels with Animal Pathosystems

M. E. Taliansky,* **J. W. S. Brown,*,†** **M. L. Rajamäki,‡** **J. P. T. Valkonen,‡** and **N. O. Kalinina§**

Contents

I. Introduction 121
II. Structure and Functions of the Nucleolus 123
III. What we have Learned about Interactions of Viruses with the Nucleolus from Animal Virology 128
A. Viruses that replicate in the nucleus 129
B. Viruses replicating in cytoplasm 129
IV. Nucleolar Functions of Plant Virus Proteins and Viroids 132
A. Nucleolus in replication of viroids and viruses 134
B. The nucleolus and plant virus movement 135
C. Nucleolar targeting for interference with host antiviral defense 140
D. Nucleolar localization of viral proteins for as yet unknown reasons 143
E. Formation of viral ribonucleoprotein complexes (RNPs) in the nucleolus 145
V. Conclusions and Perspectives 146
Acknowledgments 147
References 147

* Scottish Crop Research Institute, Invergowrie, Dundee, United Kingdom
† Plant Sciences Division, University of Dundee, United Kingdom
‡ Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland
§ A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia

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Abstract The nucleolus is a dynamic subnuclear body with roles in ribosome subunit biogenesis, mediation of cell-stress responses, and regulation of cell growth. An increasing number of reports reveal that similar to the proteins of animal viruses, many plant virus proteins localize in the nucleolus to divert host nucleolar proteins from their natural functions in order to exert novel role(s) in the virus infection cycle. This chapter will highlight studies showing how plant viruses recruit nucleolar functions to facilitate virus translation and replication, virus movement and assembly of virus-specific ribonucleoprotein (RNP) particles, and to counteract plant host defense responses. Plant viruses also provide a valuable tool to gain new insights into novel nucleolar functions and processes. Investigating the interactions between plant viruses and the nucleolus will facilitate the design of novel strategies to control plant virus infections.

ABBREVIATIONS

AGO	argonaute protein
BiFC	bimolecular fluorescence complementation
CaMV	cauliflower mosaic virus
CB	Cajal body
CBL	Cajal body-like structure
CCCVd	coconut cadang cadang viroid
CMV	cucumber mosaic virus
CP	coat protein
CP-RT	coat protein-read through portion
DCL	Dicer-like ribonuclease
DEAD box protein	Asp-Glu-Ala-Asp family protein
DFC	dense fibrillar component
DRB	dsRNA-binding protein
FC	fibrillar centers
GRV	groundnut rosette virus
GAR	glycine and arginine rich domain
GFP	green fluorescent protein
GC	granular component
HC-Pro	helper-component proteinase
HIV	Human immunodeficiency virus
IFN	interferon
IBV	infectious bronchitis virus
IRES	internal ribosome entry site
MFSV	maize fine streak virus

MP	movement protein
miRNA	microRNA
NIa (b)	nuclear inclusion protein a (b)
NES	nuclear export signal
NLS	nuclear localization signal
NMD	nonsense-mediated decay
NoLS	nucleolar localization signal
NS1	nonstructural protein 1
ORF	open reading frame
PABP	poly(A) binding protein
PD	plasmodesmata
PLRV	potato leafroll virus
PMTV	potato mop top virus
pre-rRNA	precursor rRNAs
PSLV	poa semilatent virus
RDRP	RNA-directed RNA polymerase
PSTVd	potato spindle tuber viroid
PVA	potato virus A
RNP	ribonucleoprotein
rRNA	ribosomal RNA
sDMAs	symmetric di-methyl arginines
SMN	survival motor neuron gene product
snRNA	small nuclear RNA
siRNA	small interfering RNA
snoRNA	small nucleolar RNA
SPMV	satellite panicum mosaic virus
SRP	signal recognition particle
TEV	tobacco etch virus
TGB	triple gene block
ToLCJAV	tomato leaf curl Java virus associated with DNA β satellite
TuMV	turnip mosaic virus
TYLCV	tomato yellow leaf curl virus
VIRP1	viroid RNA binding protein 1
VPg	viral genome-linked protein

I. INTRODUCTION

The interactions between a virus and its host cell play a central role in the viral infection cycle. The analysis of virus–host interactions is critical for understanding the mechanisms of viral infections and for the development of novel antiviral strategies. Viruses are obligate intracellular pathogens with small genomes and, therefore, are reliant on subverting of the cellular

functions and machineries to facilitate their own replication. The cell nucleus is one of the key features of eukaryotic cells. It is a highly dynamic, membrane-bound organelle that hosts major cellular events, including DNA replication, messenger RNA synthesis and processing, and ribosome subunit biogenesis (Trinkle-Mulcahy and Lamond, 2007). Given the pivotal role of the nucleus in cell host function, it is not surprising that viruses interact with this organelle and its compartments, and that such interactions play a crucial role in the virus infection cycle. Indeed, certain viruses including plant DNA containing begomoviruses (Rojas *et al.*, 2001; Sharma and Ikegami, 2009), RNA reverse-transcribing caulimoviruses (CaMVs) (Haas *et al.*, 2005), and negative-strand RNA rhabdoviruses belonging to the genus *Nucleorhabdovirus* (Tsai *et al.*, 2005) replicate in the nucleus, and therefore it makes sense that these viruses modify nuclear structures to usurp some of the nuclear functions and provide an appropriate environment for their own replication. In contrast, single-stranded positive-sense RNA [(+)ssRNA] viruses replicate in the cytoplasm. Therefore, the rational for RNA viruses targeting the nucleus and its compartments is not so evident. However, an increasing number of reports clearly show that cytoplasmic RNA viruses including plant viruses can also target nuclear compartments and, nucleoli, in particular (reviewed by Hiscox 2002, 2007; Greco, 2009). Thus, investigating why and how plant RNA viruses interact with the nucleolus to meet their own needs will contribute to the better understanding of molecular biology of plant viruses and facilitate the design of novel strategies for virus control. These studies may also teach us about the fundamental principles of plant nucleolar structure and functions.

The nucleolus is a prominent subnuclear compartment formed around the clusters of genes (rDNA) coding for ribosomal RNA (rRNA), and is the site of rDNA transcription, rRNA processing, and ribosome assembly (Olson, 2004; Rubbi and Milner, 2003; Sirri *et al*., 2008). Indeed, for many years the exclusive function of the nucleolus was thought to be rRNA synthesis and ribosome biogenesis. However, several insights from the past decade have dramatically changed this traditional view and implicated the nucleolus in many other aspects of cell function such as cell-cycle regulation, gene silencing, telomerase activity, senescence, stress responses, and biogenesis of multiple ribonucleoprotein (RNP) particles (Boisvert *et al*., 2007; Olson and Dundr, 2005; Sirri *et al*., 2008).

This chapter briefly reviews the structure and composition of the nucleolus and, subsequently, data implicating the nucleolus in the infection cycles of animal and plant viruses and also viroids. Most of the current knowledge on nucleolar localization and functions of viral proteins has been gained in studies on animal viruses and has also been reviewed comprehensively (Greco, 2009; Hiscox, 2002, 2007). Therefore, the main emphasis of this review will be on what is known about the

different aspects of interactions between plant virus proteins and the nucleolus, of which the functional significance in control of virus movement and interference with host antiviral defense has started to appear only recently. We also discuss recent findings on the potential role of Cajal bodies (CBs), another type of small subnuclear bodies structurally and functionally associated with the nucleolus.

II. STRUCTURE AND FUNCTIONS OF THE NUCLEOLUS

The nucleus is a highly structured organelle responsible for chromosome organization, replication and division, for gene expression and regulation, and the integration of a vast array of activities required for cellular function. The nucleus contains chromatin-rich regions, made up of condensed heterochromatin, more dispersed euchromatin, and interchromatin regions, and chromatin organization is responsible for regulating gene expression, DNA replication, and chromosome segregation (Schneider and Grosscheld, 2007; Trinkle-Mulcahy and Lamond, 2008). In addition, it contains many other structures or bodies of different numbers and sizes which vary between cell types, at different stages in the cell cycle and under different conditions. The most prominent of these structures is the nucleolus but many other bodies [e.g., CBs, splicing speckles, paraspeckles, PML (pro-myeloid leukemia) bodies, etc.] have now been identified and are being characterized on the basis of their protein and RNA components and functions (Boisvert *et al.*, 2007; Cioce and Lamond, 2005; Lamond and Spector, 2003; Matera *et al.*, 2009; Rippe, 2007).

The nucleolus is where rRNA genes are transcribed and processed, and rRNAs are assembled, along with ribosomal proteins, into the small and large subunits of the ribosome (Boisvert *et al.*, 2007; Fatica and Tollervey, 2002; Granneman and Baserga, 2004). The nucleolus contains three different regions on the basis of their appearance in the transmission electron microscope: fibrillar centers (FC), the dense fibrillar component (DFC), and the granular component (GC). In the mammalian nucleolus, the FCs are small, lightly staining structures surrounded by the more densely stained DFC. The DFC in turn is surrounded a particulate region—the GC. The plant nucleolus tends to be more regular in its structure (usually near to spherical) than animal nucleoli. The organization of the nucleolar regions is different in plant nucleoli in that the DFC is not as densely stained as in animal nucleoli and occupies much more of the nucleolar volume (up to 70%). Transcription of precursor rRNAs (pre-rRNAs) occurs at multiple sites (200–400) within the DFC regions and pre-rRNAs undergo processing in the DFC and GC. Indeed, the localization of pre-rRNAs and different small nucleolar RNAs (snoRNAs) and proteins has allowed early and late pre-rRNA cleavage events to be

correlated to the DFC and GC, respectively, suggesting a vectorial model for the production and maturation of rRNAs. Plant nucleoli also often contain a prominent central region called the nucleolar cavity (Brown and Shaw, 1998; Shaw and Brown, 2004). The function of the nucleolar cavity is currently unknown, and it remains to be seen whether the differences in organization of plant and animal nucleoli influence the type and range of other functions which nucleoli carry out and interactions with different viruses.

The major function of the nucleolus is the transcription and processing of precursor rRNAs and ribosomal subunit assembly. The dynamic assembly pathway involves a series of intermediate pre-ribosomal complexes of the 40S and 60S ribosomal subunits and in addition to ribosomal proteins, requires up to 200 accessory proteins (Grannemann and Baserga, 2004). Processing of the pre-rRNAs involves both cleavage of the precursor transcript and nucleotide modifications, the majority of which are 2′-*O*-ribose methylation and pseudouridylation. Some cleavage reactions and the modifications require snoRNPs. SnoRNPs consist of a snoRNA and associated proteins (Kiss, 2002). By the nature of the major function in rRNA production and ribosomal subunit assembly, there is a huge flux of proteins and RNA complexes into and out of the nucleolus. The highly dynamic movement of proteins and complexes has been illustrated in animal cells by quantitative nucleolar proteomic analyses (Andersen *et al.*, 2005; Lam *et al.*, 2007). In particular, ribosomal proteins are highly expressed and rapidly accumulate in the nucleolus where they are incorporated into ribosomal subunits or rapidly degraded (Lam *et al.*, 2007).

Three of the most abundant and well-studied nucleolar proteins are fibrillarin, nucleolin, and B23. Fibrillarin, one of the major proteins of the nucleolus, is a core component of box C/D snoRNPs and is required for rRNA processing (Venema and Tollervey, 1999). Fibrillarin has methyltransferase activity directing 2′-*O*-ribose methylation of rRNA (Barneche *et al.*, 2000; Cioce and Lamond, 2005; Matera and Shpargel, 2006). Fibrillarin is highly conserved in sequence, structure, and function in eukaryotes. The N-terminal region of fibrillarin comprises a glycine- and arginine-rich (GAR) domain (Barneche *et al.*, 2000). The GAR domain is methylated at arginine residues (Liu and Dreyfuss, 1995) and is responsible for interactions with various proteins including the survival motor neuron (SMN) gene product (Jones *et al.*, 2001) and the nuclear DEAD (Asp-Glu-Ala-Asp) box protein p68 (Nicol *et al.*, 2000). Fibrillarin contains a centrally located RNA-binding domain which together with the C-terminal helix domain and the intervening spacer constitutes a methyltransferase-like domain that contains an *S*-adenosyl methionine binding motif and is responsible for fibrillarin methyltransferase activity (Wang *et al.*, 2000a). The C-terminal helix domain appears to target fibrillarin to CBs (Snaar *et al.*, 2000). Although it is well established that fibrillarin plays a role in ribosome biogenesis within the nucleolus, its role in CBs is not

well understood but it is presumably responsible for the 2′-*O*-ribose methylation of small nuclear RNAs (snRNAs).

Nucleolin is an abundant, ubiquitously expressed protein, which is highly phosphorylated, methylated, and also can be ADP-ribosylated (Ginisty *et al.*, 1999). Nucleolin is found in various cell compartments, and it is especially abundant in the nucleolus. Nucleolin has three well-defined domains. The N-terminal domain with alternating acidic and basic stretches is involved in rDNA transcription by interacting with rDNA repeats and histone H1 and in nuclear localization. The central portion is the RNA-binding domain, whereas the C-terminal part contains a GAR domain involved in interaction with the ribosomal proteins (Tuteja and Tuteja, 1998). Nucleolin is involved in ribosome biogenesis (Mongelard and Bouvet, 2007), affects transcription, processing and modification of rRNA and nuclear-cytosolic transport of ribosomal proteins and ribosomal subunits by shuttling between the nucleus and the cytoplasm (Tuteja and Tuteja, 1998).

B23 is an abundant, multifunctional nucleolar phosphoprotein whose activities are proposed to play a role in ribosome assembly, binding to other nucleolar proteins, nucleocytoplasmic shuttling (Li *et al.*, 1996), and possibly regulating transcription of rDNA by mediating structural changes in chromatin (Okuwaki *et al.*, 2001). Two isoforms of B23 have been identified: the major form (B23.1) is predominately located in the nucleolus and the minor form (B23.2) resides in the cytoplasm (reviewed by Hiscox, 2002).

Small proteins of less than 40–60 kDa can enter the nucleus through nuclear pore complexes by passive diffusion (Hiscox, 2007; Nigg, 1997). RNA-binding proteins that diffuse into the nucleus may therefore non-specifically target the nucleolus as it contains a large amount of rRNA. The nuclear import of larger proteins is mediated by nuclear localization signals (NLSs), composed of one (monopartite) or two (bipartite) stretches of basic amino acid (arginine and/or lysine) residues of a given size (Hiscox, 2007; Macara, 2001). Nucleocytoplasmic shuttling proteins also contain specific nuclear export signals (NESs), usually leucine-rich amino acid motifs (Hiscox, 2007; Macara, 2001).

How proteins may be further delivered to the nucleolus is poorly understood. The nucleolus does not have apparent membrane or other barriers, and entry into it does not require energy, unlike entry to the nucleus. It seems conceivable that viral proteins localize to the nucleolus, firstly, as a result of targeting the nucleus via classical NLS followed by direct or indirect interactions between the viral molecules (via various nucleolar localization signals, NoLSs) and components that make up the nucleolus (Hiscox, 2002, 2007). The structure of NoLSs is not well defined and depends on different factors including whether the protein associates with another nucleolar-bound protein or alternatively traffics to the nucleolus on its own

or associates with RNA transcripts that are being transcribed in the nucleolus. At least in some cases, NoLS are rich in arginine and lysine residues and can overlap with NLSs (Hiscox, 2002, 2007).

The second most studied nuclear body is CBs which are frequently associated with the nucleolus and found in animal and plant nuclei. CBs are involved in snRNP and snoRNP maturation and transport, with snRNPs and snoRNPs accumulating in CBs before appearing in speckles or the nucleolus, respectively (Cioce and Lamond, 2005). As mentioned above, spliceosomal snRNAs are also modified and contain 2′-*O*-ribose methylations and pseudouridines. Modification of nucleotides in snRNAs is guided by small CB-specific RNAs (scaRNAs) (Darzacq *et al.*, 2002; Jady *et al.*, 2003). A major component of CBs is the protein, coilin, which is required for their formation. In plants, mutants are available which knock out CBs or alter their size and number, two of which are due to mutations in coilin (Collier *et al.*, 2006). The number of CBs per nucleus can vary in different cell types and is under developmental control (Cioce and Lamond, 2005).

Besides rRNA transcription and processing and the production of ribosomal subunits, the nucleolus is also involved in many other aspects of RNA processing and RNP assembly as well as cellular functions (Boisvert *et al.*, 2007; Olson, 2004; Pedersen, 1998; Raška *et al.*, 2006; Rubbi and Milner, 2003). For example, the nucleolus has a role in the maturation, assembly, and export of RNP particles. The signal recognition particle (SRP) has a nucleolar phase in its assembly with particular protein constituents of the SRP being localized to the nucleolus. Similarly, telomerase RNP, required for chromosome replication, may be assembled in the nucleolus, and the nucleolus may also have a role in sequestering telomerase RNP to avoid inappropriate nucleation of telomere structures. Spliceosomal snRNPs may also undergo part of their assembly in the nucleolus, and processing of pre-tRNAs and U6snRNA occurs in the nucleolus. In addition, the nucleolus is a site of sequestration of particular proteins to regulate, for example, the cell cycle or cell death, and acts as a sensor of cellular stress (Boisvert *et al.*, 2007; Olson, 2004; Pedersen, 1998; Raška *et al.*, 2006; Rubbi and Milner, 2003).

Characterization of the protein composition of the nucleolus under different conditions has provided support for or suggested new functions for the nucleolus. Initial proteomic analyses of human cells identified a few hundred proteins which included not only well-known nucleolar proteins such as ribosomal proteins, fibrillarin, nucleolin, B23, etc. but also, for example, splicing and translation factors (Andersen *et al.*, 2002). Advances in proteomics has now allowed the identification of around 4500 proteins in the human nucleolar proteome (Ahmad *et al.*, 2008) and the dynamic behavior of nucleolar components and complexes can now be determined (Andersen *et al.*, 2005; Lam *et al.*, 2007). In plants, a partial

proteomic analysis of the nucleolus identified many expected ribosomal and nucleolar proteins but also found splicing and translation factors (Pendle *et al.*, 2005). In particular, exon junction complex proteins (associated with mRNAs following splicing) were identified in the nucleolar proteome and in the nucleolus by fluorescence microscopy (Pendle *et al.*, 2005). One of these proteins, eIF4A-III, a core protein of the exon junction complex, was shown to redistribute from the nucleoplasm to the nucleolus and finally to splicing speckles under stress conditions of hypoxia (Koroleva *et al.*, 2009). Similar dynamic distribution, involving the nucleolus, of proteins that interact with mRNAs has been demonstrated and the nucleolus and CBs have been shown to be involved in U1snRNP production in plants (Lorković and Barta, 2008; Tillemans *et al.*, 2006).

In plants, novel functions for the plant nucleolus, CBs, and another largely nucleolar-associated body, the D-body, have been described. Firstly, the production of heterochromatic siRNAs, which are involved in transcriptional silencing, is thought to occur in a region of the nucleolus or in D bodies due to the localization of protein components of the machinery and of siRNAs (Pontes and Pikaard, 2008). Secondly, maturation of microRNAs (miRNAs) may occur in D-bodies as precursor miRNAs as well as Dicer-like ribonuclease 1 (DCL1) have been located to these structures (Pontes and Pikaard, 2008). Similarly, in mammalian cells, some precursor and mature miRNAs have recently been found in the nucleus with some being enriched in the nucleolus (Politz *et al.*, 2009; Scott *et al.*, 2009). Of particular interest was the enrichment of some precursors in the GC suggesting that miRNA processing could occur in the nucleolus, or these miRNAs may be involved in ribosome synthesis or other nucleolar functions (Politz *et al.*, 2009). The link between the nucleolus and miRNA production is illustrated by the evolutionary relationship between some snoRNAs and miRNA precursors. For example, a human snoRNA was shown to be processed by Dicer to generate small RNAs which were associated with argonaute proteins (AGOs) and caused reduced expression of gene targets (Ender *et al.*, 2008). In addition, numerous snoRNA-derived small RNAs from different organisms (including Arabidopsis) were associated with components of RNA silencing pathways (Taft *et al.*, 2009) and many miRNA precursors have retained snoRNA features (Scott *et al.*, 2009). Finally, a third novel function for the plant nucleolus is in mRNA biogenesis, surveillance, or nonsense-mediated decay (NMD). Biochemical fractionation of nucleoplasm and nucleoli and subsequent sequencing of isolated cDNAs have shown that the plant nucleolus not only contains mRNAs but that aberrant mRNAs are enriched in the nucleolus (Kim *et al.*, 2009). The aberrant mRNAs show splicing defects, the majority of which would introduce premature termination codons and therefore be expected to be substrates for NMD. Using *upf* mutants (affected UPF proteins, key components of the NMD

mechanism), the correlation between enrichment of aberrant mRNAs in the nucleolus and turnover by NMD was corroborated and was further supported by the localization of the NMD proteins, UPF2 and UPF3, to the nucleolus (Kim *et al.*, 2009).

Before the observation that the plant nucleolus contained mRNAs and aberrant mRNAs, some spliced mRNAs (e.g., c-myc) were localized to the nucleolus while their unspliced versions were found in the nucleoplasm in mammalian cells (Pedersen, 1998; Olson, 2004). The nucleolus has also been associated with mRNA export on the basis of accumulation of polyA+ RNA upon disruption of export factors, nucleolar structure, or stress conditions in yeast and animal cells, although this could reflect increased polyadenylation prior to degradation (Ideue *et al.*, 2004; Pederson, 1998; Schneiter *et al.*, 1995). More recently, the nucleolus has been proposed to be involved in the formation of mRNPs which are localized to specific regions of the cytoplasm for translation. In yeast, *ASH1* mRNA enters the nucleolus bound to specific RNA-binding proteins at which time translation repressor proteins are loaded onto the mRNA. The mRNP is then exported to the cytoplasm, transported to its final destination, and then translation is activated (Du *et al.*, 2008; Jellbauer and Jansen, 2008). A similar trafficking pathway may also operate in mammals for mRNPs associated with the nucleolar protein, Staufen, which is involved in transport of mRNAs in neurons (Jellbauer and Jansen, 2008).

Thus, the nucleolus has numerous functions related to RNA biogenesis and different RNA processing and RNP maturation pathways. It contains many RNA-interacting proteins involved in these processes, including highly abundant RNA-binding proteins (such as fibrillarin and nucleolin) involved in rRNA and ribosomal subunit production. It is therefore a dynamic hub of RNA processing activity, RNA:protein interaction and complex formation. These characteristics, in addition to the potential involvement of the nucleolus in mRNA biogenesis and, particularly, the transport of mRNAs and mRNPs to and from the nucleolus to other parts of the cell, make the nucleolus a prime target for exploitation by viruses. It is therefore not surprising that viruses have taken advantage of the nucleolus and nucleolar components for production and distribution of viral RNAs and RNPs.

III. WHAT WE HAVE LEARNED ABOUT INTERACTIONS OF VIRUSES WITH THE NUCLEOLUS FROM ANIMAL VIROLOGY

Several excellent reviews have documented various aspects of the involvement of the nucleolus in the infection cycle of animal and human viruses (Greco, 2009; Hiscox, 2002, 2007; Matthews and Olson, 2006; Stark

and Taliansky, 2009). This section therefore briefly summarizes the main findings in this research area to illuminate the nucleolar functions involved in virus infections that are conserved between plant and animal kingdoms.

A. Viruses that replicate in the nucleus

Certain animal viruses such as DNA containing adenoviruses that replicate in the nucleus interact with and disrupt the nucleolus. As a result, synthesis of rRNA is disrupted in adenovirus-infected cells. Adenovirus infection also causes the redistribution of nucleolin and B23 (Matthews, 2001). Interestingly, B23 has been shown to stimulate adenovirus replication. RNA virus replication may also be facilitated by nucleolar proteins. Indeed, the HDAg protein encoded by hepatitis delta virus (a (−)ssRNA virus which is a subviral satellite of the DNA virus, hepatitis B virus) also binds to nucleolin and B23. It has been proposed that HDAg interacts with both these proteins in a complex that promotes viral RNA replication, presumably as a result of the helicase activity of nucleolin (Huang *et al.*, 2001).

Nucleolar proteins may also be involved in virus assembly and egress. For example, accumulation and assembly of structural (Cap) proteins of adeno-associated virus take place in nucleoli (Bevington *et al.*, 2007). Further export of the assembled Cap proteins from the nucleolus is mediated by another type of virus-encoded packaging (Rep) proteins, which should form a complex with Cap proteins. Remarkably, formation of this complex is mediated by B23. After export of complexes containing Rep, B23, and Cap proteins from the nucleolus to the nucleoplasm, viral DNA encapsidation occurs (Bevington *et al.*, 2007).

Another animal virus, the (−)ssRNA containing Borna disease virus, uses the nucleolus as a site of genome replication (Pyper *et al.*, 1998). An RNA-binding protein encoded by this virus has the appropriate trafficking signals for import and export to and from the nucleus (Cros and Palese, 2003)

B. Viruses replicating in cytoplasm

Many proteins encoded by animal viruses that replicate mainly or exclusively in the cytoplasm have also been shown to localize to the nucleolus, cause the relocalization of nucleolar proteins and disruption of nucleolar architecture and function. These include the (+)ssRNA virus proteins such as nucleocapsid proteins encoded by coronavirus and arterivirus, the dengue virus core protein, the alphavirus capsid protein and nonstructural nsP2 protein, the (−)ssRNA virus proteins, including the influenza virus nucleoprotein (NP) and nonstructural protein 1 (NS1),

Newcastle disease virus matrix protein, and retrovirus proteins such as human immunodeficiency virus (HIV) Rev and the transactivator Tat proteins (reviewed by Greco, 2009; Hiscox, 2002, 2007). Although functional relevance of why these proteins localize to the nucleus or nucleolus and how this relates to their functions in virus replication in many cases is largely unknown, several reports reveal significant progress in this area as exemplified below.

Infection of cells with poliovirus (picornavirus) results in the inactivation of the nucleolar protein, RNA polymerase I upstream binding (transcription) factor, which inhibits transcription of rRNAs in the host cell (Banerjee *et al.*, 2005). Poliovirus is also able to interact with nucleolin causing its selective redistribution from the nucleolus to the cytoplasm (Waggoner and Sarnow, 1998). After relocalization, nucleolin binds to the internal ribosome entry sites (IRESs) at the 5′ untranslated region of poliovirus genomic RNA, and this interaction stimulates IRES-dependent translation (Hellen and Sarnow, 2001). This also occurs in hepatitis C virus (Izumi *et al.*, 2001) and represents an alternative translation initiation strategy as compared to the classical eukaryotic Cap-dependent translation (Hellen and Sarnow, 2001). Nucleolin is also able to interact with the 3′-untranslated region of poliovirus RNA, which controls synthesis of negative strand RNA (Waggoner and Sarnow, 1998). Interaction of picornaviruses with the nucleolus could also down-regulate host gene expression. For example, the human rhinovirus 3C protease precursors that are localized in the nucleolus at early stages of the infection inhibit cellular RNA by cleavage of vital transcription factors (Amineva *et al.*, 2004).

The nucleocapsid proteins encoded by porcine arterivirus and avian coronavirus (infectious bronchitis virus, IBV) interact with nucleolin and fibrillarin (Chen *et al.*, 2002; Yoo *et al.*, 2003), and as a result may disrupt the normal functions of these proteins. For instance, by altering the distribution of fibrillarin, viruses might be reducing polI transcription, that is, the synthesis of rRNA, as blocking fibrillarin with antibody prevented its translocation to nucleoli and resulted in the reduction or inhibition of polI transcription (Fomproix *et al.*, 1998). The IBV infection leads to disruption of the nucleolus (Dove *et al.*, 2006a) and arrest of the cell cycle and cytokinesis (Chen *et al.*, 2002; Dove *et al.*, 2006b; Wurm *et al.*, 2001). Therefore, disruption of nucleolar architecture and function might be common in cells infected with viruses interacting with the nucleolus. The loss of essential nucleolar function in its turn may play an important role during virus infection toward an active production of the virus.

One of the most studied viruses in terms of viral interactions with the nucleolus is HIV, a retrovirus. HIV RNAs are reverse transcribed in the cytoplasm of infected cells and trafficked to the nucleus. After transcription in the nucleus, progeny RNA molecules are transported back to the

cytoplasm. One of the functions of the Rev protein is to export unspliced or partially spliced viral mRNA from the nucleus (reviewed by Greco, 2009; Hiscox, 2007). Before nuclear export, HIV RNA passes through the nucleolus. Rev binds to a *cis*-acting RNA element (Rev-response element), which is found in all unspliced and incompletely spliced viral mRNAs, and this promotes the translocation of these RNAs from the nucleus (Cantó-Nogués *et al*., 2001; Michienzi *et al*., 2000). The nucleolus plays a central role in this process, and the nucleolar trafficking of Rev and viral RNA is critical for the outcome of infection.

Thus, many animal viruses, whether they replicate or not in the nucleus, have evolved a nucleolar phase for part of their infection cycle to prevent unwanted interference from the cell. Alternatively, they use nucleolar functions for their own benefit. Recruitment of nucleolar proteins is especially beneficial for viruses, and in particular for RNA containing viruses, as these proteins possess many crucial functions in cellular RNA biosynthesis, processing, translation, and trafficking. Indeed, during virus infections of mammalian cells, various viral components traffic to and from the nucleolus where they interact with different host factors. Certain nucleolar proteins are redistributed into other cell compartments or are modified, and some cellular proteins are relocalized in the nucleolus of infected cells (reviewed by Greco, 2009; Hiscox, 2002, 2007). Well-documented studies have established that several of these nucleolar modifications play a role in some steps of the viral infection cycle such as viral attachment and entry, intracellular trafficking, transcription, translation, replication, virus assembly, and regress (reviewed by Greco, 2009). The virally induced nucleolar modifications could also affect fundamental cellular pathways including the initiation of transcription from the DNA promoter of the rRNA genes, cell-cycle regulation, and apoptosis (reviewed by Greco, 2009).

Although some steps (replication, translation) of the infection cycles of plant and animal/human viruses are essentially similar, there is a fundamental difference in some other mechanisms employed to enter host cells and spread from cell to cell between viruses infecting animal cells and viruses infecting plants. This is because animal cells are separated by barriers far less formidable than the thick, rigid, and impermeable cell walls consisting of cellulose and pectin that separate plant cells from one another. Other differences relate to defense strategies employed by humans/animals and plants against viral infections. For example, in mammals the interferon (IFN) pathway plays a key role in the innate antiviral immune response, whereas plants do not display such an activity. Instead, plants primarily rely on other natural defense strategies such as RNA silencing. Interestingly, functional links between plant virus infection cycle and the nucleolus have been described for both common and plant-specific virus infection steps.

IV. NUCLEOLAR FUNCTIONS OF PLANT VIRUS PROTEINS AND VIROIDS

Certain plant virus proteins localize to the nucleolus with examples from single-stranded DNA viruses, para-retroviruses and negative-strand and positive-strand RNA viruses (Table I). The most common technique for studying the nucleolar targeting of plant virus proteins is based on the confocal microscopy localization of the proteins which have been tagged with a fluorescent fusion protein (such as green fluorescent protein, GFP). Such proteins are usually larger than the size-exclusion limit (∼40–60 kDa) and hence prevented from nonspecific protein diffusion into the nucleus. Moreover, in many cases the specific nucleolar localization of plant virus proteins has been supported by identification of NoLSs (Table II). Although no overall conserved nucleolar trafficking motif has been identified in these NoLSs, they presumably resemble host NoLSs. Thus plant viral nucleolar trafficking might use a form of molecular mimicry as has earlier been proposed for animal viruses (Rowland and Yoo, 2003). Like host NoLSs, plant virus NoLSs may contain single [ToLCJAV CP, PLRV CP (CP-RT), CMV 2b] or bipartite [GRV ORF3, PVA NIa (VPg)] motifs which are usually characterized by basic amino acid stretches (Table II). However, in the case of GRV ORF3, in addition to the arginine-rich domain (NLS), a leucine residue at position 149 (L149) residing inside the leucine-rich domain (NES) is also essential for nucleolar targeting of ORF3 protein (Table II; Kim *et al.*, 2007a). The fact that viral proteins contain NoLSs is a strong indication that viruses have evolved specific nucleolar functions.

Viral proteins might also traffic to the nucleolus through association with host proteins. One example of such an association may be the interaction of various plant virus proteins with the major nucleolar protein, fibrillarin. The first description of this interaction was the demonstration that the umbravirus GRV ORF3 long-distance movement protein (MP) binds to fibrillarin *in vivo* and *in vitro* (Kim *et al.*, 2007a,b). For example, the leucine-rich domain (and L149, in particular) of ORF3 is involved in direct interaction with fibrillarin (Kim *et al.*, 2007b). Mutations in the leucine-rich domain prevent fibrillarin from binding to ORF3 and nucleolar trafficking (Kim *et al.*, 2007b). By implication, this may relate fibrillarin binding to nucleolar trafficking.

Other plant virus MPs which interact with fibrillarin are the pomovirus PMTV triple gene block protein 1 (TGB1) and hordeivirus PSLV TGB1 (N. O. Kalinina and D. Rakitina, unpublished results). As their name suggests, MPs are involved in virus spread in infected plants, and the potential role of fibrillarin in this process will be discussed in Section IV.B. The multifunctional PVA (potato virus A)-encoded viral genome-linked protein (VPg) is also able to interact with fibrillarin (Rajamäki and

TABLE I Examples of plant virus proteins and viroids that localize to the nucleolus

Genus	Virus[a]	Protein/RNA[b]	Reference (s)
DNA (single-stranded) viruses			
Begomovirus	TYLCV	CP	Rojas *et al.* (2001)
	ToLCJAV	CP	Sharma and Ikegami (2009)
RNA reverse transcribing virus (para-retrovirus super-group)			
Caulimovirus	CaMV	P6	Haas *et al.* (2005)
Negative-stranded RNA virus			
Nucleorhabdovirus	MFSV	N and P proteins	Tsai *et al.* (2005)
Positive-stranded RNA viruses			
Potyvirus	TEV	NIa (VPg); NIb; P3	Restrepo *et al.* (1990), Baunoch *et al.* (1991), Langenberg and Zhang (1997)
	TuMV	NIa (VPg)	Beauchemin *et al.* (2007)
	PVA	NIa (VPg)	Rajamäki and Valkonen (2009)
Umbravirus	GRV	ORF3	Ryabov *et al.* (1998), Ryabov *et al.* (2004)
Polerovirus	PLRV	CP (CP-RT)	Haupt *et al.* (2005)
Satellite virus (*Panicovirus*)	SPMV	CP	Qi *et al.* (2008)
Cucumovirus	CMV	2b, 3a (MP)	González *et al.* (2010), Mackenzie and Tremaine (1988)
Pomovirus	PMTV	TGB1	Torrance (personal communication)
Hordeivirus	PSLV	TGB1	NOK (unpublished results)
Viroid			
Pospiviroid	PSTVd, CCCVd	RNA	Schumacher *et al.* (1983), Harders *et al.* (1989), Bonfiglioli *et al.* (1996), Qi and Ding (2003)

[a] Virus acronyms: TYLCV, tomato yellow leaf curl virus; ToLCJAV, tomato leaf curl Java virus associated with DNA β satellite; CaMV, cauliflower mosaic virus; MFSV, maize fine streak virus; TEV, tobacco etch virus; TuMV turnip mosaic virus; PVA, potato virus A; GRV, groundnut rosette virus; PLRV, potato leafroll virus; SPMV, satellite panicum mosaic virus; CMV, cucumber mosaic virus; PMTV, potato mop top virus; PSLV, poa semilatent virus; PSTVd, potato spindle tuber viroid; CCCVd, coconut cadang cadang viroid.

[b] CP, coat protein; P6, CaMV multifunctional protein; N protein, nucleocapsid protein; P protein, phosphoprotein; NIa (b), nuclear inclusion protein a (b); VPg, viral genome-linked protein; P3, potyviral unstructural protein with no well-characterized function; ORF3, open reading frame 3 protein; CP-RT, coat protein-read through portion; 2b, CMV silencing suppressor; MP, movement protein (3a, CMV MP); TGB1, triple gene block protein 1.

TABLE II Examples of plant virus proteins that contain nucleolar localization signals (NoLS)

Protein	Amino acid position	NoLS	Reference (s)
ToLCJAV CP	16–20	KVRRR (NLS)[a]	Sharma and Ikegami (2009)
PVA NIa (VPg)	4–9 41–50	KRQRQK (NLS I)[b] KKGKTKGKTH (NLS II)[b]	Rajamäki and Valkonen (2009)
GRV ORF3	108–122 148–156	RPRRRAGRSGGMDPR L*L*PSLLNTL (NES)[c]	Ryabov *et al*. (2004) Kim *et al*. (2007a)
PLRV CP (CP-RT)	17–31	PRRRRRQSLRRRANR	Haupt *et al*. (2005)
CMV 2b	22–27	KKQRRR (NLS1)[a]	González *et al*. (2010)

Virus acronyms and other abbreviations are as in Table I.
[a] NLSs are also required for the nucleolar localization.
[b] Nuclear and nucleolar localization is controlled independently by the same NLS regions.
[c] L149 (italic) residing inside the ORF3 NES is also essential for nucleolar localization of the ORF3 protein.

Valkonen, 2009). However, the role of this interaction is likely to be different from that in virus movement as this process is not compromised by fibrillarin depletion (Rajamäki and Valkonen, 2009; Section IV.C). Another example of a viral protein that interacts with fibrillarin in the nucleolus is a silencing suppressor of CMV (cucumber mosaic virus), the 2b protein (González *et al*., 2010; Section IV.C). Collectively, these data indicate that interaction with fibrillarin is a general property of various plant virus proteins that is not restricted to one or two virus taxonomic groups. However, such interactions may have quite diverse molecular implications for different viruses being required at various phases of virus infection cycle. This may also suggest novel, unexpected natural functions for fibrillarin that are hijacked or affected by plant viruses at different stages of infection for needs of the viruses.

A. Nucleolus in replication of viroids and viruses

Viroids are small, circular, self-replicating, and non-coding RNA molecules that cause plant diseases (reviewed by Tabler and Tsagris, 2004). Viroids do not replicate in the cytoplasm like conventional plant RNA viruses but replicate in either the nucleus or the chloroplast. Nuclear viroids such as PSTVd and CCCVd have a nucleolar phase in their replication cycle (Table I; Qi and Ding, 2003; Schumacher *et al*., 1983).

In early experiments using *in situ* hybridization, PSTVd RNA [including both (+) and (−) RNA strands] was localized in nucleoli of nuclei isolated from infected plants (Harders *et al.*, 1989), suggesting that the nucleolus is the replication site of the viroid. However, later using improved sample preparation and *in situ* hybridization protocols, Qi and Ding (2003) have found that the (−) strand of PSTVd localizes in the nucleoplasm but not in the nucleolus. By contrast, the (+) strand of PSTVd localizes in the nucleolus as well as in the nucleoplasm, with various distinct spatial patterns. These experiments are suggestive of successive stages of nuclear involvement in viroid replication: (1) synthesis of the (−) and (+) strands of PSTVd occurs in the nucleoplasm; (2) the (−) strand RNA is anchored in the nucleoplasm; (3) the (+) strand RNA is transported selectively into the nucleolus; and (4) some (+) strand RNA traffics from the nucleolus back into the nucleoplasm and further into the cytoplasm for spreading into neighboring cells. The significance of (+) strand PSTVd RNA trafficking into the nucleolus remains to be determined. However, nucleolar machineries for processing of rRNAs, snoRNAs, and tRNAs make the nucleolus an attractive candidate site for PSTVd processing. The specific intranuclear localization of (−) and (+) strands of PSTVd may have some implication for pathogenicity. Assuming that the (−) and (+) strands each can interact with specific cellular factors to disrupt normal cell functions to cause symptoms, the differential subnuclear localization of the (+) and (−) strands of PSTVd may suggest different cellular targets for these RNAs.

(+) strand of PSTVd RNA has been shown to interact with a bromodomain-containing protein (a member of a family of transcriptional regulators associated with chromatin remodeling), termed viroid RNA binding protein 1 (VIRP1) (Martínez de Alba *et al.*, 2003; Tabler and Tsagris, 2004). VIRP1 contains an NLS and therefore might transfer the viroid RNA to the nucleus and bring it into contact with transcription units associated with chromatin (Martínez de Alba *et al.*, 2003; Tabler and Tsagris, 2004). However, mechanisms for selective nucleolar trafficking of (+) strand PSTVd are still unknown.

The nucleolus and its factors may be used not only by viruses and viroids for their own needs in replication, but also by plant host defense systems. For example, one of the major nucleolar proteins, nucleolin, binds to the 3′ non-coding region of the tomato bushy stunt virus, tombusvirus, RNA (Jiang *et al.*, 2010). This leads to significant inhibition of tombusviral RNA replication and may thus represent one of the innate immunity systems of plant hosts.

B. The nucleolus and plant virus movement

Plant viruses enter cells either through sites of mechanical injury to plant tissues or during the feeding by a specific vector organism (insect, nematode, or soil microbes belonging to protocists). To induce disease, after

replication in the initially infected cells, plant viruses must spread to the rest of the plant. The systemic spread of plant viruses proceeds in two stages: (i) cell-to-cell movement through plasmodesmata (PD) and (ii) long-distance movement through vascular tissues. First, the virus (in the form of virions or nucleic acid protein complexes) moves intracellularly from the sites of replication to PD, which are unique intercellular membranous channels that span cell walls linking the cytoplasm of contiguous cells. The virus then transverses the PD to spread intercellularly. It is generally accepted that viral cell-to-cell movement involves virus-encoded MPs as well as host-encoded components (reviewed by Lucas, 2006).

Virus systemic movement between organs (long-distance movement) occurs through the phloem, the specialized vascular system used by plants for the transport of assimilates and macromolecules (reviewed by Lucas, 2006; Oparka, 2004). Viruses enter, move through, and exit from the vascular system, which is usually surrounded by bundle sheath cells and contains various cell types including vascular parenchyma cells, companion cells, and enucleate sieve elements. Thus, transport of a virus into and within vascular tissue implies movement from mesophyll cells to bundle sheath cells, from bundle sheath cells to vascular parenchyma and/or companion cells, and into sieve elements. Virus exit from vascular tissue presumably involves the same steps in reverse order. Coat protein (CP) is essential for efficient long-distance transport of plant viruses, with only few exceptions (reviewed by Lucas, 2006).

1. Umbraviruses

One of the most well-studied plant viruses in terms of viral interactions with the nucleolus is GRV, an umbravirus [(+)ssRNA virus]. Umbraviruses differ from most other viruses in that they do not encode a CP such that conventional virus particles are not formed in infected plants. Umbraviral genomes encode at least three proteins. In GRV, two ORFs at the 5′-end of the RNA are expressed by a frameshift mechanism as a single protein that appears to be an RNA replicase (Taliansky and Robinson, 2003). The other ORFs (ORF3 and ORF4) overlap each other. ORF4 encodes the MP that mediates the cell-to-cell movement of viral RNA via PD (Ryabov *et al.*, 1998). ORF3 protein is the long-distance movement factor that facilitates trafficking of viral RNA through the phloem (Ryabov *et al.*, 1999). Umbraviral ORF3 proteins (26–29 kDa) show no significant similarity with any other recorded or predicted proteins (Taliansky and Robinson, 2003). The GRV ORF3 protein interacts with viral RNA *in vivo* to form filamentous RNP particles, which have elements of regular helical structure, but not the uniformity typical of virus particles (Taliansky *et al.*, 2003). The RNPs accumulate in cytoplasmic inclusions which have been detected in all cell types and were

abundant in phloem cells (Taliansky *et al.*, 2003). They serve to protect viral RNA and move it through the phloem to cause systemic infection. Remarkably, in addition to its presence in the cytoplasm, the ORF3 protein was also found in nuclei and predominantly in nucleoli (Ryabov *et al.*, 1998, 2004).

Studies of the biology of GRV infection have provided molecular insights into how and why viruses may target the nucleolus (Canetta *et al.*, 2008; Kim *et al.*, 2007a,b). It has been demonstrated that the GRV ORF3 protein traffics to the nucleolus via a mechanism involving the reorganization of CBs into multiple Cajal body-like structures (CBL) and their fusion with the nucleolus. Nucleolar localization and further trafficking of ORF3 protein from the nucleolus back to the cytoplasm is essential for the umbravirus infection. The integral connection between nucleolar targeting of the ORF3 protein and its biological function in virus long-distance spread is demonstrated by mutagenesis of the arginine- and leucine-rich domains that block nucleolar localization or nuclear export of the ORF3 protein, and which prevent the formation of cytoplasmic viral RNPs and their long-distance movement (Kim *et al.*, 2007a).

Although the mechanisms by which the ORF3 protein targets CBs and produces CBLs are unknown, it could be suggested that targeting of CBs by the ORF3 protein may utilize elements of existing CB-trafficking pathways. For example, part of the maturation pathway of snRNPs in mammalian cells occurs in the cytoplasm and involves a complex containing the SMN protein (SMN complex) which together with the snRNP are reimported into the nucleus and targeted to CBs (Matera and Shpargel, 2006; Navascues *et al.*, 2004; Sleeman and Lamond, 1999). Particular snRNP proteins contain modified symmetric di-methyl arginines (sDMAs) which enhance the formation of snRNPs and interaction with SMN (Paushkin *et al.*, 2002). Preliminary experiments have identified sDMAs in the ORF3 protein (M. Taliansky, unpublished results), suggesting that targeting of the ORF3 protein to CBs could involve interactions with the SMN protein. Although SMN protein has yet to be identified in plants, the existence of an orthologue has been suggested (Collier *et al.*, 2006).

The formation of CBLs may involve either fragmentation of CBs into multiple bodies by the ORF3 protein or the redistribution of CB components into new structures containing the ORF3 protein. Interestingly, the multiple CBL phenotype, described here, is similar to that of the *poly Cajal bodies* (*pcb*) mutant of Arabidopsis (Collier *et al.*, 2006). As the protein normally encoded by a gene controlling the *pcb* phenotype, appears to regulate CB formation, ORF3 protein may interfere with the function of this or other proteins to affect the integrity and number of CBs in nuclei. The second possibility is that the ORF3 protein causes the redistribution of CB components, such as coilin, U2B″ and fibrillarin, to form CBLs with the ORF3 protein.

ORF3 protein trafficking to the nucleolus uses a novel pathway of nucleolar import by causing the fusion of CBLs with the nucleolus. The physical and functional association of the nucleolus and CBs is well-documented and is controlled by complex molecular interactions among CB and nucleolar proteins such as coilin, SMN, fibrillarin, and Nopp140 (Cioce and Lamond, 2005; Ogg and Lamond, 2002). Expression of mutant versions of some of these proteins has profound effects on CB structure and function causing disruption or dispersal and compositional changes (Jones *et al.*, 2001; Pellizzoni *et al.*, 2001; Tucker *et al.*, 2001). Moreover, phosphorylation of coilin is an important factor determining physical interactions and trafficking of CBs (Cioce and Lamond, 2005; Ogg and Lamond, 2002). For example, CBs form within the nucleolus of HeLa cells upon treatment with okadaic acid (an inhibitor of protein phosphatase) and with transient expression of coilin mutated at a single serine residue (Lyon *et al.*, 1997; Sleeman *et al.*, 1998). CBs have also been observed within nucleoli in human breast carcinomas (Ochs *et al.*, 1994) and in liver cells of hibernating dormice (Malatesta *et al.*, 1994). Therefore, the ORF3 protein may interfere with normal protein–protein interactions or posttranslational modifications causing the reorganization and fusion of CBs with the nucleolus.

The last stage of the nuclear voyage of the ORF3 protein is its nuclear export leading to the redistribution of some fibrillarin from the nucleolus to cytoplasm (fibrillarin normally does not accumulate in cytoplasm) (Kim *et al.*, 2007a). This redistribution is mediated by the direct interaction between the ORF3 protein and fibrillarin (Kim *et al.*, 2007b). Taking into account the long-distance movement function of GRV ORF3, it could be expected that fibrillarin is directly involved in this particular viral function. Further support of a role for fibrillarin in umbravirus systemic infection has been provided by the fibrillarin knock-down experiments (Kim *et al.*, 2007b). Silencing of the fibrillarin gene has indeed prevented formation of RNP particles and long-distance movement of GRV but does not affect viral replication or cell-to-cell movement. Thus, it has been concluded that fibrillarin is needed for formation of cytoplasmic RNP particles that are capable of long-distance movement and causing systemic viral infection such that the redistribution of fibrillarin with ORF3 protein is a prerequisite to form RNPs (Kim *et al.*, 2007a,b). Further experiments have shown that fibrillarin, in combination with ORF3 protein and viral RNA *in vitro*, produced filamentous RNP structures with structural properties similar to *in vivo* RNPs (as discussed in detail in Section IV.E). Fibrillarin, an RNA-binding protein, may bind the viral RNA or act as a chaperone to permit or catalyze the regular assembly of proteins around viral RNA. Although fibrillarin and ORF3 protein are sufficient to form functional viral RNP particles *in vitro*, additional proteins may also be associated with the *in vivo* particles. When formed in

phloem companion cells, the viral RNPs are able to enter sieve elements and move through the plant to cause systemic infection. Hence formation of the fibrillarin-ORF3 protein complexes appears to be the key prerequisite for formation of GRV RNP particles and their long-distance movement through the phloem.

2. Poleroviruses

Poleroviruses are (+) ssRNA viruses that are mainly restricted to cells in the vascular system. Besides a major CP PLRV encodes a minor CP, an extended version of the major CP produced by occasional translational ''readthrough'' of the CP gene (CP-RT) (Bahner *et al.*, 1990). Both CP and CP-RT contain the same NoLS, and are targeted to the nucleolus when they are individually expressed in plant tissues (Haupt *et al.*, 2005). However, CP-RT but not PLRV CP loses its nucleolar localization in the presence of replicating PLRV. It has been suggested that the CP-RT protein does not accumulate in the nucleolus in the presence of PLRV infection because PLRV RNA or PLRV-encoded or -induced factors retain this protein outside the nucleolus (Haupt *et al.*, 2005). Like GRV, PLRV has been unable to cause systemic infection in the fibrillarin-silenced plants, although accumulation of PLRV in the inoculated leaves was not affected (Kim *et al.*, 2007b) suggesting that fibrillarin is also involved in PLRV long-distance movement.

3. Viruses that require a TGB for movement

A role of nucleolar functions in systemic infection has also been suggested for plant viruses that require TGB for movement. The genomes of some viruses, such as potexviruses, hordeiviruses, and pomoviruses, contain so-called triple gene block, TGB that encodes three proteins required for cell-to-cell and long-distance movement (reviewed by Morozov and Solovyev, 2003).

The TGB1 protein encoded by the TGB-containing virus, PMTV (pomovirus) localizes to the nucleus and nucleolus. Deletion of 84 N-terminal amino acids abrogates its nucleolar localization. Northern blots of RNA from inoculated and upper non-inoculated leaves of plants infected with clones carrying the TGB1 N-terminal deletion mutant reveal that long distance movement of viral RNAs has also been abolished, but this mutant is still competent for cell-to-cell movement (L. Torrance, personal communication). Moreover, the PMTV TGB1 protein has been shown to interact with fibrillarin *in vitro*. Interestingly, TGB1 protein encoded by the hordeivirus, PSLV can also interact with fibrillarin both *in vitro* and *in vivo* (N. O. Kalinina, unpublished results). Thus it appears that some TGB-encoding viruses such as PMTV and PSLV may represent another example of plant viruses that require association with the nucleolus to control long distance movement of their genomes.

C. Nucleolar targeting for interference with host antiviral defense

Potyviruses form the largest group of plant-infecting RNA viruses (Rajamäki *et al.*, 2004). They have a polyadenylated (+) ssRNA genome of ca. 9500–10,000 nucleotides that is encapsidated into a 680–900 nm long, filamentous particle. The genome is translated into a large polyprotein of about 3000–3350 amino acids, which is subsequently processed into up to ten mature proteins by three virus-encoded proteinases (Rajamäki *et al.*, 2004). Additionally, a 25-kDa protein produced from the P3 cistron by frameshifting was recently identified (Chung *et al.*, 2008).

Potyviruses replicate in membranous structures in the cytoplasm (Cotton *et al.*, 2009; Schaad *et al.*, 1997a). However, two potyviral replication-associated proteins, the nuclear inclusion protein a (NIa) and nuclear inclusion protein b (NIb), accumulate in the nucleus of virus-infected cells (Baunoch *et al.*, 1991; Dougherty and Hiebert, 1980; Hajimorad *et al.*, 1996; Knuhtsen *et al.*, 1974; Restrepo *et al.*, 1990). In addition, NIa localizes in the nucleolus and CBs (Baunoch *et al.*, 1991; Beauchemin *et al.*, 2007; Rajamäki and Valkonen, 2009; Restrepo *et al.*, 1990). The NIb of TEV (tobacco etch virus) has also been detected in the nucleolus (Baunoch *et al.*, 1991; Restrepo *et al.*, 1990). Potyviruses may also induce nuclear inclusions, which consist of NIa and NIb (Baunoch *et al.*, 1991; Dougherty and Hiebert, 1980; Edwardson and Christie, 1983; Knuhtsen *et al.*, 1974; Martin *et al.*, 1992). The significance of these nuclear inclusions is unknown but they may simply represent inactivated protein complexes, because they seem to form only at late stages of virus infection (Baunoch *et al.*, 1991).

NIa is multifunctional and consists of two domains. The N-proximal half of NIa is the VPg that is covalently linked to the 5′-end of viral genomic RNA (Oruetxebarria *et al.*, 2001; Siaw *et al.*, 1985). The C-proximal part (NIa-Pro) is the main viral proteinase (Dougherty *et al.*, 1989). The two domains are separated by a suboptimal proteolytic cleavage site, the slow processing of which is essential for efficient viral replication (Carrington *et al.*, 1993; Schaad *et al.*, 1996). The majority of full-length NIa is found in the nucleus of virus-infected cells (Carrington *et al.*, 1993). However, NIa can also exist as a polyprotein with the 6K2 protein located upstream of NIa in the viral polyprotein. The 6K2 protein impedes nuclear localization of the 6K2-NIa polyprotein (Restrepo-Hartwig and Carrington, 1992) and directs NIa to cytoplasmic membrane vesicles, the sites of viral replication (Beauchemin *et al.*, 2007; Cotton *et al.*, 2009; Restrepo-Hartwig and Carrington, 1994; Schaad *et al.*, 1997a). Many lines of evidence suggest that NIa is part of the viral replication complex involving several viral and host proteins (Beauchemin and Laliberte, 2007; Fellers *et al.*, 1998; Li *et al.*, 1997; Murphy *et al.*, 1996; Puustinen

and Mäkinen, 2004; Schaad *et al.*, 1996). The VPg domain has NTP binding activity, is uridylylated by NIb, and may act as a primer for synthesis of viral RNA (Anindya *et al.*, 2005; Murphy *et al.*, 1996; Puustinen and Mäkinen, 2004; Schaad *et al.*, 1996). In addition, VPg is involved in viral cell-to-cell and long-distance movement (Nicolas *et al.*, 1997; Rajamäki and Valkonen, 1999, 2002; Schaad *et al.*, 1997b). The NLSs and NoLS of potyviral NIa map to the N-proximal part of VPg (Carrington *et al.*, 1991; Rajamäki and Valkonen, 2009; Schaad *et al.*, 1996). The signal controlling nuclear localization of NIa is bipartite (Carrington *et al.*, 1991; Rajamäki and Valkonen, 2009; Schaad *et al.*, 1996). The regions of NIa controlling nucleolar targeting of NIa have been studied in PVA and found to map to the same regions as the NLSs. However, mutations in both NLS regions are required to abolish nuclear targeting of PVA NIa, whereas mutations in either NLS prevent nucleolar localization of NIa (Rajamäki and Valkonen, 2009). The most N-terminal NLS controls also localization of PVA NIa to CBs (Rajamäki and Valkonen, 2009). Mutations that affect nuclear localization of NIa are detrimental for genome amplification of TEV and PVA (Rajamäki and Valkonen, 2009; Schaad *et al.*, 1996), suggesting that nuclear/nucleolar localization of NIa has an important role in virus infection.

Potyviral NIa (or VPg) has been shown to interact with several host proteins (Dunoyer *et al.*, 2004; Huang *et al.*, 2010; Léonard *et al.*, 2004; Rajamäki and Valkonen, 2009; Schaad *et al.*, 2000; Thivierge *et al.*, 2008; Wittmann *et al.*, 1997). The structure of VPg is intrinsically disordered (Grzela *et al.*, 2008; Rantalainen *et al.*, 2008), which may provide flexibility for many types of interactions. One of the interaction partners of VPg and/or NIa is the eukaryotic translation initiation factor eIF4E or its isoform (Robaglia and Caranta, 2006; Schaad *et al.*, 2000; Wittmann *et al.*, 1997) and the interaction appears important for virus infectivity (Léonard *et al.*, 2000; Robaglia and Caranta, 2006). Remarkably, interaction of TuMV NIa with eIF(iso)4E and the poly(A) binding protein 2 (PABP2) has been detected in the nucleus and nucleolus using bimolecular fluorescence complementation (BiFC) and colocalization experiments (Beauchemin and Laliberte, 2007; Beauchemin *et al.*, 2007). By contrast, interaction with the 6K2-NIa polyprotein targets eIF(iso)4E and PABP2 to cytoplasmic membrane vesicles (Beauchemin and Laliberte, 2007; Beauchemin *et al.*, 2007). The data suggest that these host proteins are needed in different viral processes. In healthy plants, most of PABP is cytoplasmic but some of the protein is redistributed to the nucleolus probably by the NIa (Beauchemin and Laliberte, 2007). NIa appears to mediate also eIF (iso)4E localization to the nucleolus (Beauchemin *et al.*, 2007) and could interact simultaneously with eIF(iso)4E and PABP2 as shown by *in vitro* binding assays and BiFC and colocalization experiments (Beauchemin and Laliberte, 2007). Hence, all three proteins may be part of the same

complex. Although the role of interaction between NIa and eIF(iso)4E and/or PABP2 in the nucleus is currently unclear, some possibilities can be suggested. The nuclear pool of eIF4E takes part in mRNA export from the nucleus but may also be involved in nuclear translation and NMD of RNA (Strudwick and Borden, 2002). PABP regulates the initiation of protein synthesis, nuclear export of mature mRNAs, and mRNA stability and decay (Mangus *et al.*, 2003). Interaction with NIa might disrupt some of these functions. As mentioned above, some animal viruses are known to modulate and inhibit activities of translation initiation factors in order to favor viral replication and translation (reviewed by Thompson and Sarnow, 2000).

Recent data connect nuclear/nucleolar targeting of plant viral proteins to interference with host antiviral defense. For example, the potyviral VPg protein has been observed to accumulate in the companion cells of upper leaves of a wild potato species ahead of virus infection, which suggested that VPg might interfere with host defense and hence facilitate infection of the cells that receive the virus *via* systemic transport (Rajamäki and Valkonen, 2003). Indeed, this hypothesis gained support in the experiments in which overexpression of VPg in leaf tissues temporarily interfered with cosuppression of gene silencing (i.e., RNA silencing), whereas NLS mutants of VPg, which exhibited reduced nuclear and nucleolar localization, were not able to suppress RNA silencing (Rajamäki and Valkonen, 2009).

RNA silencing is a sophisticated, sequence-specific RNA degradation mechanism operating in the cytoplasm (Ruiz-Ferrer and Voinnet, 2009). A key feature of the RNA silencing pathway is the generation of dsRNA that corresponds in sequence to the target (virus) RNA. This dsRNA is cleaved into siRNAs by DCLs and these mediate the target specificity for RNA degradation (for reviews, see Carrington, 2000; Ruiz-Ferrer and Voinnet, 2009; Vance and Vaucheret, 2001; Voinnet, 2001). RNA silencing is a natural anti-viral defense system of plants but is also involved in gene regulation in a wide range of developmental and pathogen defense processes (Ruiz-Ferrer and Voinnet, 2009). To combat host defense RNA silencing, most plant viruses encode silencing suppressor proteins. The potyviral helper-component proteinase (HC-Pro) was the first detected viral suppressor of RNA silencing (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington 1998) and the potyviral P1 protein may enhance its activity (Rajamäki *et al.*, 2005). HC-Pro acts in the cytoplasm and, therefore, the association of VPg with RNA silencing suppression when localized in the nucleolus is intriguing because many host proteins involved in RNA silencing as well as the processing centers for small RNAs are located in the nucleus, nucleolus, and CBs as discussed in Section II (Pontes and Pikaard, 2008).

In contrast, nuclear/nucleolar localization of the P19 protein of TBSV, tombusvirus, mediated by plant ALY proteins (mRNA-processing and

-export factors) may be a defense mechanism of the plant to down-regulate the silencing suppressor activity of P19 (Canto *et al.*, 2006).

Previously, suppression of RNA silencing has been found to be connected to nuclear localization of another viral protein: the 2b protein, silencing suppressor of CMV. CMV 2b localizes in the nucleus and the nucleolus where it interacts with Argonaute 1 (AGO1), the core component of the RNA-induced silencing complex (González *et al.*, 2010; Lucy *et al.*, 2000; Zhang *et al.*, 2006). More recently, it has also been shown that CMV 2b interacts with AGO4 (González *et al.*, 2010). However, these interactions are not sufficient for suppression of RNA silencing, and hence their biological relevance remains so far unclear (González *et al.*, 2010).

The VPg of PVA interacts with fibrillarin in the nucleolus and CBs as detected by BiFC experiments (Rajamäki and Valkonen, 2009). Depletion of fibrillarin reduces PVA accumulation in *Nicotiana benthamiana*, suggesting a role for fibrillarin in virus infection (Rajamäki and Valkonen, 2009). As mentioned above, in GRV, fibrillarin is recruited for viral long-distance transport (Canetta *et al.*, 2008; Kim *et al.*, 2007a,b), but in potyviruses the role is likely to be different as long-distance transport of PVA is not compromised by depletion of fibrillarin (Rajamäki and Valkonen, 2009). Taking into account the silencing suppression activity of VPg, it could be suggested that VPg-fibrillarin might contribute to such an activity. Alternatively, the fibrillarin–VPg interaction may disrupt certain nucleolar functions (e.g., host transcription or pre-mRNA processing), which might explain the observed shutdown of host gene expression during potyvirus infection (Wang and Maule, 1995).

In mammals, the IFN pathway plays a key role in the innate antiviral immune response whereas involvement of RNA silencing is still controversial (reviewed by Hale *et al.*, 2008). However, emerging evidence suggests some parallels in how plant and animal viruses could use the nucleolus to counteract host defense. For example, the NS1 protein of influenza A virus is an important factor in counteracting IFN-based cellular antiviral mechanisms (Hale *et al.*, 2008). In addition, NS1 is also able to suppress RNA silencing in plant, insect, and mammalian cells (Bucher *et al.*, 2004; de Vries *et al.*, 2009; Delgadillo *et al.*, 2004; Haasnoot *et al.*, 2007; Li *et al.*, 2004). Remarkably, NS1 localizes to the nucleolus and interacts with nucleolin (Murayama *et al.*, 2007).

D. Nucleolar localization of viral proteins for as yet unknown reasons

NIb is a viral RNA-dependent RNA polymerase of potyviruses and hence involved in the replication complex (Hong and Hunt, 1996; Schaad *et al.*, 1997a). While viral replication takes place in the cytoplasm on cellular

membranes, NIb is also targeted to the nucleus and the nucleolus (Baunoch *et al.*, 1991; Restrepo *et al.*, 1990). Nuclear targeting of NIb appears to be highly sensitive to alterations in protein conformation and is eliminated by deletions, dipeptide insertions and amino acid substitutions introduced also into parts of NIb other than the NLSs (Li and Carrington, 1993). Nuclear and nucleolar localization of NIb may be important for the viral infection cycle, because mutations in the NLSs abolish infectivity of TEV (Li and Carrington, 1995; Li *et al.*, 1997). Three host proteins, the PABP2, the heat shock cognate 70 protein (Hsc70-3), and the eukaryotic translation elongation factor 1A, eIF1A, interact with NIb (Dufresne *et al.*, 2008; Thivierge *et al.*, 2008; Wang *et al.*, 2000b). However, none of these protein interactions was found in the nucleolus, shedding no light on the role of the nucleolar localization of NIb.

Besides nuclear inclusion proteins, the P3 protein of TEV is targeted to the nucleus and nucleolus of virus-infected tobacco cells, as detected by immunogold labeling (Langenberg and Zhang, 1997). P3 is a nonstructural protein with no well-characterized function. However, it is involved in virus multiplication (Kekarainen *et al.*, 2002) and virus–host interactions (Chu *et al.*, 1997; Eggenberger *et al.*, 2008; Jenner *et al.*, 2003; Johansen *et al.*, 2001). A role for nuclear/nucleolar localization of P3 is currently unknown.

The multifunctional nucleocytoplasmic shuttling P6 protein encoded by plant para-retrovirus CaMV has also been found to localize to the nucleolus (Haas *et al.*, 2005). Although a NoLS has not been identified for this protein, its nucleolar import might be facilitated by its interaction with ribosomal proteins of the 60S ribosomal subunit (Haas *et al.*, 2008). The presence of P6 in the nucleolus, where assembly of ribosomal subunits occurs, raises the possibility that P6 might interact directly with ribosomes before their export to render them competent for translation of the CaMV polycistronic mRNA. Indeed, P6 interacts with the ribosomal proteins L18 (Leh *et al.*, 2000), L24 (Park *et al.*, 2001), and L13 (Bureau *et al.*, 2004), which hence may be the potential targets because they participate in the formation of the 60S subunit in the nucleolus (Andersen *et al.*, 2002). Another functional activity of CaMV P6 is suppression of RNA silencing. This protein interferes with RNA-directed RNA polymerase 6 (RDR6)-dependent RNA silencing via inhibition of the dsRNA-binding protein DRB4, a protein normally enhancing DCL4-mediated dicing. However, unlike nuclear targeting, the nucleolar localization of P6 is completely dispensable for its silencing suppression function (Haas *et al.*, 2008).

Transport of the viral genome into the nucleus is an obligatory step in the replication cycle of plant DNA viruses such as the begomoviruses. CPs of monopartite begomoviruses (such as TYLCV and ToLCJAV) are nucleocytoplasmic shuttling proteins thought to be involved in this process (Rojas *et al.*, 2001; Sharma and Ikegami, 2009). Interestingly, these

proteins also contain NoLSs targeting them to the nucleolus (Sharma and Ikegami, 2009). However, the biological significance of nucleolar localization of begomoviral CP is unclear.

NLSs have been identified in three of the seven proteins encoded by plant nucleorhabdovirus, MFSV. Remarkably, two of them, the nucleocapsid (N) protein and phosphoprotein (P), localize to the nucleolus but only when they are coexpressed in plant tissues (as fusions with GFP) using the *Agrobacterium* system; when expressed individually these proteins do not target nucleoli (Tsai *et al.*, 2005). This clearly indicates the interdependent character of nucleolar targeting for the N and P proteins. However, the molecular mechanisms responsible for this effect and its biological relevance remain to be explored.

Another plant virus protein with nucleolar localization is the CP of SPMV (satellite panicum mosaic virus) (Qi *et al.*, 2008). Some of the functional and biochemical properties of the SPMV CP including the nucleolar association of SPMV CP, its RNA binding activity (Desvoyes and Scholthof, 2000), and the involvement of the CP in systemic movement (Omarov *et al.*, 2005) are similar to those of GRV ORF3. By analogy therefore, the authors have suggested that nucleolar localization of SPMV CP may be essential for its role in the systemic movement of SPMV as in the case of GRV ORF3. Collectively, all of these findings support an active role of the nucleolus and fibrillarin in various aspects of the virus infection cycle and interactions with host cells promoting systemic infections with plant viruses belonging to various taxonomic groups.

E. Formation of viral ribonucleoprotein complexes (RNPs) in the nucleolus

The nucleolus contains a complex machinery for rRNA modification and rRNP assembly and may provide an environment which allows other forms of functional RNPs, such as SRP, telomerase, splicing snRNPs, and viral RNPs to exploit the nucleolus or nucleolar components in their biogenesis pathways (reviewed by Boisvert *et al.*, 2007). For example, fibrillarin is one of the four core protein components of a box C/D snoRNP complex (reviewed by Tran *et al.*, 2004). Being a methyltransferase, fibrillarin (as a component of box C/D snoRNPs) functions in the 2′-*in vitro*-methylation and processing of pre-rRNA. In addition, human fibrillarin forms a subcomplex with splicing factor 2-associated p32, protein arginine methyltransferases, and tubulins α3 and β1 that is independent of its association with snoRNPs, suggesting that fibrillarin may also coordinate protein methylation during the processes of ribosome biogenesis (Yanagida *et al.*, 2004). Furthermore fibrillarin interacts with some other cellular proteins such as SMN protein (Jones *et al.*, 2001) and the nuclear DEAD box protein p68, an RNA-dependent ATPase and RNA

helicase (Nicol *et al.*, 2000). However, the physiological role of these interactions is unclear and may be based on novel natural functions of fibrillarin that remain to be established.

The most studied viral RNP complex containing fibrillarin is the GRV ORF3 complex with elements of regular helical structure that is capable of long-distance movement via the phloem. Assembly of GRV RNP particles occurs in the cytoplasm and requires fibrillarin relocalized from the nucleolus (Kim *et al.*, 2007a,b; Taliansky *et al.*, 2003; also see above). RNP particles similar in architecture and infectivity to the viral RNPs formed *in vivo*, have been reconstituted *in vitro* from fibrillarin, the ORF3 protein and viral RNA (Kim *et al.*, 2007a,b). Taking the study further, the *in vitro* experiments have shown that the ORF3-fibrillarin interaction occurs between the leucine-rich region (L149 in particular) of the ORF3 protein and the GAR domain of fibrillarin (Kim *et al.*, 2007b) known to be responsible for interaction with other proteins such as SMN (Jones *et al.*, 2001). This interaction leads to formation of single-layered ring-like complexes of ORF3 with fibrillarin as was shown by atomic force microscopy (Canetta *et al.*, 2008). The diameter of these ORF3 protein-fibrillarin rings is 18–22 nm which correlates with that of the filamentous RNP particles (Canetta *et al.*, 2008). It thus appears that the ORF3 protein fibrillarin rings interact with viral RNA, encapsidating it and reorganizing it into helical structures, and thereby play a key role in the assembly of umbraviral RNP complexes. These results demonstrate that, in addition to traditional functions in rRNA processing and modification, fibrillarin possesses completely novel functions in mediating assembly of umbraviral RNPs. These functions are presumably based on the ability of the ORF3 protein to interact and form ring-like complexes with fibrillarin such that the virus alters and exploits the properties of fibrillarin for successful virus propagation.

Other viral proteins, such as the nucleocapsid protein encoded by porcine arterivirus also interact with fibrillarin in an RNA-dependent manner (Yoo *et al.*, 2003). However, the structure and architecture of these complexes and how they impact the viral infection cycle remain unknown.

V. CONCLUSIONS AND PERSPECTIVES

The nucleolus is a highly conserved feature of eukaryotic cells that has a key role as the site of ribosome subunit production. However, recent multiple lines of investigation have confirmed and characterized additional roles for nucleoli in other important cellular processes including cell-cycle control, stress responses, and coordination of the biogenesis of a number of other functional RNPs. The nucleus has also been shown to

play a crucial role in the infection cycle of various viruses, and the nucleolar localization of viral proteins has recently been described as a pan-virus phenomenon (Hiscox, 2002, 2007). In this regard, plant viruses are not different from other eukaryotic viruses. The past few years have brought remarkable progress in our understanding of why and how some plant viruses (in particular, umbraviruses and potyviruses) target the nucleolus and the functional role of the interaction between viral and nucleolar proteins in the plant virus infection cycle. For many other plant virus proteins, nucleolar localization and interaction with nucleolar components have also been demonstrated, and functional implications of these findings is a challenge for future research. We anticipate that more information will emerge about the mechanisms involved in regulating nucleolar function and structure in response to plant virus infections and hijacking nucleolar functions for the virus infection cycle. There are now several examples in which the plant viruses also target other subnuclear bodies, such as CBs. In particular, for umbraviruses the role of CBs in nucleolar trafficking of the ORF3 protein has been established. The potential role of sub-nuclear structures in other plant virus infections will be addressed in the future.

The study of viral interactions with the nucleolus also provides unique and valuable tools to gain new insights into novel nucleolar functions and processes. For example, as previously discussed, the major nucleolar protein fibrillarin, is involved in formation and long-distance movement of umbraviral RNP particles. These functions as well as other potential yet unrecognized natural functions of nucleolar proteins will be the focus of much future research. On a practical level, both the plant cell and viral biology of the nucleolus can, and hopefully will be exploited for the design of novel strategies to control plant virus infections.

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