Hitchhikers, highway tolls and roadworks: the interactions of plant viruses with the phloem

Svetlana Y. Folimonova[1], Jens Tilsner[2,3]

[1]University of Florida, Plant Pathology Department, Gainesville, FL 32611, USA

[2]Biomedical Sciences Research Complex, University of St Andrews, BMS Building, North Haugh, St Andrews, Fife KY16 9ST, United Kingdom

[3]Cell and Molecular Sciences, The James Hutton Institute, Invergowrie, Dundee DD2 5DA, United Kingdom

jt58@st-andrews.ac.uk

svetlana@ufl.edu

Abstract

The phloem is of central importance to plant viruses, providing the route by which they spread throughout their host. Compared with virus movement in non-vascular tissue, phloem entry, exit, and long-distance translocation usually involve additional viral factors and complex virus-host interactions, probably, because the phloem has evolved additional protection against these molecular 'hitchhikers'. Recent progress in understanding phloem trafficking of endogenous mRNAs along with observations of membranous viral replication 'factories' in sieve elements challenge existing conceptions of virus long-distance transport. At the same time, the central role of the phloem in plant defences against viruses and the sophisticated viral manipulation of this host tissue are beginning to emerge.

Introduction

For plant-infecting viruses, the phloem is of particular importance, as it provides the fastest way to spread throughout the host in a race against systemic defence responses, in order to optimize viral load and reach tissues favoring host-to-host transmission [1;2]. Perhaps because it is a gatekeeper to systemic infection, the phloem appears to be specially protected against viruses, as its successful invasion often requires additional viral proteins compared with non-vascular movement. Recent studies on the unexpectedly widespread phloem trafficking of endogenous plant mRNAs suggest that host transport systems suitable for viral exploitation may exist and viral long-distance movement (LDM) requirements may provide insights into them. A few recent studies have also raised the possibility that the xylem might function as an additional/alternative systemic transport route [3*;4]. Due to space limitations, we do not discuss these findings, but we highlight the increasing evidence that viruses actively manipulate phloem cells to their advantage. Although viruses might be safe from many plant defences within dead xylem vessels, the predominance of the phloem as the route for viral LDM could be related to its susceptibility to being manipulated, as well as the amplification along the LDM pathway possible in living companion cell (CC)-sieve element (SE) complexes, and contact with vectors.

In what form do viral RNAs move within SEs?

Many plant viruses require their capsid protein (CP) for systemic movement. However, this does not necessarily mean that virions are the entities moving in the sieve elements, as the CP can also be involved in entry to and exit from the phloem, or in suppression of host defences (e.g. [5]). On the other hand, viruses capable of systemic spread in the absence of CP are generally assumed to be transported as ribonucleoprotein (RNP) complexes involving viral movement proteins or host RNA binding proteins.

Now, two studies have addressed the general phloem mobility of mRNAs. Whilst LDM of proteins and small RNAs has long been established [6;7], observations of phloem-mobile mRNAs have been more sporadic. An elegant approach avoiding invasive phloem sampling [8*;9*] combined grafting of different Arabidopsis ecotypes or grape varieties, respectively, with whole tissue RNA-seq, identifying ~2000 (Arabidopsis) and >3000 (grape) mRNAs that were systemically mobile, indicating

that phloem mobility of mRNAs has so far been vastly underestimated. Modelling based on mRNA abundance alone successfully explained the data of [8*], suggesting that mobility of most of the mRNAs could be due to unregulated, non-sequence-specific “leaking” into the phloem stream [10]. If this was the case, one might wonder why viruses, whose RNA genomes can be highly abundant after replicating in the CCs and repeatedly amplifying along the phloem pathway, would need a phloem transport mechanism at all, especially, since the mobile mRNA dataset from [8*] included transcripts of similar size to viral genomes. Indeed, replication-incompetent RNA3 of Brome mosaic virus can move systemically independent of viral factors [11].

However, the model [10] did not take into consideration that the size exclusion limit of plasmodesmata between CCs and SEs is ~40-60 kDa [6], which is significantly smaller than the size of most mRNAs (~340 kDa/kb). Furthermore, all three studies [8*;9*;10] found indications of selective movement of certain mRNAs, such as mobility greater than expected from abundance and size, transport against the shoot-to-root phloem direction, selective trafficking into specific aboveground organs, and mobilization of immobile mRNAs by transcriptional fusion to mobile species. Generic mobility of mRNAs is also contradicted by studies which detected systemic movement of proteins, but not their corresponding mRNAs (e.g. [12;13]). It, therefore, seems more likely that a transport system for specific endogenous mRNAs exists that may permit some abundance-dependent unspecific “leakage”. This transport system may rely on RNA secondary structures conferring mobility, as [14**] demonstrated that stem-loop secondary structures in tRNAs can mobilize transcriptionally fused mRNAs, and phloem-mobile transcriptomes [8*;9*] contained significant numbers of mRNAs either containing tRNA-like structures or transcribed as dicistronic fusions with proximal tRNA genes. Importantly, some RNA viruses have tRNA-like hairpins in their 3’UTRs, and the majority of viral RNAs contain extensive secondary structures involved in regulating various infection steps, which might interact with such a system for LDM [15]. Segmented multipartite viruses may traffic as a net of inter-segmentally base-paired RNAs stabilised by proteins including CP and host proteins, in order to ensure that recipient cells receive all genome components [16]. Viroids, non-coding pathogenic RNAs, also rely on their complex secondary structures for systemic transport [17], as well as on host proteins, some of which are graft-mobile [18].Thus, intra- or intermolecular RNA stem-loop-type structures are probably available in all RNA viruses that do not move systemically as encapsidated virions.

Viral interactions with cellular RNA-binding proteins are, therefore, of general interest for phloem RNA transport. Among these, the nucleolar RNA-binding protein and methyltransferase fibrillarin (FIB) that functions in maturation of ribosomal RNAs is particularly noteworthy. FIB is involved in the LDM of at least four different viruses [19;20;21*] and interacts with the movement proteins of another two [22;23]. In the best-studied case of FIB-umbravirus interaction, FIB is recruited to the cytoplasm, where it forms ring-like oligomers together with a viral protein, which then encapsidate viral RNA, likely the systemic transport entity [19;24;25]. Recently, it was shown that FIB also aids LDM of satBaMV, a satellite RNA of Bamboo mosaic virus, but not BaMV itself [21*]. FIB co-purified with the movement complex and was required within the phloem, as satBaMV was expressed from a 35S promoter and thus, in CC.

Given its prominence for systemic movement of viruses, it will be interesting to see if FIB also plays a role in endogenous mRNA trafficking. Both FIB and its mRNA were detected in Arabidopsis phloem exudate [26;27], and the latter was identified as mobile [8*]. It remains to be tested if silencing [19]

or knock out of FIB also affects the transport of graft-mobile mRNAs or tRNA-fused reporter constructs [8*;9*;14**]. Such findings would strengthen the case for an endogenous RNA systemic transport system exploited by viruses and open the door for its characterisation.

At the other extreme of potential transport forms of viral LDM, [3*] have observed up to ~10 μm aggregations of vesicles associated with viral replicase, dsRNA, and CP in mature SEs of *Nicotiana benthamiana* stem internodes above leaves inoculated with Turnip mosaic virus (TuMV), proposing that entire virus replication complexes (VRCs) move systemically. SE occlusion-related (SEOR) protein, which forms aggregates up to several μm in size, is systemically mobile [28]. Thus, it seems possible that VRCs could also pass through sieve plates, particularly, as agglomerations of smaller membrane structures. Whether entire VRCs in SEs are just a fortuitous outcome of phloem infection, or actually required for systemic transport, or for virion assembly of aphid-transmitted viruses like TuMV in SEs, remains to be shown.

Viral re-programming of the phloem

The phloem is known as the conduit for systemic RNA silencing and systemic acquired resistance (SAR) [7;29], and the requirement of viral suppressors of RNA silencing for LDM [2] highlights that viruses have to overcome plant defences in the phloem itself. Now, a study [30**] analysing the phloem translatome in naïve and Tobacco mosaic virus (TMV)-infected Arabidopsis and *N. benthamiana* plants, found phloem responses to be several-fold higher than in non-phloem tissues, and most biological processes were only altered in the phloem. These results impressively demonstrate the importance of the phloem during virus infection. It is, therefore, not unexpected that viruses have evolved mechanisms to subvert phloem responses.

These authors also found that TMV reprograms CC transcription by disrupting nuclear localization of auxin/indole acetic acid (AUX/IAA) responsive transcription factors [31**]. TMV-interacting AUX/IAA isoforms were shown to be CC-expressed, and their recruitment to the cytoplasm by the TMV replicase had a positive effect on virus phloem loading and systemic transport. Conversely, CC over-accumulation of a stabilized AUX/IAA mutant reduced TMV phloem transport [31**]. Transcriptomic analysis of the over-accumulating plants revealed that CC AUX/IAAs down-regulate pectin methylesterase (required for TMV phloem exit) and β-glucanases (involved in opening plasmodesmata) and up-regulate salicylic acid-dependent responses involved in SAR.

Most phloem gene expression changes during TMV infection [30**] are likely to represent plant responses. But in light of the results from [31**] it seems possible that some are also induced by the virus for its own advantage, for instance, the down-regulation of three plasmodesmata-associated callose synthases (involved in closing plasmodesmata). Most differentially regulated transcription factors had auxin-responsive elements in their promoters and might, thus, be affected by the viral manipulation of AUX/IAA proteins [31**]. Considering these extensive changes to gene expression, the full extent of viral manipulation of the phloem is likely yet to be uncovered.

The surprisingly complex host interactions of phloem-limited viruses

Another extreme example of vascular manipulation are phloem-derived tumours arising from proliferation of phloem parenchyma cells and SEs, but not CCs, which are induced by phloem-limited reoviruses [32;33*;34]. These viruses replicate preferentially in the neoplastic phloem cells, which may provide a particularly favourable environment [33*]. If the insect vectors of those viruses feed from the tumours, the latter might also function as reservoirs for efficient host transmission. Elucidation of the process by which the viruses genetically control the differentiation of new SEs will provide invaluable insights into phloem development in general. First inroads in this direction have been made by [33*], which identified proteins differentially expressed in the tumours and demonstrated down-regulation of photosynthesis (tumours possibly acting as sink tissue) and up-regulation of catabolic processes in those tissues.

The restriction of some plant viruses to the phloem is not well understood (reviewed in [35]), but may either be a virus-controlled, positively selected function allowing the virus to reach high titers at the sites of its acquisition by feeding insects [36] or be due to viruses lacking proteins facilitating infection of non-phloem cells. For instance, Citrus tristeza closterovirus (CTV) was recently shown to partially overcome severe movement restriction in sour orange and the ability to escape from the phloem when its silencing suppressor was over-expressed and salicylic acid and RNA silencing defence pathways were down-regulated [37;38].

It might be expected that viruses remaining confined to a tissue that facilitates extensive RNA movement have simplified movement systems, but in fact, the opposite is true. For instance, luteo- and poleroviruses, already known to require a canonical movement protein p4, CP, and a translational read-through extension of the CP ORF (read-through domain, RTD) [2;35;39] were recently shown to use a fourth protein for LDM. p3a, discovered as the product of a small non-AUG-initiated ORF, appears to function at the CC-SE interface or at phloem unloading [40*]. Moreover, p4 and RTD of some poleroviruses are required for virus infection of some but not all hosts [41;42], indicating that transport processes might differ between host species. The most complex movement machinery is found amongst phloem-limited closteroviruses, which require at least five proteins for movement, including the major and minor coat proteins, HSP70h, a heat shock protein homolog that behaves like a movement protein and is also incorporated into virions, p64/p61, and a small non-structural hydrophobic protein p6 (reviewed in [43]). However, individual closteroviruses require different additional proteins for systemic transport, e.g., Beet yellows virus requires the viral p20 protein, which participates in virion tip formation, and the L-Pro leader protease that interferes with the host defences in the phloem [44;45], while CTV, as shown recently, absolutely needs its leader protease L2 for systemic infection of some hosts [46*], again highlighting host-specific requirements of LDM.

Some particularly interesting observations come from recent research on the CTV p33 protein, which is also required for infection of certain hosts but is dispensable in others [47;48]. This extension of the CTV host range appears to be due to the p33 C-terminal transmembrane domain [49]. p33 shows characteristics of a movement protein, including localization to plasmodesmata, the ability to form tubules, and co-localization with the p6 movement protein [50*;51], and could facilitate virus translocation in specific hosts. But additionally, p33 is also required for CTV superinfection exclusion [52;53]. This inability of viruses to invade cells already infected by a similar virus, is a widespread but little-understood phenomenon with important applications in the cross-protection of crops [54;55]. Interestingly, p33 mediates superinfection exclusion at the whole organism (i.e., whole phloem),

rather than the cellular level. It may facilitate the spread of a “protected state” from the cells where it was produced to cells that were not infected with the primary virus [56], and it will be particularly interesting in future research if there is a connection between the two p33 functions, and to dissect virus-phloem interactions in the presence and absence of p33.

Very few host factors participating in movement of phloem-limited viruses have so far been identified. Plasmodesmata-localized calcineurin B-like-interacting protein kinase 7 (CIPK7) interacts with the RTD of Turnip yellows polerovirus and promotes an over-accumulation of the virus in inoculated cells by limiting virus exit [42]. Chaperonin Containing T-Complex Polypeptide 1, subunit 8, was identified as an interactor of the RTD of Potato leafroll polerovirus [57], and, in analogy to the role of a chaperonin in intercellular transport of the transcription factor KNOTTED 1, was suggested to assist virus transport. A more detailed understanding of the complex interactions of phloem-limited viruses with their host tissue will provide new insights into the mechanisms by which all plant viruses infect the phloem and overcome its defences.

Conclusions

A wide spectrum of different viral LDM forms and multi-faceted interactions between viruses and the phloem highlight the complexity of systemic infection processes. However, the emerging central role of the phloem in plant defence responses and possible parallels between systemic transport of viral and host RNAs make the study of virus-phloem interactions an important and exciting area of current research. Progress is likely to accelerate in the near future, thanks to the increasing experimental accessibility of this tissue, exemplified by the varied approaches highlighted in this review. We expect that more examples of viruses actively manipulating gene expression in the phloem will help to pinpoint host factors crucial for systemic movement, and in turn help to answer some of the open questions relating to viral transport forms and the requirement for various virus-encoded proteins in this process. This will enable not only new strategies to combat virus infections in crops, but also contribute to a better general understanding of regulation and function of the phloem.

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Conflicts of interest: none

Figure captions

Figure 1. The complexity of virus-phloem interactions

[1] tRNA-like secondary structures can confer phloem-mobility to a β-glucuronidase (GUS) reporter construct. Predicted secondary structures of $tRNA^{Met}$ and $tRNA^{Gly\ (GCC)}$, which confer mobility, and $tRNA^{Ile\ (TAT)}$, which does not. With the exception of ΔA/T loop/stem, deletions within $tRNA^{Met}$ do not affect mobility. Viruses contain similar secondary structures that may interact with a host mRNA trafficking system. Reproduced from [14**] with permission. [2] Requirement of fibrillarin for systemic movement of Bamboo mosaic virus satellite (satBaMV) RNA. In *N. benthamiana* heterografts between 35S::satBaMV transgenic (sat) stocks and wild-type (WT) scions, satBaMV can be detected in leaf 9 above the graft union, whereas it does not move into fibrillarin-silenced (Fib-s) scions. (Northern (top three lanes) or Western (fibrillarin protein) blots; rRNA and Coomassie Brillant Blue (CBB): loading controls). Reproduced from [21*] with permission. [3] Turnip mosaic virus replication complexes in mature SEs. Aniline blue-stained sieve plate pores in magenta; viral transmembrane protein inducing replicative vesicles ($6K_2$) in green; immunofluorescence-labelled double stranded RNA (dsRNA) in red. Dashed oval highlights VRC, arrowheads point to possible SE plastids. The last panel shows the approximate outlines of SEs and sieve plates. Modified from [3*] with permission. [4] Differential gene expression in Arabidopsis and *N. benthamiana* in response to TMV infection. Numbers of genes with >10-fold expression change compared to uninfected tissue in the phloem (pSULTR2;2 and pSUC2) are significantly higher than in non-vascular tissue (p35S non-shared). Reproduced from [30**] with permission. [5] and [6] Rice black-streaked dwarf reovirus-induced phloem tumor in maize. Overview of tumor outgrowth (Ph, phloem; Xy, xylem) [5] and high-resolution TEM image [6]. Tumor tissue contains SEs and phloem parenchyma (PP) but no CCs (A) (Chl, chloroplast; Pl, SE plastid; Vp, viroplasm; Vs, vessel). Magnifications show viroplasm, crystalline arrays of virus particles (CV) (B) and virions (V) associated with tubular structures (Tub) (C) in SEs. Reproduced from [33*] under Creative Commons License.

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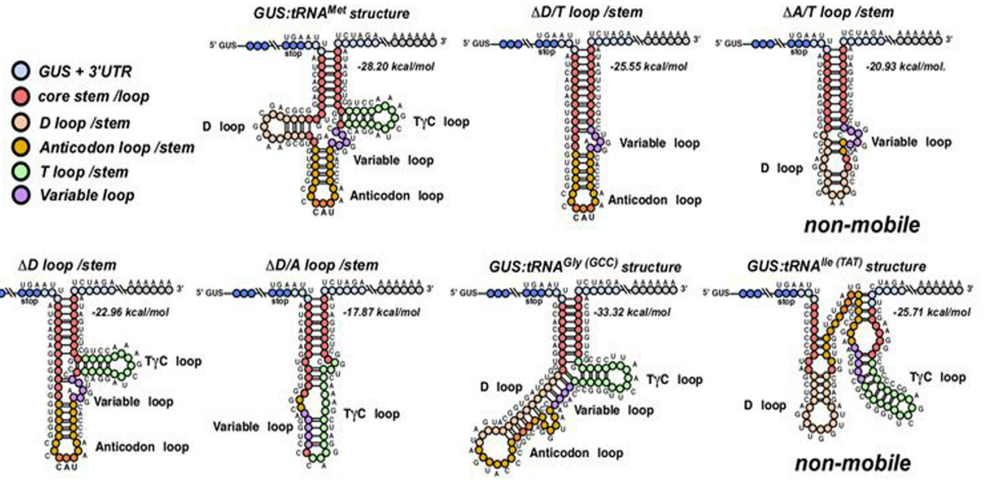
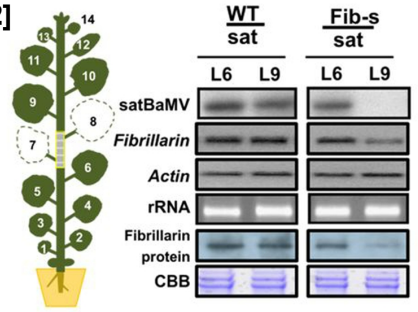
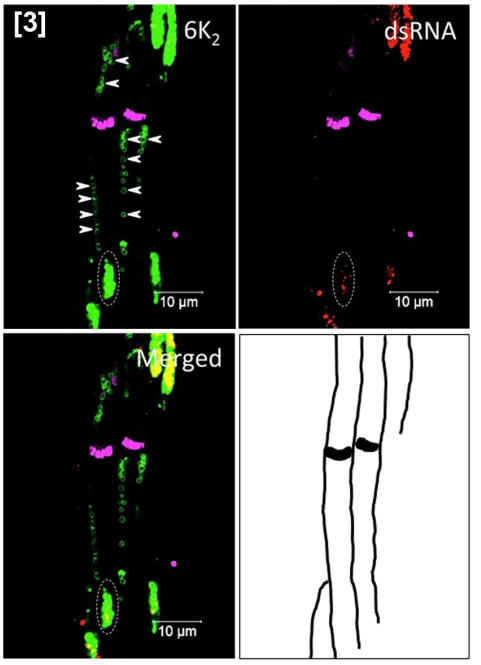
[1]


GUS + 3'UTR
core stem /loop
D loop /stem
Anticodon loop /stem
T loop /stem
Variable loop
GUS:tRNA^Met structure
-28.20 kcal/mol
ΔD/T loop /stem
-25.55 kcal/mol
ΔA/T loop /stem
-20.93 kcal/mol.
non-mobile
ΔD loop /stem
-22.96 kcal/mol
ΔD/A loop /stem
-17.87 kcal/mol
GUS:tRNA^Gly (GCC) structure
-33.32 kcal/mol
GUS:tRNA^Ile (TAT) structure
-25.71 kcal/mol
non-mobile
D loop
TψC loop
Variable loop
Anticodon loop

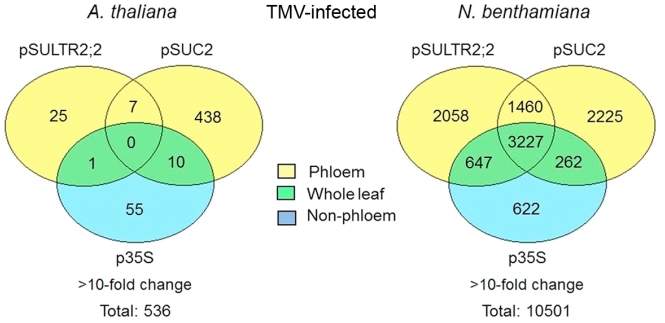
[2]


WT sat
Fib-s sat
L6
L9
satBaMV
Fibrillarin
Actin
rRNA
Fibrillarin protein
CBB

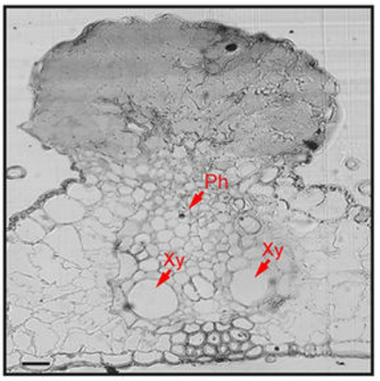
[3]


6K2
dsRNA
Merged
10 µm

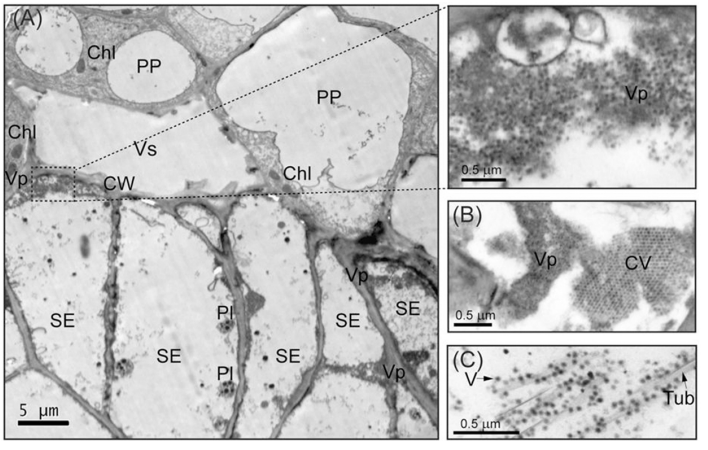
[4]


A. thaliana
TMV-infected
N. benthamiana
pSULTR2;2
pSUC2
p35S
25
7
438
0
1
10
55
Phloem
Whole leaf
Non-phloem
>10-fold change
Total: 536
2058
1460
2225
3227
647
262
622
>10-fold change
Total: 10501

[5]


Ph
Xy
Xy

[6]


(A)
Chl
PP
PP
Chl
Vs
Vp
CW
Chl
Vp
SE
SE
Pl
SE
SE
SE
Pl
SE
Vp
5 µm
Vp
0.5 µm
(B)
Vp
CV
0.5 µm
(C)
V
Tub
0.5 µm

Highlights

- *Viral RNA secondary structures may "hijack" endogenous phloem RNA transport system*
- *Entire viral replication complexes may move in sieve elements*
- *Viruses extensively modulate phloem gene expression to their advantage*
- *Phloem limitation correlates with increased complexity of virus-phloem interactions*