# **Probing the virus long-distance transport pathway**

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espite much progress in unravelling the mechanisms of intracellular and intercellular virus movement, the processes involved in systemic movement, most significantly phloem entry and exit, remain very poorly understood. For mechanically transmitted viruses, such as potato virus X (PVX), systemic infection results from the combination of both cell-to-cell and phloem-dependent movement. In the initial phase of infection, intercellular movement from epidermal cells, via mesophyll and bundle sheath cells, leads to infection of the minor vein phloem. Subsequent loading of virus into sieve elements leads to rapid dispersal of virus, through the phloem transport pathway, followed by phloem unloading of virus and cell-to-cell movement away from the vascular tissue. The pattern of virus movement mimics the translocation of photoassimilate in that both virus and solutes are exported from photosynthetic source tissues to sink tissues. Furthermore, this common pathway for phloem translocation of virus and solutes extends to the specific vein classes involved in phloem loading, which occurs in the minor veins, and unloading, which occurs exclusively from the major veins<sup>1</sup>.

We are investigating the requirements and pathways for both local- and long-distance movement of PVX through a combination of molecular and cell biological approaches. Precise, real time, analysis of viral movement processes is greatly facilitated by the use of the green fluorescent protein (GFP), as a reporter of virus-infected cells, and we have utilised a GFP-tagged PVX in order to investigate the movement phenotypes of a series of viral mutants<sup>2,3</sup>. PVX is the type member of the potexviruses and falls into a larger grouping of viruses that require the products of three overlapping open reading frames, the triple gene block (TGB), for cell-to-cell movement. Despite their central role in cell-to-cell movement, no clear function for the TGB proteins in either intracellular or intercellular transport has been established. In addition to the TGB proteins, PVX also has an absolute requirement for coat protein (CP) for intercellular movement<sup>2</sup>. In fact, the only potexvirus protein to date that has been shown to localize to plasmodesmata is the viral CP<sup>4</sup>, and available evidence suggests that PVX moves between cells as encapsidated virus particles<sup>5</sup>.

To gain a better understanding of the PVX movement process, a series of frame-shift mutations were introduced to disrupt each of the three TGB protein open reading frames. All mutations were introduced into a PVX genome tagged with the green fluorescent protein as a reporter for virus infection. Analysis of these mutants on Nicotiana clevelandii plants showed that whereas mutations in either the 25 kDa or 12 kDa TGB completely abolished cell-to-cell movement, the mutant 8 kDa protein still supported some local movement. Because the mutation introduced in the 8 kDa protein in PVX-8KFS.GFP permits the expression of the amino-terminal half of the protein, a second mutant was engineered in which translation of the entire 8 kDa protein is prevented (Fig. 1). This mutant, PVX- $\Delta$ 8K.GFP, like the mutants carrying disrupted 25 kDa and 12 kDa genes, was incapable of



#### **Figure 1** Viral genomes of 'wild-type' and mutant forms of PVX.GFP.

Schematic representation of the 'wild-type' PVX.GFP genome and the mutant genomes used in the analysis of the 8 kDa protein. The triple gene block is shown in blue with the individual open reading frames identified by the predicted sizes of their translation products (K = kDa). In the mutant PVX- $\Delta$ 8K.GFP the initiating methionine codon of the 8 kDa protein has been mutagenized to prevent translation of the protein. In PVX-8KFS.GFP a truncated protein is translated due to the introduction of a +1 frame shift mutation that introduces a stop codon after 29 amino acids.



**Figure 2** GFP fluorescence in *Nicotiana clevelandii* leaves 7 days after inoculation. Infections with either 'wild-type' PVX.GFP (a) or PVX-8KFS.GFP (b) show multicellular infection foci. Infection with PVX- $\Delta$ 8K.GFP (c) gives rise to infections that are restricted to single epidermal cells.

cell-to-cell movement and infections remained restricted to single inoculated cells (Fig. 2).

Further analysis of the attenuated movement mutant, PVX-8KFS.GFP, demonstrated that this virus was completely blocked in phloem-dependent movement. This failure to invade host plants systemically could be caused by blocks at a number of steps in the pathway either into or out of the phloem conducting tissue. In order to investigate the specific block to phloem-mediated transport exhibited by PVX-8KFS.GFP, inoculated leaf tissue was examined by electron microscopy. The results, summarised in Figure 3, showed that the inability of the mutant PVX-8KFS.GFP to move via the phloem reflected a barrier preventing infection of the minor vein phloem companion cells. The distribution of both wild-type PVX.GFP and PVX-8KFS.GFP in infected vascular tissue is illustrated in Figure 3. Thus, whereas both the wild-type and mutant viruses were equally efficient in infecting the bundle sheath cells, their ability to invade the phloem and phloem-associated cells differed. Most significant, with respect to access to the phloem transport pathway, was the absence of PVX-8KFS.GFP from the companion cells.

Figure 3 clearly shows that PVX-8KFS.GFP is able to exit the bundle sheath cells and infect both xylem and phloem parenchyma. However, the mutant was unable to invade the paired companion cells that for the wild-type virus appear to be the main route for phloem loading. Infection of phloem companion cells could result from either direct entry of virus from adjacent bundle sheath cells or, via a two-step process, in which virus first enters the phloem parenchyma and subsequently infects the companion cells. Previous studies of wild-type PVX infection have demonstrated that plasmodesmata between bundle



#### **Figure 3** Distribution of virus in different cell types of *Nicotiana clevelandii* minor vein phloem. Schematic representation of class V veins, indicating the percentage of cells showing infection within leaf tissue infected with PVX.GFP (a, 7 days post inoculation) and PVX-8KFS.GFP (b, 21 days post inoculation). The percentage of infected cells was obtained by scoring electron micrographs for either infected or uninfected cells from at least thirty independent minor veins. (c) Shows an electron micrograph of a class V vein infected with PVX.GFP and is labelled to identify the cell types shown in panels (a) and (b) (CC = companion cell, PP = phloem parenchyma, SE = sieve element, XP = xylem parenchyma).

sheath and companion cells are heavily labelled with antibody raised against the viral coat protein whereas labelling of plasmodesmata between parenchyma and companion cells is rare<sup>5</sup>. This evidence suggests that direct invasion of companion cells from adjacent bundle sheath cells is the normal route by which PVX reaches the phloem. A significant feature of the findings presented is that, although PVX-8KFS.GFP is clearly able to move beyond the bundle sheath cells and invade the phloem parenchyma, the normal route for PVX invasion of the phloem is completely blocked. The results strongly suggest that, as well as a general requirement in facilitating the cell-to-cell movement process, the PVX 8 kDa protein plays an essential rôle in allowing invasion of the companion cells to occur.

The barrier to phloem entry exhibited by PVX-8KFS.GFP, between bundle sheath and companion cells, is of particular importance in the transport pathway of Nicotiana species as it marks the boundary between symplasmic and apoplasmic transport. In many species, including members of the family Solanaceae, phloem loading occurs apoplasmically, whereas virus movement is known to be symplasmic. Apoplasmic phloem loading involves the retrieval of solutes from the apoplast into companion cells and sieve elements by specific transporter proteins. The minor vein configuration in species of the genus Nicotiana is typical of apoplasmic phloem loaders and two lines of evidence point to a functional symplasmic barrier, sufficient to prevent solute transfer, between the companion cell-sieve element complex and surrounding cells. First, phloem loading of sucrose in tobacco is completely blocked by the sucrose symport inhibitor PCMBS<sup>6</sup>. Second, experiments performed on tobacco have shown that treatment of tissues with high concentrations of solute causes plasmolysis of bundle sheath and phloem parenchyma cells but not of companion cells<sup>7</sup>. Both these lines of evidence argue against the presence of an operational symplasmic pathway into the phloem of mature (*i.e.* source) leaves. However, the immunolocalization of PVX in companion cells of the minor vein  $phloem^{1,5}$  (Fig. 3) clearly shows that PVX, and undoubtably other viruses, can exploit a symplasmic pathway that is not functional for solute transport in uninfected tissue. This in turn raises the question of whether PVX is directing the *de novo* production of intercellular channels or is opening pre-existing plasmodesmata that under normal circumstances are nonfunctional for solute transport.



Figure 4 Plasmodesmogram of cells within and surrounding a *Nicotiana clevelandii* class V vein. Plasmodesmatal frequencies at the different interfaces between cells of a class V vein were counted from serial sections of veins imaged by electron microscopy. Numbers refer to the average number of plasmodesmata per 10  $\mu$ m of cell wall.

In order to establish the distribution of plasmodesmata in the minor veins of uninfected N. clevelandii. a detailed analysis of plasmodesmatal frequency was carried out using electron microscopy. The results, shown in Figure 4, demonstrate that in non-infected tissues the companion cells are highly connected to neighbouring bundle sheath and parenchyma cells. This apparent symplasmic continuity between bundle sheath and companion cells is clearly non-functional in the mature leaf, based on the evidence discussed above. Whether the observed high frequency of plasmodesmata connecting the bundle sheath to the companion cells reflects a legacy of development or whether some symplasmic continuity is maintained for transport of small molecules and ions (e.g. for signalling purposes) cannot be resolved from this study. However, the evidence obtained does clearly demonstrate that PVX is able to exploit a symplasmic movement pathway that under normal conditions does not permit the passage of solutes. Furthermore, the failure of PVX-8KFS.GFP to invade companion cells strongly suggests that a normal role of the 8 kDa TGB protein is to unlock this gateway to phloem entry.

Phloem loading of virus, like every other aspect of the viral life cycle, requires productive interactions between host and viral proteins. Mutants, such as PVX-8KFS.GFP, that are capable of cell-to-cell but not phloem dependent movement, suggest that the specific interactions required for these two phases of the movement process are different. The demonstra-

tion that, for PVX-8KFS.GFP, the barrier to phloem loading occurs at the same cell interface, between bundle sheath and companion cells, as the symplasmic barrier to solute transport in Nicotiana species, emphasises the key importance of this boundary in regulating access to the long distance transport path-



way of the phloem. Strategies designed to exploit this natural barrier to long distance transport, reinforcing the block to symplasmic movement, could provide an effective barrier to long distance virus movement without affecting the capacity of the plant to transport essential macromolecules via the phloem.

#### References

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