Instituto Juan March de Estudios e Investigaciones

80 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Plasmodesmata and Transport of Plant Viruses and Plant Macromolecules

Organized by

F. García-Arenal, K. J. Oparka and P. Palukaitis

D. Baulcombe
R. N. Beachy
C. E. J. Botha
B. Ding
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S. Santa Cruz
R. Turgeon
A. J. E. van Bel
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INTRODUCTION

Fernando García–Arenal, Karl J. Oparka and Peter Palukaitis

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INTRODUCTION

Everything flows and nothing stays (Heraklitus, 513 B.C.)

Plasmodesmata are symplastic bridges between plant cells that define a topological unit of the plant body, limited by plasma membranes, known as the symplast. Symplastic connection is critical for processes in plant development such as morphogenesis or assimilate transport through the phloem. Thus, plant physiologists and cell biologists have made major efforts to understand the structure and function of plasmodesmata that connect different tissues.

Macromolecules do not pass through plasmodesmata, unless they provide a system that facilitates their own transport. Plant viruses fall into this category. To invade a plant systemically, most plant viruses need to move from the initially infected cells to neighbouring ones, and then, via the vascular tissues, to other parts of the plant. Thus, plant viruses encode proteins that facilitate their movement through plasmodesmata and into the phloem.

Considerable progress has been made in the last ten years in identifying virus-encoded proteins involved in various aspects of the movement process. These movement proteins have facilitated the understanding of macromolecular trafficking between cells, although less is known about how viruses access the phloem and move within and out of sieve elements. Nevertheless, the various virus movement proteins and viral genetic materials are very useful either as probes or to provide molecular markers to understand both plasmodesmatal functions and processes related to phloem transport.

Consequently the stage has been reached at which there is a convergence between the goals of plant biologists and virologists, with each being able to offer unique contributions and perspectives to the further understanding of the specific mechanisms by which viruses and other macromolecules move within plants. Thus, it was timely that there should be a meeting bringing together these two fields to foster future research and collaboration.

This meeting first reviewed what is known about plasmodesmata structure and development, and then proceeded to consider the progress made in understanding the functions of plasmodesmata through viral and developmental approaches. Processes involved in phloem-dependent transport were examined using different markers such as fluorescent dyes, phloem-specific proteins and viruses. Various viral movement strategies

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were described, as well as the role of plasmodesmata in the plant's response to pathogen invasion.

The stage is now set for genetic and molecular approaches that will result in the characterisation of the detailed structure of plasmodesmata between different cell types. This will contribute to our understanding of how the plasmodesmal components interact with viral movement proteins and other macromolecules to regulate intercellular communication within plants.

Fernando García-Arenal
Karl J. Oparka
Peter Palukaitis

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Plasmodesmata Structure, Formation and Development

Chair: William J. Lucas

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KOLLMANN, Rainer: Multimorphology and terminology of plasmodesmata

In multicellular higher plants the number and structure of plasmodesmata changes during cell differentiation. Primary plasmodesmata are formed during cytokinesis in the cell plate as single unbranched cytoplasmic strands each containing one tightly constricted ER tubule (Hepler, 1982). Depending on cell differentiation, many of these straight primary plasmodesmata become modified by various forms of branching during wall thickening (Ehlers and Kollmann, 1996; Glockmann and Kollmann, 1996) thus partially compensating for the dilution of cytoplasmic bridges during cell extension and division. It is furthermore likely that plasmodesmata are formed de novo in elongating division walls by fusion of opposite half plasmodesmata. This process resulting in branched secondary plasmodesmata occurs in wall regions with thinned and loosened matrix following a mechanism described by Kollmann and Glockmann (1991) for non-division walls. The principle of formation of primary and secondary plasmodesmata hold one fundamental mechanism in common: enclosure of ER by fusing Golgi-vesicles which deliver wall material and the plasma membrane surrounding the cytoplasmic bridge (Hepler, 1982; Kollmann and Glockmann, 1991; Monzer, 1991; Ehlers and Kollmann, 1996). Coincidence in the mechanism of formation accounts for the identical fine structure and corresponding dimensions of primary and secondary plasmodesmata. There is only one difference in the formation process between both types of cell connections: while primary plasmodesmata are continuous from the very beginning of cell plate building, secondary cell connections require fusion of plasmalemma and ER of adjacent cells. Secondary branching occurs in primary as well as in secondary plasmodesmata during wall thickening. This in connection with the identical fine structure make any distinction between primarily and secondarily formed cell bridges difficult. Unequivocally, secondary plasmodesmata can only be identified in non-division (fusion) walls of e.g. graftings (Kollmann and Glockmann, 1985), chimeras (Steinberg and Kollmann, 1994), host parasite interactions (Dörr, 1969), fusion of carpels (van der Schoot et al, 1995). In division walls, where the mode of origin of the cell bridges is unclear, the term "branched plasmodesmata" will be more appropriate as it is correct for both, primary and secondary cell connections.

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ARCHITECTURE OF PLASMODESMATA

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The architecture of plasmodesmata has been under conjecture since since they were first observed some forty yeaars ago in the electron microscope. One reason for the difficulty in the discerning the detailed structure of plasmodesmata has been that the diameter of a plasmodesma is in the order of 50nm, similar to the thickness of the thin sections used for electron microscopy. In longitudinal view, the entire plasmodesma may be included within the section and in a transverse view, one third of the plasmodesma may be superimposed. There has been disagreement about the interpretation of the distribution of stain within a plasmodesma (compare Overall et al, 1982 and Ding et al, 1992). In addition, the processes involved in preparation of material for electron microscopy are likely to have introduced artefacts.

We have used a variety of techniques in an attempt to overcome these problems. The ultrastructural details of plasmodesmata that have been conventionally fixed with those that have been freeze-substituted have been compared. Using 2-deoxy-D-glucose (DDG), an inhibitor of callose synthesis, we have demonstrated that the neck constrictions at the ends of plasmodesmata which restrict the cytoplasmic annulus are an artefact of callose deposition stimulated by chemical fixation (Radford et al, 1998). The cytoplasmic pathway through plasmodesmata may be larger than previously thought.

The development of the field emission scanning electron microscope (FESEM) has achieved the resolution available in transmission electron microsopy. High resolution scanning electron microscopy (HRSEM) has provided spectacular 3-dimensional images of the plant cytoskeleton and has been combined with immunocytochemistry (Vesk et al, 1996). We have applied these techniques to study the three-dimensional architecture of plasmodesmata in an attempt to clarify confusion over interpretation of staining patterns and superimpostion of many structures in one image. On preparations in which the walls have been lost, simple primary plasmodesmata appear as columns between cells. This technique is particularly suited to imaging the architecture of complex branched plasmodesmata such as those in *Chara corallina*.. When the wall is present, there is a consistent space between the plasmodesma and the visible wall. There are occasional spokes traversing this space, connecting the plasmodesma and the surrounding wall material. On rare occasions, the internal structure of plasmodesmata has been visible revealing a central structure surrounded by particulate material. Combining HRSEM with immunogold cytochemistry of callose has shown that callose is located around the neck regions of *Chara corallina* plasmodesmata, closely associated with the plasmamembrane rather than the cell wall.

Immunogold cytochemistry has demonstrated that actin and myosin are present in the plasmodesmata of both higher plants (White et al, 1994; Radford and White, 1988) and *Chara corallina* (Blackman and Overall, 1998). We have attempted to incorporate these cytoskeletal elements into a dynamic model for the architecture of plasmodesmata (Overall and Blackman,1996). The central axial structure is presented as a tightly curled cylinder of endoplasmic reticulum (ER), although it is possible with the wider dimensions of plasmodesmata that this pathway could be opened. The actin is thought to be the particulate matter associated

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with the ERand is depicted spiralling around the it down through the plasmodesma. The spokes seen connecting this particulate matter to the plasmamembrane may be myosin. The model also incorporates the extracellular structures observed around plasmodesmata (Badelt et al, 1994) and a putative contractile protein at the neck region.

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PLASMODESMATA - TOWARDS A RECONCILIATION OF ULTRASTRUCTURE WITH FUNCTIONAL STATE

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Robards' paper in Nature, (1968a) entitled "Desmotubule - A plasmodesmatal Substructure" - started a debate which, some 29 years later, remains largely unresolved. The concept of the desmotubule, its fine structure, and relationship to the other components of higher plant plasmodesmata remains equivocal with respect to its substructure, its presence in most higher plant plasmodesmata and, therefore, its role in cell to cell communication. Almost as many interpretations of structure exist in the literature as there are references.

In a review Robards and Lucas (1990) alluded to the difficulties associated with electron imaging of plasmodesmata. The problem remains one of interpreting the electron image as seen in transverse view and the reconciliation of this with longitudinal views of plasmodesmata from the same tissues. Thus, the statements made by Robards and Lucas (1990), remain true - it is and remains difficult to correlate transectional with longitudinal plasmodesmal structure. The difficulties are enormous difficulties, associated for the most part with noise generated within the electron image and the plane of the section, where average section thickness exceeds that of the diameter of the desmotubule foe example. In addition, plasmodesmatal function also remains equivocal. Indeed, there are many interpretations of function, and as many views on their potential regulatory role cell-cell transport. The literature contains many examples of evidence for changes in plasmodesmal functionality, which seem to be chiefly related to the roles of the tissues concerned - i.e., whether these are importers (sinks) or exporters (sources). There is direct evidence for pressure-regulated closure of plasmodesmata (Oparka and Prior, 1992), that short term osmoregulation leads to increased phloem unloading (Schulz, 1994) and that symplastic phloem unloading is inhibited during the source-sink transition in *Nicotiana benthamiana* leaves (Roberts *et al.*1997). There is direct evidence that accelerated leaf senescence may be effected through arrested development of secondary plasmodesma in transgenic tobacco plants (Ding *et al.* 1993). However, none of these papers make use of high resolution, detailed electron microscopy to substantiate argument and evidence for loss of transport capacity in the tissues investigated.

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The suggestion by Robards and Lucas (1990) that electron microscope images are "snapshots in time" and therefore only indicators of potential symplastic transport, remains for the most part, correct.

This paper will explore the relationships between plasmodesmatal substructure and function using electron microscopy and cell-cell transport of Lucifer yellow in two very different sink systems -the first 225 day old avocado fruit, and the second, mature sugar cane stem tissue. We will demonstrate that conformational changes that occur in the plasmodesmatal ultrastructure of these ageing systems preclude cell-cell transport of Lucifer yellow and that regulation is achieved through the formation of neck-associated electron-dense sphincter-like structures in avocado and in sugar cane. There is evidence that the electron-dense material may contain callose.

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Abstract: The formation of symplast domains by plugging of plasmodesmata - a prerequisite for coordinated differentiation processes?

Cultured protoplasts of many plant species develop into calluses which are able to organize adventitious embryos and shoots especially when they are transferred onto agar media with special phytohormone combinations. Electron microscopical studies on protoplast-derived microcalluses of *Solanum nigrum* revealed that within these calluses certain cells or groups of cells were most likely symplasmically isolated from the surrounding callus cells by plugging the connecting plasmodesmata (Pd) with an osmiophilic, dense material. Plugged Pd were already observed within 9- to 11-day-old microcalluses consisting of small, dedifferentiated cells which do not yet show any structurally apparent peculiarities but were never found at younger stages of culture. Moreover, it was proved in serial sections that if plugged Pd do occur between adjacent callus cells, all the Pd in this certain cell wall will be occluded. However, the Pd in other contact walls of the same callus cells might be ultrastructurally inconspicuous. Within a plugged Pd the osmiophilic blocking material is obviously located in the microchannels of the cytoplasmic sleeve which presumably represent the pathways for the Pd transport. Thus, it can be assumed that the plugged Pd are non-functional. Consequently, those cells or cell clusters which are encircled by plugged Pd obviously are symplasmically isolated from the surrounding callus cells and might therefore function as an independent symplast domain. Sometimes, the blocking material was found to be located at only one of the Pd neck regions which presumably indicates that the osmiophilic material was deposited by one of the adjacent cells, i.e. by the non-isolated surrounding callus cell.

When structural studies were carried out on embyogenic calluses derived from immature embryos of *Molinia caerulea*, plugged Pd were also observed in certain cell walls of the globular proembryogenic structures which are formed on scutellar callus and develop rapidly into somatic embryos. It will be shown in an overall view summarizing the detailed analyses of 12 ultrathin serial sections that already at a very early developmental stage the selective occlusion of Pd in certain cell walls creates a complicated pattern of symplasmic (dis)continuity and obviously leads to the formation of isolated symplast domains within the proembryogenic structures.

Plugged Pd have already been described to occur in developing antheridia of *Chara vulgaris* L. (e.g. Kwiatkowska and Maszewski 1985, 1986) between asynchronously dividing or asynchronously differentiating cells and, therefore, symplasmic discontinuity has been interpreted as a prerequisite for a desynchronisation of the cells in the course of developmental processes. In agreement with these findings the plugged Pd in the protoplast-derived calluses or in the proembyogenic structures might as well be regarded as the first symptoms indicating desynchronisation of mitotic activity and initiation of differentiation processes in the course of embryogenesis. Especially in the view of recent findings that a macromolecule transport through Pd might influence developmental processes it seem to be reasonable that, on the other hand, symplasmic isolation and the formation of symplast domains might as well be prerequisites for coordinated differentiation processes in the course of organogenesis.

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Probing Plasmodesmata Functions

Chairs: Karl J. Oparka

Robert Turgeon

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GFP LIGHTS UP THE TUNNEL: STUDIES ON PLASMODESMAL COMPOSITION AND FUNCTION AS PROBED WITH VIRAL MOVEMENT PROTEINS LABELED WITH GREEN FLUORESCENT PROTEIN.

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Plasmodesmata (PD) are specialized gatable co-axial membranous trans-wall tunnels that interconnect the cytoplasm of contiguous cells and function in a direct intercellular movement of small molecules and, in certain cases, of macro-molecules. Although the importance of these quasi-organelles is recognized, only recently have PD components begun to be identified. In addition to their normal role in symplastic transport, PD are exploited by viruses as conduits for spreading from cell-to-cell. Because plant viruses target to and modify PD, they have become important tools for the study of PD composition and function. The putative movement proteins V1 and V2 of the DNA maize streak geminivirus, MSV, and the movement protein MP^{tmv} of the RNA tobacco mosaic tobamovirus, TMV, were fluorescently labeled by translational fusion to the green fluorescent protein (GFP) of *Aequorea victoria* and the fusion gene expressed behind a 35 S promoter following biolistic bombardment. In maize leaves, V1-GFP but not free GFP moved cell-to-cell, thus establishing V1 as the MSV cell-to-cell movement protein. V2 targeted only to nuclei, and no expression could be detected in the cytoplasm in the absence of V1. Unlike V1, V2 did not move cell-to-cell. MP^{tmv} -GFP when expressed independent of the virus in *Nicotiana bentamiana* leaves targeted transiently to several subcellular sites including cytoskeleton and PD, and moved cell to cell. Employing various MP mutants, we are studying partial reactions of MP targeting and movement. By employing MP^{tmv} -GFP labeled PD, we have developed procedures for isolating and purifying PD following enzymatic digestion of isolated cell walls. Selected PD proteins in non-infected leaves and non-infected are being purified and microsequenced and their genes are being cloned and sequenced. Results from these studies should provide us with molecular information on PD composition and on mechanisms by which tobamoviruses target to, modify and exploit PD for cell-to-cell spread of infection.

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Viral and developmental clues into plasmodesmata function.

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We approach the study of plasmodesmata (PD) by monitoring their function following two general types of provocation, either following infection with plant viruses, or following alteration in developmental programming.

Regarding plant viruses, we continue to use tobacco mosaic virus (TMV) as a molecular probe for PD function. In particular, we have focused on the movement protein (MP) of TMV. To date we have mapped 5 regions of the 30 kDa TMV-MP, C-terminal domains A and B define single strand nucleic acid binding activity, domain C is essential for proper folding, domain D at the very end of TMV-MP contains sites for phosphorylation by a plant cell wall associated protein kinase, and domain E specifies gating of PD. Our most recent work delimits a 6th domain, F, for cytoskeletal binding. We have initiated studies on the MPs of turnip crinkle virus (TCV). This virus is of particular interest since it infects Arabidopsis, providing a genetically tractable host system to dissect PD structure and function. In addition, TCV encodes 2 very small MPs, p8 (8 kDa) and p9 (9 kDa), that may provide a more precise localization of signal sequences for intra- and intercellular movement.

Regarding PD function during development, we have begun to address changes in PD function in the shoot apex of Arabidopsis. The availability of fluorescent markers that monitor symplastic movement, such as HPTS (8-hydroxypyrene-1,3,6 trisulfonic acid) makes these types of studies highly feasible. HPTS is loaded into the phloem and distributes symplastically to sites far from the site of initial loading. The fluorescent tracer is monitored by confocal imaging. We are particularly interested in determining symplastic domains in the shoot apical meristem during development. We have observed that the patterns of dye movement in the vegetative meristem becomes altered during the transition to flowering. Much research in the area of floral development has focused on the induction of genes, particularly transcription factors, essential for reprogramming the meristem to produce inflorescence and floral meristems. However, our studies suggest that intercellular trafficking via PD within the meristem also undergoes changes when flowering is induced.

An additional area of research involves our attempts to identify PD components by a genetic screen for mutations in Arabidopsis with altered PD trafficking patterns/size exclusion limits. For these studies we are screening embryo lethal mutants for embryos with altered PD size exclusion limits. One mutant ise-1 (Increased Size Exclusion limit-1) allows 11 kDa dextrans to move from cell to cell, while wild type embryos generally have a size exclusion limit below 3 kDa. The ise-1 mutant also exhibits morphological defects late in embryogenesis.

For review and additional references see:

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***ise-1 (Increased Size Exclusion limit-1)* allows cell-to-cell movement of molecular probes that are typically restricted to individual cells.**

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We have initiated a screen for *Arabidopsis* mutants with altered plasmodesmal size-exclusion limits. We are using fluorescent probes including HPTS, carboxyfluorescein and F-dextrans to assay for altered size exclusion limits in embryo-defective mutants. The first characterized mutant from the screen, *ise-1*, allows 11 kDa dextrans to move from cell-to-cell, while wild-type embryos generally have a size exclusion limit below 3 kDa. *ise-1* mutant embryos also exhibit morphological defects which are most prominent late in embryogenesis. The mutant does not appear to be a cell wall, or cytokinesis-defective mutant. Mapping data and allelism tests are currently in progress.

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EVOLUTION OF MACROMOLECULAR TRAFFICKING THROUGH PLASMODESMATA

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Over the past decade we have seen an explosion of information on the novel functions performed by plasmodesmata. During this period of intense activity we have seen a paradigm shift from plasmodesmata acting as the foundation for the symplasm concept, to the emerging picture, where plasmodesmata appear to establish supracellular information highways. In the symplasmic scenario, the plasmodesmal cytoplasmic channels facilitate the cell-to-cell diffusion of metabolic substrates to permit the coordination of biochemical and physiological processes. In the supracellular information trafficking scenario, plasmodesmata potentiate the cell-to-cell trafficking of macromolecules, either proteins or protein(s)-nucleic acid complexes, to exert control over developmental and physiological processes. Although much has been discovered, with respect to plasmodesmal function, only the basic framework of the "picture" has been established at this stage.

Discoveries into the evolutionary events that ultimately gave rise to the capacity of plants to mediate cell-to-cell trafficking of macromolecules will likely add depth to the current "picture." Likewise, molecular information into the mechanisms that underlie the capacity of the plasmodesmata to engage in the selective trafficking of macromolecules will surely open the way to a more detailed characterization of the many proteins that constitute this novel supramolecular structure. These aspects of plasmodesmal biology will be discussed from the perspective of cellular, tissue and whole plant signaling systems.

There is now a significant body of evidence that both endogenous and viral proteins have the capacity to interact with the cellular machinery of the plant to mediate their movement from cell to cell. These results suggest that viral movement proteins may have acquired, from the plant, the genes that encode the capacity to interact with plasmodesmata. If this were the case, endogenous and viral movement proteins may very well interact with a common plasmodesmal receptor(s). Results consistent with this hypothesis will be discussed. The identification and cloning of this receptor(s) will provide a useful tool to perform an evolutionary analysis of the phylogenetic distribution of this gene(s). We will also discuss the strategy taken to search for plant genes that contain sequences that are homologous to viral movement proteins.

Finally, we will discuss the evolutionary development of the sieve element system of the phloem. Here we will advance the hypothesis that refinement in the trafficking of macromolecules through plasmodesmata may have opened the way for the development of the enucleate sieve tube system of higher plants. An important consequence of this advancement appears to be the emergence of long-distance macromolecular (protein and protein-RNA) information processing (signaling) systems.

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Intercellular trafficking of potato spindle tuber viroid

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Viroids are small, circular, and autonomously-replicating pathogenic RNAs that infect only plants (Diener, 1979). For a viroid that replicates in the nucleus the infection process should consist of the following distinct components: 1) import into the nucleus, 2) replication), 3) export out of the nucleus, 4) cell-to-cell movement, 5) entry into the phloem, 6) long-distance transport via phloem (Palukaitis, 1987), 7) exit from the phloem, and 8) re-entry into non-vascular tissues to establish new infection at a remote site. How a viroid accomplishes each of these steps is poorly understood.

A distinguishing feature of viroids is that they do not encode any proteins. Thus, these RNA molecules must interact directly with cellular factors to accomplish all of the functions necessary for infection, including intracellular and intercellular movement during infection. As such, viroids can also be used as unique probes to study RNA trafficking in plants. We have recently initiated projects to use potato spindle tuber viroid (PSTVd) as a model system to investigate viroid movement. PSTVd is the best characterized viroid at the molecular level. Many infectious and non-infectious mutants of PSTVd are currently available (e.g., Owens et al., 1991). Whether those non-infectious mutants are defective in replication or in any of the movement processes are not understood.

Our initial microinjection studies with fluorescently-labelled PSTVd RNA and cDNA and other RNAs indicate that 1) PSTVd moves from cell to cell via plasmodesmata and 2) this movement may be mediated by a specific sequence or structural motif (Ding et al., 1997). We are developing complementary approaches to further characterize PSTVd cell-to-cell movement. We are also developing experimental approaches to characterize nuclear transport of PSTVd and to separate PSTVd mutants defective in replication and in various movement processes. Preliminary data from these experiments will be discussed.

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Role of TMV movement protein in intra- and intercellular spread of infection

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Our studies of the P30 movement protein (MP) of TMV have focused on characterizing the role of the protein in establishing sites of virus replication within infected cells, determining the distribution of the MP in infected protoplasts and leaves, and establishing structure/function relationships of the protein. We created a number of mutations of three amino acids (TAD) in the MP, fused the wild type and mutant MP with GFP and expressed the proteins in place of w.t. MP in TMV. Subcellular localization of the fusion protein was determined through imaging live and fixed tissues (using fluorescent and confocal microscopy) and biochemical fractionation of infected tissues. Infected cells that produce MP:GFP accumulate fluorescence in selected subcellular compartments that change during the course of infection; the nature of the sites of accumulation were identified by antibody co-staining and/or co-isolation. Early in infection MP:GFP accumulates (in protoplasts) at punctate sites at, or on, the plasma membrane (PM); we suggest that some of these are sites of attachment of cortical ER and PM. Accumulation of MP:GFP in plasmodesmata (Pd) is the earliest sign of infection in inoculated leaves (Padgett et al, 1996). Soon thereafter MP:GFP is observed in irregularly shaped fluorescent bodies in the cytoplasm and with the perinuclear ER, and subsequently is associated with the vortexes of the cortical ER. Late in infection, MP:GFP is associated with microtubules (Heinlein et al, 1995) after which MP:GFP fluorescence is lost from the large irregular structures, leaving only fluorescent punctate structures at the periphery of infected protoplasts, and in Pd in infected leaves (Heinlein et al, in press). We have identified TAD mutants of the MP that do not function in cell-cell spread of infection, yet accumulate in a subset of the sites in which w.t. MP:GFP accumulates; one of the mutants appears in all known sites of accumulation of w.t. MP, yet the mutant does not enable cell-cell spread (Kahn et al, in press).

The cortical fluorescent bodies often grow in size and/or fuse to form large sheets of fluorescence. Antibody against TMV replicase is localized to these bodies, which we suggest are sites of TMV replication and protein synthesis (Heinlein et al, in press). In infected leaves the cortical ER is disrupted during the period when the sites of replication are being established, after which the ER returns to the pre-infection state (C. Reichel and R. N. Beachy, submitted). ER disruption is concurrent with the period when the size exclusion limits (SEL) of the plasmodesmata (Pd) are increased; recovery of ER precedes the time when SEL of the Pd is returned to normal (Oparka et al, 1997). Microsomes were isolated from infected leaves and shown to contain ER luminal proteins as well as the MP. While luminal proteins were released by detergent treatments, MP remained associated with the membranes treated with Triton X-100, but was released by CHAPS. When isolated microsomal membranes were treated with proteinase K the MP was degraded, while luminal proteins were resistant to digestion unless membranes were first treated with Triton X-100 (C. Reichel and R.N. Beachy, submitted). We conclude that MP is bound to cortical and cytoplasmic ER early in infection and that ER establishes and/or distributes infection sites around the cell, presumably through involvement of the cytoskeleton. During this time the infection is targeted to the Pd for intercellular spread; we propose that spread to adjacent cells may occur through movement of ER through the Pd (Grabski et al, 1993).

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Abstract Christina Kühn:

Localization and turnover of the sucrose transporter SUT1 in enucleate sieve elements

The leaf sucrose transporter SUT1 is essential for phloem loading and long distance transport of assimilates. In tomato plants SUT1 mRNA is diurnally regulated. The amount of SUT1 protein can be reduced either by prolonged dark treatment or by addition of a translational inhibitor indicating a high turnover rate of SUT1 protein.

An affinity-purified SUT1-specific antiserum has been used to localize the sucrose transporter at the light and electron microscopic level in tissue sections of different *Solanaceae* species, namely in tobacco, potato and tomato.

The antibody identifies the transporter with an apparent molecular mass of 46 kDa, in membrane preparations of yeast and plant cells. The antibody localizes the sucrose transporter specifically at the plasma membrane of enucleate sieve elements in different potato, tobacco and tomato tissues.

In situ hybridization at the EM level shows that SUT1 mRNA localizes at high levels in sieve elements, and at low levels in companion cells. The transcripts are preferentially associated with plasmodesmata, between sieve elements and companion cells.

Due to the lack of nuclei, mature sieve elements require import of RNAs or protein from neighboring companion cells. Young sieve elements still containing a nucleus already exrpress SUT1 protein. Either SUT1 protein is stable for several months, i.e. the lifespan of sieve elements, or SUT1 mRNA or protein is translocated asymetrically from companion cells toward sieve elements via plasmodesmata.

Antisense inhibition of *SUT1* expression under control of the companion cell specific promoter *rolC* indicates synthesis of SUT1 mRNA in companion cells (Kühn et al., 1996). Localization of SUT1 protein in enucleate sieve elements and preferential association of SUT1 mRNA with plasmodesmata connecting both companion cells and sieve elements, together with indications of a highly regulated turnover rate of SUT1 mRNA and protein, provide evidence for the targeting of plant endogenous mRNA, and potentially SUT1 protein through phloem plasmodesmata.

The data obtained in the three investigated *Solanaceae* are consistent with sucrose loading occuring directly at the plasma membrane of sieve elements.

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Kühn C., Quick W.P., Schulz A., Riesmeier J. & Frommer W.B. (1996) Phloem-specific inhibition of sucrose transport in transgenic potato plants. Plant Cell Environm. 19, 1115-1123.

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CHARACTERIZATION OF KNOTTED1: A PLANT PROTEIN THAT CAN SELECTIVELY TRAFFIC THROUGH PLASMODESMATA

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Our aim is to understand the function and mechanism of cell to cell trafficking of the *knotted1* (*kn1*) homeobox gene products during plant development. *kn1*, and its' Arabidopsis orthologue, *shootmeristemless*, have been shown to play a pivotal role in plant development by being essential for the initiation and/or maintenance of the shoot apical meristem. The *Knotted1* mutation was the first of a growing number of developmental mutations in plants that are known to act non cell autonomously, making it an ideal model for the study of intercellular communication during development. The *kn1* gene encodes a homeodomain protein suggesting that it acts as a transcriptional regulator. Several independent lines of evidence, including mosaic analysis, expression studies and recent microinjection experiments indicate that the KN1 protein traffics between plant cells through plasmodesmata (Jackson & Hake 1997; Lucas et al 1995). Therefore, the intercellular trafficking of KN1 may define a novel signaling pathway in plants. We will describe approaches to determine the biological function and significance of cell to cell trafficking of KN1. We are also using green fluorescent protein (GFP) tagged KN1 to study trafficking *in vivo* through the generation of mosaics and the use of cell specific promoters, and will describe our progress.

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Movement Protein / Plasmodesmata Interactions

Chairs: Aart J.E. van Bel

Patricia Zambryski

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Expression of truncated forms of the cucumber mosaic virus 3a protein in transgenic tobacco plants affects viral accumulation.

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The 3a protein encoded by RNA 3 of cucumber mosaic virus (CMV) has been identified as the cell-to-cell movement protein (Vaquero *et al.*, 1994. **J. Gen. Virol. 75**: 3193; 1996. **Arch. Virol. 141:** 987. Kaplan *et al.*, 1995. **Virology 209**: 188; Ding *et al.*, 1995. **Virology 207**: 345).

Ten deletion mutants of the 3a protein, spanning aa 124-221, have been constructed, and used to transform *N. tabacum* plants. Transgenic tobacco plants expressing the 3a protein deletion mutants were assayed for their effect upon viral accumulation. ELISA-DAS analysis showed that plants expressing certain deletion mutants exhibit a reduction of CMV accumulation, whereas others increase the viral accumulation. This effect parallels symptom appearance.

In some cases, the expression of the deleted forms of CMV 3a protein is effective against heterologous virus. It is associated to a reduction in the size of necrotic local lesions induced by TMV.

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Functional analysis of the movement protein of cucumber mosaic virus

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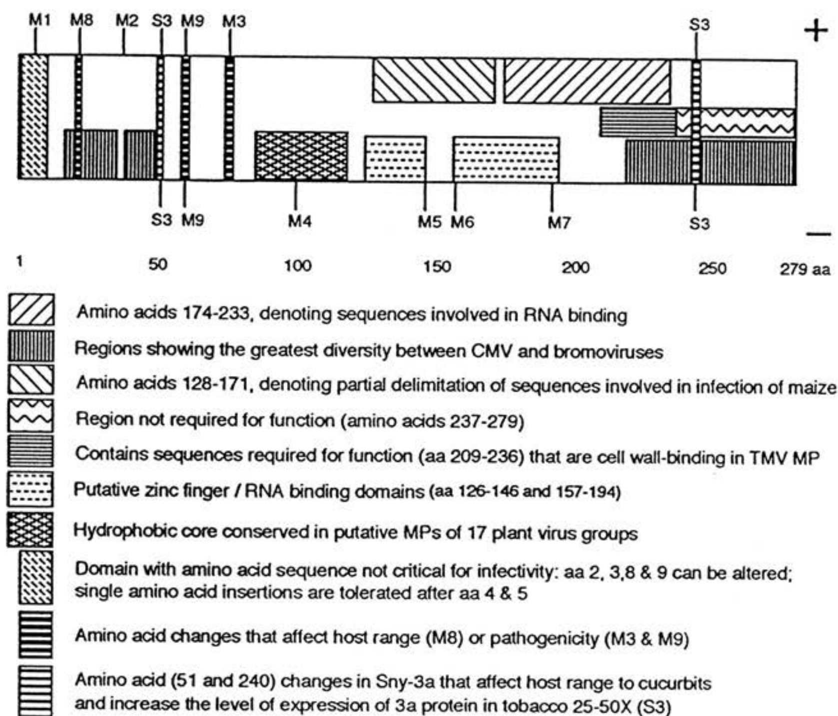
The movement protein (MP) and coat protein (CP) are both required for the cell-to-cell movement of cucumber mosaic virus (CMV) [1]. Deletions in the N-terminal region of the CMV CP were used to demonstrate that virus particle formation is not essential for movement between cells, but is necessary for movement between leaves [2]. This is consistent with other data on the absence of virus particles from plasmodesmata [3,4], although MP has been localized to plasmodesmata [4]. In microinjection experiments, the CMV MP was able to traffic itself and RNA, and promoted the cell-to-cell movement of 10-kDa fluorescent dextran [3]. The CMV MP was also shown to bind RNA and GTP *in vitro* [5].

To delimit specific sequences involved in the various functions ascribed to the MP, a series of alanine-scanning mutants (M1 - M9) was created in the MP gene of CMV and the mutants were assessed for (a) cell-to-cell and long-distance movement in eight host species, (b) the subcellular localization of the mutant MPs after fusion to the green fluorescent protein, and (c) the ability of the mutant MPs to bind RNA and GTP *in vitro*. The data from these nine mutants were combined with earlier data on the effects of other point and deletion mutants on infectivity in different hosts, and recent data mapping the *in vitro* RNA binding domain [6], to generate a functional map of the CMV MP.



M9 is ts for long-distance movement in tobacco; (-) at 34°C and (+) at 32°C or lower.

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[2] Kaplan et al. (1998) Virology (in press).
[3] Ding et al. (1995) Virology 207: 345-353.
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Tubule-guided movement of plant viruses

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Systemic spread of plant viruses is achieved by movement of an infectious complex from cell to cell through plasmodesmata followed by long-distance transport through the vascular system. Plant viruses employ different mechanisms for movement of virions or viral RNA through plasmodesmata. A common characteristic of each mechanism employed is the inevitable modification of plasmodesmal gating or even drastic alteration of the plasmodesmal structures, which is established by one or more viral proteins, to enable such translocation. An apparent mechanism for virus movement, employed by a considerable number of plant viruses from different families and genera (e.g. comoviruses, caulimoviruses, nepoviruses, tospoviruses and geminiviruses) is movement of virions through tubules. These tubules are assembled inside the plasmodesma, thereby replacing the desmotubule, and consist of the viral movement protein (MP). Identical 'movement tubules' are formed in virus-infected protoplasts or protoplasts that transiently express the MP alone, where the tubules extend from the cell surface, thus showing a functional polarity of the tubule (from the infected cell towards the uninfected cell). For several viruses it has been shown that this process of tubule formation does not necessarily rely on the presence of plasmodesmata or even requires specific plant proteins, as tubules are also formed in a similar fashion at the surface of heterologous insect cells expressing the MP. Cowpea mosaic virus (CPMV) best exemplifies this mechanism of tubule-guided movement. Systemic infection by CPMV requires the capsid proteins and the (48 kDa) MP. The MP alone is capable of inducing tubular structures when transiently expressed in protoplasts and the tubules are made up solely of the MP. In protoplasts tubule formation is recorded as early as 10 hrs p.i., but also in (synchronously) infected plant cells tubules are observed early in infection. At later time points of infection, as a result of continuous production of the MP (MP and CPs are produced in equimolar amounts), tubules often extend several microns from the surface of protoplasts or, in plant tissue, through plasmodesmata into the cytoplasm of the neighbouring cell. Similar observations have been made with other viruses from the genera comovirus, caulimovirus, nepovirus and tospovirus.
Recently, we reported that alfalfa mosaic virus (AMV) and brome mosaic virus (BMV), two viruses belonging to the family of Bromoviridae, were also capable of inducing virion-occluding tubular structures at the surface of infected protoplasts (Kasteel et al., 1997). This observation suggests that these viruses also employ a tubule-guided movement mechanism to achieve systemic infection, although up till now, no reports exist on tubules in plasmodesmata of plant tissue infected by either virus. In some aspects, however AMV movement differs from CPMV. In AMV infected mesophyll tissue the MP could only be detected in a narrow layer of 3-4 cells at the advancing front of infection and exclusively localised to plasmodesmata. Besides MP also the coat protein was found in these plasmodesmata. In cells behind the infection front, in which AMV-infection had been fully established, plasmodesmal localisation of MP or CP was absent. The transient appearance of MP and CP in plasmodesmata of cells at the infection front coincided with an obvious and also transient, nearly twofold, increase in plasmodesmal diameter (Van der Wel et al.,

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1998). Besides the increase in diameter such plasmodesmata lacked the desmotubule, however, virion-containing tubules (as were found in AMV-infected protoplasts) were not clearly discerned in these modified plasmodesmata. The mechanism by which plasmodesmata are (transiently) modified to facilitate tubule-guided virus movement is not understood. Observations of an increase in the number of plasmodesmata in mesophyll cells at the front of AMV-infection may indicate that AMV-movement involves newly formed plasmodesma-like structures rather than modification of existing ones.

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MUTATIONAL ANALYSIS OF THE MOVEMENT PROTEIN OF COWPEA MOSAIC VIRUS

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The diameter of plasmodesmata excludes molecules over 1 kDa from transport, but plant viruses, which are larger than 1 kDa, are able to make use of them to establish systemic infection. For this plant viruses encode movement proteins (MPs) that apparently modify existing plasmodesmata or induce modified plasmodesmatal structures.
The genetic information of cowpea mosaic virus is divided among two plus-strand RNA molecules. The RNA2-encoded 48K movement protein (MP) is capable of forming tubules in infected protoplasts. These tubules contain virus particles and are also found in infected plants, where they are located in presumably modified plasmodesmata. To determine whether distinct domains of the MP are involved in different steps in the cell-to-cell movement process (e.g. plasmodesmatal targeting and modification, tubule formation and incorporation of virus particles) an alanine scanning mutagenesis was performed on the MP. Two domains have been located so far. A domain involved in the formation of tubules in infected protoplasts maps between amino acids 1 and 313 of the MP. A second domain that is located in the C-terminus of the MP is not involved in tubule formation, but seems to be involved in the incorporation of virus particles within the tubule. One of the mutants is able to form tubules in protoplasts, but is not able to infect plants. This may imply the presence of a third functional domain. Upon inoculation of pinto beans, a local lesion host for CPMV, lesions which developed with most mutant viruses were smaller compared to wildtype virus.
To investigate whether we could use GFP as an intracellular marker for the MP, several MP-GFP fusions were constructed. After infection of protoplasts with these constructs, fluorescent tubules could be visualised in living cells, suggesting that intracellular targeting of these MP-GFP fusions was normal. With some of the fusions the fluorescence remained restricted to single epidermal cells on the inoculated leaf, indicating that these MP-GFP fusions were not functional in plants. With other fusions limited spread was observed and in these cases fluorescence became localized to punctuate spots on the cell wall, which presumably were plasmodesmata.

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Unravelling the PVX Cell-to-Cell Movement Process

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Cell-to-cell movement of plant viruses results from the combined processes of intracellular transport, from sites of synthesis to sites of export, and intercellular transport, via plasmodesmata, of viral nucleic acid. However, despite this common goal, the mechanisms by which these events are coordinated and executed differ substantially between groups of viruses. One clear distinction between movement strategies is seen in the requirement for viral coat protein (CP) in the movement process. Thus, whereas TMV requires the virus encoded 30 kDa protein but not CP for cell-to-cell movement, cowpea mosaic virus moves as a virion through plasmodesmata. For other viral groups both the mechanisms of cell-to-cell movement and the forms in which viral nucleic acids are transported have not yet been established.

In the case of potato virus X (PVX), type member of the potexviruses, the viral genome encodes five proteins of which four are directly involved in effecting cell-to-cell movement. Of these four proteins required for movement three are encoded by a so called triple gene block (TGB), typical of several viral groups. The fourth PVX gene essential for movement is the CP [1]. We have studied the cell-to-cell movement process of PVX using modified viral genomes expressing the green fluorescent protein (GFP) either as a free protein or as a fusion to the viral CP [1,2]. These tagged viruses allow the precise detection of infected cells and, in the case of the PVX expressing the GFP-CP fusion protein, also permit the subcellular localization of virus [2].

Using PVX mutants lacking one or more of the genes required for movement we have analyzed both functional and ultrastructural aspects of the infection process. Immunoelectron microscopy was used to investigate the distribution of virus encoded proteins, both in infected tissues and protoplasts, in order to determine the possible role(s) of the viral proteins. In addition, protoplasts infected with the PVX mutants were used to investigate the contribution of different viral proteins to the characteristic ultrastructure of PVX infected cells. These experiments demonstrated that of the PVX encoded proteins only the CP and 12 kDa TGB protein could be immunolocalized to plasmodesmata. In contrast the 25 kDa TGB protein was shown to be necessary for the formation of cytoplasmic laminate structures typically induced in potexvirus infections.

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Functional analysis of plasmodesmatal modification, using microinjection of fluorescently labelled probes, demonstrated that whereas the TGB proteins can modify the plasmodesmatal size exclusion limit the CP is not involved in plasmodesmatal modification. Furthermore, microinjection of fluorescently labelled CP showed that this protein was unable to traffic between cells in the absence of other virus-coded proteins. However, in the course of infection the coat protein is transported between cells suggesting that transport of CP between cells is a necessary feature of infection. This data, in conjunction with immunoelectron microscopy demonstrating the presence of virions in plasmodesmata, lead us to conclude that the TGB proteins orchestrate the transport of PVX virions via plasmodesmata [3].

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The potyvirus CI protein acts transiently in aiding virus movement

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A modification to plasmodesmal gating is a prerequisite to a spreading virus infection in plant tissues. This modification increases the functional size exclusion limit of plasmodesmata to allow the passage of virus nucleic acid and/or virus particles. Different viruses achieve this in different ways but, in all cases, it is through the action of virus-encoded proteins, called movement proteins (MP). Classically, these MPs have been identified as proteins, which after mutation, block virus spread from cell to cell but have no effect on virus replication in single cells, or by their ultrastructural or functional association with plasmodesmata.

The mechanism of movement of the potyviruses, the largest group of plant viruses, has not been fully resolved. These viruses have a large (approx. 10 kb) RNA genome that is translated into a single large polyprotein which releases functional gene products through the action of self-encoded proteinases. Three of the potyviral gene products (HC-Pro proteinase, coat protein and cylindrical inclusion (CI) protein) have been implicated previously in the process of virus movement. We have used a systematic ultrastructural study across the edge of an advancing infection in pea seed-borne mosaic (PSbMV) potyvirus-infected pea cotyledons to study the dynamic state of the CI protein in relation to virus movement (Roberts *et al*, 1998). Initially, the characteristic CI pinwheel inclusion bodies were positioned centrally over the plasmodesmal apertures (including those of plasmodesmata connected to the previously infected cell) in agreement with a proposed role in virus movement (Carrington *et al*, 1998). The viral coat protein was associated with these structures and was seen within the modified plasmodesmata, most notably in a continuous channel that passed along the axis of the pinwheel and through the plasmodesmata. The CI protein was not detected within the plasmodesmal cavities. Later in the infection (i.e. behind the zone of active virus replication) the CI was no longer associated with cell walls, or with coat protein, and showed signs of structural degeneration. In contrast, the coat protein remained within plasmodesmal cavities. The nature of the coat protein (i.e. free coat protein or capsids) could not be resolved although similar fibrillar aggregates in plasmodesmata have been interpreted by others as bundled virus particles. However, using *in situ* hybridisation for the electron microscope we were able to show that the coat protein aggregates were accompanied by viral (+) sense RNA. Using the same technique, viral RNA was detected in advance of cells containing discernible CIs, particularly within the plasmodesmal cavities. Coat protein was not detected in the same location. We suspect that viral RNA moves ahead of the main zone of virus multiplication and that mature CIs are not necessary for this process. The role of the CI in assisting virus movement is not known but the presence of the CI was linked with an apparent transient reduction

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in callose in the vicinity of the plasmodesmata. Other ultrastructural changes were observed exclusively in cells at the infection front. This highlighted the asynchronous nature of virus infection in intact tissue and emphasized the importance of building temporal and spatial factors into experimental design.

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Roberts, I. M., Wang, D., Findlay, K. and Maule, A.J. (1998) Ultrastructural and temporal observations of the potyvirus cylindrical inclusions (CIs) shows that the CI protein acts transiently in aiding virus movement. *Virology* In Press

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The cylindrical inclusion protein of a potyvirus contains signals for targeting to the wall of tobacco mesophyll cells.

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In a recent study of the subcellular location of viral proteins and RNA at early stages of infection of tobacco mesophyll cells by a potyvirus, tobacco vein mottling virus (TVMV) we have identified viral proteins and structures probably involved in the transport of potyviruses through plasmodesmata (Virology 236, 296-306). Our work showed that the nonstructural cylindrical inclusion protein (CI) associates with cell walls early in infection to form conical wall-bound inclusions only in areas of contact between cells. Coat protein (CP)- and RNA-containing complexes of linear shape (resembling virions) are present at the periphery of or inside these inclusions and are transferred from cell to cell. To study the targeting of the CI and CP proteins of TVMV in the absence of viral infection we have examined sections of mesophyll cells of transgenic tobacco plants expressing the individual CP or CI genes of TVMV. By immunogold labeling the CP was found in the cytoplasm and no accumulation of the protein in any organelle or near the cell wall was detected. In contrast, labeling of sections from two different CI-transgenic tobacco lines showed that the CI protein accumulated in the cell wall, including wall areas not corresponding to contacts between cells, and also accumulated in the intercellular spaces. The typical cylindrical inclusions were not observed in these transgenic cells. The CI protein also behaved as a secretory protein when expressed via a baculovirus vector in *Spodoptera frugiperda* (Sf9) cells. CI was not detected in insect cells in immunofluorescence experiments but this protein was detected by Western blot analysis, especially in the cell culture medium even though it apparently lacks a signal peptide sequence for secretion. Expression of CP in insect cells resulted in accumulation of the protein in the cytoplasm. Our results show the presence of signals on the CI protein for cell wall targeting and suggest that additional factors induced by TVMV infection may be needed for accumulation of CI in specific areas of the cell wall. The results also suggest that CP lacks signals for targeting to plasmodesmata and may be transported by interaction with other viral proteins.

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Phloem-Dependent Movement

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Plasmodesmata of Intermediary cells: Evolution of Form and Function

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There are few well-documented cases in which the structure of plasmodesmata (pd) are obviously specialized for a specific, recognized physiological purpose. In the cucurbits, and in several other families, the pd of certain companion cells (intermediary cells) in the minor vein phloem have a characteristic structure that evidently allows the cells to act as traps for sugar (1). We hope to extend our understanding of pd function by analyzing the structural and functional evolution of intermediary cells.

"Polymer trapping" occurs when sucrose diffuses from bundle sheath cells into intermediary cells through the numerous, highly branched pd that join the two cell types. The sucrose inside the intermediary cells is converted into larger sugars, raffinose and stachyose, which cannot back-diffuse into the bundle sheath because they are too large (2). Pd branches on the intermediary cell side of this interface are especially numerous and they are noticeably narrowed (3). It may be the narrowed portion of the structure that acts as a molecular size-discrimination filter.

One family which does not fit the pattern described above is the Apocynaceae. Members of this family apparently translocate raffinose, but not stachyose (4). We have characterized the transport physiology and minor vein ultrastructure of a well known member of the Apocynaceae, *Catharanthus roseus*, the Madagascar periwinkle, with the idea that such studies could provide more detailed insights into the functional and structural specialization of pd.

There are substantially fewer intermediary cell pd in *C. roseus* than in *Cucumis melo*, about one-third as many on the intermediary cell side and one-fifth as many on the bundle sheath side. A desmotubule runs through the center of each of the plasmodesmatal channels, on both sides of the common wall between the intermediary cell and bundle sheath cell, but the desmotubule is obscured at either end where the channel narrows to form a distinct neck. The neck region is much longer on the intermediary cell side than on the side closest to the bundle sheath. The cell wall surrounding the neck regions on both sides has a less fibrillar texture than the rest of the wall, forming a distinct sheath around the neck.

In several respects, the characteristics of phloem loading in *C. roseus* are midway between those of tobacco, an apoplastic phloem loader, and *Coleus blumei*, a symplastic phloem loader. For example, loading of exogenous sucrose is sensitive to the inhibitor PCMBS in tobacco, insensitive in *C. blumei*, and partially sensitive in *C. roseus*. It seems likely that *C. roseus* uses both apoplastic and symplastic modes of phloem loading and the reduced emphasis on symplastic loading is reflected in the frequency and structure of intermediary cell pd.

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The ins and outs of systemic virus transport.

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The exact points of entry and exit of systemic viruses into and from the host phloem have remained an elusive target due to the inaccessibility of vascular networks. It is commonly assumed that, once in the phloem, virus movement obeys the 'ground rules' of assimilate transport, movement from source to sink tissues occurring by presssure-driven mass flow within the sieve tubes. However, there is no *a priori* reason as to why virus movement into and out of the phloem system should be identical to that of solutes.

Using a battery of GFP-tagged viruses that differ in their movement 'strategies', we have examined the entry and exit sites along the phloem pathway using a combination of non-invasive imaging and immunoelectron microscopy. We show that for cucumber mosaic virus (CMV), the 3a movement protein (MP) is capable of trafficking directly into minor vein sieve elements (SEs) where it accumulates in elaborate formations in the SE parietal layer. Evidence will be presented that CMV traffics through the branched plasmodesmata connecting SE and CC as a linear ribonucleoprotein complex that comprises the viral RNA, coat protein and 3a MP, with subsequent viral assembly occurring within membrane-bound complexes in the SE parietal layer. In the case of potato virus X (PVX), we have followed extensively the systemic movement of a viral construct that expresses a CP-GFP fusion. By comparing the movement of this fluorescent virus with phloem-unloaded carboxyfluorescein (CF) we have been able to show that both solutes and virus are unloaded from class III veins, providing strong evidence that in sink leaves of *N. benthamiana* the exit pattern for PVX escape closely mirrors that of phloem-delivered solutes.

Recently, we have extended our studies to root systems using intact *N. benthamiana* seedlings grown on agar and infected with GFP-tagged viruses. While PVX moved poorly into root systems, we found that the nematode-transmitted virus, tobacco rattle virus (TRV), moved extensively in roots, unloading from the protophloem and subsequently displaying a remarkable pattern of cell-cell movement, 'tracking' basipetally up the root stele and targeting developing lateral primordia. Tobacco mosaic virus (TMV) also invaded the root system but showed a completely different escape pattern to that found for TRV. In the case of TMV, the virus unloaded from apparently random sites along the transport phloem. These exit points were eventually joined up by extensive cell-cell movement along the cortex. Unlike TRV, TMV failed to infect developing lateral primordia. Microinjection of TMV into single root cells was followed by extensive redistribution of the virus along the root system, exit once again occurring at apparently random sites. These marked differences in virus distribution demonstrate that virus escape in roots cannot be predicted entirely from solute unloading patterns. It appears that the exit and subsequent cell-cell spread of TRV may be an adaptation to nematode transmission.

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Mobility of Structural Proteins in the Phloem

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The phloem exudate of Cucurbitaceae contains a number of proteins that show a species-specific pattern in SDS-PAGE. The main components are two structural proteins: the phloem filament protein PP1 and the phloem lectin PP2. Localisation studies in developing phloem indicated that PP1 and PP2 are synthesised in companion cells (CC) and move into sieve elements (SE) via the pore/plasmodesma contacts where they accumulate as P-protein bodies and filaments.

Recently, we have used intergeneric and interspecific grafts to demonstrate the mobility of these structural and other exudate proteins in the phloem. The proteins were observed in SDS-PAGE of scion exudate as a group of additional bands that correspond in size exactly to prominent proteins of the stock. A possible artefactual shift of proteins across the graft interface was experimentally excluded. Transfer from stock to scion of proteins or their precursors started 7 days after grafting. Direct developmental comparison revealed that this transfer depended upon the establishment of graft-bridging sieve tubes.

Polyclonal antibodies against *Cucurbita maxima* PP1 and PP2 specifically immunolocalised these proteins to both SE and CC in the *Cucumis sativus* scion grafted on a *Cucurbita* stock. Localisation of the protein in the SE-CC of the scion could result from long distance transport of either the proteins or their mRNAs. RNA blot analyses and RT-PCR using *Cucurbita*-specific probes or primers detected *PP1* and *PP2* mRNAs only in the *Cucurbita* stock, but not in *Cucumis* scions, suggesting that the proteins are translocated. Genomic DNA blot analysis also failed to detect divergent *PP1* and *PP2* genes in *Cucumis* species with *Cucurbita* probes.

Several interspecific and intergeneric graft combinations of Cucurbitaceae were studied comparatively for the appearance of graft-transmitted phloem proteins. Interestingly, the graft partner behaved either as "donor" or "acceptor" for additional proteins. Preliminary experiments with carboxyfluorescein as transport marker showed that the protein transport parallels that of assimilates from stock into scion.

These results demonstrate that PP1 and PP2 move from the stock through sieve tubes across the graft union and accumulate in the scion not only in the bundle sieve tubes but also in the extrafascicular sieve-tube system. It remains to be determined whether their appearance in the CC is due to a free symplasmic exchange between SE and CC, or indicates the point of their proteolytic decomposition. Since the mobility of structural phloem proteins implies their accumulation in sink tissue, their degradation in nucleate cells of the acceptor plant must be postulated.

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New methods to observe sieve elements in action

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Intracellular injection of high-molecular probes is to be carried out by pressure. So far, the technical disadvantage is that the membrane potential of the cell impaled (an indicator for the cell vitality) cannot be monitored during pressure injection. For that reason, an electrode head was designed that combines controlled pressure injection and electrical microinjection with membrane potential recording. Continuous CLSM-mediated monitoring of Texas Red and 10 kDa-LYCH-dextran conjugate injected into epidermal cells of Coleus blumei leaves evidenced that the device succeeded in differential microinjection. Further, the microinjection methods functioned alongwith continuous membrane potential measurements.

As noticed before, however, both fluorochrome injection methods produced structural responses when sieve elements were injected. This reflects the major problem in observing sieve tubes i.e. any manipulation triggers a range of structural and biochemical reactions. To avoid any reaction to damage, a method was designed for in vivo observation of sieve element/companion cell complexes by using CLSM. In intact sieve tubes, the sieve plates did not present a barrier to mass flow; the translocation of phloem-mobile fluorochromes appeared to be unhindered. Two major occlusion mechanisms were distinguished. In reponse to intense laser light, the parietal sieve element proteins detached from the plasma membrane and fromed a network of minute strands and clustered material that aggregated and pressed against the sieve plate. In response to mechanical damage (a electrode with a tip diameter of 1 micron normally used for fluorochrome injection!), the evenly distributed P plastids exploded, giving rise to the formation of a massive plug against the sieve plate. In case of severe mechanical damage, the parietal proteins transformed into elastic threads that extended throughout the sieve element lumen. Our provisional assessment is that sieve plates are sealed by a "two-component glue". One component is encapsulated within the P-plastids and liberated by the explosion induced by turgor loss, the other is in the crystalline P-protein bodies. On top of the plug, the P-proteins located along the sieve element wall may be deposited.

Desorganization of the sieve element ultrastructure and occlusion of the sieve plates, the degree of which is dependent of how cautious the injection is executed, is triggered by impalement of an electrode. Currently, we are developing therefore a novel electrode type with a minuscule electrode tip (approximately 0.1 micron) to minimize the injury response of sieve elements.

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PLASMODESMATA: ORGANELLES INVOLVED IN THE CONTROL OF PHLOEM TRANSPORT

It is now well established that most if not all viruses move from cell to cell via plasmodesmata. This process requires an interaction between a specific virally encoded protein. termed movement protein (MP) and proteins within the host plant. Evidence accumulated over recent years has established that these viral MPs have the capacity to alter plasmodesmal function (Lucas and Gilbertson, 1994). Pursuant the hypothesis that cell-to-cell movement of sucrose from the site of synthesis to the site of phloem loading within the vascular bundle follows a symplasmic pathway. we have used transgenic tobacco plants that express the tobacco mosaic virus (TMV) MP to study the role of plasmodesmal function in controlling sugar transport and photoassimilate partitioning between the various plant organs. Analyses of diurnal accumulation of carbohydrates and export rates from source leaves, combined with microinjection experiments in which plasmodesmal function was determined, established that the TMV-MP has a pleiotropic effect on leaf physiology. These findings suggested that this protein have at least two sites of action: one that modifies plasmodesmal size exclusion limit and another that affects carbohydrate metabolism and allocation (Balachandran et al., 1995: Olesinski et al., 1995).

Transgenic potato plants expressing the TMV-MP under the control of several tissue specific promoters were employed to further explore the mode by which this viral protein interacts with cellular metabolism to change carbohydrate allocation. When restricted to photosynthetic tissues, the TMV-MP induced a reduction in the level of sugars and starch in source leaves compared with control potato lines (Olesinski et al., 1996). However, when the TMV-MP was expressed predominantly in phloem parenchyma and companion cells, sucrose and starch accumulated to higher levels, whereas the rate of sucrose export from excised petioles was lower than that observed with control plants (Almon et al., 1997). Perhaps the most significant result obtained from experiments performed on these potato plants was the discovery that the influence of the TMV-MP on carbohydrate metabolism within source leaves was under developmental control and was exerted only after tuber initiation.

Ultrastructural studies indicated that plasmodesmata exist between the phloem parenchyma and companion cells in all species examined. Based on these observations, together with the recent findings that plasmodesmata facilitate the cell-to-cell transport of macromolecules including proteins and nucleic acids, we advanced the hypothesis that trafficking of regulatory (information) molecules through plasmodesmata may establish a special supracellular communication network between the companion and mesophyll cells.

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which operate to regulate carbon partitioning (Lucas et al., 1996, Wolf and Lucas, 1997). According to this model, the supracellular communication system facilitates efficient orchestration of the network of biochemical and physiological processes in photosynthate metabolism and transport. The TMV-MP may interfere at several loci within this putative communication network, and therefore can induce and alteration in carbon metabolism within source leaves, either when expressed in the mesophyll or within the phloem.

The challenge now is to elucidate the mechanism(s) by which the TMV-MP affects this putative endogenous control system involved in regulating photosynthate transport in the phloem.

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A systemic, gene specific signal of gene silencing in plants: transgenic artefact or novel communication system in plants?

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Post transcriptional gene silencing in transgenic plants is mediated in part by a systemic signaling molecule (Palauqui et al., 1997; Voinnet and Baulcombe, 1997). Our recent investigations have shown that signal production is initiated by ectopic interactions of transgenes with homologous DNA in just one or a few cells of the affected plant. The signal is then able to move rapidly away from the initiation site and activate a relay of signal production that eventually affects the whole plant. The timing and pattern of spread of the signal indicates that there is movement through the phloem. There is also cell to cell movement of the signal through plasmodesmata.

The signal molecule has not yet been characterized. However, from the sequence specificity of the post transcriptional gene silencing, it can be inferred that it is a nucleic acid (Palauqui et al., 1997; Voinnet and Baulcombe, 1997). Thus gene silencing associated with a GFP transgene is targeted specifically against GFP RNAs whereas silencing of GUS is targeted specifically against GUS RNAs, and so on with other transgenes. One speculative hypothesis is that double stranded RNA is implicated in the signal of gene silencing. Supporting that speculation it has recently been described how double stranded RNA can mediate systemic gene silencing in nematodes (Fire et al., 1998).

It remains possible that this signal is an artefact of transgenic plants. Alternatively it could be that the signal is implicated in protection against viruses and transoposons. The signal could also be involved in control of flowering or other developmental switches in plants.

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RNA-BINDING PROPERTIES, IDENTIFICATION OF AN RNA-BINDING DOMAIN AND DETECTION IN INFECTED TISSUE OF THE P7 PUTATIVE MOVEMENT PROTEIN FROM CARNATION MOTTLE CARMOVIRUS (CARMV).

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The functional and structural characterisation of the p7 putative movement protein from a Spanish isolate of carnation mottle carmovirus (CarMV) has been initiated. The nucleotide and amino acid sequences of p7 from this new isolate showed high identity with those of the reference isolate (Guilley et al., 1985). The homologous protein from turnip crinkle carmovirus has been demonstrated previously to be involved in virus cell-to-cell movement (Hacker et al., 1992). Sequence alignment of all carmovirus proteins homologous to CarMV p7 showed that they have: (i) a short N-terminal sequence rich in acid amino acids, (ii) a high proportion of basic amino acids which could define an RNA-binding domain at the central region of the molecule, and (iii) a highly conserved C-terminal motif. CarMV p7 gene was fused to a sequence coding for a six histidines tag and expressed in E. coli. The sequence of histidines allowed the purification of CarMV p7 produced in bacteria in the absence of detectable contaminants, and the subsequent production of a specific polyclonal antiserum. This antiserum led to the immunological identification of CarMV p7 in Chenopodium quinoa-infected tissue. A series of studies were aimed to explore the putative RNA-binding properties of CarMV p7. Four different CarMV-specific digoxigenin-labelled riboprobes were used to demonstrate RNA-binding activity of p7 in both electrophoretic mobility-shift and protein blot assays. Apparently, binding was sequence unspecific and cooperative. In order to map the RNA-binding domain of CarMV p7, a small 19 amino acid synthetic peptide whose sequence corresponds to the basic central part of CarMV p7 was synthesized, and it was demonstrated that it binds to viral RNA probes. This peptide can be induced to fold into an amphipatic a-helix structure upon salt addition. We have previously shown that small RNA-binding peptides are able to specifically inhibit plant virus infection (Marcos et al., 1995). Following this work, it has been demonstrated that the p7 synthetic peptide partially inhibited the infection of CarMV virions upon inoculation onto the experimental host C. quinoa. Amino acid substitutions in the peptide that were shown to perturb the a-helix structure did not affect its RNA-binding or inhibition properties.

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Virus Movement Strategies
Chair: Peter Palukaitis

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Virus Movement and Phloem Development: Studies on a Phloem-Limited Geminivirus.

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Plant virus encoded movement proteins act to direct viral genomes through the cytoplasm and across the plant cell wall (1, 3). An added dimension to the cytoplasmic trafficking is provided by the ssDNA geminiviruses which replicate in the nucleus, thus requiring that a movement protein also acts as a nuclear shuttle to move replicated viral genomes to and from the nucleus (8, 10). Studies on mesophyll-infecting viruses such as tobacco mosaic virus and potato virus X, among others, have examined movement protein function in affecting plasmodesmal gating properties during viral invasion of the leaf (4, 5, 11) and in a few cases have shown that virus entry into the phloem to propagate additional rounds of infection within the leaf or move systemically follows the sink-to-source transition (2, 7). However, how a phloem-limited virus invades a leaf remains to be examined.

Our investigation of the phloem-limited bipartite geminivirus squash leaf curl virus (SqLCV) has shown that the two viral-encoded movement proteins BR1 and BL1 act cooperatively to move the viral genome(6, 8, 9, 12). BR1 is a nuclear shuttle protein that binds the viral ssDNA genome and can thus move it between the nucleus and cytoplasm. BL1 is associated with unique ER-derived tubules that appear to extend from and cross the walls of developing phloem cells. An implication of these studies is that SqLCV movement is coordinated with phloem development. To further investigate this point we have tracked SqLCV movement in systemically infected leaves of pumpkin and squash using an infectious virus engineered to express GFP. Examination of GFP fluorescence at both the whole leaf and confocal microscopic levels shows virus movement to be restricted to the phloem and supports our hypothesis that SqLCV invasion of the leaf is coordinated with phloem development. Details of these!
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studies and of the functional domains of BR1 and BL1 will be reported.

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Phloem movement of virus particles

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To stablish a systemic infection, a plant virus must move from the initially infected cells to other neighbouring cells through the plasmodesmata, and long distance through the vascular tissue. The cell-to-cell movement of several different viruses has been extensively studied but less is known about systemic movement. Most plant viruses are believed to move long distance through the phloem sieve elements. The majority of the experimental approaches that have been carried out analyze the ability of natural and engineered mutant viral strains to infect distal leaves. Inmunohystochemical analysis have also been undertaken to discover at which cell type the mutant viruses stop before reaching the sieve elements.

Direct information about how (i.e. in which structural form) virus moves through the phloem is lacking. The viral coat protein has been proven necessary for the systemic movement of most of the viruses that have been studied. Mutant strains of tobacco mosaic virus that produced a coat protein unable to encapsidate the RNA or that presented mutations in the RNA origin of encapsidation were not able to stablish a systemic infection. These and other results suggest that the virus might move long distance either as a whole virion or as a ribonucleoprotein complex formed by the viral RNA, the viral coat protein and probably other viral and host proteins.

We have approached the characterization of the form(s) in which viruses move long distance by analyzing the system cucumber/cucumber green mottle mosaic tobamovirus (CGMMV). The advantages of this experimental system are the possibility of collecting moderate amounts of phloem sap from sectioned internodes of cucumber, and the high titers that tobamoviruses reach in the tissues of their host plants.

In phloem exudates of CGMMV-infected plants, virus was readily detected in western blots. Viral RNA was also present, as shown by RT-PCR amplification. Amplification was only possible after a phenol extraction, indicating that CGMMV RNA is protected in the phloem of infected plants, most possibly through its association with proteins. The degree of protection was probed by analyzing the resistance of these putative ribonucleoprotein structures to RNAse A, and the accessibility to RT-PCR amplification under different pH conditions. Purified CGMMV virions were used as controls. It was found that CGMMV RNA in the phloem is more resistant to RNAse digestion than virion-encapsidated RNA. It was also found that the RNA in purified virions became as protected as that in the phloem after mixing virions with phloem exudates from non-inoculated plants.

The viral structure in the phloem of infected plants also shared the mass, weight and isopycnic density of the purified virions, as shown by their equal sedimentation profiles in sucrose and cesium chloride gradients. Finally, negative staining of the fractions of the sucrose gradient revealed in an electron microscope the presence of 300 nm long rod-shaped cucumber green mottle mosaic virions in the phloem exudate of infected plants.

The results suggest that virus movement in the phloem is mainly (if not only) in the form of "stabilized" virus particles.

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Long distance transport of RCNMV requires movement protein as well as capsid protein possibly in the form of a virion to cross the bundle sheath sieve element-companion cell complex plasmodesmata

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The red clover necrotic mosaic virus (RCNMV) single-stranded RNA genome is split into two largely non-homologous RNAs (Giesman-Cookmeyer *et al.*, 1995). The viral cell-to-cell movement function has been genetically and functionally mapped to the 35 kDa polypeptide, called the movement protein (MP), encoded by the smaller monocistronic RNA-2. The RNA-1 encoded capsid protein (CP) is not necessary for cell-to-cell movement but is required for long-distance transport leading to a systemic infection (Xiong *et al.*, 1993). The 35 kDa MP binds to viral single-stranded RNA *in vitro* and *in vivo* (Giesman-Cookmeyer *et al.*, 1993). The RCNMV infection moves from cell-to-cell as a genomic RNA-MP complex (Fujiwara *et al.*, 1993). This RNA-MP complex is sufficient to achieve infection up and into the bundle sheath cells on the inoculated leaf (Yang *et al.*, 1998).

We provide evidence that the MP possesses a functional domain for long distance transport that is genetically distinct from that for cell-to-cell movement. Using immunogold cytochemistry and antibodies conjugated with either enzymes or fluorescent tags, in combination with RCNMV infectious RNA transcripts expressing mutant forms of the 35 kDa MP, we show that the plasmodesmata between the bundle sheath cells and the sieve element-companion cell complex can function as a barrier to viral entry into the phloem translocation system (Yang *et al.*, 1998).

It has long been established genetically that CP is required for long distance transport and systemic infection. A portion, if not all, of the RCNMV origin of assembly sequence (OAS) has been identified. Destruction of the OAS while maintaining the expression of wild-type CP prevents both virion formation and systemic spread of the virus (Sit *et al.*, unpublished).

The RCNMV capsid protein is expressed *in vivo* from RNA-1 by the production of a subgenomic RNA (Giesman-Cookmeyer *et al.*, 1995). We have identified a 34-nucleotide element on RNA-2 which is essential for long distance transport. Using GFP as a reporter in place of the viral CP gene, we have shown that the 34-nucleotide element *trans*-activates subgenomic RNA synthesis (Sit *et al.*, 1998). Consequently, this RNA-2 *trans*-activator is required to drive the expression of CP, which is required for long distance transport.

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Collectively, these data suggest that virion formation is required to achieve movement of the infection into the sieve element-companion cell complex. We therefore hypothesize that cell-to-cell movement of RCNMV occurs as an RNA-MP complex not involving CP and long distance transport occurs as virions which includes MP interactions that are distinct from those for cell-to-cell movement.

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MOLECULAR BASIS OF THE LONG-DISTANCE VIRUS MOVEMENT FUNCTION ENCODED BY THE 2b GENE OF CUCUMBER MOSAIC VIRUS

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The tripartite ssRNA genome of cucumber mosaic virus (CMV) encodes five proteins, 1a, 2a, 2b, 3a and coat protein (CP). Proteins 1a and 2a are components of the viral RNA polymerase while both 3a and CP are required for virus cell-to-cell movement. In the last few years our research has been focused on the 2b gene we reported in 1994. The 2b gene is encoded by RNA 2, partially overlaps the 3' portion of the 2a gene, and is expressed in infected plants via a subgenomic mRNA (RNA 4A). Sequence analysis indicated that this 2b gene is conserved in all cucumoviruses sequenced to date and that it may represent a naturally occurring hybrid gene fused from two regions of distinct origin. Mutational studies revealed that 2b encodes a host-specific long-distance virus movement function and that the gene is also important for virulence determination. These observed roles for 2b were further confirmed through infectivity studies on hybrid viruses (made by exchange of gene 2b) in host plant species that either are or are not shared between parental cucumoviruses.

The molecular basis of the biological functions observed for the cucumoviral 2b gene is being investigated using several approaches. We have recently produced (i) transgenic tobacco plants expressing 2b genes encoded by different cucumoviruses and (ii) chimeric viruses based on either tobacco mosaic virus or potato virus X expressing various cucumoviral 2b genes. Plant responses under normal or stress conditions that are specifically due to the 2b gene expression from either the plant genome or chimeric viral genomes are being analysed. Localisation of various 2b-GFP fusion proteins both in tobacco suspension cells and whole plants is also being determined. Results from these experiments will be presented and their relevance to the long-distance virus movement is discussed.

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Plant Responses to Virus Movement
Chair: David Baulcombe

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TMV REPLICATION AND VIRUS MOVEMENT: LINKED PHENOMENA?

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Virus-encoded and host-encoded factors are necessary for cell-to-cell movement of the pathogen both outside and inside the vascular tissue (Deom et al., 1997; Ghoshroy et al., 1997; McLean et al., 1997; Nelson and van Bel 1998). Recent results from various laboratories have led to the conclusion that viral proteins besides the so-called "movement" proteins have a role in this process. This conclusion was derived from studies of the systemic accumulation of viruses mutated in the requisite open reading frame (orf). The cellular location where the block in movement occurred was rarely studied. Also, the molecular basis of how these "non-movement" proteins support virus spread has not been studied.

We have initiated experiments to determine how the 126 kDa protein from tobacco mosaic virus (TMV) functions in supporting cell-to-cell spread. We have utilized mutant viruses altered in the 126 kDa protein orf to achieve this goal. In so doing, we have also provided the plant the potential to respond to an altered protein and prevent virus movement.

Previous research has determined that the 126 kDa protein either alone or in combination with other proteins modulates systemic virus accumulation of TMV (Derrick et al., 1997). We have since produced viruses mutated in the 126 kDa protein orf that are affected in cell-to-cell spread. One set of mutants affected in cell-to-cell spread contains site-directed mutations in the nonconserved region of the 126 kDa protein between the conserved methyltransferase-like and helicase-like domains found in all proteins from sindbis-like viruses (Shintaku et al, 1996). Although this region as a whole is not conserved, there are certain amino acid motifs within the region that are conserved among these proteins. One such motif, WFP, is also observed in membrane-associated host proteins and in meromyosin.

We mutated the WFP motif to WAP and WYP and observed the effect of these mutations on the replication, cell-to-cell spread, and vascular-dependent accumulation of the resultant viruses. WAP (the nomeclature for the mutant virus altered from F→ A) was unable to replicate in *N. tabacum* protoplasts, while WYP accumulated. However, WYP produced only a small necrotic lesion on a hypersensitive host indicating that cell-to-cell movement of this virus was compromised. The altered accumulation of WYP and WAP was correlated with a host-specific instability of transiently-expressed 126 kDa protein fused to GFP in plant leaves. We have further characterized the movement ability of the 126 kDa protein in whole plants and the stability and intracellular location of this protein and the mutant proteins in protoplasts. Lastly, we have produced transgenic plants

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expressing the wild type or mutant proteins fused with GFP and a preliminary discussion comparing results from protoplasts and transgenic plants will be presented. These results will be discussed in the context of our postulate (supported by immunocytochemical studies) that the replication structures, which contain the 126 kDa protein, are involved in virus cell-to-cell movement.

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INVOLVEMENT OF CLASS I β-1,3-GLUCANASES IN VIRAL MOBILITY.

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Antifungal class I β-1,3-glucanases (β-GLU I) are believed to be part of the defense reaction of plants against fungus infection. Studies of β-GLU I deficient tobacco and *N. sylvestris* generated by antisense transformation suggest that β-GLU I also plays a role in viral pathogenesis. β-GLU I deficient Havana 425 tobacco showed markedly reduced lesion size, lesion number, and virus spread in the local-lesion response to tobacco mosaic virus (TMV) at 25 °C. These effects were inversely correlated with β-GLU I expression in independent antisense transformants and depended on the developmental age of the plants. Antisense transformation also markedly reduced TMV spread and disease symptoms in the response of the plants at 32°C.

One possible mechanism for these effects is suggested by the finding that callose, a potential substrate for β-GLU I, can restrict virus spread. Callose is deposited around lesions as part of the local-lesion response to TMV. This is believed to decrease the size exclusion limit (SEL) of plasmodesmata and reduce virus movement from cell to cell. Callose deposition in response to TMV is earlier and upto ca. 40-fold greater in β-GLU I deficient transformants than in wild-type plants. A similar increase was observed for wound-induced callose formation indicating that the antisense effect on callus is not limited to virus infection. Direct evidence that β-GLU I deficiency can decrease intercellular transport was obtained by measuring the movement of microinjected, FITC-labeled dextrans between adjacent leaf-trichomes cells. Taken together, the results suggest a working hypothesis for the decreased virus susceptibility of β-GLU I deficient transformants. We propose that the extent of callose deposition in response to TMV depends on the balance of β-1,3-glucan synthesis and degradation mediated by β-1,3-glucanases. Thus, β-GLU I deficiencies could reduce callose degradation and, hence, increase callose deposition. This results in decreased intercellular transport via plasmodesmata, which helps decrease the spread of virus from cell to cell.

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Alex M. Murphy

Salicylic acid and virus movement

Salicylic acid (SA) is a key component of the signal transduction pathway culminating in plant resistance to an avirulent pathogen, manifested as the hypersensitive response (HR; Staskawicz et al.,1995). Application of SA to susceptible plants can also induce resistance to normally virulent pathogens, thus facilitating studies on the mechanism of SA induced resistance to plant viruses in the absence of the HR.

SA treatment of susceptible tobacco delayed symptom development in TMV and CMV inoculated whole plants. Analysis of viral RNA's from virus-inoculated, SA pre-treated leaf disks showed that SA profoundly interfered with TMV replication (Chivasa et al., 1997) but had no effect on the replication of CMV. These results suggested that SA-induced resistance to CMV in whole plants was due to interference of virus movement.

Western analysis of whole plants demonstrated that CMV exit from inoculated leaves was retarded in SA-treated plants, indicating that SA was affecting long distance movement. Plant virus movement is known to parallel the translocation of photosynthates from source to sink leaves (Leisner, Turgeon and Howell, 1992; Roberts et al., 1997). $^{14}CO_2$-feeding experiments showed that SA treatment does not interfere with the movement of newly synthesised radiolabelled carbon compounds, nor change the boundary of the carbon sink/source transition in young, expanding leaves. Thus, SA-induced inhibition of long distance virus movement is not due to perturbation of carbohydrate translocation.

Recently, it was reported that SA interference of TMV replication is induced via a novel branch of the defensive signal transduction pathway that is sensitive to inhibition by salicylhydroxamic acid (SHAM; Chivasa et al., 1997). Surprisingly, SA-induced resistance to CMV was abolished by SHAM, implicating the SHAM-sensitive signalling pathway in the activation of the mechanism(s) underlying inhibition of virus long-distance movement.

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Plasmodesmal targeting of the maize Pathogenesis-Related PRms protein

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The PRms protein is a 15.5 kD Pathogenesis-Related protein whose expression is induced in response to fungal infection in maize seedlings. A peculiarity of the PRms gene is its remarkable cell-type specific pattern of expression (Murillo et al. Plant Cell 9, 145-156, 1997). A detailed analysis of the *PRms* mRNA and PRms protein distribution in different tissues of the fungal-infected maize seedling was conducted. In situ mRNA hybridizations revealed that the PRms gene is expressed in the epitheliar scutellum cells of the germinating embryo, and in parenchyma cells surrounding the differentiating xylem elements of the vascular system. However, no correlation was found between the PRms mRNA and the protein localization data. Immunocytochemical localization studies revealed that the PRms protein was detected not only in those cells where PRms gene expression occurred, but also in distant parenchyma cells, in which PRms gene expression was never detected. The PRms protein was always localized at the contact areas between contiguous cells in the infected maize tissues. Immunoelectron microscopy studies demonstrated that these immunoreactive regions corresponded to the plasmodesmata. These results suggest that mechanisms responsible for targeting the PRms protein to plasmodesmata must exist. For another maize protein, KNOTTED1, which has the capacity to move from cell-to-cell (Lucas et al. Science 270, 1980-1985, 1995), it has been reported that the cytoskeleton is involved in its targeting to plasmodesmata. In contrast to the KNOTTED1 protein, which is a cytoplasmic protein, PRms is a protein that enters into the secretory system of the plant cell. The molecular mechanism by which PRms might be targeted to plasmodesmata will be discussed. Finally, the plasmodesmal localization of PRms in infected maize tissues implies that plasmodesmata between these cells must have altered or modified structures and suggest that plasmodesmal regulation could well be involved in the plant response to pathogen attack. In relation to that, the possible role of the PRms protein in the plant defense response will be discussed.

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POSTERS

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LOCALISATION OF A CENTRIN-LIKE PROTEIN TO HIGHER PLANT PLASMODESMATA

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The exact mechanism and site of the regulation of cell-to-cell transport is unknown (Overall and Blackman, 1996). However, changes in the intracellular concentration of calcium is one mechanism by which intercellular transport can be controlled. Calcium-binding proteins, such as centrin, are therefore attractive candidates for components of plasmodesmata. Centrin is a calcium modulated contractile phosphoprotein which was first identified in the basal bodies of flagellar in green algae (Salisbury, 1995). This ubiquitous protein has been localised to the forming cell plate in a number of higher plant species (Del Vecchio et al., 1997). In this present study, we have used immunolocalisation techniques to determine if centrin is a component of plasmodesmata. On cryosectioned cauliflower florets, centrin was immunofluorescently localised to the cell walls in a punctate manner. In double immunolabelling experiments, centrin colocalised with callose indicating that centrin or a centrin-like protein forms part of the molecular architecture of plasmodesmata. Centrin antibodies were also localised to plasmodesmata of cauliflower florets and onion root tips using immunoelectron microscopy. In contrast, antibodies to calmodulin, which shares over 50 % sequence homology with centrin (Bhattacharya et al., 1993), did not label plasmodesmata.

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Mutagenesis of the PVX 8K protein identifies a barrier to long distance movement

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For cell-to-cell and long distance movement potato virus X (PVX) requires its coat protein and a set of three proteins called the triple gene block proteins. Mutagenesis of the 8K triple gene block protein was performed to elucidate its role in virus movement. Mutations were introdced into a modified PVX genome designed to express the green fluorescent protein (GFP) thus allowing the effects of the mutations on the virus movement to be monitored visually. A virus carrying a mutation that removed the initiating AUG codon of the 8K gene was restricted to the initial infected cell indicating that the 8K protien is essential for cell-to-cell movement. However, a virus in which more than half of the 8K protein was deleted was still capable of cell-to-cell movement at a much reduced rate. The movement of this virus was restricted to the inoculated leaf. Examination of the infected leaves by immunogold electron microscopy revealed that the virus had infected the vascular bundle but was only found in bundle sheath cells, phloem parenchyma, and xylem parenchyma. It was never found in the sieve elements, or companion cells. The 8K protein may therefore be required for the transition from cell-to-cell to long distance movement.

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Detection of several mRNA species from rice phloem sap

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Rice Phloem sap collected by the insect laser method contains a number of proteins including thioredoxin h (Ishiwatari et al. 1995). Rice phloem thioredoxin h is capable of dilating plasmodesmata in tobacco mesophyll cells upon microinjection and this capacity is associated with structural characteristics of the protein (Ishiwatari et al., in press).

Here we present evidences suggesting the presence of several mRNA spices in the rice phloem sap. Rice phloem saps were subjected to PCR amplification using three sets of primers corresponding to thioredoxin h, oryzacystatin, and actin. The thioredoxin h and actin primer sets amplified DNA fragments of expected sizes, whereas the cystatin set failed to detect any band.

Phloem sap were also collected from transgenic rice plants expressing GUS activity under the control of the rice thioredoxin h promoter. The sap did not show detectable GUS activity. GUS activities were detected strongly in companion cells. GUS mRNA, on the other hands, was detected from the phloem sap.

These findings suggest presence of several mRNA species in rice phloem sap and the complex nature of sorting process of macromolecules through plasmodesmata at the sieve element-companion cell boundary in rice.

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IN SITU AND IN VIVO SUBCELLULAR LOCALIZATION OF OLIVE LATENT OLEAVIRUS 2 PUTATIVE MOVEMENT PROTEIN

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Olive latent virus 2 (OLV-2) the type species of the genus Oleavirus (family Bromoviridae) has quasi spherical to bacilliform particles and a non polyadenylated, tripartite, positive sense ssRNA genome. Virions encapsidate four major RNA species all of which were sequenced. RNA1 (3126 nt) and RNA2 (2734 nt) are both monocistronic molecules coding for replication-related proteins, with conserved motifs of helicase, methyltransferase (RNA1) and RNA polymerase (RNA2). RNA3 (2438 nt) codes for a 36.5 kDa polypeptide with conserved motifs for the 30kDa superfamily movement proteins (MP), and the 20 kDa viral coat protein (CP). RNA4 (2078 nt) is co-terminal with RNA3 with undetermined biological function. A subgenomic RNA (c. 1042 nt) responsible for CP production is formed in infected plant but it may not be encapsidated.

To investigate the role of the putative MP in the cell-to-cell movement of the virus, the 36.5 kDa protein gene was cloned and expressed using the Escherichia coli pGEX-2T expression system. The purified fusion protein has been used to raise a polyclonal antiserum. According to time course accumulation study of the MP protein in Nicotiana benthamiana-infected leaves the maximum accumulation level was reached at the early stage of infection (c. one day post inoculation). With western immunoblots of subcellular fractions from N. benthamiana infected OLV-2 MP was detected in cell wall fractions.

Ultrathin sections of N. benthamiana infected cells showed the presence of tubular structures near the cell wall and often extending through it, protruding to a adjacent cell at the level of plasmodesmata. These tubular structures appeared two days post inoculation and in later stages of infection could contain virus-like particles (c. four days post inoculation). Immunogold labellingassays using the antiserum to OLV-2 MP shoxed that the homologous antigen was associated to the plasmodesmata and could be detected across the cell wall and into the cytoplasm of two adjacent cells. Gold labellingof virus-induced tubules showed that the antigen was not present on the surface of the sheath but appeared to be located at its ends.

OLV-2 MP coding sequences were fused with the N terminus of the green fluorescent protein (GFP5) coding sequences and cloned in a plasmid vector containing a 35S promoter (pGY1). The obtained MP-GFP fusion construct was transiently expressed in N. tabacum protoplasts producing long tubular structures protruding from the protoplast surface.

The significance of these findings on the role of the OLV-2 36.5 kDa protein in the cell-to-cell movement of the virus is discussed.

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TARGETING AND MODIFICATION OF PROCARYOTIC CELL-CELL JUNCTIONS BY TOBACCO MOSAIC VIRUS CELL-TO-CELL MOVEMENT PROTEIN

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The movement protein (MP) of tobacco mosaic virus (TMV) facilitates the cell-to-cell spread of infection by altering the structure and function of plasmodesmata, the intercellular communication channels in plants. Because the protein was shown to interfere with intercellular communication when expressed in the cyanobacterium *Anabaena* sp. strain PCC 7120, we tested whether the ability of the protein to target and to modify intercellular communication channels in plants is conserved in this prokaryote. We found that the MP localizes to the cell junctions and induces the formation of filamentous structures which traverse the septa. We propose that the protein interacts with host components that are similar between plants and *Anabaena* and which may be evolutionary related. The observations in *Anabaena* suggest that the MP modifies plasmodesmata by forming a filamentous aggregate within the pore.

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CELL-TO-CELL MOVEMENT OF THE TRIPLE GENE BLOCK VIRUS WHITE CLOVER MOSAIC POTEXVIRUS

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We have expressed white clover mosaic potexvirus (WClMV) triple gene block (TGB) transport proteins (26, 13 and 7 kDa products) individually and as multicistronic constructs in *Nicotiana benthamiana.* Functional complementation of WClMV TGB mutants in terms of cell-to-cell and long-distance movement was confirmed.

Microinjection studies were performed using bacterially expressed 26 kDa protein, FITC-labeled F-dextrans, coat protein and labeled RNA to dissect the molecular mechanism of TGB-mediated cell-to-cell movement of infectious viral RNA.

We have shown: (i) that the 26 kDa TGB protein can dilate plasmodesmata (PD); (ii) that the 26 kDa protein potentiates its own movement from cell-to-cell and (iii) that the 26 kDa protein is dependent on the remaining 13 and 7 kDa TGB proteins and coat protein for the mediated movement of labeled RNA. We present data that a viral nucleoprotein complex comprised of RNA, the 26 kDa TGB protein and coat protein moves from cell-to-cell. This is the first instance of RNA trafficking assisted by 4 identified protein products. A model encompassing these results will be presented.

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POSTER ABSTRACT

The frequency of plasmodesmata increases early in the whole apical meristem of *Sinapis alba* L. during floral transition

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In higher plants, the most common way that cells communicate among each other is through plasmodesmata (Pds). Recently, Lucas et al. (1995) have observed that Pds facilitate the cell-to-cell transport of a plant encoded transcription factor in the maize shoot apical meristem (SAM). During the floral transition, in the SAM, the synchronization of the cell divisions in several species (Bernier, 1988) and the relationships between different genes which are expressed in distinct zones (Bradley et al., 1996) have led to the idea that specific intercellular communications should occur during this developmental step and are perhaps essential for this process.

We are thus investigating the distribution and frequency of Pds in the SAM of the long-day (LD) plant *Sinapis alba* L. during the floral transition induced by a single LD.

In this system, previous works showed that (1) floral signals (sucrose, cytokinins,...) of foliar origin are transported to the SAM between 10 and 24 h after the start of the LD, (2) a first wave of cell division occurs at 26-30 h and (3) the first floral meristem is initiated by the SAM at 62-70 h (Bernier, 1989).

Apical buds of both induced and noninduced plants were collected at various times after the start of the LD, fixed with 2% $KMnO_4$ and embedded in Epon. Ultrathin longitudinal axial sections obtained from 3 to 6 apices in each batch were visualized under a TEM. The Pds of 20 non contiguous cells from each meristem were examined and periclinal and anticlinal wall length of each cell were measured.

In the whole meristem, cells were interconnected by groups of unbranched Pds. Occasionally, branched Pds were also observed.

The Pds were counted into the upper three cell layers (L1, L2 and L3) of meristems and their frequency was determined by dividing the number of Pds encountered at a cell-to-cell interface by the interface cell wall length. We observed that the frequency of Pds became more important between cells of the L3 at 28 h after the start of the LD and that Pd frequency was markedly increased in the whole meristem, including L1 and L2, 4 h later. This increase culminated at 36 h after the start of the LD. All these changes start to decrease at 48 h after the start of the LD.

This situation suggests an increase of intercellular communications in the SAM during a particular time interval of the floral transition. This change will be discussed in relation to the numerous metabolic, cellular and molecular changes that are known to occur during the floral transition of this species (Bernier, 1989).

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INTRACELLULAR LOCALIZATION OF COAT PROTEIN AND GENOMIC RNAS OF CHERRY LEAF ROLL VIRUS AND ITS INVOLVEMENT TO VIRAL MOVEMENT AND REPLICATION

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Systemic viral infection needs to get established a complex process to take place including different steps such as: the ability of entering the initial cell, synthesis of the viral components by using the cell machinery and movement, firstly, from the initially infected cell to the adjacent healthy one (short distance) and secondly, to the rest of the plant (long distance) using the plant vascular system. Two mechanisms have been described for the short-distance movement of plant viruses: The TMV-like mechanism that involves the formation of a ribonucleoprotein complex (Citovsky and Zambrysky, 1993; Annu. Rev. Microbiol. 47:167-197), and the CPMV-like mechanism which consists in the formation of tubules through cell walls and/or plasmodesmata, that transport the virions to the adjacent cell (Carrington et al. 1996, Plant Cell 8:1669-1681). Several types of plant viruses including como-, nepo-, caulimo-, gemini- and tosposviruses, induce these specific viral tubular structures. In addition to the MP, and in some virus-hosts combinations to the CP, it has been recently shown that replication proteins of several viruses are also involved in cell-to-cell transport (Traynor et al. 1991, J. Virol. 65:2807-2815; Nelson et al. 1993, Mol. Plant-Microbe Interact. 6:45-54; Gal-On et al. 1994, Virology 205:280-289) and can be considered as modulators of cell-to-cell or long-distance movement (Carrington et al. 1996). Genome replication in positive-stranded RNA viruses takes place in close association with cytoplasmic membranes. The formation of membranous vesicles are common features to many nepo- and comoviruses and it has been suggested that they are the sites for viral replication (de Zoeten et al. 1974, Virology 59:341-355; Eggen and van Kammen, 1988). Cherry leaf roll virus (CLRV) belongs to the plant nepovirus genus within the *Comoviridae* family. Its genome is made up of two positive-sense polyadenylated ssRNAs. In its interaction with tobacco plants, CLRV induces the appearance of necrotic ringspots in inoculated and systemically infected leaves. Genomic RNA and CP are mainly localized associated with these symptoms as well as with vascular tissue (Más and Pallás 1995, J. Virol. Methods 52:317-326, 1996, J.Gen.Virol. 77:531-540; Balsalobre et al. 1997, Mol. Plant-Microbe Interact. 10, 784-788).

In this study we show that CLRV induces the appearance of several characteristic cytopathological structures: cytoplasmic paramural bodies (Pb's), abundant membranous vesicles and tubular structures which are associated with the cell wall. Intracellular localization of the coat protein (CP) and genomic RNAs was studied by immunogold labeling and in situ hybridization respectively. CLRV CP was mainly concentrated inside the Pb's and genomic RNAs were localized associated with the small cytoplasmic vesicles. Both CP and genomic RNAs were also localized in the tubules being detected when they

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were sectioned in such a way that they were accessible to the antibody or to the probe, respectively. The localization of CLRV RNAs in the cytoplasmic vesicles constitutes the first direct evidence of their association to viral genome and reinforces the hypothesis that these structures are the viral replication sites.

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Expression of the *MP* genes of maize streak virus and bean yellow dwarf virus in host and non-host transgenic plants produces virus-like symptoms and affects virus infectivity.

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The single-stranded DNA-containing geminivirus, maize streak virus (MSV) infects cereals and grasses and requires both the coat protein (CP or V2 protein) and the movement protein (MP or V1 protein) for infection of plants although neither is required for replication of the virus in protoplasts [1,2]. Recently two viruses (tobacco yellow dwarf virus (TYDV) and bean yellow dwarf virus (BeYDV) have been characterised and shown to possess a genome organisation similar to MSV, although both infect dicots. The *V1* gene product of both viruses has a secondary structure similar to that of the MSV MP [2], the BeYDV and MSV proteins have 54% amino acid sequence similarity. The BeYDV V1 protein has been shown to be necessary for efficient infection of *Nicotiana benthamiana* plants [3]; the protein has been suggested to fuction as a MP. Recent studies showing the presence of the MSV protein in plasmodesmata in infected maize and the movement of a MP-green fluorescent protein (GFP), but not GFP alone, between plasmodesmata of maize epidermal cells, have confirmed the MSV product to be a MP [4,5].

To determine whether the MPs are functional in both host and non-host plants, transgenic *Nicotiana benthamiana* and *Nicotiana tabacum* plants containing the MSV or BeYDV *MP* genes under the control of the CaMV 35S promoter have been generated. All plants expressing the MSV MP are phenotypically abnormal, and the protein appears to be toxic as *N. benthamiana* plantlets expressing large amounts of the protein were unable to survive transplantation to the glasshouse. In *N. benthamiana*, leaf distortion and curling and veinal chlorosis, resembling the symptoms of geminivirus infection, are apparent, plants are female sterile and must be used as the pollen donor to produce a further generation. Fewer abnormalities are seen with *N. tabacum* and the plants are fertile. Most plants transformed with the BeYDV *V1* gene were phenotypically normal except for one plant which showed leaf curling and chlorosis. For one line in which the F_0 plant had a normal phenotype, approximately 30% of the F_1 plants showed leaf distortion and chlorosis. Antiserum to the BeYDV V1 protein is not yet available and this precludes us from determining whether the "symptom" phenotype correlates with the presence of the BeYDV protein.

Plants containing the BeYDV or MSV *V1* gene failed to complement BeYDV genomes mutated in the V1 gene. This was not because of the presence of a mutation in the transgene as PCR-based sequence analysis confirmed the inserts to be wild-type. However, agroinculation of plants expressing the MSV MP with a wild-type BeYDV construct resulted in a lower proportion of the plants becoming infected compared to plants transgenic for vector sequences only (approx 17-30% infection compared to 70-80% for control lines). The reason for the decreased susceptibility of the plants is currently under investigation. Such decreased susceptibility has not been seen with plants transgenic for the BeYDV gene.

Experiments are in progress to determine whether expression of the MP genes in the plants affects plasmodesmatal size exclusion limits or the movement of a BeYDV V1- (or MSV MP-) GFP construct through plasmodesmata.

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Transgenic expression of oilseed rape mosaic tobamovirus movement protein in *Arabidopsis thaliana* confers resistance to ORMV

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The movement function is one of the most characteristic features of plant viruses. This function is provided by a protein (MP), or set of proteins depending on the virus familiy, that normally allows the virus to modify plant plasmodesmata in their own benefit. The so-called short-distance movement can thus be performed. The expression of the movement proteins of different plant viruses in transgenic tobacco plants has deep effects on some important traits of plant physiology, such as the size of cell-connecting plasmodesmata, or carbon metabolism and photosynthate partitioning. Mutated versions of the MP are able to confer resistance to virus infections in the transgenic plants. Many of these studies have been carried out by transformation of the tobacco plants with the MP gene of tobacco mosaic tobamovirus (TMV).

Several tobamoviruses have been identified that are able to infect *Arabidopsis thaliana*. We have focused on the study of oilseed rape mosaic virus (ORMV), a crucifer tobamovirus that we have previously described as an experimental Arabidopsis pathogen. We have determined the complete nucleotide sequence of ORMV genomic RNA. The analysis of this sequence highlights a different gene array, as compared to TMV. This array seems cahracteristic of crucifer tobamoviruses, since it has also been found by other authors in tobamoviruses different to ORMV.

Transgenic Arabidopsis plants expressing the MP of ORMV have been generated in our laboratory. Several independent transgenic lines were obtained differing in both gene dosage and gene orientation. All the transgenic lines analyzed by Southern hybridization showed the presence of the transgene. However, when analyzed by Northern hybridization, different levels of MP transcript

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accumulation were found. Moreover, different transgenic lines showed different behaviour, including resistance, upon challenge with the homologous virus, ORMV. The analysis of the biological effects of ORMV MP on transgenic Arabidopsis should allow to exploit the potential of this model plant to identify and study host components involved in the interaction.

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The helper component protein of plum pox virus as a limiting factor for virus long distance movement in *Nicotiana tabacum* plants.

Plum pox virus, a member of the *Potyvirus* genus, is unable to infect systemically some herbaceous hosts such as *Nicotiana tabacum* althouth it can replicate in the inoculated leaves. Some other potyviruses, as tobacco vein mottling virus (TVMV) and tobacco etch virus (TEV), can establish a systemic infection in the same host. We have followed several approaches aimed to identify the PPV genome regions responsible of the movement constraint. Tobacco plants were co-inoculated with PPV+TVMV or PPV+TEV. In both cases, PPV was able to replicate in the inoculated leaves, but at lesser extent than in single infections. Although TEV and TVMV systemically spread very efficiently, they were not able to complement the movement defect of PPV. HC and CP proteins have been shown to be involved in potyvirus long distance movement, and it has been suggested that the high sequence divergence of P1 migth be related with host specificity. For these reasons we focussed the next research steps on these proteins. A PPV-TVMV chimeric virus containing the P1 cistron 5' half from TVMV was infectious, but it resembled wild type PPV in its inability to infect systemically tobacco. *N. tabacum* transgenic plants expressing the 5' end of the TEV genome, including the P1 and the helper component (HC) cistrons (U6B line*), the coat protein (CP) gene of the same virus (FL3.3 line*), the 5' end of the TVMV genome, including the P1, HC, P3 and part of $6K_1$ cistrons (B8 line*), or the TVMV CP gene (CP4 line*) were inoculated with PPV. U6B plants, but not the rest of the transgenic plants resulted systemically infected. Transgenic plants expressing the same TEV 5' end fragment, but mutated in different positions of the transgene (B, C, E, I, K, L lines*) were also analyzed. PPV infected systemically transgenic plants with mutations in the P1 gene or in the amino-terminal part of the HC gene. In the plants with transgenes mutated in the central part of HC, PPV replicated only in inoculated leaves. We can conclude that the long distance movement function of the potyvirus HC protein is not strictly virus specific, and that, in the case of PPV, HC is a limiting factor for *N. t abacum* plant infection.

* TEV transgenic plants were a gift from James C. Carrington. TVMV transgenic plants were kindly provided by Emilio Rodriguez-Cerezo and were obtained by Arthur Hunt .

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