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# Plasmodesmata: composition, structure and trafficking

Bernard L. Epel
*Botany Department, Tel Aviv University, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel*

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## Abstract

Plasmodesmata are highly specialized gatable trans-wall channels that interconnect contiguous cells and function in direct cytoplasm-to-cytoplasm intercellular transport. Computer-enhanced digital imaging analysis of electron micrographs of plasmodesmata has provided new information on plasmodesmatal fine structure. It is now becoming clear that plasmodesmata are dynamic quasi-organelles whose conductivity can be regulated by environmental and developmental signals. New findings suggest that signalling mechanisms exist which allow the plasmodesmatal pore to dilate to allow macromolecular transport. Plant viruses spread from cell to cell via plasmodesmata. Two distinct movement mechanisms have been elucidated. One movement mechanism involves the movement of the complete virus particle along virus-induced tubular structures within a modified plasmodesma. Apparently two virus-coded movement proteins are involved. A second movement mechanism involves the movement of a non-virion form through existing plasmodesmata. In this mechanism, the viral movement protein causes a rapid dilation of existing plasmodesmata to facilitate protein and nucleic acid movement. Techniques for the isolation of plasmodesmata have been developed and information on plasmodesma-associated proteins is now becoming available. New evidence is reviewed which suggests that plasmodesmatal composition and regulation may differ in different cells and tissues.

## Introduction

In higher plants, cell-to-cell communication as well as nutrient transport may be symplastic, via junctional structures called plasmodesmata or apoplastic, via membrane-associated receptors, trans-membranous gatable channels and/or transporters. Plasmodesmata are highly specialized gatable cytoplasmic trans-wall channels that interconnect contiguous cells and function in the direct cytoplasm-to-cytoplasm intercellular movement of water, nutrients, small signalling molecules and, in certain cases, of macromolecules.

Plasmodesmata were thought for many years to be nonselective pores, passively allowing the bi-directional movement of molecules between adjacent cells. During the past few years this static concept of the plasmodesmata has been changing and it is now clear that plasmodesmata are dynamic selective entities with the capacity to 'gate'. Evidence is now emerging which suggests that plasmodesmatal composition and regulation may differ in different cells and tissues. In this review, we consider new studies on plasmodesmatal structure, composition, on the regulation of gating, and on virus-induced alterations in plasmodesmatal conductance.

**Plasmodesmatal structure**

Early electron micrography studies of plasmodesmata led to a simple general consensus model describing the plasmodesma as a wall-embedded plasmalemma-lined unbranched cylinder that contains a central axial component generally termed the desmotubule. For a review of early structural aspects of plasmodesmata, see the book by Gunning and Robards [29]. The desmotubule was considered to be derived from and continuous with the endoplasmic reticulum of adjoining cells. Recent studies suggest that the endoplasmic reticulum (ER) component of a plasmodesma is a derivative of and continuous with cortical ER [30, 31, 52]. In some cells, the outer regions of the plasmodesma are constricted and form what has been termed the neck region, a structure which is not a general feature of all plasmodesmata [56]. The cylindrical space between the desmotubule and the plasmalemma has been referred to as the cytoplasmic annulus or sleeve. Most models consider the cytoplasmic annulus as the pathway through which transport occurs. Electron micrographs show that in the orifice region, the ER was apparently constricted and no lumen was detected. Lucas *et al.* [38] have suggested that, since it is now clear that the desmotubule is not a tubule but a modified ER, its nomenclature should be changed to *appressed ER*. This new nomenclature may not be appropriate for a number of reasons. The term 'appressed' refers to laminar structures while the ER within the plasmodesma is more like a cylinder. A more appropriate descriptive term might be *constricted ER* rather than *appressed ER*. Furthermore, within the more interior regions of the plasmodesmata, the ER often balloons and a lumen is present, i.e. it is neither appressed or constricted. Most importantly, we feel that one must be cautious in interpreting static electron micrographs. The ER within the plasmodesmatal channel may be a dynamic structure, constricting and dilating as signalling and function dictate. We feel that the term 'desmotubule' could be retained. However, if one feels the need to use a functional neutral term, then one can refer to the 'plasmodesma ER component' (PERC). It is entirely conceivable that within the plasmodesmata the ER has undergone major modifications, and that different parts of the plasmodesmatal ER component have different compositions.

The substructural detail of plasmodesmata in higher plants has been examined by various workers and a number of models have been proposed (see review by Robards and Lucas [55]; [7, 14, 68]). Recently, two models of plasmodesmatal structure were proposed based on computer-enhanced digital imaging analysis of electron micrographs of plasmodesmata from a dicotyledonous plant [14] and from a $C_4$ grass [7].

Ding *et al.* [14] examined the substructure of the plasmodesmata of the $C_3$ dicot *Nicotiana tabacum* following cryofixation and freeze substitution. According to their model, the plasmodesma is depicted as a complex pore-containing proteinaceous particles embedded in the inner leaflet of the plasma membrane and in the outer leaf of the desmotubule. Spoke-like filamentous strands apparently connect the globular proteins of the outer leaf of the desmotubule to the proteins embedded in the encasing PD plasma membrane. The central region of the desmotubule, the so-called rod, an electron-dense region, is depicted as being composed of a series of particles, probably protein, that are embedded in the lipid of the fused inner leaflet of the ER membrane. In their model, the desmotubule is constricted and no lumen is present. It was suggested that the electron-dense particles embedded in the inner leaflet of the plasmalemma and the outer leaflet of the desmotubule form a convoluted channel that functions as a molecular sieve, determining the size exclusion properties. It was further hypothesized that the radial spokes may function in dynamically altering the effective radius of the cytoplasmic sleeve, thus regulating the size exclusion limit of the plasmodesma.

Botha *et al.* [7] employed similar computer enhancement techniques with plasmodesmata at the Kranz mesophyll bundle sheath interface of *Thermeda trianda*, a $C_4$ grass. Their model showed many similarities to that of Ding *et al.* [14] but with a number of significant differences. Associ-

ated with the inner plasmalemma leaflet and the outer desmotubule wall were particles ca. 2.5–3 nm in diameter, presumably proteins that abutted into the so-called cytoplasmic sleeve. In the central region of the desmotubule were electron-dense particles also about 2.5 nm in diameter. In contrast to Ding *et al.* [14], Bothe *et al.* [7] detected the presence of a lumen of about 2.5 nm in diameter between the central protein core and the desmotubule wall. Furthermore, filamentous connections were observed between the central rod particles of the desmotubule and the desmotubule wall. This was in contrast to the model of Ding *et al.* [14] for the plasmodesma for *N. tabacum*, in which the spoke-like filamentous strands connected proteins of the outer leaf of the desmotubule to the proteins embedded in the encasing PD plasma membrane. The data and model present by Bothe *et al.* [7] brings into question the general consensus that desmotubules are constricted and can not function in cell-to-cell transport. Additional studies are needed to address this question.

Other workers have reported on the presence of structures, termed 'sphincters', either external to [49] or within [56] the neck region of plasmodesmata. It was suggested that these 'sphincters' function in the regulation of plasmodesmatal conductance. It should be noted that these conclusions were based on structural evidence alone and since these structures are not universal, it is difficult to assess their involvement in the regulation of plasmodesmatal conductance.

## Transport through plasmodesmata

Transport through plasmodesmata has generally been studied by measuring the cell-to-cell movement of plasma membrane-impermeable fluorescent dyes of different sizes and properties. The classical studies of Goodwin and co-workers [23, 27], of Tucker [71] and of Terry and Robards [67] led to a consensus that only molecules of less then about 800–1000 Da pass freely through plasmodesmata. Recent studies, however, are leading to a re-evaluation of the size exclusion limit (SEL) and selectivity of plasmodesmata. These new studies indicate not only that the plasmodesma can be gated, but that the size exclusion limit can be modulated by environmental and developmental signals.

The diffusion selectivity of plasmodesmata to a series of fluorescein labelled probes was re-examined quantitatively by Tucker and Tucker [73] employing fluorescein labelled (F-) mono-, di-, tri- and quatra-amino acids. Their data suggested that the plasmodesmata of the stamenal hairs of *Setcreasea purpurea* have a size exclusion limit of about 800 Da and select for small hydrophilic molecules with a charge from −2 to −4. Molecules, however, that have attached aromatic amino acid such as phenylalanine and tryptophan exhibited low mobility and their kinetic curves, as well as those of F-$(meth)_2$ and F-$(his)_2$ generally did not fit simple diffusion kinetics. When carboxyfluorescein (CF), a highly mobile probe was micro-injected into the cytoplasm of cells previously micro-injected with probes whose transport was apparently blocked, the CF passed readily from cell to cell. This implied that the aberrantly mobile probes had neither blocked nor closed the permeation pores but that the plasmodesmata exhibited specific selectivity against these molecules.

Azide treatment markedly altered the conductance properties of *Setcreasea purpurea* plasmodesmata [74]. In untreated cells, probes such as F-Phe, F-Try, F-$(meth)_2$, F-$(his)_2$ and the FITC-labelled octa-peptide angiotensin II did not pass through plasmodesmata or passed with non-diffusion kinetics. However, following azide treatment all probes passed with ease with their diffusion coefficients increasing by about 400%. The data indicated that azide treatment may have caused the plasmodesmatal pore to dilate.

Further support for the notion that the size exclusion limit of plasmodesmata is much more extended then previously believed and that size selectivity can be modulated comes from studies by Cleland *et al.* [10]. They found that under aerobic conditions or in the absence of inhibitors of respiration, the SEL of wheat root PD, as probed with LYCH or F-dextrans, was about 1 kDa. However, after either azide (1 or 10 mM)

treatment or flushing with nitrogen, the SEL increased within 30–60 min to 3–4 kDa and sometimes to as high a mass as 7–10 kDa.

The effects of azide and anaerobiosis are pleiotropic, causing an inhibition in oxidative respiration, a decrease in ATP, an increase in $Ca^{2+}$ and a lowering of cytoplasmic pH. Evidence has previously been presented which suggests that the gating of plasmodesmata [3, 42, 72] and of animal junctional pore structures, the gap junctions [12, 57, 58, 65, 66, 70] may be regulated by phosphorylation. Changes in $Ca_2^+$ and ATP levels possibly alter protein kinase and/or phosphatase activity resulting in a change in the phosphorylation level of plasmodesmatal proteins and thus altering the structure of the conducting channels within the plasmodesmata and resulting in a modulation of the SEL. I hypothesize that the phosphorylation of plasmodesmatal components results in the down-regulation of plasmodesmatal conductance while dephosphorylation results in a dilation of the conducting channel and up-regulates conductance.

## Regulation of closure by differential pressure

The gating of plasmodesmata has been shown to be affected by various environmental and developmental signals [11, 18, 50, 51, 53, 60, 61]. In giant algae such as *Chara* and *Nitella*, pressure gradients between cells increased electrical resistance across the node and inhibited intercellular transport of radioactively labelled assimilates [11, 53]. In higher plants, direct evidence for pressure-generated closure was recently provided in a study by Oparka and Prior [50]. In this study, a pressure gradient was generated between adjacent cells of leaf tricomes of *Nicotiana clevelandii* with a micropressure probe and its effect on intercellular transport followed by measuring the cell-to-cell movement of the fluorescent probe Lucifer Yellow. A pressure differential in excess of 0.2 MPa was required to close plasmodesmata completely. The degree of plasmodesmatal closure was dependent on the magnitude of the pressure differential between the two cells. Just raising or lowering the internal pressure was not sufficient, a pressure differential between two adjacent cells was essential. If a cell was wounded by puncturing, cells acropetal to the wounded cell lost pressure but remained in communication with each other and with the wounded cell. However, cells basipetal to the punctured cell which were in direct contact with the plant body (leaf) retained turgor, and communication with the wounded cell was terminated.

The above findings have obvious implications for wounding. It was suggested that one of the first responses during cell wounding would be a pressure-generated closure of plasmodesmata in the cells adjacent to the wound, provided that the tissue had an adequate water supply and there was no general loss of turgor in the cell adjacent to the wound site. If there was a loss of turgor in tissues adjacent to the wound site then these cells would remain in symplastic communication with the wounded cell and leakage out into the wound region would occur. Based on these observations, we suggest that if excised tissue would be placed in an isotonic solution, no pressure differential would be established between the wounded cell and adjacent intact cells and the plasmodesmata in the wounded cells would not close. The plasmodesmata of the wounded cell would then continue to communicate with adjacent cells. This phenomenon could be exploited to introduce non-membrane permeable molecules into cells without the necessity of microinjection. This hypothesis could explain the uptake of polar tracers into intact cells of fresh cut sections as reported by Burnell [8] for bundle sheath strand from $C_4$ plants, by McCaskill *et al.* [40] for glandular trichomes from peppermint leaves, and by Tucker and Tucker [73] for severed stamenal hairs of *Setcreasea purpurea* and by Wang and Fisher for fresh crease tissue of developing wheat grains [77].

## Regulation of gating by light

Plasmodesmatal gating can also be regulated by light. Epel and Erlanger [18] measured symplas-

tic transport in the mesocotyl tissue of dark grown maize seedlings using the fluorescent symplastic probe carboxyfluorescein. They reported that lateral symplastic transport from the stele into the mesocotyl cortex was inhibited by a prior white-light irradiation. The inhibitory effect of a prior white-light irradiation was completely photomodulatable by terminal far-red and far-red/red irradiations, suggesting the involvement of phytochrome. It was suggested that the modulation of plasmodesmatal conductance by light, and possibly by other environmental and/or developmental signals, might modulate growth and development, in part by establishing or altering symplastic domains and by channelling cell-to-cell transport of nutrients and growth regulations. As we have previously pointed out, large gradients of growth regulators and solutes would exist at interfaces between domains and sub-domains with resultant effects on growth and differentiation [17].

**Cell- and tissue-specific differences in SEL**

There is accumulating evidence that the size exclusion limit of plasmodesmata may differ in different cells and tissues. It was found that when fresh-cut sections of the crease tissue of developing wheat grains were incubated in fluorochrome solutions of normally impermeable apoplastic probes such as Lucifer Yellow, dye was rapidly absorbed via unsealed plasmodesmata [77]. Once absorbed, the dye moved symplastically. Using a size-graded series of probes, the size exclusion limit (SEL) for the post-phloem pathway was estimated. In most, perhaps all, cells of the crease tissue with the exception of the pericarp, the effective molecular diameter of the conducting channel was estimated to be about 6.2 nm vs. about 2.5–3 nm from other studies with other plants and tissues.

A number of recent studies suggest that plasmodesmata between sieve elements and their companion cells may traffic in proteins [6, 24]. In higher plants, mature sieve elements are generally enucleated and lack ribosomes. Since these cells can function for prolonged periods, either the proteins in these cells have a longer lifetime or proteins may be transported into these cells, most likely from adjacent companion cells via plasmodesmata. Aphids and other sap-sucking insects obtain food by inserting their stylet into a functional sieve element. The content of sieve elements can be analyzed by severing the stylet and collecting the pressure-mediated exudation of the phloem sap. Fisher *et al.* [24] investigated protein turnover in sieve tubes of wheat by $^{35}$S-methionine labelling of leaves and by the use of aphid stylets to sample the sieve tube contents. About 200 different soluble proteins were present in the sieve tube, many of which underwent rapid labelling. Given the constant protein composition along the path, it was concluded that most proteins were loaded at the source (the leaf) and unloaded at the sink (the grain). Certain proteins showed very rapid turnover suggesting some selectivity of movement from companion cells into sieve tubes. If movement through these plasmodesmata is indeed specific for particular proteins, then it must be concluded that these plasmodesmata do not function as simple molecular sieves. It is unclear whether the potential to transport proteins between cells is a unique property of sieve tubes and their companion cells or whether protein transport can normally occur between all cells. Molecular studies must be designed to test whether the potential for protein transport requires special targeting sequences. In order to examine whether signal sequences are involved in selective plasmodesmatal transport of proteins, genes of proteins able to transverse plasmodesmata should be cloned and sequenced and putative consensus sequences for plasmodesmatal targeting identified. By employing chimeric reporter proteins it may be possible to identify small targeting sequences. Micro-injection experiments with fluorescently labelled phloem proteins could be employed to test whether plasmodesmata in other tissues are competent to transport these phloem-resident proteins.

Since membrane lipids and proteins within the sieve tubes probably also undergo turnover, there must be some mechanism for the transport of

membranes or membrane components, both lipids and proteins, into sieve tubes. This could be accomplished through migration within the plasma membrane of the plasmodesmata or via budding-off of desmotubule membranes and incorporation into the plasma membrane of the sieve tube. A recent study using the technique of fluorescence redistribution after photobleaching (FRAP) has provided evidence that the ER membranes of plasmodesmata can serve as a dynamic diffusion pathway for the movement of lipids and lipid signalling molecules between contiguous cells [28]. In this study, either the plasma membranes or the ER membranes of cultured soybean suspension cells were labelled with a range of fluorescent lipids or phospholipid analogues. After photobleaching of the fluorescent probe in a target cell, the transport of the lipid from adjacent cells into the target cell was monitored under a confocal microscope. In the case of ER-located fluorescent probes, there was clear evidence for intercellular communication between contiguous cells. No detectable intercellular communication was observed for fluorescent probes residing exclusively in the plasma membrane despite the fact that plasma membrane-located probes showed considerable lateral mobility within the plasma membrane of a single cell.

Further evidence that plasmodesmata between companion cells and sieve elements can selectively transport proteins was provided by experiments with the phloem exudate from pumpkin. The phloem exudate from pumpkin contains two abundant basic proteins, termed PP1 and PP2 that are involved in slime plug formation. These two proteins are expressed exclusively in developing and mature sieve elements and their companion cells [62]. P-protein synthesis is thought to occur either in the immature sieve elements or in the companion cells prior to transport into sieve elements. This transport from companion cells into sieve elements has been postulated to occur via plasmodesmata. Thompson and coworkers [6] used a combined molecular and structural approach to investigate the temporal and spatial appearance of these two P-proteins. To obtain evidence for the site of P-protein synthesis, PP1 and PP2 mRNA were localized by *in situ* hybridization (PP1 [6], PP2, personal communication). PP1 and PP2 antisense transcripts hybridized to mRNA only in companion cells within the phloem of hypocotyl tissues in both the bundle and extrafascicular phloem tissue. Not withstanding the above data, the exact site of P-protein synthesis in the differentiating phloem tissue is still unclear. Thompson suggests three possible scenarios regarding the site of P-protein synthesis: (1) protein synthesis occurs exclusively in the companion cells and proteins are transported into sieve elements; (2) immature nucleated sieve elements synthesize stable proteins which are also synthesized in companion cells; and (3) a combination of 1 and 2 where protein synthesis occurs in both cell types and protein transport occurs from the companion cells to the enucleated mature sieve element. Developmental studies using *in situ* localization and/or using transgenic plants containing reporter constructs under the transcriptional regulation of the PP promoter should aid in determining the correct model. If indeed P-proteins are synthesized in companion cells and transported via plasmodesmata to contiguous sieve elements, this could indicate that the plasmodesmata between the companion cells and sieve element have different properties and possibly different composition then other plasmodesmata. Alternatively, it could be hypothesized that plasmodesmata connecting the companion cells with a sieve element have a unique regulatory mechanism for passage of large molecules. As indicated above, under conditions of low ATP, plasmodesmata can dilate, allowing passage of large molecules. If this regulation is due to phosphorylation/dephosphorylation of plasmodesmatal proteins, regulation of plasmodesmatal dilation may be by cell-specific protein kinases or phosphatases.

Kikuyama *et al.* [35] examined the SEL of plasmodesmata in the characean plant *Nitella* using a number of fluorescent probes included dextrans and proteins. In experiments measured in the time frame of minutes, they obtained results similar to those observed with higher plants, i.e. the SEL was about 1000. However, if transport

was measured after 24 h, it was found that myoglobin (20 kDa) and egg albumin (45 kDa) were transported from cell to cell, but bovine serum albumin 70 kDa was not transported. It would be of importance to quantify the transport rates for these proteins and determine whether the energy charge of the cell has any effect on the transport capacity for these large molecules.

### Virus plasmodesma interactions

Most, if not all, plant viruses spread from cell to cell via plasmodesmata [1, 32, 39]. Several studies over the past few years have focused on the process of viral cell-to-cell spread and have revealed the involvement of virus-encoded movement proteins (MP). Studies with different classes of viruses indicate at least two distinct movement mechanisms. One mechanism, exemplified by tobacco mosaic virus [16], red-clover necrotic mosaic virus [25] and bean dwarf mosaic geminivirus [48], involves a virus-encoded movement protein that facilitates the passage of a non-virion form of the virus. The second mechanism, exemplified by the comovirus cowpea mosaic virus (CPMV), involves the movement of the complete virus particles along tubular structures through plasmodesmata [75, 78]. In this type of mechanism the desmotubule is absent and specific virus-induced tubules are assembled in the plasmodesma, through which virus particles move from one cell to the other [76]. Two virus-encoded movement proteins are involved, one of which becomes incorporated into the virus-induced tubules; the function of the second protein which is not incorporated into the tubule is unclear. It is still an open question whether existing plasmodesmata are modified or whether new connections between cells are induced by the virus. Cell-to-cell movement through virus-induced tubules has also been reported for a number of other important viruses from different virus groups [76].

The mechanism for the movement of the non-virion form apparently does not entail a modification in the composition of existing plasmodesmata. Recent data indicate that the virus movement protein apparently interacts directly with an intact plasmodesma, causing it to dilate and to potentiate the passage of itself and other large biomolecules [25, 48]. Previous immunological studies suggested that MP may become incorporated into plasmodesmata [2, 15, 45, 69]. It was suggested that in transgenic tobacco plants transformed with MP30, the MP was incorporated only into secondary plasmodesmata during their formation and caused a significant increase in the SEL from 800 Da (control) to $>10$ kDa [15]. It was hypothesized that only these modified secondary plasmodesmata have altered SEL and function in viral transport [15].

Recent experimental evidence suggests that the hypothesis that *de novo* synthesis of modified secondary plasmodesmata containing MP as a prerequisite for virus spread is incorrect. In these experiments, various putative viral movement proteins were directly introduced in mesophyll cells and their cell-to-cell movement measured. Evidence from these microinjection studies indicated that the injected movement protein altered the state of existing plasmodesmata almost immediately, potentiating the trafficking of macromolecules [25, 48]. When FITC-labelled 35 kDa movement protein of the red-clover necrotic mosaic virus (RCNMV) was microinjected into cowpea mesophyll cells, it spread from cell to cell within seconds. By contrast, an alanine scanning mutant movement protein (mutant protein 278 which was unable to promote virus spread) did not spread out of the injected cell. The wild-type RCNMV movement protein also promoted cell-to-cell spread of fluorescently labelled RCNMV RNAs. Upon co-injection of a 10 kDa Fluorescently labelled dextran (F-dextran) with native MP, the F-dextran moved from cell to cell. In the absence of the MP only F-dextran molecules of less than 1 kDa moved. It was reported that only those alanine scanning mutant proteins that promote virus movement in infected plants increased the SEL with but one exception: mutant 280, which did not promote virus movement *in vivo* [26] but which did increase the SEL. The finding that this mutant protein up-regulated the SEL of plasmodesmata but did not facilitate viral move-

ment suggested that there may be a distinct domain responsible for opening plasmodesmata that is different from that for mediating viral spread.

Similar results were obtained with the movement protein for the bean dwarf mosaic geminivirus [48]. This virus possesses a bipartite genome divided between two circular single-stranded (ss) DNA molecules, DNA-A and DNA-B. Encapsidation and replication are encoded by DNA-A while systemic spread functions are encoded by DNA-B. Frame-shift mutations or single-amino-acid substitutions in either the BL1 or BR1 open reading frames encoded on DNA-B-abolished systemic movement of the mutated form of the virus but did not affect DNA-B replication in protoplasts. These results led to the suggestion that these genes are essential for the systemic spread of the infectious form of BDMV. When FITC-labelled BL1 protein was microinjected into bean cells, the movement protein spread from cell to cell within seconds. Upon co-injection of BL1 with fluorescently labelled dextrans, it was found that 10 kDa but not 29 kDa F-dextran moved from cell to cell within 1–2 min. The BL1 movement protein could also facilitate cell-to-cell movement of double- but not single-stranded DNA. The BL1 movement protein potentiated both its own movement and that of ssDNA but did not potentiate the movement of BR1, a second viral protein that mediates the movement of ssDNA and dsDNA out of nuclei. It is unclear whether the BL1 movement protein could potentiate the movement of proteins other than itself.

The finding that MP up-regulates plasmodesmatal conductance within seconds is not in accord with the hypothesis that viral movement occurs only through *de novo* synthesized MP-modified secondary plasmodesmata. The large-scale accumulation of MP in secondary plasmodesmata is apparently unrelated to the movement mechanism. These results suggest that MP, in some unknown manner, almost immediately up-regulates the plasmodesmatal SEL, with no need for *de novo* secondary plasmodesmata formation. It is suggested that the MP interacts with regulatory factors associated with plasmodesmata that normally function in gating plasmodesmatal conductivity and/or selectivity. In order to understand how viruses alter plasmodesmata, basic information is required on the composition of plasmodesmata.

## Plasmodesmatal composition

First biochemical and molecular information on plasmodesmata came from studies demonstrating the presence of animal connexin-homologous proteins in plants [33, 34, 36, 41, 42, 43, 44, 59, 81, 82]. Schindler and co-workers noted numerous functional homologies between plasmodesmata and between animal gap junctions, structures involved in cytoplasmic cell-to-cell communication in animals. These functional analogies included similar behavior with regard to SEL tracer dyes, similar electrical properties and similar modes of down-regulation by effectors of protein kinase C. It was suggested that there may be biochemical homologies between gap junction proteins and plasmodesmatal proteins. Immunological data suggested that dicotyledonous plants contain peptides immunologically related to the mammalian gap junction proteins, connexin32 [33, 41, 42, 44, 81], and connexin26 [33, 34, 59]. Indirect evidence suggested that these cross-reacting proteins [33, 34, 59, 81] may be plasmodesma-associated. More definitive evidence for the presence of a connexin homologous protein in plants was provided by immunocytochemical studies in maize by Yahalom *et al.* [82]. Using affinity-purified antibodies against two different gap junction proteins, connexin32 and connexin43, it was shown by indirect immunogold labelling of thin sections that maize mesocotyl plasmodesmata contain two different proteins that cross-react with connexin gap junction antibodies. A connexin32 antiserum cross-reacted with a 27 kDa maize plasmodesma-associated protein termed PAP27, while an affinity-purified antiserum against connexin43 labelled a 26 kDa protein termed PAP26. PAP26 immunolocalized along the entire length of the plasmodesma as well as to plasmalemma regions sur-

rounding the plasmodesma orifice. PAP27 immunolocalized to outer regions of the plasmodesma and is apparently a peripheral membrane protein. After tissue homogenization and differential centrifugation it was found not only associated with the wall fraction but also with other membrane fractions as well as the soluble fraction [36, 82]. After repeated passages through a nitrogen pressure bomb and repeated washes in a buffer containing high chelator concentrations it dissociated from the wall fraction. In contrast, PAP26 was concluded to be an integral membrane protein; it was found associated only to the wall fraction and could not be extracted with chelators, high salt, high pH or Triton X-100 (Yahalom and Epel, unpublished results).

Meiners *et al.* [44] isolated and sequenced a cDNA clone, termed CX32, from an *Arabidopsis* expression library that encoded for a protein that cross-reacted with an animal connexin32 antibody. They attempted to align its deduced amino acid sequence with that of a rat connexin and concluded that the *Arabidopsis* CX32 protein showed significant homologies to that of the animal connexin. It was suggested that the plant protein was a connexin homologue. Mushegian and Koonin [47] re-analyzed the alignment employing a more statistically rigorous alignment program and suggested that the alleged plant connexin from *Arabidopsis* was unrelated to animal connexins but was more closely related to a protein kinase-like protein.

At present no sequence data are available either for the two confirmed plasmodesma-associated maize proteins PAP26 and PAP27 [82] or for the *Vicia faba* 21 kDa wall-associated protein that cross-reacted with antibodies to mouse liver connexin26 [33]. It would be of great interest to see if these proteins are truly connexin-homologous or only show very limit homologies at a small number of epitopes.

Further characterization of plasmodesma-associated proteins was made possible by the development of techniques for plasmodesma isolation. Initial work was performed with only semiclean cell wall fractions that contained embedded plasmodesmata. [46, 82] but which contained contaminating subcellular organelles and membranes [36]. Yahalom *et al.* [82] detected well over 20 proteins associated with such a semiclean maize mesocotyl wall fraction including PAP26 and PAP27. Monzer and Kloth [46] examined proteins associated with a wall fraction obtained from shoot tips of *Solanum nigrum*. A comparison of the proteins extracted from the various cell fraction with those associated with the wall revealed that 2 proteins with apparent molecular masses of 28 and 43 kDa were highly enriched in this wall fraction. Turner and Roberts (personal communication) have examined proteins associated with isolated walls from a 2 mm region of the root tip of maize seedlings. This meristematic region has very high concentrations of primary plasmodesmata. Turner found that there were three major wall associated proteins with apparent molecular weights of 100, 70 and 40 kDa that could be extracted with Triton X-100 or with CHAPS, and that the extraction with these detergents apparently removed plasmodesmata from the walls. Blackman and Overall (personal communication) have taken a unique approach to examining proteins associated with plasmodesmata. In studies with the higher alga *Chara*, they isolated the internode wall which contain no plasmodesmata and walls of nodal cells which contain very high concentrations of plasmodesmata. It should be noted that the plasmodesmata in these cells are structurally different from those of higher plants in that they do not contain a desmotubule. Associated with the walls containing plasmodesmata, they identified 4 unique proteins with apparent molecular masses of 53, 27, 26 and 20 kDa that were absent in the walls of the internode cells which are devoid of plasmodesmata. Monoclonal antibodies are being generated against these proteins and will be used in immunolocalization studies.

Kotlizky *et al.* [36], in order to enrich for plasmodesmatal proteins and to reduce or eliminate spurious non-plasmodesmatal proteins, developed a procedure for preparing a clean wall fraction that contained wall-embedded plasmodesmata and that was devoid of contaminating cytoplasm, organelles and non-relevant mem-

branes. At least 10 proteins were associated with this clean wall fraction [21, 22, 36], including at least two calcium-dependent protein kinases [22, Yahalom and Epel, unpublished data].

With the advent of this wall isolation procedure, it became feasible to attempt to isolate plasmodesmata by enzymatic digestion of the encasing cell wall. Since most if not all commercial cellulase preparations contain proteases and lipases which cause a partial degradation of the plasmodesmata, it was necessary to include a cocktail of protease inhibitors in order to reduce the degree of proteolysis [22]. After overnight enzymatic hydrolysis of the encasing cell wall with a low-protease cellulase from *Trichoderma reesi* and after differential centrifugation of the hydrolysate, a fraction was obtained which contained free plasmodesmata, and plasmodesmata aggregates enmeshed in an apparently cellulase resistant network [22, Epel and Van Lent, unpublished data]. About 8 putative plasmodesmata-associated proteins (pPAP) with apparent molecular masses of 64, 51, 41, 32, 26, 21, 17 and 15 kDa as determined by SDS-PAGE were identified and isolated [20, 21].

Epel and co-workers generated polyclonal antibodies against the 17, 26, 32, 41, 51 and 64 kDa wall-associated polypeptides and showed by western blot analysis of different cell fractions that the 17, 26, 32, and 51 kDa proteins were detectable only in the wall fraction, suggesting that these proteins are probably unique to the plasmodesmata (Epel, Levi and Erlanger, unpublished results). An unequivocal and final confirmation that these proteins are truly plasmodesma-associated proteins requires that each protein be immunolocalized to the plasmodesma. Immunolocalization studies, in addition to those performed with PAP26 and PAP27, have now been performed with the 41 kDa protein [21]. Silver-enhanced immunogold light microscopy showed that the 41 kDa protein was associated with the walls of cells both in the stele and cortex. The immunolabelling pattern was trans-wall and punctate. Electron microscopic immunogold labelling localized the polypeptide to plasmodesmata and to electron-dense cytoplasmic structures which are apparently Golgi membranes. The observation of gold particles over all parts of the plasmodesmata suggested that the protein was spread over the whole length of the structure. Unfortunately, immunogold labelling does not have sufficient resolution to determine more precisely where in the fine structure of the plasmodesmata the protein is located.

PAP41 is apparently a peripheral membrane protein. Following tissue homogenation, the 41 kDa protein was found not only to be associated with the wall fraction but was also present in the soluble fraction and in heavy- and light-membrane fractions. Treatments that release peripheral-bound membrane protein such as 3 M NaCl or 100 mM $Na_2CO_3$ pH 11, released PAP41 as a soluble protein from the wall/plasmodesmatal fraction (Yahalom, Katz and Epel, unpublished results). Interestingly, overnight incubation of the wall fraction in the presence of 2% Triton X-100 did not solubilize this protein. The protein has been isolated and an internal peptide isolated and microsequenced. Katz, Kotlizky and Epel (unpublished results) screened a $\lambda$ZAP maize cDNA library with antibodies to PAP41 and with oligonucleotide probes synthesized according to a microsequenced internal peptide. One clone has been partially sequenced and shown to contain the sequence for the internal peptide. A full-length sequence should soon be available.

The finding that 41 kDa protein was also associated with Golgi-like structures suggested that this protein is probably transported to plasmodesmata via Golgi membranes. Primary plasmodesmata are apparently formed in part as the result of fusion of Golgi at the phragmoplast at the laying down of the primary cell wall [30]. The region of the mesocotyl sectioned and probed was from the 5 mm region below the coleoptile node, a region undergoing rapid elongation. In this region secondary plasmodesmata formation would be occurring [19].

In animals, the connexins, a family of related gap junction proteins [37], exhibit heterologous expression in various tissues [4, 5, 13, 79]. It was suggested that the diversity of connexins may be

to serve the different functional needs of the cell type; differences in conductance and differences in regulatory mechanisms have been shown [9, 63, 64]. Kotlizky, in the laboratory of Epel, obtained data that suggest that plasmodesmata, much like gap junctions, may be composed of different molecular subunits, depending on the source tissue or plant organ [20]. They showed by western blot analysis that PAP26, PAP41 and pPAP17 varied in the level of expression in different organs. PAP26 was present in leaf and mesocotyl but was essentially absent in the root. pPAP17 was present in root, and mesocotyl but was undetectable in the leaf. In contrast to the other 2 proteins, PAP41 was found in all organs tested. In the cortex and stele of the mesocotyl, all three proteins exhibited a qualitatively similar pattern of expression, albeit the specific concentration of all three was higher in the cortex than in the stele. It should be noted that the quantitative differences in expression between the cortex and the stele were more pronounced in both pPAP17 and PAP26 than in PAP41. This variation in expression both in plant organs and in plant tissues hints at a possible specialization of plasmodesmata in different tissue and organs.

Accumulating evidence suggests that in animal cells the regulation of transport through gap junction channels is by a phosphorylation mechanism. Several gap junction proteins (connexins) are phosphorylated by protein kinase C, calmodulin-dependent protein kinase and c-AMP dependent protein kinase [57, 58, 65, 66, 70]. Correlations were found between the phosphorylation of gap junction proteins and the gating of the gap junction channel [12, 54, 57]. Available evidence suggests that the gating of plasmodesmatal conductance may also be via a phosphorylation/dephosphorylation of as yet unidentified regulatory elements [3, 10, 72, 74].

Support for this hypothesis comes from *in vitro* phosphorylation studies with isolated clean wall fraction and isolated plasmodesmata [83, Yahalom and Epel, unpublished results]. In the presence of labelled ATP, several proteins including PAP41 and pPAP17 were phosphorylated by one or several endogenous $Ca^{2+}$-dependent protein kinase(s). *In situ* phosphorylation on nitrocellulose paper revealed the presence of at least 2 polypeptides that undergo autophosphorylation with an apparent MW of 51–56 kDa. The cell wall/plasmodesma-associated protein kinases were activated by $Ca^{2+}$, but not by phospholipids or calmodulin. They are probably tightly bound to the cell wall/plasmodesma fraction since proteins were phosphorylated even after overnight extraction with either 3 M NaCl, 2% Triton X-100 or 100 mM $Na_2CO_3$ (pH 11). While LiCl concentrations of up to 4 M increased phosphorylation, overnight extraction with 8 M LiCl released the endogenous kinases from the cell wall/plasmodesmata and completely abolished phosphorylation. It should be pointed out that direct evidence that these protein kinases function *in vivo* in phosphorylating plasmodesma-associated proteins is still lacking. Furthermore, strategies must be designed which will allow for the testing of the hypothesis that these kinases function in the regulation of plasmodesmatal conductance.

## Prospects

Plasmodesmata regulate the movement of nutrients, ion, signalling molecules and, in certain cases, macromolecules. This movement can be gated and is under developmental and environmental control. Indirect evidence indicates the involvement of protein phosphorylation mechanisms. The nature of the signalling mechanisms is unclear and needs to be explored. Biochemical and molecular studies must be extended to identify and characterize plasmodesmatal components and regulatory elements. Studies must also be extended to cell- and organ-specific differences in plasmodesmatal structure, composition and regulation. Most studies to date have been devoted to maize. Studies should be extended to other organisms. Once we have identified and characterized plasmodesmatal components and characterized their genes we will have molecular tools for exploring mechanisms by which plants coordinate intercellular and tissue-tissue interac-

tions. These molecular tools will also allow us to explore how viruses interact with plasmodesmata and exploit them as conduits for virus spread.

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