CHAPTER 3

# Tissue Preparation and Substructure of Plasmodesmata

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A. J. E. van Bel et al. (eds.), *Plasmodesmata*

## 1 Introduction

Research on the plasmodesma seeks to address two basic issues: what kinds of molecules are transported through this intercellular organelle, and how are these molecules transported? Elucidation of the transport mechanisms requires that the substructure of the plasmodesma be understood. This understanding may include at least two aspects: ultrastructural features and molecular compositions.

Views of the plasmodesmal substructure have mainly been obtained through the use of transmission electron microscopy (TEM). This chapter attempts to discuss in general terms the prevailing methods used to prepare tissue samples for TEM studies of plasmodesmata, focusing on the limitations of these methods and on the interpretation of data obtained with these methods. I will also discuss briefly the use of integrated approaches to study the plasmodesmal structure at the molecular level.

## 2 Structure of the Plasmodesma

The structure of the plasmodesma is reviewed extensively in Chapter 9 and only some general features are outlined here to facilitate subsequent technical discussions. Through the exploration of many workers over the past 20 years (e.g. López-Sáez et al. 1966; Robards 1968, 1971; Zee 1969; Olesen 1979; Hepler 1982; Overall et al. 1982;

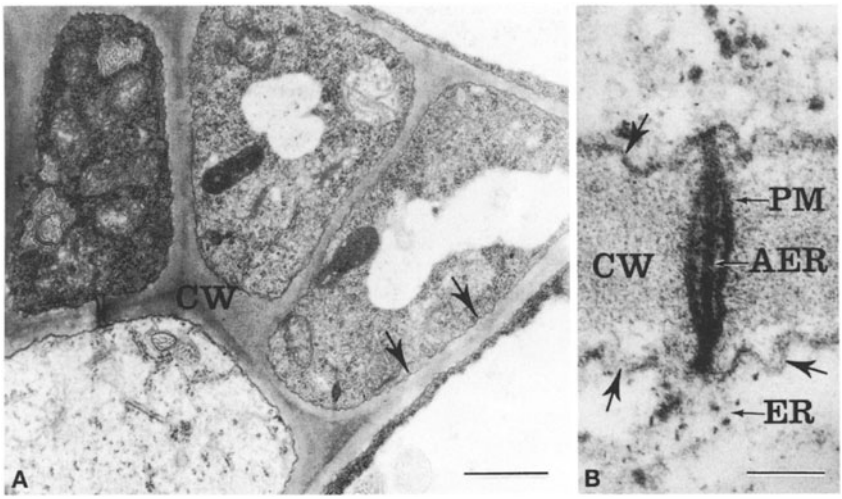


**Fig. 1 A, B. A** Vascular cells from a tobacco leaf fixed first with 4% glutaraldehyde/0.1% tannic acid and then with 2% osmium tetroxide. Note that the plasma membrane appears wavy and pulled away from the cell wall (*CW*), as indicated by arrows. *Bar* 1 μm. **B** A plasmodesma between bundle-sheath cells of a tobacco leaf fixed with the same fixation protocol as in **A**, showing major structural components of the organelle at this resolution: the plasma membrane (*PM*) and the appressed endoplasmic reticulum (*AER*). The AER is continuous with the endoplasmic reticulum (*ER*) in the cytoplasm. *Unlabelled arrows* indicate the plasma membrane that is pulled away from the cell wall (*CW*). This is a typical artifact of chemical fixation. *Bar* 100 nm (Ding et al. 1992a)

Thomson and Platt-Aloia 1985; Olesen and Robards 1990; Tilney et al. 1991; Ding et al. 1992b; Botha et al. 1993; Badelt et al. 1994; Ehlers and Kollmann 1996; Glockmann and Kollmann 1996), it is now established that a plasmodesma consists of the plasma membrane (PM) which is continuous between adjacent cells and forms a porous structure across the cell walls, and that the appressed endoplasmic reticulum (AER) cylinder (formerly desmotubule; see Lucas et al. 1993 for terminology) is positioned longitudinally in the center of the pore (Fig. 1A, B). The AER is structurally continuous with the rest of the cytoplasmic endoplasmic reticulum (ER) system. Within the plasmodesma, the space between the plasma membrane and the AER cylinder are thought to form microchannels for intercellular transport (Ding et al. 1992b). The AER itself may also play a role in intercellular transport of lipids (Grabski et al. 1993) and photoassimilates (Gamalei et al. 1994; Glockmann and Kollmann 1996). In particular, an open ER lumen is postulated to be the pathway for intercellular transport of at least photoassimilates (Gamalei et al. 1994).

Despite so many intensive investigations, some key issues of plasmodesmal substructure still remain to be resolved. Different views have been expressed concerning various aspects of the substructure of the plasmodesma. It is possible that variations in the plasmodesmal structure as proposed by different workers are dependent on plant species, tissue or cell types, and developmental stages in some cases. However, these variations may have also arisen when different methods were used to prepare tissues for microscopy. The latter is the focus of discussion of this chapter.

## 3 Methodologies of Tissue Preparation for Studying the Plasmodesmal Structure

Two methods have been used to fix plant tissues for studying the plasmodesmal structure: chemical fixation and cryofixation. Chemically fixed tissues were usually processed to obtain thin sections for TEM examination. Cryofixed samples have either been freeze-fractured to obtain replicas or freeze-substituted to obtain thin sections for TEM examination.

### 3.1 Chemical Fixation

Generally speaking, a typical chemical fixation protocol to prepare samples for structural studies at the TEM level consists of fixing small pieces of samples first in a primary fixative such as glutaraldehyde and/or paraformaldehyde, and then in a secondary fixative which is usually osmium tetroxide. Glutaraldehyde/paraformaldehyde cross-links proteins and osmium tetroxide fixes lipids (Glauert 1975; Bozzola and Russel 1992). Chemical fixation was the primary method used by many workers to prepare plant materials for plasmodesmal structure studies (e.g. López-Sáez et al. 1966; Robards 1968, 1971; Zee 1969; Olesen 1979; Overall et al. 1982; Olesen and Robards 1990; Tilney et al. 1991; Botha et al. 1993; Badelt et al. 1994; Ehlers and Kollmann 1996; Glockmann and Kollmann 1996). These studies have provided a general and fundamental understanding of the structure of the plasmodesma. In particular, that the plasmodesma is basically composed of the plasma membrane and the AER which is

continuous with the cytoplasmic ER is elegantly demonstrated (e.g. López-Sáez et al. 1966; Hepler 1982; Overall et al. 1982; Ehlers and Kollmann 1996; Glockmann and Kollmann 1996).

In a number of studies, tannic acid of 1–2% was included in the primary fixative (e.g. Olesen 1979; Overall et al. 1982; Thomson and Platt-Aloia 1985; Tilney et al. 1991; Badelt et al. 1994). Since tannic acid stains microtubules negatively (Fujiwara and Linck 1982), it was suggested that tannic acid could also stain plasmodesmal structures negatively. In particular, "electron-lucent" (negatively stained) particles were proposed to exist between the AER and the plasma membrane (Overall et al. 1982; Thomson and Platt-Aloia 1985; Olesen and Robards 1990).

However, Ding et al. (1992b) suggest that tannic acid does not stain plasmodesmal structures negatively. First, in tissues processed with and without the use of tannic acid, the staining patterns of plasmodesmal structures are the same. Second, overstaining by high concentrations of tannic acid could produce spurious images that appear negatively stained. As discussed by Fujiwara and Linck (1982), it is extremely important to use tannic acid of low-molecular weight and at low concentrations. When used at a concentration of more than 1%, tannic acid can cause extensive precipitation inside and outside a cell, thereby obscuring fine cell structures (Fujiwara and Linck 1982). For example, the diameter of microfilaments is increased from 6–7 nm to 13 nm by the use of 1% tannic acid (Seagull and Heath 1979). Ding et al. (199la, 1992b) used 0.1% tannic acid and found that the 6–7 nm diameter of microfilaments was not increased by this treatment. Therefore, use of low concentrations of tannic acid results in minimal distortion of the native dimension of cell structures. On the basis of these considerations, Ding et al. (1992b) suggest that the electron-lucent areas between the AER and the plasma membrane represent spaces, rather than particles. Their study further indicates that these spaces are created by electron-dense particles embedded in the plasma membrane and the AER cylinder of the plasmodesma (see below).

A number of workers have reported that "sphincter"-like structures are present in the cell wall region surrounding the orifice of the plasmodesma. These structures are particularly evident when 1–2% tannic acids were used in the primary fixative (Robards 1976; Olesen 1979; Mollenhauer and Morré 1987; Badelt et al. 1994), and when Driselase was used to digest the cell walls (Badelt et al. 1994). It has been suggested that these sphincter structures may function to regulate the opening or closing of the plasmodesmal transport channels between the plasma membrane and AER by some sort of contraction and expansion mechanism. However, a close examination of some of the published micrographs (Overall et al. 1982; Badelt et al. 1994) reveals that similar globular structures are also present at the interface between the plasma membrane and the cell wall, when the former is pulled away from the latter, in regions where plasmodesmata are absent. It is important to keep in mind that the effect of Driselase treatment on cell structures other than the cell walls is unknown. As discussed above, use of high concentrations of tannic acid can also be problematic. Thus, it is possible that these sphincter structures are pure artefacts induced by a combination of poor chemical fixation (evidenced by the distortion of membrane structures), Driselase treatment and tannic acid precipitation. Even though Badelt et al. (1994) used Freon to cryofix some samples for freeze substitution, the images presented (Fig. 2 in their paper) showed gross distortion of the cell structure - the plasma membrane is pulled far away from the cell wall. Thus, the freezing technique used in that particular study

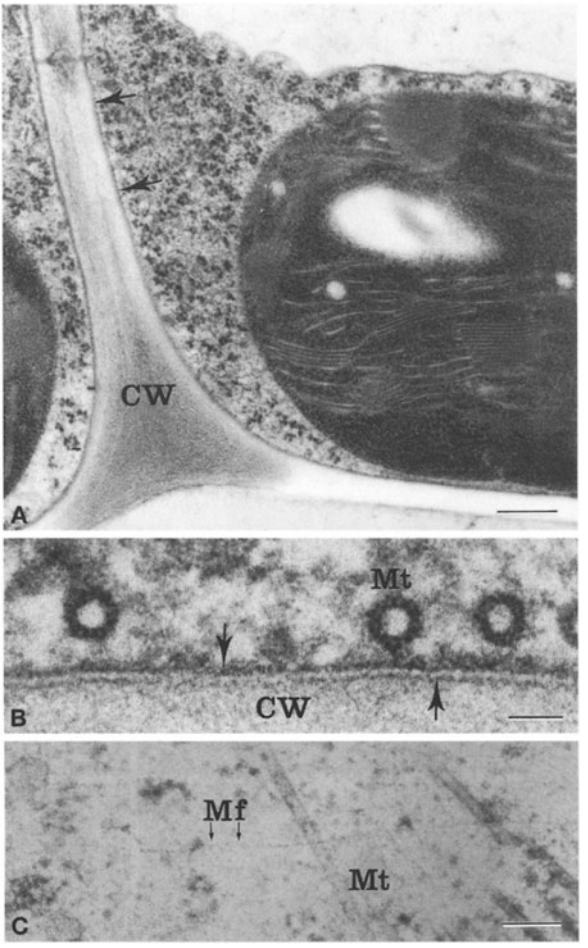


**Fig. 2 A–C.** Tobacco cells prepared by cryofixation and freeze substitution. The leaf samples were cryofixed by propane jet freezing (**A**) and (**C**) or high pressure freezing (**B**), freeze substituted in acetone containing 0.1 % tannic acid and then in acetone containing 2% uranyl acetate/2% osmium tetroxide. **A** Tobacco mesophyll cells. There is no visible ice damage at this resolution level. Note that the plasma membrane (*unlabelled arrows*) *is* smooth and tightly appressed to the cell walls (*CW*). *Bar* 0.2 µm (Ding et al. 1991a). **B** A tobacco mesophyll cell, showing good preservation of the subunit structures of the microtubules (*Mt*). *Unlabelled arrows* indicate the plasma membrane that is smooth and tightly appressed to the cell wall (*CW*). *Bar* 30 nm (Ding et al. 1991b). **C** A tobacco root cortical cell showing good preservation of a single microfilament (*Mf*), in addition to microtubules (*Mt*). *Bar* 80 nm (Ding et al. 1991a)

apparently did not preserve cell structures as well as expected. In conclusion, the images published so far can hardly be considered convincing evidence for the presence of sphincters, due to inferior fixation quality. This conclusion, however, does not exclude the possibility that some special structures are present. It is possible that the presence of such structures is species-dependent. Tannic acid may indeed stain some important and unique structures. The nature of these structures needs to be reevaluated using alternative fixation protocols that cause minimal distortion of cell structures. Furthermore, if such structures indeed exist, their functions need to be carefully studied.

In addition to the sphincter structures, spiral structures have also been suggested to exist in the cell wall and encircle the whole length of the plasmodesma (Badelt et al. 1994; Overall and Blackman 1996). These structures were proposed to function together with sphincters to control opening and closing of the plasmodesma. As for sphincters, whether such spiral structures indeed exist in nature requires reevaluation

using the best-preserved plant samples and innovative TEM techniques. Even if such structures are confirmed to exist by various means possible, one has to wonder about their real functions. If they were involved in regulating plasmodesmal transport, then how does a cytoplasmically localized molecule for transport efficiently communicate with complicated cell wall-localized sphincter and/or spiral structures to act upon the plasmodesma?

A recent study suggests that an open tubular structure is present in place of an AER cylinder in plasmodesmata of trichome cells of *Nicotiana clevelandii* (Waigmann et al. 1997). Waigmann et al. (1997) suspect that this tubular structure may be composed entirely of proteins, as suggested earlier by Tilney et al. (1991). Direct evidence for this assumption remains outstanding.

When it comes to substructural studies, it is important to keep in mind that chemical fixatives penetrate cells slowly, take several minutes to immobilize cellular structures, and induce many artefacts in cellular structures (Mersey and McCully 1978; Gilkey and Staehelin 1986). A notable example of such artefacts is illustrated in Fig. 1, which shows that the plasma membrane is wavy and is pulled away from the cell wall. This is of special significance when considering the fact that the plasma membrane and the ER are major components of the plasmodesma and that plasmodesmata are dynamic entities. Therefore, caution should be exercised in interpreting the details of the plasmodesmal substructure in chemically fixed materials.

## 3.2 Cryofixation

Cryofixation, or ultrarapid freezing, can physically stabilize cellular structures in a few milliseconds (Plattner and Bachmann 1982; Gilkey and Staehelin 1986; Menco 1986). In simple terms, a sample is cryofixed by bringing it rapidly into contact with cryogens such as Freon or liquid propane or with a cooled metal (usually copper) surface that has a temperature of approximately $-180°C$ or lower. There are a variety of freezing techniques, all developed to achieve the fastest freezing speed and the greatest depth of good freezing possible (Plattner and Bachmann 1982; Gilkey and Staehelin 1986; Menco 1986). The depth of good freezing is the sample thickness that can be frozen without visible ice damage at the TEM level. The simplest freezing method is to plunge the samples into a cryogen manually. At the best, this method can yield good freezing of a sample thickness of 10–15 μm (e.g. Tiwari et al. 1984). A propane jet freezer is able to freeze up to 80 μm in the presence of appropriate cryoprotectants (Ding et al. 1991a). A high-pressure freezer can freeze a sample well up to 600 μm (Gilkey and Staehelin 1986; Dahl and Staehelin 1989).

Because of its ultrarapid and purely physical action, cryofixation is superior to chemical fixation in preserving cell structures close to their native state (Plattner and Bachmann 1982; Fernandez and Staehelin 1985; Gilkey and Staehelin 1986; Menco 1986; Staehelin and Chapman 1987). As shown in Fig. 2A and B, the plasma membrane in cryofixed and freeze-substituted plant materials is smooth and tightly appressed to the cell wall. Cryofixation is also the best approach to preserve the cytoskeleton, in particular the actin filaments which are labile and difficult to preserve by chemical fixation in plant cells (Fig. 2C; Tiwari et al. 1984; Lancelle et al. 1986, 1987; Tiwari and Polito 1988; Lichtscheidl et al. 1990; Ding et al. 1991a). Because of its distinct advantages

over chemical fixation in preserving dynamic cell structures, cryofixation has also been used in combination with other methods to process tissue samples to study the plasmodesmal substructure.

### 3.2.1 Freeze Fracture

In freeze fracture, a very sharp and cooled knife is used to cut the frozen tissue at −100°C. The frozen tissue is so brittle that the knife passage does not usually yield a clean-cut surface of the tissue; rather, the tissue fractures during knife passage. Because the hydrophobic membrane interior requires less energy to fracture than the cytoplasm, the membrane lipid bilayer is often split open during fracturing. Platinum/carbon is then evaporated onto the fractured tissue surface to form a replica, which essentially copies the topography of the surface. The replica, made free of cell debris, is examined directly in the TEM. Readers interested in the technique are referred to Bozzola and Russell (1992) for a general description of the technique and references cited therein for detailed information.

The freeze-fracture technique was used by Willison (1976) and Thomson and Platt-Aloia (1985) to study the plasmodesmal structure. Robards and Clarkson (1984) also made observations of plasmodesmata in freeze-fractured maize root cells. These studies revealed distinct particles as the plasmodesmal components. The main advantage of the technique is that the replica offers a three-dimensional view of cell structures. Such a view cannot be gained directly from thin sections, which produce only a two-dimensional view of any structures. In terms of gaining insight into the substructure of the plasmodesma, the freeze-fracture method has a number of drawbacks. First, the fracture plane occurs randomly and rarely exposes structures of interest. In particular, it is difficult to expose and visualize the internal structure of the plasmodesma. Although Thomson and Platt-Aloia (1985) presented some longitudinal views of freeze-fractured plasmodesmata, the resolution is very low. Second, the platinum particles evaporated onto the fractured tissue surfaces are approximately 2 nm in diameter, which is similar to the diameter of plasmodesmal particles (see Fig. 3 and discussion below), as revealed by TEM examination of thin sections. Thus, the details of the plasmodesmal structure may well be buried in a replica. Furthermore, when the evaporated platinum/carbon coats the fractured cell membranes, the cell structures are inevitably augmented in dimension in the replicas. For instance, the smallest cell structure that can be clearly identified in a replica above platinum particle background, such as an intramembrane protein particle, has a replica diameter of approximately 8–10 nm (Robards and Clarkson 1984; Bozolla and Russell 1992). Thus, when one observes a particle of 10 nm in a replica of a plasmodesmal structure, it is uncertain whether such a particle represents a 2-nm plasmodesmal particle augmented in the replica, or two or three closely spaced 2-nm plasmodesmal particles showing up as one big particle when coated with platinum/carbon. Despite these drawbacks, freeze fracture could be a useful technique in studying the plasmodesmal structure when data generated with this technique are corroborated by data obtained with other techniques.

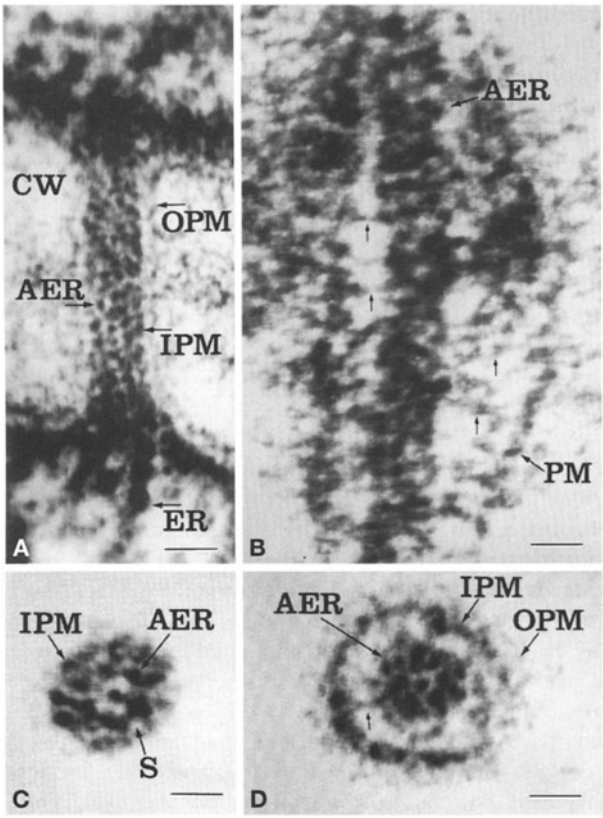


**Fig. 3 A–D.** Computer-enhanced high-resolution images of plasmodesmata in cryofixed/freeze-substituted tobacco leaves. **A** Longitudinal view of a newly formed primary plasmodesma between mesophyll cells. Electron-dense particles are embedded in the appressed endoplasmic reticulum (*AER*) and the inner and outer leaflets of the plasma membrane (*IPM* and *OPM*, respectively); *CW* cell wall; *ER* endoplasmic reticulum. *Bar* 18 nm. **B** A developing primary plasmodesma between phloem parenchyma cells, showing enlarged space between the plasma membrane (*PM*) and the AER that contains electron-dense "spoke-like" structures (*unlabelled arrows*). *Bar 13* nm. **C** Transverse view of a plasmodesma between phloem parenchyma cells. The spaces (S) between the electron dense particles of the AER and the IPM are presumably microchannels for intercellular transport. *Bar* 10 nm. **D** Oblique-transverse view of the middle portion of a developing primary plasmodesma between phloem parenchyma cells as shown in **B**. The *unlabelled arrows* indicate "spoke-like" structures interconnecting the *IPM* and *AER*. Because the section plane is oblique, several layers of electron-dense particles of the AER can be seen. *Bar* 12 nm (Ding et al. 1992b)

### 3.2.2 Freeze Substitution

An alternative approach to freeze fracture is freeze substitution, which has been shown to yield superior preservation of even the very labile plant cytoskeletal elements, microfilaments, in addition to other cellular components (Fig. 2; Tiwari et al. 1984; Lancelle et al. 1987; Tiwari and Polito 1988; Lichtscheidl et al. 1990; Ding et al.

1991a). In freeze substitution, the frozen samples are usually placed in acetone or ethanol at −90°C for a period of 1 to several days to allow substitution of ice by the dehydrant. The dehydrated samples are gradually brought to room temperature, infiltrated with and then embedded in plastic for obtaining thin sections. Because the cell structures are already physically stabilized by freezing, chemical fixatives such as glutaraldehyde and osmium tetroxide can be used in the substitution fluid to chemically stabilize the structures. This chemical fixation step presumably alters very little the cell structures.

Ding et al. (1992b) obtained high-resolution electron microscopic images of freeze-substituted tobacco plasmodesmata, which revealed that distinct particles (presumably proteinaceous) of 2–3 nm are embedded in both the plasma membrane and the AER membranes of the plasmodesma (Fig. 3). Spoke-like structures are found to extend from the AER to the plasma membrane in the central cavity region of a plasmodesma. The biochemical and functional nature of these extensions remains to be determined. The spaces between the plasma membrane and AER particles are thought to form microchannels for intercellular transport (Ding et al. 1992b; Botha et al. 1993). These microchannels may be tortuous (Ding et al. 1992b).

Thus far, thin sections of cryofixed tissues are probably the most suitable samples for high-resolution TEM studies of the plasmodesmal substructure. The main limitations are the same as with any thin sections. The images are two-dimensional and a section thickness of 60–70 nm imposes severe limitations on the resolution of the precise spatial arrangement of overlapping structures, such as the 2–3 nm particles, within the plasmodesma. The resolution can be partially improved by examining a section at various tilt angles in the microscope so that overlapping structures can be optically separated (Ding et al. 1992b), but it works only to a limited section depth.

In previous studies using the cryofixation method (Willison 1976; Thomson and Platt-Aloia 1985; Ding et al. 1992b), plant materials were cut into small pieces necessary to accommodate cryofixation. Thus, one cannot exclude the possibility that some artefacts may have already been introduced during the cutting procedure. Nevertheless, the superior structural preservation by cryofixation as compared to chemical fixation is widely recognized. The ultimate goal of preserving the plasmodesma in a completely native and undisturbed state is to find a plant system which can be cryofixed without cutting or pretreating the materials.

## 4 Elucidating the Molecular Structure of the Plasmodesma by Integrative Approaches

Recent studies have indicated that the cytoskeleton may interact with the plasmodesma. White et al. (1994) localized actin to the plasmodesma via immunolabelling. However, the labelling intensity is disappointingly low. If actin filaments are indeed present in the plasmodesma, then the low intensity of labelling could be explained in a number of ways. First, actin filaments, especially single filaments, are notoriously labile in plants and only the best cryofixation protocols can preserve them well (e.g. Tiwari et al. 1984; Lancelle et al. 1987; Lichtscheidl et al. 1990; Ding et al. 1991a, 1992c). Second, an actin filament has to be exposed, longitudinally, on the very surface of a tissue section for maximal access by antibodies. Any oblique orientation of an actin filament

with respect to the section surface will allow only partial labelling of the filament and thus make the labelling signal very low. Clearly, other methods need to be developed to improve the accessibility of actin filaments to antibodies in tissue sections. For example, samples may be embedded in diethylene glycol distearate (DGD; Capco et al. 1984; Nickerson et al. 1990) or polyethylene glycol (PEG; Wolosewick 1984). The advantage of this technique is that DGD and PEG can be removed from sections so that all cellular structures, including the cytoskeleton and plasmodesmata, are fully exposed to antibodies. Indeed, such sections have been shown to give superb immunolabelling of the cytoskeleton (Wolosewick et al. 1983; Wolosewick 1984; Nickerson et al. 1990). If necessary, soluble proteins may be extracted from sections to improve visualization of the cytoskeleton (Nickerson et al. 1990).

White et al. (1994) found that treatment of cells with cytochalasin B to disrupt actin filaments led to an enlargement of the diameter of the plasmodesma. Ding et al. (1996) showed that disruption of actin filaments with cytochalasin D or maize profilin (Staiger et al. 1994) increased the size exclusion limit (SEL) of plasmodesmata in tobacco mesophyll cells from a basal value of 1 kDa to at least 20 kDa, whereas treatment of these cells with phalloidin, a drug that stabilizes actin filaments, had no such effect. These results, taken together, suggest that actin filaments may control the permeability of plasmodesmata in tobacco mesophyll in some manner. It will be of great interest to determine whether or not the spoke-like structures as shown in Fig. 3B represent any cytoskeletal elements (i.e. actin or myosin?). The identity of those electron-dense particles is also of special interest.

Isolation and functional characterization of the biochemical components of plasmodesmata will be a major focus in elucidating the molecular structures of these intercellular organelles. There has been some progress made in this direction (e.g. Epel 1994; Epel et al. 1996; Waigmann et al. 1997; Ding 1998). In order to make further progress, molecular, biochemical, genetic and electron microscopic approaches need to be integrated. Immunolabelling will become a critical tool in this effort. The minute amount of some of the plasmodesmal proteins may render immunolabelling ineffective, especially when using thin sections, which limit immunodetection of antigens exposed on the section surface. When the labelling density is low, uncertainty arises as to whether the labelling truly represents the presence of antigen or is merely background labelling. Furthermore, indirect immunolabelling, where a gold-conjugated secondary antibody is used to detect the location of the primary antibody, may not be the ideal method to localize plasmodesmal proteins in some cases. The problem is that the antibodies are a few nanometers long (e.g. a 150 000 kDa-IgG molecule is approximately 8 nm long) and the observed location of an antibody does not necessarily correspond exactly to the location of the antigen. Such position shift is further amplified when a secondary antibody is used. Thus, the observed localization of a gold particle conjugated to the secondary antibody may be many nanometers away from the antigen location. While this may not be a serious problem in localizing antigens in most cases, it is a critical factor in evaluating the localization pattern of putative plasmodesmal proteins by indirect immunolabelling due to the minute size of the plasmodesma. When gold particles are observed in the cell wall areas immediately surrounding the plasmodesma, it becomes uncertain whether the antibody indeed decorates the plasmodesma or the cell walls. Thus, it may be desirable to use gold-conjugated primary antibodies alone so as to improve the spatial resolution of localization in such situations. Clearly,

innovative methods need to be developed for the detection or localization of plasmodesmal proteins.

## 5 Concluding Remarks

Our ultimate goal of elucidating the structure of the plasmodesma is to facilitate an understanding of its function in mediating cell-to-cell transport. Future studies in this direction will benefit from a wealth of new information that has been accumulated in recent years about the function of the plasmodesma. In particular, the discovery that the plasmodesma has the capacity to transport macromolecules (Mezitt and Lucas 1996; Ding 1998; Ding et al. 1999) provides a new incentive for research on the molecular architecture of this intercellular organelle. Pure TEM investigation, especially using chemically fixed samples, will probably yield limited new information on the plasmodesmal substructure. Cryofixation is clearly the choice of method at present to preserve samples for high resolution structural and immunolabelling studies. When integrated with molecular, biochemical and genetic approaches, TEM will be more powerful than ever.

It will be ideal to establish a plant system where multidisciplinary approaches are possible. Such a system should allow easy investigation of transport functions by such cell biological means as microinjection, structural analysis by cryofixation with minimal pretreatment, and biochemical isolation of plasmodesmal components. Furthermore, it should be amenable to genetic analysis of mutants defective in plasmodesmal transport functions. Once such a system is established, research on the molecular structure and function of plasmodesmata will be greatly facilitated.

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