DARWIN REVIEW

The cytoskeleton in plasmodesmata: a role in intercellular transport?

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Abstract

Actin and myosin are components of the plant cell cytoskeleton that extend from cell to cell through plasmodesmata (PD), but it is unclear how they are organized within the cytoplasmic sleeve or how they might behave as regulatory elements. Early work used antibodies to locate actin and myosin to PD, at the electron microscope level, or to pitfields (aggregations of PD in the cell wall), using immunofluorescence techniques. More recently, a green fluorescent protein (GFP)-tagged plant myosin VIII was located specifically at PD-rich pitfields in cell walls. Application of actin or myosin disrupters may modify the conformation of PD and alter rates of cell–cell transport, providing evidence for a role in regulating PD permeability. Intriguingly, there is now evidence of differentiation between types of PD, some of which open in response to both actin and myosin disrupters, and others which are unaffected by actin disrupters or which close in response to myosin inhibitors. Viruses also interact with elements of the cytoskeleton for both intracellular and intercellular transport. The precise function of the cytoskeleton in PD may change during cell development, and may not be identical in all tissue types, or even in all PD within a single cell. Nevertheless, it is likely that actin- and myosin-associated proteins play a key role in regulating cell–cell transport, by interacting with cargo and loading it into PD, and may underlie the capacity for one-way transport across particular cell and tissue boundaries.

Key words: Actin, cell–cell transport, cytoskeleton, myosin, plasmodesma.

The plant cytoskeleton

The plant cytoskeleton is a dynamic network of protein filaments that, together with protein motors and a wide range of accessory proteins that modify its behaviour, is involved in every aspect of cell growth and development. As in animal cells, one major function is to shuttle cargo within cells, a particularly important function in mature, vacuolate plant cells. Actin filaments, assembled from actin monomers, are responsible for this cytoplasmic streaming, and in the cortical regions of plant cells are extremely dynamic, with individual filaments mostly short lived (<30 s; Staiger et al., 2009). The second major component of the plant cytoskeleton, microtubules, are hollow cylinders assembled from αβ tubulin heterodimers which form actively organized arrays in the cell cortex (Shaw et al., 2003) whose primary function in interphase cells is to guide the direction of cellulose microfibril deposition in the cell wall (Paredes et al., 2006). Microtubules are also dynamic, with turnover half-times of 30–90 s in interphase plant cells (Hush et al., 1994; Shaw et al., 2003). Despite their stochastically dynamic behaviour, actin filaments and microtubules form tracks along which the motor proteins myosin and kinesin, respectively, traffic cellular cargo. Cytoskeleton organization and turnover are constantly modified by a highly diverse and still-expanding array of actin-binding proteins (ABPs) and microtubule-associated proteins, including nucleating, severing, end-binding, capping, or bundling proteins, as well as proteins that form cross-bridges within and between these filament systems (e.g. Wasteneys and Yang, 2000).
In addition to its primary role in intracellular transport, the cytoskeleton is implicated in intercellular transport of cytoplasmic molecules via plasmodesmata (PD), the membrane-lined channels linking plant cells across the cell wall (Fig. 1). Both actin and myosin have been immunolocalized to these intercellular channels (White et al., 1994; Blackman and Overall, 1998; Radford and White, 1998; Reichelt et al., 1999; Baluška et al., 2001a, 2004), and cytological evidence indicates a role for both in cell–cell transport. Interestingly, although there is no specific evidence for microtubules or tubulin within PD, both microtubules and actin filaments can direct certain cargo towards PD, a process that appears to be high-jacked by viruses during infection of plant cells (reviewed by Harries et al., 2010). Here, PD structure is described, then similar intercellular connections in other kingdoms are considered. The evidence for cytoskeletal proteins as components of PD is then reviewed in detail and their role in cell–cell transport is assessed (see also earlier reviews by Overall et al., 2000; Aaziz et al., 2001).

Fig. 1. Ultrastructure of plant plasmodesmata (PD), revealed by TEM analysis of intact tissues. (A) Longitudinal section showing a young Allium cepa root PD with electron-dense material (arrows) in the cell wall around the neck region (with kind permission from Springer Science+Business Media: Protoplasma Callose deposition at plasmodesmata. 201, 1998. 30–37 Radford JE, Vesk M, Overall RL. Fig. 4b). (B) Longitudinal section of a single PD, showing cytoplasmic endoplasmic reticulum (ER) narrowing into the desmotubule (DT; large arrowhead), which links the ER of two adjacent cells. The plasma membrane (PM) and cytoplasmic sleeve (small arrows) are also continuous from cell to cell. (courtesy of JE Radford, unpublished). (C) Diagram highlighting PD components, showing where cytoskeletal elements may fit into this structure. An alternative model is possible, with actin closest to the PM, and myosin attached to the DT. (D) Longitudinal sections of some PD show constrictions only at the neck region, with the PM widely separated from the DT in the region deep within the cell wall. Note electron-dense material (arrows) on the cytoplasmic side of the PM, and occasional strands (small arrow) connecting DT and PM. (Hordeum vulgare root, courtesy of JE Radford, unpublished). (E) Longitudinal sections of PD from high-pressure frozen and freeze-substituted tobacco leaf tissue showing open DT (arrowheads) with strands linking them to the PM (small arrows). Microtubules (MT) can be seen adjacent to the PM. (courtesy of MEA Schoenwaelder, unpublished). (F) Transverse section of tobacco leaf PD, showing particles possibly attached to or embedded in both desmotubule wall (DW) and plasma membrane (IPM, inner plasma membrane) and connected by strands across the cytoplasmic sleeve (with kind permission from Springer Science+Business Media: Protoplasma Substructure of freeze-substituted plasmodesmata. 169, 1992. 28–41. Ding B, Turgeon R, Parthasarathy MV. Fig. 4b). (G) Transverse section in which particles (arrowhead) are mainly associated with the DT, and strands (arrows) appear to link the DT and PM (double arrow). (Egeria densa leaf, JE Radford; reprinted from Trends in Plant Science 1, Overall RL, Blackman LM. A model of the macromolecular structure of plasmodesmata. 307–311, 1996, with permission from Elsevier). Bars=50 nm in A–E; 10 nm in F, G.
Tunnels linking cells—plasmodesmata

Plasmodesmata are minute cell wall channels connecting the plasma membrane and cytoplasm of neighbouring plant cells, and contain a central core of tightly compressed endoplasmic reticulum (ER), the desmotubule, which links the ER networks of most cells (Fig. 1A–C). Newly formed PD commonly have proteinaceous material at the neck (Fig. 1A) and a narrow channel between the ER and enclosing plasma membrane (Fig. 1A–C). In rapidly frozen and freeze-substituted tissue, this channel, and occasionally the desmotubule, may widen in places (Fig. 1D, E), with connections between the two membranes (Fig. 1D–G). Cytoplasmic components, including metabolites, signals, or developmental regulators such as transcription factors and RNA molecules (reviewed in Xu and Jackson, 2010), move from cell to cell through the small cylinder of cytoplasm between the plasma membrane and desmotubule, and this narrow space is where cytoskeletal components of PD are likely to be found. Cytoskeletal activity at the neck regions of PD may also direct and/or regulate movement of molecules into the cytoplasmic channel. While small cytoplasmic molecules [including the 27 kDa green fluorescent protein (GFP)] diffuse freely between many plant cells, transport of larger molecules is restricted and may require interaction with PD-specific proteins to facilitate movement (Roberts and Oparka, 2003). This size exclusion limit (SEL) is higher between cells in developing tissues and lower in more mature tissues (reviewed in Xu and Jackson, 2010), suggesting a greater degree of intercellular transport regulation for older PD. Deposition of callose in the cell wall at the neck regions of PD (Fig. 1A) can modify their SEL and ultimately close the cytoplasmic pathway. Actin and myosin are also implicated in regulating the SEL of PD, but the mechanics of this are as yet, unknown.

Small proteins in the ER lumen (Barton et al., 2011) and both lipids (Grabski et al. 1993; Martens et al., 2006) and transmembrane proteins in the ER membrane (Guenoune-Gelbart et al., 2008) can move between cells via the desmotubule. However, there appears to be no intercellular exchange of either lipid or protein within the plasma membrane through PD. As both actin and myosin are associated with other ER domains in plant cells, it is possible that they play a role in regulating intercellular transport via the desmotubule. They may also help to maintain the structure of the narrow cytoplasmic sleeve by forming links between the ER and plasma membranes via other PD proteins.

Tunnels linking cells—from Anabaena to animals

The lower members of the plant kingdom also show a range of PD-like connections (Cook et al., 1997; Cook and Graham, 1999; Raven, 1997, 2005), many of which have a central strand of ER surrounded by a connecting tube of plasma membrane. Certain groups of fungi form cell-like compartments linked by a diverse range of intercellular connections, a small subset of which are identical in structure to plant PD (Hawker et al., 1966; Markham, 1994; Lutzoni et al., 2004). Smaller and less complex connections, termed ‘microplasmodesmata’, are found between cells in cyanobacterial filaments (Lang and Fay, 1971; Giddings and Staehelin, 1981; Flores and Herrero, 2010).

In the animal kingdom, structures called tunnelling nanotubes (Rustom et al., 2004; Gerdes et al., 2007) are detected between cells in an ever-increasing range of tissues. As in plants, these intercellular connections are formed from narrow tubes of plasma membrane and generally contain a bundle of actin microfilaments. They allow direct transfer of macromolecules from cell to cell, including intercellular virus transfer, similar to PD (Sowinski et al., 2008). Tunnelling nanotubes also traffic lipid-bound organelles (Gurke et al., 2008), seen very rarely in PD (Fig. 4.11 in Waters, 2004), and they are routes for migration of plasma membrane lipids and proteins, which has not been detected in PD.

These cell–cell channels differ in evolutionary origin and composition, but converge on the same essential function, which is to facilitate the exchange of large signalling or information molecules, particularly nucleic acids, between cells while protecting their contents from exposure to the environment outside the cell membrane. It can be concluded, therefore, that direct cytoplasmic connections between neighbouring cells are essential to most multicellular organisms. So far, cytoskeletal elements have been detected only in PD and tunnelling nanotubes, but this may change with further investigation.

Cytoskeletal components of plasmodesmata

A number of different approaches have been used to locate cytoskeletal proteins in or near PD. Proteomic analysis of relatively pure cell wall fractions has identified a number of putative PD proteins (e.g. Epel, 1994; Epel et al., 1996; Blackman et al., 1998; Faulkner et al., 2005; reviewed in Faulkner and Maule, 2011), but cytoskeletal proteins are not prominent in PD-enriched cell fractions, although amino acid sequences with some similarity to animal tropomyosins have been identified (Faulkner et al., 2009). A more specific approach is to use antibodies against targeted cytoskeletal proteins in pull-down assays, on protein blots, or in immunolocalization studies.

Actin

Actin was first associated with PD in transmission electron micrographs by immunogold labelling (Fig. 2A, B, Table 1; White et al., 1994; Blackman and Overall, 1998) using a monoclonal antibody to chicken gizzard actin that recognizes an epitope (amino acids 23–28; McLean et al., 1990b) on the external surface of actin monomers and filaments (Lessard, 1988; McLean et al., 1990b; Lorenz
Fig. 2. Distribution of actin, myosin, and other cytoskeletal proteins by immunofluorescence and immunoelectron microscopy. In C, G, and H, PD are oriented horizontally on the page; in all other panels, PD are oriented vertically on the page. (A) ImmunoEM using an antibody to chicken gizzard actin shows sparse labelling of PD in *Hordeum vulgare* root tips (with kind permission from Springer Science+Business Media: *Protoplasma*, Actin associated with plasmodesmata, 180, 1994, 169–184. White RG, Badelt K, Overall RL, Vesk M. Fig. 2d). (B) *Chara corallina* PD are also labelled by the same antibody to chicken gizzard actin (from Blackman LM, Overall RL. 1998. Immunolocalisation of the cytoskeleton to plasmodesmata of *Chara corallina*. *The Plant Journal* 14, 733–741, with permission from Wiley-Blackwell) (C) Rhodamine–phalloidin labelling shows fluorescence at pitfields and in actin strands leading up to the pitfields in *H. vulgare* coleoptiles (with kind permission from Springer Science+Business Media: *Protoplasma*, Actin associated with plasmodesmata, 180, 1994, 169–184. White RG, Badelt K, Overall RL, Vesk M. Fig. 8b,c). (D) Occasionally, single filaments are detected crossing cell walls of *Nicotiana plumbaginifolia* suspension-cultured cells (with kind permission from Springer Science+Business Media:...
Table 1. Location of cytoskeletal proteins to cell plates, PD, and/or pitfields

<table>
<thead>
<tr>
<th>Cytoskeletal protein</th>
<th>Localization technique; antibody used; species tested</th>
<th>Reference</th>
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<tr>
<td>Actin</td>
<td>IEM; C4 anti-chicken gizzard actin; <em>Hordeum vulgare</em>, <em>Nepenthes exultata</em>, <em>Azolla pinnata</em> PD in roots and rhizomes</td>
<td>White et al. (1994)</td>
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<td></td>
<td>IEM; monoclonal C4 anti-chicken gizzard actin; <em>Chara corallina</em> PD</td>
<td>Blackman and Overall (1998)</td>
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<td></td>
<td>Fluorescent phalloidins; PD in <em>H. vulgare</em> coleoptiles, <em>Nicotiana plumbaginifolia</em> suspension cells</td>
<td>White et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>IF; monoclonal C4 anti-chicken gizzard actin; <em>C. corallina</em> PD</td>
<td>Blackman and Overall (1998)</td>
</tr>
<tr>
<td>Actin-related proteins</td>
<td>IF; anti-Dictyostelium discoideum Arp3; <em>Zea mays</em> pitfields</td>
<td>van Gestel et al. (2003)</td>
</tr>
<tr>
<td>Myosin</td>
<td>IEM; polyclonal anti-bovine smooth and skeletal; <em>Allium cepa</em>, <em>H. vulgare</em>, <em>Zea mays</em> root PD</td>
<td>Radford and White (1998)</td>
</tr>
<tr>
<td></td>
<td>IF; polyclonal anti-bovine smooth and skeletal; <em>Z. mays</em> myosin</td>
<td>Radford and White (1998)</td>
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<td></td>
<td>IEM; IF; monoclonal anti-mung bean myosin; <em>C. corallina</em> PD</td>
<td>Blackman and Overall (1998)</td>
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<td>IEM; IF; anti-mysin VIII; <em>Lepadium sativum</em> roots—cell plates</td>
<td>Reichelt et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>IF; <em>Z. mays</em> roots—cell plates</td>
<td>Reichelt et al. (1999)</td>
</tr>
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<td></td>
<td>IF; anti-175 kDa myosin from BY-2 cells; <em>Nicotiana tabacum</em> BY-2 cell plates</td>
<td>Yokota et al. (2009)</td>
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<td></td>
<td>FP; ATM1–GFP myosin in <em>N. tabacum</em> BY-2 cell plates</td>
<td>van Damme et al. (2004)</td>
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<td></td>
<td>FP; GFP–myosin VIII (ATM1) in pitfields of <em>Nicotiana benthamiana</em> leaf epidermis</td>
<td>Golomb et al. (2008)</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>IF; polyclonal anti-chicken gizzard tropomyosin; <em>C. corallina</em> PD, pitfields in <em>Arabidopsis</em> roots and <em>Allium porrum</em> leaf epidermis</td>
<td>Faulkner et al. (2009)</td>
</tr>
<tr>
<td>Centrin</td>
<td>IEM; IF; anti-Chlamydomonas centrin; roots and florets of <em>Brassica oleracea</em>, <em>A. cepa</em> roots—cell plates, PD, pitfields</td>
<td>Blackman et al. (1999)</td>
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</tbody>
</table>

IEM, immuno-EM: shows localization to individual PD; IF, immunofluorescence: labelled antibodies, labelled phalloidin show localization at pitfields; FP, GFP-tagging: shows localization at pitfields.

et al., 1993). More general localization of actin to PD clusters, or pitfields, was detected using fluorescent phalloidins [fungal toxins that bind specifically to filamentous actin (Barden et al., 1987; Cooper, 1987; White et al., 1994)], or by immunofluorescence, using an antibody to human actin (C4 clone; Baluśka et al., 2001a, 2004). However, it is curious that in many publications showing well-preserved, immunofluorescently labelled actin filaments there is no obvious labelling of pitfields, even in elongate cells with pronounced filaments (e.g. Collings and Wasteneys, 2005; Collings et al., 2006; Barton and Overall, 2010), or when pitfields are specifically examined (Chaffey and Barlow, 2001).

One reason for this may be that visualization of fluorescently tagged actin in PD pushes against current optical microscope resolution limits. In the first place, the sizes of individual PD, at 40–50 nm diameter and 100 nm long in young cell walls, and actin filaments, at 4–8 nm diameter, are well below the resolution limits of conventional fluorescence or confocal microscopes. Therefore, fluorescently labelled actin at or within PD may not be obvious unless the PD are clustered into pitfields. Secondly,
since individual actin filaments are difficult to preserve in plant cells using conventional aldehyde fixation treatments (Goodbody and Lloyd, 1990; Baskin et al., 1996; Collings et al., 2001; Meagher and Fechheimer, 2003; Barton and Overall, 2010) they may be reduced or absent from PD in aldehyde-fixed tissues. In some cases, immunolabelling protocols incorporate a cell wall digestion step, which may also disrupt pitfield actin. Thirdly, there are a number of different actin isoforms in plants, including eight in Arabidopsis (from 10 genes; reviewed in Meagher et al., 1999; Meagher and Fechheimer, 2003), and it is conceivable that actin in PD represents one or more specific isotypes in vegetative or reproductive tissues (cf. McLean et al., 1990a), so may not be recognized by antibodies raised against animal actins. Even when plant-specific antibodies to highly conserved epitopes are used, they may not recognize all actin isoforms (Kandasamy et al., 1999; Meagher et al., 1999; Meagher and Fechheimer, 2003). If the actin in PD is in an unusual conformation, the epitope on the filament surface may be hidden, and inaccessible for antibody binding. However, before abandoning this approach, it would be worthwhile to use plant- and isotype-specific antibodies in immunoelectron microscopy (EM) analyses to provide further information about the nature and location of actin filaments at PD, especially since there has been only one such study to date!

It is surprising that even with improved imaging technologies that push beyond the theoretical resolution limits, such as super-resolution microscopy or electron tomography (Fitzgibbon et al., 2010; Bell and Oparka, 2011), we are still ignorant as to how actin filaments physically associate with PD. Are they present at PD neck regions only, or do they traverse the length of the PD channel? If the latter, do they form a continuous cytoskeletal pathway between cells? Globular structures seen in the PD channel in transmission electron microscopy (TEM) images (Overall et al., 1982; Ding et al., 1992) appear similar to actin filaments in vitro (Ding et al., 1991; Maciver et al., 1991). Together with the immunolocalisation evidence above, this has given rise to models incorporating actin filaments within PD channels (e.g. White et al., 1994; Overall and Blackman, 1996; Overall et al., 2000; Faulkner and Maule, 2011; Tilsner et al., 2011), although some question whether any cytoskeletal elements could fit into the cytoplasmic sleeve (Bell and Oparka, 2011; Tilsner et al., 2011). Actin microfilaments are closely associated with cortical ER tubules (Quader et al., 1987; Lichtscheidt et al., 1990; Boevink et al., 1998; Runions et al., 2006) and microfilament removal disrupts the cortical ER network (Wright et al., 2007), indicating a role for actin in maintaining the ER network (Lichtscheidt and Weiss, 1988; Lichtscheidt et al., 1990; Boevink et al., 1998). Actin filaments may bind to, and help maintain, the ER-derived desmotubule within PD.

Alternatively, actin filaments may associate with the plasma membrane (Ding et al., 1991), even within PD, since it is unknown whether actin comprises the inner or outer electron-dense or electron-lucent protein-sized structures seen in TEM. Direct association of filamentous actin with membranes occurs when the actin interacts with lipids at specific sites (Gicquaud and Wong, 1994), and undergoes a major conformational change to form highly ordered arrays of actin filaments on the membrane surface (Gicquaud, 1993, 1995). These arrays partially penetrate the membrane surface (St-Onge and Gicquaud, 1990) and would appear similar to interpretations of the ER- and plasma membrane-associated proteins in PD (Ding et al., 1992; Botha et al., 1993). If actin (or other proteins) in PD were ‘recessed’ into one or other membrane, it would help explain how all of the putative PD proteins might fit into this tiny intermembrane space. A recent re-analysis of actin filament structure suggests that it is quite flat (Oda et al., 2009; Dominguez and Holmes, 2011), and in this conformation filaments would be more likely to fit into PD, especially at the neck. Occasionally, PD with very wide cytoplasmic sleeves (but narrower necks) can be seen, especially in freeze-substituted tissues (e.g. Dolzmann, 1965; Burgess, 1971; Tinline et al., 1991; Ding et al., 1992; Fig. 1b in Overall et al., 2000), and here, perhaps, the actin is not constrained to either membrane. At present, there are no convincing data either way to indicate how actin is arranged in PD.

Detection of fluorescently tagged actin within living PD would provide more substantial evidence for its location there, but development of stable lines expressing any plant actin isoform tagged with a fluorescent protein has proven intractable. Introduction of readily available derivatized animal actins has not solved this problem, as Oregon green-tagged animal injected into plant cells becomes aggregated and does not integrate with plant actin filaments (Jing et al., 2003). Instead, stable Arabidopsis lines expressing fluorescent protein-tagged actin-associated proteins, such as fimbrin (Kovar et al., 2001; Wang et al., 2004), actin-binding domain protein (McCurdy et al., 2001), mouse talin (Kost et al., 1998), human talin (Takemoto et al., 2003), or LIM-domain proteins (Papuga et al., 2010), all highlight actin filaments and filament bundles within cells. Interestingly, in none of these lines are there specific PD- or pitfield-associated fluorescent structures, implying that if actin is located there, it may not be in a standard filamentous form with side-binding-associated proteins. Currently there is no evidence for intercellular movement of actin or fluorescently tagged ABPs between plant cells, although rhodamine-phalloidin, and other fluorescently tagged variants of these small actin-binding peptides, can spread into neighbouring cells following injection ( Cleary, 1995; Molchan et al., 2002).

A role for actin in plasmodesmal function?

With the assumption that cytoskeletal proteins in PD will be regulated similarly to the cytoskeleton elsewhere in the cell, a range of actin-disrupting or actin-stabilizing drugs have been used to identify a role for actin filaments in regulating PD transport between cells. Effects on transport are measured by observing effects on cell–cell transmission of cytoplasmic signals, such as electrical currents or
fluorescent probes of different sizes. The time for the signal to move into neighbouring cells, or the number of cells traversed within a certain time, is compared following different treatments and assumed to indicate the permeability of the PD cytoplasmic sleeve. Co-injecting the actin disrupter, cytochalasin D, with a fluorescent 10 kDa dextran into *Nicotiana tabacum* (Ding et al., 1996; Table 2) or *Nicotiana benthamiana* (Su et al., 2010) mesophyll cells substantially increased cell–cell transport of the dextran. Conversely, co-injecting the actin-stabilizing fungal toxin, phalloidin, with the dextran almost completely prevented its movement. This suggests that actin filament dynamics may play a role in regulating the pore size of PD between these cells and, when filaments are disrupted, larger molecules can move from cell to cell. When phalloidin favours filament assembly and bundling over disassembly, perhaps the PD pore is physically blocked, or binding of regulatory proteins is perturbed, preventing transport. Supporting these findings, treatment of *Nephrolepis exaltata* root tips with cytochalasin D induced widening of PD neck regions (Fig.

### Table 2. Effects of cytoskeleton disrupters on cell–cell transport

<table>
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<tr>
<th>Cytoskeletal inhibitor</th>
<th>Effects on PD or transport; specific tissue tested</th>
<th>Detection method</th>
<th>Reference</th>
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<td>Actin filament disrupters; cytochalasin B or D; actin polymerization inhibitors; latrunculin A or B</td>
<td>Widening of neck region; <em>Nephrolepis exaltata</em> rhizomes</td>
<td>TEM analysis</td>
<td>White et al. (1994)</td>
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<tr>
<td></td>
<td>Increased transport, <em>Nicotiana tabacum</em> mesophyll</td>
<td>Dye transport</td>
<td>Ding et al. (1996)</td>
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<tr>
<td></td>
<td>Increased transport; <em>Nicotiana benthamiana</em> mesophyll</td>
<td>Dye transport</td>
<td>Su et al. (2010)</td>
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<td></td>
<td>No effect; <em>Setcreasea purpurea</em> stamen hairs</td>
<td>Dye transport</td>
<td>Tucker (1987)</td>
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<tr>
<td></td>
<td>No effect on structure; * Hordeum vulgare* roots, <em>Azolla pinnata</em> roots</td>
<td>TEM analysis</td>
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<td></td>
<td>No effect; <em>N. tabacum</em> leaf trichomes</td>
<td>Dye transport</td>
<td>Christensen et al. (2000)</td>
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<tr>
<td></td>
<td>No effect; <em>Tradescantia virginiana</em> stamen hairs</td>
<td>Dye transport</td>
<td>Radford and White (2011)</td>
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<tr>
<td></td>
<td>No effect; <em>Arabidopsis</em> root tissues</td>
<td>Dye transport, GFP transport</td>
<td>RG White et al. (unpublished)</td>
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<td></td>
<td>No effect; <em>Egenia densa</em> leaf tissues</td>
<td>Dye transport</td>
<td>Enwee and Goodwin (1983)</td>
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<td>Reduced transport due to reduced cytoplasmic streaming; <em>Chara sp.</em></td>
<td>Chloride transport</td>
<td>Bostrom and Walker (1976)</td>
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<td></td>
<td>Reduced transport; <em>N. tabacum</em> mesophyll</td>
<td>Virus transport</td>
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<td>Increase or decrease depending on initial conductance; <em>Salvinia auriculata</em> trichomes</td>
<td>Cell–cell electrical conductance</td>
<td>Krasavina et al. (2001)</td>
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<td>Profilin; actin-sequestering protein</td>
<td>Increased transport; <em>N. tabacum</em> mesophyll</td>
<td>Dye transport</td>
<td>Ding et al. (1996)</td>
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<td>VIGS against actin</td>
<td>Increased transport; <em>N. benthamiana</em> mesophyll</td>
<td>Virus transport</td>
<td>Liu et al. (2005)</td>
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<td>Actin filament stabilizers; phalloidins</td>
<td>Reduced transport; <em>N. tabacum</em> mesophyll</td>
<td>Dye transport</td>
<td>Ding et al. (1996)</td>
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<td></td>
<td>Reduced transport; <em>N. benthamiana</em> mesophyll</td>
<td>Dye transport in MP-expressing tissue</td>
<td>Su et al. (2010)</td>
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<td>BDM, myosin inhibitor</td>
<td>Narrowing of neck region; <em>Zea mays</em> roots</td>
<td>TEM analysis</td>
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<td></td>
<td>Reduced transport; <em>Arabidopsis</em> root tissues, <em>Eloeda canadensis</em> leaf epidermis</td>
<td>Dye transport, GFP transport</td>
<td>RG White et al. (unpublished)</td>
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<td>No effect on transport; <em>N. tabacum</em> mesophyll</td>
<td>Virus transport</td>
<td>Kawakami et al. (2004)</td>
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<td>Increased transport; <em>T. virginiana</em> stamen hairs</td>
<td>Dye transport</td>
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<td></td>
<td>Reduced transport; <em>Nitella translucens</em></td>
<td>$^{11}$C and $^{14}$C transport</td>
<td>Dale et al. (1983)</td>
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<td>Reduced transport; <em>T. virginiana</em> stamen hairs</td>
<td>Dye transport</td>
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<tr>
<td>NEM, myosin inhibitor</td>
<td>Increased transport; <em>Arabidopsis</em> root tissues, <em>E. canadensis</em> leaf epidermis</td>
<td>Dye transport, GFP transport</td>
<td>RG White et al. (unpublished)</td>
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<tr>
<td>Myosin antibodies</td>
<td>Increased transport; <em>Arabidopsis</em> root epidermis, <em>N. tabacum</em> mesophyll</td>
<td>Dye transport</td>
<td>Volkmann et al. (2003)</td>
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<td></td>
<td>Increased transport; <em>T. virginiana</em> stamen hairs</td>
<td>Dye transport</td>
<td>Radford and White (2011)</td>
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<td></td>
<td>Reduced transport; <em>Arabidopsis</em> root epidermis</td>
<td>Dye transport</td>
<td>RG White et al. (unpublished)</td>
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Actin-binding proteins

It is puzzling that despite the large number of ABPs in plants, few have been localized to PD. While this could be partly due to the difficulty in preserving and immunolabelling actin in plants, it is also possible that PD-specific actin-associated proteins may not be detected by currently available antibodies, or by protein isolation or fluorescent tagging techniques, leading to the impression of absence. Nevertheless, it can be speculated that actin-depolymerizing factors or proteins such as villins that sever actin filaments (Khurana et al., 2003) could be important in regulating intercellular traffic, if located at PD. One ABP localized to pitfields is the actin-related protein Arp3 (van Gestel et al., 2003). The Arp2/3 complex nucleates actin filaments (Deeks and Hussey, 2005; Fiserová et al., 2006) when activated by SCAR/WAVE (Zhang et al., 2008), and its presence in PD may indicate a role for actin filament turnover in their dynamic regulation. Since virus MPs are located at PD and show actin-severing capacity only in this location, there may be additional endogenous protein(s) with similar functions at PD. Research into actin-binding proteins is at an early stage, and further investigation may identify specific associations with PD, especially with recent discoveries of a range of new proteins involved in actin nucleation (e.g. Campellone and Welch, 2010).

Plant myosins

The motor protein, myosin, is the most prominent ABP found in PD. Of the 24 recognized types of myosin found in eukaryotes, the four myosins VIII and 13 myosins XI are found in plants, with myosin XIII found only in Acetabularia (Mooseker and Foth, 2008). They are multicomponent complexes identified by their heavy chains, which comprise
an actin-binding head, hinged neck, and variable length, cargo-binding tail domain. These heavy chains are usually accompanied by regulatory calmodulin-like myosin light chains. The myosin head binds actin strongly in the absence of ATP (rigor state), whereas ATP releases myosin from the actin filament. The neck undergoes an immediate conformational change, the recovery stroke, and upon binding to actin several monomers further along the filament, the bound ATP is immediately hydrolysed, enabling somewhat tighter binding. Following the release of ADP, release of Pi causes the now tightly bound myosin head to undergo a conformational change, the power stroke, which moves its cargo relative to the actin filament, and the cycle begins again (see Spudich, 2001; Holmes, 2008).

Like actin, myosin was first localized to PD with immuno-EM using polyclonal antibodies to animal myosins (Fig. 2E, F; Table 1; Blackman and Overall, 1998; Radford and White, 1998), which recognize highly conserved motifs in the myosin head, as well as an antibody to the C-terminal tail of plant myosin VIII (Reichelt et al., 1999). Immunofluorescence analyses with the same antibodies showed labelling of individual, multilobed PD (Blackman et al., 1998), pitfields (Radford and White, 1998; Reichelt et al., 1999; Baluška et al., 2001a, 2004) and occasionally fine strands crossing young cell walls (Fig. 2G; Radford and White, 1998).

Apart from these earlier studies, there have been no further analyses of myosin (or actin) in or near PD at the EM level. There is also a lack of any analysis of changes in the location of myosin or other proteins as PD change their structure from an initially simple pore to more complex synapses linking neighbouring cells. With the wider availability of plant myosin-specific antibodies, it would now be useful to identify whether myosin is located more towards the neck region or deeper in the cytoplasmic sleeve, and confirm, if possible, co-localization with actin. Other large, multicomponent structures which regulate traffic across double-membrane boundaries, such as the nuclear pore complex, undergo substantial changes in composition during cell development (Doucet et al., 2010), and there are distinct differences in pore protein isoform composition between cell types (Meier and Brkljacic, 2010). It remains to be elucidated whether specific myosins are components of PD at different developmental stages, and whether they are also restricted to certain cell or tissue types.

Fluorescence analysis of living cells has expanded myosin analysis, and several groups have used GFP fusions to parts of the myosin protein to localize myosin in both transient and stably expressing Arabidopsis and Nicotiana lines. These analyses show that none of the fluorescent protein-tagged myosin XI tail regions localizes to PD (Reisen and Hanson, 2007; Walter and Holweg, 2008; Avisar et al., 2009; Sattarzadeh et al., 2009), and it seems likely that no myosin XI plays a direct role as an internal component of PD. One of two known myosin XI isotypes is strongly localized to the coalescing cell plate in tobacco BY2 cells and appears critical for localization of the ER there, suggesting a key role in bringing and holding the trapped ER in place between cell plate vesicles, although it is not localized to PD in mature cell walls (Yokota et al., 2009). Intriguingly, activity of the tobacco myosin XI, and not myosin VIII, appears essential for ER tubule formation (Yokota et al., 2011); perhaps one role is tightening the desmotubule in nascent PD. Of the Arabidopsis myosin VIII family, one GFP fusion with the IQ-tail zone of ATM1 appears to localize to sites of ER attachment as well as pitfields when expressed in N. benthamiana leaves (Golomb et al., 2008). Furthermore, overexpression of the tail regions, including the IQ motif, of three orthologues of Arabidopsis myosin VIII sequences from N. benthamiana prevented PD localization of a closterovirus hsp70 homologue, a viral protein essential for cell-cell virus movement, whereas overexpression of myosin XI tails had no effect (Avisar et al., 2008). However, when transiently expressed as tail–GFP fusions in N. tabacum, three of the four Arabidopsis myosin VIII family were detected at the plasma membrane, with the fourth located in the nucleus (Avisar et al., 2009).

The actin-binding myosin head may be required to locate the myosin correctly, if located at PD, or indeed anywhere else in the cell. For example, a class I myosin requires the head domain and correct conformation of the tail domain for localization to actin-enriched membrane projections in animal cells (Komaba and Coluccio, 2010). Furthermore, free GFP or GFP-tagged myosin can impair actin–myosin interactions in highly excitable muscle cells (Agbulut et al., 2006, 2007; Nishimura et al., 2006; Sekar et al., 2007) where actomyosin interactions occur in highly structured tissues. Although there is no evidence that GFP–protein fusions cause similar problems in other cell types, it is possible that in the tightly packed PD neck region, the GFP–actomyosin interaction may affect localization of the GFP–myosin tail fusions. As for analysis of the role of actin, local cell or tissue silencing would be useful to confirm roles for specific myosins in regulation of PD.

Tropomyosin

Of particular interest is the recent localization of a tropomyosin-like protein in PD, and identification of a putative endogenous plant tropomyosin in Chara and Arabidopsis (Fig. 2H; Faulkner et al., 2009). In animals and yeast, tropomyosin forms head–tail dimers that bind along, and stabilize, actin filament structure and regulate its activity and interaction with other ABPs, including myosin (Meagher and Fehheimer, 2003; Gunning et al., 2008). Tropomyosin research in plants has lagged because tropomyosins are almost entirely α-helical proteins and BLAST homology searches cannot identify specific tropomyosin sequences (Pruyne, 2008; Gardiner et al., 2011). The sequence similarities identified in plant genome databases tend to be short and are often to coiled-coil regions of diverse other proteins (Meagher and Fehheimer, 2003; Staiger and Hussey, 2004; Gunning, 2008), so there is residual doubt that plants contain a true tropomyosin homologue. However, since a major function of tropomyosin is to modulate the interaction between the motor...
domain of the myosin head with filamentous actin (Perry, 2001; Gunning, 2008; Gunning et al., 2008), it is a puzzle how plant myosins function in the absence of actin-bound tropomyosin (and associated proteins such as troponins) which provide calcium-regulated access to the actin filament. A different approach is needed; for example, yeast tropomyosin was only identified through biochemical purification (Liu and Bretscher, 1989; reviewed in Pruyne, 2008), which may yet be a pathway for identification of plant tropomyosins. Recently, Gardiner et al. (2011) identified two putative Arabidopsis tropomyosin homologues using an algorithm that predicts protein homology. It will be of interest to see whether these localize along actin filaments or to PD in vivo.

In PD, a tropomyosin-like protein could stabilize actin against turnover by agents that normally lead to disassembly of the highly dynamic cortical actin cytoskeleton (Wachstock et al., 1994; Staiger et al., 2009), or regulate actin–myosin interactions there. PD are quite resistant to proteases and detergents (Tilney et al., 1991; Turner et al., 1994), and additional cytoskeleton-binding proteins may underlie this stability. Tropomyosin is not indispensable for myosin ‘walking’ along actin filaments or for movement of (non-tropomyosin) stabilized actin filaments on a bed of anchored myosin heads, but, without tropomyosin, the sliding velocity is much lower (Higashi-Fujime and Nakamura, 2009). Perhaps tropomyosin in PD is a stabilizing agent, not an accelerator. In animal epithelial cells, actin filaments in lamellipodia lack tropomyosin and are highly dynamic, whereas the more stable filament bundles further from the leading edges of cells, in the lamella, are stabilized with tropomyosin (Gupton et al., 2005). Tropomyosin is also concentrated along the very robust peripheral actin filament bundles in Chara cells (Faulkner et al., 2009), which are locations requiring stable actin bundles as well as high myosin activity. Arp2/3 binds more readily to actin filaments lacking tropomyosin (van Gestel et al., 2003; Gupton et al., 2005), and is found at the PD neck, but more sparsely (if at all) down the length of PD (van Gestel et al., 2003), providing some indirect support for tropomyosin-stabilized actin within PD but not more generally in the cytoplasm.

Myosin function in plasmodesmata

The role of myosin in PD has been less well characterized, and, as with actin, the effects of chemical inhibitors vary with the tissue examined. Inhibitors of myosin function generally arrest myosin either attached to actin in the rigor conformation, or detached from actin and unable to hydrolyse ATP prior to reattachment or the power stroke (Spudich, 2001; Holmes, 2008). If myosin inhibitors interfere with actomyosin-based activities, such as cytoplasmic streaming, they are generally assumed to inhibit all other processes relying on actomyosin dynamics.

Two drugs commonly used to inhibit myosin function are 2,3-butanedione monoxime (BDM) and N-ethylmaleimide (NEM). BDM blocks myosin ATPase activity, such that the myosin head is bound loosely to actin, and NEM binds to a conserved cysteine residue near the myosin head, altering the ATP-binding site, which prevents ATP binding and prevents myosin detachment from actin after the power stroke (Kohama et al., 1987, Patel et al., 1998). Although BDM may not affect actomyosin activity in some plant (McCurdy, 1999; Forer and Fabian, 2005) or animal (e.g. Coluccio, 2008) tissues, the consensus is that both inhibitors are useful probes of myosin function in plants (Tominaga et al., 2000; Molchan et al., 2002; Funaki et al., 2004; Forer and Fabian, 2005).

BDM induced constrictions in the neck region of Zea mays and Hordeum vulgare PD (Fig. 2J; Radford and White, 1998); it also reduced intercellular transport of TMV replication complexes in tobacco mesophyll tissue (Kawakami et al., 2004), and transport of fluorescent tracers in Arabidopsis root cells (RG White, MJ Talbot, JE Yarnold, JE Radford, unpublished results). The implication is that constriction of the PD neck results in reduced transport, yet myosin is found all along the cytoplasmic sleeve, not solely at the neck, suggesting that myosin is only active in this specific zone in PD. Furthermore, in animal tissues, BDM detaches the myosin II (Packer et al., 1988; Higuchi and Takemori, 1989; Bagnti et al., 1992; Herrmann et al., 1992; Steele and Smith, 1993; Backx et al., 1994; Hajjar et al., 1994; Zhao et al., 1995) and V (Cramer and Mitchison, 1995) heads from actin, suggesting that disengagement of actin and myosin, each putatively on different PD membranes, causes PD closure. PD also open when ATP levels are reduced (Tucker, 1993; Cleland et al., 1994; Wright and Oparka, 1997) and close in the presence of additional ATP (Radford and White, 2011), which would produce either firmly attached or detached myosin, respectively, supporting the idea that detached myosin correlates with closed PD. If the default status of PD is open, and reduced SEL or complete closure requires energy, then perhaps myosin forms ‘struts’ between the ER and plasma membrane (one of which is lined with actin), which are no longer active if myosin is detached.

The PD-located plant myosin VIII is more specifically inhibited by injection of antibodies to its unique coiled-coil tail region, which increased transport between Arabidopsis root epidermis or tobacco mesophyll cells (Volkmann et al., 2003). Similarly, injection of polyclonal anti-myosin antibodies, in this case presumably interfering with a conserved site on the myosin head, also increased cell–cell transport between T. virginiana stamen hairs (Radford and White, 2011). However, when the same antibody was injected into Arabidopsis root epidermal cells, PD transport was reduced (RG White, MJ Talbot, JE Yarnold, JE Radford, unpublished results). Furthermore, BDM treatment increased and NEM reduced cell–cell transport in T. virginiana (Radford and White, 2011), as seen previously in Nitella (Dale et al., 1983), but opposite to Z. mays or H. vulgare (Radford and White, 1998), again suggesting that interfering with the head and tail regions can produce different results, and that cell- and tissue-specific factors regulate PD. It would be profitable to screen other small molecules for
their potential to inhibit specific plant myosins, as has been done for skeletal myosin II (Cheung et al., 2002).

Myosin regulation of plasmodesmatal actin

A final consideration is that myosin inhibitors may indirectly cause changes in cell–cell transport by affecting other aspects of cell physiology. For example, both NEM (Karlsson and Lindberg, 1985; Menzel and Elsner-Menzel, 1989; Liebe and Quader, 1994; Quader and Liebe, 1995) and BDM (Samaj et al., 2000; Holweg et al., 2003) have been shown to cause fragmentation, re-arrangement, and bundling of actin, although 30 mM BDM, while inhibiting cytoplasmic streaming, did not affect actin filaments in T. virginiana stamen hairs (Molchan et al., 2002; Radford and White, 2011). When muscle myosin II is bound to actin the myosin head interacts strongly with regions on two adjacent actin monomers (Milligan, 1996). This strong interaction can stabilize the actin filament and even induce the polymerization of actin monomers into filaments (Carlier et al., 1994). Thus disruption of the actin–myosin interaction with chemical treatments or antibody injection could disrupt the actin filaments within PD (White et al., 1994), which could then affect cell–cell transport (Ding et al., 1996), at least in expanding Arabidopsis root cells. Yarrow et al. (2003) found that BDM blocked actin incorporation into sites of rapid actin turnover in cultured animal cells, by delocalizing actin-polymerizing proteins at the cell membrane. Myosin I proteins interact directly with the Arp2/3 complex in yeast, Dictyostelium, and other species to promote actin polymerization (Ridley, 2000), and this interaction is disrupted by BDM (Yarrow et al., 2003). If specific sites of rapid actin turnover in plant cells also depend upon a suite of plasma membrane-associated, BDM-sensitive polymerization factors, and such active sites include PD, then BDM may have a secondary effect on PD through this mechanism.

Related cytoskeletal proteins; centrin, calmodulin, tubulin

Antibodies to the contractile protein, centrin, label developing cell plates (Del Vecchio et al., 1997; Blackman et al., 1999; Harper et al., 2000) and the neck region of PD (Fig. 2K; Blackman et al., 1999), leading to speculation that a centrin-like protein may link the ER and plasma membrane. Centrins decorate repeated sites on centrin-binding proteins, and initiate twisting and contraction of these long proteins when they bind calcium and change their conformation (Salisbury, 2004; Gogendeau et al., 2007). Centrins are also components of the nuclear pore complex in yeast (Rout et al., 2000) and vertebrates (Resendes et al., 2008), where they play an essential role in export of mRNA and protein. Nuclear pores are useful comparisons with PD, as they have similar features, particularly the regulated transport of macromolecules through a pore which traverses a double membrane. Perhaps, similar to the actin-severing role of some virus MPs, centrin may act in the regulation and turnover of PD proteins at the neck region, similar to its role in yeast (Chen and Madura, 2008).

Since changes in calcium levels clearly regulate PD permeability, it is surprising that other calcium-binding regulatory proteins, such as calmodulin, are not localized there (Blackman and Overall, 1998), and this absence suggests that the calmodulin-like myosin light chains may not regulate rapid myosin dynamics at PD. Antibodies to tubulin label PD rather sparsely, only slightly more than in the cytoplasm (Blackman and Overall, 1998), and treatment with a microtubule inhibitor, colchicine, had little effect on cell–cell conductivity in Trianea bogotensis roots or S. auriculata trichomes (Krasavina et al., 2001). Microtubules can deposit cargo, especially viruses, at PD (Harries et al., 2009b), but they do not seem to continue into the cytoplasmic channel. Other viruses traffic along actin filaments within cells (Harries et al., 2009b, 2010). Perhaps the common element is myosin, since some myosins, for example myosin V, can bind to either actin filaments or microtubules, and while the true motor function operates only with actin, this myosin can slide for some distance along microtubules (see Moosiker and Foth, 2008). Further investigation may reveal a similar dual function in one or more of the plant myosins XI or VIII, which share features in common with myosin V (Yamamoto, 2008).

Cytoskeleton organization in and regulation of plasmodesmata

Ultrastructural elements seen in and around PD, revealed by TEM staining and thought to be individual proteins, suggest a spiral arrangement of single filament(s) around the desmotubule (White et al., 1994; Overall and Blackman, 1996; Blackman et al., 1999; Overall, 1999; Benitez-Alfonso, 2010), although supporting evidence is sparse. In comparison, it was thought that helically arranged elements decorating microtubules in developing photoreceptor cells were actin derived (Obata and Usukura, 1992), but this is probably centrin decoration (Wolfrum, 1995; Trojan et al., 2008), with the actin filaments playing a role adjacent to the centriole-derived microtubules (Obata and Usukura, 1992; Trojan et al., 2008). In any event, it is difficult to envisage how myosin could traffic along actin filaments in the confined space of the cytoplasmic sleeve, especially at the neck region.

Most plant actin is highly dynamic (Staiger et al., 2009), and actin filaments are probably not stiff enough to shape the desmotubule by themselves (Kole et al., 2004, 2005; Gupton et al., 2005). In bacteria, MreB and Mbl are two of several bacterial homologues of actin which form spiral structures on the inner surface of the bacterial inner membrane, and are essential for maintenance of cell shape and assembly of the bacterial peptidoglycan matrix (Gupton et al., 2005; Divakaruni et al., 2007; White et al., 2010). MreB filaments form thick, spiral bundles that require auxiliary cross-linking factors to reinforce their stiff cortex-like structure and to anchor these assemblies to the inner
membrane (Esue et al., 2005; White et al., 2010). If plant actin is similarly stabilized and associated with a membrane, association with the plasma membrane rather than the desmotubule would produce a looser actin spiral, similar to those seen in bacteria.

However, instead of actin monomers or filaments, the particles associated with the desmotubule may be myosin heads, arranged in a spiral as they are in muscle, with the heads pointing outwards to actin lining the plasma membrane. Myosin heads may interact with plasma membrane-bound actin only at the PD neck, whereas, through the cytoplasmic sleeve, myosin may interact with actin or actin-like proteins on certain specific types of cargo. In any event, further high resolution analyses of PD ultrastructure are clearly needed to reveal the position of actin filaments and myosin in PD, especially at the neck where cargo transfers from the cytoplasmic to the PD cytoskeleton.

Conclusions and prospects

Exploration of the role of cytoskeletal proteins in PD structure and regulation has only just begun, and future progress will depend on targeting these proteins in specific cell types, rather than using general inhibitors. For example, injection of RNAi to actin (cf. VIGS; Liu et al., 2005) or myosin could be very informative, together with cell-targeted inducible expression of RNAi, and complementation analyses (e.g. Abu-Abied et al., 2006). Perhaps then we will be able to answer the questions raised here and shed a brighter and clearer light on the intercellular cytoskeleton in plants.

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References


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