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SHORT COMMUNICATION

Evidence that actin filaments are involved in controlling the permeability of plasmodesmata in tobacco mesophyll

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Summary

The role of actin filaments in regulating plasmodesmal transport has been studied by microinjection experiments in mesophyll cells of tobacco (*Nicotiana tabacum* L. cv. Samsun). When fluorescent dextrans of various molecular sizes were each co-injected with specific actin filament perturbants cytochalasin D (CD) or profilin into these cells, dextrans up to 20 kilodalton (kDa) moved from the injected cell into surrounding cells within 3–5 min. In contrast, when such dextrans were injected alone or co-injected with phalloidin into the mesophyll cells, they remained in the injected cells. Phalloidin co-injection slowed down or even inhibited CD- or profilin-elicited dextran cell-to-cell movement. Dextrans of 40 kDa or larger were unable to move out of the injected cell in the presence of CD or profilin. These data suggest that actin filaments may participate in the regulation of plasmodesmal transport by controlling the permeability of plasmodesmata.

Introduction

Plasmodesmata are the intercellular organelles that provide the major pathways for direct cell-to-cell communication in plants (reviewed in Beebe and Turgeon, 1991; Ding and Lucas, 1996; Gunning and Robards, 1976; Gunning and Overall, 1983; Lucas and Wolf, 1993; Lucas *et al.*, 1993; Oparka, 1993; Robards and Lucas, 1990). Structurally, each plasmodesma represents a plasma membrane-lined cylindrical pore across cell walls, with appressed endoplasmic reticulum (ER) in the center of the pore. Proteinaceous particles are embedded in both the plasma membrane and the appressed ER membrane (Ding *et al.*, 1992b; Tilney *et al.*, 1991), and the spaces formed between these proteinaceous particles constitute the major transport channels within the plasmodesma. These channels establish a basal size exclusion limit (SEL) of 1 kilodalton (kDa) for cell-to-cell diffusion of molecules (e.g. Goodwin, 1983; Terry and Robards, 1987; Tucker, 1982; Wolf *et al.*, 1989). This SEL, however, can be down- or up-regulated.

Several factors have been shown to be able to down-regulate the plasmodesmal SEL. These include elevated levels of free Ca^{2+} (Erwee and Goodwin, 1983; Tucker, 1990), inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3) (Tucker, 1988), and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (Baron-Epel *et al.*, 1988), formation of callose around the orifice of the plasmodesma (Wolf *et al.*, 1991), and a pressure gradient, of at least 200 kPa, between neighboring cells (Oparka and Prior, 1992).

Virus–plasmodesma interactions provide the best examples for the up-regulation of the plasmodesmal SEL. The genomes of many plant viruses encode a specific protein, called movement protein (MP), that is indispensable for viral cell-to-cell movement during infection (Atabekov and Taliansky, 1990; Deom *et al.*, 1992). The MPs of several viruses have been shown to be able to increase the SEL of plasmodesmata to larger than 10 kDa (Derrick *et al.*, 1992; Ding *et al.*, 1995; Fujiwara *et al.*, 1993; Noueiry *et al.*, 1994; Poirson *et al.*, 1993; Vaquero *et al.*, 1994; Waigmann *et al.*, 1994; Wolf *et al.*, 1989). In addition, the SEL of plasmodesmata can be up-regulated by azide treatment (Cleland *et al.*, 1994; Tucker, 1993) and by anaerobic stress (Cleland *et al.*, 1994) to between 5 and 10 kDa (Cleland *et al.*, 1994).

Despite the impressive repertoire of factors that have been shown to be able to regulate the permeability of plasmodesmata, the cellular basis of such regulations remains largely unknown. An important question to be answered is what cellular factors physically control the permeability of plasmodesmata?

Recently, White *et al.* (1994) presented immunolabeling images showing that actin was localized to plasmodesmata. The density of labeling, however, is too low to be considered conclusive evidence. Nevertheless, White *et al.* (1994) did present more convincing evidence that treatment of plant cells with cytochalasin B (CB) appeared to cause an enlargement of the orifice region of plasmodesmata, as revealed by electron microscopy. Because one of the major actions of CB is to disrupt the organization of actin filaments, the enlargement of plasmodesmal orifice in this case is presumably a consequence of actin filament disorganization. Thus, there is a possibility that actin filaments are involved in the regulation of plasmodesmal transport. This is further supported by the finding that tobacco mosaic virus (TMV) MP co-localizes with actin filaments as well as

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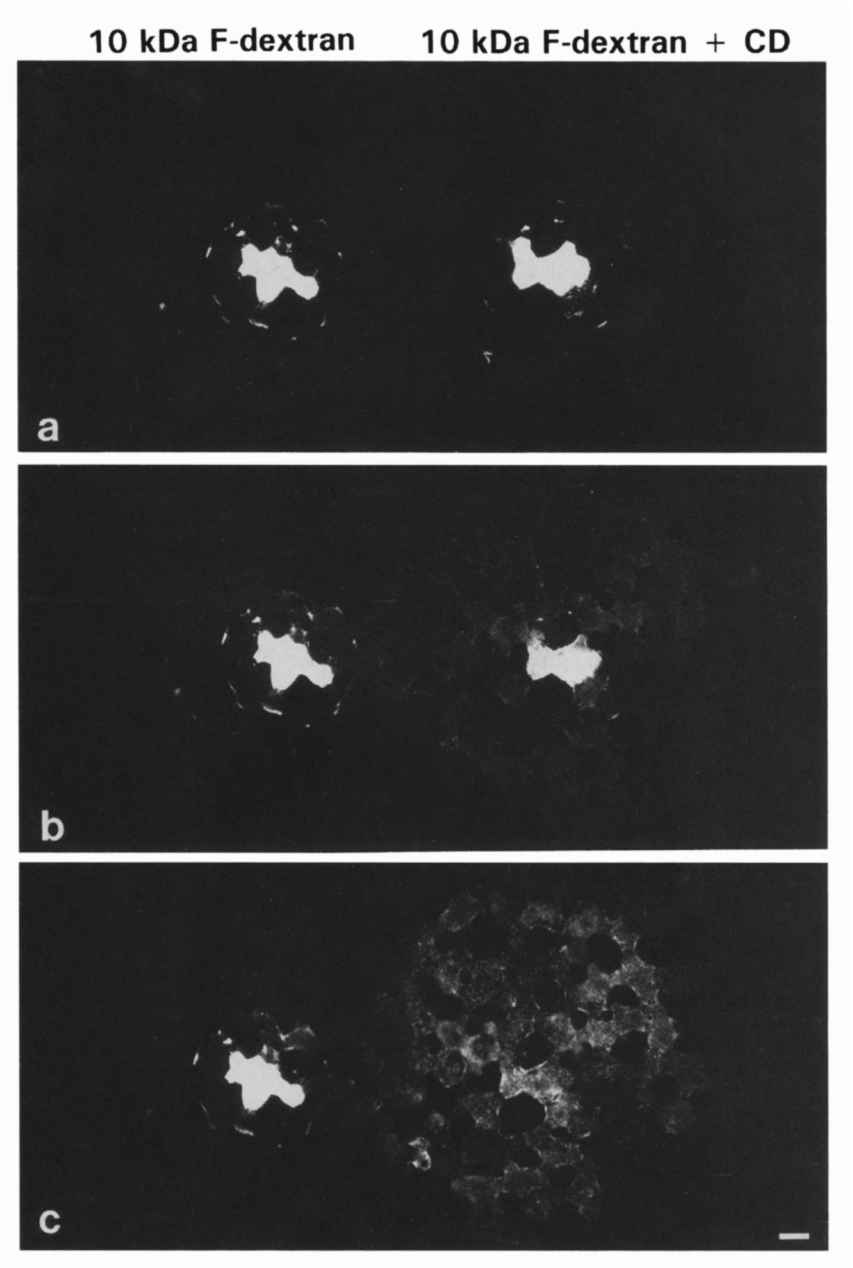

Figure 1. Cell-to-cell movement of 10 kDa F-dextran after co-injection with cytochalasin D (CD) in tobacco mesophyll.
(a) The left cell was first injected with 10 kDa F-dextran alone, and the right cell was then injected with a mixture of 10 kDa F-dextran and 10 μM CD. The photograph was taken 1 min after the right cell was injected with the mixture.
(b) Five minutes after the right cell was injected with the F-dextran/CD mixture, the F-dextran fluorescence was clearly visible in several surrounding cells. Note that the fluorescence intensity decreased in the injected (right) cell.
(c) Thirty minutes after the right cell was injected. The F-dextran fluorescence in this injected cell decreased substantially, whereas the surrounding cells had accumulated distinct F-dextran fluorescence. Note that the left cell still retained the bright F-dextran fluorescence and there was no sign of F-dextran movement out of this cell. The bar represents 30 μm.

microtubules in tobacco cells (McLean *et al.*, 1995). McLean *et al.* (1995) suggested that plasmodesmal-trafficking macromolecules track along microtubules to plasmodesmata, where the macromolecules interact with actin filaments to translocate across the plasmodesmata. Presumably, such interactions include enlargement of plasmodesmal transport channels.

We have used microinjection methods to test whether actin filaments do interact with plasmodesmata. In this communication, we present data from such studies showing that treatment of cells with cytochalasin D (CD), a drug that is more specific than CB for actin filaments, and profilin, a specific actin-binding protein, leads to an increase in the permeability of plasmodesmata to 20 kDa. The significance of these findings is discussed.

Results

The effect of cytochalasin D on plasmodesmal permeability was tested first. As illustrated in Figure 1, when a 10 kDa F-dextran alone was injected into mesophyll cells, it remained in the injected cells and never moved out (Figure 1, Table 1). However, when CD at a tip concentration of 10 μM was co-injected with the 10 kDa F-dextran, the F-dextran moved from the injected cell into surrounding cells within 3–5 min. In the second type of experiments, the 10 kDa F-dextran was

Table 1. Cell-to-cell movement of fluorescent probes in leaf mesophyll of tobacco (*N. tabacum* L. cv. Samsun NN)

Injected material	Number of injections showing movement[a]	Overall movement
10 kDa F-dextran	3 (25)	–
10 kDa F-dextran +CD	20 (27)	+
10 kDa F-dextran +profilin	7 (8)	+
20 kDa F-dextran	2 (19)	–
20 kDa F-dextran +CD	15 (18)	+
20 kDa F-dextran + profilin	8 (10)	+
40 kDa F-dextran	0 (5)	–
40 kDa F-dextran +CD	1 (16)	–
40 kDa F-dextran + profilin	0 (7)	–
70 kDa F-dextran + CD	0 (6)	–
70 kDa F-dextran + profilin	0 (4)	–
10 kDa F-dextran + 2% DMSO	0 (10)	–
10 kDa F-dextran + phalloidin	2 (15)	–

Four to six different plants were used for the experiments for most of the 'injected materials' listed.
[a]For each 'injected material', the number in parentheses represents the total number of injections performed, and the number preceding the parenthesized number represents the number of injections showing cell-to-cell movement of the dextran.

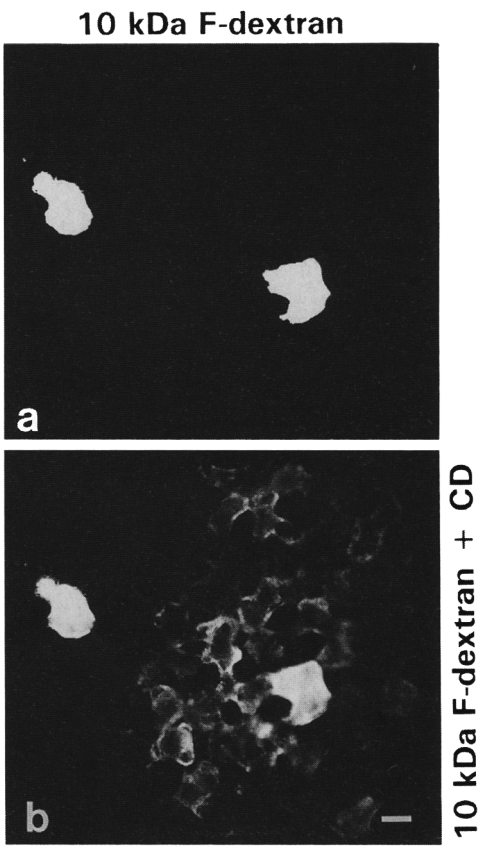

Figure 2. Cell-to-cell movement of 10 kDa F-dextran under the influence of cytochalasin D (CD) in tobacco mesophyll cells.
(a) Two mesophyll cells were injected with 10 kDa F-dextran. After 30 min, the F-dextran remained in the two injected cells.
(b) CD was then injected into the right cell. Within 2 min, the F-dextran fluorescence was visible in the neighboring cells. The photograph was taken 10 min after CD injection into the right cell. The bar represents 30 μm.

first injected into target mesophyll cells. After 30 min or longer, these F-dextrans still remained in the injected cells. CD was then injected into such cells. Within 3–5 min, F-dextran fluorescence started to accumulate in the surrounding cells (Figure 2). Finally, in some other experiments, the 10 kDa F-dextran was injected into one cell, and CD was injected into an immediate neighboring cell. The CD was able to induce movement of the F-dextran out of the initially injected cell (data not shown). It should be noted from Figures 1 and 2 that, under CD treatment, the F-dextran moved into distant cells that were three to four cells away from the injected cell. This fact indicates that the injected CD very likely has moved cell to cell to elicit increases in the plasmodesmal SEL in other non-injected cells. Alternatively, if actin filaments indeed form a continuous network between neighboring cells via plasmodesmata (Li and Zhang, 1994; White *et al.*, 1994), it is possible that disorganization of actin filaments in the injected cell triggers similar events in the neighboring cells. This, however, remains to be established.

Close examination of these figures, especially Figure 2(b), reveals that the F-dextran fluorescence was clearly localized in the cytoplasm. Therefore, plasmodesmata, but not the plasma membrane and cell walls, were the pathways for cell-to-cell movement of the dextran. Because CD was dissolved in 2% DMSO, the effect of DMSO on plasmodesmal permeability was also examined. In all of the 10 experiments in which DMSO and 10 kDa F-dextran were co-injected, the dextran did not move from cell to cell (Table 1). These results indicated that in CD/F-dextran co-injections, cell-to-cell movement of dextran was due to the presence of CD, but not due to DMSO.

Although CD is generally considered to be very specific for actin, we were still concerned about its possible effects on other cellular targets. Therefore, the actin-binding protein profilin, derived from maize plant, was used in separate experiments. Profilin has recently been shown to depolymerize plant actin filaments very rapidly after being injected into cells, presumably by sequestering actin monomers and thereby promoting actin filament depolymerization (Staiger *et al.*, 1994). As shown in Figure 3, when profilin of a tip concentration of 60 μM was co-injected with the 10 kDa F-dextran, the F-dextran moved from the injected cell into surrounding cells within 5 min. Like CD, profilin could be injected into a cell pre-injected with 10 kDa F-dextran to induce dextran cell-to-cell movement (data not shown).

The fact that the dextrans moved several cells away from the injected cell appears to be an indication that profilin itself also moved from cell to cell to exert its function in

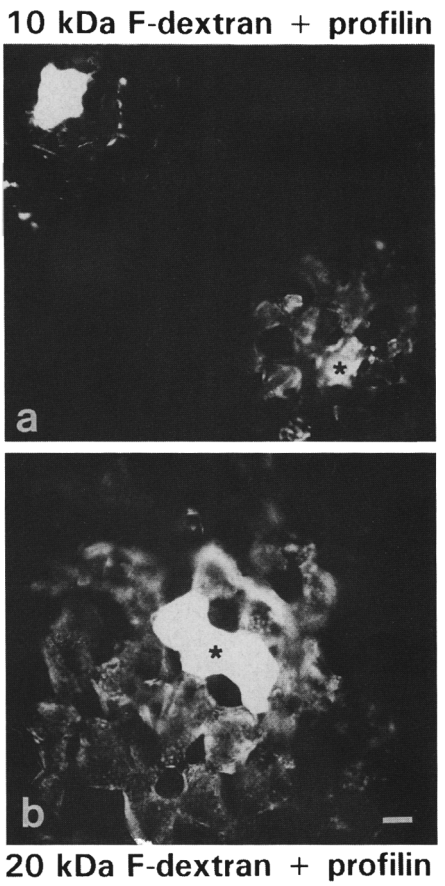

Figure 3. Cell-to-cell movement of dextrans under the influence of maize profilin in tobacco mesophyll.
(a) The cell in the upper left corner was injected with 10 kDa F-dextran alone, and the cell labeled with '*' on the lower right was injected with a mixture of 10 kDa F-dextran and profilin. Profilin co-injection resulted in cell-to-cell movement of the F-dextran. The photograph was taken 30 min after injection.
(b) The cell labeled with '*' was injected with a mixture of 20 kDa F-dextran and profilin. Profilin co-injection resulted in cell-to-cell movement of the F-dextran. The photograph was taken 20 min after injection. The bar represents 30 μm.

neighboring cells. However, this remains to be established because profilin could be sequestered by binding to phosphoinositides and actin in the injected cell. Further studies are required to determine whether enough free profilin is indeed available to move from cell to cell, or whether reorganization of actin filaments induced by profilin and other treatments (e.g. CD) in the injected mesophyll cell can trigger reorganization of actin filaments in neighboring cells via mechanisms that have yet to be uncovered.

To determine the maximal SEL of plasmodesmata that could be induced by CD and profilin treatments, dextrans of increasing sizes were used as probes. A 20 kDa F-dextran was able to move cell to cell when co-injected with CD or profilin at the same concentrations as used above (Figures 3 and 4, Table 1). However, dextrans of 40 kDa and larger were unable to move from cell to cell under such conditions (Table 1, Figure 4). To exclude the possibility that the failure of dextrans of 40 kDa or larger to move cell to cell is due to the inability of CD or profilin to modulate the SEL of plasmodesmata in specific cells, or due to vacuolar injection, the following control experiments were performed. As shown in Figure 4, a mixture consisting of 20 kDa F-dextran, 40 kDa rhodamine B-isothiocyanate (RITC)-conjugated dextran (R-dextran), and 7 μM of CD was injected into a mesophyll cell; the 20 kDa F-dextran moved cell to cell, but the 40 kDa R-dextran remained in the injected cell over long periods of time (hours).

In order to confirm further that cell-to-cell transport of large dextrans under the influence of CD or profilin was due to actin filament reorganization, experiments were also conducted in which 10-kDa F-dextran was co-injected with phalloidin of a tip concentration of 6.6 μM into mesophyll cells. As shown in Figure 5 and Table 1, phalloidin co-injection did not result in cell-to-cell movement of the dextran. Furthermore, the presence of phalloidin of 6.6 μM slowed down or even inhibited CD- or profilin-elicited dextran cell-to-cell movement. In a total of 25 co-injections of 10 kDa F-dextran, 10 μM CD, and phalloidin into tobacco mesophyll cells, nearly half of the injections showed dextran cell-to-cell movement to one or two neighboring cells after 10 min. The other half did not show detectable cell-to-cell movement of the dextran (Figure 5). Similar results were obtained with dextran, profilin, and phalloidin co-injections.

Discussion

We have demonstrated that treatment of tobacco mesophyll cells with actin filament perturbants such as CD and profilin leads to an increase in the permeability of plasmodesmata. Cytochalasins B (CB) and D (CD) are the most commonly used actin-antagonists for studying the functions of actin filaments in plants (e.g. Goodbody and Lloyd, 1990; Grolig, 1990; Lloyd and Traas, 1988; McCurdy and Gunning, 1990; Mineyuki and Palevitz, 1990; Palevitz, 1987; Schmit and Lambert, 1988; Witztum and Parthasarathy, 1985). CD is always preferred over CB because it is more specific for actin, is more effective in disrupting actin filaments, and induces fewer artifacts (Cooper, 1987). Although CD may affect cellular structures other than actin filaments, all of the data accumulated suggest it is specific for actin (Cooper, 1987). Profilin, on the other hand, is known to bind to phosphatidylinositol(4,5)bisphosphate (PIP_2), the precursor of the two second messenger molecules IP_3 and DAG (Aderem, 1992; Drobak *et al.*, 1994), in addition to binding to and depolymerizing actin filaments (Staiger *et al.*, 1994). Phalloidin binds and stabilizes actin filaments (Andersland and Parthasarathy, 1992; Cooper, 1987; Wulf *et al.*, 1979). Because CD and profilin both disrupt the integrity of actin filaments, both influence the SEL of plasmodesmata in a

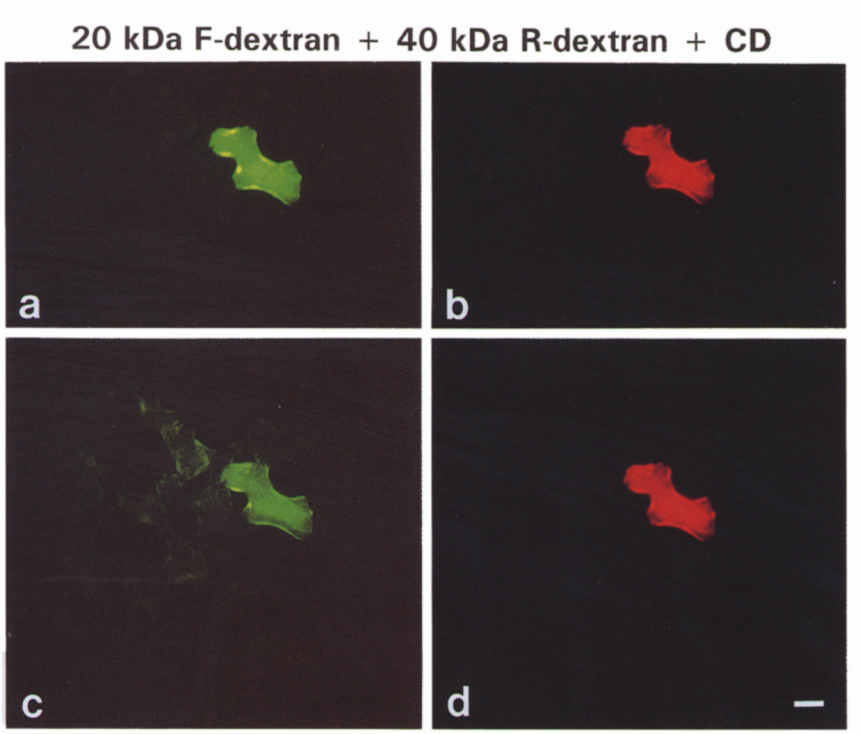

Figure 4. Upper size exclusion limit of plasmodesmata in tobacco mesophyll under the influence of cytochalasin D (CD).
(a and b) The same cell was injected with a mixture of CD, 20 kDa F-dextran (a) and 40 kDa R-dextran (b). The photograph was taken 1 min after injection.
(c and d) Thirty minutes after injection, the 20 kDa F-dextran was clearly visible in cells surrounding the injected cell (c), but the 40 kDa R-dextran still remained in the injected cell (d). The bar represents 30 μm.

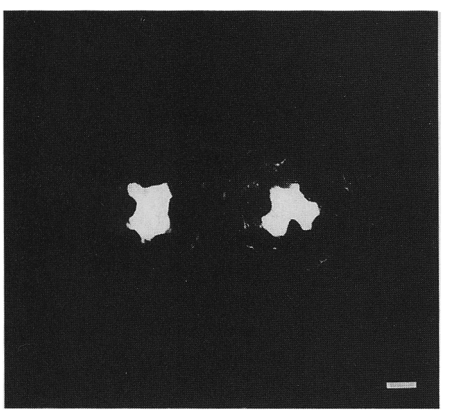

Figure 5. Effect of phalloidin on cell-to-cell movement of 10 kDa F-dextran in tobacco mesophyll.
The cell on the left was injected with a mixture of 10 kDa F-dextran and phalloidin. The cell on the right was injected with a mixture of 10 kDa F-dextran, CD, and phalloidin. The image was taken 20 min after injection. The bar represents 30 μm.

consistent manner, and phalloidin counteracts the effect of CD and profilin, the increase in the plasmodesmal SEL under CD and profilin treatment is most likely due to disorganization of actin filaments, rather than due to other factors.

The finding that CD or profilin treatment resulted in dextrans of up to 20 kDa, but not of 40 kDa, to move cell to cell is remarkably similar to the situation in which tobacco mosaic virus MP also opened plasmodesmata to allow 20 kDa dextran to move cell to cell (Waigmann *et al.*, 1994). More recently, it was also found that the Knotted 1 protein of maize plant potentiated cell-to-cell movement of 20 kDa dextran, but not larger dextrans (Lucas *et al.*, 1995). Thus, under special physiological or experimental conditions, the SEL of plasmodesmata in tobacco mesophyll can be increased to larger than 20 kDa, but smaller than 40 kDa, for cell-to-cell diffusion of molecules.

The detailed mechanism(s) underlying actin–plasmodesma interactions remains to be elucidated. One possibility is that actin filaments physically link the appressed ER to the plasma membrane at the orifice of the plasmodesma, and localized reorganization of actin filaments, induced by specific physiological signals, leads to changes in the physical dimensions of the transport channels, and thus the permeability of plasmodesma. The data obtained in this study should establish a technical and conceptual framework to pursue such issues in detail in the future.

In plants, actin filaments mediate many fundamental cellular processes such as cytoplasmic streaming, organelle movement, nuclear positioning, and tip growth (reviewed in Lloyd, 1989; Seagull, 1989; Staiger and Lloyd, 1991; Williamson, 1993). All of these functions are possible largely because of the dynamic nature of actin filaments. In animal cells, turnover of actin filaments involves the participation of many actin-binding proteins (Stossel, 1989). Interestingly, reorganization of actin cytoskeleton is linked to the production of second messenger molecules 1,2-diacylglycerol (DAG) and inositol(1,4,5)trisphosphate (IP_3) via the 12–15 kDa actin-binding protein, profilin (Aderem, 1992; Goldschmidt-Clermont and Janmey, 1991). In other words, cell signaling at the plasma membrane level

can be transduced to actin via profilin. Many components of the second messenger system exist in plants (Cote and Crain, 1993), and profilin has been isolated from higher plants (Staiger *et al.*, 1993; Valenta *et al.*, 1991). A possible role for profilin to couple the production of the second messenger molecules IP_3 and DAG to actin reorganization in plant cells has been suggested by recent studies (Drobak *et al.*, 1994; Staiger *et al.*, 1994). Given that the second messenger molecules can regulate plasmodesmal function as well as actin dynamics, one cannot rule out the intriguing possibility that actin filaments serve as the link between second messenger production and some aspects of plasmodesmal function.

Experimental procedures

Plant material

Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were grown in a growth chamber under a temperature regime of 24°C (14 h)/18°C (8 h) (day/night). During day time, the light level was approximately 260 μmol m^{-2} sec^{-1}. Six-week-old mature plants were used for the experiments.

Reagents

Cytochalasin D (CD), fluorescein isothiocyanate (FITC)-conjugated dextrans (F-dextrans), and rhodamine B isothiocyanate (RITC)-conjugated dextrans (R-dextrans) were purchased from Sigma Co. (St Louis, Missouri, USA). Phalloidin was purchased from Molecular Probes (Eugene, Oregon, USA). Solutions of dextrans (1 mM) were prepared in 5 mM $KHCO_3$ (pH 8.0), sterilized through a 0.2 μm filter membrane, filtered through an Amicon microconcentrator to remove fractions of small molecular masses (e.g. a membrane with a molecular cutoff of 3000 Da was used to filter the 10 kDa F-dextran, and a membrane with a molecular cutoff of 10 kDa was used to filter the 20 kDa F-dextran), and stored at 4°C.

A 20 mM stock solution of CD was prepared by dissolving CD in dimethylsulfoxide (DMSO) and stored at −20°C. Working solutions of CD were prepared by diluting the stock solution in 2% DMSO and stored at 4°C. Phalloidin was dissolved in methanol to give rise to a stock solution of 6.6 μM and stored at −20°C. A working solution of 6.6 μM phalloidin was prepared by evaporating the methanol at room temperature and then dissolving the nearly dried phalloidin in 100 mM KCl.

The maize profilin was a gift from Christopher Staiger, Purdue University. The profilin was maintained in a buffer containing 20 mM Tris–HCl, pH 7.4, 150 mM KCl, and 0.2 mM DTT.

Microinjection and photography

The microinjection set-up consisted of a hydraulic micromanipulator (Model MMO-203, Narishege Co., Tokyo, Japan) attached to a coarse positioner (Model MMN-1, Narishege Co., Tokyo, Japan) that was mounted on to a Nikon Optiphot-2 epifluorescence microscope (Nikon Corp., Tokyo, Japan). A Pneumatic PicoPump (Model PV820, World Precision Instruments Inc., Sarasota, Florida, USA) was used for pressure injection. Micropipettes with a tip size of approximately 1 μm were fabricated from borosilicate pipettes (World Precision Instruments) using a micropipette puller (Model P-97, Sutter Instruments Co.,Novato, CA, USA).

The microinjection procedures were essentially as previously described (Ding *et al.*, 1992a, 1995). A mature leaf, still attached to the plant, was taped (abaxial-side up) on to a glass plate which was in turn mounted on to the microscope stage. A small portion of the lower epidermis was removed with a forceps. The revealed mesophyll tissue was immediately bathed in distilled water. All of the injections were performed with the mesophyll cells. Cell-to-cell movement (or non-movement) of dextrans was monitored with the Nikon epifluorescence microscope. The F-dextrans were visualized with the following combination of filter sets: a blue excitation filter (420–490 nm), a 510 nm dichroic mirror, and a green barrier filter (520–560 nm). The R-dextrans were visualized with the following combination of filter sets: a green excitation filter (546/10 nm), a 580 nm dichroic mirror, and an orange-red barrier filter (590 nm). Fluorescent images were photographed with a Nikon UFX-DX camera system, using either Kodak Tmax p3200 black and white negative films, or Fujichrome Provia 1600 color slide films.

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