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CELL-TO-CELL COMMUNICATION VIA PLANT ENDOMEMBRANES

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Cell-to-cell communication was investigated in epidermal cells cut from stem internodal tissue of *Nicotiana tabacum* and *Torenia fournieri.* Fluorescently labelled peptides and dextrans were microinjected using iontophoresis into the cytoplasm and cortical endomembrane network of these cells. The microinjected endomembrane network was similar in location and structure to the endoplasmic reticulum (ER) as revealed by staining with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$). No cell-to-cell movement of dextrans was observed following cytoplasmic injections but injection of dextrans into the endomembrane network resulted in rapid diffusion of the probes to neighbouring cells. It is proposed that the ER acts as a pathway for intercellular communication via the desmotubule through plasmodesmata.

KEYWORDS: endoplasmic reticulum; plasmodesmata; dextrans; microinjection.

INTRODUCTION

The endoplasmic reticulum (ER) forms a continuous network throughout the cytoplasm of eukaryotic cells and has the roles of synthesizing, processing and sorting proteins and lipids as well as regulating cytosolic calcium levels (Staehelin, 1997). In plants, an ultrastructural relationship between the cortical ER and plasmodesmata has been recognized since the earliest EM images of plasmodesmata (see Robards, 1976 and references therein). The nature of the desmotubule link between the ER of adjacent cells has been the subject of much speculation. The desmotubule has been modelled as a solid proteinaceous rod (Tilney *et al.*, 1991), a membrane cylinder (Gunning and Overall, 1983) or as an integration of both features (Ding *et al.*, 1992). The possibility that the desmotubule plays a role in intercellular communication has also been the topic of much debate (Robards, 1976; Robards and Lucas, 1990).

The lipid-packing calculations of Overall *et al.* (1982) demonstrated the possibility that ER lipids could be constricted into a tubular strand that passes through the centre of plasmodesmata. With this model, the desmotubule lumen was reduced to the diameter of a few water molecules and as a result the spaces between protein subunits within the cytoplasmic sleeve became the preferred route for cell to cell communication (Lucas *et al.*, 1993; Overall and Blackman, 1996). More recently, new evidence has revived the idea of the desmotubule as a pathway of cell to cell communication. The intercellular diffusion of ER specific lipids has been demonstrated (Grabski *et al.*, 1993), and the potential of the ER network to act as a pathway for phototsynthate or water transfer from cell to cell (Gamalei *et al.*, 1994; Zhang and Tyerman, 1997) has also been indicated. Strong evidence also exists for the cell to cell movement of tobacco mosaic virus movement protein within the membrane of the ER (Reichel and Beachy, 1998).

Evidence is presented in this paper that a microinjectable subcellular compartment exists in addition to the familiar cytoplasmic, nuclear and vacuolar compartments. This compartment has the appearance of a lacy network. It resembles cortical ER in both form and location and is a potential avenue for cell to cell communication in higher plants.

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MATERIALS AND METHODS

Plant material

Explants of *Nicotiana tabacum* L. cv Wisconsin 38 (CSIRO Plant Industry, Canberra, Australia) were obtained from vegetative stock plants. Stock plants were maintained under sterile conditions on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with vitamins according to Linsmaier and Skoog (1965), 30 g/l sucrose, 8 g/l of bacto-agar and with the pH adjusted to 5.7. Light was provided by fluorescent tubes (40W, Crompton, Australia) (26 W/m^2) for 4-h intervals, each separated by 2 h of darkness. Temperatures during the light and dark periods were 27°C and 24°C, respectively. Explants of stem epidermis were cut from the second and third extended internodes below the apex of 6 to 8 week old stock plants (modified from Tran Thanh Van *et al*., 1974). Each explant was approximately 5 mm long and 2 mm wide and consisted of one layer of epidermal cells and 3–7 layers of subepidermal cells. For microinjection, tobacco stem explants were bathed in liquid MS medium.

Stock plants of *Torenia fournieri* Lind. cv Clown Mixed (Yates, Australia) were cultured on modified MS media according to Tanimoto and Harada (1979). These were incubated in a controlled environment room at a temperature of 30°C under continuous illumination (6.8 W/m^2) provided by fluorescent tubes (40W, Crompton, Australia). Explants were cut from the second internode below the apex of 6 to 8 week old stock plants and were approximately 5 mm long and 3 mm wide, consisting of the epidermis and many layers of subepidermal and cortical cells including some vascular tissue (modified from Tanimoto and Harada, 1979). For microinjection, *Torenia* stem explants were bathed in liquid modified MS medium.

Anchoring explants

Injection slides and anchoring of stem explants was adapted from Hepler and Callaham (1987). Injection slides were constructed from 1.2-mm thick glass slides (American Scientific McGaw Park, IL, U.S.A.) each drilled with an 18-mm diameter hole. Each hole was covered by a 22 × 22-mm coverslip (#1 thickness) glued around the edges with dilute nail polish to form a well. The injection slides were warmed to 37°C on the heated stage of a binocular dissecting microscope prior to anchoring the explants. Explants were blotted of excess medium prior to anchoring and were placed one per slide and epidermis up. A few drops of molten 1% agarose solution (type VII; Sigma) +0.02% Triton X were spread around the sides of the injection slide well and left to cool to 37°C for 30 s on the heated stage. Agarose was pushed up to the edges of the explant and allowed to run under it and across the base of the well. The slide was then placed on a freezer brick for 10 s to solidify the agarose. The explants were then immersed once more in the appropriate culture medium and allowed to equilibrate for at least 30 min prior to the first injection.

Probes

F(Glu)$_3$ was synthesized according to the method of Oparka and Read (1994) by conjugating fluorescein isothiocyanate isomer I (FITC) (Sigma Chemicals, St Louis, MO, U.S.A.) and triglutamic acid [(Glu)$_3$] (Sumac, University of Sydney, Australia). FITC-labelled dextrans (FD 3000, FD 10000 and FD 40000) were obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). Prior to use, the dextrans were reprecipitated in 80% ethanol according to Derrick *et al.* (1992) to eliminate unreacted FITC. 6-Carboxyfluorescein (Eastman Chemicals, Rochester, NY, U.S.A.) was initially dissolved in 0.3 M KOH. All probes were made up to 1-mM solutions in millipore filtered (0.45 µm pore size) distilled water and the fluorescent conjugates were tested for low-molecular weight contaminants using thin layer chromatography (Kieselgel 60, Merck Darmstadt, Germany) according to Goodwin (1983). Solutions of all fluorochrome conjugates were discarded after 1 day to avoid low molecular weight contaminants from microbial degradation.

Microinjection

Micropipettes were pulled from 1.2-mm borosilicate glass capillaries with inner filaments (World Precision Instruments (WPI), Sarasota, FL, U.S.A.) on a P87 puller (Sutter Instrument Company, Novato, CA, U.S.A.). Tips were filled with 5 µl of the fluorescent probe, back filled with 0.1 mM KCl and secured in a microelectrode holder (MEH2SFW12, WPI) attached to the active probe of the electrometer (Intra 767, WPI). A chloridized silver wire placed in the bathing solution was used as a reference electrode and all metal components of the set up were electrically grounded. Tip integrity of the micropipettes was monitored via the built in electrode resistance test function. Resistances of around 1000 MΩ indicated that the tip

was undamaged and suitable for microinjection. Injections were performed via an hydraulic micromanipulator (WR-30, Narishige, Japan) mounted on a coarse manipulator (M-35, Narishige) attached to the microscope stage. Cells were impaled at a 45 degree angle in the corner of a cell or close to the nucleus. The micropipette tip was positioned so as to push gently on the cell wall/membrane and the side of the microscope was tapped to gain entry to the cytoplasm. The membrane potential was noted both before and after iontophoretic injection. Injections where membrane potential fluctuated or large changes were seen after microinjection were rejected. Probes were injected by iontophoresis using a current pulse of -1 nA for 15–30 s. The current was generated as a square wave from a function generator (CFG253, Techtronix, Beaverton, OR, U.S.A.) and was delivered via the breakaway box on the electrometer.

$DiOC_6$ staining

$DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) (Sigma) was dissolved in a small quantity of dimethyl sulfoxide (DMSO; final concentration no more than 0.1%) and made up into a 5 mg/ml stock solution with distilled water according to the protocol of Oparka and Read (1994). Internodes of whole *Torenia* and tobacco stems were gently abraded with a lint-free tissue to remove cuticle so as to allow $DiOC_6$ to penetrate and stain epidermal cells. This step also removed trichomes from the tobacco epidermis which would have otherwise interfered with observations due to fluorescence from non specific staining of the trichome cell walls. Explants were cut from the abraded area and these were bathed in a staining solution containing the appropriate MS medium and $DiOC_6$ (5 μg/ml) for 2 min. Stained explants were washed briefly in fresh MS medium to remove excess $DiOC_6$ and mounted on slides.

Microscopy

Microinjections were monitored as they occurred with an Axiophot microscope (Zeiss) equipped with a $\times 40$ water immersion objective. $DiOC_6$ staining was observed with a standard $\times 40$ and a $\times 63$ oil immersion lens. The FITC probes and $DiOC_6$ were observed under blue illumination (excitation 450 to 490 nm). Experiments were recorded on Fujichrome 400 ASA colour reversal film (Fuji Photo Film Co, Tokyo, Japan) and Ektachrome 400 or 1600 ASA colour reversal film (Eastman Kodak). Photographic slides were scanned by a Nikon LS-1000 Super Cool Scan (Nikon Inc., Tokyo Japan). The digitized images were printed out on a Codonics NP-1600M dye sublimation printer (Codonics Inc. Middleberg Heights, OH, U.S.A.).

RESULTS

Small molecule microinjections

The presence of a microinjectable endomembrane system was first observed when microinjecting CF and $F(Glu)_3$ into tobacco and *Torenia* explants. Figure 1a shows a typical cytoplasmic injection of CF into a *Torenia* internodal epidermal cell and compares it to an endomembrane injection (Fig. 1b). The cytoplasmic injection shows a characteristically evenly distributed fluorescence in addition to the bright nucleus in each of the cells and this contrasts with the punctate fluorescence of the endomembrane injection. The higher magnification image (Fig. 1c) reveals that the distinct bright points which underlie the cell wall of the injected cell seem to be linked by fluorescent strands. These bodies and strands are similar to the strands and lamellae of cortical ER revealed by $DiOC_6$ staining of *Torenia* cells (Fig. 1d).

Injections of this type using CF and $F(Glu)_3$ were observed at a low frequency in the two cell types tested (less than 10% of injections; see Table 1) and this probably reflects the chance of locating the micropipette tip within a structure of small volume relative to the rest of the cell. Cytoplasmic injections of CF and $F(Glu)_3$ moved from cell to cell in the majority of cases although a percentage of these injections resulted in no cell to cell movement (see Table 1). In contrast, all cases of endomembrane injection of CF and $F(Glu)_3$ resulted in cell to cell movement (see Table 1). Furthermore, these small fluorescent probes seemed to travel more rapidly from cell to cell via endomembrane injections than via cytoplasmic injections, although this was not possible to quantify, as in most cases fluorescence vanished from the endomembrane network within a few seconds of microinjection.

Macromolecule microinjections

Injections of FD 3000 into the cytoplasm of tobacco cells resulted in no cell to cell movement of this dextran probe (see Table 1; Fig. 2a,b). Figure 2a is focused at the level of the external cell wall of

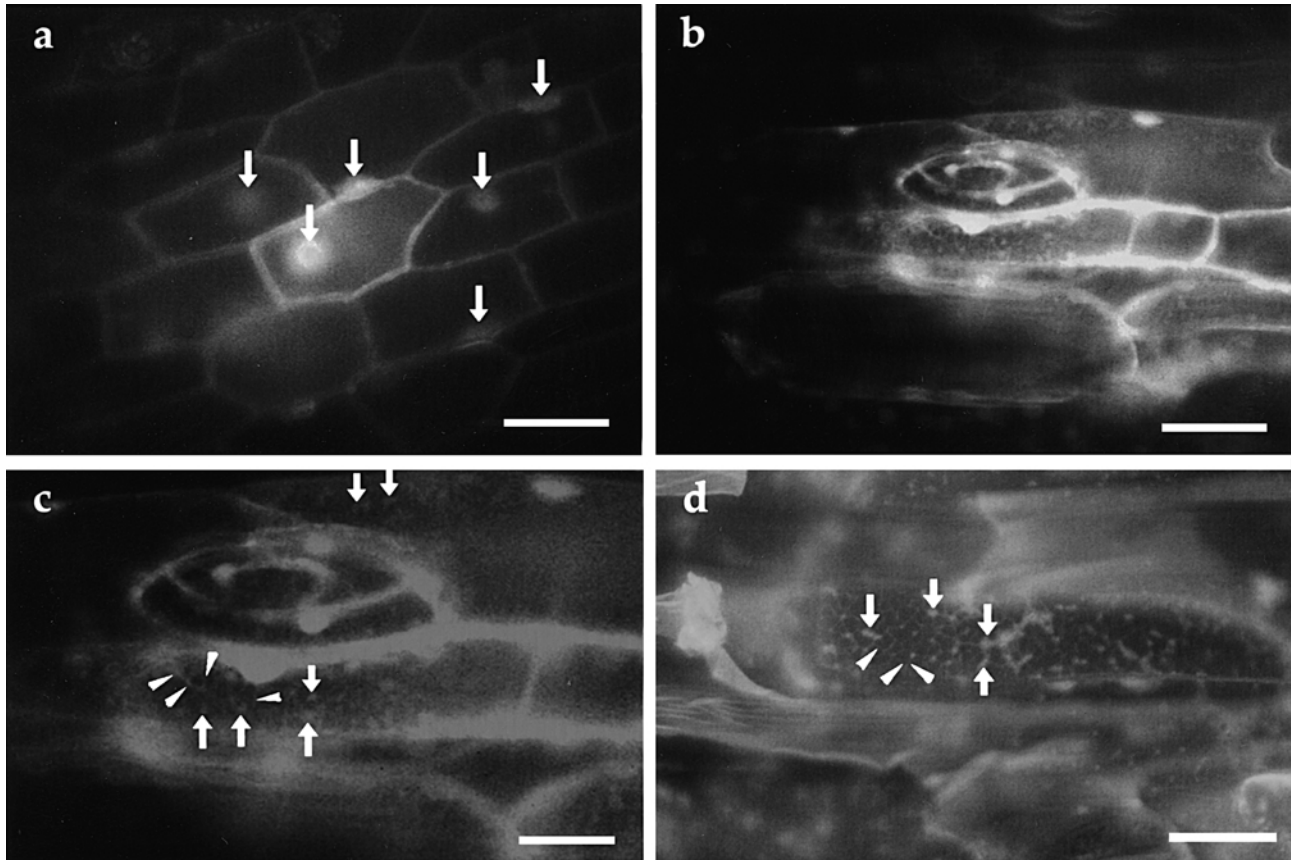


Fig. 1. Fluorescence images of internodal epidermal cells of *Torenia fournieri*. (a) A microinjection of CF showing the characteristic cytoplasmic distribution of the fluorescent probe. Fluorescence is observed in the cell nuclei (arrows) and is present in an even band inside the cell wall. Bar = 50 μm. (b) A CF injection showing the punctate fluorescence of an endomembrane network. Bar = 50 μm. (c) At a higher magnification, the small bodies are more evident (arrows, →) and in places these seem to be linked by small fluorescent strands (darts, ▲). Bar = 25 μm. (d) $DiOC_6$ staining of ER in an internodal epidermal cell of *Torenia fournieri*. Fluorescent bodies (arrows) joined by strands (darts) are similar to those observed in 1c. Bar = 25 μm.

Table 1.
Frequency of intercellular movement observed during endomembrane and cytoplasmic injections into tobacco and *Torenia* explants

Explant type	*Tobacco*				*Torenia*		
Probe	$F(Glu)_3$	FD 3000	FD 10000	CF	$F(Glu)_3$	FD 3000	FD 10000
Molecular weight	799	~3000	~10,000	376	799	~3000	~10,000
Total number of injections	14	18	8	64	67	6	33
Cytoplasmic injections	13	17	8	61	64	6	30
Intercellular movement observed	11 (84.6)*	0	0	55 (90.1)*	46 (71.9)*	0	0
Endomembrane injections	1	1	0	3	3	0	3
Intercellular movement observed	1 (100)†	1 (100)†	0	3 (100)†	3 (100)†	0	3 (100)†

*% of cytoplasmic injections where intercellular movement observed.
†% of endomembrane injections where intercellular movement observed.

a tobacco epidermal cell and FITC fluorescence is concentrated in the cytoplasm which is seen very brightly in the cell periphery but also exists just below the cell wall in view and which appears as a fluorescent haze all over the central part of the cell. This fluorescence is uniform and is not located in

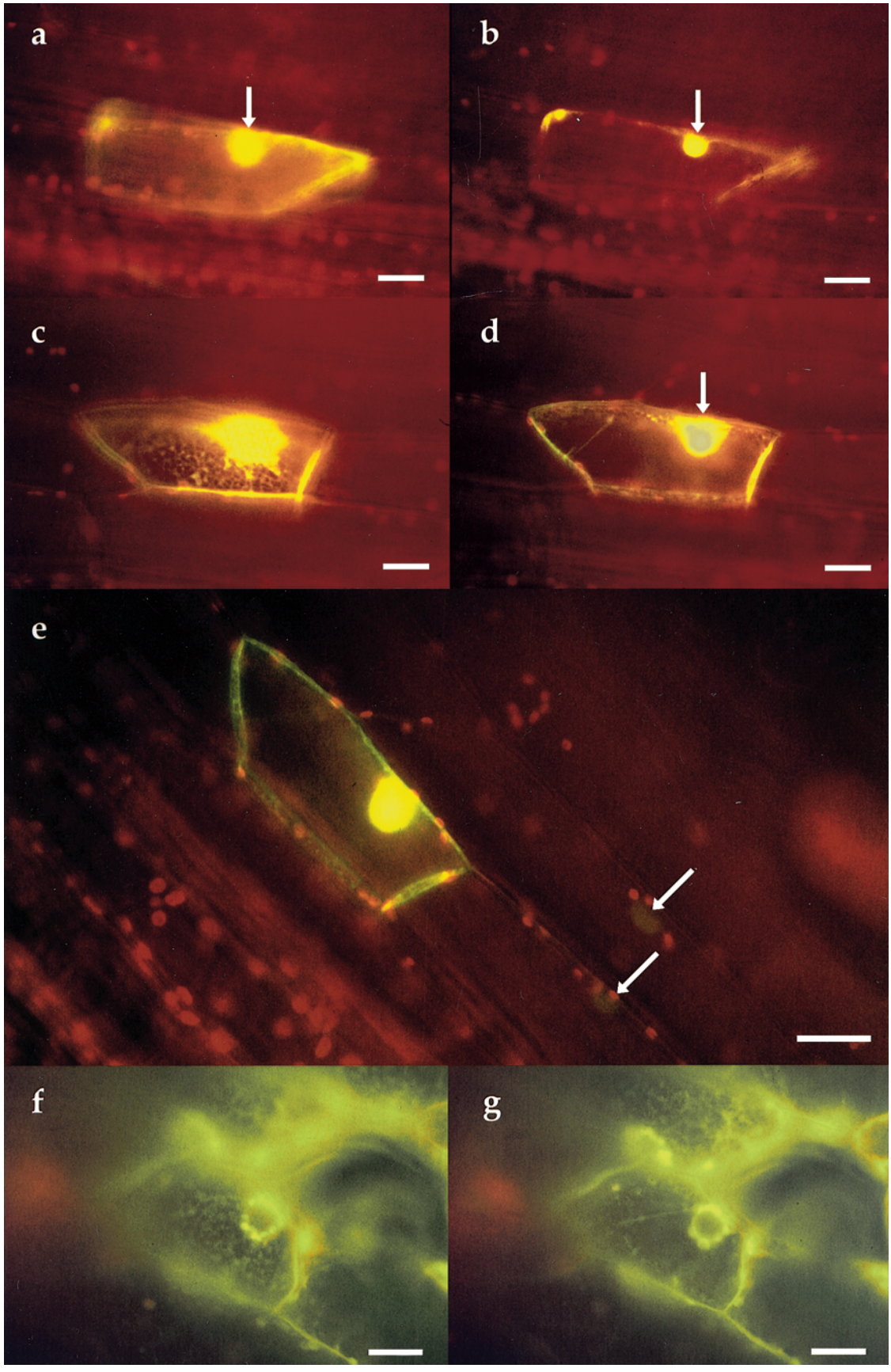
small bodies just below the cell wall. Observing the same cell at a lower focal plane (Fig. 2b) provides a similar picture, with the nucleus in focus this time and the peripheral cytoplasm surrounding a dark central area (the vacuole) which contains little or no fluorescence. However, Fig. 2c presents a tobacco cell in which FD 3000 was injected into a compartment that was different from the usual cytoplasmic injections. A lacy intracellular network appeared to be located just below the cell wall in the cell cortex. Not only was there a distinct localization of fluorescence to this network but there was also a lack of the normal all-over fluorescence seen at similar focal planes in cytoplasmic injections (Figs 1a and 2a). Dye was distributed within a network of small fluorescent bodies which appeared to be interlinked by fine strands. At a mid plane focus, this network is shown to be confined to the area just inside the cell wall (Fig. 2d). Figure 2e reveals the most striking feature of this injection; a large fluorescent body in each of the neighbouring cells. These were the nuclei of the neighbouring cells and the presence of fluorescence within them indicated that FD 3000 had moved from cell to cell. The similarity in form and location between cortical ER in a tobacco cell stained with $DiOC_6$ (Fig. 2f and g) and the microinjected endomembrane network (Fig. 2c and d) suggests that they are identical structures.

Similar observations were made with injections of FD 10000 into freshly prepared *Torenia* explants and as with the other probes already discussed, only a small proportion (<10%) of the macromolecule injections demonstrated the punctate network (Table 1). No endomembrane injections were observed using FD 3000 in *Torenia* or FD 10000 in tobacco and this was most likely due to the small sample size. However, as with the other cytoplasmic macromolecule injections, no cell to cell movement was observed (Table 1).

DISCUSSION

Tobacco and *Torenia* stem internodal epidermal cells do not normally permit macromolecular movement after cytoplasmic injection but evidence has been presented here for the intercellular diffusion of synthetic macromolecules following endomembrane injections. The appearance of the microinjected endomembrane network is also similar to ER in cells stained with $DiOC_6$. These observations have important implications for our assumptions about the location of the micropipette tip within the cell during microinjection and for our understanding of where the channel or channels of intercellular movement are located within plasmodesmata. If microinjection of the ER produces results that are different from injections into the main body of the cytoplasm, then care must be taken in evaluating cell to cell movement of microinjected probes. Other workers have already reported low levels of cell to cell movement of macromolecules in control injections (12% for 4.4-kDa dextrans, Vaquero *et al.*, 1994; 9% for 9.4- and 20-kDa dextrans, Lucas *et al.*, 1995; 12% for 20-kDa dextran, Ding *et al.*, 1996) and these might have involved some type of interaction with the ER. It is interesting that these 'anomalous' injections also occurred in approximately 10% of experiments, which is similar to the frequency reported here for endomembrane injections. It is impossible to know at this stage whether any of these observations are part of the normal functioning of plasmodesmata and ER, or if they are artefacts of the microinjection process. It is possible that the passage of current (or pressure) into the confines of the ER damages the plasmodesmata or stimulates the gating of the channels. Alternatively, enzymes within the ER lumen might have broken down the dextrans. However, the movement from cell to cell was visible within a few seconds of injection and was probably too rapid for substantial enzymic degradation to have occurred.

Endomembrane injections of CF and $F(Glu)_3$ resulted in similar endomembrane networks appearing in neighbouring cells, although the dye was only observed very briefly in this compartment. Continuity of ER membranes to the desmotubule and through plasmodesmata has been established by other authors (Grabski *et al.*, 1993; Oparka *et al.*, 1994). The observations here could be taken as indicating a continuity of the ER lumen through the cell wall via the desmotubule. In contrast to CF and $F(Glu)_3$, FD 3000 and FD 10000 were observed only in the nuclei of neighbouring cells after injection into an ER-like structure. However, it is known that ER and nuclear membranes, and the ER lumen and the lumen between the double nuclear membranes are continuous (Boevink *et al.*, 1996; Staehelin, 1997 and references therein). It may be that superimposition of the top and bottom of the nuclear envelope surrounding the large nucleus made the dye in this region visible, whereas the much smaller cisternae and tubules of the endomembrane network were not visible, particularly given the red autofluorescent background of the cells and the limits of epifluorescence microscopy. These low levels of macromolecular-dye fluorescence in neighbouring cells after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endomembrane injection might also indicate that there is gating or control over the molecular size exclusion limit in this pathway.

With epifluorescence it was difficult to determine if the dye was in the nuclear envelope or nucleoplasm of neighbouring cells. If dye was in the nucleoplasm after an endomembrane injection, it could mean that a previously unobserved pathway for transport exists between the ER/nuclear envelope lumen and the nucleoplasm. Indeed, Singh *et al.* (1998) have described a trans-nucleoplasmic reticulum that significantly increases the surface area of the nuclear envelope by invaginating deeply into the nucleoplasm and which is continuous with the endomembrane system.

Intercellular movement through subcellular membrane networks has been observed or proposed in other systems. Shepherd *et al.* (1993*a*,*b*) reported that a system of tubules and vacuoles moved intercellularly through the dolipore in the wall between the terminal and penultimate cells of fungal hyphae. Lazzaro and Thomson (1996) reported an intercellular vacuolar system within secretory trichomes of chickpea that transported Lucifer yellow hydrazide from the basal to the tip cells via plasmodesmata. These latter authors speculated on a possible relationship between the chickpea vacuoles and the ER network seen in other plant cells and the possibility that the ER lumen might also provide an avenue for cell to cell transport. Recent evidence suggests that vacuoles are derived from specialized regions of the ER (Staehelin, 1997 and references therein) and thus the relationship could be very close. Waigmann *et al.* (1997) have also reported 'open' desmotubules in EM images of trichome cells of *Nicotiana clevlandii* and that these cells possessed a basal size exclusion limit in the macromolecular range (Waigmann and Zambryski, 1995). However, these authors speculated that in trichome cells, the desmotubule might be open to the cytoplasm rather than being continuous with the ER.

Viral movement in several systems has been proposed to involve ER membranes. Gemini viruses recruit ER to form specialized tubules containing movement protein that enable cell to cell movement of the virus particle via the tubule lumen (Ward *et al.*, 1997). Tobacco mosaic virus movement protein has also been localized to ER membranes suggesting that it and the viral RNA might be transported from cell to cell via the movement of ER through plasmodesmata (Reichel and Beachy, 1998). Indeed, the ER also appears to be ready made for many of the active macromolecule trafficking functions that are now attributed to plasmodesmata. ER undergoes continual movement and reorganization (Knebel *et al.*, 1990) and is closely association with cytoskeletal elements including actin and myosin (Liebe and Quader, 1994; Reuzeau *et al.*, 1997) which have also been localized to plasmodesmata (White *et al.*, 1994; Blackman and Overall, 1998; Radford and White, 1998). Furthermore, the protein targeting (Bar-Peled *et al.*, 1996) and protein folding functions attributed to ER (Boston *et al.*, 1996) make these endomembranes very attractive sites for further studies of macromolecular cell-to-cell communication. It is clear that the technique reported here of microinjection into plant endomembrane systems has reinforced this evidence of the ER playing an active role in cell to cell communication through plasmodesmata.

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Fig. 2. Fluorescence images of internodal epidermal cells of tobacco. (a) Dye distribution of a cytoplasmic injection of FD 3000 into an internodal epidermal cell of tobacco seen at the focal plane of the external wall. The yellow haze observed across the cell shows the presence of an even layer of cytoplasm just below the cell wall. The nucleus is indicated (arrow). Bar=25 μm. (b) The same cell focused at the level of the nucleus (arrow) shows that most of the cell appears clear due to the presence of the large central vacuole. A narrow line of fluorescence around the cell is the probe contained within the thin layer of peripheral cytoplasm. No neighbouring cells contain fluorescent nuclei or cytoplasm indicating that this probe does not move from cell to cell in this tissue type. Bar=25 μm. (c) An endomembrane injection of FD 3000 into an internodal epidermal cell of tobacco. In the cell wall focal plane shown here, the cell appears to contain a lacy network of fluorescent bodies linked by fluorescent strands. Bar=25 μm. (d) The same cell focused at the level of the nucleus (arrow). Some fluorescent bodies are visible at the edges of the cell but the central area is free of fluorescence indicating that the network is likely to be located in the cell cortex. Bar=25 μm. (e) A wider field view of the cell in (c) and (d). Note the fluorescent nuclei in neighbouring cells (arrows) indicating the intercellular movement of FD 3000 after an endomembrane microinjection. Bar=25 μm. (f) $DiOC_6$ staining of tobacco internodal epidermal cells showing the presence of interlinked fluorescent bodies of the ER in the cell cortex. These are similar to the fluorescent bodies in Fig. 2c. Bar=25 μm. (g) The same cell at a lower focal plane showing the absence of ER in the central region of the cell. Bar=25 μm.

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