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Organization of microtubules and microfilaments in protoplasts from suspension cells of *Nicotiana plumbaginifolia*: a quantitative analysis

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SUMMARY

The cortical cytoskeleton of suspension cells and protoplasts of *Nicotiana plumbaginifolia* was studied by means of fluorescent probes by a light microscope and by means of dry-cleaved preparations by an electron microscope. No differences in the organization of the cortical cytoskeleton of the suspension cells and protoplasts were observed. Generally, the cytoskeleton showed highly organized domains within superficially random-like patterns. Microtubules formed extensive bundles with up to 10 elements per cross-linked bundle. About 65 cross-bridges occurred per micrometre. The cross-links were 25–50 nm long and 5–20 nm in diameter. On average, 56% of the entire microtubular length was present in the cross-linked bundles. The length and abundance of microtubules ranged between 0·8 and 14·28 μm and 0·93–4·29 μm μm^{-2}, respectively. Microtubules were regularly aligned with microfilaments, either as parallel or as 'stitched' filaments. These alignments did not occur over distances longer than 1·46 μm. The distribution of the coated pits appeared to depend on microtubular distribution. Coated pits were often connected with the microtubules by means of putative actin filaments.

Key-words: dry-cleaving, immunofluorescence, microfilaments, microtubules, *Nicotiana plumbaginifolia*, protoplasts.

INTRODUCTION

The cytoskeleton in interphase cells of higher plants consists mainly of cortical microtubules and actin filaments. The cortical microtubules appear to be interconnected and may form large arrays. Actin filaments occur in different forms: large bundles, probably involved in generating plasma streaming, and fine cortical filaments. The existence of intermediate filaments, as found in animal cells, has not been demonstrated yet, although there is evidence that the constituents of intermediate filaments are present (review: Derksen *et al.* 1990).

Parts of the cortical actin filaments and intermediate filaments co-localize with microtubules (e.g. Tiwari *et al.* 1984; Lancelle *et al.* 1987; Goodbody *et al.* 1989; Pierson *et al.* 1989).

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However, as many co-localizations have been observed merely at the light-microscopic level and most electron-microscopic preparations show only a small part of the cell, information on the interactions between cytoskeletal elements is limited. Moreover, most observations have been carried out on highly specialized cells like pollen tubes (e.g. Pierson *et al.* 1989). To study the cortical cytoskeleton at the electron-microscopic level, dry-cleaving has proved useful, allowing the measurement of cytoskeletal properties (Traas *et al.* 1984, 1985). Protoplasts are suitable objects for dry-cleaving studies, as they lack a cell wall and thus large cleaved areas are easily obtained. However, the cytoskeleton in protoplasts may not be representative of that in intact cells. For example, the abundance of microtubules in protoplasts seems to be much lower than in walled cells, as judged from the data presented in many papers (Lloyd *et al.* 1979; Doohan & Palevitz 1980; Van der Valk *et al.* 1980).

The present study aims to describe the physical connections between associated cytoskeletal elements and to quantify the extent of these associations. Therefore, in addition to protoplasts from a cell culture of *Nicotiana plumbaginifolia*, we also studied non-enzyme treated cells and cells during wall degradation by means of fluorescent probes at the light-microscopic level and by using dry-cleaved preparations at the electron-microscopic level.

MATERIALS AND METHODS

Plant material and protoplast isolation

Suspension cells of *Nicotiana plumbaginifolia* were maintained on B5 medium (Gamborg *et al.* 1968) supplemented with 0·5 mg l^{-1} 2,4-D on a rotary shaker at 200 rpm in the dark at 28°C. Cells were subcultured once every 3–5 days by adding fresh medium to the cells and by a twofold dilution. The enzyme mixture used for protoplast isolation consisted of 5% cellulase Onozuka R10 (Yakult, Japan), 0·5% macerozyme R10 (Serva, Heidelberg) and 0·4 M mannitol at pH 5·6. Protoplasts were isolated from log-phase cultures by incubation of suspension cells in the enzyme solution for 3–4 h at 28°C on a rotary shaker (30 rpm). The protoplasts were collected on 0·4 M sucrose after rinsing with W5 solution (Sidorov *et al.* 1981). Preparations were made from non-enzyme treated cells, cells after 2 or 4 h incubation in the enzyme mixture and from protoplasts immediately before or after collection on sucrose medium. Cells and protoplast were handled with extreme care so as to avoid mechanical damage.

Fixation and preparation for immunofluorescence

Protoplasts were fixed in 3% formaldehyde, 10 mM ethylene glycol-bis (β-aminoethylether)-N,N,N′,N′-tetracetic acid (EGTA), 5 mM $MgSO_4$, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10% dimethylsulphoxide (DMSO) in 100 mM 1,4-piperzinediethane-sulphonic acid (PIPES) buffer, pH 6·8 for 1 h. After fixation the protoplasts were rinsed in PIPES for 15 min, attached to poly-L-lysine coated coverslips, and immediately processed. Procedures were as described by Wick *et al.* (1981). The primary antibody was a monoclonal rat anti-tubulin (MAS 077b, Sera Lab. Ltd, Crawley Down), the secondary antibody was a fluorescein isothiocyanate (FITC)-conjugated goat anti-rat antibody (Nordic BV, Tilburg). F-actin was visualized using rhodamine–phalloidin as a probe (Wieland 1977). For double staining of microtubules and actin filaments, rhodamine–phalloidin was added to the second antibody as described by

Pierson *et al.* (1989). In order to avoid fading, preparations were mounted on glass slides in Citifluor Glycerol/PBS (Agar Scientific Ltd, Stansted, UK). Observations were made using a Leitz Orthoplane microscope, equipped with appropriate filters. Micrographs were taken with a Leitz Vario Orthomat combination on either Agfapan professional film 400 ASA or Fujichrome DX colour reversal film 400 ASA. Selected preparations were also studied in the Bio-Rad MRC-500 Confocal Imaging System and photographed from a high resolution flat screen monochrome monitor with Agfa APX 100 film.

Staining of F-actin by means of extraction procedure

Cells and protoplasts were added to equal amounts of extraction buffer (100 mM PIPES buffer, pH 6·9, containing 10% (v/v) DMSO, 10 mM EGTA, 10 mM $MgSO_4$, 0·05% (v/v) Nonidet P-40 and 0·4 M mannitol as osmoticum) and rhodamine-conjugated phalloidin for 5 min (Hussey *et al.* 1987). Preparations were screened and photographed immediately as described above.

Fixation and preparation for dry-cleaving

Cells and protoplasts were fixed in 0·5% glutaraldehyde, 10 mM EGTA, 5 mM $MgSO_4$ in 100 mM PIPES buffer, pH 6·8 for 1 h. Non-enzyme treated cells were washed twice in PIPES buffer, treated with Cellulase (Onozuka R10) for 30 min, washed again three times and extracted for 1 h in buffer (Traas 1984). Further treatments were the same for both cells and protoplasts. After rinsing twice with distilled water, cells and protoplasts were allowed to settle on poly-L-lysine coated grids. Post-fixation took place for 30 min in 0·5% osmium tetroxide, followed by staining in 0·5% uranyl acetate, also for 30 min. Protoplasts were dehydrated in ethanol and prepared for critical-point drying. Dry-cleaving was performed as described earlier (Traas 1984). The material was photographed in a Jeol JEM 100 CX II.

Data collection

The exact magnification of micrographs of cleaved cells was established with a grating replica. Microtubules, microfilaments and coated pits were traced from the micrographs on plastic sheets. Protoplast areas, lengths of microtubules and microfilaments, as well as distances between these elements and between coated pits and microtubules were measured using a Kontron Videoplan computer. Measurements were carried out on 10 protoplasts selected for the largest possible cleaved areas. They were derived from three independent experiments.

To test whether the distribution of microtubule-coated pit distances has a structural basis or results from randomly distributed elements, the SAS statistical programme was used to generate a number of sheets displaying randomly distributed markers. The sheets were enlarged to give a number of markers $\times$ area^{-1} similar to the number of coated pits on each original micrograph, projected over the traces of the microtubules and the distances between microtubules and markers were measured.

RESULTS

Light-microscopic observations

All preparations studied showed more or less irregular patterns of cortical microtubules (Figs 1 and 2). Individual protoplasts and cells differed considerably in the distribution of

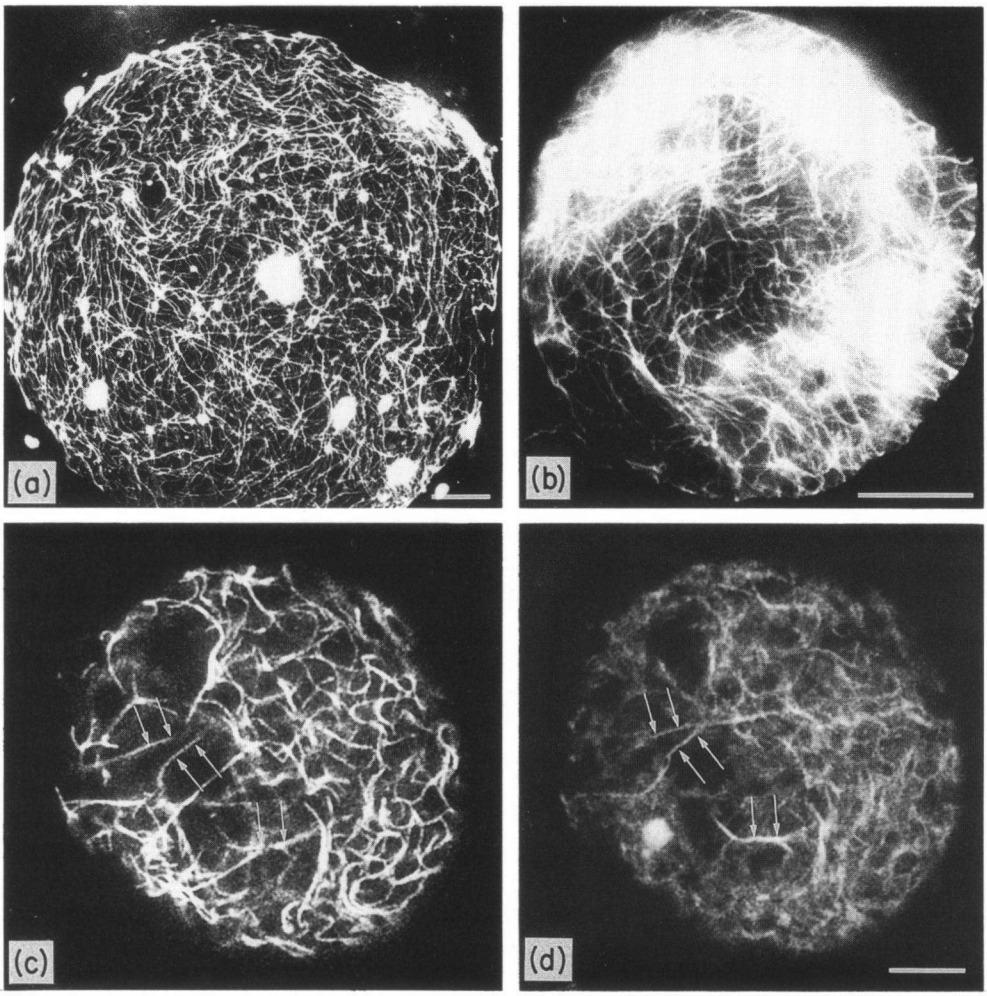

Fig. 1. Immunofluorescence microscopy of protoplasts showing random orientations of cortical microtubules. (a) Protoplast fragment. (b) Whole mount. Confocal images of protoplast stained for tubulin (c) and F-actin (d). Arrows indicate partial co-localization of microtubules and F-actin. Bars = 10 μm.

their cortical microtubules. In addition to criss-cross patterns, many protoplasts and cells showed domains with wave-like or parallel configurations. Only the non-enzyme treated cells showed regular patterns (Fig. 2a). These patterns were limited on one to three sides of the polygonal cells. No indication of other differences between the various preparations was found. Protoplasts often cleaved spontaneously during mounting. The distribution of microtubules in whole mounts did not differ from that in cleaved parts. However, whole mounts gave considerably more background fluorescence in the cell (Fig. 1b), mainly as a result of antibody binding outside the focus level.

F-actin was stained with rhodamine–phalloidin using both fixation and extraction procedures. Although the latter procedure was more reliable and yielded preparations with more detail, no essential differences were observed between the two procedures. Actin filaments formed a basket around the nucleus from where they ran in bundles to the

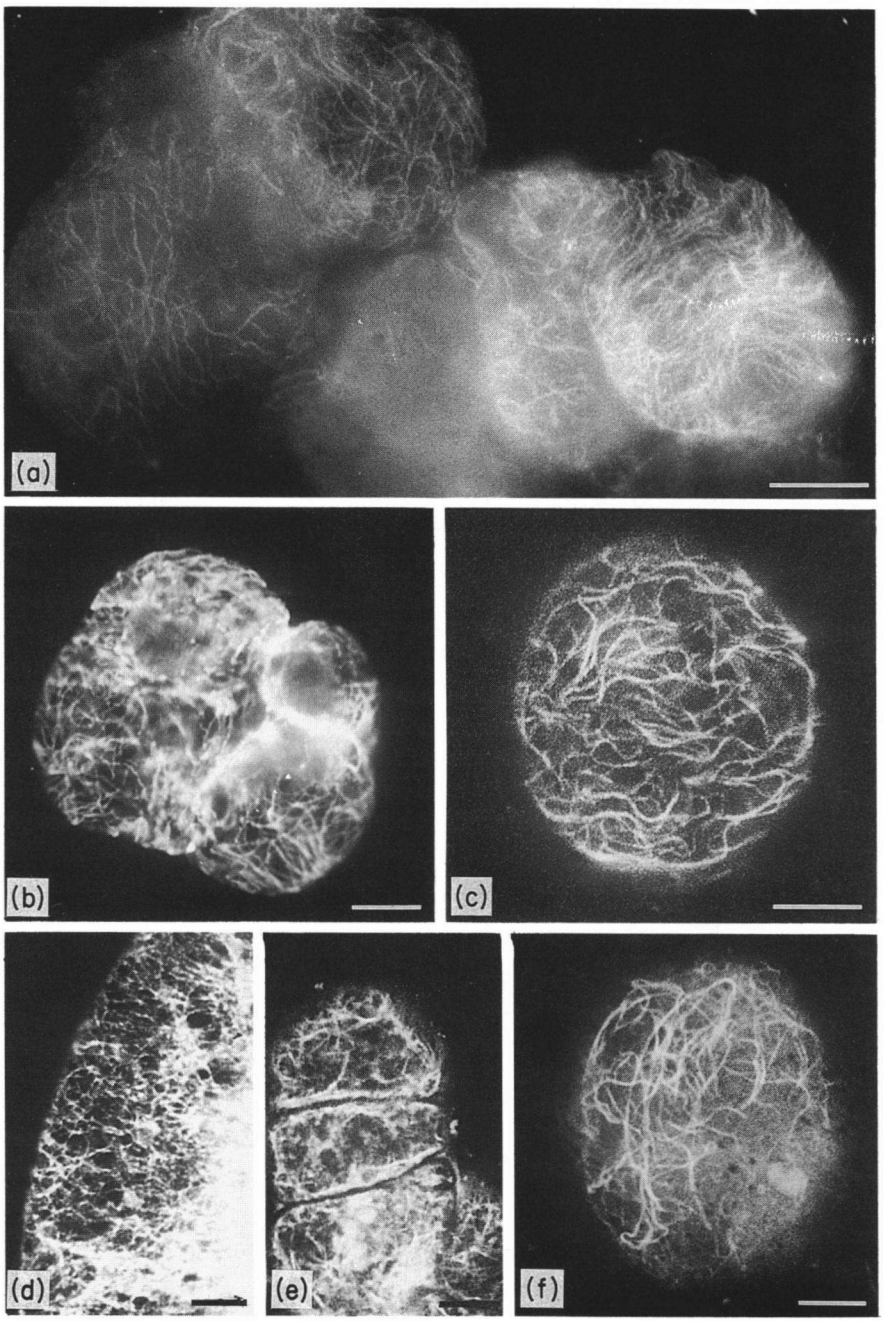

Fig. 2. Immunofluorescence microscopy of suspension cells stained for tubulin. (a) Non-enzyme treated cells. (b) Cells after 2 h in enzyme mixture. (c) Protoplast (4 h in enzyme mixture). Confocal fluorescence microscopy of cells stained for F-actin. (d) Fine cortical actin filaments in a non-enzyme treated cell. (e) Group of non-enzyme treated suspension cells. (f) Protoplast (4 h in enzyme mixture); note hoop-like arrangement of F-actin filaments. Bars = 10 μm.

periphery of the cell or protoplast. Occasionally, hoop-like arrangements were observed in the cortical cytoplasm (Fig. 2f). Due to the strong fluorescence of the bundles and the deformation of the cell residues during their preparation, individual filaments were difficult to trace. Even in non-enzyme treated cells fine cortical filaments could seldom be observed (Fig. 2d). In addition to the difference in the numbers of cells with cortical actin filaments, no differences between cells and protoplasts were observed (Fig. 2e and f). Double labelling tubulin and F-actin revealed, in part, a co-distribution of actin bundles and cortical microtubules as shown for protoplasts (Fig. 1c and d).

Dry-cleaving

After dry-cleaving, non-uniformly sized patches of the critical-point dried protoplasts (Fig. 3a) or cells remained on the grid. The size of the patches left on the grid was not proportional to the size of the original cells or protoplasts. Measurements in 23 protoplasts revealed patch sizes varying between 2 and 63% of the original plasma membrane area. The size of patches from suspension cells was much smaller, and did not allow extensive quantitative measurements.

After dry-cleaving, the cytoskeletal elements attached to the plasma membrane were visible (Figs 3 and 4). If cleaving occurred through the vacuole, a small but undisturbed layer of cortical cytoplasm remained on the grid. After dry-cleaving, microtubules showed nearly the same patterns as observed in immunofluorescence preparations. In the 10 cleaved protoplasts that were selected, the relative abundance of microtubules ranged from 0·93 to 4·29 $\mu m\ \mu m^{-2}$, with an average of 2·75 $\mu m\ \mu m^{-2}$. The length of the microtubules varied from less than 1 μm to 14·28 μm, with an average length of 2·35 μm. Microtubules appeared to form bundles of up to 10 elements running parallel to each other (Figs 3b, c and 4a). In the bundles many cross-bridges interconnecting the microtubules could be seen (Figs 3b–d). The lengths of these cross-bridges varied between 25 and 50 nm, their diameters ranged from 5 to 20 nm (Fig. 3b–e). Measurements on 21 randomly chosen parts of cross-bridged microtubules (of 1·5 μm length each) showed an average 65 cross-bridges per micrometre. If microtubules are considered to form bundles only when interconnected by cross-bridges, bundle formation can be measured unambiguously. The portion of microtubules in bundles averaged 56% (see Table 1). Measurements of a few cleaved preparations from the variously treated cells showed results within the range of those obtained for the selected protoplasts.

In preparations of suspension cells and protoplasts many microtubules showed free, uncapped endings (Fig. 3g, thick arrow). Some microtubules, however, were seen ending in a tangle of filamentous structures (Fig. 3f), or in a cluster of globular particles, giving the microtubule-ending a 'club'-like appearance (Fig. 3g). Crossing microtubules were also regularly present. The vertical distance between these microtubules appeared to be short, probably less than 50 nm (Fig. 4c).

Although numerous F-actin filaments, either in bundles or as fine filaments, could be seen with rhodamine–phalloidin as a probe, the dry-cleaved preparations of cells and protoplasts showed only a few broad hoops of microfilaments. Putative microfilaments could be observed, either connecting coated pits and microtubules (Fig. 3b and h), or co-aligned with microtubules (Fig. 3e). In the latter case, they either followed the microtubules at a fixed distance of about 10 nm, or they showed a periodically spaced attachment (Fig. 3e). Occasionally, individual filaments were observed running along a microtubule over distances up to 1·46 μm. As the diameter of the filaments appeared to be

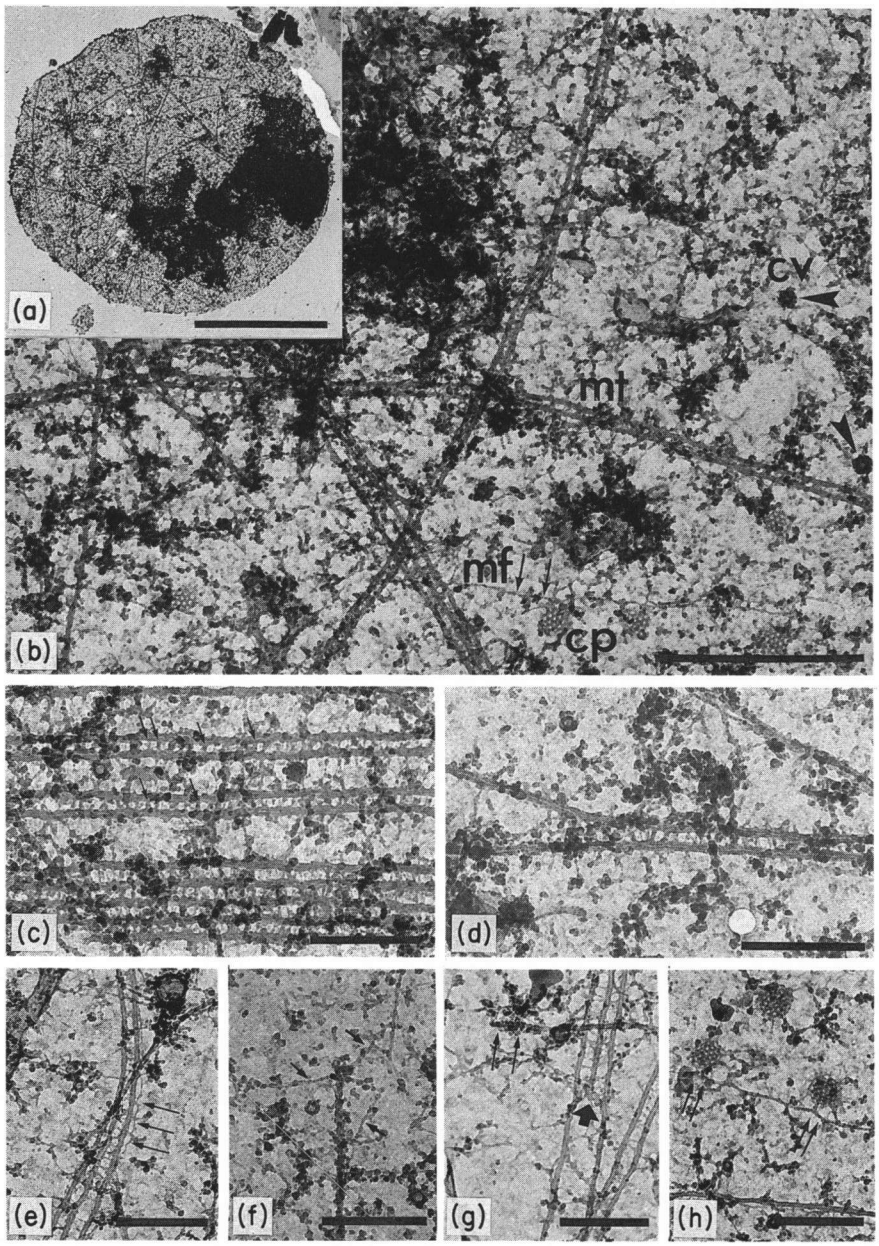

Fig. 3. (a) Dry-cleaved preparation of a protoplast of *Nicotiana plumbaginifolia*. Bar = 5 μm. (b) The cortical cytoplasm of protoplast in (a) at higher magnification, showing microtubules (mt), microfilaments (mf, arrows), coated pits (cp) and coated vesicles (cv, arrowheads). Bar = 0·5 μm. (c) Several bundles of parallel-oriented microtubules in the cortical cytoplasm of protoplast. Note cross-bridges (arrows) between microtubules. (d) Cross-bridges between microtubules appear when their distance is less than 50 nm. (e) Periodically spaced interconnections between microfilaments (arrows) and microtubules. (f) Microtubule-ending closely associated with non-directionally oriented microfilaments (arrows). (g) Microtubule-endings closely associated with small globular structures (arrows) forming 'club'-like appearances. In (g) an uncapped microtubule ending is indicated by a thick arrow. (h) Coated pits situated along and connected to filaments (arrows). Bars in c–h = 0·25 μm.

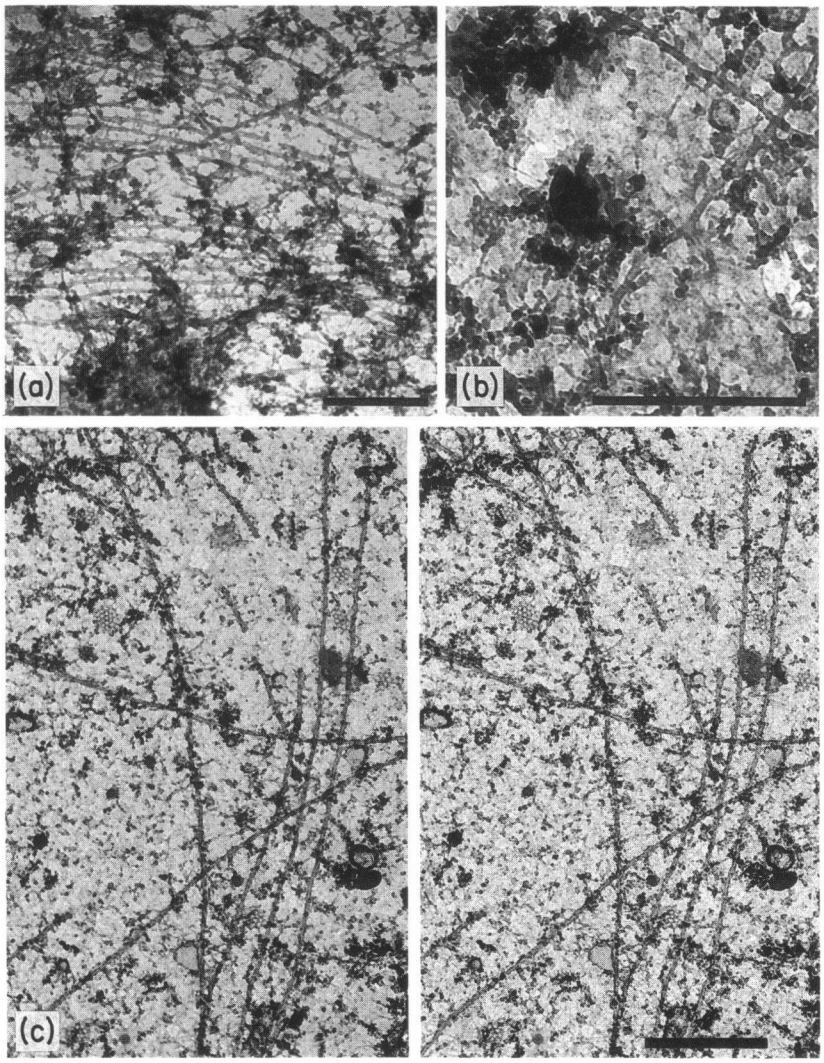

Fig. 4. Dry-cleaved preparation of a non-enzyme treated suspension cell of *Nicotiana plumbaginifolia*. (a) Parallel microtubules in cortical cytoplasm. Bar = 0·5 μm. (b) Microtubules, coated pit and microfilaments in cortical cytoplasm. Bar = 0·25 μm. (c) Stereomicrographs of cortical cytoplasm of protoplast, showing three-dimensional distribution of microtubules. Bar = 0·5 μm.

variable (from 5 to 15 nm), they cannot be classified unambiguously as microfilaments. Numerous coated pits and coated vesicles were present. Measurements on 10 selected protoplasts showed a density of coated pits varying between 0·23 and 2·40 μm^{-2} of membrane. Measurements on a few cleaved preparations from the variously treated cells showed similar results.

Connective threads, possibly microfilaments, between microtubules and coated pits were regularly seen in all preparations, sometimes via another filament. Sometimes coated pits occurred in rows along the filaments (Fig. 3b and h). Only occasionally, coated pits

Table 1. Quantitative measurements carried out on microtubules of 10 protoplasts using a Kontron Videoplan Computer

Number of protoplasts	Area*	Length‡	Relative abundance§	Bundles¶
1	29·77	77·36	2·60	61·36
2	115·30	494·24	4·29	62·08
3	104·88	323·40	3·17	60·76
4	35·12	121·25	3·45	52·38
5	108·06	323·05	3·07	55·10
6	74·84	162·59	2·17	49·97
7	270·48	1137·79	4·21	60·94
8	12·22	24·72	2·02	35·53
9	91·62	85·07	0·93	61·55
10	280·20	445·57	1·59	63·17

*Membrane area of protoplast patches remaining on the grid after dry-cleaving (μm^2).
‡Total length of microtubules (μm).
§Relative abundance of microtubules ($\mu m\ \mu m^{-2}$).
¶Percentage of total microtubular length appearing in bundles (i.e. being cross-bridged).

and coated vesicles were connected to the filaments accompanying microtubules. Stereomicrographs showed that the connections seen were real and did not result from superimposition of the various elements; the structures were definitely lying in the same plane of focus (Fig. 4c). The distances between coated pits and the nearest microtubule were measured in 10 protoplasts. The pooled results are shown in Fig. 5a. More than 75% of the coated pits were situated within 200 nm from the nearest microtubule. Measurements on the randomly generated markers projected over the traces of the microtubules resulted in different distributions (Fig. 5b). Using the chi-square test the difference was shown to be highly significant with $P < 0{\cdot}0001$.

DISCUSSION

The cortical cytoskeleton was essentially the same in all preparations and was not changed by protoplast formation. However, due to mechanical distortions, abundancy and lengths of microtubules in the protoplasts decreased and microfilaments disappeared if the preparations were not handled with extreme care.

The more or less random patterns of microtubules seen here are similar to those observed in other protoplasts (Doohan & Palevitz 1980; Lloyd *et al.* 1980; Van der Valk *et al.* 1980; Hasesawa *et al.* 1988). However, since microtubules often form domains with ordered patterns or bundles, as is especially clear from the dry-cleaved preparations, the overall random organization must be considered to be a superficial view. Protoplasts showing these domains may derive from the polygonal cells, which thus may have preserved their microtubular organization. The occurrence of these random-like patterns may relate to the more or less isodiametric or polygonal morphology of the cells and protoplasts (Lloyd *et al.* 1980).

The organization of the actin skeleton is similar to that in carrot protoplasts (Traas *et al.* 1987). Occasionally, cortical actin filaments and microtubules co-localized in double-

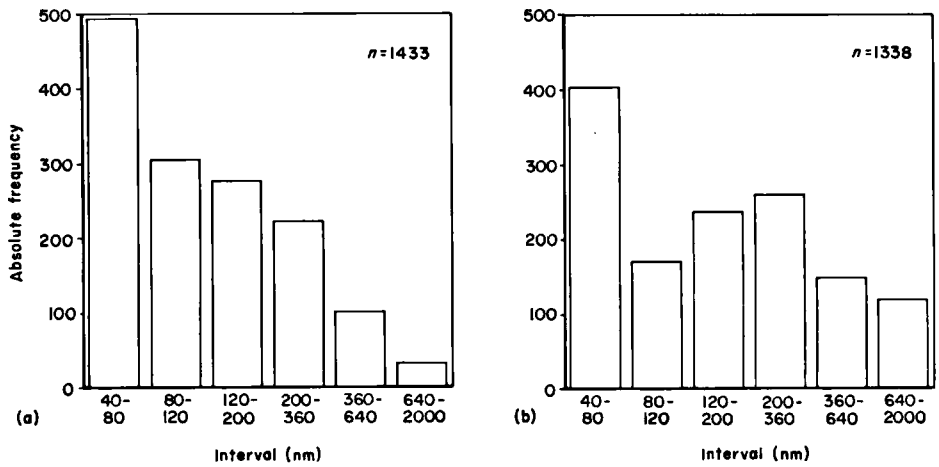

Fig. 5. (a)(b) The SAS statistical programme was used to create a number of sheets displaying randomly distributed markers. The sheets were enlarged to achieve a number of markers area^{-1}, comparable to the concentration of coated pits on individual electron micrographs. The distances between coated pits and the nearest microtubule on the electron micrographs (a) are compared to the distances between the artificially generated, randomly distributed markers and their nearest microtubule (b). In both groups of measurements, identical intervals were compared. Chi-square test was 109·855 and $P<0·0001$. Because of inaccurate measuring in the lower interval of 0–40 nm, inherent in the Kontron Videoplan System, interval 0–40 nm was omitted from the graphs.

stained preparations. Extensive co-localization has been demonstrated in pollen tubes (Pierson *et al.* 1989) and root hairs (Emons 1987). No essential differences were observed between extraction and fixation procedures (Pierson 1988).

Quantitative measurements on the dry-cleaved preparations showed that length and relative abundance of microtubules are similar to previous counts in other cells (Traas *et al.* 1984, 1985). The extent to which microtubules are present in bundles is impressive, even when the very strict criterion of extant cross-bridging is used. Such extensive bundle formation has not been observed in other dry-cleaved material (Traas 1984; Traas *et al.* 1984, 1985). Sometimes, the bundles contained as many as 10 elements and are thus the largest bundles ever reported in plant cells. In dry-cleaved seed hairs of *Cobaea* (Quader *et al.* 1986), the parallel microtubules are densely packed, giving the impression of huge bundles. As only a few cross-bridges between the microtubules in *Cobaea* seed hairs were observed, by our definition they should not be considered as true bundles. The cross-bridges seen in the present study have regularly been observed in freeze-prepared material of plant cells (Tiwari *et al.* 1984; Hawes & Martin 1986; Lancelle *et al.* 1987). The length, density and diameter of the cross-bridges as seen here, were similar to those calculated from micrographs published in these papers. The dimensions of the cross-bridges are, however, not consistent with data reported for specific microtubule-associated proteins as MAP1 (Olmstedt 1986) and MAP2 (Vallee *et al.* 1984; Centonze & Sloboda 1986; Olmstedt 1986; Lewis *et al.* 1989) and adligin (Aamodt *et al.* 1989). From their length, they could represent the tau factor (Hirokawa *et al.* 1988; Lewis *et al.* 1989) or the bundling proteins isolated from suspension-cultured carrot cells (Cyr & Palevitz 1989).

In dry-cleaved preparations, cortical microfilaments were much less abundant than expected from the results in rhodamine–phalloidin stained cells or protoplasts. They were not degraded during fixation as they were present in the same amount in freeze-

substituted, cleaved protoplasts (unpublished results). Thus, the rhodamine–phalloidin stained filaments may have been removed during cleaving.

Microtubules aligned with microfilaments have been observed in dry-cleaved preparations (Traas *et al.* 1985), in deep-etched whole mounts (Hawes & Martin 1986) and in sections of freeze-substituted material (Emons 1987; Lancelle *et al.* 1987; Tiwari & Polito 1988). This alignment has been thought to indicate a possible cortical transport function at the plasmalemma (Traas *et al.* 1987). In dry-cleaved preparations the microfilaments often appear to be 'stitched' to microtubules, a configuration that probably can only be seen by means of cleaving techniques. The possibility is not excluded that part of these filaments, which often show diameters larger than those reported for F-actin, may represent intermediate filaments (Dawson *et al.* 1985; Goodbody *et al.* 1989), as suggested by Derksen *et al.* (1990).

The relative abundance of coated pits did not differ from previous counts in protoplasts (Emons & Traas 1986). The highly significant difference in distribution between coated pits and randomly generated markers with the same density (Fig. 5a and b) indicates that microtubules might determine the site of formation of the coated pits or of the coated vesicles. Coated pits were often connected to filaments, which in turn were connected to microtubules. Bridges between microtubules and coated vesicles that were persistent during microtubule isolation were observed in animal cells (Imhof *et al.* 1983). These bridges putatively contained actin and MAP2 (kinesin ?) leaving the hypothesis that actin and microtubules drive movement.

However, the physiological reason for the specific coated pit distribution in plants and the mechanism used to achieve it are not yet known.

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