Taxol-induced structures derived from cytoskeletal elements of the *Nicotiana* pollen tube

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Summary

***Nicotiana* pollen tube extracts were treated with 20 μM-taxol and structures derived from the cytoskeleton were collected and observed in the electron microscope. We have identified three different groups of elements with characteristic size and morphology: wide filaments (WF) probably formed by assembled microtubules, thin filaments (TF) interacting with WF and debris-like material principally associated with TF. SDS–PAGE and Western blot analysis provide some preliminary evidence of the biochemical composition of the taxol-induced structures.**

Key words: plant microtubules, pollen tube, *Nicotiana*.

Introduction

The presence of microtubules in the generative and vegetative cells of growing pollen tubes has been demonstrated by both electron microscopy and immunofluorescence labelling (Cresti *et al.* 1984; Derksen *et al.* 1985; Tiezzi *et al.* 1986; Pierson *et al.* 1986). The various observations have revealed a characteristic longitudinal distribution of the microtubules in the pollen tube, suggesting that the microtubular system, together with other cytoskeletal structures, might be involved both in the shaping of the cell and in the movement of cytoplasmic components, vegetative nucleus, generative cell and gametes.

Recently, Lancelle *et al.* (1987), using freeze substitution for the preparation of *Nicotiana* pollen tubes for electron microscopy, have reported the presence of fine filaments running parallel with the microtubules in the vegetative cell, and have shown that microtubules in the generative cell are linked by an extensive system of cross-bridges. Similar structures have been repeatedly observed in plant material (Franke *et al.* 1972; Seagull & Heath, 1979; Tiwari *et al.* 1984; Lancelle *et al.* 1986), but in the absence of biochemical data it is not clear whether the cross-binding structures can be identified with microtubule-associated proteins (MAPs) previously studied in animal cells (Herzog & Weber, 1978; Vallee & Bloom, 1984; Dustin, 1984).

In this paper we report on the effects of taxol, an anti-mitotic drug, on the complex network of thin filaments associated with microtubules in the pollen tubes of *Nicotiana*.

Materials and methods

Pollen of *Nicotiana alata* L. was germinated in standard medium (Brewbaker & Kwack, 1963), and the tubes were harvested after 3 h.

Samples for electron microscopy were prepared according to the method used by Cresti *et al.* (1984). Material was embedded in Spurr's resin by standard procedures, and sections were stained with uranyl acetate and lead citrate before examination with a JEOL JEM100B electron microscope.

For the investigation of taxol-induced aggregates, pollen grains and 3-h pollen tubes were washed in TBS (Tris-buffered saline) at pH 7·2, and processed following the procedure of Vallee (1982).

Germinated pollen was washed in TBS and homogenized in 2 vol. of PEM buffer (0·1 M-Pipes, pH 6·6, 5 mM-EGTA, 1 mM-$MgSO_4$, 1 mM-phenylmethylsulphonyl fluoride, 1 mM-dithiothreitol, 10 μg ml^{-1} leupeptin). The homogenate was first centrifuged at 30 000 ***g*** for 30 min at 4°C, and the supernatant was centrifuged again at 140 000 ***g*** for 90 min at 4°C. After adding GTP and taxol to the supernatant to final concentrations of 1 mM and 20 μM, respectively, the material was incubated for 20 min at 23°C. The solution was then centrifuged at 27 000 ***g*** for 15 min at 4°C. The supernatant was discarded and the microtubular pellet resuspended in

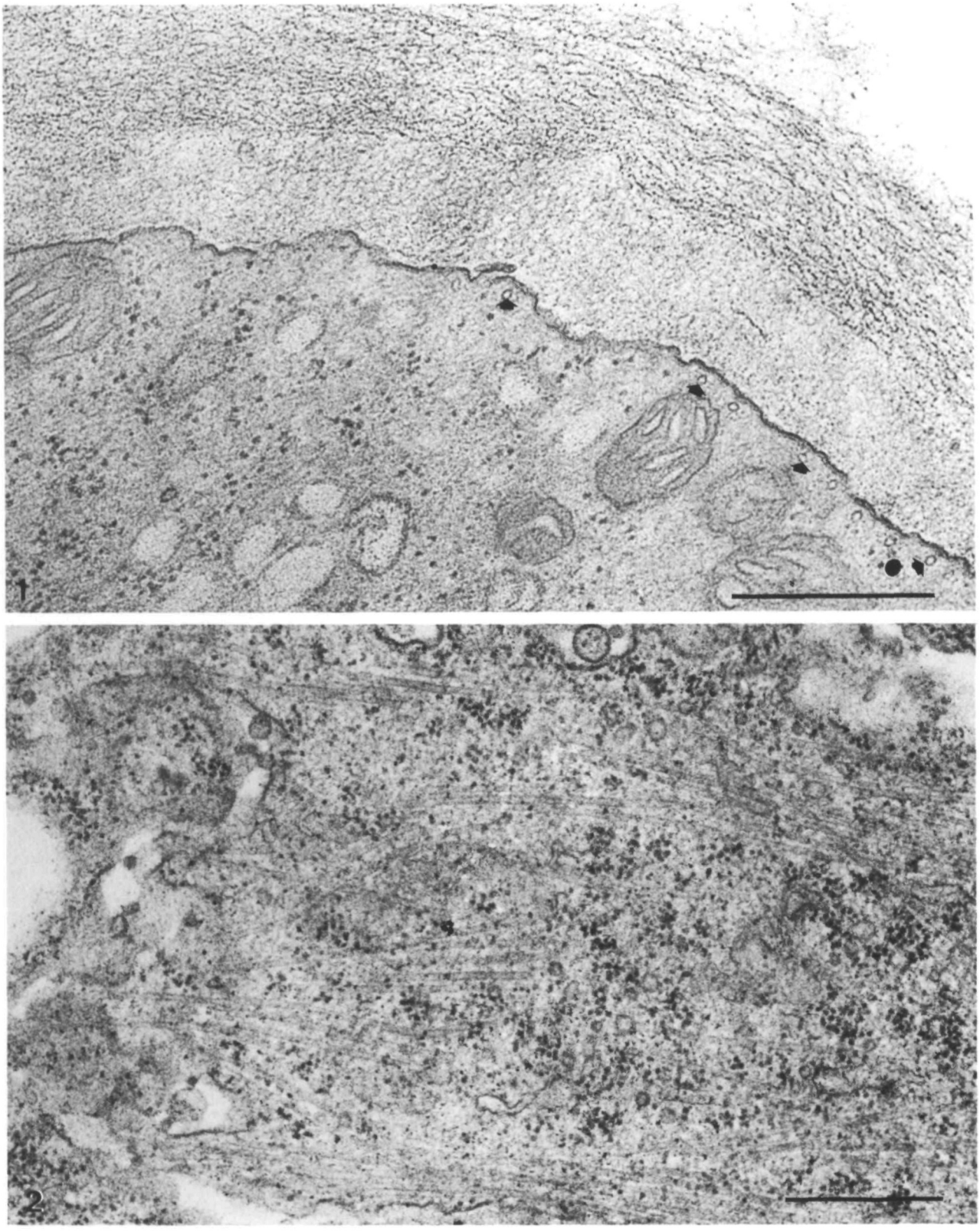

Fig. 1. Cortical microtubules (arrows) in the vegetative cell; their orientation is longitudinal. ×70 000.
Fig. 2. Network of microtubules in the generative cell: the orientation is longitudinal and mainly they are aggregated in bundles. ×54 000.

PEM buffer containing 1 mM-GTP and 20 μM-taxol, and then centrifuged through a 2-ml cushion of 15 % sucrose in PEM buffer containing 1 mM-GTP and 20 μM-taxol at 27 000 g for 1 h at 4 °C. The pellet was then washed gently in PEM buffer containing 1 mM-GTP and 20 μM-taxol and, after centrifugation, resuspended in Laemmli (1970) sample buffer, boiled and stored at −20 °C.

Taxol-induced aggregates were observed with the electron microscope after staining with 2 % uranyl acetate.

Protein analysis of the taxol-induced aggregates was carried out by SDS–PAGE using chicken ovalbumin ($43\times10^3 M_r$), purified calf brain tubulin ($55\times10^3 M_r$) as standards on 4 % to 16 % gradient gels (Laemmli, 1970) and stained with Coomassie Blue R 250.

Western blots (Towbin *et al.* 1979) were prepared using an anti-tubulin polyclonal antibody and an anti-actin monoclonal antibody (provided by Dr R. Cyr and J. Lessard, respectively) and peroxidase-coupled second antibodies (Cappel Laboratories).

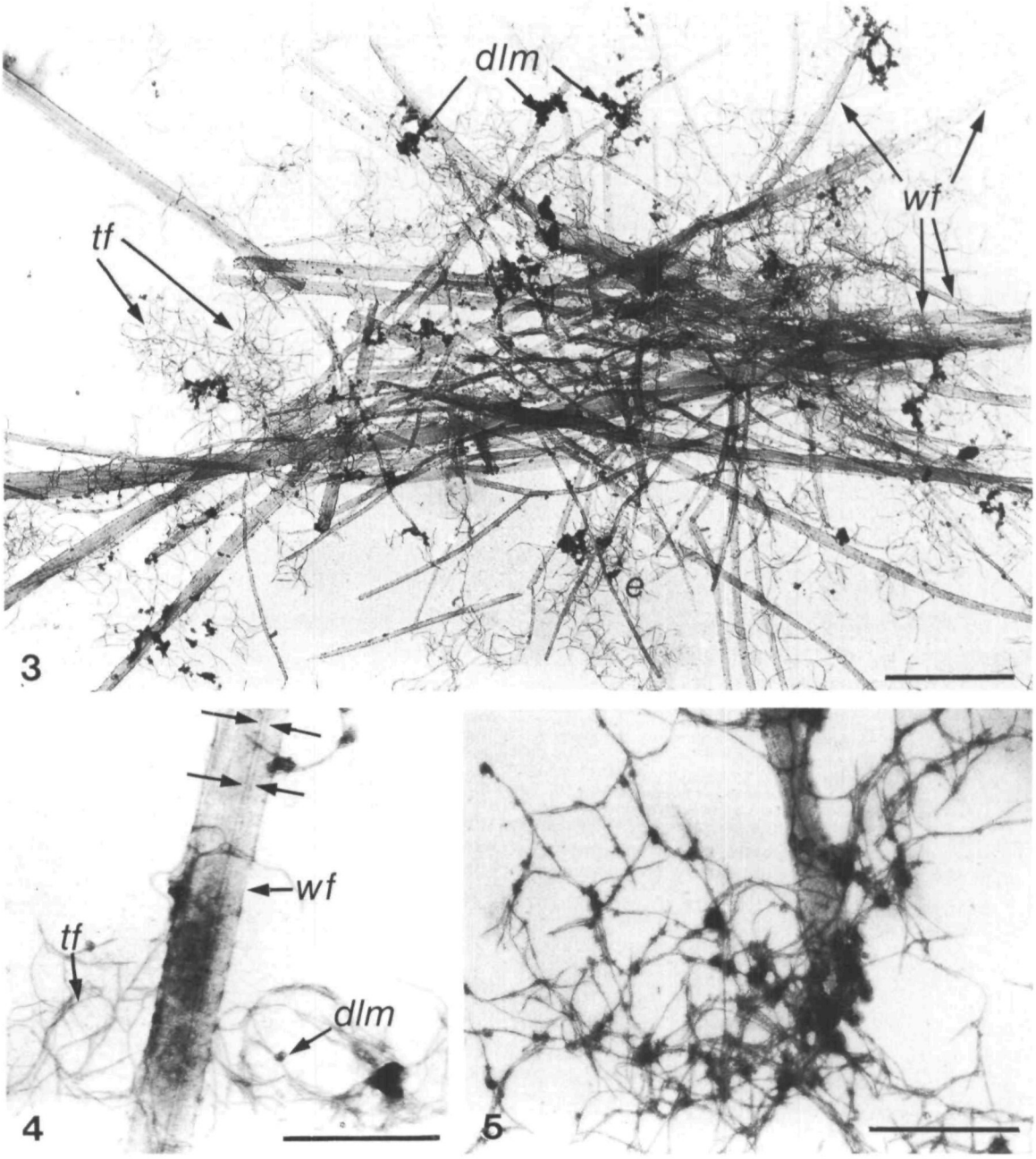

Fig. 3. Aggregates after taxol treatment: three different classes of structure are visible. *wf*, wide filaments; *tf*, thin filaments; *dlm*, debris-like material. ×9500. Bar, 2 μm.

Fig. 4. High magnification of *wf*. The arrows show thin structures, 22 nm in diameter. ×45 000. Bar, 0·5 μm.

Fig. 5. Close association between *dlm* and *tf*. ×45 000. Bar, 0·5 μm.

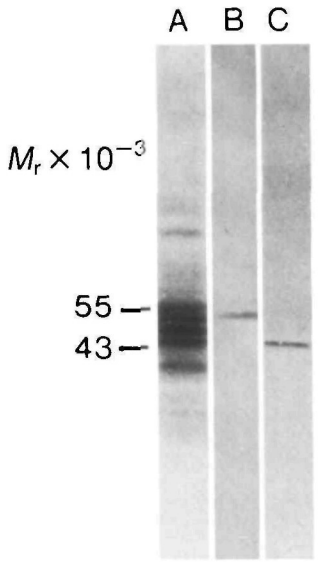

Fig. 6. SDS–PAGE (A) and Western blot analysis (B,C) of pollen tube extracts. Lane A, taxol-induced aggregates; lane B, Western blot using a polyclonal antibody to tubulin; lane C, Western blot using monoclonal antibody to actin.

Results

Microtubules in intact pollen tubes

In the vegetative cell, microtubules are restricted to the cortex and their orientation is primarily longitudinal (Fig. 1). In the generative cell the microtubules are also longitudinally oriented, and organized mainly in bundles (Fig. 2).

Taxol-dependent aggregates

In the presence of taxol, complex aggregates are obtained from pollen tube extracts. Fig. 3 is a micrograph of an aggregate observed after staining. Three different, well-defined classes of structure are present: (1) wide filaments (WF), of varying diameters (80–370 nm); (2) thin filaments (TF), with a mean diameter 5–8 nm and not apparently derived by fraying of the ends of the WF (but often seen to be connected to the WFs along their lengths); and (3) debris-like material associated with the TF system.

At high magnification the WFs appear to contain thinner structures with a diameter approximately 22 nm (Fig. 4). This micrograph and Fig. 5 show that the debris-like material is made up of more or less globular particles of varying diameter (20–100 nm), associated with the TF network.

Electrophoresis and Western blot analysis

Fig. 6 shows the protein composition of the taxol-induced aggregates as revealed by SDS–PAGE and Coomassie Blue staining (lane A). Several polypeptides of different molecular weights are present. By electrophoretic comparisons with standards, tubulin ($\approx 55\times10^3$) and a band with an M_r of 43×10^3 are evident.

The proteins in the A lane were investigated further by the Western blot technique, using a polyclonal antibody to tubulin and a monoclonal antibody to actin. Specific reactions were observed at the 55×10^3 M_r and 43×10^3 M_r polypeptides, respectively (lanes B, C).

Discussion

Most studies on the effects of taxol have been carried out with tubulin from animal sources (Schiff *et al.* 1979; Vallee, 1982; Brady, 1985; Vale *et al.* 1985), although plant tubulin has been used in some earlier studies (Morejohn & Fosket, 1982; Dawson & Lloyd, 1985). In addition, the action of taxol on microtubule arrays in cultured higher plant cells has been investigated as well (Falconer & Seagull, 1985; Weerdenburg *et al.* 1986). The present results show that by the use of taxol it is possible to produce wide filaments and associated thin filaments in extracts derived from tobacco pollen tubes and, although we are not really able to explain the formation of this complex, some analysis can be done on the basis of our electron-microscope results and SDS–PAGE and Western blot data.

(1) Wide filaments are well-defined structures of various sizes and consist of thinner elements (≈22 nm in diameter) (Fig. 4). On the other hand, from Western blots we also know that tubulin is present in these structures. It therefore seems likely that they represent a form of tubulin polymer or aggregate.

(2) The thin filament system contains structures similar in size to microfilaments. Since Western blots clearly show the presence of actin in the taxol-induced aggregates, it is possible that these filaments contain that protein.

(3) As biochemical studies have not been carried out on debris-like material, it is difficult to say what it represents. Ultrastructural images of thin filaments running parallel to and interacting with microtubules have been obtained with pollen tubes and other plant material (e.g. see Tiwari *et al.* 1984; Lancelle *et al.* 1987). These structures may be homologous with the thin filament system shown here. However, it is also possible that they could be a form of microtubule-associated protein closely arranged on the microtubule surface.

The results reported here are preliminary in nature, and obviously further work is now needed to characterize better the structures we have described. The procedures applied to *Nicotiana* pollen tube extracts are based on methods used previously in animal material (Vallee, 1982). The large microtubular structures appear to be produced by taxol treatment only *in*

vitro, since similar bodies have not been seen in intact pollen tubes. Nevertheless, taxol has proven useful in animal and plant cytological studies (Bajer *et al.* 1982; Weerdenburg *et al.* 1986), and it will probably provide a valuable tool for biochemical investigations of the plant cytoskeleton as well.

We thank Dr R. Cyr of the University of Georgia for providing the polyclonal anti-tubulin, and Dr R. Meagher of the University of Georgia and Dr J. L. Lessard, Children's Hospital Research Foundation, Cincinnati, Ohio, for the monoclonal anti-actin. We also thank Drs J. Heslop-Harrison, B. Knox, P. K. Hepler and B. A. Palevitz for helpful comments during the preparation of the manuscript.

This research was carried out in the framework of contract no. BAP-0204-I of the Biotechnology Action Programme of the Commission of the European Communities.

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(Received 13 April 1987 – Accepted, in revised form, 7 September 1987)